

Linglin Fu · Bobby J. Cherayil
Haining Shi · Yanbo Wang · Yang Zhu

Food Allergy

From Molecular Mechanisms to Control
Strategies

 Springer

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Abbreviations

AA	Arachidonic acid
Ab	Antibody
ABPs	Auxin-binding proteins
Ag	Antigen
AhR	Aryl hydrocarbon receptor
AK	Arginine kinase
Ala	Alanine
APC	Antigen-presenting cell
Arg	Arginine
BALF	Bronchoalveolar lavage fluid
BAT	Basophil activation test
BCG	Bacillus Calmette-Guerin
BCR	B-cell antigen receptor
BLG	β -lactoglobulin
bTJ	Bicellular tight junction
CLR	C-type lectin receptor
CT	Cholera toxin
C _T	Cycle threshold
CTA	Cholera toxin A subunit
CTB	Cholera toxin B subunit
DAMP	Damage-associated molecular pattern
DBPCFC	Double-blind placebo-controlled food challenge
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin
DHA	Docosahexaenoic acid
DHPM	Dynamic high-pressure micro jet
DNP	Dinitrophenyl
DNP-BSA	Dinitrophenyl-bovine serum albumin
DPPH	1,1-diphenyl-2-picrylhydrazyl
DSS	Dextran sulfate sodium

EAACI	European Academy of Allergy and Clinical Immunology
EAST	Enzyme allergosorbent test
EC	Epicutaneous
EFSA	European Food Safety Authority
EIS	Electrochemical impedance spectroscopy
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EPA	Eicosapentaenoic acid
EPIT	Epicutaneous immunotherapy
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FCA	Freund's complete adjuvant
FcεRI	High-affinity IgE receptors
FDA	Food and Drug Administration
FIA	Freund's incomplete adjuvant
Foxp3	Forkhead box protein 3
FSA	Food Standards Agency
FSAI	Food Safety Authority of Ireland
FSANZ	Food Standards Australia New Zealand
GALT	Gut-associated lymphoid tissue
GAP	Goblet cell-associated antigen passage
GF	Germ-free
GLPs	Germin-like proteins
GM	Genetically modified
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Good manufacturing practice
GPR	G-protein-coupled receptor
HACCP	Hazard analysis and critical control point
HDAC	Histone deacetylase
HDM	House dust mite
HMC	Hemocyanin
HMC-1	Human mast cell line
HRP	Horseradish peroxidase
HRT	Histamine release test
IAEA	International Atomic Energy Agency
IBD	Inflammatory bowel disease
IDST	Intradermal skin testing
IEC	Intestinal epithelial cell
IFN	Interferon
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IG	Intragastric
IgE	Immunoglobulin E
IL	Interleukin
ILC	Innate lymphoid cell

IN	Intranasal
iNOS	Inducible nitric oxide
IP	Intraperitoneal
JAK2	Janus kinase
JNK	Jun N-terminal kinase
LAD2	Laboratory of allergic diseases 2
LC-MS	Liquid chromatography-mass spectrometry
Leu	Leucine
LFIA	Lateral flow immunoassay
LFS	Lateral flow strip
LGG	<i>Lactobacillus rhamnosus</i> GG
LPS	Lipopolysaccharide
LTP	Lipid transfer protein
M cell	Microfold cell
mAb	Monoclonal antibody
MAMP	Microbe-associated molecular pattern
mBMMC	Mucosal-type bone marrow-derived mast cell
MCP-1	Mast cell protease-1
Met	Methionine
MHC	Major histocompatibility complex
MIM	Multiple ion monitoring
MLC	Myosin light chain
MLN	Mesenteric lymph nodes
MLNL	Mesenteric lymph node lymphocyte
MR	Mannose receptor
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NDO	Nondigestible oligosaccharides
NLR	NOD-like receptor
NMR	Nuclear magnetic resonance
nsLTPs	Nonspecific lipid transfer protein
nTreg	Natural regulatory T
OFC	Open food challenge
OFC	Oral food challenge
OIT	Oral immunotherapy
OPS	Oyster-derived polysaccharides
OVA	Ovalbumin
pAb	Polyclonal antibody
PAMP	Pathogen-associated molecular pattern
PAR	Proteinase-activated receptor
PCA	Passive cutaneous anaphylaxis
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane
PMD	Piecemeal degranulation
PP	Peyer's patch

PPO	Polyphenol oxidase
PR-10	Pathogenesis-related protein family 10
PRR	Pattern recognition receptor
PTM	Post-transcriptional modification
PUV	Pulsed ultraviolet
PV	Parvalbumin
PVDF	Polyvinylidene fluoride
QCM	Quartz crystal microbalance
Q-IT	Quadrupole-ion trap
QQQ	Triple quadrupole
Q-TOF	Quadrupole-time of flight
RALDH2	Retinaldehyde dehydrogenase-2
RAST	Radioallergosorbent test
RBL	Rat basophilic leukemia
RIE	Rocket immune-electrophoresis
RLR	RIG-I-like receptor
SBPCFC	Single-blind placebo-controlled food challenge
SC	Subcutaneous
SCFA	Short-chain fatty acid
SCP	Sarcoplasmic calcium-binding protein
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis
SEB	Staphylococcal enterotoxin B
SERS	Surface-enhanced Raman spectroscopy
SFB	Segmented filamentous bacteria
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
sIgA	Secretory immunoglobulin A
SIM	Selected ion monitoring
SLIT	Sublingual immunotherapy
SPR	Surface plasmon resonance
SPT	Skin prick tests
SRM	Selected reaction monitoring
SSOP	Sanitation standard operating procedure
TAMP	Tight junction-associated MARVEL proteins
TCR	T-cell antigen receptor
TED	Transepithelial dendrite
TG	Transglutaminase
TGF- β	Transforming growth factor- β
Th	Helper T cell
TIM	Triosephosphate isomerase
TIR	Toll/interleukin-1 receptor homology
TIRF	Total internal reflection fluorescence
TLR	Toll-like receptor
TM	Tropomyosin
Tn C	Troponin C

TNF- α	Tumor necrosis factor- α
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
tTJ	Tricellular tight junction
UHP	Ultrahigh pressure
UV	Ultraviolet
Val	Valine
WHO	World Health Organization

Chapter 1

Overview of the Immunology of Food Allergy



Abstract Food allergies are caused by an abnormal immune response to ingested antigens. They manifest as urticaria, nausea, vomiting, abdominal pain, or anaphylaxis, usually within a short period after consumption of the relevant food, and can be either IgE-dependent or IgE-independent. They affect large numbers of people, particularly children in industrialized countries, and represent an important and increasing public health problem. The pathogenesis of food allergy involves a failure of the mechanisms that normally prevent or control immune reactivity to orally administered antigens. Treatment strategies include avoidance of the offending dietary component, control of symptoms with the appropriate pharmacologic agents, and immunologic approaches directed at restoring nonresponsiveness to the relevant allergen. This chapter will review current concepts in the immunology and treatment of food allergy.

According to a National Institute of Allergy and Infectious Diseases Expert Panel, food allergy is defined as “an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food” (Boyce et al. 2011). This definition includes a variety of IgE-mediated disorders that usually have a relatively rapid onset after exposure to the offending food and that can manifest with urticaria; gastrointestinal symptoms such as nausea, vomiting, and abdominal pain; and difficulty breathing because of bronchoconstriction or laryngeal edema and even systemic anaphylaxis and cardiovascular collapse (Sicherer and Sampson 2017). It also covers more chronic non-IgE-mediated conditions such as celiac disease, food protein-induced enterocolitis, and eosinophilic esophagitis that have specific clinical presentations related to the tissue affected. This chapter will focus on IgE-mediated food allergy, and the reader interested in the non-IgE-mediated problems is referred to several recent reviews (Bingemann et al. 2018; O’Shea et al. 2018; Sicherer and Sampson 2017). It is important to mention that the term food allergy should not be used to describe food-associated adverse effects that are not mediated by an immune response, e.g., lactose intolerance or symptoms caused by ingesting toxin-containing foods.

IgE-mediated food allergies are common and are estimated to affect up to 10% of the population in industrialized countries, predominantly children (Osborne et al. 2011). The prevalence appears to have increased over the last 10–20 years, an impression that is supported by the results of several studies, including those that have used stringent diagnostic criteria (Jackson et al. 2013; Sicherer et al. 2010; Supinda et al. 2014). The reasons for this increase are not well understood, but it is clear that food allergies represent a significant public health burden. Most of this burden is attributable to a relatively small number of dietary components: peanut, tree nuts, fish, shellfish, egg, milk, wheat, soy, and seeds. Some allergies, particularly those to milk, egg, wheat, and soy, have a high likelihood of resolving in childhood, whereas others, such as those to peanut and tree nuts, are more persistent (Savage et al. 2016).

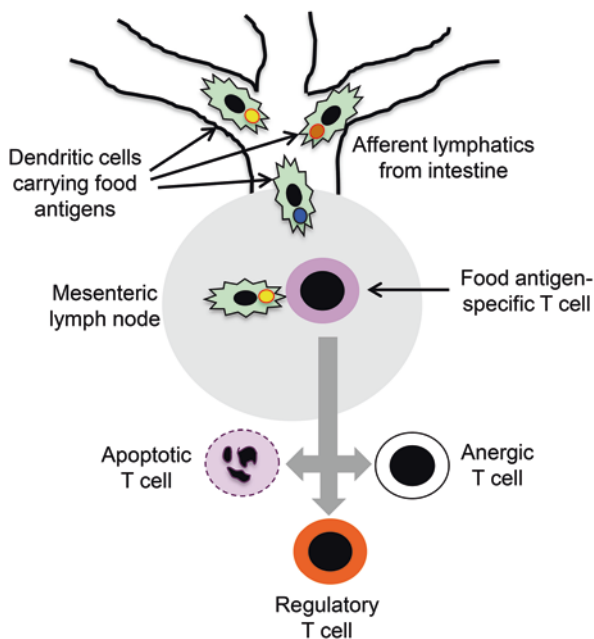
1.1 Immunopathogenesis of Food Allergy

Orally administered antigens, including components of food, normally induce a state of antigen-specific immune unresponsiveness known as oral tolerance (Tordesillas and Berin 2018). Broadly speaking, food allergies occur when oral tolerance to one or more dietary antigens fails to develop. Thus, in order to understand the pathogenesis of food allergy, it is important to know something about the mechanisms involved in oral tolerance.

1.1.1 Oral Tolerance

The process of oral tolerance is illustrated in Fig. 1.1. Under steady-state conditions, antigenic material in the gastrointestinal tract is constantly sampled by phagocytic cells located in the lamina propria (Joeris et al. 2017). These cells include CD103⁺ dendritic cells (DCs) and CX3CR1⁺ macrophages that are closely associated with the intestinal epithelium. Antigen can reach these cells by several routes: transcytosis across specialized epithelial cells known as microfold or M cells, transcellular or paracellular transport across enterocytes, movement through specialized goblet cell-associated passages (GAPs), and following capture by DC and macrophage cytoplasmic processes that extend between epithelial cells into the lumen (Ménard et al. 2010; Mcdole et al. 2012; Niess and Reinecker 2005; Ohno 2016; Rescigno et al. 2001). Protein antigens taken up by DCs and macrophages are processed into short peptides that are displayed on the cell surface in the cleft of major histocompatibility complex (MHC) molecules, making them available for recognition by T lymphocytes (Rock et al. 2016). Some nonprotein antigens may be processed and presented similarly. DCs that have acquired antigen from the gut migrate via local lymphatic vessels to the mesenteric lymph node (MLN). Macrophages do not generally migrate from the lamina propria, so antigens phagocytosed by these

Fig. 1.1 Induction of oral tolerance. Under basal conditions, food antigens are taken by dendritic cells from the intestinal lamina propria to the mesenteric lymph nodes, where peptides derived from the antigens are presented to naïve T cells with the appropriate antigen receptor. The T cells undergo apoptosis, become anergic or differentiate into antigen-specific regulatory T cells, thus ensuring that the food antigen does not activate a productive immune response



cells may have to be transferred to DCs through gap junctions before they can be taken to local lymph nodes (Elisa et al. 2014).

T cells in the MLN normally recognize peptide-MHC complexes derived from food antigens in the absence of co-stimulatory signals that are required for the T cells to become activated (Lieping and Flies 2013). Thus, instead of being activated, the food antigen-specific T cells either die by apoptosis or become nonresponsive to subsequent stimulation, a phenomenon known as anergy (Kalekar and Mueller 2017). A third potential outcome is the differentiation of the T cells so that they acquire the ability to suppress immune responses in an antigen-specific manner. Several types of suppressive T cells have been described, each of which uses slightly different mechanisms to inhibit the functions of other cells. The best studied of these cells are regulatory T cells (Tregs), which are characterized by the expression of the transcription factor Foxp3 and suppressive mechanisms involving both secreted factors (e.g., the cytokine IL-10) and cell surface molecules (Kitagawa and Sakaguchi 2017). The importance of Tregs in tolerance to food antigens is highlighted by the observation that individuals with mutations in the FOXP3 gene have a severe form of food allergy (Torgerson et al. 2007). Other types of suppressive T cells include Th3 cells, which produce TGF- β , and Tr1 cells, which express both TGF- β and IL-10 (Daniel et al. 2005; Tordesillas and Berin 2018). T-cell apoptosis, anergy, and the acquisition of a suppressive or regulatory phenotype all contribute to the development of tolerance to food antigens, with the specific mechanism involved being influenced by the type and dose of antigen (Tordesillas and Berin 2018). The induction of these tolerogenic mechanisms is probably dependent on specific characteristics of the DCs that present the antigen (Domogalla et al. 2017).

1.1.2 Oral Tolerance Failure and the Development of Food Allergy

The development of food allergy is associated with failure of the mechanisms that promote tolerance to orally administered antigens. Instead of inducing a state of unresponsiveness, food antigens aberrantly activate a Th2-type effector T-cell response that leads to the production of antigen-specific IgE (Fig. 1.2) (Wambre et al. 2017). This type of abnormal Th2 activation can occur during exposure to antigen through the gastrointestinal tract, the skin, or the respiratory tract. During this initial sensitization phase, the antigen-specific IgE becomes bound to the high-affinity IgE receptor on tissue mast cells. On subsequent exposure to the food

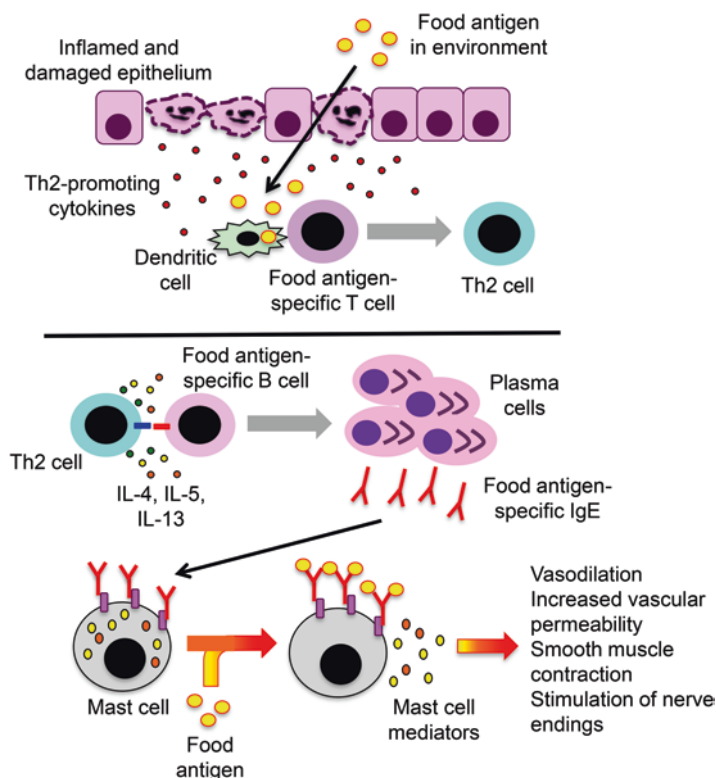


Fig. 1.2 Induction of food allergy. Food antigens presented by dendritic cells in the presence of Th2-promoting cytokines (e.g., in the context of atopic dermatitis) activate T cells to become Th2-type helper T cells. These Th2 cells migrate to other tissues and interact with food antigen-specific B cells to promote their differentiation into plasma cells secreting IgE against the antigen. The IgE binds to the high-affinity IgE receptor on tissue mast cells and triggers mast cell degranulation on subsequent encounter with the antigen. The released mast cell mediators cause the vasodilation, vascular leak, bronchoconstriction, and other changes that give rise to the clinical manifestations of food allergy

antigen that activated this response, the mast cell-bound IgE is cross-linked by the antigen, leading to mast cell activation and the rapid release of various preformed and newly synthesized mediators, including histamine, prostaglandins, tryptase, and inflammatory cytokines. These mediators cause dilation and increased permeability of the local vasculature, with associated fluid leak into the surrounding tissue, recruitment of immune cells, and stimulation of nerve endings. They can also induce contraction of intestinal and bronchial smooth muscle. Depending on the site and extent of these responses, the affected individual can manifest with relatively mild symptoms such as urticaria, abdominal pain, vomiting, or diarrhea or more serious problems such as narrowing of the airways caused by bronchoconstriction or laryngeal edema and complete cardiovascular collapse (systemic anaphylaxis) (Church et al. 2018; Reber et al. 2017).

Why do some individuals develop this maladaptive and potentially life-threatening allergic response to food? Although we do not have a definitive answer to this question, we now know that both genetic and environmental factors can contribute to the occurrence of food allergy. With respect to genetics, sex (male gender) and race/ethnicity (Asian or African descent) are known to play important roles, although the underlying mechanisms are yet to be elucidated. Other genetic factors that have been implicated in the pathogenesis of food allergy involve variants or mutations in genes that affect the T-cell response to food antigens. As mentioned above, mutations in the FOXP3 gene impair the differentiation of Tregs, leading to failure of oral tolerance and the consequent development of food allergy (Torgerson et al. 2007). Similarly, certain MHC alleles and epigenetic modifications (DNA methylation) in genes involved in Th1- and Th2-type immune responses increase the risk of food allergy, presumably by favoring food antigen-induced T-cell responsiveness rather than unresponsiveness (Hong et al. 2015, 2016). Another well-defined genetic risk factor for food allergy is represented by loss-of-function mutations in the filaggrin gene (Devasmitha et al. 2014). Filaggrin is a protein that is involved in maintaining the normal barrier properties of the skin. Individuals with filaggrin mutations that interfere with this function are predisposed to developing atopic dermatitis (eczema), which in turn increases the risk of subsequent food allergy (Devasmitha et al. 2014; Han et al. 2017). It is believed that exposure to environmental antigens, including food antigens, through a compromised and inflamed skin barrier activates antigen-specific T lymphocytes as a result of upregulation of co-stimulatory molecules on cutaneous innate immune cells. Moreover, since the encounter with antigen occurs in an inflammatory environment that is rich in cytokines such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) produced by epithelial cells, as well as IL-5 and IL-13 produced by local innate lymphoid cells (ILCs), the activated T cells are induced to differentiate along the Th2 pathway (Han et al. 2017). The Th2 cells then migrate to other tissues, including the mucosal surfaces and associated lymphoid structures of the lung and gastrointestinal tract, where they are available to interact with food antigen-specific B cells and promote the production of IgE to the antigen by secreting cytokines such as IL-4, IL-5, and IL-13 (Czarnowicki et al. 2017).

Nongenetic (environmental) factors also play a role in determining whether an individual will develop an allergic response to food antigens. Cesarean delivery, formula feeding, and residence in urban areas are some examples of the nongenetic influences that increase the risk of developing food allergy (Sicherer and Sampson 2017). Dietary deficiencies of vitamin D and omega-3-polyunsaturated fatty acids have also been implicated, although the exact mechanisms involved are not clear (Sicherer and Sampson 2017). The time of introduction of potentially allergenic foods is another factor. Delayed introduction in the diet provides time for exposure to food antigens in the environment via routes (e.g., inflamed skin) that lead to T-cell activation instead of tolerance. The primed pre-existing food antigen-specific T cells can then respond in an allergenic fashion on subsequent dietary exposure to the antigen. Thus, early introduction of antigens via the gastrointestinal tract can prevent this sequence of events, presumably by promoting oral tolerance. This concept has received strong support from a clinical trial in which infants at high risk for allergy were randomized to consume or avoid peanuts until the age of 5 years. The results showed clearly that the infants who were introduced to peanut-containing foods between the ages of 4 and 11 months had a significantly lower prevalence of peanut-specific allergy, along with a lower proportion of individuals with elevated peanut-specific IgE titers (Du et al. 2016, 2017; Du Toit et al. 2015). It remains to be seen whether similar results will be obtained with other food allergens, but meanwhile the American Academy of Pediatrics and other organizations have issued a guidance statement indicating that “health care providers should recommend introducing peanut-containing products into the diets of high-risk infants early on in life” (Fleischer et al. 2015).

Many of the environmental effects on food allergy risk are likely to be mediated by changes in the microbiota, the community of commensal microorganisms that resides on cutaneous and mucosal surfaces and profoundly influences the development and function of the immune system (Gensollen et al. 2016). A number of studies in humans and experimental animals have linked variations in microbiota composition, which occur as a result of differences in a host of environmental factors such as diet, mode of delivery, residence, etc., to the development of allergic disease (Huang et al. 2017). Although the exact mechanisms that are involved in this connection are not completely clear, it is becoming increasingly appreciated that the microbiota is an important component of the immunopathogenesis of food allergy.

1.2 Diagnosis of Food Allergy

The gold standard for making a diagnosis of food allergy is the oral food challenge (OFC), preferably conducted in a double-blind, placebo-controlled fashion (Bird et al. 2016; Nowak-Węgrzyn et al. 2009). However, because of the difficulty of carrying out this procedure in routine clinical practice and because of the small but real risk that is associated with it, other approaches are usually used. A clear history of typical symptoms following the ingestion of specific foods is very helpful in making

the diagnosis. It can also aid in identifying the relevant allergen and can provide a framework for further investigation. Once clinical suspicion is raised by the history, two of the most commonly used methods for confirming food allergy are the skin prick test (SPT) and measurement of food-specific IgE in the serum. In the SPT, the skin surface is pricked with various candidate food allergens. A wheal and flare response of a specific size developing within 15 min is indicative of the presence of cutaneous mast cells with bound IgE specific for the allergen. The detection of serum IgE reactive with whole food extracts is a sensitive indicator of possible food allergy but has relatively low specificity. Measurement of circulating IgE to specific food protein antigens (known as component-resolved diagnosis) improves the predictive value of the assay, and commercial tests using this approach are now available for over 100 different allergens. Finally, a recently developed method for diagnosing food allergy is the basophil activation test (BAT), which measures the *in vitro* degranulation of basophils in response to crude allergen extracts or purified antigens. The BAT has high sensitivity and specificity for various food allergies but is currently being used mainly for research purposes.

1.3 Management of Food Allergy

The mainstays of food allergy management are avoidance of the relevant food and prompt treatment of allergic manifestations (Sicherer and Sampson 2017). Strict avoidance can be difficult, especially if multiple allergens are involved, and requires the cooperation and compliance of the patient, as well as parents and other caregivers where applicable. In some cases the avoidance does not have to be absolute. For example, many children who are allergic to milk and egg are able to tolerate these components if they are incorporated into baked goods that are subjected to extensive heating (Leonard et al. 2015). OFCs can be helpful in determining the kinds and amounts of foods that can be tolerated and the degree to which avoidance has to be implemented. Children on a strict avoidance diet should be evaluated regularly to ensure that the diet has not adversely affected their growth or nutritional status and may also benefit from counseling and supportive services to help them cope with social problems (Boyce et al. 2011).

Mild allergic symptoms such as urticaria usually resolve spontaneously or can be treated with antihistamines. More severe allergic reactions to food, such as bronchoconstriction, laryngeal edema, and anaphylaxis, require the immediate use of injected epinephrine in order to counteract the potentially life-threatening effects of released mast cell mediators on the vasculature and airways (Commins 2017; Farbman and Michelson 2016; Lebowhl et al. 2017). Individuals with a history of food allergy should be provided with an auto-injection device for emergency self-administration of epinephrine along with detailed instructions on its use (Lebowhl et al. 2017).

In addition to avoidance and emergency epinephrine treatment, food allergy manifestations can be modified by using immunotherapy. Although current forms of

immunotherapy rarely, if ever, lead to a state of permanent tolerance that allows intake of normal amounts of the offending food, they do offer the possibility of altering the immune response to the point that small amounts of the relevant food component can be consumed safely. Thus, they can provide some relief from the burden of strict avoidance and can reduce the potentially dangerous consequences of accidental ingestion. The strategy behind most forms of immunotherapy is the administration of gradually increasing amounts of the allergen under very carefully monitored and supervised conditions (Burks et al. 2018). In the most common form of immunotherapy—oral immunotherapy (OIT)—this escalation of allergen consumption generally starts with a dose of 10–25 mg per day, which is then slowly increased, usually once a week over the course of 3–9 months, to a daily maintenance level of 500–4000 mg. The maintenance dose is then continued for months to years and allows a significant proportion of treated individuals (50–90% in various studies) to become sufficiently desensitized that they can consume 5–6 g of the allergen (the equivalent of about 20 peanuts) in OFCs without clinical problems. A smaller proportion of individuals achieve sustained unresponsiveness to the allergen, i.e., lack of clinical reactivity in an OFC after therapy has been discontinued. Some trials of OIT have incorporated the use of an anti-IgE monoclonal antibody (omalizumab) as a way of reducing side effects and allowing a more rapid escalation of allergen intake. This approach has been successful in achieving desensitization in a few weeks rather than the many months required for conventional OIT (Bégin et al. 2014; Macginnitie et al. 2017; Nadeau et al. 2011; Schneider et al. 2013). Other forms of immunotherapy have used different routes of administration of the allergen. In sublingual immunotherapy (SLIT), the allergen is applied under the tongue for 2–3 min before being swallowed, while epicutaneous immunotherapy (EPIT) makes use of allergen-impregnated patches that are applied to the skin. SLIT and EPIT require relatively low doses of allergen and have a lower incidence of side effects, but also a lower success rate, than OIT (Burks et al. 2018). The exact mechanisms involved in the beneficial effects of OIT, SLIT, and EPIT are not clearly understood. Various studies of these therapies have demonstrated decreased allergen-specific IgE levels, increases in allergen-specific IgG4 and regulatory T cells, or attenuated basophil activation (Burks et al. 2018).

Since existing forms of immunotherapy have limitations with respect to success rate, side effects, acceptability, and cost, a number of additional strategies for the treatment of food allergy are being evaluated. One approach involves the administration of the food allergen in combination with adjuvants that modify the allergen-specific Th2 response. Several such adjuvants are under investigation, including interferon- γ , Toll-like receptor ligands, and probiotic bacteria (Loh and Tang 2018). Another approach that has shown some promise, mainly in animal models but also in small human studies, is the use of herbal remedies, including formulations from Chinese traditional medicine (Li 2018). Data on all these treatment modalities are currently insufficient to draw firm conclusions about their efficacy. Further study will be required before they can be considered for addition to the therapeutic armamentarium.

1.4 Conclusion

Although we now have a reasonable conceptual understanding of the immunopathogenesis of food allergy, much remains to be learned about the mechanisms that are involved. In particular, it will be important to clarify in cellular and molecular detail how the normal process of oral tolerance is altered by genetic and environmental factors so as to produce a food-specific Th2 response. That level of fine-grained insight will provide the foundation for a whole new generation of strategies to prevent and treat food allergy.

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Chapter 2

Species and Structure of Food Allergens: Epitopes and Cross-Reactivity



Abstract In recent years, with the improvement of food production and industrialization as well as the development of transgenic technology and the popularization of genetically modified food, the incidence of food allergic diseases is on the rise and has become a common chronic disease that endangers people's physical and mental health, especially those of children's. The World Health Organization (WHO) has listed food allergy as the sixth problem affecting people's health, and the research on food allergen has become one of the public issues of global concern. This chapter will discuss multiple allergens in the "big eight" categories of food allergens. Biochemical characteristics associated with food allergens like the presence of multiple, linear IgE-binding epitopes seem to predominate among food allergens, more so than common structural features. Here, the recent studies on the identification of structure and potential epitopes of food allergens are also presented. Due to the high sequence homology and structural similarity of food allergens in different species, especially the major allergens, cross-reactivity is common; therefore a comprehensive introduction to the cross-reactivity among different origins of allergens is also reviewed.

Food allergies are immunoglobulin E (IgE)-mediated immunological reactions, which differ from other types of food sensitivities (food intolerance or toxic food reactions), and estimated to affect about 2% of the adult population in industrialized countries and about 6–8% of infants and children (Mills et al. 2007; Poms et al. 2004). To date, over 180 allergenic food proteins have been identified, yet more than 90% of the recognized food allergens belong to eight food categories, including milk, egg, fish, crustaceans, peanut, soybean, wheat, and tree nuts (Poms et al. 2004). In sensitive individuals, intake of trace amounts of food allergens can cause severe and deadly reactions, such as digestive disorders (emesis, diarrhea), respiratory symptoms (rhinitis, asthma), circulatory symptoms (edema, hypotension), skin reactions (urticaria, atopic dermatitis/eczema), and anaphylactic shock. Thirty microgram hazelnut can provoke an allergic reaction, and the calculated threshold values for the allergenic protein in milk, egg, peanut, and soy were 0.07 µg, 0.003 µg, 0.5 µg, and 0.3 mg, respectively (Pedrosa et al. 2015).

Epitope also known as antigenic determinant refers to a specific chemical group in an antigen molecule that determines antigen specificity. Epitope is the basic structural unit of T-cell antigen receptor (TCR), B-cell antigen receptor (BCR), or antibody-specific binding and usually consists of 5–17 amino acid residues or 5–7 polysaccharide residues or nucleotides. Epitopes can be divided into B-cell epitopes and T-cell epitopes according to their binding to cells and can be divided into linear epitopes and conformational epitopes according to their structural characteristics. Linear epitopes are composed of continuous amino acids, conformational epitopes are composed of amino acids of different segments, and the amino acids of these different segments are structurally close to each other and finally form a conformational epitope due to the protein folding. Generally, linear epitopes are mainly present in T-cell epitopes and partial B-cell epitopes, conformational epitopes are only present in B-cell epitopes, and 90% of B-cell epitopes belong to conformational epitopes (Hopp and Woods 1981). Epitope is the basis of the antigen-antibody reaction. In a food allergic reaction, the allergen protein does not act through its intact molecule but directly reacts with the antibody via an allergen epitope. Therefore, the first task of researching allergic foods is to study the epitopes of allergens.

Cross-reactions originate from two antigens of different origins, which can have the same antigenic epitope with each other, whereby the epitopes stimulate the production of antibodies that can not only bind to their own epitopes but also to the same epitope of another antigen. The formation of cross-reaction phenomena may be related to the following factors: (1) the allergen is a common protein, which have the same antigenic structure in different organisms; (2) the allergens contain a common allergenic epitope that presents in different organisms; (3) the allergens contain a similar partial spatial conformation that can be matched with the complementarity-determining region of the same antibody.

Food allergens are usually stable molecules that resist to processing, cooking, and digestion and are capable of stimulating immune responses. Besides, food can be contaminated with foreign ingredients containing allergenic components during shipping, storage, and processing. Therefore, it's sometimes difficult for sensitive individuals to totally avoid allergenic proteins (Huggett and Hischenhuber 1998). On the basis of these, a knowledge of the species and structures of food allergens are necessary and essential not only for the allergenic protein detection but also for the improvement of safety measures for allergenic individuals.

2.1 Plant Food Allergens and Corresponding Epitopes

In the past few decades, structural features of hundreds of plant-derived and animal-derived food allergens have been identified, which mainly fall into a few protein families. According to the Pfam protein database, all plant-derived food allergens fall into 31 of 8296 protein families (Radauer and Breiteneder 2007). Among them, 65% belong to the prolamin superfamily, the cupin superfamily, the Bet v 1 superfamily, and the profilin family (Table 2.1).

Table 2.1 The main plant food allergens

Allergen name	Allergen source	Protein family	References
Ara h 1	Peanut	Cupin	Mills et al. (2004)
Ara h 3	Peanut		
Jur r 2	Walnut		
Ses i 1	Sesame		
Ber e 2	Brazil nut		
Fag e 1	Buckwheat		
Caj c 1	Red gram		Misra et al. (2010)
Caj c 2	Red gram		
Caj c 3	Red gram		
Caj c 4	Red gram		
Caj c 5	Red gram		
Vig r 2	Green gram		Misra et al. (2011)
Vig r 3	Green gram		
Vig r 4	Green gram		
Vig r 5	Green gram		
Pru p 3	Peach	Prolamin	Egger et al. (2010)
Mal d 3	Apple		
Pru ar 3	Apricot		
Pru d 3	Apricot		Javier and Alfonso (2008)
Sin a 1	Yellow mustard		
Ber e 1	Brazilian walnut		
Jug r 1	British walnut		
Ara h 2	Peanut		
Ara h 6	Peanut		
Pyr c 4	Pear	Profilin	Asero et al. (2003)
Pru av 4	Cherry		
Pru p 4	Peach		
Mal d 4	Celery		Lópeztorrejón et al. (2010) and Ma et al. (2010)
Cuc m 2	Celery		

2.1.1 The Cupin Superfamily

Cupin is derived from the Latin “cupa,” which refers to a barrel structure formed by at least six β -folded sheets. Cupin is a family of multifunctional proteins widely distributed in fungi, higher plants, and animals, with at least six β -barrel structures (Dunwell 1998). Cupin superfamily can be divided into different cupin subgroups based on whether the proteins comprise a single cupin domain or a duplicated (bicupin) or multicupin (>2 cupin domains) structure (Dunwell et al. 2004). A minimum of 18 different functional subgroups have been estimated according to their enzymatic and nonenzymatic functions. The majority of cupin proteins are comprised of monocupins or single-domain cupins which are either monomeric,

dimeric, or oligomeric. Monocupins mostly comprise enzymes, such as dioxygenases and phosphomannose isomerases (Dunwell et al. 2004). Auxin-binding proteins (ABPs), a family of dimeric monocupins from plants, are involved in a variety of plant growth responses through interacting with the plant hormone auxin (Woo et al. 2014). Germins and germin-like proteins (GLPs) as oligomeric monocupins are the largest family of cupins in plant. Germin is a hydrogen peroxide-generating oxalate oxidase with extreme thermal stability; it is responsible for defending against biotic and abiotic stress in plants. Structural analysis revealed that the germin protein is a disc-shaped homohexamer (a trimer of dimers) containing manganese (germin is a manganese-containing homohexamer with oxalate oxidase and superoxide dismutase activities). The structure of ABP1 from corn is similar to that of germin, and they share 24% sequence identity. Besides, ABPs display considerable sequence conservation across a wide range of plants, including apple and strawberry (Woo et al. 2014).

Proteins with two cupin domains originally found in higher plant seed storage proteins comprise 7S globulin trimer (vicilin) and 11S globulin hexamer (legumin), and these proteins are thermostable and have nutritional value to human beings. Although 7S and 11S globulins only have about 35–45% identity in sequence, they share high similarity of structure (Radauer and Breiteneder 2007). 7S globulin trimer has a molecular weight of about 150–190 kDa, and peanut allergen Ara h 1, walnut allergen Jur r 2, and sesame allergen Ses i 1 all belong to this protein; peanut allergen Ara h 3, Brazil nuts Ber e 2, and buckwheat allergen Fag e1 belong to 11S globulin (Mills et al. 2004). Recently, five allergens Caj c 1, Caj c 2, Caj c 3, Caj c 4, and Caj c 5 found in red gram were also classified to the cupin superfamily (Misra et al. 2010). Caj c 1 has a high sequence similarity with β -conglycinin α chain of soybean, while Caj c 2, Caj c 3, Caj c 4, and Caj c 5 showed high homology with β -conglycinin α prime subunit of soybean. In addition, all these proteins showed homology to known allergens lentil (Len c 1 and Len c 2), peanut (Ara h 1), and pea (vicilin) (Misra et al. 2010). Potential allergens Vig r 2, Vig r 3, Vig r 4, and Vig r 5 identified as members of cupin superfamily and seed albumin in green gram displayed significant sequence similarity with known allergens of soybean, lentil, pea, lupin, etc. (Misra et al. 2011).

2.1.2 The Prolamin Superfamily

Prolamins were initially named on the basis of their solubility in alcohol/water mixtures and their high content of proline and glutamine. The prolamin superfamily mainly includes three groups of plant-derived food allergen proteins, namely, non-specific lipid transfer proteins (nsLTPs), α -amylase/trypsin inhibitors, and 2S albumin. This superfamily is characterized by little sequence homology to each other, the presence of a conserved skeleton of eight cysteine residues, highly similar α -helical structure linked by disulfide bonds, and stability to thermal processing and proteolysis.

nsLTPs are a class of small molecule soluble proteins, which account for 4% of all soluble proteins in higher plants (Liu et al. 2015). nsLTPs are the major food allergens present in Rosaceae fruits, such as peach (Pru p 3), apple (Mal d 3), apricot (Pru ar 3) and (Pru d 3), etc. (Egger et al. 2010). nsLTPs are also widely present in other plant-derived foods, such as vegetables, grains, and nuts, and these allergens have high homology. According to the molecular weight, the plant nsLTPs can be divided into two categories, nsLTP1 and nsLTP2, with the molecular weight of 9 kDa and 7 kDa, respectively. These proteins can promote phospholipid transportation between cell membranes. nsLTPs have good stability, they can be stored at 4 °C for a long time, their structure will not change at 100 °C, also they have high stability for enzyme digestion, and their immunogenicity and sensitization can be remained even after digestion in gastrointestinal tract. nsLTPs are characterized of compact structure formed by four α -helices with internal four disulfide bonds for the stability of spatial structure. Patients allergic to nsLTPs are often provoked by specific IgE-mediated allergic reactions and produce severe allergic symptoms. Therefore, nsLTPs-specific IgE is considered a hazard and is currently being explored as a diagnostic marker for allergic reactions. Recently, it was reported that damage in the structure of Pru p 3 significantly reduced the stability for digestion, antigenicity, and sensitization and that the structurally unfolded Pru p 3 had lower allergenicity, which may be a candidate vaccine for the treatment of peach-allergic patients (Toda et al. 2011).

α -Amylase/trypsin inhibitors are mainly found in cereals and can cause allergies through the respiratory tract, therefore triggering occupational allergies such as baker's asthma (wheat, barley, and rye) or food allergies through the gastrointestinal tract (wheat, barley, and rice). α -Amylase inhibitory protein is the most important allergen in rice grains with a molecular weight of about 16 kDa (Nakase et al. 1996). The allergens of corn and beer (origin from barley) also belong to the α -amylase inhibitory protein family (Curioni et al. 2010; Pastorello et al. 2003). Trypsin inhibitors widely exist in legumes and grains and are mainly distributed in the seeds of these crops, especially in soybeans and mung beans.

2S albumin is present in many monocotyledonous and dicotyledonous species and has a certain protective effect on plants against attack of fungi. In recent years, some members of this protein family have been identified as major food allergens with the ability of highly binding to IgE in the serum of allergic patients (Moreno and Clemente 2008), such as yellow mustard Sin a 1, Brazilian and British walnut Ber e 1 and Jug r 1, and peanuts Ara h 2 and Ara h 6.

2.1.3 *The Bet v 1 Superfamily*

The Bet v 1 superfamily is found throughout the plants and composed of eight sub-families with approximately 154–160 amino acid residues (Radauer et al. 2008). This superfamily has low levels of sequence similarity between each other and includes the pathogenesis-related protein family 10 (PR-10), the family of major

latex proteins and ripening-related proteins, the norcoclaurine synthases, and cytokinin-binding proteins from legumes. The birch pollen allergen Bet v 1 is a representative member of this family, which accounts for 90% of birch pollen allergy (Jarolim et al. 2010).

PR-10 protein family is the major birch pollen allergen Bet v 1 (Van et al. 2006), and ubiquitously distributed among dicotyledonous plants (Wen et al. 1997), and also present in monocots (Wang et al. 1999) and gymnosperms (Yu et al. 2000). PR-10 proteins are members of small gene families characterized by differentially expressed isoforms. Thirteen gene loci were detected in birch, seven of them were encoded proteins which were specifically expressed in pollen (Schenk et al. 2006). The regulation of PR-10 protein expression can be divided into two types: stress-induced and developmentally regulated. The stress-induced PR-10 protein expression is triggered by pathogen infection (Swoboda et al. 2010), plant hormones (Wang et al. 1999), wounding (Liu et al. 2005), salt stress (Jain et al. 2006), or cold (Yu et al. 2000). Developmentally regulated PR-10 proteins are highly expressed in pollen, seeds, fruits, or storage organs and identified as allergens cross-reactive with Bet v 1 (Vieths et al. 2010). The major latex protein/ripening-related protein family comprises latex proteins expressed in laticiferous plants, such as opium poppy and plantain (Nessler et al. 1985), and proteins from ripening fruits, including wild strawberry (Nam et al. 1999) and soybean (Strömvik et al. 1999). (S)-Norcoclaurine synthases cloned from meadow rue (*Thalictrum flavum*) and poppy catalyze the first committed step in the biosynthesis of benzylisoquinoline alkaloids (e.g., morphine) (Gammon et al. 2010; Liscombe et al. 2005). Cytokinin-binding proteins forming a distinct family from PR-10 and (S)-norcoclaurine synthases were found in the legumes, mung bean, and lupine (Pasternak et al. 2006).

Bet v 1 homologous proteins are expressed in birch pollen, mature fruits (apples, pears, cherries, and other members of Rosaceae), plant roots (carrots, etc.), plant bulbs, celery, and so on. In addition, the expression of Bet v 1 homologous protein may be upregulated in plants that are infected by pathogens or grow in stress. Meanwhile, Bet v 1 homologous protein is also a potential inhaled allergen, and most patients who are initially allergic to pollen often become allergic to Bet v 1 homologous proteins in fruits (apples, pears, cherries, kiwis, jackfruit), nuts (hazel-nuts), and vegetables (carrots, celery, and coriander) (Geroldinger-Simic et al. 2011), but the allergic symptoms are usually mild and limited to the oral cavity and are summarized as oral allergic syndrome.

2.1.4 The Profilins

All prolamins are allergenic; Rasanen et al. studied wheat allergy and found that more than half of children's sera produced IgE that can specifically bind to prolamin, indicating prolamin is the main allergic protein of wheat. However, its sensitization mechanism is still unclear. The profilins are present in all eukaryotic cells and are cytosolic proteins. As a binding protein of actin (Kreis et al. 1985), it may play a key role in regulating intracellular transport, cell morphology, and division. The

genes encoding the profilins form a gene family (Maruyama et al. 1998) and thus show high sequence homology with each other (more than 75% of the sequence similarity), with conserved sequence length around 14 kDa. Although the sequence similarity between profilins from vertebrate and other organisms is low, 3D structures of all profilins are significantly similar, which are all compact globular mixed α/β structures. Due to their largely conserved sequence, they constitute a family of highly cross-reactive allergens in monocot and dicot pollens, plant foods, and *Hevea* latex (Radauer and Breiteneder 2007). A large number of profilin genes have been cloned from different plants, such as wheat, kidney bean, tomato, and pasture, and their immunological properties have also been studied. In 1991, the secondary allergen of birch Bet v 2 was identified as a profilin, attracting the broad attention among allergy researchers. The profilins as allergens are mainly present in plant pollen, fruits, and vegetables, such as pear Pyr c 4, cherry Pru av 4, peach Pru p 4, and the like. It is generally estimated that 10–20% of patients allergic to birch pollen show the allergy symptoms of profilins; therefore profilin allergy is considered a potential risk of food allergy associated with pollen. Asero et al. (2003) found a correlation between profilin sensitization and clinical allergies caused by melon, citrus fruits, bananas, and tomatoes by studying a group of patients allergic to fruits and vegetables. The sequential and structural similarities of profilins between various plants can easily lead to an IgE-mediated allergic cross-reaction. The in vitro experiments of heating, radiation, and ultrahigh pressure treatment to celery profilins Mal d 4 and Cuc m 2 had no effect on the binding activity of IgE. However, with the low pH and digestive ability in gastric juice, the profilins were easily degraded and showed no further allergic activity (Lópeztorrejón et al. 2010; Ma et al. 2010).

2.1.5 Other Plant-Derived Allergen Families

Other plant-derived allergens belong to a family of less-distributed allergens. Oleosins are believed to be protein components that contribute to stabilize the plant storage structures and determined to result in the allergy caused by beans, nuts, and seeds. On the other hand, when a plant is infected by a pathogen, the number of related proteins will increase during the pathogenesis. Surprisingly, a significant number of food allergens are derived from a variety of protein families associated with pathogenesis, such as various chitinases, β -1,3-glucanases, and thaumatin-like proteins (Hoffmannsommergruber 2000). Chitinase mainly distributes in Brazilian rubber latex, avocado Pers a 1, chestnut Cas s 5, and grape Vit v 5 (Alenius et al. 1996), whose activity is determined by the catalytic domain formed by 220–230 amino acid residues (Mauch and Staehelin 1989). Another type of plant defense protein is composed of β -1,3-glucanase. These glycosyl hydrolases have an ($\alpha\beta$) 8 barrel structure with a size of 25–35 kDa. Their main function is to catalyze the hydrolysis of the 1,3- β -D-glycosidic bond in β -1,3-glucan and therefore is considered to play a role in the prevention of phytopathogenic microorganisms. They also act on the physiological and developmental processes of uninfected plants, such as pollen formation and pollen tube growth, nutrition, fruit ripening, and seed

germination. In addition, they can respond to radiation, cold, ozone, and ultraviolet radiation. Allergens of this protein family are found in avocados, bananas, chestnuts, figs, and kiwi (Blanco et al. 1994), inhaled allergens such as olive pollen (Untersmayr et al. 2006), and latex (Yagami et al. 2002). Thaumatin-like proteins are a class of antifungal proteins that accumulate in harsh conditions and can enhance the ability of plants to resist freezing and drought stress, but their full functions are still unclear. The thaumatin-like protein has a molecular weight of about 20 kDa and forms a structure of antiparallel β -sheet which is further stabilized by eight disulfide bonds (Leone et al. 2006). Due to the rigid structure formed by disulfide bonds, they can resist heat treatment and protein degradation (Breiteneder 2004). Allergens of the disease-related protein family have been found in a variety of fruits, such as cherries (Pru av 2), apples (Mal d 2), kiwis (Ac d 2-green kiwi, Act c 2-golden kiwi), orange, grapes, and bell peppers (Cup a 1) (Breiteneder 2004).

2.1.6 Epitopes in Plant Food Allergens

Peanut allergens have become a major health concern for the public. Most of the IgE-binding epitopes of 7S globulin trimer that have been identified or predicted have similar amino acid sequences, and these epitope amino acids are well exposed on the surface of the pea globulin trimeric homologue, and most of them belong to positively charged region rich in arginine and lysine. Some epitopes in Ara h 1, such as epitopes 11 (SYLQGFSSNT), 15 (NNFGKLFEVK), 18 (ELHLLGFGIN), 19 (HRIFLAGDKD), and 20 (IDQIEKQAKD), show a very similar three-dimensional conformation to other tree nuts and strong IgE-binding activity (Barre et al. 2008). The amino acids in these epitopes were classified, and results indicated that hydrophobic amino acids accounted for 32%, the neutral amino acid accounted for only 10%, and the hydrophilic amino acid accounted for 58%.

By analyzing the immunological activity of the overlapping peptides of Ara h 3, eight highly active IgE-binding epitopes were revealed, and they are ALSRLVLRNALRRP (1), EPAQQGRRHQSQ2RPP (2), LRYQQQSRRRSLPY SPYS (3), SPRGQH2GRR (4), RILSPDRKR (5), DRRRGRGSR (6), ICTASFKKNIGRNRSPDIYNP (7), and PREQAR2QLKNNNPFKFFVPPSEQS (8). Most of these epitopes (taking up about 23% of the whole amino acid sequence) contain positively charged amino acid residues (K, R), which are located on the both sides of surfaces of Ara h 3 monomer and dimer molecules (Rougé et al. 2009). Similarly, statistical analyses were carried out for these amino acids in the eight epitopes, and results showed that hydrophobic amino acids occupied only 14%, neutral amino acids occupied 9%, and the hydrophilic amino acids occupied as high as 77%.

Allergens that are very similar to peanuts also appear in tree nuts. Jason et al. (Robotham et al. 2009) detected the IgE-binding ability of the 62 overlapping peptides of the walnut allergen (Jug r 4) using the serum of 17 walnut allergy sufferers and found that 4 epitope fragments exhibited strong binding ability with IgE, 7 fragments showed mild binding ability, and 12 fragments had weak binding ability. The

four strong binding epitopes are VFSGFDADFLADAFN, QSENDHRRSIVRVEG, WSREEQEREERKERE, and VVKRARNEGFEWVSF. Classification of the four epitopes of walnut allergens was carried out by statistical methods. It was found that the hydrophobic amino acids accounted for 27%, the neutral amino acids were 8%, and the hydrophilic amino acids reached 65%.

Later, the linear IgE-binding epitopes of the scorpion allergen (Cor a 9) were identified using the serum of 12 scorpion allergic patients. Among the 63 overlapping peptide fragments synthesized, 3 epitope fragments have strong binding activity to serum IgE, and they are NPDDQHQRQGGQQQFG, FSGFDAEFLADAFNV, and QVVRPERSRQEWERQ, 2 epitope fragments reacted mildly, and 20 epitopes showed weak or no binding activity. Similarly, the amino acid analysis of the three epitopes with the strongest binding activity of the scorpion allergens revealed that the hydrophobic amino acids accounted for 22%, the neutral accounted for 9%, and the hydrophilic amino acids accounted for as high as 69% (Robotham et al. 2009).

With the extensive use of buckwheat, there are more and more reports on the allergy of buckwheat, and many people suffer from allergic symptoms such as asthma, skin disease, and shock after eating buckwheat. At present, research on buckwheat allergy and its sensitization mechanism has received worldwide attention. As major allergens in common buckwheat, the epitopes of Fag e 1 and Fag 13S were studied. To determine the epitope region in Fag e 1 that can specifically recognize IgE, scientists synthesized 90 overlapping peptides and detected their immunological activity with serum from patients with buckwheat allergy. Key regions responsible for IgE binding were identified by replacing different amino acids in the epitopes with alanine (Ala), and some epitopes lost IgE-binding activity when the initial amino acids were substituted by Ala or methionine (Met), indicating that these amino acids play a major role in the binding of allergen epitopes to IgE and were considered as key amino acids in the epitope. The amino acid analysis on eight epitopes of the buckwheat allergen Fag e 1 revealed that hydrophobic amino acids occupied 26%, neutral amino acids occupied 13%, and hydrophilic amino acids occupied 61%.

Eleven consecutive IgE epitope synthetic peptides of Fag 13S were identified by dot blot, including positive and negative charge residues. These 11 epitopes are SSTMRARQCRLDQLTSSQ (1), KIRSSEGGTIEVWDEE (2), MRVTVPDSL-SLPSYYSSPRLV (3), DAHQPTRRVKGDVVALP (4), QGGSKEGKSQKLNS (5), ESDERGPIVKARKNMRQMVT (6), NMRFRHNLGPRTEADIASRQAGRIH SVD (7), NAMLAPAWPLSGHRVFYVLRGEAQRQI (8), PQFYISTCRAGR (9), HASVFKGMPIPVLSNSYQISPR (10), and QTRSHEHGLFSPFGGRS (11). Epitopes 1, 3, 4, 7, 10, and 11 have strong binding activity with IgE of patient serum, while epitopes 2, 5, 8, and 9 have weak interactions with patient serum. These epitopes are located in the exposed area of the molecule except for epitope 8, which is located inside the molecule. In total, 4 of the 11 epitopes (epitopes 2, 3, 8, 9) are located in the core structural region of the Fag 13S, and 5 (epitopes 1, 5, 6, 10, 11) are scattered throughout the allergy, and the remaining 2 (epitopes 4, 7) overlap in the core area and the additional core area. The surface-exposed amino acids of 11 consecutive IgE linear epitopes were analyzed, and results showed that

hydrophobic amino acids account for 22%, neutral amino acids account for only 5%, and hydrophilic amino acids account for as high as 73%.

Similarly, the epitopes of the allergen in tartary buckwheat with a molecular weight of 24 kDa (TBa) were predicted, and the full length of TBa (195 amino acid) sequence was divided into 6 epitope segments. After analysis, E1 (a fragment of TBa sequence) was identified as one of the major epitopes in this allergy because it has the strongest binding activity with the serum of buckwheat allergy sufferers. On the basis of these data, the sequence of E1 was compared with other plant seed storage proteins of high homology using DNASTar software. The results showed that leucine (Leu) at the sites 39, 42, 47, and 54 was highly conserved. Then, E1 was subjected to site-directed mutagenesis, and the Leu at the sites 39, 42, 47, and 54 and valine (Val) at the site of 52 were replaced with arginine (Arg) and named L39R, L42R, L47R, V52R, and L54R, respectively. ELISA and dot blot were used to detect the IgE-binding activity of recombinant protein to serum of allergic patients and healthy volunteers, and results showed that the immunoreactivity of the five mutants was reduced, and the activities of L42R, L47R, and L54R were reduced by about 50%, indicating that Leu at the site of 42, 47, and 54 in the TBa molecule play important roles in its allergenicity (Ren et al. 2010).

Tanabe (2004) found two epitopes in wheat prolamin, QQFPF and PQQPF, which can bind to serum IgE. According to the binding activity to serum IgE of wheat allergy sufferers, Battais (Battais et al. 2015) identified epitopes QQLVPQ and QQSFPQ in γ -gliadin and epitopes QQPIQQ and QQPFPQQ in ω 2-gliadin. Similarly, Matsuo (Matsuo et al. 2004) identified QQXPQQ and QQSPEQQ in ω 5-gliadin.

Ganglberger et al. (Cai et al. 2010) screened allergen epitope mimic peptides of the main allergen Bet v 1 of birch pollen from phage peptide libraries through different schemes and further studied their therapeutic value in allergies. After purification of Bet v1-specific IgE from the serum of allergic patients, human IgE mimotope (Bet mim E, CQQFLSVRALC) was obtained through panning of phage peptide library (pVIII 9aa Cys). Comparing with Bet v 1 primary structure and computer-modeled three-dimensional conformation, it is speculated that Bet mim E may mimic the surface region of the Bet v 1 molecule (located at the site of 9–22 and 104–123, respectively), which are IgE-binding regions.

In conclusion, epitopes are often in areas that are highly exposed to solvents and contain hydrophilic side chains that facilitate binding to B-cell antigen receptors or antibodies; besides, epitopes usually have different degrees of flexibility because the binding of antigen to receptor or antibody is a chimeric process.

2.2 Animal Food Allergens

Animal food allergens have a lower diversity than plant food allergens. They can be classified into three major families: calcium-binding proteins (parvalbumin), tropomyosins (crustaceans and mollusks), and caseins; besides, there are also many

emerging families of only a few reported allergens (Jenkins et al. 2007). It seems that their ability to act as allergens is related to their closeness to human homologues. Interestingly, proteins with a sequence identity of 54% to human homologues are probably allergenic, but those with a sequence identity more than 63% to human homologues were rarely allergenic, which indicates the relationship between the similarity with human homologues and the ability to amount an immune response. Therefore, it has been argued that a low degree of similarity to a host's proteome is indispensable in immunogenicity (Kanduc et al. 2001). The most important animal food allergens are distributed in seafood, milk, and egg (Table 2.2).

2.2.1 Calcium-Binding Proteins

Calcium-binding proteins are characterized by the possession of the EF-hand domain, a calcium-binding loop composed of 12 amino acid residues and another 12 amino acids of 2 adjacent α -helices forming a conserved domain, participating in intercellular signaling transmission or transportation. In vertebrates, this protein is largely expressed in muscle tissue due to its ability of loosening up muscles by binding to Ca^{2+} . A representative allergen of calcium ion-binding protein is parvalbumin with a molecular weight of about 12 kDa, which was first found in squid in 1971 by

Table 2.2 The main animal food allergens

Allergen name	Allergen source	MM (KDa)	References
Tropomyosin	Seafood	31.7	Jeong et al. (2006)
Parvalbumin		12	Cai et al. (2010)
Arginine kinase		40	Mao et al. 2013
Sarcoplasmic calcium-binding protein		20	Ayuso and Grishina Gibanez (2009)
Myosin light chain		18–20	Ayuso et al. (2008) Bauermeister et al. (2011)
Troponin C		17	Chen et al. (2013) Abdel Rahman et al. (2013)
Triosephosphate isomerase		28	Knobeloch et al. (2010)
Hemocyanin		60–80	Piboonpocanun et al. (2011)
Paramyosin		100	Suzuki et al. (2011)
Enolase		47	Liu et al. (2011)
Creatine kinase		41	Liu et al. (2011)
β -Lactoglobulin	Milk	18.3	Chapman and Wood (2001)
α -Lactalbumin		14.1	Osuga and Feeney (1968)
Ovomucoid	Egg	28	Yamashita et al. (1982)
Ovalbumin		44	Perlmann (1950)
Ovotransferrin		78	Aisen et al. (1966)
Lysozyme C		14	Amo et al. (2010)

Elsayed et al., and is widely present in various fish species (Cai et al. 2010; Tyrer et al. 2002). The parvalbumin of salmon and trout is a typically α -helix structural protein, which can maintain the stability even when heated to 95 °C. However, the parvalbumin is unstable under acidic conditions; therefore, the allergic activity of the main fish allergen can be degraded by the gastric enzyme. However these allergens can be preserved in the case of impaired physiological digestion conditions, thereby increasing the risk of allergies (Untersmayr et al. 2005). Although parvalbumin is the main allergen of fish and cross-reacts with the parvalbumin derived from amphibians, but so far, parvalbumin of higher vertebrates has not been found to cause allergy.

Parvalbumin is generally divided into two types, α -parvalbumin and β -parvalbumin; the difference is that α -parvalbumin has characteristic Lys-Ala sequence and β -parvalbumin has Ala-Ala at the corresponding position in substitution. Besides, α -parvalbumin has a higher isoelectric point than β -parvalbumin but has a weaker affinity to Ca^{2+} than β -parvalbumin. It was reported that most fish muscles contain only one type of parvalbumin. However, two parvalbumin subtypes belonging to α -parvalbumin were separated and purified from *Dasyatis akajei* with isoelectric points of 5.4 and 5.0, respectively (Cai et al. 2010). And three parvalbumin subtypes belonging to β -parvalbumin were purified from *Hypophthalmichthys molitrix*, and it was found that the ability of different subtypes to form multimers after heat treatment was significantly different and closely related to their sensitizations.

2.2.2 Tropomyosin

Tropomyosin presents in all eukaryotic cells and is a highly conserved α -helix protein. Two identical α -helix structure subunits are intertwined to form a supercoiled structure containing seven interacting actin-binding sites, promoting the interaction between troponin and actin in striated muscle cells to regulate muscle contraction (Brown and Cohen 2005). Tropomyosin food allergens are mainly distributed in crustaceans, mollusks, fish parasites, and inhaled allergens (Jeong et al. 2006). In the 1980s, Hoffman et al. identified a heat-stable IgE-reactive allergen in shrimps, which was later identified in the brown shrimps as tropomyosin by Lehrer and colleagues (Daul et al. 1994; Leung et al. 1994; Yu et al. 2003). Shrimp tropomyosin, which has an acidic isoelectric point, is the major heat-stable shrimp allergen and has a highly conserved amino acid sequence among various invertebrate organisms and is present in muscle and non-muscle cells (Hoffman et al. 1981). So far, only invertebral tropomyosin produces an allergic reaction with a high degree of cross-reactivity. The main food allergens represented by tropomyosin only cause mild inhalation allergic reactions in most cases. They retain their ability to bind to IgE even after heat treatment or enzymatic digestion due to structural characteristics.

2.2.3 Caseins

Casein is widely present in mammalian emulsions and shows a random helical structure. The casein fraction constitutes up to 80% of the total protein in milk. Casein is mainly composed of α 1-casein, β -casein, and a small amount of α s2-casein and κ -casein. Besides, three γ -caseins are derived from the hydrolysis of β -casein. γ 1, γ 2, and γ 3 represent, respectively, the sequences 29–209, 106–209, and 108–209 of β -casein. In contrast, casein of goat milk is mainly composed of β -casein with a small amount of α 1-casein, κ -casein, and α s2-casein. In addition, it has been reported that there is an IgE cross-reaction between milk casein and goat and sheep casein, while horse milk casein has a low IgE cross-reaction (Spuergin et al. 1997).

2.2.4 Other Animal Food Allergens

2.2.4.1 Allergens in Seafood

Arginine kinase is also an important crustacean allergen with a molecular weight of approximately 40 kDa and an isoelectric point of approximately 6.0–6.5. It has been found that thermal processing and acid and alkali treatment can reduce their allergenicity (Chen et al. 2013; Pedrosa et al. 2015). According to reports, the arginine kinase of *Penaeus monodon* structurally consisted of an N-terminal spherical structure (composed of α -helix) and a C-terminal domain (eight antiparallel β -sheets surrounded by seven α -helices).

Sarcoplasmic calcium-binding protein (SCP) is a new type of allergen present in crustacean sarcoplasmic proteins with a relative molecular weight of approximately 20 kDa, which has similar structure and function to parvalbumin in vertebrates. It was reported that, as compared to parvalbumin, SCP in *Penaeus monodon* had a lower rate of positive allergic reaction and higher reaction intensity. Besides, the SCP in *Litopenaeus vannamei* also has a strong IgE-binding activity (Ayuso and Grishina Gibanez 2009). SCP was found to have polymorphism; the three types of SCP in *Procambarus clarkii* termed SCP-I, SCP-II, and SCP-III were composed of two subunits α/β in the form of $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$, respectively. In addition, the IgE-binding activity of SCP-II was significantly lower than that of SCP-I and SCP-III (Chen et al. 2013).

Myosin light chain (MLC) was proved to be a novel allergen of *L. vannamei* (Ayuso et al. 2008), *Crangon crangon* (Bauermeister et al. 2011), and *P. monodon*, with a relative molecular weight of approximately 18–20 kDa. The MLC is divided into a basic light chain (MLC1) of about 18 kDa and a regulatory light chain (MLC2) of about 20 kDa. It has been reported that MLC2 of *P. monodon* was only detected in IgE-binding activity in cooked crude shrimp extract, while the IgE-binding activity of MLC2 of *L. vannamei* could be reduced by heat treatment.

Apart from the major allergens described above, there are many newly found allergens. Troponin C (Tn C) is a minor allergen found in *C. crangon* (Chen et al. 2013) and *Pandalus borealis* (Abdel Rahman et al. 2013) in recent years, with a molecular weight of about 17 kDa. Tn C is the calcium-binding subunit of troponin, which contains four Ca^{2+} -binding regions, while the troponin subunit Tn I can bind to actin, inhibiting the interaction between actin and myosin; besides, subunit Tn T can combine with tropomyosin. Triosephosphate isomerase (TIM) is an important catalytic enzyme in the glycolysis process, which catalyzes the reversible transformation between dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. TIM is a glycoprotein with a molecular weight of about 28 kDa, it exists in the form of a stable dimer, and its peptide chain is regularly folded into ($\alpha\beta$) 8 barrel structure (Knobeloch et al. 2010). In recent years, it has been found that TIM in *C. crangon* (Bauermeister et al. 2011), *Scomber japonicus* (Wang et al. 2011), and *Blattella germanica* (Jiingguang et al. 2011) can bind to serum IgE; therefore it was considered as a novel allergen. Hemocyanin (HMC) was first purified from *Macrobrachium rosenbergii* as an IgE-binding activity subunit with a molecular weight of about 60–80 kDa (Piboonpocanun et al. 2011) and has no cross-reactivity with *P. monodon*. Suzuki et al. isolated and purified paramyosin with a molecular weight of 100 kDa from the muscle of *Haliotis discus discus*, which was a heat-labile allergy and could cross-react with various mollusks (Suzuki et al. 2011). Hamada et al. found that the collagen of *Thunnus obesus* has immunological binding activity and first proposed that fish collagen protein may be a new type of allergen (Hamada et al. 2001). Pan et al. purified a type of collagen and its two subunits with IgE-binding activity from the muscle of *Tilapia zillii*, which were judged to be new allergens (Pan et al. 2012). Two new allergens, enolase with a molecular weight of 47 kDa and creatine kinase with a molecular weight of 41 kDa, were found in the muscle of *Megalobrama amblycephala* (Liu et al. 2011). Nucleoside diphosphate kinase B purified from *Micropterus salmoides* was also identified as a new allergen (Liu et al. 2014).

2.2.4.2 Allergens in Milk

Compared with casein and β -lactoglobulin (BLG) (up to 50% of whey proteins), other whey proteins are less abundant (20% of the total protein) in milk. BLG is a globular protein consisting of 162 amino acid residues with a molecular mass of 18.3 kDa, belonging to the apolipoprotein family. As a carrier of small molecules, this protein family shares a conserved three-dimensional structure but has comparative low similarity in the overall sequence (Chapman and Wood 2001). Its tertiary structure is composed of nine antiparallel β -sheet structures forming a so-called β -barrel stabilized by the formed two disulfide bonds (Stanic-Vucinic et al. 2012). BLG is highly stable to denaturation and resistant to proteolytic hydrolysis in solution due to its compact fold (Lonnerdal and Lien 2010; Maruyama et al. 1998). Bos d 4 (α -lactalbumin, ALA) is a single-chain polypeptide consisting of 123 amino acids with a molecular weight of 14,178 Da and belongs to the C-type

lysozyme/ α -whey protein family, which can bind calcium and participate in the milk lactose synthesis. ALA normally binds one calcium ion, which dramatically changes the tertiary structure of the molecule from an open flexible form to a tight, compact globular structure. ALA serves as a regulator of the enzyme galactosyl-transferase within the mammary gland, which is responsible for the synthesis of lactose from galactose and glucose (Osuga and Feeney 1968).

2.2.4.3 Allergens in Egg

Egg is cheap and nutritious food and is one of the most important and available protein source all over the world. One egg consists of about 60% egg white, 30–33% egg yolk, and 9–12% eggshell. The proteins in egg are of high quality, and the protein absorptivity rate in human body is 98% (Seussbaum et al. 2017). However, eggs are also one of the eight major allergic foods. Epidemiological surveys show that egg allergies account for 35% of food allergies in children and infants and 12% in adults (Rona et al. 2007). Ovomucoid (28 kDa, Gal d 1), ovalbumin (44 kDa, Gal d 2), ovotransferrin (78 kDa, Gal d 3), and lysozyme C (14 kDa, Gal d 4) are four allergens identified in hen's egg (*Gallus gallus*) and major proteins in egg white, representing 11%, 54%, 12%, and 3.4% of the egg white proteins, respectively. In a study by Sampson (1992), egg hypersensitivity was found in two thirds of children with severe atopic dermatitis. Studies by Bleumink and Young (1971) and Bernhisel-Broadbent et al. (1994) have shown that Gal d 1 is a major allergen of egg white, whereas Hoffman (1983) concluded that ovalbumin, ovomucoid, and ovotransferrin are all strong allergens and that lysozyme is a weak allergen.

Gal d 1 is a very stable serine protease inhibitor with nine disulfide bonds and no free -SH groups under in vivo and in vitro conditions (Yamashita et al. 1982). It contains 186 amino acid residues and 25% carbohydrate (Kato et al. 1987). Penta-antennary, heterogeneous, and partially sialylated carbohydrate chains are the cause of substantial mass and charge heterogeneity of native ovomucoid (Jacobsen et al. 2008; Yet et al. 1988).

Gal d 2 contains 385 amino acid residues (Perlmann 1950) and 3% carbohydrate. The dimerization of Gal d 2 results from its structure of one disulfide bond and four free -SH groups. Sequence variations and phosphorylation in two sites with a specific ratio lead to considerable charge heterogeneity of native ovalbumin. During storage in the atmosphere, Gal d 2 rearranges to a different conformation S-albumin, which exposes an additional carboxylate group (Williams et al. 2010).

Gal d 3 contains 686 amino acids with 15 disulfide bonds (Aisen et al. 1966) and 3% of carbohydrates, which is a readily soluble amorphous albumin and a highly sensitized egg allergen (Caubet and Wang 2011). Sequence variations and variations in bound Fe^{3+} result in the charge heterogeneity. Gal d 3 can bind two Fe^{3+} together with a bicarbonate anion (Amo et al. 2010), generating one extra negative charge per bound ferric ion. However, Gal d 3 in egg white is normally without ferric ions. The dominant form has a theoretical pI of 6.69 and a molecular mass of

75,828 Da. And glycosylated Gal d 3 has a molecular mass of approximately 77,000 Da (Nisbet et al. 2010).

As a weaker allergen, Gal d 4 contains 129 amino acid residues with 4 disulfide bonds and no free -SH groups (Amo et al. 2010). Gal d 4 has a theoretical pI of 9.3 and a molecular mass of 14,313 Da.

Gal d 5 (α -livetins) incorporates 1 potential glycosylation site and 35 cysteine residues. According to similarity with other serum albumins, the -SH groups might be linked in 17 disulfide bridges leaving 1 free -SH group resulting in dimerization. Gal d 5 has a theoretical molecular mass of 66,815 Da and a pI of 5.31 (Nisbet et al. 2010).

Gal d 6 is the yolk glycoprotein YGP42, a fragment of vitellogenin (VTG), which can produce the components of the water-insoluble yolk granular lipoproteins. Besides, as the main components of the yolk plasma, yolk glycoprotein YGP42 comes from the C-terminal part of VTGs (Martos et al. 2012).

It was reported that the riboflavin-binding protein (RfBP) is a minor IgE-reactive protein in the egg yolk and white (Sampson and Cooke 1990). It consists of 219 amino acid residues, containing carbohydrates that account for about 14% of its molecular mass (30–35 kDa). Its phosphate moieties are conducive to its pI of about 4.0, and its nine disulfide bonds benefit to its thermal stability.

2.2.5 Epitopes in Animal Food Allergens

Changes in the microenvironment where proteins are located, such as temperature, pH, and ionic strength, have a large effect on their secondary structure and spatial conformation, which can alter the structure of the epitopes and their antigenicity. Therefore, the composition, number, property, and spatial configuration of the epitope determine the specificity of the antigen. And systematic knowledge of antigenic epitopes is the key to reveal the role of allergens in food allergic reactions and is the foundation for allergenicity elimination. The current researches mainly focus on B-cell epitopes. The main methods for epitope analysis are bioinformatics prediction, overlapping peptide library technology, hydroquinone exchange-mass tandem analysis, genetic engineering of site-directed mutagenesis, and phage display technology.

The trypsin degradation fragment of the codfish parvalbumin, mainly including residues 33–44, residues 65–74, and residues 88–96, has been identified to be immune active (Elsayed and Apold 2010). Further, peptide (49–64), peptide (88–103), and peptide (13–32) have been determined to be immune active peptides with the technology of overlapping peptide library, which are all linear epitopes. With the usage of phage-displayed peptide library technology, three conformational epitopes of the carp parvalbumins, which can bind to human serum IgE and IgG specifically, have been identified as epitopes: they are epitope 1 (amino acids at the site of 23, 25–29, 33–37), epitope 2 (amino acids at the site of 77–79), and epitope 3 (amino acids at the site of 87, 89–92, 94) (Untersmayr et al. 2006). It was found that the

epitopes of the codfish parvalbumin and the carp parvalbumin overlap with each other. Similarly, based on the technology of overlapping peptide library, peptide (21–40) has been determined to be the major linear epitope region of parvalbumin Sco J 1 in *Scomber japonicus* (Yoshida et al. 2008). Also, Ser²³, His²⁶, Lys²⁷, Lys²⁸, Lys³¹, Cys³³, Leu³⁵, and Lys³⁸ are picked up as the key amino acid residues among the epitopes by site-directed mutagenesis.

Shanti et al. (1993) identified trypsin degradation peptide fragments (amino acids at the site of 50–66, 153–161) as the linear epitopes in the tropomyosin of *Penaeus indicus* using inhibitory ELISA and Western blot. Ayuso et al. (2002a, b) identified five linear epitopes in the tropomyosin of *Penaeus aztecus* using the technology of overlapping peptide library, that is, Pen a 1 (43–57), Pen a 1 (85–105), Pen a 1 (133–148), and Pen a 1 (187–202), which were also found to be cross-reactive with other invertebrates such as lobster and scorpion. Likely, epitope 1 (1–36), epitope 2 (37–63), epitope 3 (61–81), epitope 4 (82–105), epitope 5 (115–150, 142–162, 157–183), epitope 6 (190–210), and epitope 7 (246–284) have been determined to be the linear epitopes in the tropomyosin of *Litopenaeus vannamei* (Ayuso et al. 2010). As for the tropomyosin of *Penaeus monodon*, linear epitopes, including peptide 2 (45–59), peptide 3 (89–105), peptide 4 (115–128), peptide 5 (131–142), peptide 6 (145–164), peptide 7 (177–190), peptide 8 (210–224), peptide 9 (243–259), and peptide 10 (263–280), were determined to have IgE-binding activity using inhibitory dot blot (Zheng et al. 2011). In addition, Ishikawa et al. (2010) approved that three linear epitopes (amino acids at the site of 77–112, 148–160, 269–281) were identified in the tropomyosin of *Octopus vulgaris* through the degradation of lysyl endopeptidase. The above results validated the immunological cross-reactivity of tropomyosin in different species, and the information on the dominant epitope and its key amino acids remains to be explored.

Similarly, the epitopes in arginine kinase were also explored. Using the technology of overlapping peptide library, eight linear epitopes in arginine kinase of *Litopenaeus vannamei* were identified; they are fragments at the site of 1–18, 25–42, 64–96, 121–141, 142–159, 160–192, 232–255, and 319–342, respectively (Ayuso et al. 2010). It was determined that amino acids at the site of 174–181 and 253–256 were two major epitope regions of arginine kinase in *Scylla paramamosain*, and amino acids at the site of 127–141, 141–155, and 211–225 were identified as linear epitopes (Mao et al. 2013). Besides, Chen et al. (2013) predicted six linear epitope regions in the arginine kinase of *Procambarus clarkii*, amino acids at the site of 39–43, 89–103, 146–152, 252–256, 311–317, and 325–330.

Ayuso et al. (2010) demonstrated the amino acids at the site of 10–36, 49–72, and 130–147 as the linear epitopes of sarcoplasmic calcium-binding protein in *Litopenaeus vannamei*. In addition, epitope 1 (13–30), epitope 2 (22–48), epitope 3 (49–66), epitope 4 (58–90, 79–99), and epitope 5 (118–141) were identified as linear epitopes in the myosin light chain of *Litopenaeus vannamei* (Ayuso et al. 2010).

Selo et al. used a serum from cow milk allergy patients to study the IgE-binding ability of the cyanogen bromide cleavage or trypsin-hydrolyzed fragment of β -lactoglobulin and found multiple epitopes including short linear sequences and conformational epitopes consisting of large fragments in β -lactoglobulin, wherein the

most recognizable amino acid residues are 41–60, 102–124, and 149–162, which can be recognized by more than 90% of patients' sera, and the immunoreaction caused by each fragment account for approximately 10–15% of the whole allergic reactions of β -lactoglobulin. Fragments at the site of 41–60 and 102–124 were stabilized by hydrogen bonding or disulfide bonding and located on the surface of the molecule; thus they could be easily recognized by antibody. The fragment at the site of 149–162 was located at the N-end of molecule and formed a short spiral structure which had a strong mobility and thus could also be easily recognized by antibody (Sélo et al. 2010). The hypoallergenic peptide chains are divided into two categories: the amino acid residue sequences at the site of 1–8, 25–40, and 92–100 (could be recognized by about 52–65% of the patients' sera), the amino acid residue sequences at the site of 9–14 and 84–91 (could be identified by approximately 40% of patients' sera), and the sequences at the site of 78–83 and 125–135 (almost unrecognizable). The peptide chain at the site of 84–91 was not located on the surface of the molecule and was considered as a minor epitope by Ball et al. (2010). Peptide chains at the site of 9–14 and 92–100 were deep inside the molecule and could not be easily hydrolyzed and thus have low immunological activity (Sélo et al. 2010).

Maynard et al. studied the binding ability of the trypsin cleavage fragment of natural α -lactalbumin and denaturing α -lactalbumin to IgE; results indicated that 11 of the 19 patients' sera were able to recognize the complete α -lactalbumin, 8 of them could bind to the trypsin cleavage fragments (Maynard et al. 1997). Hopp and Woops believed that the ring formed by peptide chains at the site of 60–80 and 91–96 with disulfide linkage was the main antigenic region by animal model studies; however, Maynard et al. found that they were not the main IgE-binding epitopes through serological studies (Hopp and Woods 1982; Maynard et al. 1997). In addition, Maynard et al. found that some fragments in the hydrophobic region of the molecule such as sequence at the site of 99–108 also showed IgE-binding activity, but their sensitization was difficult to predict because of their location (Maynard et al. 1997). Sequence at the site of 17–58 and C-terminal sequence at the site of 109–123 exhibited strong IgE-binding ability, although they had 81% and 87% similarity to the corresponding sequence of human lactalbumin, respectively. In some serum, the degraded fragment (59–94) appeared to have similar or higher IgE-binding ability than the natural fragment. Maynard et al. believed that some epitopes hidden deep inside the molecule were exposed during denaturation. In addition, IgE can bind to natural α -lactalbumin and some large fragments, indicating that α -lactalbumin also has conformational epitopes (Hopp and Woods 1982).

Allergic reactions of bovine serum albumin are relatively independent, as 50% of allergic patients with bovine serum albumin are not allergic to other proteins (Wal 1998). Restani et al. studied the characteristics of bovine serum albumin epitopes and their role in allergic reactions (Dunwell et al. 2004), and found that there existed at least one epitope in bovine serum albumin (sequence at the site of 524–542), and indirectly demonstrated the presence of conformational epitopes in bovine serum, suggested that the presence of a loop in the molecule played an important role in maintaining the conformational epitope of the molecule. Soich Tanabe et al. found that the major epitope structure of bovine serum albumin contains a segment of

EXXV motif. The recombinant sequence obtained by substituting Asp for Glu in the EXXV motif has a significantly reduced binding ability to IgE and can induce the proliferation of T cells. This sequence is expected to induce the formation of immune tolerance (Tanabe et al. 2004).

Otani et al. studied the epitopes of α S1-casein using cyanogen bromide cleavage and trypsin-hydrolyzed fragments and found six antigen fragments, wherein the main one is the fragment at the site of 61–123, and considered that molecular phosphorylation regions played an important role in molecular sensitization (Wal 1998). Spuergerin et al. identified 7 IgE-binding epitopes of α S1-casein using 15 cow milk allergy patients' sera and synthetic peptide chain (Spuergerin et al. 1996). Results showed that the peptide chain sequences at the site of 19–30, 86–103, and 145–150 were the most important IgE-binding site, wherein the sequence at the site of 86–103 had the strongest immunologically activity. The common feature of these three peptide chains is that they contain a large number of nonpolar aromatic amino acids. The three peptide chains are located in the hydrophobic region of the molecule; therefore they are not easily accessible by antibodies unless they are denatured or degraded. Chatchatee et al. found 6 major and 3 minor IgE-binding regions as well as 5 major and 1 minor IgG-binding regions of α S1-casein using 24 bovine milk allergy children's sera and overlapping peptide chains (Chatchatee et al. 2001). The sequences at the site of 17–36, 69–78, 109–120, and 173–194 in the peptide chain were proved to be the highly binding zone of IgE, which can be recognized by 75%, 46%, 53%, and 63% of patients' sera, respectively. Besides, persistent allergic patients and temporary allergic patients showed some differences in recognizing IgE-binding fragments (Chatchatee et al. 2001; Vila et al. 2010). Although human milk contains a large amount of β -casein and the casein in cow milk has a relatively high sequence similarity to β -casein in human milk, β -casein still induces a strong IgE response (Wal 2002). The conserved regions of human milk casein and cow milk casein that were composed of a large number of phosphoserine residues were thought to be involved in the cross-reactivity between them (Bernard et al. 2000; Cantisani et al. 1997). Otani et al. have extensively studied the epitope of β -casein and found that the β -casein had at least six linear epitopes, and the motif KXKE played an important role in the sensitization of the peptide chain at the site of 94–102 and 103–109; the similar sequence was also present in the antigenic region of lysozyme (Wal 1998).

At present, synthetic peptide technology is mainly used to localize the linear epitopes of ovalbumin. In 1992, amino acid sequences at the site of 41–172 and 301–385 were identified as key B-cell epitopes of ovalbumin by dot blot (Kahlert et al. 1992). Then the amino acid sequences at the site of 38–49, 95–102, 191–200, 243–248, and 251–260 were identified as main B-cell epitopes of ovalbumin one after another; besides, the main allergenic amino acids mainly consisted of hydrophobic amino acid and a structural region with a β -sheet or β -turn composed of partially polarized and charged amino acid residues, wherein the amino acid sequence at the site of 95–102 formed an α -helix structure alone (Mine and Rupa 2003). These epitopes provide useful information for the role of amino acid residues in ovalbumin and provide a strong basis for the correlation between structural

changes and sensitization of ovalbumin. In addition, amino acid sequence at the site of 323–330 was identified as ovalbumin T-cell epitopes (Mackenzie et al. 2013). In recent years, Jankovicova used biochemical-capable magnetic beads to locate ovalbumin epitopes through microfluidic channels and found that HAVINAVLFGR (at the site of 371–382) in ovalbumin is the main B-cell epitope of ovalbumin, and this epitope is expected to become a vaccine for egg allergy immunotherapy (Jankovicova et al. 2008).

Mine et al. (Mine and Zhang 2002) found 8 specific IgG-binding linear epitopes and 9 IgE-binding epitopes of ovomucoid using synthetic peptide technology, wherein the IgG-binding epitopes have 5–11 amino acids in length and the IgE-binding epitopes have 5–16 amino acids in length. Besides, charged amino acids aspartate, glutamate, and lysine; hydrophobic amino acids leucine, phenylalanine, and glycine; and polar amino acids serine, threonine, tyrosine, and cysteine were demonstrated to play important roles in binding to antibodies by replacing the main amino acids in the epitopes. Furthermore, three main IgE-binding epitopes, amino acid sequence at the site of 32–42, 40–50, and 56–66, were identified in the domain I of ovomucoid. Four IgE-binding epitopes, amino acid sequence at the site of 71–75, 80–90, 101–105, and 121–130, were identified in the domain II. Two secondary IgE-binding epitopes, amino acid sequences at the site of 159–174 and 179–186, were found in domain III. Meanwhile, the IgG-binding epitopes were discovered in the three domains: they were amino acid sequences at the site of 6–15, 31–35, and 46–50 in domain I, amino acid sequences at the site of 71–75 and 101–105 in domain II, and amino acid sequences at the site of 160–167, 165–174, and 177–183 in domain III, respectively.

There are many reports on the lysozyme epitopes of eggs, mainly focusing on the study of T-cell-binding epitopes but little research on their B-cell epitopes. Epitopes that bind to T cell were mainly located in the N-terminus and C-terminus of lysozyme, including amino acid sequence at the site of 1–17, 6–127, and 120–129 (Maizels et al. 2010). Zouhair et al. analyzed the amino acid residues, conformations, and precise boundaries of lysozyme; combined with surface simulation synthesis, three epitopes of the enzyme are recognized, including epitope 1 (Arg-5, Glu-7, Lys-13, Arg-14, and Arg-125), epitope 2 (Thr-8, Trp-62, Asp-87, Lys-97, Lys-96, and Asn-93), and epitope 3 (Lys-33, Phe-34, Lys-116, Asn-113, and Arg-114) (Atassi and Lee 1978). Padlan et al. used X-ray crystallography to locate the epitopes consisting of amino acid residues that are spatially adjacent but not continuous when egg lysozyme binds to the anti-lysozyme monoclonal antibody HyHEL-10 Fab fragment, wherein epitope 1 (His-15, Tyr-20, Arg-21, and Gly-26) was located on one side of the α -helix, epitope 2 (Thr-89, Asn-93, Lys-96, Lys-97, and Ile-98) was located on the outer surface of the α -helix, epitope 3 (Ser-100, Asp-101, and Gly-102) was not in the helical structure, and epitope 4 (Trp-63) and epitope 5 (Arg-73 and Leu-75) were located on both sides of the lysozyme active site, respectively. In addition, the binding region of the egg lysozyme to the anti-lysozyme monoclonal antibody HyHEL-10 Fab fragment contains a number of hydrophobic amino acid residues, which form hydrogen bonds with each other, so that solvent molecules such as water can be excluded, thereby making the antigen

and the antibody closely bound (Padlan et al. 1989). When studying the T-cell binding epitope of lysozyme, Jang et al. used the conjugate of phosphorus phthalein choline (PC-HEL) and egg lysozyme complex to immunize C57BL/6 mice that were low allergic to lysozyme and found some T-cell hybridomas could recognize peptide fragments at the site of 81–93, suggesting that amino acid sequence at the site of 81–93 may be a potential linear epitope for lysozyme (Jang et al. 2010). Arnon and Takagaki et al. also found that polypeptide fragments of egg lysozyme at the site of 38–54 and 64–80 and some polypeptide fragments in helix structures also reacted strongly with anti-lysozyme antibodies (Arnon et al. 1971; Takagaki et al. 1980). In addition, Garvin identified four T-cell epitopes of lysozyme using continuous overlapping peptide chains; they were amino acid sequences at the site of 17–29, 34–47, 49–62, and 96–110, respectively (Gammon et al. 2010). On the other hand, Gammon et al. located another five T-cell epitopes; they were amino acid sequences at the site of 13–35, 30–53, 46–61, 74–96, and 106–129, respectively (Gammon et al. 2010). Ibrahimi et al. used phage inhibition assays and micro-complement binding assays to find that the site-directed mutagenesis replacement of Arg-68 with Lys could affect the binding ability of lysozyme to antibodies (Ibrahimi et al. 1980). Smith et al. also mapped Arg-68 as a key amino acid of the epitope using HyHEL-5 monoclonal antibody (Smith-Gill et al. 1982), indicating that Arg-68 is a key amino acid of lysozyme.

Overall, in addition to the allergens mentioned above, other new allergens in foods are constantly being discovered. However, the epitopes of these novel allergens and their effects on allergic reactions need to be systematically studied. Many structural studies have determined the mechanisms of allergen structural stability that make them resistant to digestion or heat inactivation. Understanding these structural features of food allergens provides a theoretical basis for preventing food allergies and further development of allergenicity elimination approaches.

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Chapter 3

Intestinal Permeability and Transport of Food Allergens



Abstract Serving as the predominant site for food digestion and nutrition absorption, the gastrointestinal tract separates the inner environment of the human body from the outer environment by epithelial barrier. The highly selective barrier favors fluxes of nutrients but limits host contact with the massive intraluminal load of dietary antigens and microbes. Under normal physiological conditions, controllable small amounts of food-derived antigens and microorganisms transport through the barrier with proper processing, inducing a homeostatic immune response and resulting in immune tolerance to dietary antigens. Conversely, primary or secondary intestinal barrier defects can lead to excessive entrance of lumen macromolecules, contributing to the pathogenesis of a wide range of human diseases including food allergy and inflammatory bowel disease (IBD). Consequently, the intestinal barrier dysfunction could be a promising target for treating food allergy; however, limited mechanical researches and practical applications are available nowadays, and far more investigations are required for reliable clinical practices.

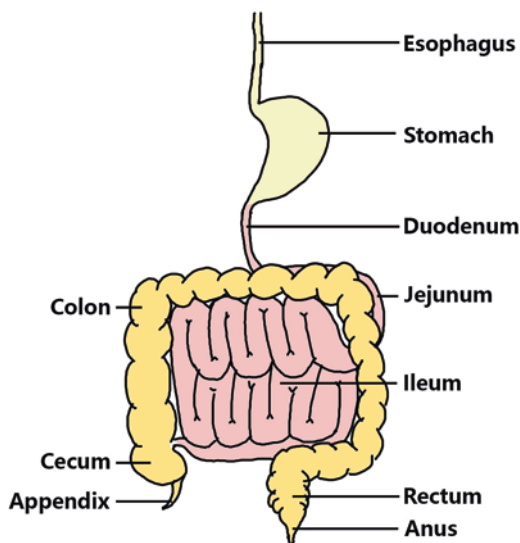
3.1 Protein Digestion in Gastrointestinal Tract

3.1.1 *The Structure of Gastrointestinal Tract*

The gastrointestinal tract is an open-ended long tubular organ throughout the body that serves as the site for food digestion and nutrition absorption. Human gastrointestinal tract started from the oral cavity, followed by esophagus, stomach, small intestine, and large intestine, and ended with anus (Fig. 3.1). Specifically, the small intestine further divides into the duodenum, jejunum, and ileum, and the big intestine includes the cecum, colon, rectum, and anal canal (Singh and Gallier 2014).

Although food digestion presented all over the gastrointestinal tract, the small intestine is the place where most food digestion and absorption take place, which result from the presence of a large amount of digestive enzymes and the huge surface area. Additionally, due to the direct interaction with intact and degraded allergens as well as the presence of abundant immune cells, the small intestine is also a major location for food allergy triggering.

Fig. 3.1 The structure of the human gastrointestinal tract. The human gastrointestinal tract started from the oral cavity, followed by esophagus, stomach, small intestine, and large intestine, and ended with anus. Specifically, the small intestine further divides into the duodenum, jejunum, and ileum, and the big intestine includes the cecum, colon, rectum, and anal canal



The large intestine is the biggest habitat of commensal microorganisms (microbiota), where trillions of bacteria live in. Gut microbiota has been considered as a part of the gastrointestinal tract, which can communicate with the host and regulate host physiological activities including immunity and allergy. More details on the role of microbiota in regulating food allergy are introduced in Chap. 8.

The gut-associated lymphoid tissue (GALT) is the largest reservoir of the immune cells in the body, where various kinds of immune cells, such as T cells, B cells, dendritic cells (DCs), mast cells, and so on, are located in. These cells act cooperatively to orchestrate the overall immune responses. More details on the gut mucosal immunity are introduced in Chap. 1.

The gastrointestinal tract is covered by mucosa layer that can be further divided into three layers: the epithelium, the lamina propria, and the muscularis mucosae (Cubells-Baeza et al. 2015) (Fig. 3.2). The epithelium covers the wall of the gastrointestinal tract, directly contacts with food ingredients, and separates inner cells from the intestinal lumen. Microfold cells (M cells) are a type of specialized enterocyte distributed on the epithelium that participate in antigen uptake. Lamina propria is a layer of connective tissue next to the epithelium and infiltrated by lymphoid and myeloid cells, which is the predominant location for food allergen recognition and allergic response establishment (Fu et al. 2018).

3.1.2 Protein Digestion Process

After being splintered and macerated in the mouth, food entered the gut and started the chemical digestion. In the stomach, proteins contact with the low-pH hydrochloric acid and unfold, leading to the disruption of protein advanced structure and the

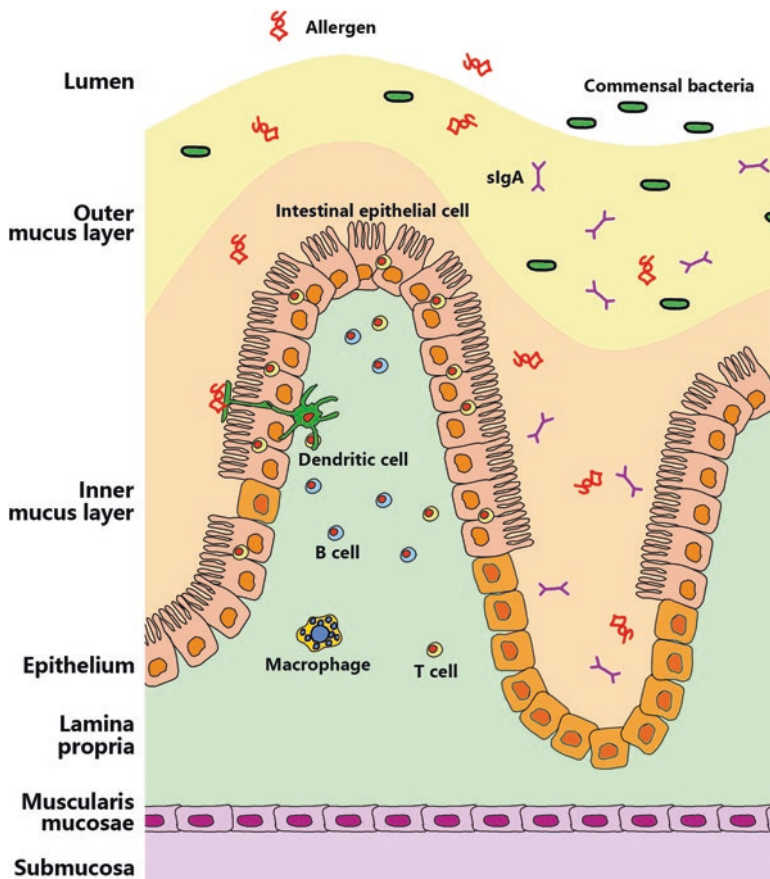


Fig. 3.2 The structure of human intestinal barrier. The intestinal barrier is the combination of mucus layer with the commensal microbiota and sIgA and the mucosa layer that can be further divided into epithelium, lamina propria, and muscularis mucosae. The lamina propria contains various immune cells that are responsible for mucosal immunity and food allergy

increment of protease pepsin accessibility, the latter further breaking the proteins into peptides and thus destroying the protein primary structure. Protein structure is the fundament of allergenicity; as a result, structure disruption may eliminate the potential allergenicity of protein. It has been reported that the application of antacid that neutralize peptic acid and hinder peptic digestion will enhance food allergy (Untersmayr et al. 2003; Untersmayr and Jensen-Jarolim 2008). In the intestine, the proteins are further degraded into short peptides and amino acids by pancreatic proteases, trypsin, etc. and absorbed. During this process, the remained allergenicity of the undigested or partially digested proteins may be recognized by intestinal epithelium and GALT and then trigger the food allergy responses.

3.1.3 Digestion Resistance of Allergens

It is proposed that the ability of food allergens to reach the intestinal mucosal is a prerequisite for allergenicity, and therefore most food allergens should show digestion resistance, which is indeed true (Astwood et al. 1996). On the other hand, some food allergens are also resistant to conventional processing methods such as heating, which further ensures their accessibility to intestinal mucosa (Liu et al. 2018; Scala et al. 2018; Ruethers et al. 2018). Consequently, several novel food process approaches are utilized to denature food allergen so as to eliminate the allergenicity (Houska et al. 2013). However, it should be noted that not all the allergens are resistant to digestion. For example, though to be major allergens, glycinin from soybean and 11S legumin-like protein from peanut have been reported to be sensitive to peptic digestion (Pali-Schoell and Jensen-Jarolim 2011). As a result, digestion resistance is an important hint of allergenicity, but cannot be used as the decisive characteristic of food allergens.

3.2 Intestinal Permeability and Transporting of Food Proteins

The gastrointestinal tract constitutes the selective intestinal barrier that protects the internal environment from external harmful substances such as toxins, pathogens, and allergens but favors nutrient uptake. The intestinal barrier is the combination of mucus layer with the commensal microbiota and secretory immunoglobulin A (sIgA), the single intestinal epithelial cell (IEC) layer, and the lamina propria that contains various immune cells (Vancamelbeke and Vermeire 2017) (Fig. 3.2). In the normal physiological state, only a small portion of food proteins can pass the barrier, which induces the immune tolerance to food proteins. However, the permeability increases under intestinal barrier defects, which is potentially associated with a broad range of diseases such as food allergy, infection, IBD, irritable bowel syndrome, obesity, and celiac disease (Konig et al. 2016). Consequently, the understanding of intestinal permeability and transporting of food proteins can provide insight in the prevention and treatment of food allergy.

3.2.1 Mucus Layer Permeation

The mucus layer is important to protect the gastrointestinal tract; it has a well-established two-layer structure in the stomach and colon, which is composed of a loose outer layer and a firm inner layer. However, in the small intestine, the mucus layer is discontinuous and less well defined (Johansson et al. 2011) (Fig. 3.2). The major components of mucus layer are the highly glycosylated protein mucins,

especially MUC2 that produced by goblet cells. In addition, Fcgbp, Clca3, Zg16, Agr2, immunoglobulins, and some other proteins are also detected in mucus layer. In addition to those free components, there are also anchored mucins associated with the IEC such as MUC3 and MUC17, which all together formed the glycocalyx that covers the apical membrane (Kim and Ho 2010). Although the commensal microorganisms are associated with the outer mucus layer, the inner mucus layer totally blocked these organisms. However, it should be noted that the mucus cannot totally preclude the access of biomolecules such as secreted bacterial metabolites, toxins, and food allergens (Capaldo et al. 2017).

3.2.2 Intestinal Epithelial Cell Permeation

Compared with the mucus layer, the intestinal epithelia cell monolayer is a much powerful barrier to food allergens, and the permeability of macromolecules as well as micromolecules (including water and ions) is tightly regulated in normal physiological conditions. The increased permeation of IEC layer may lead excess undigested food allergen or partially digested allergenic peptides to contact with immune cells located in the lamina propria and thus induces food allergy responses. There are two major routes for food proteins transport through the IEC layer: the paracellular transport through tight junctions between adjacent IECs and the transcellular transport involving transcytosis (Ménard et al. 2010). Both routes show high specificity and are tightly regulated by several signaling pathways.

3.2.3 Paracellular Transport

The IECs connect with each other by complex protein structures including tight junction, adherens junction, and desmosome, among which tight junction that located near the apical member is the predominant barrier for paracellular transport (Fig. 3.3).

Tight junctions are big protein complexes that consist three major groups of proteins: the transmembrane proteins, the cytoplasmic plaque proteins, and the cytoskeletal proteins (Yu and Turner 2008). The transmembrane proteins such as claudins and occludin serve as the direct linkage between two IECs and form the pores for paracellular transport. The cytoplasmic plaque proteins such as zonula occludens (ZO)-1/2/3 are usually considered to be the scaffold proteins that interacted with both transmembrane proteins and cytoskeletal proteins especially actin and myosin II. These proteins also participate in the transport of transmembrane proteins and the regulation of tight junction structure. In addition, there are also regulatory proteins such as the kinase PKC ζ localized to the tight junction.

According to the permeability, the epithelia can be divided into leaky ones and tight ones (Diamond 1974). However, the leaky epithelia are not more permeable to all the subjects but show high selectivity; different leaky epithelia have diverse

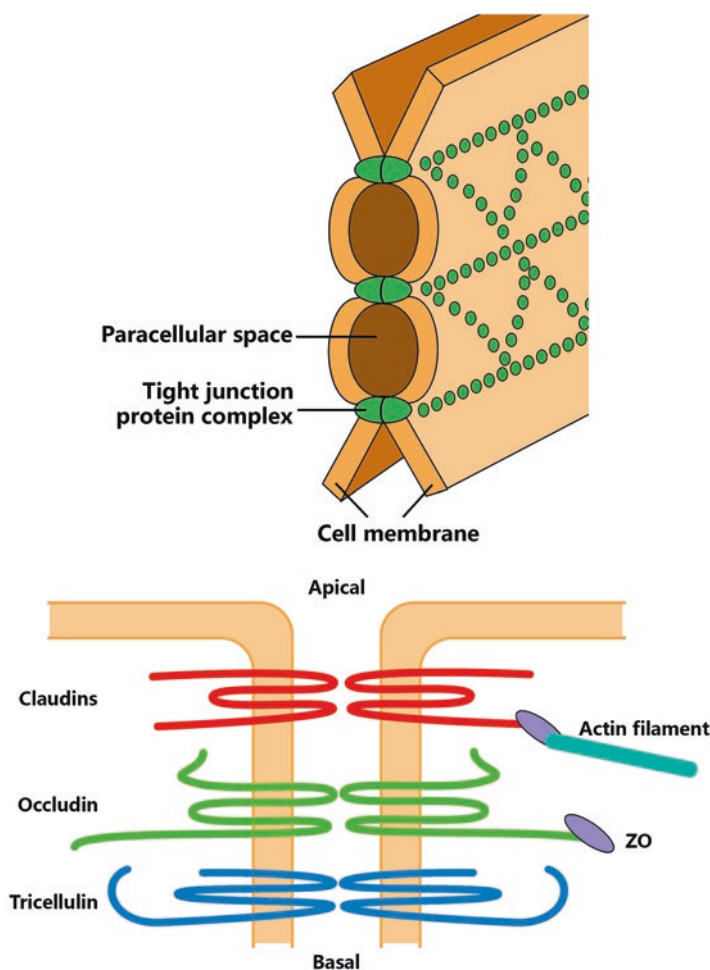


Fig. 3.3 The structure of tight junction. Tight junction that is located near the apical membrane is the predominant barrier for paracellular transport, which is a big protein complex that consists of three major groups of proteins: the transmembrane proteins, the cytoplasmic plaque proteins, and the cytoskeletal proteins. The transmembrane proteins such as claudins, occludin, and tricellulin serve as the direct linkage between adjacent IECs and form the pores for paracellular transport. The cytoplasmic plaque proteins such as ZO-1/2/3 are usually considered to be the scaffold proteins that interacted with both transmembrane and cytoskeletal proteins especially actin and myosin II

permeability to specific molecules such as water, ions, and proteins. The selectivity of small molecules usually comes from the specific charge-selective tight junction channels formed by different claudin proteins, such as the cation channels by claudin-2, claudin-10b, or claudin-15, the anion channels by claudin-10a or claudin-17, and the water channel by claudin-2 (Krug et al. 2014). On the other hand, the selectivity of macromolecules usually comes from size-selective channels formed by

tight junction-associated MARVEL proteins (TAMP) including occludin, tricellulin, and MarvelD3 (Raleigh et al. 2010).

In addition to common bicellular tight junction (bTJ) that forms between two adjacent cells, tricellular tight junction (tTJ) that seals three adjacent cells is also present in the epithelial barrier. Compared with bTJ, tTJ is a dispensable route for small ions transport (Krug et al. 2009), and is considered as a weak point of the total TJ network (Krug et al. 2014). However, because the size-selective channel formation protein tricellulin mainly localized in, tTJ could be an important route for macromolecules, though it is unknown to date whether bTJ or tTJ for macromolecules transport predominantly (Krug et al. 2014).

Occludin is the first discovered tight junction protein with four transmembrane domains (Furuse et al. 1993; Balda et al. 2000), but its detailed role for tight junction barrier function and paracellular transport is not fully elucidated yet (Krug et al. 2014). It has been reported that overexpression of occludin inhibited ion transport (Balda et al. 1996). Additionally, occludin knockdown in cell models increased the permeability of macromolecules including proteins (Al-Sadi et al. 2011; Buschmann et al. 2013). On the other hand, inflammation diseases such as IBD (Amasheh et al. 2009), Crohn's disease (Zeissig et al. 2007), and ulcerative colitis (Heller et al. 2005) were reported to downregulate occludin expression and thus lead to macromolecular barrier dysfunction, which is potentially regulated by cytokines IL-13, IFN- γ , and TNF- α . In addition, some pathogens including bacteria, e.g., pathogenic *Escherichia coli* (Strauman et al. 2010; Roxas et al. 2010), and viruses, e.g., norovirus (Troeger et al. 2009), can also downregulate occludin expression to loosen the intestinal barrier and so as to promote pathogen infection.

Tricellulin plays a regulating and tightening role in tTJ to establish the paracellular barrier. Unlike occludin, tricellulin knockdown increases not only the macromolecule but also the ion permeability, manifest as the decrease of transepithelial electrical resistance, which could be due to the breakdown of tight junction, indicating the important role of tricellulin in maintaining tight junction structure (Ikenouchi et al. 2005). The consistent phenomena were also observed in overexpression models: while low overexpression level even increased the permeability of macromolecules but not ions, high overexpression level strengthened the whole tight junction barrier function and blocked both macromolecules and ions. Occludin and tricellulin are partially redundant in forming barrier, because occludin knockout mouse shows similar non-syndromic deafness as in tricellulin knockout mouse due to the degeneration of hair cells (Nayak et al. 2013; Kitajiri et al. 2014). The phenocopy could result from the migration of tricellulin from the tTJ to the bTJ because of the lack of occludin (Ikenouchi et al. 2008; Kitajiri et al. 2014).

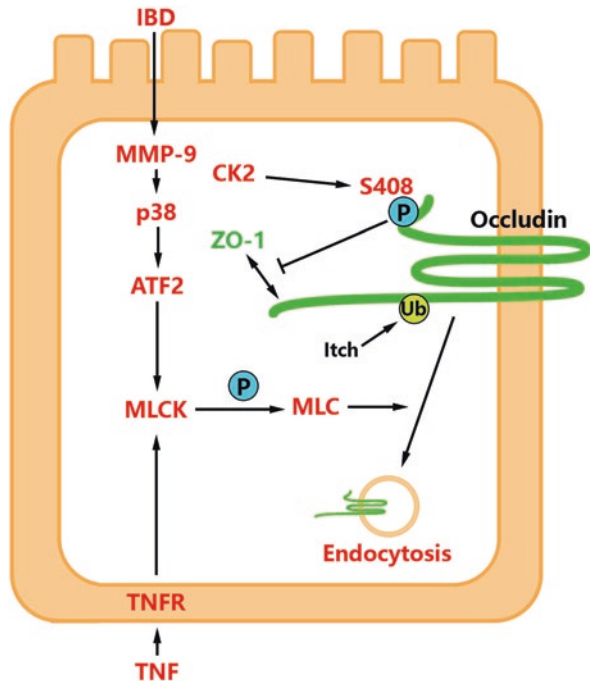
Similar with occludin and tricellulin, MarvelD3 is also a transmembrane component of tight junctions, which showed partially overlapping activity with the formers (Raleigh et al. 2010). Recent studies demonstrated the activity of MarvelD3 in coupling tight junction and JNK pathway as well as inhibiting JNK pathway to regulate cell migration, proliferation, survival, and development (Steed et al. 2014; Vacca et al. 2018). However, the detailed role of MarvelD3 in maintaining tight junction structure and regulating barrier permeability is unknown.

3.2.4 Regulation of Paracellular Transport

Several physiological and pathological processes can regulate protein paracellular transport by changing the dynamics of channel-making proteins in tight junction (Fig. 3.4).

Previous studies have identified several post-transcriptional modifications (PTMs) of tight junction proteins in regulating their function and thus paracellular transport (Shigetomi and Ikenouchi 2018). In addition to claudins whose PTMs are well described (González-Mariscal et al. 2010), PTMs of occludin and tricellulin are also critical for barrier function. Immunofluorescence microscopy revealed that non- or less phosphorylated occludin is distributed on the basolateral membranes and highly phosphorylated occludin is selectively concentrated at tight junctions (Sakakibara et al. 1997), and phosphorylation also regulated occludin association with ZO-1 and membrane assembly (Sheth et al. 2000). A more recent molecular study showed that phosphorylation of occludin at S408 site by casein kinase 2 disrupted the barrier integrity and dephosphorylation of this site reduced occludin exchange and increased the exchange of ZO-1, claudin-1, and claudin-2, causing transepithelial resistance elevation and reducing paracellular cation flux (Raleigh et al. 2011). In addition, both occludin and tricellulin can be ubiquitinated by the E3 ubiquitin ligase Itch, and tricellulin can also undergo phosphorylation, but the

Fig. 3.4 Regulation of paracellular transport of food proteins. Occludin is the predominant tight junction protein responsible for macromolecule paracellular transport. The activity of occludin can be regulated by PTMs such as ubiquitination and phosphorylation. MLCK is a key regulator of occludin, which induces occludin endocytosis by phosphorylating MLC



function of these modifications remains unclear to date (Traweger et al. 2002; Ikenouchi et al. 2005; Susanne et al. 2017).

Furthermore, accumulated results have shown that myosin light chain (MLC) and its kinase MLCK are core mediators for regulating protein paracellular transport (Turner et al. 2014). In detail, the phosphorylation of MLC by MLCK will induce contraction of the perijunctional actomyosin ring and thus disrupts tight junction and increases the permeability of epithelial barrier (Cunningham and Turner 2012; Chen et al. 2012). Additionally, TNF-induced MLCK activation was reported to trigger caveolin-1-dependent endocytosis of occludin to affect structural and functional tight junction regulation (Marchiando et al. 2010). Notably, MLC phosphorylation is sufficient to increase tight junction permeability since the expression of constitutively active MLCK resulted in the redistribution of tight junction protein ZO-1 and occludin but not claudin-1 and claudin-2 in a kinase-activity-dependent manner (Shen et al. 2006). Given that occludin but not claudin is critical for macromolecular permeability, the main effect of MLCK should be on the macromolecules such as food allergens.

Several effective factors have been reported to regulate intestinal barrier function through modulating MLCK activity. In Caco-2 cells, matrix metalloproteinases 9 (MMP-9), a key pathogenic factor in IBD, was demonstrated to disrupt intestinal barrier function. Detailed studies revealed that MMP-9 activated p38 kinase pathway, which further triggered the transcription factor ATF-2, thus promoted MLCK expression, and finally increased tight junction permeability in Caco-2 cells (Al-Sadi et al. 2018). Similarly, in mouse models, MMP-9 also induced increase in mouse intestinal tight junction permeability in a MLCK-dependent way (Al-Sadi et al. 2017). On the other hand, regulating MLCK activity by membrane-permeant peptide, proton pump inhibitors, or TNF signaling can also modulate intestinal barrier function (Zolotarevsky et al. 2002; Clayburgh et al. 2006; Su et al. 2013; Nighot et al. 2017).

On the other hand, due to the direct interaction with intestinal barrier and the strong effect on intercellular junction proteins, digestive proteases are also reported to regulate paracellular transport, and the dysregulation of intestinal protease/anti-protease balance contributes to the epithelial damage and increased permeability and finally results in gastrointestinal diseases such as IBD (Van Spaendonk et al. 2017). In addition to direct degrading junctional proteins, proteases also trigger proteinase-activated receptors (PARs) and downstream signaling pathways (Hollenberg et al. 2014). For example, SLIGRL, a PAR-2-activating peptide, can increase colonic permeability via the activation of MLCK by calmodulin (Cenac et al. 2004). Interestingly, a recent study showed that the probiotic strain *Escherichia coli* Nissle 1917 (EcN) recovered occludin and ZO-1 expression as well as barrier permeability under SLIGRL or TNF- α stimulation, which was correlated with MLCK expression and can explain the efficacy of the strain in alleviating ulcerative colitis (Barbaro et al. 2016).

3.2.5 Transcellular Transport

In addition to undergo paracellular transport through cell junctions, food proteins can also directly penetrate through epithelial cells into the lamina propria by transcellular transport. Unlike paracellular transport, due to the exist of additional intracellular protease, most proteins are partially degraded into immunogenic peptides or fully degraded into amino acids during transport, and only a small portion remained intact (Heyman et al. 1982, 1987). M cells, columnar enterocytes, goblet cells, and some myeloid cells are able to transport and process food proteins.

M cells are a kind of specialized epithelial cells located in the follicle-associated epithelium (FAE) of Peyer's patches (PPs) covering mucosal lymphoid follicles and can transport luminal allergens or even bacteria in large particles termed endosomes by transcytosis across the FAE to the antigen-presenting cells (APC) underneath and thus initiate mucosal immune responses (Keita et al. 2006). M cell has a large intraepithelial "pocket" formed by the invagination of basolateral membrane that inhabited by lymphocytes and mononuclear phagocytes. This special morphology not only narrowed the distance between apical and basal surface to make the allergens easier to translocate throughout the cell but also insure the immune cells contact with transported cargos in the first place. Moreover, M cells also lack the rigid brush border as well as the glycocalyx or microvilli, making the cells more easier to access to and take up the antigens (Kimura 2018).

On the other hand, it has been reported that PPs are not mandatory for the development of oral tolerance, a process that requires allergen transporting through intestinal barrier, so other cell types other than M cells can also transport and sample food proteins (Ménard et al. 2010). For example, the transport of food proteins in normal human IEC (columnar enterocytes) has been observed and reported (Heyman et al. 1988; Wheeler et al. 1993). Furthermore, goblet cells, which produce mucins to form the mucus layer, can also participate in allergen transcellular transport. In vivo imaging approach showed that small intestine goblet cells transport small molecular weight antigens from the intestinal lumen to lamina propria and selectively deliver the antigens to tolerogenic CD103⁺ DCs but not inflammatory CD103⁻ DCs and thus maintain intestinal immune homeostasis (McDole et al. 2012). This process, which was named goblet cell associated antigen passages (GAPs), was further demonstrated to be involved in the secreting and processing of mucin proteins by goblet cells (Gustafsson et al. 2017).

In addition to relying on the transcellular transport in other cell types, some myeloid cells, especially the CX3CR1⁺ monocytic DCs, can directly contact with lumen allergens by penetrating the epithelium with their transepithelial dendrites (TEDs) (Knoop et al. 2013). In a co-culture cell model, bone marrow-derived DCs expressed tight junction proteins and extended their dendrites through the epithelium to sample bacteria (Rescigno et al. 2001), which is further demonstrated to be regulated by the Toll-like receptor (TLR) signaling (Chieppa et al. 2006). Both lamina propria DCs and PP DCs can form TEDs, and cytokines including CX3CL1 and CCL20 can regulate that process (Niess et al. 2005).

3.2.6 *Antibody-Mediated Transport*

Accumulated evidence has shown that antibodies, including IgA, IgG, and IgE, are involved in antigen transcellular transport.

IgA is the most abundant antibody in the human body that is produced 5–15 g every day in adult and performs a very specialized role which involves mucosal immunity, development of tolerance, and protection against infection. Though a small portion of IgA exists as a monomer and is presented in serum, it is mainly secreted into the intestinal lumen as a dimer (sIgA) (Singh et al. 2014); consequently, IgA is the most representative Ig isotype at the mucosal surface and plays important role in mucosal immunity. Though the main function of IgA is considered to be restricting the entry of exogenous antigens in the intestinal mucosa, it can also help to transport the antigens by retrotransport. In gluten-induced celiac disease, when recognized by sIgA, the gliadin peptides were subsequently retrotransported from apical surface to basal surface into the intestinal mucosa, and the process is dependent on the transferrin receptor CD71 as well as the interaction between sIgA and CD71 (Matysiak-Budnik et al. 2008; Heyman and Ménard 2009).

Though normally present in serum, IgG have also been reported to be secreted into the intestinal lumen and play a role in allergen transepithelial transport. By interacting with Fc receptor FcRn on the epithelium membrane, IgG can transfer through the epithelial barrier on both directions (Dickinson et al. 1999). When binding with specific IgG, antigen can be transported across the epithelial barrier in a FcRn-dependent way and processed in the endosome, then the IgG-antigen complex is released from the receptor due to the change of pH, and the antigen is presented to downstream cells and thus triggers subsequent immune responses (Jones and Waldmann 1972; Berin 2012).

IgE is the most important antibody in food allergy development, which is produced by the specific Th2-induced B cells. The most well-known function of IgE is binding to the FcεRI receptor on mast cell surface and triggering mast cell degranulation when contact with the specific allergen again. However, IgE receptors, including the high affinity FcεRI as well as the low affinity FcεRII (CD23), are also expressed by IECs (Kaiserlian et al. 1993; Bannert et al. 2012), and IgE is also present in intestinal lumen, which was suggested to be produced from pancreas (Freier et al. 1983). It has been reported that IgE can be transported through IEC via both directions (basal-to-apical and apical-to-basal) in a CD23-dependent manner (Tu et al. 2005; Li et al. 2006). During this process, the IgE-associated antigen in the intestinal lumen is also transported into the basolateral media, with the ability of triggering FcεRI-mediated mast cell degranulation retained (Li et al. 2006). Interestingly, the interaction between the antigen-IgE complex and CD23 can activate downstream inflammatory pathways within IECs and induce the release of chemokines such as CCL20 and thus could chemoattract cells with the correlated receptor CCR6 (such as memory T or B cells and immature DCs) (Li et al. 2007). In addition, it has also been proved that IgE facilitates the delivery of antigen from B cell to DC and T cell, which is also dependent on CD23 (Henningsson et al. 2011).

3.3 Allergen Recognition and Presentation in Intestine

Once food allergens get over the intestinal barrier, they come into the inner environment of the human body and contact directly with the immune cells. Antigen recognition, uptake, processing, and presentation by innate immune cells (especially the APCs) are the first steps of specific adaptive immune responses that may finally be developed into food allergy in the intestine. During this process, DC has been demonstrated to be the predominant APC, which is able to recognize a diverse set of conserved motifs on allergens (termed pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs)) by corresponding pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs). After that, by producing cytokines and presenting processed allergen peptides to downstream T cells, APCs promoted T cells differentiation and triggered subsequent immune responses. In addition to DCs, epithelia, mast cells, and B cells have also been reported to serve as APCs in food allergy process (Fig. 3.5).

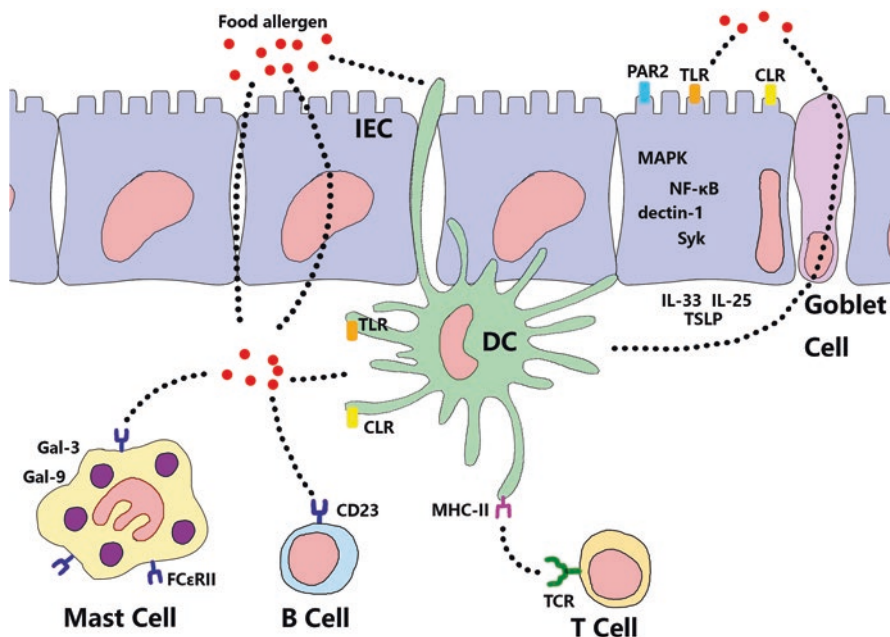


Fig. 3.5 Allergen recognition and presentation in the intestine. Antigen recognition, uptake, processing, and presentation are the first steps of specific adaptive immune responses. DC is the predominant APC that recognizes a diverse set of conserved motifs on allergens by specific receptors such as TLRs and CLRs and finally presents the allergen information to T cells. In addition, epithelium, mast cell, and B cell have also been reported to participate in allergen recognition and presentation

3.3.1 Allergen Recognition and Presentation by DC

DC is the most common APC in human bodies. Once taken up by DC, the allergenic protein is processed into peptides and presented on the surface of cell membrane by major histocompatibility complex (MHC)-II. After that, the peptide-MHC-II complex is presented to T cells, which together with the cytokines and co-stimulatory molecules drives naïve T cell differentiation into distinct effector T cells such as Th1, Th2, and Treg depending on both the nature of the allergen and the microenvironment (Salazar and Ghaemmaghami 2013).

In addition to capture allergens transported through the intestinal epithelial barrier, some DCs can also directly capture allergens in the gut lumen by intercalating between IECs with dendrites. During this process, DC expresses proteins such as occludin and claudin, so as to open and reform the tight junction with adjacent IECs without the disruption of epithelial barrier (Rescigno et al. 2001; Sung et al. 2006). Compared with other DC subtypes, CD103⁺ DC is the predominant one for the process, which shows approximately tenfold activity in capturing luminal antigen over other CD103⁻ or plasmacytoid DCs (McDole et al. 2012). Recent studies also revealed the role of goblet cell in delivering allergens from intestinal lumen to DCs. By using a minimally disruptive in vivo imaging approach, it was shown that small intestine goblet cell uptake the low molecular weight soluble antigens from the intestinal lumen to underlying lamina propria DCs. Interestingly, that antigens are preferred to be transported to anti-allergenic CD103⁺ DCs rather than allergenic CD103⁻ DCs, resulting in the polarization of Treg against Th17, and finally induced oral tolerance and maintained intestinal immune homeostasis (McDole et al. 2012). Further studies with mucus defect mouse models showed that both the processing and secretion of the mucus play important roles in regulating antigen delivery (Gustafsson et al. 2017).

DCs recognize conserved motifs on allergens by a set of PRRs especially TLRs and CLRs. TLRs are the most well-identified PRRs, which contains more than ten members in human. Each TLR recognizes ligands by the N-terminal leucine-rich repeat motif and transduces the signaling to downstream molecules by the C-terminal Toll/interleukin-1 receptor homology (TIR) domain (Botos et al. 2011). As the receptor of lipopolysaccharide (LPS), TLR-4 can also be activated by lipocalin family allergens such as Fel d 1 and Can f 6, which is dependent on MD2, an essential protein for TLR-4 function. Interestingly, Fel d 1 did not interact with TLR-4 directly but through the mediation of LPS, suggesting that a lipid transfer mechanism may be involved in (Herre et al. 2013). On the other hand, house dust mite (HDM) allergen Der p 2 activates TLR-4 as well as TLR-2 in a totally different mechanism. Due to the high homology with the MD2 molecule, Der p 2 serves as an auto-adjuvant and interacts with TLR4 to form a complex that signals similarly to MD2/TLR4 complex, inducing innate immune activation in the absence of LPS, which is independent of MD2 (Trompette et al. 2009; Chiou and Lin 2009).

Compared with TLRs, CLRs are receptors that recognize the oligosaccharide portion of other molecules including allergenic proteins (Sancho and Reis e Sousa 2012).

In human DCs, CLRs especially dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), mannose receptor (MR), and Dectin-2 have been shown to recognize allergens (Salazar et al. 2013). MR was identified to be a positional candidate gene for allergen-induced airway hyperresponsiveness by gene-mapping linkage analyses (Hattori et al. 2009). Further studies demonstrated that MR is a common receptor for allergens including HDM (Der p 1 and Der p 2), cockroach (Bla g 2), dog (Can f 1), and peanut (Ara h 1) (Salazar et al. 2013). When activated, MR drives DC to promote Th2 cell polarization, which is mediated by indoleamine 2,3-dioxygenase activity (Salazar et al. 2015). Similarly, it has been reported that DC-SIGN can recognize allergens from peanut (Ara h 1), pollen (BG-60), HDM (Der p 1, Der p 2), and dog (Can f 1) (Salazar et al. 2013). However, DC-SIGN normally inhibit allergic responses, which is opposite to MR. Knockdown of DC-SIGN led to a bias toward Th2 polarization, and the HDM allergen Der p 1 has been reported to cleave DC-SIGN protein and downregulate DC-SIGN expression. On the other hand, knockdown of MR led to a bias toward Th1 and against Th2 polarization. Moreover, it has been reported that Dectin-2 expressed by DC is also a receptor of allergens including HDM and mold (*Aspergillus fumigatus*). Once triggered by allergens, Dectin-2 promotes DC producing cysteinyl leukotriene, so as to trigger type 2 immune responses against allergens (Barrett et al. 2011).

In addition, the sugar moiety of glycosylated proteins is another common target for allergen recognition. It has been reported that deglycosylation of allergens by either chemical or enzymatic approaches can significantly decrease DC uptake (Al-Ghoul et al. 2012) and recombinant nonglycosylated allergen also showed less allergenicity compared with the native counterpart (Iacovacci et al. 2002). Based on this observation, recombinant allergen protein could be used as hypoallergen to prevent and treat type I hypersensitivity (Himly et al. 2003).

3.3.2 Allergen Recognition and Presentation by Epithelia

Directly interacting with allergens, the epithelia express multiple receptors to recognize allergens, present them by MHC-I, and produce specific cytokines to trigger downstream immune responses. Allergens with protease activity such as the main HDM allergen Der p 2 can activate the PAR-2, which further triggers subsequent signaling such as MAPK, NF- κ B, and TNF pathway and modulates immune responses (Salazar and Ghaemmaghami 2013). Consistently, PAR-2 has also been proved to be required for several allergic diseases (Day et al. 2012; Lianto et al. 2018).

Similar with the case of DC, TLR-4 of epithelium also plays an important role in food allergy; however, the influence is not consistent but highly antigen- and strain-dependent (Berin et al. 2006). Interestingly, the activating level of TLR-4 by LPS is crucial for driving either Th1 or Th2 response, with lower level of LPS inducing Th2 response and higher levels of LPS inducing Th1 response, which may reflect the role of microbiota homeostasis in regulating host immunity (Eisenbarth et al. 2002).

Epithelia also express specific receptors to recognize the sugar moiety of allergens. It has been reported that HDM-induced CCL20 production by air epithelial cell is dependent on the β -glycan moiety of HDM extract, which could be mediated by the Dectin-1/Syk signaling pathway (Nathan et al. 2009). Additionally, the increase in TSLP secretion by lung epithelia upon natural Der p 1 stimulation was also carbohydrate dependent, since the deglycosylated preparation of Der p 1 showed less efficiency (Al-Ghoulleh et al. 2012).

3.3.3 Allergen Recognition and Presentation by Other Cell Types

Besides DCs and epithelia, there are several other cell types such as mast cells and B cells that can recognize and response to allergens.

Mast cell expressed high affinity Fc ϵ RII receptor that binds to IgE during allergy sensitization phase and further activated by binding with specific allergen during effector phase, resulting in mast cell degranulation and allergic reactions. However, in addition to specific allergens, the IgE bound on mast cell surface may also interact with the autocrine regulator Gal-9, leading to the blockage of mast cell degranulation and alleviation of allergic reactions (Niki et al. 2009). Moreover, Gal-9 also directly bound the CD44 adhesion molecule and inhibited interactions with hyaluronan on T cells. As a result, Gal-9 was considered to be a potential therapeutic approach for Th2-associated inflammation (Katoh et al. 2007). By contrast, besides blocking mast cell degranulation, Gal-9 may serve as a recruiter of eosinophil, hence promoting dominant Th2 responses (Sziksz et al. 2010). Similarly, Gal-3 is also important for mast cell activity; the deletion of Gal-3 gene in mast cells or the whole mice will alleviate Th2 responses by promoting Th1 polarization (Chen et al. 2006; Saegusa et al. 2009). Interestingly, Gal-3 was substantially increased in intraepithelial gut lymphocytes of food allergic patients compared with non-allergic subjects, suggesting a potential role of Gal-3 during allergic inflammatory processes (Mercer et al. 2009). However, though expressing MHC-I molecule and secreting several allergenic cytokines, how mast cell presents the allergen signaling to T cells is largely unknown and requires far more investigations (Stelekati et al. 2009).

On the other hand, B cells express the low affinity IgE receptor Fc ϵ RI (CD23), which accompanies specific IgE to function as an antigen focusing mechanism, including the uptake and presentation of allergens (Kehry and Yamashita 1989; Pirron et al. 1990). It has been demonstrated that antigen presentation to T cells by IgE is dependent on CD23-expressing B cells; however, antigen presenting to T cells is performed by DCs rather than B cells in vivo. Consequently, B cells should be antigen-transporting cells that deliver antigens to DCs for presenting to T cells (Henningsson et al. 2011).

3.4 Intestinal Barrier Defects and Food Allergy

There is a long-standing hypothesis that a primary defect of the intestinal barrier might promote a shift from tolerance to sensitization state due to the excessive absorption of antigens and therefore initiate digestive diseases such as food allergy, celiac disease, and IBDs (Ménard et al. 2010). However, some studies have concluded that constitutive modification of epithelial permeability participates in a self-perpetuating cycle that maintains allergic inflammation rather than the development of food allergy and suggested the increased epithelial permeability being more the consequence rather than the cause of food sensitization (Heyman 2005). A vast body of studies have investigated the bidirectional relationship between intestinal barrier defects and food allergy.

On the one hand, because intact allergens can hardly gain access to the gut lamina propria due to exclusion by the tight junctional barrier, the defect of intestinal barrier and the increase of intestinal permeability can greatly promote the interaction between allergen and immune cells, resulting in the allergic responses. Rat model study showed that chronic psychological stress promoted the production of factors such as corticotropin-releasing hormone to induce intestinal barrier defect, which increased the uptake of luminal proteins and predisposed animals to sensitization of orally delivered antigens (Yang et al. 2006). However, these results cannot conduct the causal relationship between intestinal barrier defect and food allergy, as psychological stress may directly induce allergic responses in a mast cell-dependent way (Wijngaard et al. 2009). Another study demonstrated that mast cell-dependent intestinal barrier defect can promote antigen sensitization, as the administration of cromolyn, a mast cell stabilizer, blocked the barrier defect as well as the sensitization. However, the causal relationship is still uncertain because both the barrier defect and sensitization could be the direct downstream of mast cell activity and independent of each other (Forbes et al. 2008). Yet a contradictory study showed that the treatment of dextran sulfate sodium (DSS), a compound that impairs intestinal barrier function, remitted ovalbumin-induced food allergy. However, the coinstantaneous onset of colitis induced by DSS made the conclusion fallible, and the decrease of Th1-type cytokine IFN- γ after DSS stimulation also implied the complexity of the underlying mechanisms (Ino et al. 2016).

On the other hand, several kinds of allergic cells and cytokines are produced during food allergy process, which can impair the intestinal barrier in either direct or indirect ways. Clinical studies in children with cow's milk allergy demonstrated that intestinal permeability rise after, rather than before, allergen challenge (Jalonen 1991). And biopsy specimens exposed to food allergen *in vitro* showed decreased expression of tight junctional protein in tissues obtained from patients with food allergy compared to those from normal subjects (Pizzuti et al. 2011). *In vitro* cell models have demonstrated the destructive effects of Th2-type cytokines on intestinal barrier. With IL-4 stimulation, the transepithelial resistance decreased, and the apical-to-basal movement of protein indicator (such as the macromolecular horseradish peroxidase (HRP) and small molecular peptide) increased. Interestingly, both

the transcellular and paracellular transport pathways are influenced, and anti-allergenic cytokines IL-10 and TGF- β inhibit the IL-4-induced macromolecule but not small peptide flux, while only TGF- β but not IL-10 attenuated the transepithelial resistance drop, implying complex underlying mechanisms (Berin et al. 1999; Di Leo et al. 2002). Similarly, IL-13 also impaired the transepithelial resistance through the induction of cell apoptosis and pore-forming claudin-2 expression (Heller et al. 2005, 2008). Moreover, PI3K pathway has been demonstrated to be a major proximal signaling event in the process, which provided potential pharmacological target for manipulating epithelial permeability (Ceponis et al. 2000; Di Leo et al. 2002). In addition to Th2-type cytokines, the inflammatory mediators released by mast cell degranulation also result in the intestinal barrier defects. It has been reported that mast cell tryptase cleaves PAR-2 on colonocyte to decrease transepithelial resistance, increase transepithelial flux of macromolecules, and induce redistribution of tight junction proteins. Further investigations showed that the process is dependent on downstream β -arrestin-mediated activation of ERK1/2, as the inhibition of ERK1/2 abolished the effects of PAR-2 agonist on permeability and the redistribution of F-actin (Jacob et al. 2005). In addition, the mast cell-produced TNF- α can also impair the intestinal barrier through the disruption of tight junctions, which could be counteracted by diosmectite, a common medicine for gastrointestinal diseases (Mahraoui et al. 1997).

Overall, it should be noted that though tightly correlated, the direct regulation between intestinal permeability rise and food allergen sensitization remains to be established to date and therefore requires far more investigations.

3.5 Food Allergy Control by Changing Allergen Permeability

Due to the tight correlation between intestinal barrier defect and food allergy, targeting and restoring the epithelial barrier function could be a promising approach to treat food allergy. Unfortunately, no clinical therapy for intestinal barrier defect is available to date, and a promising candidate (larazotide acetate) was also failed in clinical trials (Odenwald and Turner 2017). Nevertheless, several potential approaches to target the epithelial barrier have been proposed.

3.5.1 *Direct Treatment of Intestinal Barrier Defect*

Several molecular and cellular therapeutic methods for directly regulating intestinal barrier defect are under investigation nowadays. Although each one still shows some inadequacy that impaired the clinical applications, they are promising candidates and could be reliable after far more investigations and improvements in the future.

As a core mediator for regulating protein paracellular transport, MLCK could be a potential target for regulating tight junction and restoring intestinal permeability. It has been demonstrated that the long isoform of MLCK mediated the TNFR2-induced tight junction dysregulation and subsequent colitis, although the disease will progress to MLCK-independent at advanced stage (Su et al. 2013). Similarly, MLCK-dependent epithelial barrier dysfunction is also required for the development of T cell-mediated acute diarrhea. Notably, the application of a highly specific MLCK inhibitor peptide prevented the tight junction disruption and diarrhea, suggesting the inhibitor to be a potential drug for treating intestinal barrier defect and diarrhea (Clayburgh et al. 2005). On the other hand, the activation of MLCK is also correlated with allergic responses. Chemokine CCL11 activates MLCK through both ERK1/2 and p38 MAP kinases, resulting in the subsequent eosinophil migration, which can be blocked by the MLCK inhibitor ML-7 (Adachi et al. 2003). Interestingly, MLCK also participated in IgE-mediated cell degranulation, as the inhibition of MLCK by the specific inhibitor wortmannin or KT5926 blocked the IgE-mediated histamine release from RBL-2H3 and human basophils cell models (Kitani et al. 1992). Moreover, the anti-allergic drug disodium cromoglycate, whose mechanism partially relies on the stabilization of mast cells but not totally cleared yet, is structurally related to quercetin, a drug that can enhance intestinal barrier function of human intestinal cell; however, the direct effect of disodium cromoglycate on epithelial barrier has not been studied yet (Corey 2010). Unfortunately, current inhibitors show low specificity to different MLCK isoforms; however, these isoforms may play important roles in other physiological processes; therefore the use of MLCK inhibitors may result in side effects. For example, MLCK is necessary for maintaining smooth muscle function, and the deletion of MLCK in smooth muscle will result in hypotension and impaired smooth muscle contraction (He et al. 2008, 2011). Moreover, MLCK deficient mice also lacked bronchial constrictions in both normal and asthmatic airways, although the asthmatic inflammation response was not affected (Zhang et al. 2010). Consequently, more specific inhibitors for targeting long MLCK isoform must be developed before the application in treating human diseases.

In addition to MLCK, the other tight junction proteins such as claudin2 and occludin could also be potential therapeutic targets for restoring barrier dysfunction. By upregulating the expression of occludin, a short peptide derived from milk casein (NPWDQ) adjusted the intestinal barrier and thus inhibited the permeation of ovalbumin (Tanabe 2012). Nevertheless, yet only limited proper inhibitors for these tight junction proteins are available (Odenwald and Turner 2017).

Besides the tight junction proteins, the intestinal epithelial permeability can also be regulated by cellular signaling. For example, the EGF receptor EGFR and ErbB1 inhibited TNF-stimulated intestinal cell apoptosis and mediated TNF-induced cell survival (Yamaoka et al. 2008). The epithelial mitogen R-spondin 1 was also reported to reduce DSS-induced inflammation by preserving the mucosal barrier function and stimulating crypt cell growth (Zhao et al. 2007). However, it should be noted that due to the multi-effects of these proteins, the hyperactivation of these

signals can upregulate other important pathways such as the Wnt signaling and thus may finally result in diseases including cancer (Odenwald and Turner 2017).

In addition to directly regulating critical genes, the engraftment of intestinal stem cells has been proposed as another therapy for repairing damaged intestinal barrier. Advanced intestinal organoid culture technology has made long-term culture and expansion of intestinal stem cells in vitro possible, which shed light on the isolation, expansion and transplantation of intestinal stem cells in aiding epithelial regeneration and barrier restoring (Sato et al. 2009). Notably, the transplanted fetal-derived organoids can be partially differentiated into functional goblet cells (Fordham et al. 2013). Based on this, colonic organoids were introduced into mice with DSS-induced colitis, and the results showed that the organoids localized to the injured areas; moreover, they engrafted and remained as a transplant with proper differentiation and no tumor formation was observed, and finally the symptoms of colitis were relieved (Yui et al. 2012). However, due to the potential tumor-genesis ability of stem cells and the unclear repairing mechanisms, far more investigations and improvements are required before safe and effective clinical applications.

Notably, because tight junction permeability is physiologically regulated to facilitate nutrient transport, simply strengthening the barrier function may not only block pathogen and allergen transport but also inhibit nutrient absorption. Overall, only when revealed the detailed mechanisms of all the cellular and molecular approaches and totally wiped out the possibility of destructive side effects can these promising methods be applied in clinical therapy.

3.5.2 Restoring Barrier Defect by Treating Diseases

Some diseases other than food allergy such as IBD (including ulcerative colitis and Crohn's disease) can result in intestinal barrier defect, which may further exacerbate food allergy. Consequently, treating these underlying diseases could be an ideal therapy for alleviating epithelial barrier dysfunction. Clinical data has indicated that dairy allergy was tightly correlated with ulcerative colitis (Judaki et al. 2014). Another research with Finnish children also indicated that cow's milk allergy in infancy was associated with ulcerative colitis and Crohn's disease, but childhood asthma was only associated with Crohn's disease (Virta et al. 2013). On the other hand, compared with healthy control, patients with Crohn's disease showed significantly stronger allergic responses to diverse food allergens, indicating the induction of food allergy by Crohn's disease. However, only marginally difference was observed between healthy controls and ulcerative colitis patients. Combined with mouse models, the correlation between Crohn's disease and food allergy was suggested to be involved in IL-10 (Kawaguchi et al. 2015). On the contrary, administration of food allergen may also initiate colitis in mouse models, and elimination of the allergen will ameliorate the development of colitis, which is suggested to be independent of intestinal microbiota (Kawaguchi et al. 2015; Li et al. 2016).

Overall, though only limited evidence of the direct causality between food allergy and IBD has been obtained nowadays, the tight correlation between them is significant. Taking the underlying relationship of intestinal barrier defect into consideration, the combination therapy of food allergy and IBD could be a promising clinical application.

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Chapter 4

Detection and Quantification Methods for Food Allergens



Abstract Food allergy represents an important issue in the field of food safety in industrialized countries because of the severity of allergenic reactions for affected persons even with small amounts of food. Recently, the food industries and legislative and regulatory agencies in different countries have formulated new regulations about displaying food allergen on food labels. Therefore, consumer protection and food labeling require reliable detection and quantification methods of allergens in food products. Several analytical approaches, with the ability of quantifying and detecting traces of allergenic ingredients, which target either the allergenic proteins or allergen markers (peptide fragment or gene segment) have been developed. The most popular methods for allergen detection can be mainly divided into two large groups: the protein-based approaches and the DNA-based approaches. Protein-based methods include enzyme-linked immunosorbent assays (ELISA), MS methods, and biosensors. On the basis of the amplification of specific DNA fragments, DNA-based methods can trace the potential presence of culprit allergen, which include polymerase chain reaction (PCR) and real-time PCR. In this chapter, we present the advances about specific and highly sensitive analytical methods in the field of food allergens detection, which can avoid the influence by the presence of matrix components.

Food allergies represent an important food safety concern in industrialized countries on account of the severity of allergenic reactions even with small amounts of food. Approximately 1–2% of the general population suffer from some form of food allergy, with a higher prevalence in children than in adults (Chafen et al. 2010). Food allergy mainly is mediated by allergen-specific immunoglobulin E (IgE), usually against certain food antigens. Generally, even trace amounts of an allergen can cause a very strong reaction in sensitive individuals, with a wide variety of symptoms in the skin (urticaria, atopic dermatitis), respiratory system (rhinitis, asthma), and circulatory system (edema, hypotension), and even life-threatening reactions such as IgE-mediated anaphylactic shock (Berin and Sicherer 2011). In most countries, 90% of allergic reactions occur by eight foods: cow's milk, wheat, chicken egg, fish, soy, tree nut, shellfish, and peanut; among them, reactions to egg

and cow's milk are common in children, while fish and shrimp are more frequent in adults (Boyce et al. 2011). Most food allergens are proteins or glycoproteins with molecular mass of 5–100 kDa, which usually represent the predominant ingredients with the ability to cross-link IgE receptors in a particular allergenic food (Cucu et al. 2013).

Currently, the only effective management for food allergy is the complete avoidance of the allergen-containing food, relying on providing allergen information on food labels. Nevertheless, it is difficult for allergic individuals to totally avoid, since food products contain a large variety of allergenic ingredients from allergenic food materials and cross-contamination during shipping, storage, and processing. Therefore, allergen control measures are implemented to decrease the risk of allergen contamination, for instance, frequent monitoring of manufacturing surfaces and foods for allergen residues. Sensitive individuals may inadvertently have contact with allergenic ingredients by taking food products which are supposed to be free of a certain allergen. Additionally, trace amounts of allergens can induce severe even fatal reactions in sensitive individuals. Generally, the threshold of hazelnut is 10 μg taken by individuals, while the threshold values are 0.07 μg of milk, 0.003 μg of egg, 0.5 μg of peanut, and 0.3 mg of soybean (Bindslev-Jensen et al. 2002; Cochrane et al. 2015). In accordance with the new EU Directive 2007/68/EC, a list of 14 groups of allergenic foods are required to declare on their labels, including gluten-containing cereals, crustaceans, eggs, fish, peanuts, soybeans, milk, nuts (almond, hazelnut, walnut, cashew, pecan nut, Brazil nut, pistachio nut, macadamia nut, and Queensland nut), celery, mustard, sesame seeds, lupine, mollusks, and sulfites (Taylor and Baumert 2015).

With the main aim of helping food industries and food manufacturers to guarantee the correct labeling of food products and protect allergic consumers, several methods have been developed for the detection and quantification of food allergens. The employed analytical methods should be specific and highly sensitive and can avoid the interference by the presence of matrix components. These methods can target either the allergenic proteins or allergen markers (peptide fragment or gene segment). Protein-based methods, mostly relying on immunological (antibody-based) techniques, are widely applied for determination of allergen itself. Protein-based methods include enzyme-linked immunosorbent assays (ELISA), immunoblots, lateral flow immunoassay (LFIA), MS methods, and biosensors. On the basis of the amplification of specific DNA fragments originated from the offending food, DNA-based methods include polymerase chain reaction (PCR), endpoint quantitative PCR, real-time PCR, and PCR-ELISA.

4.1 Immunoanalytical Methods

Immunoassays are analytical techniques based on the specific and high-affinity interactions of antibody with target antigen to obtain information about antigen concentration in samples. Normally, the antibodies utilized in immunoassays are mainly

of two categories: polyclonal antibodies, when antiserum comprises immunoglobulins used from any immunized animal that bind different epitopes on the antigen, or monoclonal antibodies, which are produced from a clone of a single hybridized B cell and therefore bind antigen with only a single epitope. Immunoassays are one of the most popular methods of the quantitative allergen detection because of their high sensitivity, specificity, and versatility.

Historically, the most common techniques for allergen detection based on immunochemistry include radio-allergosorbent test (RAST), enzyme allergosorbent test (EAST), rocket immunoelectrophoresis (RIE), enzyme-linked immunosorbent assay (ELISA), dot immunoblotting, and protein chip. However, RAST, EAST, and ELISA are quantitative methods, and RIE and dot immunoblotting render only qualitative or semiquantitative results.

The RAST is a radioimmunoassay test to diagnose specific IgE antibodies to the clinically suspected or known food allergens in the sera from allergic patients. This method is widely used to diagnose food allergies in vitro and in clinic. In this procedure, the specific anti-IgE antibodies bound to food allergen-recognized IgE on the surface of a solid phase carrier are detected. On the basis of the established DNA microarray technology, protein chips generally utilize covalent linking of protein ligands to the activated silica surface of microscope slides. In comparison with other immunoassays, protein chips offer the greatest advantage for detecting hundreds of allergens simultaneously. Li et al. (2010) developed a protein chip based on sandwich immunoassay, with the limit of detection (LOD) being 0.054 mg tropomyosin/kg food, to quantify shrimp allergen tropomyosin. Lin et al. (2011) designed a 3D aldehyde protein chip for the detection of fish allergen parvalbumin in food matrices, with the LOD and limit of quantitation (LOQ) being 0.048 and 0.087 mg parvalbumin/kg food. In addition to these conventional methods, the other classical immunoanalytical methods will be introduced in detail.

4.1.1 ELISA

The ELISA is the gold standard and most widespread method employed in industries and official food control agencies for food allergen detection, offering a simple, reliable experiment with suitable sensitivity for allergic ingredients in different sample matrices. The analytes could be any specific allergens, either one allergen or a mixture of more than one allergen. In ELISAs, two enzymes, horseradish peroxidase and alkaline phosphatase, are by far the most commonly used labels. The labeled enzyme converts a colorless substrate to a colored soluble product in the reaction mixture. Therefore, the intensity of color product directly assesses the amount of target protein antigen present in the sample extract. Interestingly, two formats of ELISA are chosen as typical approaches for the quantification of food allergens: competitive ELISA (Fig. 4.1a) and sandwich ELISA (Fig. 4.1b).

In the sandwich ELISA, two different specific antibodies make a sandwich with the antigen, and these antibodies can recognize different epitopes of this antigen.

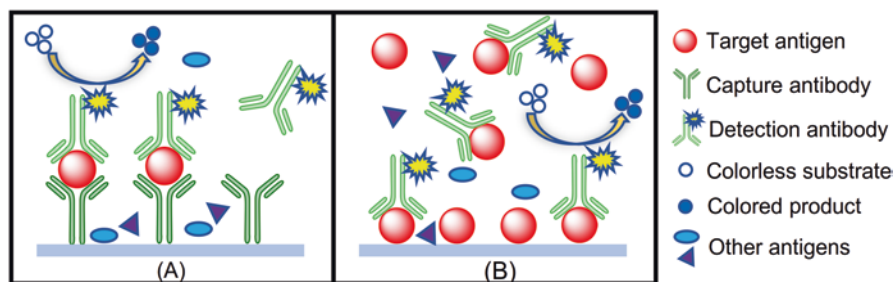


Fig. 4.1 Two formats of ELISA. (a) Competitive ELISA. (b) Sandwich ELISA

Generally, the first antibody (capture or coating antibody) is immobilized onto a hydrophobic solid phase such as plastic, and excess first antibody is removed by washing with a washing solution. Then, as the “meat” of the antibody sandwich, the target antigen is immobilized with the first antibody. After washing away the remaining unbound molecules, the second antibody (detection antibody) labeled with an enzyme is introduced. Thus, enzyme will only be present if the second antibody has been immobilized by binding to the immobilized target antigen. With a sandwich ELISA format, amount of color seen is directly related to the amount of target antigen present in the extracted food sample. Fæste and Plassen (2008) developed a novel sandwich ELISA with the LOD being 0.01 mg parvalbumin/kg food for the quantitation of fish allergen parvalbumin in food matrixes, using a polyclonal rabbit anti-cod parvalbumin antibody for capture and a biotinylated conjugate of the same antibody for detection. Zhang et al. (2014) established a sandwich ELISA for measuring the concentration of shrimp allergen tropomyosin in food and food materials with the LOD being 0.09 ng/mL, using a specific monoclonal antibody (mAb), CE7B2. Kamath et al. (2014) described an IgG antibody-based immunoassay to enable the specific and sensitive quantification of the major food allergen tropomyosin present in the environment of two crab processing facilities, with an excellent LOD being 60 pg/m³. Peng et al. (2013) designed a highly sensitive sandwich ELISA based on two mAbs with the LOD being 0.34 ng/mL for monitoring the major peanut allergen in food products by measuring Ara h 1 content. Hei et al. (2012) presented a sandwich ELISA to trace the amount of β -conglycinin in soybean and soybean products with the LOD being 1.63 ng/mL, where mouse anti- β -conglycinin monoclonal antibody (mAb 5C5) was used as coating antibody and rabbit anti- β -conglycinin pAb was used as secondary antibody. Tao et al. (2015) developed a sandwich ELISA for the determination of the major soybean allergen Gly m 4 with the LOD being 2.1 ng/mL, using a mouse anti-Gly m 4 mAb and a goat anti-Gly m 4 pAb as the capture and detection antibodies, respectively. Morishita et al. (2008) described a novel sandwich ELISA with the LOD being 0.47 ng/mL for the detection and quantification of the major soybean allergenic protein Gly m Bd 30K in processed foods, employing pAbs raised against p34 as a soybean marker protein and a specific extraction buffer for extract.

In the competitive ELISA, standard of target allergen is first immobilized to a solid surface by hydrophobic interactions. Next, a competitive reaction is created between the bound allergen and the free target antigen molecule in the sample for binding to the specific antibody labeled with an enzyme. Therefore, in competitive ELISA format, there is an inverse relationship between the amount of color seen and the amount of target antigen present in the sample. Cai et al. (2013) developed a competitive ELISA to quantify the amount of fish allergen parvalbumin in silver carp using a mAb (B2-E1) against silver carp parvalbumin, while the LOD of this method was 0.04 mg parvalbumin/kg food. Wang et al. (2014) described an indirect competitive ELISA with the LOD being 2.2 ng/mL for the detection and quantification of walnut soluble proteins in processed foods. Ma et al. (2010) established a competitive ELISA based on mAb 4B2, a mAb against glycinin, to measure soybean allergen glycinin. The LOD of this method was 0.3 ng/mL.

The disadvantage of ELISA is that it provides a significant rate of false positive results because of antibodies cross-reactivity. Additionally, it is also prone to false-negative results due to the conformational change, denature, or degradation of epitopes during thermal food process (Gomaa and Boye 2013).

4.1.2 Immunoblots

Generally, immunoblot methods for food allergen analysis include Western blot and dot blot analysis. Western blot, a routine method, combines two technologies: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoassay. Figure 4.2 briefly describes the procedure of Western blot. In the first

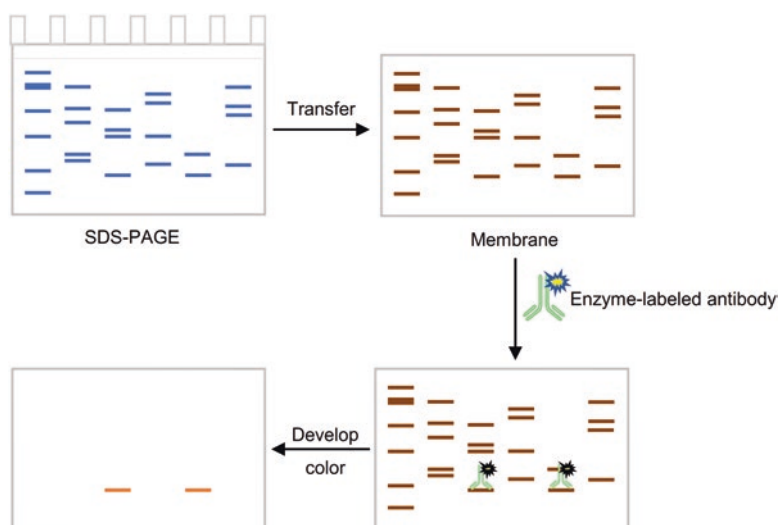


Fig. 4.2 The procedure of Western blot technique

step, the proteins in food sample are separated by SDS-PAGE according to their molecular mass. Then, the separated proteins are transferred from the polyacrylamide gel to a membrane (polyvinylidene fluoride, PVDF, or nitrocellulose) and afterward subjected to an immunoassay to analyze the target antigenic proteins. After blocking the nonspecific binding site, an enzyme-labeled antibody is immobilized on the membrane, where the target allergen can react with the antibody and a colored band forms at the site. Unlike ELISA, the color intensity and width of the band together indicate the concentration of the target allergen in the food sample extract. The greatest advantage of Western blot is that target allergens can be separately analyzed by the changes of protein bands. However, Western blot suffers from many disadvantages: (1) conformational epitopes and new IgE-binding epitopes of allergenic proteins may not be tested in denatured form, and (2) allergenic proteins with too big or too small molecular weight may not be properly evaluated for the resolution of the gel or blotting membrane.

The dot blot analysis represents a simplified version of Western blot. The sample is directly adsorbed onto a membrane (i.e., nitrocellulose or PVDF) via adding a drop of food sample extract. Unlike Western blot, target allergenic protein is directly analyzed by specific antibodies without separating electrophoretically. A general guideline describes a commonly used antigen bound dot blot assay for detecting an allergenic protein in food samples is shown in Fig. 4.3. Dot blot, therefore, is a method of immunoblotting that does not involve denaturing conditions, and confor-

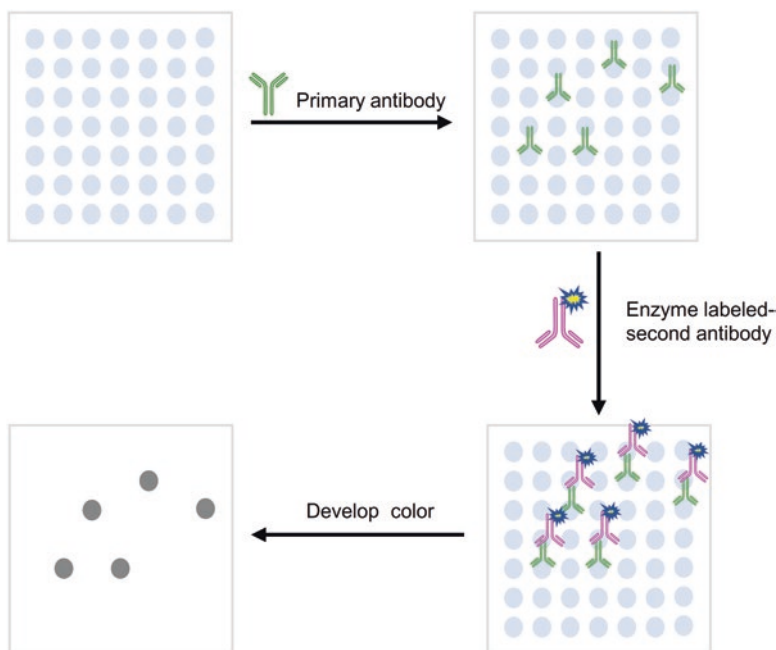


Fig. 4.3 The procedure of Dot blot technique

mational epitopes are preserved. Because proteins are not separated by molecular weight as in the case of Western blotting, the immunogenicity of the entire sample is analyzed; however, if the sample consists of an isolated protein, then single proteins may also be analyzed.

4.1.3 *Lateral Flow Immunoassay*

Lateral flow immunoassay (LFIA), also known as lateral flow strip (LFS), was developed in the 1950s. On the basis of ELISA method, the LFS is commonly used for food allergen detection in recent years because of its characteristics including low cost, speediness, reliability, and simplicity. There, home pregnancy test, the best known LFS method, is described a urine-based diagnostic test for pregnancy.

Recently, LFSs have been applied in detection of food allergens. Wang et al. (2017) established a rapid lateral flow colloidal gold immunoassay strip in a sandwich format, relying on the colloidal gold-labeled mouse anti- β -conglycinin mAb and the rabbit anti- β -conglycinin pAb for the determination of soybean allergen β -conglycinin. The LOD of the test strip was calculated at 1.66 mg/kg in powdered milk using an optical density scanner that measures relative optical density. Wang et al. (2015) presented a rapid immunochromatographic lateral flow test strip in a sandwich format, with the colloidal gold-labeled mouse anti-glycinin mAb and rabbit anti-glycinin pAb to specifically identify glycinin, a soybean allergen. This test strip has high sensitivity, and results can be obtained within 10 min without sophisticated procedures. The LOD of the test strip was calculated to be 0.69 mg/kg.

Masiri et al. (2016) developed a rapid LFIA in both sandwich and competitive format with a sensitivity of 1 ppm and selectivity values of 3–5 ppm in nondairy beverages, employing a panel of pAbs directed against the modified proteins present in almond, cashew, coconut, hazelnut, and soy milks in order to detect modified allergens. Zheng et al. (2012) described a quantitative LFIA, employing superparamagnetic nanoparticle (SPMNP) probe based on mAb against allergen parvalbumin to detect fish major allergen parvalbumin. The LOD for qualitative detection was 0.046 μ g/mL.

Among these studies, test strips in the sandwich immunoassay format are becoming a widely used diagnostic tool for the determination of food allergens. Figure 4.4a depicts the typical configuration of sandwich LFS. In a test strip, several zones made of different materials are designed. These parts overlap onto one another and are mounted on a backing card using a pressure-sensitive adhesive. To perform the detection, first, the sample is added to the sample pad and migrates to the conjugate pad, where numerous small, primary antibody-coated colored particles have been localized. These colored particles (usually 20–40 nm diameter) can usually be colloidal gold or a colored, fluorescent, or paramagnetic monodisperse latex particle. Then, the target allergen in liquid samples is recognized with primary antibody-coated colored particles, and both migrate into the next section of the strip by capillary action, while other large molecules in the sample can be filtered out by a fiber

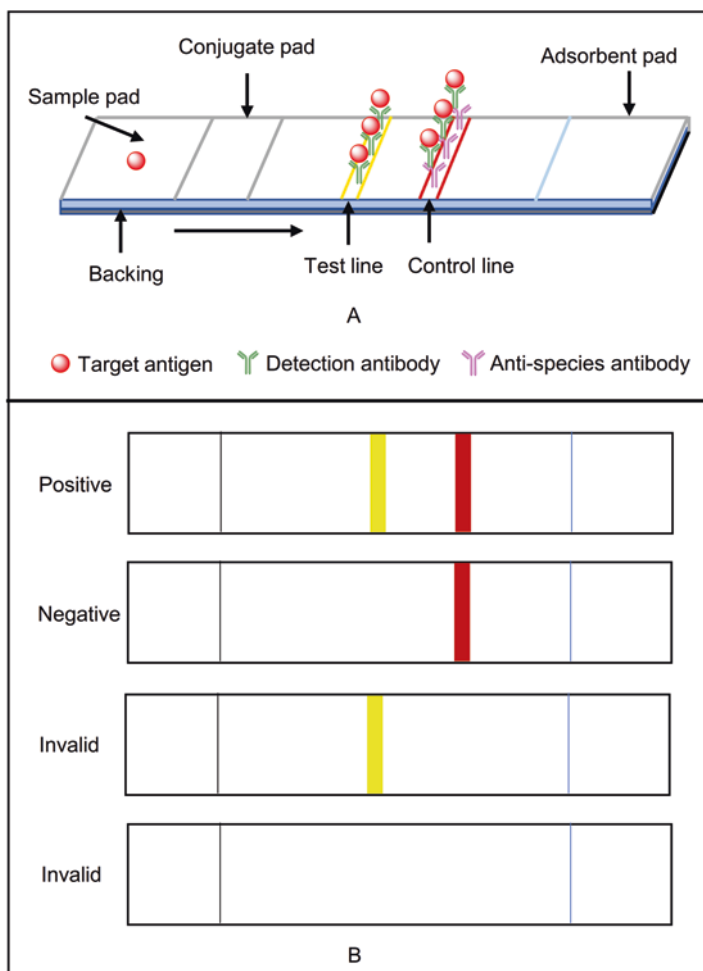


Fig. 4.4 Lateral flow immunoassay. (a) The typical configuration of lateral flow immunoassay. (b) Schematic view of a sandwich-type lateral flow test strip

filter placed in the sample pad area. Next, the target allergen binds to a second primary antibody, which has been immobilized at the test line (zone) on the surface of the fibers of the porous nitrocellulose membrane. In this case, a positive result is indicated by the presence of a test line. The excess antibody-colored particle conjugates move past the test line and bind to an anti-species antibody at the control line, serving to indicate that the test ran appropriately. As shown in Fig. 4.4b, four situations usually occurred when testing an allergenic protein with a sandwich LFS.

As a semiquantitative tool for allergens, the advantages of the LFIA are well known: (1) ease of use, minimal operator-dependent steps and interpretation, and (2) good stability, low, cost and sensitivity. Meanwhile, traditionally designed LFIA

also suffers from performance limitations: (1) it is difficult to simultaneously analyze multiple allergens in food samples; (2) the quantification of food allergens is limited.

4.2 DNA-Based Methods

Since DNA is more stable than other macromolecules to physical and chemical treatments, DNA-based methods have become efficient and powerful approaches for tracking down the presence of trace amounts of allergens in food materials and food products. DNA-based methods involve the detection of a defined DNA sequence of the genome of the allergenic food, which serves as a surrogate for the allergen. To utilize the DNA fragment as a good surrogate marker, the particular allergenic food must be inherently high in DNA content. On the basis of PCR, DNA-based detection of food allergen has been carried out with several methodologies, namely, endpoint quantitative PCR, real-time PCR, and PCR-ELISA. PCR assays do not detect allergenic proteins directly, so they are best used only in cases where the DNA is an accurate indicator of the presence of allergenic proteins.

In PCR assays, DNA can be efficiently extracted from difficult food matrices with less damage than proteins under harsh laboratory extraction conditions, since DNA is generally more stable than proteins in food processing. Besides, PCR assays contribute to the determination of allergenic ingredients at every stage of food processing. Furthermore, PCR assays can be applied to distinguish particular species or to detect broad groups of organisms, relying on gene targets chosen in accordance with desirable sensitivity and specificity characteristics.

4.2.1 *Endpoint Quantitative PCR*

In endpoint quantitative PCR, the number of target sequences in the samples is reflected by the amount of PCR product. In order to perform the quantitative analysis of DNA target sequence, three primer fragments (two senses and one antisense oriented) are used. The pair of short primer fragments, which can recognize the DNA target sequence characteristic for a given allergen source, are used for the PCR amplification. The other primer fragment as a probe is used to detect specific PCR amplification products. In the procedure, there are several key elements to obtain quantitative results, such as the amount of Mg^{2+} , forward and reverse primers, dNTPs, and DNA polymerase for PCR. Besides, it is crucial to determine PCR amplification conditions, which are related to the amount of DNA and the number of amplification cycles. The logarithm (log) of the quantity of DNA keeps a linear relationship with the log of the specific amplified gene product. This relationship is valid in a range, depending on the quantity of starting nucleic acid and the number of cycles of the PCR. Taguchi et al. (2011) carried out endpoint PCR to discriminate

allergenic ingredients of shrimp and crab. Both PCR methods could detect 5 pg of DNA extracted from target species and 50 ng of genomic DNA extracted from incurred foods.

4.2.2 *Real-Time PCR*

Currently, real-time PCR, a golden standard for qualitative and quantitative detection of both DNA and RNA targets, is increasingly being utilized in the precise quantitative determination of food allergens. In real-time PCR, the target allergenic protein is analyzed through monitoring the accumulation of fluorescent signal produced during the amplification of its specific DNA fragment in real time. In food analysis, the real-time PCR depends on DNA-binding dyes or fluorescent probes for amplification signal detection. The non-sequence-specific SYBR Green is widely applied as DNA-binding dyes to increase greatly fluorescence through binding to double-stranded DNA. With the extension of PCR products of specific DNA fragment, the fluorescence intensity increases, which is related to the amount of generated PCR products of every double-stranded DNA (dsDNA), including primer dimers and other nonspecific products. SYBR Green assay is more cost-effective than the fluorescent probe-based assays. However, SYBR Green is not the best choice when the concentration of target DNA fragment is too low (<1000 copies) due to nonspecific amplification and the increase of primer dimer products.

TaqMan probe, an oligonucleotide probe, exploits reporter attached to its 5' end and the quencher at the 3' end. When the probe pair-bonds with the target sequence, the fluorescence emitted by reporter is quenched because it is close to the quencher at the 3-prime end. During the elongation step of PCR, TaqMan probe exploits the 5'-nuclease activity of the DNA polymerase to hydrolyze a dual-labeled hybridization probe. Hydrolysis causes uncoupling of fluorescent labels and results in fluorescence emission. In a real-time PCR reaction, the generation of new PCR products is related to the production of a fluorescent signal, while the amount of PCR products is measured at each cycle using TaqMan probe. The use of TaqMan probe is beneficial to increase the sensitivity and specificity of real-time PCR assay due to the reduction of the interference of nonspecific products and the formation of primer dimers.

In real-time PCR, the entire process of amplification is monitoring and continuous analysis by the detection of fluorescent signals, which can be plotted into a curve. As shown in Fig. 4.5, there are no significant changes in the level of fluorescence at the beginning of PCR reaction. Subsequently, the fluorescence signals enter the exponential, linear, and final stationary phase. Therefore, the amount of PCR products can be detected in the exponential phase, and the initial content of the target DNA can be calculated from this. The C_T value (cycle threshold), a given sample generates signal, is determined during the exponential phase of the reaction, which is related to the amount of target DNA in the sample. A linear standard curve is established by plotting C_T values as y-axis, and the function of (log) target DNA

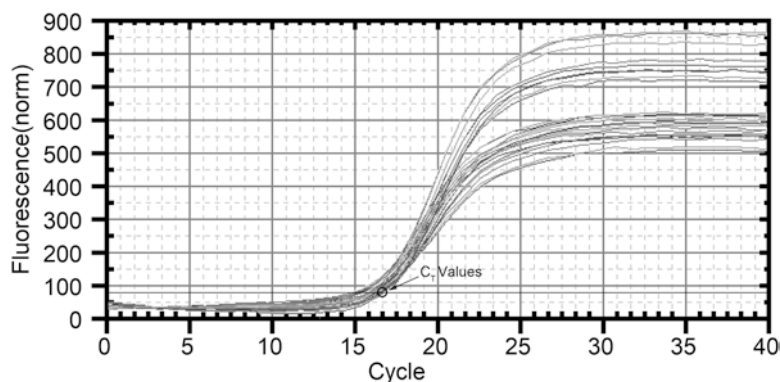


Fig. 4.5 Amplification plot of a target mRNA

amount is set as x-axis. The disadvantage of real-time PCR is that it may produce false-negative results, due to the impurities and other inhibitors which are present during DNA extraction of food samples.

Conventional, real-time PCR can be used for both detection and quantitation of crustacean allergens in complex food matrices (Sakai et al. 2013). Tetzlaff et al. (Tetzlaff and Mäde 2017) presented a real-time PCR assay, employing TaqMan probes, to detect the fish potential allergen in food. Eischeid et al. (Eischeid 2016) developed a real-time PCR method, using a fluorescent probe for detection of lobster, a crustacean shellfish allergen. A two probe-based, quantitative real-time PCR assay is reported on the development for the detection of penaeid shrimp and blue crab, crustacean shellfish allergens (Eischeid et al. 2013). Zhang et al. (2015) designed specific primer sets based on the DNA sequence of Ara h 1 and a competitive internal amplification control (IAC). A real-time PCR method with IAC was established for detecting peanut allergen Ara h 1.

4.2.3 PCR-ELISA

PCR-ELISA is advantageous to detect specific DNA among several PCR products. PCR-ELISA deals with the quantification of specific PCR products using a simple and economical ELISA-based detection system. PCR-ELISA is an important tool for semiquantitative analysis of food allergen in food products due to its high specificity the high stability of DNA (Holzhauser et al. 2002). In PCR-ELISA, a specific DNA fragment of a target allergenic protein is amplified. After that, the amplification DNA fragment is linked with a probe (biotin- or digoxigenin-labeled), which can couple with a specific enzyme-labeled antibody. The probe for PCR-ELISA is designed to specifically recognize the target PCR product for reduction of rate of false positive results. The concentration of the target DNA fragment is positively correlated with color depth resulting from an enzyme-substrate reaction.

Furthermore, coupling an ELISA analysis to traditional PCR is beneficial to ensure the specificity of the sequence of an amplified PCR product of target DNA in sample. With this method, Holzhauser et al. (2002) determined the presence of hazelnut allergens in processed foods at a level below 0.001% (w/w).

4.3 Mass Spectrometry-Based Techniques

In the last decade, mass spectrometry (MS) technology and methodology have had enormous progress in the field of proteomics, including the identification, characterization, and determination of food allergens. Especially, liquid chromatography-mass spectrometry (LC-MS) has been developed for both on- and off-line quantitation of food allergen (Bereszczak and Brancia 2009). In LC-MS assays, allergenic proteins extracted from food products are reduced, alkylated, and enzymatically digested into peptides. Trypsin, the most common endopeptidase, is used for the selective cleavage of proteins C-terminal to lysine and arginine generates peptides which are analyzed by MS. After that, this complex mixture of peptides is temporally separated through liquid chromatography, relying on the differences in relative affinity of the peptides for the column (stationary phase) and solvent (mobile phase). Next, the eluted peptides are ionized using electrospray ionization and subsequently analyzed by the mass spectrometer.

Currently, various types of mass spectrometer approaches have been employed for the quantitation of food allergens. Among them, triple quadrupole (QQQ) instruments running in multiple reaction monitoring (MRM) mode are most commonly used to detect the specific ions, which are the products of selected precursor ions fragment through collision-induced dissociation. Additionally, the full scan mode, multiple ion monitoring (MIM), and selected ion monitoring (SIM) also have been used. Furthermore, quadrupole time-of-flight (Q-TOF) and quadrupole-ion trap (Q-IT) are regularly applied in semiquantitative proteomics.

Prandi et al. (2013) described LC-MS/MS analysis of proteolytic peptides in wheat extracts for determining the content of the allergen amylase/trypsin inhibitor CM3. Korte et al. (2016) established a mass spectrometric approach, a MRM method, for the detection of proteotypic peptides and contamination with shrimp and lobster in complex food matrices at concentrations down to 0.1%. Kuppannan et al. (2014) presented a 2DLC-UV/MS assay for the simultaneous quantification of two intact soybean allergens, hydrophobic protein from soybean (HPS) and Gly m 4. Azarnia et al. (2013) developed a liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) method for the detection of egg allergy ovalbumin in egg white, whole egg, and incurred pasta. Tolin et al. (2012) showed a simple and straightforward mass spectrometric approach to reliably detect egg and milk allergens in commercial bottled wines. In this assay, 25 different wines were tested by liquid chromatography coupled with tandem MS (LC-MS/MS), demonstrating that the proposed method can be used to monitor the presence of potential allergenic proteins in fined commercial wine. Sealey-Voyksner et al. (2016) selected

two peptides as markers to represent peanut, almond, pecan, cashew, walnut, hazelnut, pine nut, Brazil nut, macadamia nut, pistachio nut, chestnut, and coconut. And novel multiplexed LC-MS method was developed to determine the presence of trace levels of peanut and tree nuts in food. Pilolli et al. (2017) proposed an optimized, sensitive, label-free LC-MS/MS method for multiplex detection of five allergenic ingredients in a processed food matrix. In this assay, a cookie base was chosen as a complex food matrix, and homemade cookies incurred with whole egg, skimmed milk, soy flour, ground hazelnut, and ground peanut were prepared at laboratory scale. New et al. (2017) presented an LC-tandem MS method to simultaneously screen or quantify the signature tryptic peptides of egg white, skimmed milk, peanut, soy, and tree nuts (almond, Brazil nut, cashew, hazelnut, pecan, pine nut, pistachio, and walnut) at a LOD of 10 ppm in incurred bread and cookies.

Overall, food allergen quantitation using MS is accomplished in four stages: peptide target selection, peptide specificity verification, targeted method development, and quantitation of food allergen. The advantage for MS is that MS methods are especially suitable for quantitation of food allergens in the process of food processing based on indifference toward conformational protein epitopes. However, there are assumptions made in MS when calculating protein quantity from peptide signal intensity, because protein quantity is the clinically relevant metric.

4.3.1 *Selection of Specific Peptides*

Unique peptides of allergenic proteins are selected as specific biomarkers for their stability in sample preparation with regard to selectivity, sensitivity, and reproducibility of MS. Currently, there are four common approaches to select specific peptides of target food allergens: shotgun proteomics, proteotypic peptide identification, peptide filtering, and public repositories of shotgun.

Classically, shotgun proteomics is commonly employed to identify peptides within an allergenic protein in the absence of established proteotypic peptides. These peptides may be utilized in targeted proteomics. In shotgun proteomics, target peptide sequences of allergenic proteins are subsequently analyzed by MS in food samples. Besides, several search algorithms are used to assign target peptide through comparison of these spectra against a protein database, such as Mascot, Sequest (Eng et al. 1994), and X!Tandem (Craig and Beavis 2004). Those optimal-identified peptides for quantitation of food allergens are selected, which have reproducible fragmentation profiles, strong signal intensities, and consistent retention times. During this phase, those peptides are avoided, including peptides with missed cleavage sites and easily modified amino acids and those which lack uniqueness to the target allergenic protein. However, with the aim of selection of specific peptides for targeted allergen detection through targeted allergen detection, a large number of targeted assays are firstly developed to select peptide targets. This step is costly, time-consuming, and generally reliant on commercial database searching software.

Proteotypic peptides are developed to standardize target peptides when the same peptides of a given allergenic protein can be detected in multiple food matrices (Johnson et al. 2014). In proteotypic peptides, it is unnecessary to select peptide targets of allergenic protein firstly. Therefore, proteotypic peptides can eliminate the costs, time, and complexity while strengthening comparability between studies and facilitating assay standardization.

Peptide filtering is usually used in combination with detectability scoring to simplify later targeted method development, relying on reducing the number of potential target peptides for a certain allergenic protein. This method can be applied to select specific peptides of many certain allergenic proteins that it is unnecessary to require shotgun proteomics for selecting peptides of allergenic proteins lacking proteotypic peptides. Furthermore, tryptic peptides derived from the same protein often differ in signal intensity by orders of magnitude, on account of physiochemical differences of target peptides, such as hydrophobicity, charge, and size, as well as tryptic digestion efficiency. Machine learning algorithms often are employed to observe peptide detectability scores (Mallick et al. 2007).

Public repositories of shotgun, the fourth approach to select specific peptides, include the Global Proteome Machine, PRIDE, and SRM Atlas. However, public MS data submission in the field of allergen detection and quantitation is low so far.

4.3.2 *Peptide Specificity Verification*

Before the development of targeted method, the specificity of each peptide should be verified. Peptide specificity can be verified by querying each peptide sequence against public databases of nucleotide and/or protein sequence data. In this method, the non-specificity of target peptide is revealed by matches to species other than that of the allergenic parent protein. For some highly homologous allergenic proteins from these species, non-specificity may not be a concern. Additionally, those non-specific peptides can be acceptable, when the likelihood of a species being present in a food is sufficiently low.

Currently, Allergen Peptide Browser is used to simplify the procedure of determining peptide specificity, which includes BLAST results of all tryptic peptides derived from allergenic proteins, with links to species and protein accession data hosted on NCBI. Although this bioinformatics approach provides a simple and rapid method to verify the specificity of target peptides, the inherent limitations of the known sequence space within databases may lead to uncertainty of peptide specificity. The comprehensiveness of these databases is continuously improved; however, the absence of a peptide should be confirmed in the allergen-free (blank) food matrix of interest during the development of targeted method.

4.3.3 Targeted Method Development

The development of targeted method in MS requires a suitable input that can derive appropriate peptide transitions and other assay parameters. Multiple reaction monitoring (MRM) and selected reaction monitoring (SRM) have been widely used for the consistent detection and accurate quantification of specific, predetermined sets of allergenic proteins in a complex background and in multiple food samples (Costa et al. 2014; Newsome and Scholl 2013; Parker et al. 2015). The development of SRM and MRM method requires the selection of transitions for each target peptide. SRM and MRM exploit full MS/MS spectra on a triple quadrupole (QQQ) to selectively isolate precursor ions which are corresponding to the mass of the specific synthetic peptides or an allergen tryptic digest of a given allergenic protein and to selectively monitor peptide-specific fragment ion(s) (Picotti et al. 2010). After acquisition of the full MS/MS spectra, database search algorithms are used to assign peptide sequences in the typical fashion, according to established quality criteria. SRM and MRM method refinement is an iterative process to optimize peptide-specific parameters by determining the most suitable SRM transitions for each target peptide, along with other associated LC-MS parameters. It is beginning of selection of the precursor ion charge state for each peptide, while ten of the most intense product ions is recommended for early stage refinement for each selected precursor ion.

For selecting product ions, it is optimal that the $m/z > 5$ above the precursor m/z to improve specificity, excepting for some special ions with high intensities, such as those with N-terminal proline. To evaluate of any drift in peptide retention time and the definition of an appropriate retention time window for each peptide, subsequent replicates are required using a scheduled SRM method. Furthermore, these replicates allow for the elimination of any unreliable at least two peptides should be retained for detection and quantitation of allergenic proteins. Consequently, for each target peptide, 3–5 of the highest intensity and most reproducible transitions are used for food allergen quantitation.

4.3.4 Quantitation of Food Allergen

After the development of MRM or SRM method, allergen quantitation can be achieved using synthetic, stable isotope-labeled peptides. These peptides with a different mass usually behave identically to their unlabeled target analogues chromatographically and in fragmentation profile. Quantitation of stable isotope-labeled peptides is preferable to quantitation at the protein level because the identification of allergenic proteins in complex matrixes may be substantially limited by ion

suppression of coeluting proteins and peak superposition of the mass spectra. For food allergens, the marker peptides should be additive in both native and processed food products. The absolute quantitation of allergenic proteins and specific peptides is achieved by determining the correlation between a measured standard curve and the ion signal. This calibration curve is generated by testing a serial dilution of endogenous peptide, containing the same concentration of marker peptides (stable isotope-labeled peptides). In this assay, high-purity peptides should be employed for calibration, and the calibration curve should be matrix-matched. The ratio of endogenous to area sum of marker peptides is then compared against a predetermined calibration curve. For sample quantitation, the amount of allergenic proteins on column is estimated by the amount of endogenous peptide obtained by comparison of the measured peak area ratio against the calibration curve. Finally, the data must then be corrected by considering recovery estimates, amount of extractable protein in a food, protein molecular weight, and any dilutions during sample preparation (Parker et al. 2015).

Currently, LC-MS approach has been accepted for the quantitative detection of food allergens in matrices as a protein-based confirmatory technique, relying on measuring marker peptides (biomarkers) using a calibration curve for the determination of relative concentration of target proteins. MS-MS is obligatory for quantitative analysis, quantitative MS of food allergens is a field under development, and more methods will be developed in the near future.

4.4 Sensor-Based Technique

Biosensors provide an important tool for the detection of various ingredients in food products, including food allergens. Biosensors have been shown to provide many exceptional performance capabilities, such as rapid response, low cost, high specificity and sensitivity, and user-friendly operation. In addition, biosensors can be made into relatively compact size, for instance, small portable real-time biosensors are designed for on-site analyses. By the immobilization of a sensor onto a chip surface, antibodies and specific DNA fragment (aptamer) are widely used for recognizing food allergens in conjunction with different detection modes (optical, electrochemical, mechanical, etc.). Biosensors can transform the binding interaction between allergens and antibodies or aptamer into a measurable chemical-physical signal proportional to the concentration of target food allergens. For food allergens, the appropriate bioreceptor should be chosen according to the changes in protein structure during food processing or sample extraction before the actual allergen analysis. According to the physical change accompanying the reaction, biosensors can be classified into optical and electrochemical types.

4.4.1 Optical Biosensors

Optical biosensors usually have two testing principles: (1) detection changes in light absorption when food allergen is recognized by the receptor immobilized on the surface of biosensor chips and (2) detection differences in light output because of a luminescent process. Recently, various kinds of optical techniques, relying on optical fiber or planar waveguide transducers, are employed to develop optical changes, including fluorescence, luminescence, surface plasmon resonance (SPR), total internal reflection fluorescence (TIRF), and surface-enhanced Raman spectroscopy (SERS).

Up to now, various optical biosensors are designed for the determination of food allergens, most of which are based on SPR that exploits the changes in the refractive index close to a sensor surface. In SPR techniques, gold nanoparticles are primarily exploited to immobilize bioreceptors (antibodies and aptamer) that are the most extended strategies to amplify SPR signals and enhance sensitivity. Besides, conjugates of antibodies and gold nanoparticles have been employed to improve the stabilization and functionality of SPR approach. Figure 4.6b briefly describes the schematic view of SPR gold sensor chip. In the past decade, electrostatic adsorption onto gold surfaces has been the most effective, simple, and implementable antibody immobilization approach. However, electrostatic adsorption (physical adsorption) is a nonspecific process which may lead to the denaturation of immobilized antibodies.

Unlike immunoanalytical techniques, SPR can directly detect food allergens in real time without labeling. For analytes with several food allergens, SPR biosensors commonly use mAbs as bioreceptors to recognize its complementary antigen using a direct assay and sandwich assay (Fig. 4.6a). Tran et al. (2013) developed an aptamer-based fiber optic surface plasmon resonance (FO-SPR) biosensor platform for detecting Ara h 1 protein in both buffer and food matrix samples. Ashley et al. (2018) developed a direct SPR technology for allergens analysis with the LOD being 0.127 ppm, using α -casein as the biomarker for cow's milk detection and SPR gold sensor chip to amplify SPR signals. Lu et al. (2015) reported a SPR biosensor for kinetic analysis of major fish allergen parvalbumin employing antibodies immobilized onto a carboxymethyl dextran (CMD) surface. Additionally, aptamer is another bioreceptors to recognize its complementary antigen in direct approach.

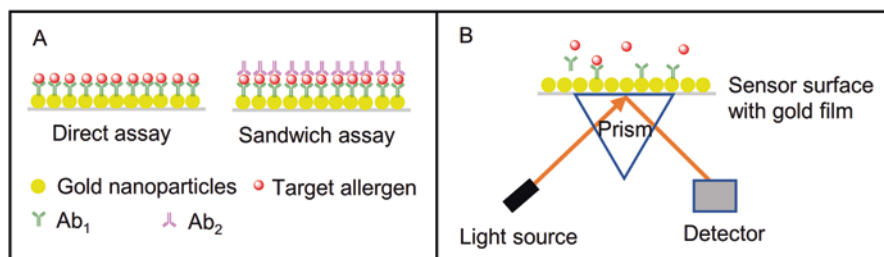


Fig. 4.6 Surface plasmon resonance. (a) Two formats of SPR techniques. (b) Schematic view of SPR gold sensor chip

SERS is an emerging and promising technique that can improve the Raman scattering coming from molecules which have been adsorbed on metallic nanosubstrates, such as Au, Ag, and Cu. In Raman signal enhancement, the type of metal and the shape of the features of the nanostructure are important influencing factors (Zheng and He 2014). Gezer et al. (2016) present a SERS sensor that is made out of a corn protein, zein, to detect the peanut main allergen, Ara h 1. In this biosensor, nanophotonic structures on zein films consisted of gold-coated pyramid structures.

4.4.2 *Electrochemical Biosensor*

Electrochemical biosensors provide analytical information using a biological-recognition element (biochemical receptor), which is retained in direct spatial contact with an electrochemical transduction element. On the basis of the mode of the physicochemical signal transduction, electrochemical biosensors can be classified into amperometric, potentiometric, voltammetric, and impedance types.

Amperometric biosensors measure the current generated/consumed result of redox reactions on the surface of the working electrode. Generally, three electrode systems with separate reference electrode are designed to control potential in amperometric biosensors (Warriner et al. 2014). Amperometric biosensors employ antibodies immobilized onto the electrode surface to analyze food allergens; in addition, enzyme labeling with horseradish peroxidase (HRP) or alkaline phosphatase (ALP) are usually required. Montiel et al. reported a highly sensitive disposable amperometric magnetoimmunosensor employing sandwich configuration for the determination of the major peanut allergen Ara h 2 (Montiel et al. 2016) and Ara h 1 (Montiel et al. 2015). Liu et al. (2010) developed a AuNP-peptide sensor to determine model chicken anti-peanut antibodies (IgY) in serum. Liu et al. (2014) designed an amperometric sensor for BoNT/A using gold nanoparticles as electronic bridges and signal amplifiers. AuNPs are tethered with mixed PEG-aryl-diazonium salts and attached to the glassy carbon (GC) electrode by forming Au-C bonding. Compared to Au and NH-Au-S bonds, Au-C bonding displayed greater stability. Tethered AuNPs were then functionalized with the capture antibody, and then a similar procedure was used to functionalize tethered AuNPs with HRP-labeled anti BoNT/A antibody for the sandwich assay.

Voltammetric biosensor is another electrochemical biosensor, which involves measuring the changes in electrical potential between the working and reference electrodes in a certain range. Additionally, voltammetric biosensor also refers to studying the current responses, such as cyclic voltammetry, differential pulse voltammetry, and square wave voltammetry. The changes in current intensity are related to the amount of allergen in food samples. For example, Eissa and Zourob (2017) presented a label-free voltammetric immunosensor utilizing graphene-modified screen-printed carbon electrodes to detect milk allergen β -lactoglobulin. Alves et al. (2015) developed a voltammetric immunosensor for Ara h 6 (a peanut allergen) with the detection and quantification limits of 0.27 and 0.88 ng/ml, relying

on a modified screen-printed electrodes with AuNPs and a sandwich-type assay using two-monoclonal mouse IgG antibodies against Ara h 6. Cao et al. (2011) proposed a novel electrochemical immunosensor based on gold nanoparticles and poly(l-Arginine)/multiwalled carbon nanotubes (P-l-Arg/MWCNTs) composite film for the determination of the presence of casein in cheese samples through differential pulse voltammetry. This electrochemical immunoassay has a low LOD of 5×10^{-8} g/ml.

Generally, electrochemical impedance spectroscopy (EIS) is applied to describe the interfacial electron transfer resistance at the functionalized transducer surfaces in the presence of a redox active probe while measuring the response of an electrochemical cell to a small amplitude sinusoidal voltage signal as a function of frequency. In electrochemical impedance experiments, the electron transfer resistance alters at the electrode solution interface when the target allergen binds to its bioreceptor. The changes are in direct relation with the amount of allergen in food sample. Jiang and coworkers (2013) developed a novel cell-based electrochemical biosensor, in which mast cells were immobilized on l-cysteine/gold nanoparticle-modified gold electrodes to quantify major shrimp allergen Pen a 1. Sun et al. (2012) presented a novel electrochemical DNA sensor using a stem-loop probe for peanut allergen Ara h 1 detection, while the changes in electron transfer efficiency between probe and electrode were proved by EIS. Jiang et al. (2015) described an electrochemical rat basophilic leukemia cell (RBL-2H3) cell sensor, employing fluorescent magnetic beads, designed for the detection of different allergens (shrimp allergen Pen a 1, soybean allergen β -conglycinin, and peanut allergen Ara h 1) in foodstuffs. Peeters et al. (2014) presented an aptamer-based sensor that amino (NH₂)-terminated Ara h 1 aptamers were covalently attached to carboxylated gold surfaces to detect Ara h 1.

Quartz crystal microbalance (QCM) biosensors, a bulk acoustic wave (BAW) sensor, measures the change in the resonant frequency of the microbalance induced by a mass change of the sensing layer (Warriner et al. 2014). The resonance of the quartz crystal produced by the immobilization of antibodies or DNA fragments on the transducer surface is monitored using EIS. Sun et al. (2010) carried out a QCM-based immunosensor, involving the use of self-assembly with 1,6-hexanedithiol (HDT) and nanogold for electrode modification, to detect the presence of shrimp allergen in food.

In recent years, food allergy has become a crucial food safety issue; therefore, the development of reliable, sensitive, and rapid methods for the detection and quantification of food allergens in complex food matrices has become important for food-allergic consumer protection and food labeling. Due to the diverse nature of food allergens and the complexity of food matrices, an enormous challenge still exists in the choice of detection methods and the achievement of reliable results. Meanwhile, with the development of advanced technologies and the increasing requirement of better detection methods, more detection and quantification approaches for food allergen will be developed in the near future.

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Chapter 5

Allergenicity Evaluation of Food Proteins



Abstract In the past decade, food allergies have occurred frequently, and the number of allergic people has been increasing, making it a serious problem that jeopardized public health. A large number of researchers are dedicated to the exploration of food allergy mechanisms and how to prevent and treat food allergies. Allergenicity evaluation of food proteins is the basis of all food allergy-related researches. At present, there are many methods and strategies for evaluating the allergenicity of food proteins, such as bioinformatics analysis, serological analysis, cell experiment, and animal experiment, and each has its own advantages and disadvantages. In addition, there are currently no globally accepted standards for assessing the allergenicity of food proteins. Different methods often lead to differences in conclusions, making the data on food allergies highly variable. The search and construction of an effective standard for allergenic assessment of food allergens will help to reduce the incidence and slow the trend of food allergies.

In 2001, the Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology published a decision tree approach to assess the allergenic potential of foods with the following steps (Fao et al. 2001) (Fig. 5.1): First, the source of the gene is judged, and different analysis steps are taken depending on whether the gene is derived from a species that is known to sensitize humans. Second, the sequence homology is analyzed, and if homologous to known allergens, the protein will be regarded as allergenic. Otherwise no sequence homology is observed, a serum screening test is required, and the positive result means that the protein may cause allergy. If the test result is negative, the protein needs to be tested for pepsin resistance, and animal model experiments will also be conducted if possible. Finally, protein allergenicity is evaluated based on the results of each stage of the test. This approach is currently the most commonly used strategy for evaluating food protein allergenicity. Although this strategy mainly applies to evaluate the allergenicity of genetically modified foods, it has certain reference value for evaluating the allergenicity of other foods. More commonly, there are a variety of food allergenicity evaluation methods including bioinformatics analysis, in vitro experiments, and in vivo experiments, which are also described in the decision tree

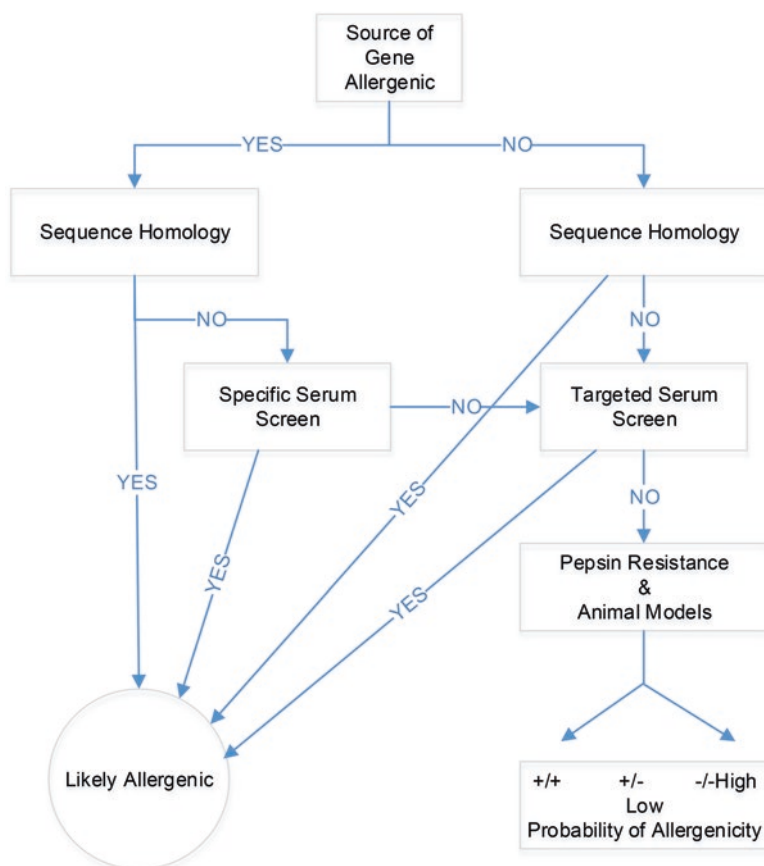


Fig. 5.1 Decision tree approach to assessment of the allergenic potential of foods derived from Biotechnology FAO/WHO in 2001

approach from the FAO/WHO and will be introduced in this chapter (Veličković and Gavrović-Jankulović 2014).

5.1 Bioinformatics Analysis

Bioinformatics is an interdisciplinary field that develops methods and software tools for understanding biological data based on the analysis of genomic DNA sequence information (Jin et al. 2017). We can elucidate the nature of information in noncoding regions of the genome and decipher the laws of genetic language hidden in DNA sequences by finding coding regions that represent protein and RNA genes in genomic sequences and then summarize and sort the data of transcription and protein profile to understand the laws of metabolism, development,

differentiation, and evolution of organisms. Nowadays, bioinformatics is commonly used to assess the allergenicity of unknown proteins, especially for genetically modified foods. It cannot directly judge whether a protein is an allergen or not but can predict its allergenicity through the homology and cross-reactivity compared with known allergens (Fu and Lin 2017).

Some research showed that the higher amino acid sequence homology of two proteins, the higher probability of cross-reaction between the two proteins (Chrysostomou and Seker 2014; Dinakarpandian and Dinakar 2017; Randhawa et al. 2011). It is generally believed that the protein with more than 70% similarity to the overall sequence of a known allergen may show cross-reaction; however, when below 50%, no immunoglobulin (Ig) E cross-reaction will occur. The documents of the FAO/WHO and the Codex Alimentarius Commission stipulated that if any 80-amino acid fragment of a foreign protein has 35% homology with a known allergen, it is considered to be potentially allergenic. Allergen databases are essential for allergens homology analysis, which can be divided into two major categories: the biologic databases and the molecular databases. The former includes clinical, physiological, and other information related to allergens, and the latter focuses on the sequences and structural characteristics of allergens. The widely used allergen databases are detailed in Table 5.1.

FASTA and BLAST are frequently used for the alignment of allergenic protein sequences. FASTA is the first widely used tool for sequence alignment and search,

Table 5.1 Common databases used in bioinformatics analysis

Database	Internet site
Allergen Online (AO)	http://www.allergenonline.org/
Structure Database of Allergenic Proteins (SDAP)	http://fermi.utmb.edu/SDAP/sdap_ver.htm
AllerMatch (AM)	http://www.allermatch.org/
Allergen Database for Food Safety (ADFS)	http://allergen.nihs.go.jp/ADFS/
The Allergen Database at the Central Science Laboratory (CSL)	http://www.csl.gov.uk/allergen/
International Union of Immunological Societies (IUIS)	http://www.allergen.org/
Bioinformatics for Food Safety (BIFS)	http://www.iit.edu/~sgendel/fa.htm
AllerDB (ADB)	http://sdmc.i2r.a-star.edu.sg/Templar/DB/Allergen/
Allergome	http://www.allergome.org/
Informall	http://www.foodallergens.info/
Protall	http://www.ifr.bbscr.ac.uk/Protall
AllAllergy	http://allallergy.net/
Allergen ATLAS	http://biodb100.apbionet.org/
Evaller	http://bioinformatics.bmc.uu.se/evaller.html
WebAllergen	http://webballerqen.bii.a-star.edu.sg/
AllerTool	http://research.i2r.a-star.edu.sg/AllerToll/
AlgPred	http://www.imtech.res.in/raaghava/alqpred/

and its analysis report provides the best comparison results and statistically significant E estimates. Based on FASTA, BLAST is more efficient with improvements in all aspects (Song et al. 2014). Although the use of bioinformatics analysis can quickly assess whether a protein is potentially allergenic, the high homology does not mean high allergenicity. It needs to be combined with serological methods or cell experiments to detect whether the target protein can bind antigen-specific IgE, so as to evaluate its allergenicity.

5.2 In Vitro Experiments

5.2.1 *Physiological and Biochemical Tests*

5.2.1.1 Pepsin Resistance Test

It is generally believed that if a natural protein or denatured protein is to maintain its allergenicity, the amino acid fragment, where the IgE binding site of the protein is located, must be resistant to food processing and in vivo digestion (Baderschneider et al. 2002; Pickles et al. 2014). That's because unlike drug allergy and respiratory allergenicity, food allergies only occur after the allergen is being processed through the digestive system (low pH, bile salts, protease hydrolysis) and reaches the intestinal mucosa to be absorbed (Bøgh and Madsen 2015). Therefore, pepsin resistance test in vitro is considered to be an important indicator for assessing food allergenicity. In order to make it clear whether there is an inevitable relationship between allergenicity and digestive stability, a lot of relevant research have been performed in this area. Early studies have shown that the occurrence of food allergies by oral exposure to allergens in BALB/c mice is related to the anti-digestive properties of food protein (Bowman and Selgrade 2008a). Regardless of decision tree approach to assessment allergenicity proposed by the FAO/WHO in 2001, or the allergenicity evaluation strategy proposed by the CAC in 2009, pepsin resistance experiment has been recognized as a method for assessing allergic protein.

Pepsin digestion assay usually uses simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). Allergen protein is incubated with SGF or SIF (containing NaCl and various proteases) in vitro and then sampled at different time points for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The degradation degree of the protein is determined by the molecular weight of the protein band in the gel to reflect the allergenicity of the sample. SGF test is mostly used in the researches of soybeans, peanuts, nuts, and other crops as well as eggs and milk. In 1996, Astwood and colleagues found that, compared with non-allergenic proteins, allergens in common allergic foods such as soy, mustard, eggs, and milk have strong resistance to SGF (Astwood et al. 1996). In addition, there are some studies on aquatic products. Untersmayr and colleagues performed the digestion tests of SGF on finfish extracts at different pH values (pH 1.25–5.0) and immunodetected the enzymolysis products, finding that parvalbumin was degraded rapidly at pH

1.25–2.5 and its IgE binding capacity decreased as the digestion time increased (Untersmayr et al. 2005). But some scholars questioned whether allergenic proteins are really more resistant to digestion than non-allergenic proteins. SGF test and SIF test on rubber emulsion, potato, and five fruit proteins found that in SGF, most of latex and vegetable food proteins were degraded in short time and that many allergic proteins are degraded within 8 min. While in SIF, most of the food proteins in vegetable are relatively stable, the kiwifruit allergen protein is almost completely degraded after 16 h (Yagami et al. 2000). However, the results of SGF and SIF tests on proteins in milk, soybeans, peanuts, and eggs indicated that the major allergenic proteins were not more resistant to enzymatic hydrolysis than the lesser allergenic proteins and that allergenic proteins have no higher digestibility than non-allergenic proteins (Fu et al. 2002). Therefore, the allergenicity of the protein cannot be judged directly by the pepsin resistance test, and further verification by serological experiments or other experiments is needed.

5.2.1.2 Serological Analysis

As shown in Fig. 5.1, serological analysis includes specific serum screen and targeted serum screen, which evaluates the allergenicity of food proteins by observing the binding ability of food-specific allergens to specific IgE antibodies in sensitized human serum. Because allergic sera from humans are difficult to obtain, animals, such as mice and rabbits, are also used to obtain allergic serums for immunoassay. At present, the commonly used serological methods include enzyme-linked immunosorbent assay (ELISA) and immunoblotting.

1. ELISA

ELISA is a technology developed in the late 1990s. It is a qualitative and quantitative method in which a soluble antigen or antibody is bound to a solid-phase carrier such as polystyrene, and the corresponding antibody or antigen is captured by specific antigen-antibody interaction and thus is detected. The method has high sensitivity, good specificity, and convenient operation and is therefore widely used for routine detection and screening of food allergens. According to different operations, ELISA is mainly divided into double-antibody sandwich ELISA, indirect ELISA, capture ELISA, and competitive ELISA, of which the most widely used are double-antibody sandwich ELISA and competitive ELISA (Fig. 5.2). Nowadays, many researchers also use ELISA to assess the allergen allergenicity through the specific binding of antigens in food and antibodies obtained from allergy sufferers' sera or immunized animals. For example, an ELISA performed by immunizing rabbits with native glycinin to obtain antisera was used to assess allergenicity changes of allergen after heat treatment or acid-base treatment (M. Venkatachalam et al. 2002). The results of ELISA, used to evaluate the allergenicity changes of tropomyosin in *Scylla serrata* (mud crab) after digestion in SGF or SIF, also showed that the IgE antibody-based ELISA can effectively evaluate the reduced level of allergenicity (Huang et al. 2010). Before, sandwich ELISA can be combined with animal models

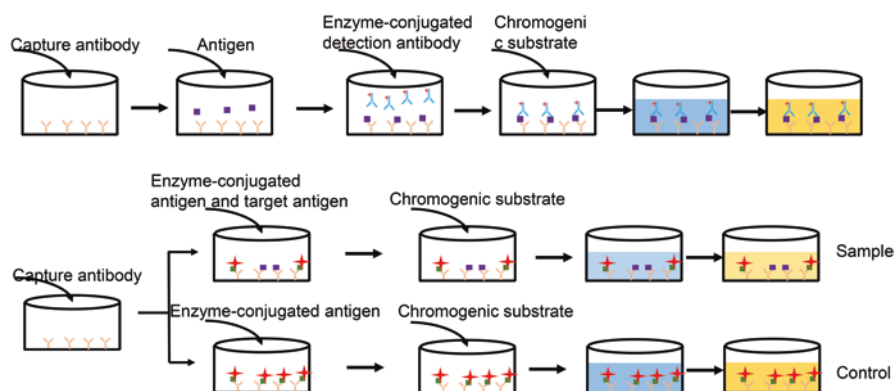


Fig. 5.2 Diagram of ELISA operation procedures. (a) Double-antibody sandwich ELISA; (b) Competitive ELISA

of food allergy to detect residues of strong allergens in commercially available wine clarifying agent residues (Lifrani et al. 2009). Later researchers have developed a more sensitive indirect ELISA for the detection of casein in white and red wines to investigate the risk of allergic residues in fine wines (Deckwart et al. 2014). Multiple comparison trials revealed that the main factors affecting ELISA analysis result were the type of food matrix, processing levels, and analytical methods (i.e., the type of ELISA kit) in the detection of food allergens (Török et al. 2015). Therefore, researchers need to carefully select ELISA strategies to assess the allergenicity of food proteins according to the nature of the food, and it should be understood that different sources of ELISA kits may have different test results.

2. Immunoblotting

Immunoblotting, also known as western blotting, is a method for detecting a certain protein in a complex sample based on the specific antigen-antibody binding. This method is an immunochemical technology developed on the basis of gel electrophoresis and solid-phase immunoassay. The mixed antigen sample is subjected to unidirectional or two-dimensional electrophoresis for separation, and then a blotting membrane is attached to the gel. The proteins in the gel are transferred to the blotting membrane under the action of natural adsorption force, electric field force, or other external forces. Finally, the antigen-immobilized membrane is detected and analyzed by using an immunoisotope probe or an immunoenzyme probe. Immunoblotting combines the high resolution of SDS-PAGE and the high specificity and sensitivity of antibody. It shows large analytical capacity, high sensitivity, and high specificity. Therefore, it is widely used in the evaluation of antigen-antibody immune activities (Grishina et al. 2017). For example, immunoblotting was performed using serum from allergic patients to evaluate immunogenicity changes of sausage batter after treatment at high hydrostatic pressure and concluded that high hydrostatic pressure treatment can eliminate immunogenicity of sausage batter (Hajos et al. 2004). Western blotting was performed on the allergenicity eval-

uation of peanut allergens (Ara h 1, Ara h 2, Ara h 3) after different heat treatment based on the serum of patients. The binding capacity of IgE and peanut allergens after 30 min of high temperature sterilization was found significantly reduced (Cabanillasa et al. 2012). Quantitative Western blotting can also be used to detect residues of ovalbumin and casein in white and red wines (Meyer and Zanetti 2015). Furthermore, immunoblotting is often combined with ELISA to detect the allergenicity of allergic foods.

Using patient serum in serological analysis for evaluating allergenicity is quite intuitive and convincing. However, the scarcity of sera from allergic patients limits the popularization of this approach. Consequently, diagnostic and evaluation methods in vitro such as ELISA and immunoblotting based on specific animal-derived antibodies have developed rapidly.

5.2.2 Cell Model

The occurrence and severity of food allergy depend on the degree of mediators released from the granules via the interaction of basophils, mast cell-bound IgE with the corresponding allergen, and the subsequent responsiveness of the target organs to these mediators. As a result, the level of histamine and β -hexosaminidase in serum is directly related to the severity of allergic reactions. Therefore, cell immunological methods based on mediator release assays using cell model in vitro can directly reflect the allergenicity of food allergens. In general, the ideal cell model should satisfy the following aspects: (a) The active medium in the cell can be released after IgE/antigen stimulation; (b) cell is easy to obtain and has good reproducibility; (c) cell has high specificity, so it can distinguish different allergenic allergens; and (d) cells are highly sensitive, and slight allergens can induce cellular degranulation (Chen and Jia 2012). At present, there are several cell models that have been used for allergenicity evaluation and sensitization mechanism studies, including mast cell model, human basophil model, and rat basophilic leukemia (RBL) cell model.

1. Mast Cell Model

The mast cells, first discovered by German scholar Paul Ehrlich in 1877, are important immune cells involved in allergic reactions. Mast cells originated from bone marrow stem cells and migrated to peripheral tissues during the progenitor cell period and matured in situ. Mast cells mainly reside around microvessels in the skin, in the submucosal connective tissue, and underneath the submucosa of the internal organs. They play an important role in inflammation and allergic reactions and express high-affinity IgE receptors (Fc ϵ RI) on the surface (Kraft and Novak 2006). The IgE-/antigen-mediated degranulation reaction releases pre-synthesized mediator and newly synthesized mediator in the particles such as histamine, leukotrienes, tryptase, and prostaglandins, directly acting on target cells, target tissues, and target organs, causing smooth muscle contraction, dilation of blood vessels,

leakage of small blood vessels, and secretion of mucus, resulting in type I hypersensitivity (Amiya et al. 2012; Bischoff 2016).

Mast cells play a central role in the pathogenesis of food allergy. It is a key link between the induction phase and the allergic response phase, which can amplify the signal of allergens (Polukort et al. 2016). Many researchers have used the mast cell model for allergenicity evaluation by different indicators, including changes in cell morphology and release of particulate mediators. For example, when rat peritoneal mast cells were stimulated *in vitro* with OVA, it was found that the cells become larger, the cell membrane shrinks, the cytoplasm was filled with air bubbles, and the cells were surrounded by particles. With transmission electron microscopy performed, it was observed that cell membranes were damaged and cell contents were reduced and uneven (Yang et al. 2013). Furthermore, induction of mast cells in the bone marrow *in vitro* also revealed the role of splenomegaly in food allergic diseases (Toyoshima et al. 2017). In addition to rat-/mouse-derived mast cells, the human mast cell line 1 (HMC-1), which can be passaged indefinitely, also plays an important role in the evaluation of food protein allergenicity (Butterfield et al. 1988).

Since mast cell degranulation is the most important cause of allergic reactions to various clinical symptoms, granular mediators that released during the process are the most common indicators for assessing allergenicity. Common granular mediators include histamine, tryptase, IL-5, and IL-13, wherein histamine is the most important one. Histamine release test (HRT) in mast cells has been used for allergenicity evaluation in a large number of food allergy studies (Fig. 5.3). The main experimental method is as follows (Chen and Jia 2012): First, an animal model of food allergy is prepared. This model can be prepared by using BALB/c mice, C57/BL6 mice, or other experimental animals. After subcutaneous or intraperitoneal injection of allergens to test animals, peritoneal mast cells are collected. Then, the collected mast cells are induced *in vitro* to release histamine, and the histamine release rate is measured. However, the half-life of histamine under physiological conditions is only a few minutes, resulting in the poor repeatability and unstable detection results (Mayorga et al. 2010). Alternatively, tryptase is a preformed proteinase in mast cells that is released from granulocytes by mast cells and participates in allergic reactions with other mediators. In addition, the detection method of tryptase is simple and can more accurately and stably reflect the degree of granulation of mast cells. Therefore, tryptase may be a replacement of histamine as a mast cell degranulation marker.

For allergenicity assessment using mast cells, the primary thing is the isolation and purification of mast cells, but this is not a simple matter. Low yield and unstable purity were obtained in the isolation of rat peritoneal mast cells by density-gradient centrifugation (Guo et al. 2009a). The magnetic bead sorting method has been used to gradually remove lymphocytes, monocytes, and the like by a plurality of antibodies to separate mast cells, although the purity is largely improved; however the cost is much higher and more blood sample is required (Gong et al. 2010). Consequently, although HRT in mast cell has a high specificity and sensitivity in the allergenicity evaluation, due to the limits of primary mast cells described above, commercially

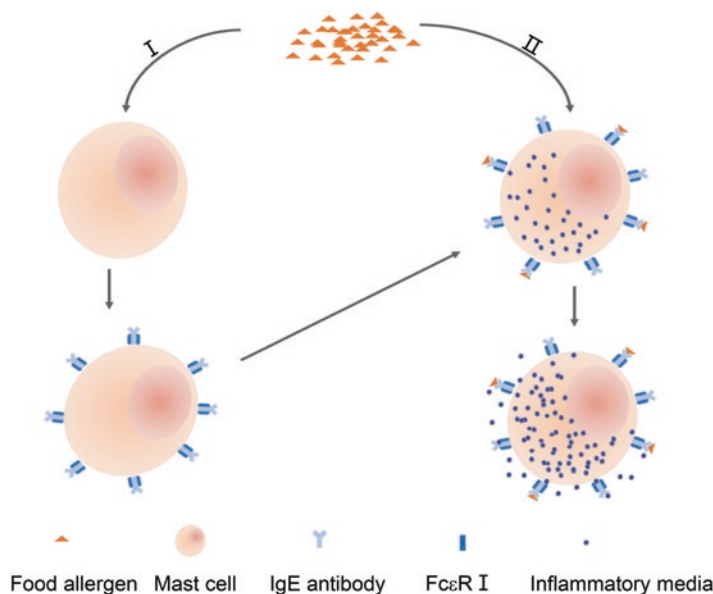


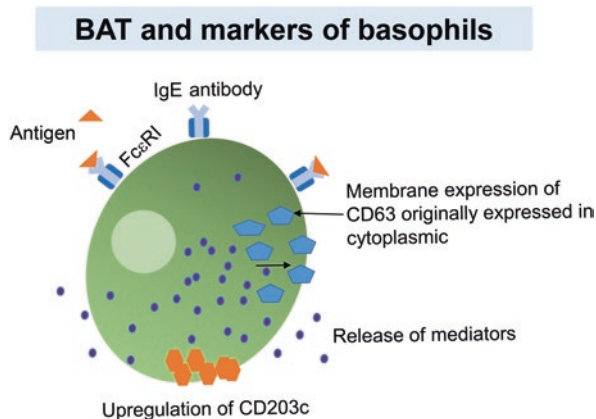
Fig. 5.3 Schematic diagram of mast cells degranulation. I: Food allergens first enter the person's body who are prone to allergies through various routes, stimulating the production of antigen-specific IgE, which binds to FcεRI on the surface of mast cells. II: The food allergens enter the body of the sensitized person again and directly bind to the surface IgE of the mast cells, leading to degranulation of mast cells

available cell lines are the most commonly used cell models, such as the HMC-1 and Laboratory of Allergic Diseases 2 (LAD2). For example, the HMC-1 and LAD2 cell models have revealed a relationship between hyperglycemia and hypersensitivity (Nagai et al. 2012). In addition, HMC-1 cells have identified the relationship between cell degranulation and JAK2/STAT5 signaling pathways, further elucidating the intracellular pathways involved in anti-allergy activities (Qian et al. 2016). Similarly, HMC-1 cells and rat peritoneal mast cells can also screen for anti-allergic components (Han et al. 2016). The aforesaid researches showed the important role of mast cells in assessing food allergenicity. However, some studies also showed that HMC-1 cells have low expression of high-affinity IgE receptors on the surface and cannot degranulate after IgE/antigen stimulation (Nilsson et al. 2010).

2. Human Basophil Model

Basophils, originating from CD34⁺ bone marrow precursor cells and maturing in the bone marrow, are distributed in the peripheral blood, bone marrow, and spleen. Mature basophils present in the blood account for less than 1% of peripheral blood leukocytes and only migrate outside the blood vessels when induced by chemokines (Karasuyama et al. 2011). Although the content is low, basophils play an important role in allergic reactions. Like mast cells, FcεRI-expressed human basophils are the main effector cells involved in type I allergies. The basophilic granules contained in

Fig. 5.4 Basophil activity test (BAT) and the expression of surface markers after basophil activation



the cytoplasm are degranulated after being stimulated by antigen and release bioactive mediators such as histamine. In addition to participating in the IgE-mediated chronic allergic reactions, basophils also play an important role in regulating the immune response, enhancing the immune response memory, and inducing the helper T-cell (Th) 2 response (Chen and Jia 2012; Karasuyama et al. 2011).

In recent years, a flow cytometer-assisted cellular allergen stimulation test (flow-CAST) termed the basophil activity test (BAT) has been developed, making basophils more widely used in clinical and scientific research (Fig. 5.4). The *in vitro* activation of peripheral blood basophils measured by flow cytometry is dependent on the change of basophil surface markers upon stimulation with specific allergens. There are two main pathways for basophil activation, the anaphylactic degranulation (AND) and the piecemeal degranulation (PMD). The main characteristics of AND are the release of extracellular secretory granules, mediators, and rapid changes in cell morphology, while PMD is a slow morphological change without extracellular secretory granules. When the allergen stimulating cells causes cross-linking of IgE bound to basophils, this not only activates basophils to produce and secrete a variety of biologically active mediators but also upregulates a variety of expression of surface markers (Ebo et al. 2010b), which mainly include CD63 and CD203c. CD63 is a member of the transmembrane protein 4 superfamily and is expressed in many types of cells such as basophils, tissue mast cells, macrophages, and platelet cells. When the cells are in a steady state, CD63 is mainly expressed on the granule membrane in the cytoplasm rather than the plasma membrane. When basophils are activated, particles in the cytoplasm migrate to the plasma membrane and fuse with the plasma membrane, allowing CD63 to be highly expressed on the cell surface (Chomiciene et al. 2014; Ebo et al. 2010a; Gangwar et al. 2017). At present, the biological function of CD63 still needs further researches to determine, but some studies have shown that anti-CD63 antibody can inhibit mast cell adhesion and activation. On the other hand, CD203c belongs to a polygenic family of pyrophosphatase/phosphodiesterases. In peripheral blood, CD203c is stably expressed on the basophilic membrane surface (Moscoso et al. 2012; Moscoso et al. 2014;

Sudheer et al. 2005). When basophils were stimulated with specific allergens, anti-IgE antibodies or anti-Fc ϵ RI antibodies, the expression of CD203c on the cell membrane surface was rapidly upregulated within 5 min.

At present, BAT has been widely used in the diagnosis of food allergy. Clinically, it is generally recommended that food allergies should be diagnosed in the combination of clinical history, skin prick tests (SPT), specific IgE tests, and BAT to get a reliable result and minimize the use of oral irritation tests. In some cases, when some individuals with specific IgE or SPT-positive reaction but do not have an allergic symptom, BAT is a powerful tool to distinguish them from being in a sensitized state to a true allergic state. In addition, BAT can also be used for allergen detection, which is a sensitive and specific tool for the detection of trace allergens such as the peanut allergens in biscuits and chocolate. In general, CD203c expression assay of allergen-stimulated granulocyte is more sensitive than the allergen-induced histamine release assay. Based on this, it has been found that the level of basophils activation in vitro in children sensitive to peanut or egg were significantly higher than those in control children after stimulation with peanut or egg allergens (Kahlert et al. 2010; Ocmant et al. 2009; Sabato et al. 2011). Studies also found that nuts not only cross-react with peanut-specific IgE but also activate peanut-sensitized basophils (de Leon et al. 2010), which explained why patients who are allergic to peanuts are also allergic to nuts.

Overall, basophil activation experiments based on CD63 and CD203c expression are highly sensitive and specific and thus widely used in clinical diagnosis of food allergy. However, due to the disadvantages such as the difficulty in extraction, the rapid decline in cell activity in vitro, and the large requirement of human sera, basophilic clinical application is greatly limited, and further improvements are urgent and necessary.

3. RBL Cell Model

Rat basophil leukemia cells were originally isolated and cloned by Eccleston in 1973 from tumor-bearing Wistar rats and can be indefinitely passaged (Eccleston et al. 1973). RBL cells have many similar properties and functions to mucosal mast cells, both of which express Fc ϵ RI on cell surface, and can be activated by IgE/antigen complexes to degranulate and release inflammatory mediators (Barsumian et al. 2010; Siraganian et al. 1982). Normally, the biomarkers for degranulation of RBL cells are histamine and β -hexosaminidase, and their release tends to be nearly parallel. Histamine is present at a lower level in RBL cells and is rapidly degraded after release, while β -hexosaminidase is hard to degrade and easy for detection, so β -hexosaminidase is a better biomarker than histamine in most situations. Since RBL cells have many similar functional properties as mast cells, many researchers have used RBL cells as an in vitro model instead of mast cells to assess the allergenicity of allergenic food extracts and purified allergens (Liu et al. 2017; Lv et al. 2018; Passante and Frankish 2009).

RBL-2H3 cells are a subline of RBL that express high level of Fc ϵ RI on their surface and can be activated by IgE/antigen complex to release inflammatory factors. RBL-2H3 cells are widely used in IgE-mediated cell degranulation experiments,

and their dose-response curves are bell-shaped (Dearman et al. 2005). Compared with the classical methods (IgE-binding test, histamine release test), RBL-2H3 cells show good consistency in the detection of allergenicity (Kaul and Hoffmann 2001). The procedure of RBL-2H3 cell sensitization model test is mainly divided into two steps: First, sensitize rats or mice to produce serum containing specific IgE antibodies; then, the obtained serum is added to RBL-2H3 cell suspension for incubation. After incubating for a period of time, the allergen is added to the cell suspension, and the released sensitization mediators and appeared morphological changes are determined. Commonly used indicators for RBL-2H3 cell sensitization model are histamine, β -hexosaminidase, cytokines, Annexin V-positive cell rate, and cell morphology changes.

RBL-2H3 cells have the advantages of good homogeneity, small test variation, indefinite passage, and strong operability and therefore are often used for the research of allergic and immune reactions (Liu et al. 2017; Passante and Frankish 2009). For example, RBL-2H3 cells have been used to identify the main allergen of fish, parvalbumin, by detecting fluorescent signals (Jiang et al. 2014b). A number of RBL cell lines transfected with human Fc ϵ RI have been established, such as RBL-hEla-2B12 expressing the human Fc ϵ RI α -subunit and RBL-30/25 and RBL SX-38 expressing human Fc ϵ RI α -, β -, and γ -subunits. These humanized RBL cell lines play an important role in the detection of allergen-specific IgE, the isolation and identification of allergens, and the diagnosis of clinical allergic reactions. For example, huFc ϵ RI α -RBL-2H3 cells constructed by Knipping allow allergenicity evaluation in vitro of whey protein hydrolysates. Humanized RBL-2H3 cells have also been used to identify the allergenicity of peanuts and hazelnut proteins in complex food matrices (Vogel et al. 2015). To some extent, RBL-2H3 degranulation test can replace animal model for allergenicity evaluation, reducing the applications of experimental animals in allergy researches (Knipping et al. 2016). However, the use of RBL-2H3 cell model is also controversial. Although RBL-2H3 cells have the characteristics of mast cells and basophils, there are many differences between RBL-2H3 cells and mucosal mast cells and connective tissue mast cells. RBL-2H3 cells do not express Toll-like receptor (TLR) 2 like mast cells do; nor do they express mast cell TLR4 signaling pathways (lacking CD14 and MyD88 molecules); some nonimmune stimuli such as Ca²⁺ concentration and cell culture environment also make RBL-2H3 cells degranulation. RBL-2H3 cells also differ from basophils; as a tumor cell line, RBL-2H3 is obviously abnormal compared with basophils. The phenotype and function of RBL-2H3 cells are also susceptible to culture conditions and test conditions (Passante and Frankish 2009). Therefore, RBL-2H3 cells are not able to completely replace mast cells or basophils, and the produced results of food allergy studies need to be treated with caution.

4. Emerging technologies

In vitro cell models possess the characteristics of simple operation, rapid detection, and strong repeatability, so many researchers are committed to construct a new cell model to maximize the simulation of the body's immune response. Since many active substances cannot directly act on immune cells, but indirectly through

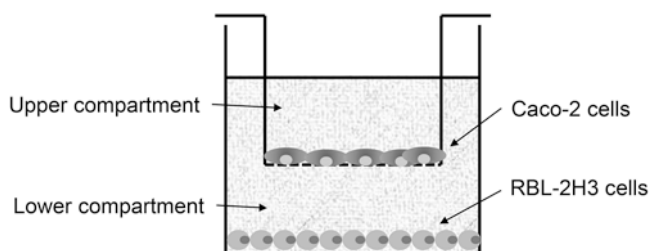


Fig. 5.5 Schematic of the transwell Caco-2/RBL-2H3 co-culture cell model

intestinal epithelial cells to induce multiple immune responses, researchers have constructed co-culture cell models of human intestinal epithelial cells with mast cells (Yamashita et al. 2016). It places Caco-2 cells as human intestinal epithelial cells on the apex of a trans-well membrane and rat basophilic leukemia cell line RBL-2H3 cells as mast cells on the basolateral side. Then, the release of β -hexosaminidase by RBL-2H3 cells after stimulating with anti-dinitrophenyl (DNP) IgE and DNP-bovine serum albumin (DNP-BSA) was measured as an indicator of allergic activity. In the presence of exogenous substances, the inhibition potential on β -hexosaminidase release was used to evaluate the anti-allergic activity. Moreover, Caco-2/RBL-2H3 co-culture model can also assess the allergenicity of the protein by replacing DNP-BSA with a specific antigen in food (Fig. 5.5).

In recent years, microfluidic chip technology has attracted a great deal of attention from researchers in biological and food safety monitoring field. It not only creates new opportunities for researches on biological and food safety detection but also has extensive applications in clinical examination and disease treatment. The microfluidic chip shows several advantages including less sample requirement, low pollution, short response time, and high analysis efficiency. The integrated microfluidic device can support high-throughput bioassay and has widespread applications in cell culture, cell metabolism, and contamination analysis. In noninvasive and sensitive detection techniques of living cells, measuring electrochemical impedance spectroscopy (EIS) is a commonly used and effective method, showing broad application prospects in monitoring adhesion, viability, and environmental changes within cells. Based on microfluidic core technology and electrochemical impedance spectroscopy, a microfluidic chip based on mast cell-macrophage co-culture was constructed by Jiangnan University of China (Jiang et al. 2016). The microfluidic chip co-cultured with macrophages and mast cells is made of four groups of gold electrodes and uses electrochemical plating electrodes to make the bottom. It contains two cell culture layers connected by a “capillary.” By co-culture of ANA-1 macrophages as antigen-presenting units and RBL-2H3 as mast cells on PDMS channels to form a sensitive immunosensor system, cells were observed for allergic reactions when introduced into antigen stimulation. Cells were stimulated with DNP-BSA without anti-DNP-IgE incubation for allergic reaction detection. Once the cells secrete extracellular cytokines, the inflammatory cytokines can be measured by ELISA, and the changes in cell impedance are detected by cell-based

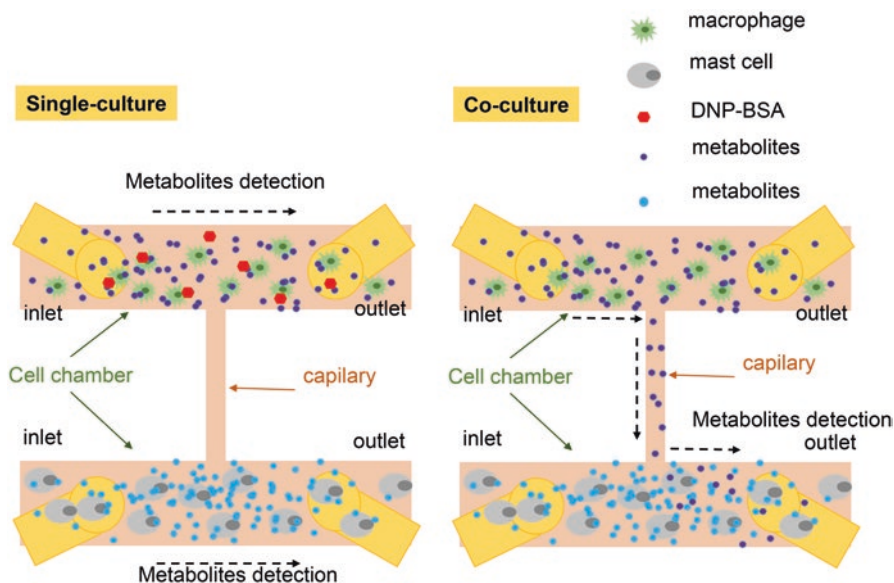


Fig. 5.6 Schematic of a bioreactor on the microfluidic device

electrochemical assays. The electrochemical microfluidic core, which can accurately monitor the real-time cellular allergic reaction, is a rapid detection biosensor with a low detection speed and low cost (Fig. 5.6).

5.3 In Vivo Experiments

Though have great advantages, the in vitro experiments can only partially reflect allergen information and cannot fully show the effects of allergens and organisms, so it is still necessary to use in vivo experiments to evaluate allergen-induced allergic reactions.

5.3.1 Human Experiment

5.3.1.1 Skin Test

The skin test is based on the immunological reaction between antigens and antibodies in the skin. There are two kinds of skin tests, one is SPT and the other is intradermal testing (IDST). SPT sensitivity is highly consistent with clinical results and has good specificity (Kaliner 2012). It is regarded as the most convenient, economical, safe, effective, and commonly used diagnostic method for allergens in the

medical community (Griffiths et al. 2017). The specific operating procedure is as follows: First, after routine skin disinfection, put a drop of each highly purified allergen liquid (about 20 kinds), negative control solution (normal saline), and positive control solution (histamine, commonly used concentration is 10 mg/mL) onto the patient's forearm skin with a distance of 3–5 cm; then, gently vertically prick the droplets into the skin surface with a needle; and finally, the patient's skin is observed about 10–20 min after the stabbing. There will appear a red bump, skin itching, or color change in the sting site if the patient is allergic to the allergen. By comparing the size of the wheal result from the allergen with the positive control, the patient's response to the allergen is determined (Gao et al. 2014). However, not all patients are suitable for SPT, such as those with severe urticaria. It should be noted that prediction of aeroallergens is more accurate than of food allergens by SPT, and the SPT result should be combined with serological analysis to ensure the accuracy of the conclusion. Compared with positive results, the SPT-negative results have better accuracy in excluding food allergies (more than 95% confidence), and positive results may be more related to clinical skin allergic reactions (Antunes et al. 2009; Rlm et al. 2017).

Traditional SPT depends on the manual operation, which cannot precisely control the depth, and the result is excessively dependent on the proficiency level of the operator. On the other hand, when dropping allergens, it is easy to make the dropper to come into contact with the skin thus causing contamination of the remaining liquid as well as a great waste of the high-cost reagent. In addition, because multiple stabbings are required and the patients are able to observe the process, it is easy to cause the fear of patients, especially children, which is not suitable for the determination and treatment of diseases. In addition, successive operation of SPT takes a long time, and the work efficiency is low. Therefore, many researchers have explored the establishment of automated SPT devices and have achieved certain results. However, it should be noted that since SPT is an in vivo experiment, emergency medicines such as diphenhydramine, dexamethasone, and epinephrine must be prepared before testing. Moreover, if the results of SPT cannot be obtained, a food challenge test should be conducted to confirm the diagnosis.

5.3.1.2 Food Challenge Experiment

To obtain more reliable results, SPT or specific IgE test results can be confirmed by food challenge experiments. Food challenge experiments are the most reliable way to diagnose food allergy, which includes the open food challenge (OFC), single-blind placebo-controlled food challenge (SBPCFC), and double-blind placebo-controlled food challenge (DBPCFC), and DBPCFC is the “gold standard” for the diagnosis of food allergy.

DBPCFC is used to stimulate patients with indicated food and then determine whether the food has allergenicity based on the patient's symptoms. This method can also be used to determine the threshold of sensitized foods (Sánchez-Monge and Salcedo 2005). In the absence of an allergic reaction, it is not sufficient to exclude

diet based on the results of serological analysis alone, and the use of DBPCFC to determine food allergens can effectively stop unnecessary dietary avoidance in most children. DBPCFCs are generally required to be performed in hospitals and outpatient departments (Fleischer et al. 2011) and require medical staffs and equipments capable of handling severe allergic reactions and are operated and tested by experienced nursing staffs (Dambacher et al. 2013). The most critical points of the experiments are the control of double-blind strategy, experimental doses, and the use of placebo. Controlling double-blind requires that the untested differences between the test food and placebo are eliminated as much as possible. That is, the vehicle should mask the taste, texture, and odor of the food, thus minimizing the influence of subjective factors on the experimental results but limiting the experimental dose. For most foods, patients, and clinical conditions, it is appropriate to use 3–3000 mg of the tested food every 20 min (Sampson et al. 2012), while in another study, it was recommended that the dosage should not exceed 250 mL for liquid and pasty foods and 125 g for solid foods (Vlieg-Boerstra et al. 2011).

DBPCFC is mainly based on the excitation dose and the degree of allergic reaction to assess allergenicity. The lesser food required to produce an allergic reaction and the more intense allergic reaction, the more allergenicity of the tested food (Gao et al. 2014). However, DBPCFC needs further standardization since the experimental results of this method are influenced by many factors. For example, the clinical tolerance caused by anti-allergic drugs or short-term specific oral tolerance during the oral test may result in false-negative results. Furthermore, strict dietary control was not achieved during the oral test. Therefore, false-positive results may be generated due to the questionable food provided by visitors, or the intake of eczema-related food by patients that also suffer from atopic eczema. In addition, due to the risk of DBPCFC, food challenge experiment is not recommended for patients with severe immediate allergic reactions. For patients with less severe symptoms, it is recommended that they are admitted to the hospital for provocation tests and close observation. At the same time, adequate first aid measures for emergency must be prepared.

5.3.2 *Animal Experiments*

Because human experiments have some risks and are often subject to moral and ethical issues, animal experiments play an alternative role in studying the direct relationship between the target protein and the body's immune response. The ideal animal model should show the pathogenesis of human diseases, the route of allergen exposure, and the symptoms of the disease. Various animals such as BALB/c mice, C3H/HeJ mice, BN rats, guinea pigs, dogs, and young pigs were used for food allergy experimental studies (Fig. 5.7).

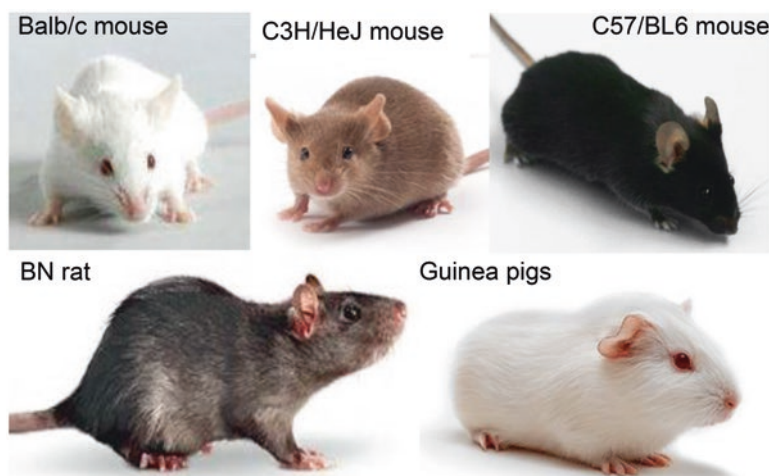


Fig. 5.7 Different strains of animals commonly used in food allergy. The illustrated animal may have a variety of coat colors and the color in the figure are not unique

5.3.2.1 Mouse Model

Mice have some unique features that help them to be more widely used in human biology than any other organism. Although it is necessary to consider the differences of innate and adaptive immune systems between mouse and humans, mouse, the second mammal that completes the whole genome sequencing after humans, is greatly similar to humans in physiology, genetics, and immunology (Liu et al. 2016). The mice own a set of unique technological advantages that provide a rich genetic resource that allows for intensive genetic manipulations such as transgene, targeted mutation, knock-in, and knockout (Fernandes et al. 2015; Justice et al. 2011). As the applications of these genetic engineering methods in mice, a large number of humanized mouse strains with targeted modifications have been widely used in human disease tests. In addition, with the superiority of a short reproductive cycle of about 18–20 days, cheap price, and easy feeding method, mice have become a priority research object for various disease models. The evaluation criteria for the success establishment of a food allergy mouse model is the degree to which mouse produces a human allergic reaction, including clinical symptoms and humoral and cellular responses to food allergens. Although the mouse model shows many advantages in food allergenicity evaluation, there are still many uncertain factors such as age, gender, strain, sensitization, and other effects. The mouse strains commonly used for food allergenicity evaluation are BALB/c, C3H/HeJ, and C57BL/6.

BALB/c mice, inbred high IgE response strains, are used in sensitization model studies for the preferential generation of Th2 immune responses and IgE antibodies

(Dearman and Kimber 2007). Currently, BALB/c mice have been widely used to construct animal models of eggs, fish, crustacean aquatic products, nuts, plant agglutination, and other allergenic foods, but they have been reported to be resistant to the induction of milk or peanut hypersensitivity reactions (Liu et al. 2016). In animal models of peanut and milk allergy, sometimes, BALB/c mice that produce a high level of specific IgE do not show a clinical allergic symptom; instead stimulation of allergens may promote Th1 cell-mediated anti-allergic immune responses. C3H/HeJ mouse is another high IgE response strain with TLR-4 gene mutation, which blocks the intestinal lipopolysaccharide (LPS) signaling, making the mouse prone to allergic reactions, so it is often used to establish allergy models to food such as peanut (Berin et al. 2010; Qureshi et al. 1999). Sensitized C3H/HeJ mouse developed significant clinical allergy symptoms similar to human after allergen challenge. Studies have shown that BALB/c mice and C3H/HeJ mice have different immune responses to different food allergens. In the construction of some food allergy models for peanuts and milk, C3H/HeJ mice produce higher levels of IgE and histamine than BALB/c mice, whereas BALB/c mice have no allergy symptoms or other immune responses. This suggests that C3H/HeJ and BALB/c mice may produce different immune response patterns for different food allergens. C57/BL6 mouse is also a common allergic animal model, but it is more common in the studies of respiratory allergies. Its allergic response to allergens is relatively weak, so it is generally considered that its Th2 bias is slightly lower than that of BALB/c mice (Gueders et al. 2009). However, due to the advantages of possessing large genetic variants, C57BL/6 mice can be a technological platform that is more convenient to study the basic elements of multiple immune systems involved in pathogenesis. In addition, C57BL/6 mice showed more pro-inflammatory and eosinophilic inflammation in bronchoalveolar lavage fluid compared with BALB/c mice, which may reflect its Th1/Th2 mixed pattern (Liu et al. 2016). The genetic background of selected mouse strains can affect the output hyperreactive and allergic inflammation. It is essential to correctly select experimental mouse strains in food allergy studies based on different types of food allergens, allergic diseases, and research purposes.

5.3.2.2 Rat Model

As an animal model of food allergy, rat models have the following advantages: The size of rat is suitable for kinetic analysis of serum-specific antibodies on individual animals, it can be orally sensitized without adjuvant, and sensitized rats may produce similar allergic symptoms to humans after challenge (Dearman and Kimber 2010). BN rats are high immunoglobulin (especially IgE) response strains and have been extensively studied as an allergic animal model. Researchers have conducted in-depth studies on multiple influencing factors of OVA sensitization model in BN rats, including dosage, feeding mode, feeding frequency, diet control, and output indicators (Knippels et al. 1999; Ménard et al. 2014). In an optimized procedure, BN rats were given OVA (1 mg/day) orally for 42 consecutive days without

adjuvant. ELISA and passive cutaneous anaphylaxis testing (PCA) showed that more than 80% of BN rats produced OVA-specific IgE, and sensitized rats had increased intestinal permeability after OVA challenge, while a few individuals had slight changes in respiratory system or blood pressure. In addition, BN rats are similar to humans in assessing peanut allergen Ara h 1, shrimp allergen Pen a 1, potato allergen Sol t 1 (rare allergen), and beef tropomyosin (non-allergen) (Ladics et al. 2010). Because a rat model can reliably reflect the sensitization rate and human responses of different allergens in the population, it is considered as an ideal food allergy animal model.

5.3.2.3 Other Animal Models

In addition to mice and rats, there are other animals have been used to build food allergy models such as guinea pigs, young pigs, and dogs. Guinea pigs with susceptibility are one of the most common allergic animal models. In an established allergy model of guinea pigs, after sensitized with oral administration of whole milk or its main allergen casein and β -lactalbumin, the guinea pigs were re-exposed to the same allergens by injection or oral administration (Devey et al. 1976). The results showed that guinea pigs suffered from death or stress shock after challenge, suggesting that guinea pigs have similar sensitization pathways to humans and are an ideal milk allergy animal model. Shrimp protein and OVA-sensitized guinea pigs have also shown that it is a suitable allergy model with a human-like allergen-recognition mechanism. Matsunaga and colleagues applied a guinea pig model to assess the allergenicity of acid hydrolyzed wheat protein using epidermal sensitization (Matsunaga et al. 2015). In addition, allergic sensitization induced allergic contractile response of guinea pig isolated ileal smooth muscle (Schultz-Dale reaction) similar to the symptoms of systemic allergic reaction of humans. On the other hand, although non-rodents such as young pigs and dogs are not commonly used to build food allergy models, there are also some interesting trials. An allergic animal model of basenji dog was established by using oral and subcutaneous injections. The sensitized dog can develop an allergic reaction to a series of food allergens, resulting in specific IgE producing and allergic clinical symptoms such as nausea, diarrhea, erythema, increased permeability of the gastrointestinal vascular, and elevated blood pressure (Ermel et al. 1997). Hardy and colleagues used a dog model to evaluate whether there is a correlation between skin disease and the production of food-specific serum IgE and IgG (Hardy et al. 2014). Helm and colleagues sensitized pregnant Landrace pigs by intraperitoneal injection of peanut protein and found that most produced young pigs present positive results of food challenge test and PCA, as well as severe gastrointestinal mucosal lesions with vomiting, diarrhea, and dyspnea (Helm et al. 2002). Overall, although young pigs and dogs have very similar allergic reactions to humans, due to the large body size, the long sensitization cycle, the large dosage of allergens, the high experimental costs, and the lack of related molecular immunological reagents, these models are more commonly used for allergic mechanism research instead of allergenicity evaluation.

5.3.2.4 Sensitization Route

The food allergic reaction is divided into sensitization period and effect period. The sensitization period puts organism in a sensitized state, and the effect period causes the body to produce various allergic clinical symptoms. Since organism has no symptoms during the sensitization period, it is difficult to identify the sensitization route for food allergies. The main routes of sensitization include oral intake, skin contact, respiratory inhalation, and maternal delivery (Liu et al. 2016). Sometimes the body is sensitized by multiple allergen exposure routes rather than by a single one. More than 50% of children allergic to nuts are unaware of allergen exposure route (Sicherer et al. 1998). Peanut allergy sufferers are similar, and many patients report that the time point when they developed the allergic reaction is the first observed exposure to the allergen. In allergic animal models, sensitization route depends on the susceptibility and specificity of animal species and allergen molecules. Allergen characteristics, immunization dose, frequency, and route of administration can all influence the direction of the immune response (sensitization or tolerance). In addition to the sensitization of proteins themselves, food media (lipids, carbohydrates, other proteins, etc.) and food contaminants (LPS, lectins, etc.) can also affect the immunogenicity and allergenicity of proteins. Different sensitization routes have been established in food allergy animal models, including intragastric (IG), intraperitoneal (IP), epicutaneous (EC), intranasal (IN), and subcutaneous (SC) administrations (Liu et al. 2016). Since the clinical allergic effects of proteins and immune responses may vary greatly depending on the route of exposure and allergen properties, the selection of a reasonable sensitization pathway is important for the construction of a best mouse model of food allergy. For different animal models, the sensitization method should also undergo corresponding changes. Generally, it is necessary to choose multiple ways to combine and compare the effects so as to obtain the best sensitization route.

The gastrointestinal tract is considered to be the most common exposure site for food allergens. Therefore, intragastric administrations of allergens to animals is the most widely used sensitization route in food allergy models. However, similar to humans, animals are prone to oral tolerance for a variety of ingested food proteins, causing difficult in constructing animal models of food allergies (Tordesillas et al. 2017). Therefore, it is necessary to use mucosal adjuvants to break oral tolerance. As a famous mucosal adjuvant for oral gavage, cholera toxin (CT) potentiates Th2 immune responses in mice. CT is a toxin produced by *Vibrio cholerae*, consisting of one A subunit (CTA) and five B subunits (CTB) in the form of AB₅. Due to the toxicity of cholera toxin, investigators explored the possibility of using CTA or CTB instead of the whole protein complex as mucosal adjuvants. It was found that both subunits take on mucosal adjuvants effect, but CTB is more versatile due to the non-toxicity. Staphylococcal enterotoxin B (SEB) is another frequently used mucosal adjuvant. It also can stimulate Th2 lymphocyte activity in a food allergy model but has lower adjuvant activity than CT (Ganeshan et al. 2009). Similar as CT, SEB overcomes the oral tolerance by oral administration of food allergens in mouse. In particular, compared with CT/allergen-sensitized mice, SEB/allergen-sensitized

BALB/c mice showed a marked eosinophils increase in the blood and intestinal tissues, which is a same pathological feature observed in food allergic patients. In addition, *Staphylococcus aureus* easily grow on the damaged parts of the skin and other mucous membranes, then producing SEB to overcome oral tolerance, which may be related to the prevalence of human food allergies (Martin et al. 2015). In addition, contrary to overcoming oral tolerance, some researchers went in the opposite direction to judge the allergenicity of foods according to whether or not the food proteins can induce oral tolerance without adjuvants. The results indicated that non-allergenic foods were more likely to induce tolerance than foods containing strong allergens (Bowman and Selgrade 2008b).

Dense blood vessels and lymphatics reside in creatural peritoneum due to its vast area. Absorption capacity of the peritoneum is extremely strong, which can absorb matters equal to 3–8% of animal's body weight per hour. Intraperitoneal injection of allergens obtains significant sensitization effects by inducing systemic allergic reactions and producing high levels of IgE and IgG antibodies in mice. Compared with some other routes, intraperitoneal injection requires only a small dose of allergens but can gain good sensitization effect (Liu et al. 2016), resulting in a wide range of applications. Similar as oral gavage, intraperitoneal injection also requires adjuvant such as Freund's adjuvant, alum adjuvant, aluminum hydroxide, and so on. Freund's adjuvant is divided into Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA). FCA is a water-in-oil emulsion containing bacillus calmette-guerin (BCG) (final concentration 3–20 mg/mL) or cell wall components of *Mycobacterium tuberculosis*. Adjuvant activity results from the sustained release of immunogen in the oil droplets to stimulate the local immune response. FCA is commonly used for primary immune stimulation; however, to reduce side effects, FIA that without *M. tuberculosis* components is used for boosting immune stimulation instead. Alum (potassium aluminum sulfate) usually stimulates macrophages by establishing a high concentration of allergic sites, thereby increasing sensitivity to allergens (Lambrecht et al. 2009). Alum is the only adjuvant that can be used in human experiments in the United States. The aluminum hydroxide adjuvant is generally prepared by adding sodium hydroxide into aluminum sulfate under strong stirring. As an adjuvant, aluminum hydroxide has no toxic effects and can induce high levels of antibody responses, but the deficiency is the inability to induce strong T cell immunity.

Skin exposure to allergens is considered as an important route to sensitize organisms. In particular, persons with skin diseases, such as eczema and urticarial, are prone to hypersensitivity, which may be due to the growth of *Staphylococcus aureus* in the eczema area to produce SEB that overcomes the tolerance to make the body sensitized by low doses of allergens. In response to increasing clinical evidence of the crucial role of skin sensitization in peanuts, enormous epidermal sensitization models have been developed, including adjuvant models and adjuvant-free models by using tape detachment, topical vitamin D analogues, or intradermal injection of cytokines thymic stromal lymphocytes (TSLP) to imitate eczema skin (Han et al. 2014; Leyva-Castillo et al. 2013; Liu et al. 2016; Oyoshi et al. 2010; Tordesillas et al. 2016). The main operation of the non-adjuvant BALB/c mice allergy model by

epidermal sensitization is as follows: First, remove the hair on a certain area of the mouse's back or abdomen, and smear the allergen to the area; then a layer of non-latex, nonocclusive bandages is applied to the body to induce sensitization with repeated exposure to allergens.

5.3.2.5 Measurement Indicators

Though food allergy is mainly divided into IgE-mediated, non-IgE-mediated, and IgE- and non-IgE mixed-mediated allergic responses (Tordesillas et al. 2017), most allergic reactions are mediated by IgE. The complicated mechanism of allergic reactions involves multiple indicators. A single index cannot fully reflect the body's allergic status; therefore multiple indicators need to be combined to get convincing results. Commonly used indicators include clinical symptoms, specific antibodies, immune cells, and cytokines.

1. Clinical Symptoms

Once the sensitized organism again ingests allergens, it can quickly trigger different symptoms in multiple parts of the body, such as the skin (itching, redness, urticarial), respiratory tract (asthma, cough), and gastrointestinal tract (nausea, vomiting, diarrhea) (Tordesillas et al. 2017). Therefore, the clinical allergic symptom score that involves all the symptoms is considered as a crucial index for evaluating the occurrence and severity of food allergy. The experimenter scores according to the severity of animal symptoms (0–6 points, six levels) in the mouse model, such as edema, diarrhea, wheezing, even convulsions, and death, to achieve quantitative detection. Increased respiratory rate, increased gastrointestinal permeability, decreased rectal temperature, and pathological lesions in the gastrointestinal tract, these animal local allergic reactions can all be detected by appropriate instrumentation or histological observations, making the symptoms possible to be quantified objectively (Birmingham et al. 2007; Gizzarelli et al. 2010). In addition, ear swelling tests and skin tests are also commonly used to detect allergic reactions in animals (Birmingham et al. 2007).

2. Allergen-Specific Antibodies

Allergen-specific IgE in serum is the paramount indicator for food allergy. The binding of IgE to mast cells through their FcεRI results in the release of large quantities of inflammatory mediators and proteases into the surrounding tissues. The IgE can be detected by electrophoresis, ELISA, and radioallergo-sorbent test (RAST) inhibition assays. ELISA is a classical and frequently used method to quantify IgE but is susceptible to IgG interference. In practice, most researchers use sandwich ELISA after labeling proteins with digoxigenin or biotin or remove serum IgG before employing indirect ELISA (Aldemir et al. 2009). Western blotting is operated to identify specific IgE-reactive components in a complex protein mixture. PCA is commonly used in rodent models to reflect the amount of biologically active IgE that bound to the surface of mast cells.

IgG is the main immunoglobulin produced by the humoral immune response, possessing high content and extensive distribution in the body. It is also the exclusive immunoglobulin that can pass through the placenta. Therefore, allergen-specific IgG has important clinical implications. Belonging to Th2-type antibodies regulated by Th2-type cytokines, IgG1 in mice, IgG2a in rats and guinea pigs are often used as indicators for allergy detection due to the association with delayed hypersensitivity reactions. The allergen-specific IgG1 and IgG2a are commonly detected by indirect ELISA. However, only in certain experimental systems, the use of IgG subtype antibodies as an alternative indicator of IgE is appropriate, while in some other cases, there is no correlation between the production of IgG1 and IgE (Aldemir et al. 2009). In certain animal sensitization models, not only serum allergen-specific IgG1 and IgE levels are significantly elevated but also the level of IgG2a (Th1 type antibody), though the increased degree is lower. In addition, the level of specific IgA is also increased. All these above imply that an allergic reaction in an animal model may be a reaction involving multiple specific antibodies (Van et al. 2010).

3. Immune Cells and Cytokines

In type I hypersensitivity reactions, such as asthma and food allergies, it is generally believed that skewing Th1/Th2 balance to Th2 is the key mechanism of allergy occurrence, in spite of another opinion about the break of Th17/regulatory T cells (Treg) balance (Chinthrajah et al. 2016). Th2 cells and CD4⁺CD25⁺ Treg play an important role in the sensitization and challenge phases of allergic reactions. Food allergies involve diversiform Tregs, such as Tr1 cells, Th3 cells, and Foxp3⁺ Treg, that produce interleukin IL-10, of which Foxp3⁺ Tregs are currently considered to play a crucial role in food allergy (Pabst and Mowat 2012). Th2 cells induce IgE production through the secretion of some cytokines, causing allergic reactions. The major Th2 cytokines are IL-4, IL-5, and IL-13. In addition, Th1 cells secrete interferon- γ (IFN- γ) to inhibit the production of IgE, whereas antigen-specific Foxp3⁺ Treg inhibit the adverse reactions to food in the intestinal lamina propria by producing immunosuppressive factors TGF- β , IL-10, and IL-35 (Bollrath and Powrie 2013).

Cytokines participate in immune response and immune regulation. Th1 cells produce tumor necrosis factor- α (TNF- α), IFN- γ , and granulocyte macrophage colony-stimulating factor (GM-CSF) to stimulate macrophage activation for increasing phagocytosis and killing activity. IL-10 is an important cytokine involved in the induction of oral tolerance and the maintenance of intestinal homeostasis, which reduce the expression of co-stimulatory molecules such major histocompatibility complex II (MHC II) and B7 to inhibit antigen presentation. T lymphocytes and cytokines have been shown to play a pivotal role in the induction of IgE-mediated allergic reactions. Therefore, in allergic animal models, spleen cell proliferation test and related cytokine analysis as a complement to serum analysis are very useful and valuable detection indicators. For example, in the oral peanut sensitization model of C3H/HeJ mice, the spleens from early and late peanut-sensitized mice were respectively cultured *in vitro*; stimulated separately with peanut extract, Ara h 1, Ara h 2, Ara h 3, and Ara h 6; and then quantified cytokines production. The content of IL-4,

IL-10, IL-13, and IFN- γ in the culture supernatant was detected by ELISA. The results showed that both in the early and late stages of sensitization, the contents of these four cytokines were increased in a dose-dependent manner compared with the control group (Van et al. 2010). Similarly, in peanut allergy patients, in addition to multiple subtypes of peanut-specific antibody responses including the elevated levels of serum IgG1, IgG4, and IgA, Th1/Th2 mixed cytokine responses were also observed, and evidences of allergic animal models have also demonstrated that allergic reactions in experimental animals are not strictly mediated by Th2 responses and may be a Th1/Th2 mixed response (Smart and Kemp 2010).

4. Active Media and Other Indicators

One of the most important characteristic of type I hypersensitivity reaction is the mast cell degranulation and the release of active media after allergen challenge. Major active mediators are histamine, tryptase, β -hexosaminidase, and mast cell protease 1 (MCP-1), which can be detected by ELISA or enzyme activity assay (Jiang et al. 2014a; Guo et al. 2009b). Histamine is a classical allergy medium but with short half-life, making other protein allergic media highly concerned and valued. In addition, the expression levels of cell surface molecules related to the activation allergy-related cells such as Th2 or related specific transcription factors such as GATA-3 are also effective indicators.

5.4 Conclusion

There is no absolute standard for evaluating the sensitization of food allergens. However, once a standardized evaluation process is developed, existing technologies can be quickly applied to evaluate the allergenicity of food allergens. Different evaluation techniques have their own advantages and disadvantages, so it is usually necessary for combining multiple methods. Bioinformatics methods are mainly utilized for the analysis of allergenicity of genetically modified foods relying heavily on the allergen database updated in real time. The serological method is safe and feasible, but cannot give an intuitive result, as it cannot truly reflect the sensitization of allergens but only reflects the binding of immunoglobulins to allergens, so it is imperative to be combined with other methods for further analysis. The cytological method owns reliable result but needs complicated and time-consuming analysis operation, so it requires to be standardized and optimized to make it a mainstream method. DBPCFC and SPT can intuitively reflect the food allergens sensitization, but the pain to the subjects, the high false-positive rate, as well as the requirement of strictly controlling the subjects' diet impaired the application. The animal model of food allergy can directly and accurately reflect food allergens information. With the development of some ideal animal models in recent years, this method has received extensive attention as an effective way to evaluate and identify food allergens. However, the existing animal models also have some limitations, such as poor

reproducibility and big artificial error. And no animal model has been tested and validated for a wide range of allergens and non-allergens to date. Therefore, the establishment of a reliable animal model should strictly be according to the standardization of allergens, sensitization processes, and detection indicators. Current research shows that the correct application of animal models, combined with other evaluation methods, can effectively detect and evaluate food sensitization. In summary, further researches are required to establish a rapid, efficient, and accurate assessment method for the allergenicity of food allergens.

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Chapter 6

Food Processing to Eliminate Food Allergens and Development of Hypoallergenic Foods



Abstract Food allergy is an adverse systemic immune response against food. With the continuous progress of social progress and globalization, the incidence of allergic diseases has been escalating. About 30–40% of people in the world suffer from various allergic diseases, and morbidity and mortality show rising trend year by year. Unfortunately, there are no effective methods to prevent and eradicate food allergy nowadays. So the only safe and effective way available nowadays is to avoid eating or contacting with allergens. Therefore, eliminating food allergens by food processing methods is an effective way. Although most allergens and antigenic determinants can tolerate various forms of food processing methods to a certain extent, many researches have shown that particular food processing can still affect the allergenicity. In the field of food processing, physical, chemical, enzymatic, and fermentation methods can modify the activity of allergens. Protein is one of the main components of food, and it is also an important nutrient as well as the predominant allergen category in people's diet. Food processing methods can cause the change of spatial structure and physicochemical properties of protein macromolecules and therefore destroy the allergenic epitopes of protein. This chapter will introduce the common and advanced application of physical, chemical, and biological approaches in the elimination of allergens in food.

6.1 Physical Modification

Physical modification processes food by physical approaches such as heat, electricity, magnetism and mechanical energy. Physical modification generally induces allergen intermolecular aggregation and changes the protein high structure, so as to reduce food allergenicity to a certain extent, but protein primary structure is normally not changed during the process. Physical modification has the advantages of low cost, short action time, no toxic side effects, and little influence on the nutritional properties of products. Commonly used physical modification methods include heat treatment, ultrasonic, ultrahigh pressure, irradiation, and microwave.

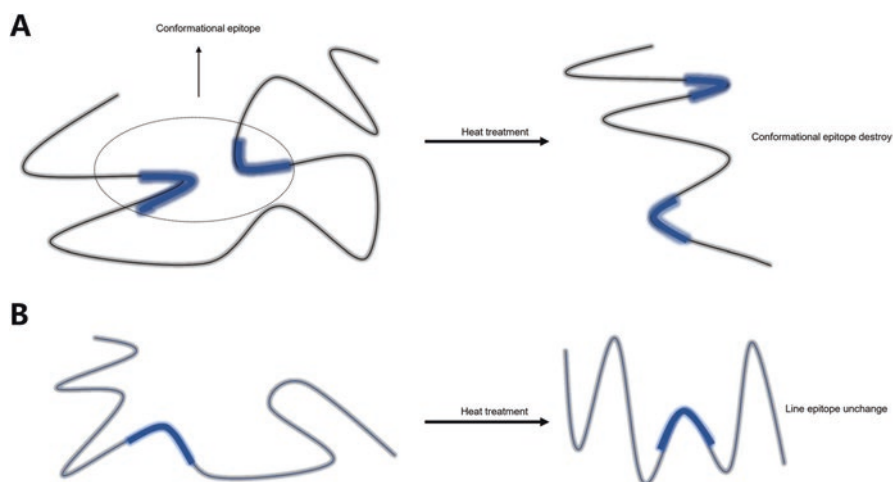


Fig. 6.1 Schematic diagram of heat-induced changes of antigenic epitopes. (a) Heat-induced changes of conformational epitopes. After heat treatment, the conformational epitope of the protein is destroyed; thereby, the allergenicity is reduced. (b) Heat-induced changes of linear epitopes. Linear epitope of the protein still existed after heat treatment, and thereby the allergenicity was not influenced

6.1.1 Heat Treatment

Heat treatment is a common method in food processing. The influence of processing methods on food allergens varies in food and protein types. Some of the food proteins that cause severe allergies are often very stable, usually contain more than two sulfur bonds, and show strong acid and digestive enzyme resistance. The stability of some other allergens is common, but the daily food heat processing methods may produce new epitopes, which can affect the allergenicity of food. For example, thermal processing can destroy the three-dimensional conformational epitopes and reduce the binding activity to human IgE, so as to eliminate their allergenicity; however, thermal processing may also cause the production of a protein-sensitized neoepitope or the exposure of previously hidden protein epitopes, increasing their ability to bind to IgE and thus leading to an increase in food allergenicity (Kuang 2013) (Fig. 6.1).

Due to the universality of heat treatment in food processing, a lot of researches have been done on the elimination of food allergenicity by this method, and the effect on various types of food such as milk, fish, grains, eggs, shellfish, and nuts has been investigated (Bavaro et al. 2018; Gomaa and Boye 2013).

The myosin is an important regulatory protein in the muscle contraction and exists widely in vertebrates and invertebrates; it exists in the myosin in parallel with the actin double helix and can regulate the interaction of actin (Jeong et al. 2006). Tropomyosin in muscle tissues of shrimp is considered to be the main allergens of shrimp, and TM from different crustacean species has high cross reactivity. Studies

showed that both normal hot processing and hot drying (105 °C oven baking 2 h) could significantly reduce the allergenicity of shrimp (Zhang et al. 2011). And the immune activity of shrimp after drying (105 °C oven baking 2 h) reduced (Liu et al. 2011). The IgE-binding ability of the milk allergen lactoglobulin was not completely destroyed after heating, but the digestibility of whey protein *in vitro* is increased, which could be another mechanism for allergenicity elimination by heat treatment; in the mouse model, it was found that the release of the medium in the rat eosinophils and the activation of the basophil could be reduced (Martos et al. 2011). Vissers and colleagues (2011) designed two kinds of heat processing methods to treat peanuts, boiling and baking methods, and compared the allergenicity of peanut allergen Ara h 1, Ara h 2, and Ara h 6 before and after processing. It was found that the boiling method could lead to the self-polymerization of Ara h 1, thus reducing the activity of binding and cross-linking with IgE, but the activity of the sensitized T cells was not affected. However, although the Ara h 1 extracted from baked peanuts was highly denatured and formed more spherical small polymers, its binding activity with IgE remained unchanged. For Ara h 2 and Ara h 6, boiling heat processing had no effect on the allergen-induced activity of T cells, but it reduced the IgE-binding and cross-linking ability, thus reducing the allergenicity of the two kinds of allergens, but the soluble Ara h 2 and Ara h 6 extracted from roasted peanuts were not different from those from unprocessed peanuts on allergenicity. Other common allergenic food ingredients, such as soybean extract including seed storage 11S and 7S globulin, are usually heat-resistant. These allergens usually contain beta-barrel motifs and show highly stable domain characteristics, so only partial and minor conformation changes occur under heat treatment (Mills et al. 2003, 2009). Heat transfer of 7S globulin occurs at 70–75 °C, while the structure of 11S globulin is released at temperatures above 94 °C. Bohel and other studies showed that cooking different recombinant Bet v 1 (a birch pollen allergen)-related allergens for 60 min could completely eliminate the binding ability to IgE, but the allergen sensitized specific T cell viability was not observed to be reduced (Bohle et al. 2006). Some food allergen proteins are very stable, such as the lipid transfer protein (LTP) in peach, apple, and other fruits, whose molecular weight is only 9 kDa but contains four pairs of two sulfur bonds. The results showed that the natural purified LTP of apple (Mal d 3) had no change in IgE-binding activity after heat treatment (90 °C, 20 min) in the matrix with or without glucose. After a higher temperature and a longer time (100 °C, 2 h) treatment, a little structural change was observed, but the IgE-binding activity decreased significantly (nearly 30 times). Specifically, the heat treatment in the presence of glucose led to the combination of four glucose molecules and 1 Mal d 3 protein molecules, which reduced the IgE-binding activity to 10–50% (Sancho et al. 2005).

Overall, heat processing may not only reduce the allergenicity of food allergens but also enhance the allergenicity of food allergens in some situations, which is dependent on the differences of food protein, processing methods, and heat processing types. Norgaard and other (1992) studies found that the allergenicity of milk boiled for 2–5 min did not change obviously. After boiling for 10 min, β -lactoprotein and albumin were inactivated, but casein remained stable and positive in the skin

pricking test (SPT). Therefore, the elimination of allergens in milk requires a longer heating time, but casein allergenicity is almost impossible to remove. Ovalbumin and ovomucin are the main allergens of eggs. When ovalbumin was heated for 3 min at 100 °C, the allergenicity reduced by 90%, while ovomucin remained allergenicity at 100 °C for a long time. Therefore it can be speculated that egg mucin in eggs has a very strong thermal tolerance (Hefle 1999). Beyer and colleagues (Beezhhold et al. 2001) found that the ability to combine IgE with peanuts was half lower than that of raw peanuts. This is not related to the changes in the structure of peanut allergens, but mainly due to the increased dissolution of some allergens such as Ara h 1 and Ara h 2, as well as some low molecular weight protein or peptide fragments of molecular weight of 10–16 kDa. The content of Ara h 1 monomers and trimers decreased in fried peanuts, resulting in a decrease in the binding capacity of IgE, while the anaphylaxis to roasted peanuts was higher than that to raw peanuts. Because Asian countries tend to consume fried peanuts, and European countries used to consume roasted peanuts, this could express the reason why the incidence of peanut allergy in Western countries is much higher than that in Asian countries. Naqpal (Naqpal et al. 1989) found that after cooking at 100 °C, the anaphylaxis-inducing activity of the shrimp remained unchanged, and the complete shrimp allergen could still be purified from the shrimp. On the other hand, the effects of heat treatment on allergens in fresh fruits and vegetables are complicated. Immunoblotting showed that storage could reduce the allergenic activity of celery, and the effect of heat treatment on allergens was even stronger (Norgaard et al. 1992). According to the study of Dube (Dube et al. 2004), heat treatment and pasteurization had no obvious damage to the allergens in mango pulp and fruit juice; similarly, Brenna (Oreste Brenna et al. 2000) reported that continuous heating for 30 min at 121 °C has no effect on the allergens in peach fruit pulp and its juice.

6.1.2 *Irradiation Treatment*

Food irradiation technology is a process that uses gamma rays produced by ionizing radiation sources ⁶⁰Co or ¹³⁷Cs, X rays produced by mechanical sources, or electron beams to treat packaged or bulk food at a controlled level by ionizing radiation for a certain period of time (Vaz et al. 2011). At present, irradiation is mainly used in the processing of food including dehydrated vegetable, root vegetable, garlic, mushroom, grain, fruit, meat, seafood, honey and so on.

In the 1970s, the International Atomic Energy Agency (International Atomic Energy Agency, IAEA), the United Nations Food and Agriculture Organization (Food and Agriculture Organization, FAO), International Health Organization (World Health), and other international organizations coordinated the health and safety of irradiated food by scientific research institutions in various countries. In October 1980, it was announced that “there is no toxicological risk for any food when its overall average absorption dose is not more than 10 kGy, without toxicological tests, and also safe in nutrition and microbiology.” Various studies showed

that the effect and molecular mechanism of irradiation on protein is complex. It changes the high structure of protein molecules and the aggregation mode between protein fractions, mainly by inducing the deamination, decarboxylation, amino acid oxidation, fracture or reconstruction of two sulfur bonds, and degradation or cross-linking of peptide chains in protein molecules (Kinsella and Melachouris 1976; Kuan et al. 2013; Molins 2001; Zayas 1997; Zhu et al. 2012). The denaturation of the functional properties of protein molecules is closely related to the free radicals produced by the irradiation of water in food components, and the amino acid with sulfur and aromatic residues including tryptophan, cysteine, methionine, tyrosine, and phenylpropyl acid is more susceptible to irradiation.

At present, soy protein isolate, bovine serum albumin, ovalbumin, peanut protein, etc. are often used as model proteins to study the effects of irradiation on protein in food components. Studies have shown that the structural changes of proteins after irradiation can lead to changes in properties. When the radiation dose is 4 kGy, the structure of soybean protein isolate changes, and its oil absorption and emulsification reach the lowest and highest value, respectively. Similarly, the increase of radiation dose promotes emulsification stability of soybean protein isolate, as well as the changes in the secondary structure of the albumin (Dian et al. 2011). The larger the amount, the greater the degree of structure change, especially conversion from the microstructures to the flake protuberance of bovine serum albumin, and the denaturation of bovine serum albumin results in the reduction of the dissolubility (Geng et al. 2015). The structure and physicochemical properties of ovalbumin were greatly affected by irradiation. With the increase of irradiation dose, the soluble protein content, free sulfhydryl content, DPPH radical scavenging capacity, and reducing power of ovalbumin were increased to varying degrees (Zong-Cai et al. 2016). When the peanut protein was irradiated, its hydrophobic group was exposed, and the secondary structure changed, with the increase of irradiation dose, the allergenicity of the within allergenic proteins decreased (Luo et al. 2013). Another study showed that the increase of irradiation dose made the protein structure of fish myofibrillar fiber change, and its emulsifying, surface hydrophobicity and free sulfhydryl content were decreased (Shi et al. 2015). It can be seen that irradiation mainly induces a chain reaction by inducing the conformational change and polymerization of protein molecules, thus improving the functional properties of the protein (Wihodo and Moraru 2013). Zhang et al. (Zhang 2013) used different electron beam doses to treat liquid protein, powdered protein, and fish meat of turbot; by detecting the change of carbonyl content, total sulfhydryl content, hydrophobicity and thermal stability, and texture, the different irradiance doses and the state of protein irradiation were detected. It is found that the electron beam irradiation changes the original structure of turbot protein and the increase of the irradiation dose will lead to the increase of the carbonyl content, decrease in the total sulfhydryl content, hydrophobicity, and thermal stability. Specifically, the effects of irradiation on the content of carbonyl groups and total mercapto content are most obvious. Irradiation has the most obvious influence on the structural properties of liquid proteins. At the same time, the texture analysis showed that the electron beam radiation of medium and high doses (more than 7 kGy) could have a weaker influence on the adhesion

and mastic ability in fish texture than the other methods. The results showed that electron beam irradiation can effectively reduce the allergenicity of fish without significant change of the texture which may affect the quality of fish products. Therefore, by optimizing the parameters of electron beam irradiation, it is hopeful to develop low-allergy fish products while maintaining the original quality of fish and provide technical support for improving the safety of fish products and protecting the health of consumers.

The researchers have also used irradiation to desensitize peanut products in their solutions. The results showed that small doses of less than 1 kGy had little effect on allergens. When the irradiation dose was above 1 kGy, the protein content in the solution, except for those with the molecular weight greater than 96.9 kDa, was generally reduced, indicating the irradiation can reduce the allergenicity of food to a certain extent (Wang et al. 2009). In the study of lectin allergens, it has been shown that irradiation can remove lectin allergens and thus can be recommended for the removal of protein allergens. In the synergistic study of the processing technology of irradiation combined with other allergen removal approaches, the researchers used irradiation and heat processing to remove allergens in eggs, milk, kidney beans, peanuts, black beans, and four season beans, respectively. It was considered that irradiation had a certain effect on the allergen structure of egg and milk. However, the reduction of allergenicity was not significant (Gomaa and Boye 2015; Lee et al. 2007). In the study of kidney beans, peanuts, black beans, and four soybean proteins, the combination of heat treatment and irradiation could reduce the allergenicity of soluble and insoluble proteins and reduce the induced anaphylaxis responses, but the effect of radiation treatment alone was not very obvious (Kasera et al. 2012). The study and application of irradiation in removing or reducing allergens are still under development, and the related processes and desensitization mechanisms need to be studied continuously. How to achieve efficient desensitization without destroying food components needs to be explored in depth. In addition, the influence of strong irradiation on the anaphylaxis activity is complex, and the intensity of irradiation may produce dose residue; moreover, there are still many other unsolved problems that need to be further explored.

6.1.3 Ultrasound Treatment

Ultrasound is an elastic mechanical wave in material medium with a frequency range of 2×10^4 – 2×10^9 Hz (Fig. 6.2). When the ultrasonic wave is propagated in the medium, it will produce thermal effect, mechanical effect, or cavitation effect, which can degrade biological macromolecules. It was found that temperature had a great influence on the effect of ultrasonic treatment of allergens. For example, Li (2006) found it can significantly reduce the allergenicity of *Litopenaeus vannamei* at 50 °C by ultrasound but almost no effect at 0 °C. This may be due to the fact that the allergen can absorb the energy of ultrasound and convert it into heat energy, which can degrade allergen.

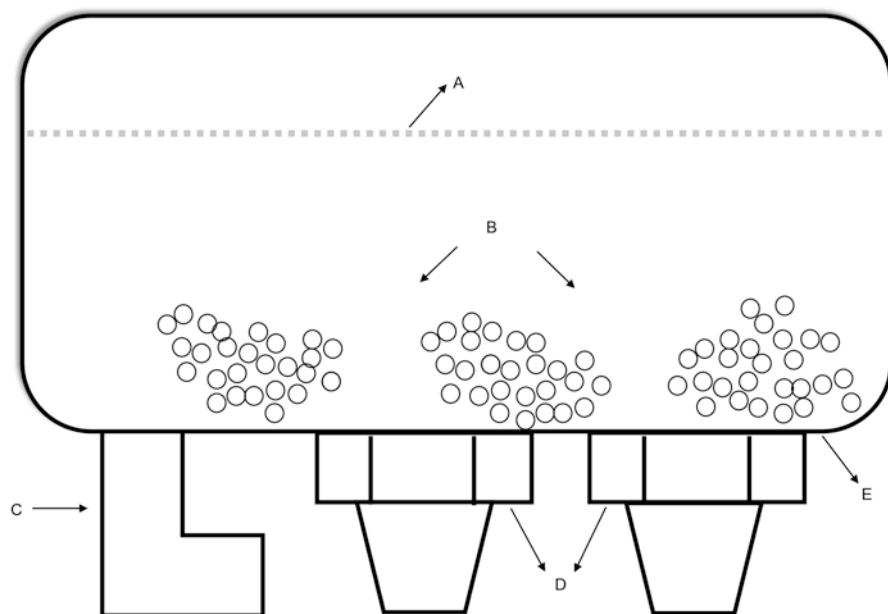


Fig. 6.2 The working principle of ultrasonic. The function of the ultrasonic apparatus is mainly based on the transducer, which converts the sound energy of the ultrasonic frequency source into mechanical vibration and radiates the ultrasonic wave to the liquid in the tank by vibrating plate. Due to the radiation of ultrasonic wave, the microbubbles in the liquid in the tank can keep vibration under the action of acoustic wave. (a) Liquid surface; (b) cavity bubble; (c) ultrasonic transducer; (d) vibrating plate; (e) outlet

On the basis of studying the effect of ultrasonic treatment on allergens of the acupoint green crab, Liu Guangming (2011) extracted the crude protein after treatment with ultrasonic, microwave, and ultrasonic combined cooking. The digestive stability of treated TM was analyzed by simulated gastrointestinal digestion, SDS-PAGE, Western blotting, and inhibitory ELISA. The results showed that compared with untreated samples and non-allergic proteins, the degradation of TM was faster after ultrasonic treatment (200W, 30) and the digestive stability and allergenic of TM were not significantly changed after microwave treatment. Furthermore, the digestive stability and allergenicity of TM after ultrasonic treatment alone or ultrasonic combined with cooking were obviously reduced.

6.1.4 Ultrahigh Pressure

Ultrahigh pressure technology (Fig. 6.3) is an emerging nonthermal technique in food industries to inhibit the growth of microorganism as well as increase the shelf life of food without affecting its organoleptic properties. It is a new type of food

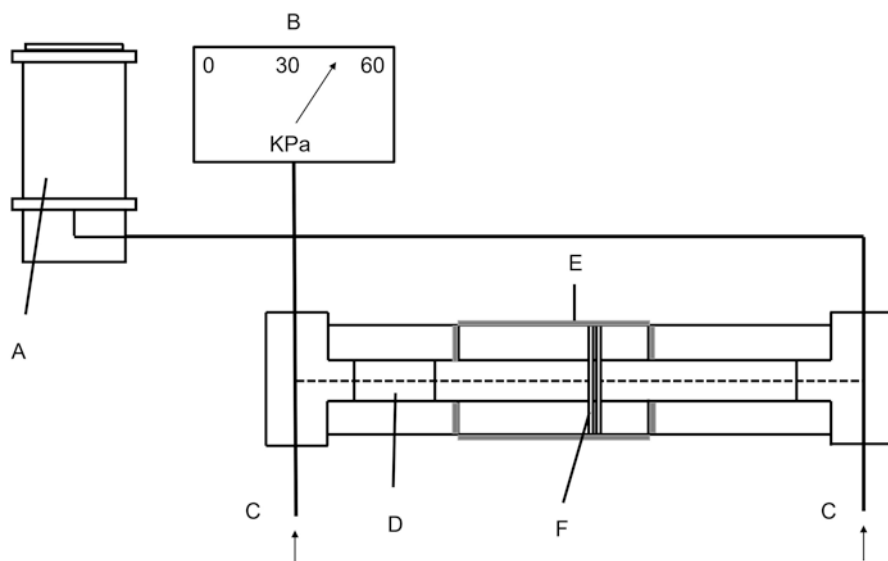


Fig. 6.3 The working principle of UHP. In a sealed ultrahigh pressure vessel, water is used as a medium; different pressures are set by the supercharger and applied to food. (a) Ultrahigh pressure cylinders; (b) pressure gauge; (c) intake; (d) liquid chamber; (e) supercharger; (f) piston

processing technology which is different from traditional hot processing. During processing, pressure has no effect on changing the covalent bonds of proteins and other high molecular substances, as well as small molecular substances such as vitamins and pigments, which can maximize the nutritional value of foods. At the same time, the ultrahigh pressure can cause changes in the microstructure of every single system in dairy products, such as white cheese and whey protein, resulting in the change of physical properties of dairy products, which further extends the application of ultrahigh pressure technology. The application of ultrahigh pressure as a food processing technology in shrimp preservation and processing mainly includes chemical sterilization, the improvement of the quality of muscle, the modification of the physicochemical properties of protein, the reduction of allergens, the discarding of the shell, and the extraction of astaxanthin. At present, ultrahigh pressure is considered as a new desensitization technique. By destroying the non-covalent bond of protein, the advanced structure of protein is changed, causing the denaturation or inactivation of protein. In Fig. 6.4, the possible mechanism of ultrahigh pressure treatment to regulate the allergy of food proteins is summarized.

The polypeptide formed by the combination of amino acids in a certain order is called the primary structure, and it is also the most basic structure of protein. It is reported that the pressure below 8 GPa does not change the primary structure of protein, so it is generally believed that the current pressure has not yet affected the primary structure (Xia 2012). Under high pressure, the secondary structure will be changed, leading to irreversible degeneration. Protein tertiary structure is mainly

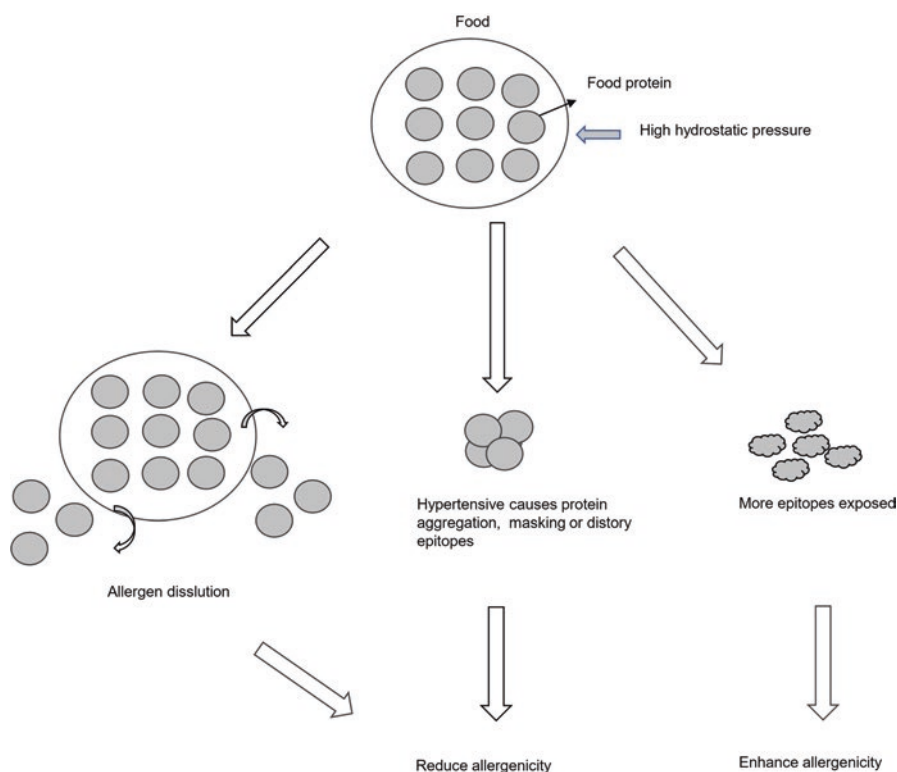


Fig. 6.4 Schematic diagram of UHP-induced changes of allergenicity. Ultrahigh pressure treatment reduced or enhanced the allergenicity of protein. On the one hand, allergenicity decreased due to the dissolution of allergens as well as the mask and destruction of antigen epitopes after high-pressure treatment. On the other hand, UHP can also produce new epitopes that enhance the allergenicity

maintained by Van Edward force, hydrogen bond, hydrophobic bond, and electrostatic interaction. The quaternary structure of protein refers to the space structure formed by two or more independent tertiary polypeptide chains and maintained mainly by hydrogen bonds. The tertiary and quaternary structures of proteins are greatly affected by pressure. Ultrahigh pressure changes the non-covalent bonds of proteins by compressing the molecular volume of proteins, causing protein depolymerization and molecular structure extension. In addition, when the protein molecules are compressed under ultrahigh pressure, the intermolecular distance of the hydrogen bond is decreased, and the hydrogen bond is enhanced. At the same time, the water molecules enter the protein and enhance the hydration of the protein, resulting in the decrease of the hydrophobic interaction between the molecules. Specifically, when the pressure exceeds 200 MPa, the change of the tertiary structure can be observed.

Milk allergy is the human immune response to one or several kinds of milk proteins, mainly mediated by IgE. UHP can change the spatial structure of allergens to some extent and make the activity of allergens change or even inactivate, so as to reduce or eliminate the allergenicity. Therefore, it has been widely used in food allergy reduction. Zhong J (Zhong et al. 2011) used dynamic high-pressure micro jet technology to treat beta lactoglobulin at different temperatures and analyze the allergenicity by indirect competitive enzyme-linked immunosorbent assay. At 90 °C, DHPM treatment had a significant effect on the reactivity of beta globulin antigen, and at 160 MPa, the antigen response increased when temperature increased. Compared with the control group, the allergenicity of DHPM-modified beta globulin significantly increased at 80 MPa, and the allergenicity decreased when the pressure was higher than 80 MPa. When exploring the relationship between β -lactoprotein and the degree of denaturation caused by ultrahigh pressure, Kleber (Kleber et al. 2007) found that increasing stress and time can lead to an increase in allergenicity. They believed that the result may be due to the unfolding of protein structure under the effect of high pressure, which may produce a new epitope.

Due to the high nutritional value, whey protein is usually used as high-energy and nutrition supplement for infants or elderly people. Additionally, after reducing the allergenicity by hydrolysis, whey protein can be applied to people with food intolerance or allergy. Ambrosi (Ambrosi et al. 2016) verified the effectiveness of UHP technology to accelerate the hydrolysis reaction of whey protein with selected enzyme (alpha pancreatic chymotrypsin, bromelain) and influence the ability of protein allergen. The experimental data showed that when UHP was combined with enzyme hydrolysis, the degree of hydrolysis increased not only with the applied pressure level but also with the treatment time. These results indicate that even if the exposure of hidden epitopes upon protein unfolding increased the allergenicity of whey proteins, further peptide bonds cleavage also took place after hydrolysis. This effect could modify whey protein antigenic epitopes and thus their antigenic activity.

Han (Han et al. 2017) studied effect of high pressure on the allergenicity of tropomyosin in shrimp. The results showed that the allergenicity of TM was fluctuant by UHP treatment. Under room temperature, the allergenicity of TM decreased and reached the lowest at 300 MPa when the fixed pressure time was 10 min.

6.1.5 Pulsed Ultraviolet Light

Pulsed ultraviolet technology is a powerful and extensive industrial technology, which can be applied to many fields, such as water treatment, air sterilization, and food processing, and in pharmaceutical, agricultural, and sideline products. The intensity of PUV is a few thousand times of the traditional ultraviolet intensity. It can instantaneously emit high-intensity pulse light energy to kill all kinds of

microbes, and its photothermal effect, polymerization, and catalytic effect can change the structure of allergens and polymerize proteins. Nooji (2011) found that pulsed ultraviolet (45 s) alone or combined heat treatment could reduce the allergenicity of wheat gluten protein, which will not be affected only by the heat treatment at this temperature. However, the excessive PUV radiation time will increase material temperature and cause water loss; moreover, the penetration has its limitations, so in the actual operation process, reasonable process parameters should be worked out during the actual operation.

Meinlschmidt (Meinlschmidt et al. 2016) investigated the effect of nonthermal processing technologies on soy immunoreactivity. Soy protein isolate was treated with PUV light; SDS-PAGE analysis revealed reduced protein intensity bands corresponding to major soy allergens β -conglycinin (Gly m 5) and glycinin (Gly m 6). Sandwich ELISA using specific mouse monoclonal anti-Gly m 5 antibodies confirmed a loss of soy immunoreactivity following PUV light treatment. Overall, this innovative technology might have great potential for industrial application due to their effectiveness in reduction of protein allergenicity.

6.1.6 Combination Method

It was found that the combination of several desensitization methods could produce better desensitization effect. For example, Liu and others compared the effects of ultrasonic, microwave, and ultrasonic combined with cooking methods on eliminating crab allergic protein allergenicity. It was found that ultrasonic combined with cooking treatment could significantly reduce its sensitization (Liu 2011). Li found that the combination of box irradiation and heating could significantly reduce the allergenic allergens (Li 2006). Long Fangyu (Long et al. 2015) treated allergen proteins by high hydrostatic pressure combined with heat treatment. It was found that thermostatic pressure combined with high hydrostatic pressure technology could better reduce the allergenic activity of allergens. Therefore, the combined action of several treatments can reduce the sensitization of postal meat to a greater extent.

6.2 Chemical Modification

Chemical modification means changing the structure, electrostatic charge, and hydrophobic groups of proteins by chemical means, thereby improving the functional properties of proteins. At present, chemical modification is mainly based on amide bond, amino group, carboxyl group, hydroxyl group, and mercapto group. By chemical modification, the allergenic epitopes of allergen protein molecules can be concealed or destroyed, and their allergenicity can be changed.

6.2.1 Glycosylation

Food allergen is related to allergenicity only by several to dozens of amino acid residues in its antigenic determinant. Therefore, if such groups are concealed, destroyed, and removed, the epitopes that cause allergenicity are no longer recognized by the body's immune system, which is the key to developing hypersensitive foods. Many allergens are glycoproteins with molecular weight of 10–80 kDa and acidic isoelectric point. Studies have shown that carbohydrate structure plays an important role in the formation of antigenic determinants. Therefore, modification of glycoproteins through glycosylation is an important direction to reduce allergy.

Maillard reaction is one of the ways of food protein glycosylation, which is a widespread nonenzymatic browning reaction in food processing, and is mainly a reaction between carbonyl group of reducing sugar and amino group of protein. In 1912, Lewis Camilla Maillard, a French chemist, discovered the Maillard reaction and named it. The reaction process was complex and accompanied with color changes. During the period, many intermediates with different odors, such as reducing ketones, aldehydes, and heterocyclic compounds, were formed. These substances could provide food with tasty flavor and attractive color. Maillard reaction is widely used in food processing because of its simple operation and significant influence on food flavor and color.

The selection of sugar in glycosylation is very important. In recent years, glucose, maltose, lactose, and chitosan have been used frequently. Oligosaccharides are straight chain or branched oligosaccharides indirectly formed from 2 to 10 monosaccharides through glycosidic bonds. Due to the high solubility in water, oligosaccharides can also be applied in glycosylation. There are many important oligosaccharides, such as maltose, lactose, and trehalose, which are widely distributed in food and vegetable crops. There are also some functional oligosaccharides, such as fructo-oligosaccharides, xylooligosaccharides, oligosaccharides, and cyclic oligosaccharides. Oligosaccharides changed protein property not only by glycosylation but also by direct interaction (Jia et al. 2014). Polysaccharides are monosaccharide polymers similar to oligosaccharides, consisting of glycosyl groups arranged in straight or branched chains, but most of them are composed of more than 20 monosaccharides. There are many kinds of polysaccharides, such as chitin, glucan, mannan, xylan, pectin, etc., among which, glucosamine and chitosan can be used for glycosylation. It has been reported that the combination of polysaccharide and protein can improve the emulsifying property and emulsification stability, as well as the solubility and the antioxidant activity of protein, and finally change the protein structure and alter the allergenicity.

Zhao (Zhao et al. 2017) investigated the influence of Maillard reaction on the structural and immunological properties of parvalbumin (PV), the major allergen in fish. Recombinant silver carp PV (rPV) was employed and incubated with glucose at 60 °C for 72 h. The IgG/IgE-binding properties of rPV were weakened after Maillard reaction as demonstrated by dot blotting. Allergenicity decrease of rPV by Maillard reaction was further confirmed on sensitized RBL-2H3 cell with the

decreasing release of β -hexosaminidase and histamine and suppressing the production of interleukin-4 (IL-4) and tumor necrosis factor- α (TNF- α). Comparison of the glycation sites with documented epitopes suggested the direct blocking of conjugated carbohydrates (K88, K97, and K108) on IgE-binding epitopes.

However, opposite results were also observed. On studying the effect of glycosylation modification on the allergen properties of *Scylla paramamosain*, after glycosylation by Maillard reaction, it was found that the rate of glycosylation of TM and AK with ribose was quicker than with glucose and the glycosylation of TM was more rapid than that of AK. Compared to dry method, Maillard reaction in wet method would be slower. The glycosylated products of Maillard reaction would lead to the decrease of IgE-binding activity of TM and AK and enhance their digestibility of trypsin (for TM) or pepsin (for AK). Finally, the ability of AK binding to antibody was enhanced during Maillard reaction of crab crude reacted with glucose (Ruan 2012).

6.2.2 Acid-Base Treatment

In addition to complex glycosylation reactions, the allergenicity of protein will also be changed after simple alkali treatment. Brenna et al. (Oreste Brenna et al. 2000) found that soaking with sodium hydroxide at 60 °C can reduce the allergic activity of peach flesh and juice. The allergenicity of soft albumin and soft mucin in eggs was also reduced after sodium hydroxide treatment. Because allergens are acid proteins that are stable under acidic conditions, most allergens have resistance to acid, and alkaline conditions make it easy to change its structure and allergenic activity.

Acid hydrolysis is a chemical method to reduce the allergenicity of proteins such as wheat flour, which used acid to alter the structure of the allergen in wheat to produce low allergenicity products. Funatsuki (Funatsuki et al. 2007) used lactic acid and hydrochloric acid to treat gluten protein. Because of the action of acid, glutamate residue in gluten protein function has been changed when the degree of deamidation is 30%. Moreover, when the degree of deamidation is 50%, the binding ability of gluten protein to IgE is significantly reduced.

6.3 Biological Modification

In recent years, biotechnology has developed rapidly and been widely used in food processing, which is not only conducive to the development of various functional food but also can reduce or eliminate the harmful substances in food and ensure the consumer's safety. Enzyme processing and microbial fermentation have shown great potential in the development of low allergenicity food.

6.3.1 Enzymatic Technology

6.3.1.1 Enzymatic Hydrolysis Modification

Enzymatic hydrolysis of food is a more widely used method to reduce food allergenicity. It is a more specific method than heat treatment. For food allergy only with linear epitopes, it is suggested that allergens of food can be reduced only when the allergen epitope is eliminated. But for most food allergens, enzymatic hydrolysis may lead to a significant reduction, because the small peptide segments after digestion cannot cross-link the IgE-binding receptors on the surface of mast cells. Moreover, their potential hapten activity will inhibit IgE-mediated responses.

Compared with chemical modification, enzymatic modification has mild reaction conditions, high efficiency, strong transferability, and less toxic and side effects. Enzymatic hydrolysis can alter the tertiary structure of the antigenic determinant or break the amide bond to reduce the molecular weight of the allergen, thereby reducing its activity. With the development of enzyme preparation industry, it is possible to select highly specific enzymes for directional decomposition of allergic proteins or to use complex enzymes for restrictive hydrolysis with controlled and optimized parameters, which would be a direction of future hypoallergenic food development.

Protein enzymatic hydrolysis is the process of protein degradation into peptides and smaller molecular amino acids in the action of enzyme (Skanderby 1994). The physicochemical and structural characteristics of the protein after enzymatic hydrolysis are changed, and the solubility and other functional properties can be obviously improved. Enzymatic modification generally involves the change of the primary structure of protein, which is one of the most effective ways to modify the spatial structure of protein, realize the diversity of protein function, and increase the added value of protein (Lee and Kim 2005). However, there are various by-products in the process of enzymatic hydrolysis, and the reaction is difficult to control. For instance, it is easy to produce bitter peptides due to the increase of the degree of hydrolysis. The effects of enzyme hydrolytic protein on allergens have two main aspects: on the one hand, by limited hydrolysis of food allergens, changing the tertiary structure of the allergenic epitopes, removing some allergenic epitopes on the surface of the allergens, or exposing the allergenic epitopes inside the protein; on the other hand, breaking peptide bonds to make the protein partial hydrolysis of the substance into small peptides or amino acids, reducing the molecular weight of allergens as well as its allergenic (Xin and Hongbing 2005).

Pepsin, trypsin, alkaline protease, neutral protease, and papain are the most commonly used proteases. Pepsin is a peptic proteinase from the stomach that belongs to the aspartate proteolytic enzyme group (Wang and Zhang 2008), which mainly acts on the peptide bonds containing phenylalanine or tyrosine in protein and polypeptide molecules, and its main decomposing product is peptone, and the production of peptides or amino acids is less. Trypsin is a commonly used proteolytic enzyme with the molecular mass of 23.8 kD, which can act exclusively on peptide

bond formed by the carboxyl group of arginine and lysine (Zhang et al. 2009). Trypsin is widely used in food processing to hydrolyze proteins. Therefore, its effects on food allergens have attracted widespread attention. Papain is a class of cysteine protease (Azarkan et al. 2003). It has a good hydrolysis effect on various proteins and can catalyze the hydrolysis of peptide bond, ester bond, and amide bond (North et al. 1990) and can also affect the allergenicity of food. Alkaline (Zheng et al. 2007) is a kind of microbial protease which has a wide range of sources, high activity, fast hydrolysis speed, and low cost. Alkaline protease is an endopeptidase, which catalyzes the formation of amide bonds from the carboxyl groups of hydrophobic amino acids as well as hydrolyzes peptide bonds within the protein, so as to produce peptides with relatively small molecular weight, destroying the antibody binding sites within the protein and effectively reducing the allergenicity of the protein (Shen et al. 2006). Chymotrypsin (Huang et al. 2005) is a proteolytic enzyme extracted from bovin or pig pancreas. It mainly hydrolyzes peptide bonds formed by the carboxyl group of aromatic amino acids and amino acids of other amino acids (Xu et al. 2006).

Cashew and pistachio allergies are considered as a serious health problem. Previous studies have shown that thermal processing, pressurization, and enzymatic hydrolysis may reduce the allergenic properties of food by changing the protein structure. Carmen Cuadrado found enzymatic hydrolysis under sonication separately induced a measurable reduction in the IgE-binding properties of pastes made from treated cashew and pistachio nuts. This treatment was more effective with pistachio allergens (Cabanillas et al. 2012).

Some other proteases were used to hydrolyze the allergens of shrimp, and the results showed that shrimp allergens were sensitive to enzymatic hydrolysis. Zhang (2015) used seven enzymes, such as alkaline protease and pepsin to hydrolyze the shrimp allergen, and the allergenicity of resulting enzymatic hydrolyte was determined by SDS-PAGE and OPA. The results showed that the effect of alkaline protease was the most significant. The allergy mice model was established with shrimp allergen and enzyme-hydrolyzed protein, and the effects of enzyme hydrolysis on allergenicity were analyzed by measuring the indexes of sIgG, sIgE, and histamine, spleen index, and the pathological section of the small intestine. The results showed that the level of sIgE in the proteolytic group decreased by 40.44%, the histamine content decreased by 5%, and the spleen index and the sIgG level had no significant difference. However, the small intestine slices showed stronger inflammatory response in the enzyme-hydrolyzed protein group for unrevealed reasons. In another study, the shrimp allergic proteins were treated with pepsin, alkaline protease, neutral protease, flavor protease, and papain. It was found that the hypersensitive effect of flavor protease and neutral protease to shrimp allergen was the most significant (Dong et al. 2010).

Enzymatic hydrolysis is also used in the production of hypersensitive rice and can destroy the antigenic determinants, so as to achieve the purpose of removing allergens. The hypoallergenic rice produced by this method is more viscous than the untreated rice and can be accepted by consumers. In addition, when the enzyme acts

on rice protein, it produces some amino acids and small peptides, which can improve the taste of rice to a certain extent, especially for aged rice. However, because of the large amount of enzyme used, the production cost is increased. Chen (2004) studied the conditions for the decomposition of rice allergens by papain. The results showed that with the condition of 37 °C, pH 4.5, 132.93 mg/5 g, and 25 h, the removed allergen protein was mainly globulin in the outer layer of rice endosperm. However, enzymatic hydrolysis did not affect all allergens; many allergens such as those in peach can tolerate the hydrolysis of enzymes, which may be related to the specificity of the selected enzyme. On the other hand, proteolysis may also lead to the deterioration of food taste. Danish Aria and New Zealand Fonterra have developed different degrees of hydrolysate whey protein products, which provide raw materials for hypoallergenic infant formula milk. Different hydrolytic patterns and degrees of proteins may lead to the difference in peptide composition and taste. Moreover, although the allergenicity of hydrolyzed products was significantly reduced, it may not be completely eliminated. Overall, the mechanism of the allergenicity and flavor of each product in the hydrolysis process needs further study for the improvement of the methods.

6.3.1.2 Enzymatic Cross-Linking Modification

Enzymatic cross-linking modification of protein method usually refers to the intramolecular or intermolecular cross-linking reaction under the action of enzymes, which can induce the polymerization of protein. The modification method is helpful to improve the gel property and thermal stability of proteins. However, the formation of insoluble polymers is easily caused by molecular cross-linking, which reduces the solubility of the products and affects their application to a certain extent. In addition, cross-linking also alters the sensitivity of proteins to gastrointestinal digestion and their absorption in the mucosa, therefore influencing their allergenicity. In Fig. 6.5, the possible mechanism of cross-linking on regulating the allergenicity of food proteins is summarized.

Enzymes that are commonly used to catalyze protein cross-linking include transglutaminase, polyphenol oxidase, and peroxidase. Transglutaminase (TG) can catalyze the reaction between the amidyl groups, which can catalyze the junction between the epsilon amino acid on polypeptide lysine and the gamma hydroxyl amide group on the glutamic acid, thereby promoting covalent cross-linking between protein and polypeptide, polymerizing them into a covalent compound. Clare (Clare et al. 2008) treated peanut powder containing casein at 37 °C and formed a polymer. The allergenicity was detected by immunoblotting and found that the IgE-binding activity of the main allergen Ara h 2 in peanut powder decreased. The transglutaminase was also used to modify the trypsin hydrolysate of the tilapia foot. The results showed that with optimized conditions, the emulsification and foaming property of the TG cross-linked products were improved, which were 1.35 times and 1.20 times higher than that before the cross-linking, respectively. Sephacryl S-200 gel filtration chromatography shows that TG cross-linking changes the structure of

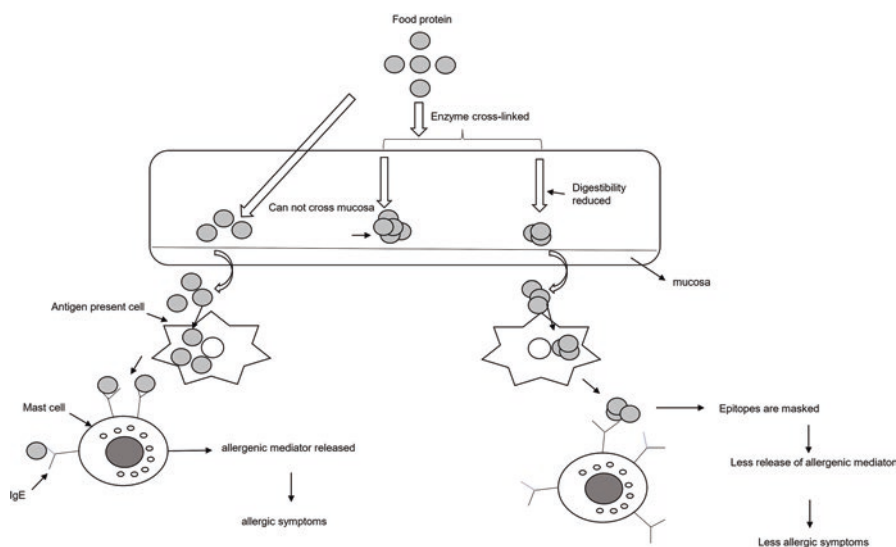


Fig. 6.5 Schematic diagram of cross-linking-induced changes of allergenicity. Cross-linking can change the protein allergenicity through multiple ways. On the one hand, the cross-linked protein may form high polymer, which was too large to pass through intestinal mucosa and cannot interact with host immune system. On the other hand, the protein antigen epitope may be masked after cross-linking, which reduced the IgE-binding activity and then decreased subsequent mast cell degranulation

protein molecules and connects the protein molecules with each other to form a space network structure, which help to improve the emulsification and foaming of the products (Ren and Zhou 2014). Han (Han 2013) evaluated the digestibility of peanuts and their cross-linked products. At the same time, microscopy was used to observe the microstructure of cross-linked products and the products after gastrointestinal digestion. It was found that the protein was resistant to stomach digestion but was easily digested by intestine. On the other hand, the cross-linked products were digested by the stomach, but the intestinal digestion rate slowed down.

Compared with the study of TG-catalyzed protein cross-linking, there are only a few studies on transglutaminase-catalyzed protein glycosylation. Yan (Yan and Wold 1984) used TG from animal origin to catalyze the cross-linking of maltotriose and beta casein. It is the first confirmation that protein glycation can be achieved by TG pathway; Davide Gottardi and Pui Khoo Hong (Davide et al. 2014; Hong et al. 2014) used TG to introduce glucosamine into wheat bran protein and fish gelatin polypeptide, and the antioxidant activity of glycosylated products significantly enhanced. Jiang Shujuan (Jiang and Zhao 2010) studied the conditions for TG to catalyze the cross-linking reaction of casein and soy protein isolate with glucosamine and found that the solubility, foaming, and emulsifying properties of the glycosylated products were improved in varying degrees. Zhou Limin (Zhou et al. 2014) used TG to import glucosinol into zein, and the solubility of glycosylated proteins was improved in all pH ranges.

In recent years, it has been found that enzyme-catalyzed glycosylation can effectively reduce the allergenicity of allergens. Yuan (Yuan et al. 2016) evaluated the effect of TG-catalyzed glycosylation on the potential allergenicity and conformational structure of TM in *Metapenaeus ensis*. Results showed that glycosylation of TM induced unfolding of primary protein structure followed by loss of the secondary structure. Cleavage of certain free amino groups was observed during TG-catalyzed glycosylation, and the glycosylation rate correlated with reaction temperature. Western blotting and indirect ELISA with TM-specific polyclonal antibodies from rabbit and sera from patients allergic to shrimp demonstrated that the antigenicity and potential allergenicity of TM decreased, which correlated well with the conformational changes in its structure. Considering TG is widely utilized in the food industry, these results indicate that TG-catalyzed glycosylation has the potential to serve as a mild method for reducing the allergenicity of shrimp products.

Peroxidase is a common type of oxidoreductase, which can catalyze a variety of enzymatic reactions, including the oxidation of various phenolic compounds and the cross-linking of proteins (Kinsella 1973). However, there are relatively few studies on peroxisome-linked proteins, so its mechanism is not completely clear. At present, most of the studies suggested that when peroxidase and H_2O_2 act on protein, the peroxidase can catalyze the transformation of tyrosine residues in the protein into free radicals and then further polymerize to dityrosine and trityrosine, thereby promoting the cross-linking reaction. According to Chung (Chung et al. 2005), in the cross-linking reaction, the major allergens in peanuts were significantly reduced after 1 h of peroxidase reaction with roasted peanuts, and the major allergens in peanuts were reduced obviously. At the same time, the binding ability of the allergen to IgE in the serum of allergic patients was weakened, which may be caused by the production of polymer which masked the antigen epitopes.

Polyphenol oxidase (PPO), also known as tyrosinase, is widely distributed in plants and fungi. It is one of the important causes of browning in fresh fruits and vegetables. Polyphenol oxidase firstly oxidizes small molecules of phenolic compounds into aldehydes and further oxidizes quinones, while quinones have strong electrophilic properties, which lead to the formation of cross-linked polymers by covalent bonds between molecules. This characteristic of polyphenol oxidase enables it to catalyze cross-linking reaction and change its original protein space structure and many functional properties. Peanut allergen Ara h 1 and 2, milk allergen casein, and lactoprotein were cross-linked by tyrosinase, and the binding activity of IgE was decreased (Jr et al. 2004; Laemmli 1970). Radosavljevic (Radosavljevic et al. 2014) found that the reaction of tyrosinase to peanut allergen could effectively reduce the potential allergenicity of peanut allergens and increase the bioavailability of peanut allergen. Cheng Wei (Cheng et al. 2012) studied the structure and allergenicity changes of lactalbumin after cross-linking by polyphenol oxidase. The cross-linked dipolymer had higher hydrophobicity and more loose protein structure. The digestibility of the cross-linked products of milk alpha lactalbumin was evaluated systematically by simulating gastric juice and intestinal liquid in vitro. It was found

that milk alpha lactalbumin was easily digested by simulated gastric liquid and intestinal juice, while the cross-linked product was relatively resistant to digestion, especially to resist enzyme hydrolysis.

6.3.2 Fermentation

Microbial fermentation is an important food processing technology. The fermentation method mainly uses microorganisms to decompose or denature some proteins with antigen characteristics, so as to eliminate or reduce allergenicity. Although the fermentation method does not play a role in all the sensitizing foods, it will not destroy the other nutrients compared as the methods of heat treatment and chemical processing. The establishment of fermentation process is a new idea for improving the food allergenic characteristics, and it could be a new technology for the desensitization process of food allergens.

Fermentation can change the content of various components in foods. Through the action of microorganisms and their secreted enzyme system, high molecular substances including proteins can be decomposed into soluble low molecular compounds such as small molecule polypeptides and amino acids; thus, the structure and properties are changed. Therefore, the large-molecule proteins that cause an allergic reaction to the body are bound to be affected, and then the allergenicity can be eliminated or reduced.

The application range of microbial fermentation is limited, which is only suitable for fermented food, but the protein in the food is decomposed into peptides and amino acids, which not only helps to absorb but also increases the flavor of fermented food to some extent (Li et al. 2006; Mills et al. 2009). The effect of fermentation on food allergens is mainly reflected in some special foods, such as peanut, soybean, milk, and so on. Studies have shown that the use of fermentation methods to treat allergenic proteins in peanut foods can make peanuts safe to eat. This method can reduce the allergenicity of peanut allergen Ara h 1 and Ara h 2 to 30% but has no serious impact on the quality of the products (Wang et al. 2014).

Recent studies have shown that eating fermented foods, especially dairy products, can reduce some of the symptoms of hereditary allergy and limit the development of disease. Therefore, the application of probiotics in milk and the development of new fermentation products are effective technology to alleviate allergy.

After fermentation, the allergenicity of milk will be changed by two possible mechanisms. One is that the active ingredient in the fermented milk enters the body and regulates the balance between the Th1 and Th2 immune responses in the auxiliary T cells, thus preventing the anaphylaxis. Matsuzaki (Matsuzaki and Farnworth 2008) and other studies had found that cow's milk allergy may be caused by an imbalance of Th1 and Th2 immune responses. Oral intake of *Lactobacillus casei* fermented milk can inhibit the organism to produce immunoglobulin and stimulate the body to

produce natural killer cell (NK cells), so as to regulate Th1 and Th2 balance in the body and then to prevent allergy. Another possible mechanism is that lactic acid bacteria can produce protease and then hydrolyze the allergenic epitope of milk protein. The protease that exists on the cell surface first cuts the protein into peptide, which then seeps into the cell and is decomposed into amino acids by the protease in the cell. *Lactobacillus fermentum* RA3, isolated from Xinjiang yogurt which can produce lactic acid bacteria in large quantities, was used to make yogurt, and the results showed that with the optimized fermentation conditions, the allergenicity of β -lacto-protein was significantly reduced without affecting the flavor of yogurt.

6.4 Conclusion

Food allergy is a public health issue as well as an important food safety issue of global concern. It is reported that more than 40% of people have suffered from food allergies, and the number is growing rapidly (Sampson 2014). At present, there are no effective methods to prevent and eradicate food allergy. Commonly used drugs such as antihistamine receptors can only temporarily relieve allergic symptoms (Leurs et al. 2002). The only safe and effective way is to avoid eating or contacting allergens, but most foods containing allergens are the main source of human nutrition, and the development of the modern food industry makes every food product contain many food ingredients that can hardly be distinguished, so it is unrealistic and difficult to avoid food ingredients completely, and reducing food allergenicity by food processing methods is the first choice to reduce the risk of allergy.

This chapter is mainly about the modification methods of protein allergens in food, including physical, chemical, and biological methods that can reduce the allergenicity of food proteins (Table 6.1). Due to the great stability of most allergens in food processing, researches have not yet found an effective method for totally desensitizing all the foods; therefore, improved methods are required to achieve the complete desensitization of allergens with strong allergenicity and a certain resistance to conventional processing.

Deep processing of food will have a certain impact on allergens, but the impact is uncertain and cannot be easily measured and controlled. In addition, several allergens often exist in a single food, so it is necessary to combine several methods to reduce or remove the allergic activity. It will be a hot research direction in the field of food science in the future to comprehensively understand the possible allergens in various foods as well as the detailed changes of these allergenic proteins during processing and to develop new methods to eliminate their activities, so as to develop high-quality hypoallergenic foods.

Table 6.1 The advantages and disadvantages of major food processing methods in eliminating food allergen

Main method	Advantage	Disadvantage
Heat treatment	Simple and convenient	New antigen epitopes may be produced
Ultrasound treatment	Simple and convenient	Greatly influenced by temperature
Ultrahigh pressure	Less influence on nutritional value	Inefficient when used alone
Pulsed ultraviolet light	Extensive application area	Dose residual
Enzymatic hydrolysis modification	Mild reaction conditions and high efficiency	Change flavor and produce by-products
Fermentation	Efficiency and helpful for absorption	Limited application scope
Enzymatic cross-linking modification	Mild reaction conditions and high efficiency	Inefficient with resistant proteins
Glycosylation	Simple and may improve protein properties	Tricky in reaction condition

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Chapter 7

Modulation of Food Allergy by Bioactive Natural Compounds and Development of Functional Foods



Abstract Chemically diverse bioactive compounds with numerous health benefits have been taken more seriously. Food allergic inflammation mediated by several types of immune cells can also be affected by the naturally occurring bioactive compounds. Various compounds show immunomodulating effects, including polyphenols, polysaccharides, vitamins, peptides, fatty acids, amino acids, minerals, as well as prebiotics. Some components boost immune responses, resulting in host defense against infection, whereas others suppress immune responses, thus inhibiting allergy and inflammation. A variety of food components act on the varying immune cells, and the effects are mainly mediated via the intestinal immune system, as well as the intestinal microbiota. This chapter focuses on anti-allergic agents derived from microorganisms, plants (phytochemicals), animals, and marine algae and presents an overview of their potential application in functional foods for the treatment of allergic disorders. The molecular mechanisms and scientific validity of some bioactive natural compounds in the treatment of food allergies will also be explored.

7.1 Modulation of Food Allergy by Bioactive Natural Compounds

7.1.1 *Screening for Bioactive Natural Compounds*

Bioactive natural compounds are those chemicals produced by living organisms that exert certain biological effects on other organisms (Colegate and Molyneux 2007). Human beings have always been dependent on natural compounds for improving our lives in various aspects. In respect of food allergy treatment, extensive research efforts have been paid to seek effective natural products. Consequently, new data are emerging in terms of the influence of various natural compounds on the development of the food allergy in humans. Bioactive natural compounds for alleviating food allergy from various sources like microorganisms, plants, and marine algae have been explored (Wang et al. 2017; Vo and Kim 2013; Thomas and Kim 2011; Sanjeeva et al. 2016).

One of the first steps in seeking for bioactive compounds is to test a myriad of extracts or compounds to determine if they show biological effects, in which diverse *in vitro* assays or *in vivo* animal models are required. As for the screening for anti-food allergic agents, allergy models usually employed include mice (Zhang et al. 2015), rats (Vijayalakshmi et al. 2011), rat mast cells (Kralovec et al. 2005), RBL-2H3 cells (Kimiya et al. 2008), KU812F cells (Shim et al. 2017), and mouse eosinophils (Lucas et al. 2015). To date, a large number of mouse models for food allergy research have been established, providing valuable insights into the mechanisms of food allergenicity and allergic inflammation as well as the development of therapies (Liu et al. 2016b). However, comparisons of research results between different models are difficult to be performed in that a variety of protocols have been developed and employed for establishing relevant mouse models. In addition, the phenotypes of mouse models are markedly affected by genetic background, route of allergen exposure, gender, the nature and concentration of food allergens, and the use of adjuvants. Apart from mouse and rat models, pigs, dogs, or sheep are used in food allergy research, all dependent on diverse sensitization methods (Bøgh et al. 2016). For instance, epicutaneous sensitization was developed in Beagles, in which case peanut paste was employed to normal and atopic dogs. After oral challenge, allergic symptoms including pruritic dermatitis, clinical symptoms scores, eosinophilic dermatitis, and IgE-positive cells in skin were significantly higher in atopic dogs (Marsella 2015). This model is of great use to study food-aggravated atopic dermatitis instead of gastrointestinal symptoms.

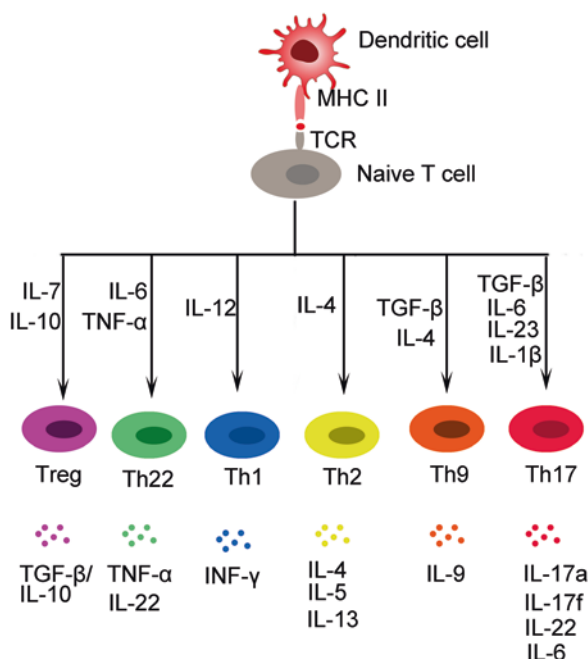
Up to now, with the established models, plenty of natural compounds possessing anti-allergic effects or potentials have been screened and identified such as polyphenols (Gorzynik-Debicka et al. 2018), flavonoids (Ahmad et al. 2017), fucoidans (Vo and Kim 2013), fatty acids (Korinek et al. 2017), essential oils (Mitoshi et al. 2014), and phlorotannins (Wang et al. 2017; Sanjeewa et al. 2016).

7.1.2 Anti-food Allergic Effects of Bioactive Natural Compounds

In most cases, food allergies are immunoglobulin E (IgE)-mediated reactions (also known as true food allergies), which encompass a series of abnormal reactions resulting from adverse immune responses to harmless proteins. Allergic diseases are initiated upon the generation of allergen-specific CD4⁺ Th2 cells (Akdis et al. 2005). Then, the effector Th2 cells produce interleukin (IL)-4, IL-5, IL-9, and IL-13 which lead to the production of IgE by effector B cells (Fig. 7.1). Subsequently, allergic reactions commenced once allergens are cross-linked with IgE, which is bound to high-affinity IgE receptors on the surface of mast cells and basophils. Following that, a range of intracellular events occur, involving the increase of Ca²⁺ level; the release of preformed inflammatory mediators, e.g., histamine and β -hexosaminidase; and the secretion of newly synthesized substances including

Fig. 7.1 Differentiation of a naïve T cells. (Adapted from Saidova et al. 2017).

Allergic diseases are initiated upon the generation of allergen-specific CD4⁺ Th2 cells, which then produce a series of cytokines such as IL-4, IL-5, IL-9, and IL-13, leading to the production of IgE by effector B cells. Finally, allergic reactions commenced once allergens are cross-linked with IgE bound to the high-affinity receptors on the surface of mast cells and basophils



prostaglandins, cytokines, and leukotrienes, which finally cause allergic inflammatory responses (Galli et al. 2008). According to the above mechanism, some steps are especially crucial for the regulation for food allergy development; therefore, the allergic reaction route may be suppressed at some points by other factors, e.g., bioactive substances. Currently, different anti-allergic mechanisms of bioactive natural compounds have been revealed, for instance, inhibiting expression of pro-inflammatory molecules and chemokines (Yang et al. 2018), preventing Th1 and Th17 cell differentiation (Zhang et al. 2015), suppressing binding of IgE to cell surface FcεRI (Shim et al. 2016), and suppressing FcεRI expression (Shim et al. 2017). The detailed anti-allergic mechanisms of some bioactive natural agents will be discussed in Sect. 7.2.5.

7.2 Anti-food Allergic Bioactive Natural Compounds from Different Sources

7.2.1 Anti-food Allergic Agents from Plants

Natural compounds from plants have been traditionally used by humans for their primary healthcare needs. There is a long history of the use of herbal medicines in Asian countries. According to the World Health Organization (WHO), nearly 20,000

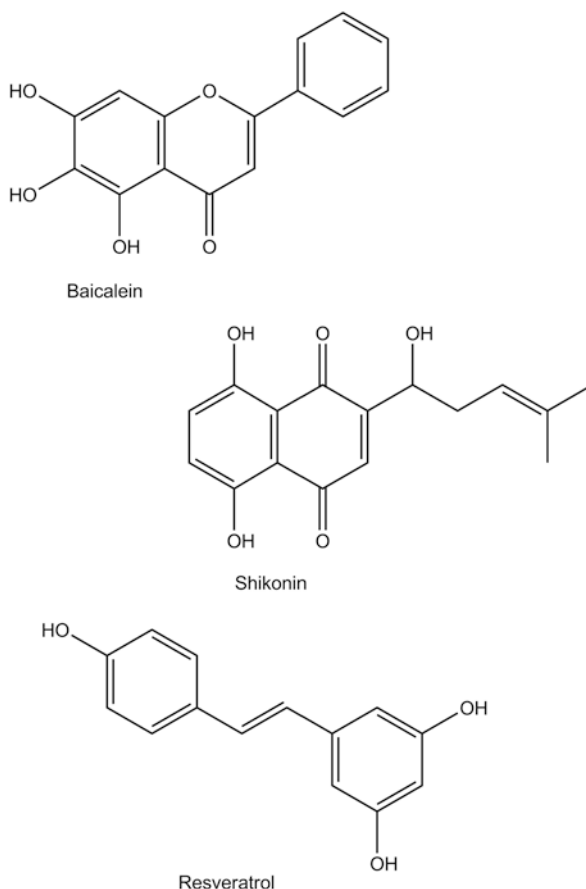
medicinal plants exist on this planet (Sasidharan et al. 2011). A wide range of substances that could be used to treat certain disorders or that possess health-promoting properties occur in plants. Plants products, primary and secondary metabolites, have served as a rich resource for anti-allergic agent exploration.

7.2.1.1 Dietary Polyphenols

Polyphenols are a class of compounds of various chemical structures, which may exist in vegetables, roots, fruits, bark, nuts and seeds, and leaves of different plants as well as in corresponding processed products (Gorzynik-Debicka et al. 2018). Health-promoting effects of plant polyphenols on humans have been indicated by plenty of scientific researches and have been well reviewed (Devi et al. 2014). Polyphenol compounds are the best studied natural substances possessing an anti-inflammatory and anti-allergic potential (Chirumbolo 2014), and there is an array of researches presenting the ability of dietary polyphenols to modulate human immune system by influencing the factors in the immunological defense (Chirumbolo 2014; Singh et al. 2011).

Flavonoids (C6-C3-C6 polyphenols) are a group of polyphenolic compounds most commonly encountered in our daily diet and exist ubiquitously in plant-derived foods and beverages. There is a large body of chemicals in this group, for example, baicalein, quercetin, kaempferol, apigenin, and chrysin. The immunomodulatory properties of dietary flavonoids have been widely studied (González-Gallego et al. 2014). Baicalein (Fig. 7.2) induced CD4⁺Foxp3⁺ T cells and enhanced intestinal barrier function in animal studies (Bae et al. 2016). The administration of baicalein ameliorated the symptoms of food allergy and reduced the production of serum IgE and effector T cells. Furthermore, baicalein enhanced intestinal barrier function via the regulation of tight junctions. Besides, baicalein induced the differentiation of Treg cells via aryl hydrocarbon receptors (AhRs). Consequently, baicalein, as an agonist of AhR, can induce Treg differentiation and enhance barrier function. A diet supplemented with apigenin and chrysin suppressed the upregulation of ovalbumin (OVA)-specific serum IgE through the suppression of Th2-type immune response in animal models (Yano et al. 2007). Polyphenol-enriched fruit extracts containing epicatechin showed a significant anti-allergic effect in mice sensitized to OVA after being fed diets containing polyphenol-enriched extracts or purified epicatechin for 8 days (Singh et al. 2014). This effect was definitely ascribed to epicatechin, since oral administration of this compound to sensitized mice via inclusion in their diets modulated allergy symptoms in a dose-dependent way. Besides, administration of epicatechin also affects immune parameters. Bioavailability measurements indicated that the alleviation of allergy symptoms could be attributed to the higher concentrations of bioavailable epicatechin metabolites. Hesperidin, a major flavonoid compound found in lemons, oranges, and some other fruits and vegetables, also showed immunoregulatory activities in food allergy animal models (Camps-Bossacoma et al. 2017a). Rats, intraperitoneally immunized with OVA together with *Bordetella pertussis* toxin and alum as the adjuvants, were orally given 100 or

Fig. 7.2 The structures of three typical dietary polyphenols. Baicalein (5,6,7-trihydroxyflavone) is a flavone, a type of flavonoid, originally isolated from the roots of *Scutellaria baicalensis* and *Scutellaria lateriflora*, as well as *Oroxylum indicum* and *Thyme*. Shikonin is a major component of a Chinese herbal medicine zicao (purple gromwell, the dried root of *Lithospermum erythrorhizon*). Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a naturally occurring polyphenol found in the skin of grapes, blueberries, raspberries, and mulberries



200 mg/kg hesperidin. The results showed that hesperidin administration altered the composition of mesenteric lymph node lymphocyte (MLNL), elevating the TCR $\alpha\beta$ + cell percentage and reducing that of B-lymphocytes. Moreover, hesperidin boosted the production of IFN- γ in the stimulated MLNL. In another experiment conducted by the researchers, the rats, which were orally sensitized with OVA plus cholera toxin (CT), were fed a diet supplemented with 0.5% hesperidin. The results showed that hesperidin intake influenced the composition of lymphocytes in the intestinal epithelium and lamina propria. However, the study indicated that immunoregulatory properties of hesperidin did not involve the change of the level of serum anti-OVA antibodies.

Citrus tachibana leaf extract (CLE) has been found being capable of mitigating OVA-induced food allergy symptoms, e.g., increased rectal temperature, diarrhea, and anaphylaxis (Chung et al. 2016). This mitigation was likely ascribed to CLE-mediated reduction in cytokine release from Th2 cells in mesenteric lymph nodes (MNLs). Furthermore, they found that higher levels of CLE alleviated systemic Th2

cell-mediated responses in splenocytes of mice sensitized with OVA+alum. This was verified by CLE-mediated decreases in Th2 cytokine release, including IL-4, IL-5, and IL-13, without the Th1 cytokines IL-12 and IFN- γ , which was ascribed to decreased levels of gene expression. In addition, they also found that kaempferol was the most potent chemical in decreasing Th2-associated responses in splenocytes.

Resveratrol (Fig. 7.2), a naturally occurring polyphenol found in the skin of grapes, blueberries, raspberries, and mulberries, has been found to be able to prevent the development of food allergy in mice (Okada et al. 2012). The mechanism of action could be the inhibition of dendritic cell (DC) maturation and subsequent early T-cell activation and differentiation. As is reported, mice fed resveratrol showed reduced OVA-specific serum IgE production and reduced OVA-induced IL-13 and IFN- α production by MNLs and spleens. Resveratrol inhibited the production of IFN- γ , IL-4, and IL-13 from splenocytes in association with the inhibition of GATA-3 and T-bet expression. Meanwhile, resveratrol inhibited CD25 expression and IL-2 production in splenocytes associated with reduced CD80 and CD86 expression levels. Finally, resveratrol suppressed CT-induced cAMP elevation associated with decreased CD80 and CD86 expression levels in mouse bone marrow-derived DCs.

Shikonin (Fig. 7.2), a naphthoquinone dye from *Lithospermum erythrorhizon*, has been found possessing a potent inhibitory effect on RBL-2H3 mast-like cell degranulation (Wang et al. 2014). Furthermore, shikonin markedly suppressed the IgE-/antigen-induced and calcium ionophore-induced upregulation of TNF- α in mucosal-type bone marrow-derived mast cells (mBMMCs). Global gene expression analysis and real-time PCR indicated that shikonin extremely suppressed the IgE-/antigen-induced and calcium ionophore-induced upregulation of the nuclear orphan receptor 4a family, i.e., Nr4a1, Nr4a2, and Nr4a3 in mBMMCs. Moreover, knock-down of Nr4a1 or Nr4a2 inhibited the IgE-/antigen-induced upregulation of TNF- α . Computational docking simulation showed that the assumed binding sites of shikonin to immunophilins, namely, cyclophilin A and FK506-binding protein (FKBP) 12, were almost the same to those of immunosuppressants (cyclosporin A and FK506) to immunophilins. Actually, shikonin suppressed the calcineurin activity to a similar degree as cyclosporin A that greatly inhibited the IgE-/antigen-enhanced expressions of TNF- α and the Nr4a family in mBMMCs. These findings imply that shikonin inhibits mucosal mast cell activation by decreasing Nr4a family gene expression via suppressing calcineurin activity (Wang et al. 2014).

7.2.1.2 Polysaccharides

Sulfated polysaccharide from *Gracilaria lemaneiformis* was found to be able to attenuate food allergy symptoms in mice, reducing TM-specific IgE and IgG1, suppressing Th2 cell polarization, and promoting the function of regulatory T (Treg) cells (Liu et al. 2016a). In addition, it was able to inhibit the function of RBL-2H3 cells and the activation of KU812 by suppressing p38 mitogen-activated protein

kinase (MAPK). In conclusion, the immunosuppression and reduction of p38 MAPK may contribute from the putative anti-allergic activity of *Gracilaria lemaneiformis* sulfated polysaccharide. In another research, a new low molecular weight sulfated polysaccharide (GLP-2) was isolated from *G. lemaneiformis* (Ren et al. 2017). Its immunomodulatory activities were examined using murine macrophage RAW 264.7 cells. The results revealed that GLP-2 greatly improved the proliferation and pinocytic capability of RAW 264.7 cells and boosted the production of reactive oxygen species, nitric oxide, IL-6, and TNF- α by activating gene expressions.

A polysaccharide isolated from Chinese yam has been reported for its immunomodulatory activities on RAW 264.7 macrophages in vitro (Li et al. 2017). It has been found that the polysaccharide was mainly composed of mannose, galacturonic acid, glucose, galactose, and arabinose. The polysaccharide increased the phagocytic activity of RAW 264.7 macrophages, NO release, and transcription and production of IL-6 and TNF- α . It has also been found that the polysaccharide induced I κ B α degradation and stimulated NF- κ B activation in RAW 264.7 cells. Notably, inhibiting Toll-like receptor 4 resulted in the suppression of NO production induced by the polysaccharide in RAW 264.7 cells. These findings suggested that the polysaccharide exhibited the potential as a functional food or therapeutic stimulant. A study was performed to investigate the effects of the polysaccharide isolated from *Citrus unshiu* peel (CPE-II) on the production of cytokine and inflammatory mediators in RAW 264.7 macrophages. Moreover, signal transduction experiments were conducted to verify the pathways involved in this process (Veronese et al. 2018). The results indicated that CPE-II possessed macrophage-stimulatory activity through the induction of IL-6, TNF- α , and NO production in RAW 264.7 macrophages, and signal transduction experiments exhibited that CPE-II could phosphorylate MAPK and nuclear factor (NF)- κ B in RAW 264.7 cells in a concentration-dependent way. They also found that the effects of CPE-II on the production of IL-6/IL-12 and TNF- α were completely inhibited by a specific inhibitor of c-Jun N-terminal kinase. Moreover, the results showed that Toll-like receptor 2 (TLR2) and TLR4 were related to the stimulation of IL-6 and NO production by CPE-II. Collectively, their results indicated that the TLR2/TLR4 and JNK pathways were crucial for the CPE-II immune activity in RAW 264.7 cells.

It has been reported that polysaccharides from *Collybia radicata* mushroom (CRP) possess various beneficial functions such as antiviral, antiaging, and hypolipidemic activities (Gao et al. 2017). Recently, the immunomodulatory activity of CRP has been revealed (Wang et al. 2018). The research indicated that a 14,942 Da polysaccharide markedly improved the proliferation and phagocytosis of macrophages and induced the secretion of inducible nitric oxide synthase (iNOS), NO, and cytokines such as TNF- α and interleukin factors. The results showed that, at a concentration of 850.0 μ g/mL, the polysaccharide prompted macrophage proliferation and phagocytosis to 2.1 and 3.4 times, respectively, compared to the negative control. Besides, it enhanced the production of NO via the induction of iNOS in a concentration-dependent manner. Moreover, it boosted the release of these cytokines to multiples. As an inhibitor of TLR4, TAK242 (resatorvid) inhibited the secretion of

iNOS, NO, and cytokines above 51%. Consequently, the polysaccharide possesses an effective immunomodulatory activity by prompting macrophages and can be deemed as a potential immunopotentiator in medical and food industries.

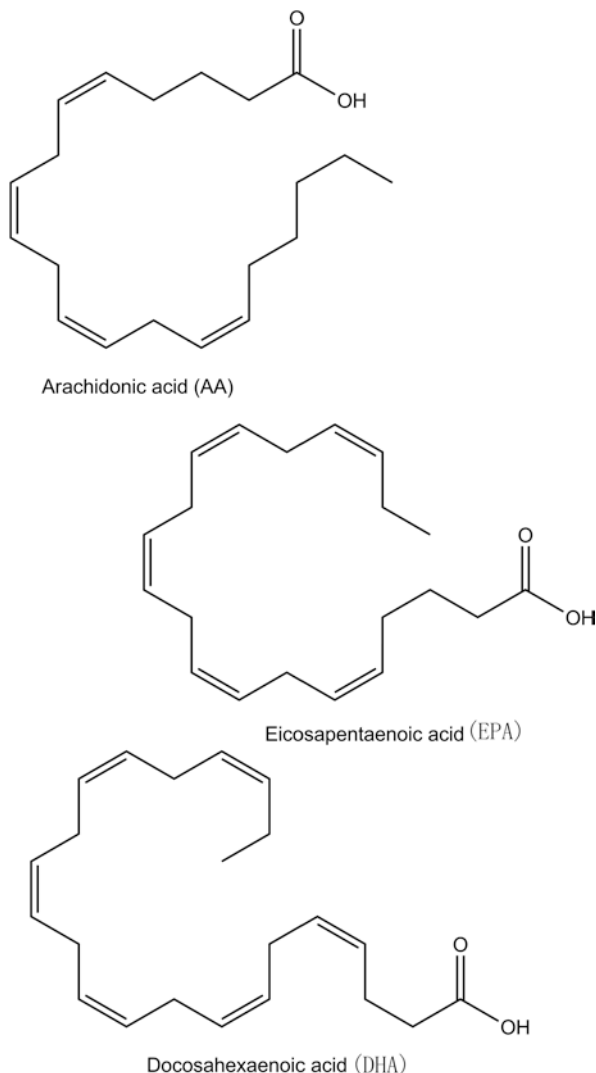
7.2.1.3 Polyunsaturated Fatty Acids

Long-chain polyunsaturated fatty acids (LCPUFA), especially the arachidonic acid (AA) and docosahexaenoic acid (DHA), are considered having important immunomodulatory roles during the immune system development. Intervention studies have shown improvement in many indicators of immune function in infants fed formula containing AA and DHA (Fig. 7.3) compared to unsupplemented formula, including decrease in the risk of developing allergic and atopic disease in early life (Richard et al. 2016).

There is an extensive pool of research findings on the potential health-improving effects of dietary polyunsaturated fatty acids. It has been reported that n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA, also known as ω -3 long-chain polyunsaturated fatty acids) may have beneficial effects on social behavioral defects caused by food allergies in mice, either by attenuating allergic responses and restoring intestinal 5-HT signaling or by DHA incorporation into neuronal membranes, affecting the dopamine system (de Theije et al. 2015). In another research, n-3 LCPUFA supplementation exhibited an apparent reduction in primary outcomes of any allergy (IgE mediated) in children aged 12–36 months (Gunaratne et al. 2015). For the secondary outcomes of specific allergies, a clear decrease was observed for children in their first 12 months with n-3 LCPUFA.

Studies have also performed to investigate the immune-metabolic progression from dietary oil of ω -3 polyunsaturated fatty acids in mice models. In the study performed by Kunisawa et al. (2015), they found that 17,18-epoxyeicosatetraenoic acid (17,18-EpETE) was an anti-allergic metabolite produced in the intestine from dietary ω -3 α -linolenic acid (ALA). Increased levels of ALA and its metabolites, especially eicosapentaenoic acid (EPA), were found in the intestines of mice receiving linseed oil rich in ALA by biochemical and imaging mass spectrometry analyses. In the murine food allergy models treated with ALA-rich linseed oil, the reduced incidence of allergic diarrhea was ascribed to the impairment of mast cell degranulation instead of affecting allergen-specific serum IgE. Mediator lipidomics based on liquid chromatography-tandem mass spectrometry revealed that 17,18-EpETE was a major ω -3 EPA-derived metabolite produced from dietary ALA in the intestine. Moreover, 17,18-EpETE showed anti-allergic property when administered in vivo. The results suggest that metabolizing dietary ω -3 polyunsaturated fatty acids results in the production of 17,18-EpETE, which is found to be an endogenous anti-allergic metabolite and potentially serves as a therapeutic agent to treat intestinal allergies.

Fig. 7.3 The structures of three typical dietary polyunsaturated fatty acids. Arachidonic acid (AA, sometimes ARA) is a polyunsaturated omega-6 fatty acid and is a carboxylic acid with a 20-carbon chain and four *cis*-double bonds. Eicosapentaenoic acid (EPA, also icosapentaenoic acid) is an omega-3 fatty acid and is a carboxylic acid with a 20-carbon chain and five *cis*-double bonds. Docosahexaenoic acid (DHA) is an omega-3 fatty acid and is a carboxylic acid with a 22-carbon chain and six *cis*-double bonds



7.2.1.4 Dietary Fiber

Dietary fiber, found mainly in fruits, vegetables, whole grains, and legumes, is a term that is referred to plant-based carbohydrates that, unlike other carbohydrates, are not digested in the small intestine. Dietary fiber is associated with numerous well-documented health benefits (Veronese et al. 2018). It has been found that high-fiber feeding improved oral tolerance and protected mice from food allergy by increasing retinal dehydrogenase activity in CD103+ DC (Tan et al. 2016). And this protection relies on the presence of vitamin A in the diet. The high-fiber feeding also

enhanced IgA production and boosted T follicular helper and mucosal germinal center responses. Besides, high-fiber diet altered the composition of short-chain fatty acid (SCFA)-producing microbiota and increased the release of SCFAs, particularly acetate and butyrate. Mice, which lack GPR43 and GPR109A, receptors for SCFAs, show severer food allergy symptoms and fewer CD103⁺ DCs. Therefore, dietary elements, e.g., fiber and vitamin A, could regulate various protective pathways in the gastrointestinal tract in mice, which are necessary for immune nonresponsiveness to food antigens. On the other hand, it was demonstrated that dietary fiber and SCFAs induced the expression of vitamin A-converting enzyme RALDH1 in intestinal epithelial cells both *in vivo* and *in vitro* (Goverse et al. 2017). Moreover, the RALDH1 expression levels in small intestinal epithelial cells were associated with the activity of vitamin A-converting enzymes in MLN DCs, along with boosted numbers of intestinal regulatory T cells and luminal IgA. In conclusion, the research has illustrated that dietary fibers could affect small intestinal epithelial cells and can be employed to modulate the mucosal immune system.

7.2.1.5 Bioactive Peptides

Peptides are an important category of functional chemicals, and a large list of functional peptides with various health-improving properties have been found from diverse sources (Ji et al. 2018; Abeyrathne et al. 2018; Leeb et al. 2014). A lot of peptides have been found possessing anti-allergic and immunomodulatory functions (Chung et al. 2017; Ko et al. 2016). For instance, the immunomodulatory effect of amaranth peptides on epithelial cells, which was activated via the NF- κ B signaling pathway, was revealed (Moronta et al. 2016a). The results exhibited that extensive protein hydrolysis from amaranth decreased the emission of light in Caco-2 CCL20:luc cells (Caco-2 cells transfected with a luciferase reporter under the control of the CCL20 promoter) activated by bacterial flagellin in comparison with the non-hydrolyzed protein. After the purification of the most active peptides by high-performance liquid chromatography and sequencing, the peptide SSEDIKE showed a modulatory capacity on activated cells, suppressing the expression of mRNA coding for CCL20. Moreover, this peptide was nontoxic for cells. These findings showed that the peptide SSEDIKE derived from proteins of amaranth alleviated the activation of human intestinal epithelial cell. Furthermore, the *in vivo* immunomodulatory effect of the peptide SSEDIKE was investigated in an IgE-mediated food allergy mouse model (Moronta et al. 2016b). The synthetic SSEDIKE peptide was orally administered, and upon oral challenge with milk allergens, an attenuation of hypersensitivity symptoms with negativization of skin test was observed, and the secretion of specific IgE and IgG1 was significantly inhibited. A substantial decrease in the IL-5 and IL-13 secretion by antigen-stimulated spleen cells and a reduction of transcripts coding for ccl20, GATA-3, and NF- κ B in mucosa were also observed. In addition, the mRNA levels of transforming growth factor- β (TGF- β) and foxp3 were upregulated in the jejunum. The charged SSEDIKE peptide derived from amaranth proteins inhibited the allergy reactions in a food allergy mouse model, with inhibition of specific IgE secretion and control of the inflammation in the intestine

preventing the activation of NF- κ B, which might contribute to the induction of local tolerance. Hence, the results indicated that amaranth proteins could be used in functional foods as a supplier of bioactive peptides with health-promoting properties.

7.2.1.6 Other Plant-Derived Agents

It has been reported that curcumin ingestion during oral allergen exposure could modulate the development of food allergy in a murine model of OVA-induced intestinal anaphylaxis (Kinney et al. 2015). The results showed that intragastric exposure to OVA in sensitized BALB/c mice triggered a potent IgE-mediated response along with elevated OVA-IgE levels, intestinal mastocytosis, increased serum mMCP-1, and acute diarrhea. On the contrary, mice exposed to oral curcumin through the whole of the experimental regimen appeared to be normal and did not show intense allergic diarrhea or a significant increase of OVA-IgE and enhancement of intestinal mast cell expansion and activation. Moreover, mast cell activation and expansion, allergic diarrhea, and Th2 responses were all inhibited in mice orally exposed to curcumin during the OVA-challenge phase alone, though the levels of OVA-IgE elevated, suggesting that curcumin may exert a direct inhibitive effect on the activation of intestinal mast cells and relieve food allergy symptoms. This effect was verified by observations that curcumin alleviated the expansion of adoptively transferred bone marrow-derived mast cells (BMMCs) and suppressed their survival and activation during cell culture. Finally, the inhibition of intestinal anaphylaxis by curcumin intake was directly related to the suppression of NF- κ B activation in allergic mice, and curcumin suppressed the phosphorylation of the p65 subunit of NF- κ B in BMMCs. The research demonstrated a protective role of curcumin during allergic responses caused by food antigens, indicating that frequent intake of curcumin may be beneficial for susceptible individuals.

7.2.2 *Anti-food Allergic Agents from Marine Organisms*

The oceans on this planet, which covers more than 70% of the earth's surface, are really an enormous resource for the discovery of potential bioactive compounds for humans. During the last decades, plenty of novel chemicals have been found from marine organisms, which usually possess attractive biological activities (Blunt et al. 2017, 2015; Hu et al. 2015).

7.2.2.1 Marine Algae-Originated Anti-allergic Compounds

Marine algae are among the most encountered marine organisms and are known as one of the most important biomass producers in the marine environment (Vo et al. 2012). They can produce various bioactive metabolites in their surroundings,

and the bioactive compounds have been revealed to possess antioxidant, anti-allergic, anticancer, anti-inflammatory, anticoagulant, antiviral, and anti-obesity activities (Fan et al. 2014; Rahelivao et al. 2015).

A study aiming at isolating R-phycocyanin (RPC) from *Porphyrha haitanensis* and determining the anti-allergic potential of RPC demonstrated that RPC reduced allergic sensitization against crab (*Scylla paramamosain*) tropomyosin (TM) by suppressing Th2 cell polarization and inhibited the release of allergic mediators in mast cells stimulated by antigen (Liu et al. 2015). It was found that, in animal studies, RPC could effectively decrease the levels of crab TM-specific IgE and histamine, attenuate jejunum tissue inflammation and allergy symptoms in mice, and suppress the expression and release of cytokines (IL-4 and IL-13) in peritoneal lavage fluid. In cell studies (spleen lymphocyte), it was reported that RPC of high purity skewed the immunological function of CD4⁺ T cells to Th1 direction. A higher expression of IFN- γ was induced by a synergistic effect of TM and RPC, via the Jun N-terminal kinase and Janus kinase 2 signaling pathways. RPC was evaluated for its anti-allergic effect using IgE-mediated rat mast cell line RBL-2H3, and the results showed that RPC suppressed the release of β -hexosaminidase, histamine, and ROS in RBL-2H3 cells sensitized with antigen. Besides, RPC also inhibited the production of pro-inflammatory factors (IL-4 and TNF- α).

The sulfated polysaccharide from *Porphyrha haitanensis* (PHPS) was also found to possess immunoregulatory activities (Liu et al. 2017). The treatment with the sulfated polysaccharide increased phagocytosis of RAW264.7 macrophages and boosted secretion of IL-6, IL-10, and TNF- α . Meanwhile, PHPS induced the production of nitric oxide through the JNK and JAK2 signaling pathways in RAW264.7 macrophages. Moreover, PHPS increased the proliferation of lymphocytes in mice, inducing the TNF- α and IL-10 generation, along with the subpopulation of CD4⁺ splenic T lymphocytes, DCs, and Tregs. PHPS also suppressed crab (*Scylla paramamosain*) TM-induced allergic reaction in mice, possibly via modulation of the imbalance of the Th1/Th2 immune response (Shi et al. 2015). Intraperitoneal injection of PHPS inhibited the allergic reaction by modulating serum IgE, IgG1, and IgG2a levels. Particularly, if PHPS was injected before the first immunization with TM, the IgE level reduced by 34.2% compared to the control. On the other hand, oral administration of PHPS to TM-sensitized mice reduced histamine release and repaired the pathology in the jejunum of the small intestine. Meanwhile, mRNA expressions of the TM-induced Th2 cytokines (IL-4, IL-5, and IL-13) in splenic lymphocytes were reduced by PHPS in vitro studies. Nevertheless, the gene expressions of Th1 and regulatory cytokines (IFN- γ and IL-10) were upregulated in splenic lymphocytes treated by PHPS. Moreover, IFN- γ secretion via the Jun N-terminal kinase (JNK) and Janus kinase 2 (JAK2) signaling pathways was induced by PHPS.

In addition to polysaccharide, some oligosaccharide has also been found possessing immunoregulatory activity and anti-food allergy function. The sulfated oligosaccharide (ESO) from *Eucheuma cottonii* was investigated for its anti-food allergy activity (Fu et al. 2017b). ESO reduced the levels of mast cell protease-1 and histamine and suppressed the levels of antigen-specific IgE by 77.7% in vivo. In addition, the production of IL-4 and IL-13 was decreased in the ESO-treated groups

compared with the non-ESO group. Furthermore, ESO increased Treg cells by 22.2–97.1%. In conclusion, ESO attenuated the food allergy responses in mice by decreasing basophil degranulation, releasing IL-10, and upregulating Treg cells via Forkhead box protein 3 (Foxp3). Among the oligosaccharides, fructo-oligosaccharides (FOS) are considered as prebiotic agents possessing immunomodulatory effects encompassing improvement of the intestinal microbiota and metabolome. In a study, the cellular mechanisms through which FOS modulate CD4⁺ T-cell responses in food allergy were investigated using the OVA23-3 mice model (Tsuda et al. 2017). The mice were fed a diet supplemented with either OVA or OVA plus FOS for 1 week. The results showed that dietary intake of FOS could provide moderate protection from the intestinal inflammation caused by OVA-containing food supply. The results also showed that FOS dramatically decreased Th2 cytokine responses in intestinal tissues induced by food allergy, but the phenomenon was not discovered in systemic tissues. FOS reduced the number of IFN- γ +IL-4⁺ double-positive CD4⁺ T cells induced by OVA-containing diet and the level of early activated CD45RB high CD69+CD4⁺ T cells in MLN. Furthermore, it was confirmed that these CD45RB high CD69+CD4⁺ T cells could produce high levels of IFN- γ and moderate level of IL-4, IL-10, and IL-13. The research indicated that dietary ingestion of FOS during the development of food allergy alleviated the induction of intestinal Th2 cytokine responses via the regulation of early activation of naïve CD4⁺ T cells, which could produce both Th1 and Th2 cytokines.

Some researchers have investigated the synergistic actions of different oligosaccharides in modulating immune responses during food allergy development. Diet containing short-chain galacto-oligosaccharides (scGOS), long-chain fructo-oligosaccharides (lcFOS), and *Bifidobacterium breve* M-16V (Bb) (GF/Bb) was found to inhibit food allergic symptoms in mice, assumedly via galectin-9 derived from intestinal epithelial cell (IEC) (Kivit et al. 2017).

Furthermore, in vitro studies indicated that galacto- and fructo-oligosaccharides (GF) enhanced the immunomodulatory capacity of a TLR9 ligand representing bacterial CpG DNA. In animal studies, BALB/c mice were fed GF/Bb during the development of oral sensitization induced by OVA. The result showed that GF/Bb attenuated allergic symptoms and boosted serum galectin-9 levels while inhibiting activation, restoring phagocytic capacity, and normalizing CD103 expression of small intestine lamina propria DCs in OVA-allergic mice. In vitro, galectin-9 inhibited the activation markers induced by LPS and cytokine secretion by BMDCs, and IEC-conditioned medium inhibited moDC activation in a galectin-9-dependent way. Apart from the inhibition of small intestine lamina propria DC activation, diets supplemented with GF/Bb also reduced the frequency of activated Th2 cells and enhanced Treg in the small intestine lamina propria in OVA-allergic mice in comparison to the control diet. The research indicated that dietary intervention with GF/Bb could boost galectin-9 and inhibit allergic symptoms in OVA-allergic mice associated with the decrease of intestinal DC and Th2 activation and the increase of Treg frequency in mice. In another study, it has been found that oral administration of specific β -lactoglobulin-derived peptides (PepMix) and a specific blend of fructo-oligosaccharides and *Bifidobacterium breve* M-16V (FF/Bb) partially prevented

Cow's milk allergy development in mice (Kostadinova et al. 2017). Female C3H/HeOuJ mice of 3-weeks-old were fed PepMix±FF/Bb prior to a 5-week long oral sensitization with whole whey and CT. The results showed that exposure to PepMix+FF/Bb in advance partially protected mice from the acute allergic skin response compared to control and enhanced Tregs and activated T cells. And it also boosted the expression of T-bet over GATA-3 in the Peyer's patches (PP) of whey-sensitized mice. Directly upon the 6-day oral tolerance phase, FF/Bb ingestion increased the levels of propionic and butyric acid in cecal of PepMix-fed mice, and propionic acid was found positively correlated with the numbers of Foxp3+ cell in the colon. In the PP of mice exposed to PepMix+FF/Bb, IL-22 expression enhanced, and IL-10 showed the same tendency. In the colon, the T-bet expression enhanced over GATA-3, while IL-22 reduced. Besides, the ratios of Foxp3+/GATA-3+ and regulatory/effector T cell in the MLN and the CD11b+/CD11b- conventional DC ratio all increased. The research indicated that the FF/Bb diet facilitated the ability of the specific β -lactoglobulin-derived peptides to partially inhibit the allergic response in mice sensitized to whole whey protein, probably by creating a tolerance-prone environment during the oral tolerance phase.

Currently, most of the natural polyphenols has been found from terrestrial sources such as tea, fruits, and grape seeds, among others, but there are some polyphenols developed from algae for their anti-allergic activity. A study has been conducted to screen commercial seaweed for natural compounds possessing anti-allergic activity (Charve et al. 2011). Five algae, namely, *Laminaria japonica*, *Porphyra* sp., *Spirulina platensis*, *Chlorella pyrenoidosa*, and *Scytosiphon* sp., were extracted, and the extracts were assessed for total polyphenol contents and their anti-allergic activity. The results indicated that total polyphenol contents in the five algae ranged from 1.67% to 8.47%, and the highest was found from *Scytosiphon* sp. Hyaluronidase inhibition assay indicated that the extract from *Scytosiphon* sp. possessed the lowest IC₅₀, while the extract from *Chlorella pyrenoidosa* had the highest one. Interestingly, the anti-allergic activity of *Scytosiphon* sp. extract was found higher than the typical anti-allergic drug disodium cromoglycate and was similar to natural polyphenols from epigallocatechin gallate. These results showed that the *Scytosiphon* sp. extract contains a high content of polyphenols with high anti-allergic activity, which may be developed to a natural anti-allergic agent.

7.2.2.2 Oyster-Derived Polysaccharides

Pacific oyster is of nutritional value, containing amino acids, proteins, carbohydrates, polyunsaturated fatty acids, lipids, and minerals (Cheng et al. 2016). Moreover, further studies have demonstrated that extracts of the Pacific oyster exhibited a lot of potential beneficial properties for humans, including antihyperlipidemia, antioxidation, and immunomodulation (Watanabe et al. 2012; Fuda et al. 2015). It has been found that direct exposure of OVA-primed murine splenocytes to oyster-derived polysaccharides (OPS) boosted the cell metabolic activity and proliferation and the expressions of cytokine and transcription factors in Th1 cell, i.e.,

IFN- γ and T-bet. On the contrary, OPS alleviated the expression of the Th2 cell-related cytokine IL-4 (Cheng et al. 2013). Then, the researchers confirmed the OPS-mediated Th1-enhancing effects by in vivo studies, which showed the upregulation of IFN- γ and T-bet expression in splenocytes from mice sensitized with OVA and then administered with OPS. These results demonstrated that the OPS exerted its immunomodulatory effects by skewing T-cell responses to the Th1-dominant direction. Furthermore, OPS was found to help to relieve food allergy symptoms in that administration with OPS alleviated OVA challenge-elicited diarrhea and the infiltration of mast cells in the intestine (Cheng et al. 2016). OPS exhibited a protective effect on the reduced ratio of villus length over crypt depth of the intestine in allergic mice. Moreover, OPS administration distinctly alleviated the intestinal expression of IL-4, a Th2 signature cytokine, in allergic mice. These results indicated the anti-food allergic activity of OPS, which related with the inhibition of allergen-induced intestinal Th2 responses and mast cell activation.

7.2.2.3 Deep-Sea-Derived Microorganisms

To discover anti-food allergic compounds, microorganisms derived from deep sea have also been explored. A systematic chemical research of the hydrothermal fungus *Graphostroma* sp. MCCC 3A00421 in the Atlantic has been performed (Niu et al. 2018). In total, 9 new and 19 known polyketides were obtained. The planar structures of the new isolated chemicals were illuminated mainly by detailed analysis of the nuclear magnetic resonance (NMR) and high-resolution mass spectrometry data. Their absolute configurations were established using the modified Mosher's method associated with electronic circular dichroism spectra. The results showed that Graphostrin A was a novel derivate of chlorinated polyketide with an oxazole moiety. All the isolates were investigated for anti-food allergic activities in IgE-mediated RBL-2H3 cells. Especially, reticulol significantly reduced the levels of degranulation and histamine release, implying that this chemical could be a potential anti-food allergic agent. In another recent study, the compound deep-sea-derived butyrolactone I (BTL-I), a type of butanolide, was isolated from *Aspergillus* sp. (Liu et al. 2018). The anti-food allergic activity of BTL-I was explored by using the OVA-induced BALB/c anaphylaxis. The results showed that BTL-I was able to attenuate OVA-induced allergy symptoms, decrease the number of histamine and mouse mast cell proteinases, suppress OVA-specific IgE, and reduce the levels of mast cells in the spleen and MNLs. BTL-I also significantly inhibited mast-dependent passive cutaneous anaphylaxis. In addition, the maturation of bone marrow-derived mast cells decreased as BTL-I resulted in downregulation of c-KIT receptors. Moreover, molecular docking analyses elucidated that there existed interactions between BTL-I and the inhibitory receptor Fc γ RIIB. Consequently, deep-sea-derived BTL-I may exert its protection against food anaphylaxis by reducing the functions of mast cell and via the interactions with the inhibitory receptor Fc γ RIIB.

7.2.3 *Anti-food Allergic Agents from Microorganisms*

Microorganisms in the nature are important sources of bioactive compounds (Gouda et al. 2016). Especially, the intestinal microbiota plays an indispensable role in the health of humans partly via the production of various metabolites, some of which possess immunoregulatory effects. It is suggested that the host immune system and the commensal microbes influence each other and have been coevolved for mutual regulation (Kim 2018). The intestinal microbes have a fundamental role in the energy homeostasis of the host and are essential for proper “education” of the host immune system (Diesner et al. 2016). A large body of evidence shows that diverse microbial metabolites greatly regulate the host immune system via target molecules (Van Den Elsen et al. 2017; Mariño et al. 2017; Goverse et al. 2017; Blacher et al. 2017). Immune cells have various receptors specific for certain metabolites such as aryl hydrocarbon receptor precursor (AhR), pregnane X receptor (PXR), and farnesoid X receptor (FXR). The microbial metabolites and corresponding receptors form a considerable array of signals to respond to changes in immunological status.

In a research performed by Diesner (Diesner et al. 2016), they repeatedly observed nonresponder mice protected from food allergy. In the analysis of the reasons for this protection, comparison of fecal microbiota showed differences of bacterial composition between the nonresponder and food allergic groups, indicating that the protection from food allergy was in association with a distinctive microbial composition in a nonresponding phenotype in this mouse model. The gut microbiota is able to ferment dietary fiber to release SCFAs, which can regulate innate and adaptive immune cell generation, trafficking, and function (Koh et al. 2016). For instance, butyrate has an anti-inflammatory effect by suppressing the recruitment and pro-inflammatory activity of neutrophils, macrophages, DCs, and effector T cells and by enhancing the number and activity of regulatory T cells (Gonçalves et al. 2018). These effects could pave the way to more effective strategies for food allergy prevention and treatment (Paparo et al. 2015). In another study, the immunological mediators involved in the effects of probiotic VSL#3 oral administration on anaphylaxis and Th2 inflammation in a mouse model of peanut allergy was investigated (Barletta et al. 2013). The results showed that VSL#3 supplementation to peanut-allergic mice was effective in attenuating anaphylaxis and Th2-mediated inflammation, by increasing regulatory responses in the jejunum mucosa and in the MNL. In further study, they found that TGF- β , which was induced in the gut by VSL#3 supplementation, was able to reduce the Th2 inflammation in association with food anaphylaxis in a mouse model of peanut allergy. TGF- β exerted its function through the induction/maintenance of Treg cells expressing FOXP3 and/or latency-associated peptide. The research indicated that probiotics supplementation may be an effective and safe strategy for food allergy treatment.

In addition to the gut microbiota, some environmental microorganisms may also produce anti-food allergic compounds. The lactic acid bacteria *Bifidobacterium longum* KACC 91563 and *Enterococcus faecalis* KACC 91532 have been found possessing the ability to suppressing food allergy by producing an extracellular protein

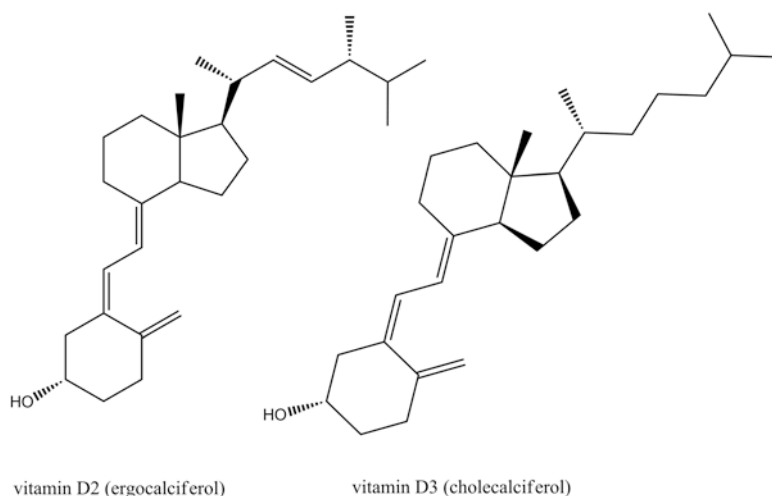


Fig. 7.4 The structure of vitamin D. Vitamin D is a group of fat-soluble secosteroids, and for humans, the most important compounds from this group are vitamin D₃ (cholecalciferol) and vitamin D₂ (ergocalciferol)

(Kim et al. 2016). In the study, *B. longum* KACC 91563 and *E. faecalis* KACC 91532 were administered to BALB/c wild-type mice, which were rendered food allergy by using OVA and alum. The results showed that *B. longum* KACC 91563, but not *E. faecalis* KACC 91532, attenuated food allergy symptoms. Extracellular vesicles secreted from *B. longum* KACC 91563 specifically bound to mast cells and induced apoptosis without influencing T-cell immune responses. Moreover, injection of family 5 extracellular solute-binding protein, the major component of extracellular vesicles, into mice greatly decreased the occurrence of diarrhea in a mouse model of food allergy. The research indicated that *B. longum* KACC 91563 could specifically induce apoptosis of mast cells and attenuate food allergy symptoms.

7.2.4 Anti-food Allergic Agents from Animal Sources and Human Milk

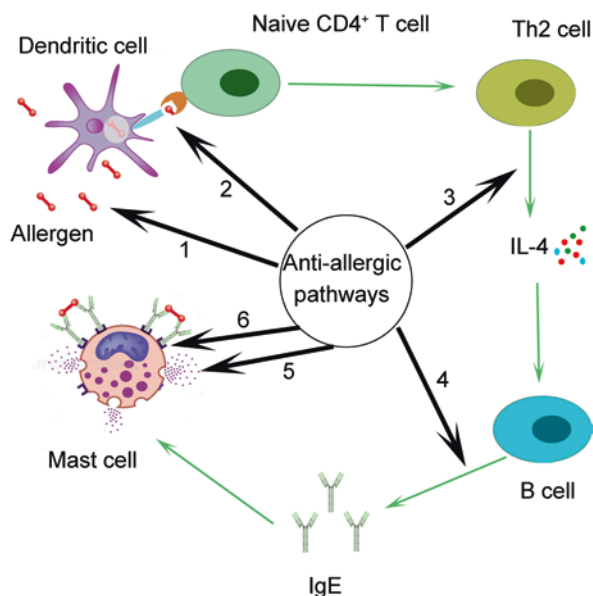
Compared to the other sources, the research on bioactive compounds from animals has been paid less attention. However, vitamin D (Fig. 7.4) has been extensively investigated for its relationship to food allergy. In addition to its classical function in the metabolism of bone and calcium, vitamin D is also found in relation to immune regulation and has been linked to immune disorders and allergic diseases (Suaini et al. 2015). Evolving evidence has indicated that vitamin D insufficiency may lead to food allergy, but research findings vary between populations. Lower vitamin D-binding protein (DBP) levels enhance the bioavailability of serum

vitamin D. Genetic polymorphisms explain almost 80% of the variation in binding protein levels (Koplin et al. 2016). The research showed that genetic polymorphisms in association with lower DBP level alleviated the association between low serum 25(OH)D3 level and food allergy, in line with better vitamin D biological availability in those with a lower DBP level. This enhances the plausibility of a role of vitamin D in the development of food allergy. Another study was performed to investigate the association between vitamin D insufficiency during infancy and challenge-proven food allergy at 1 year (Molloy et al. 2017). The research concluded that there was no evidence that vitamin D insufficiency during the first 6 months of infancy is a risk factor for food allergy at 1 year of age.

Human milk is the most important and usually the sole dietary source during the first several months in our life. It contains not only all the necessary nutrients but also chemicals that may provide health benefits. For instance, human milk oligosaccharides (HMOs) are among these functional ingredients (Bode 2006). HMOs are a group of unique glycans that are highly abundant and diverse, being capable of promoting the development of beneficial intestinal microbiota and preventing undesirable interactions of pathogens with mucosal epithelia (Gonia et al. 2015). The prebiotic nature of HMOs and accumulating evidence of immunomodulatory effects of these carbohydrates suggest that they may have some therapeutic function in allergy treatment. Consequently, a study was performed to assess the effects of two HMOs, 2'-fucosyllactose and 6'-sialyllactose, on the symptoms and immune responses in an OVA-sensitized mouse model of food allergy (Castillo-Courtade et al. 2015). In addition, the functions of mast cell in response to oral HMOs treatment were also measured in a passive cutaneous anaphylaxis model, and the direct effects on IgE-mediated degranulation of mast cells were investigated. The results showed that daily oral administration with 2'-fucosyllactose or 6'-sialyllactose alleviated food allergy symptoms including hypothermia and diarrhea. Treatment with HMOs also inhibited antigen-triggered increases of mast cell protease-1 in serum and the numbers of mast cells in the intestine. These effects were linked to increases of the cell populations of CD4+CD25+IL-10+ in the PP and MLN, while 6'-sialyllactose also contributed to the increase of IL-10 and the decrease in TNF production in antigen-stimulated splenocytes. They also found that both 2'-fucosyllactose and 6'-sialyllactose decreased the passive cutaneous anaphylaxis response. However, only 6'-sialyllactose, at high concentrations, directly suppressed mast cell degranulation in vitro. The research suggested that 2'-fucosyllactose and 6'-sialyllactose could attenuate food allergy symptoms by inducing IL-10+ T regulatory cells and indirect stabilization of mast cells. Therefore, human milk oligosaccharides may possess therapeutic potentials in allergic disease treatment.

In a more recent study, the researchers investigated nondigestible oligosaccharides (NDO), which have less complex structures than HMOs and have been used as ingredients in some dietary products to resemble the beneficial effects of HMOs, on their ability to enhance oral tolerance caused by partial hydrolyzed whey protein (pWH) (van Esch et al. 2017). Mice were administered with PBS, pWH, NDO, or pWH+NDO for 6 days and then were fed a control diet while sensitized to whey protein. Acute allergic skin responses and the activation mast cells activation were

Fig. 7.5 Potential pathways and targets for anti-allergic bioactive compounds. (1) Neutralizing allergens. (2) Inhibiting the action of antigen-presenting cells. (3) Downregulating the secretion of Th2 cytokines. (4) Inhibiting the production of IgE. (5) Preventing degranulation and suppressing the release of allergic mediators. (6) Impeding the binding of IgE and FcεRI receptors



measured after whey challenge. Acute allergic skin responses and mast cell activation were prevented by pWH+NDO, with lower whey-specific IgE induced compared with NDO-fed mice. In mice supplemented with pWH, Foxp3⁺ regulatory T cells were enhanced, while in those supplemented with NDO, galectin-9 was boosted. Increased CD103⁺DC percentages were found in the MLN of mice administered with pWH+NDO. These results showed that NDO combined with a pWH was capable of preventing the onset of allergic symptoms. NDO could serve as adjuvants in attenuating allergic responses to harmless food proteins.

7.2.5 Mechanisms of Action of Anti-food Allergic Chemicals

Generally speaking, anti-food allergy bioactive compounds exert their functions via the inhibition of the release of allergic media or by antagonizing the effects of allergic media at diverse stages of food allergic reactions, indicating a multi-target and multilevel property. Specifically, natural bioactive substances modulate the immune response with various molecular targets, as mentioned above, throughout the development of allergic responses, mainly encompassing the following pathways, neutralizing allergens, inhibiting the action of antigen-presenting cells, downregulating the secretion of Th2 cytokines, inhibiting the production of IgE, impeding the binding of IgE and FcεRI receptors, preventing degranulation, and suppressing the release of allergic mediators (Fig. 7.5). In addition, many natural bioactive compounds have an immunomodulatory effect and have anti-allergic effects by inhibiting Th2 cells and promoting the activity of Th1 cells, skewing allergic responses to the Th1 direction.

7.3 Development of Functional Foods with Anti-food Allergic Activities

In view of the dramatic increase in the prevalence of global food allergy, effective prevention strategies have become a top priority in public health work (Heine 2018). Oral immunotherapy has been deemed as a promising way for food allergy treatment. But, the current oral immunotherapy strategy is limited regarding the long-term efficacy and safety. Up to now, the best way for food allergy treatment is the avoidance of the allergens. Conventional strategies for the dietary treatment of food allergy such as extensively hydrolyzed formula and amino acid formula have been successful. However, the increasing food allergy prevalence globally has stimulated research on the possibility to find and develop effective tolerance to food allergens in prevention and dietary management strategies. With the accumulating evidence of anti-food allergy effects, some functional foods have been found or developed.

7.3.1 *Naturally Occurring Bioactive Foods*

A lot of studies have demonstrated that cocoa or cocoa-enriched diets showed immune modulating effects in vivo and in vitro (Camps-Bossacoma et al. 2017b; Jang et al. 2016; Abril-Gil et al. 2016; Massot-Cladera et al. 2014). In the study performed by Abril-Gil (Abril-Gil et al. 2016), which was aim to study cocoa action in depth, particularly focusing on its effect on a rat model of food allergy, as well as on an anaphylactic response. In the study, intestinal immune system was investigated to explore the action mechanisms. Brown Norway rats were fed reference diets or diets containing conventional cocoa or nonfermented cocoa. Food allergy to OVA was induced, and then an allergic response was provoked. The results showed that the synthesis of OVA-specific IgE and Th2-related antibodies was suppressed by diets containing conventional cocoa. In addition, the release of protease II from mast cells after anaphylaxis was partially inhibited by diets containing conventional cocoa, though other variables were not influenced. The diets containing conventional cocoa also alleviated the elevation of certain Th2-related cytokines released from MLN and spleen cells and influenced the intestinal gene expression of metabolites related to allergic response. The results indicated the influence of diets containing conventional cocoa locally and systematically. Meanwhile, the effects of the nonfermented cocoa diet were weaker than those of conventional cocoa-containing diets, showing that it was cocoa components rather than flavonoids that play a role in cocoa's action. Consequently, the cocoa-enriched diet showed a protective effect against food allergy and partially against anaphylaxis in rats, conferring this food potentially to be used in the fields of health and immune nutrition.

To obtain a full understanding of cocoa-induced mechanisms of action at small intestinal level, another research was performed (Camps-Bossacoma et al. 2017c). Female Lewis rats of 3-weeks-old were fed a standard diet or a diet containing 10%

cocoa for 4 weeks with or without concomitant oral sensitization with OVA. The results showed that in animals fed cocoa, there was a five-time decrease in the percentage of intestinal IgA+ cells together with a reduced proportion of TLR4+ intestinal epithelium. In the analysis of the lymphocyte composition, increases of the proportion of TCR $\gamma\delta$ + cells and NK cell percentage in PP and intestinal epithelium were observed. Besides, an increase in the proportions of CD25+, CD103+, and CD62L- cells was found in PP from cocoa-fed rats, as well as a reduction in gene expression of CD11b, CD11c, and IL-10. These results implied that changes in PP and small intestine epithelium composition and in gene expression induced by cocoa-enriched diets could be involved on its tolerogenic effect.

It has been reported that consumption of oily fish or fish oil, during the time of pregnancy, lactation, and infancy, is related to a decrease in the occurrence of allergic diseases in childhood (Hansen et al. 2017; Bisgaard et al. 2016). An observational study has been recently performed to investigate the potential relationship between postnatal fish oil consumption and food allergy in infants (Clausen et al. 2018). In this study, 1304 Icelandic children were prospectively followed in the first 2.5 years from birth with detailed questionnaires conducted at the time points of birth, 1 year of age and 2 years of age, including questions about fish oil consumption. The study investigated the development of sensitization to food and confirmed food allergy based on the age and frequency of postnatal fish oil supplementation by the use of a proportional hazards model. The incidence of food allergies diagnosed by children receiving regular fish oil supplementation is significantly reduced. Although it was not statistically significant, the incidence of challenge-confirmed food allergy also decreased. The research has also indicated that children who started to receive fish oil in their first half year of life got a significantly lower chance to undergo food allergy than those who began later. Increased fish oil consumption results in the decrease of Indicators of allergy severity. Moreover, the results remained the same when parent education and allergic family history were adjusted. Therefore, postnatal fish oil consumption is linked to decreased food sensitization and food allergies for infants and may serve as an intervention strategy for allergy prevention.

7.3.2 *Prebiotics and Probiotics*

A large body of clinical evidence has showed that prebiotics and probiotics may have beneficial effects for infants, which are at the risk of living with food allergies. Supplementation with certain prebiotic agents and probiotics has been considered a promising strategy in improving immune system, and balanced intestinal microbiota is part of the strategies for the nutritional management of food allergy. Oral administration of the strain *Bifidobacterium infantis* 14.518 (Binf) profoundly inhibited allergic responses induced by TM in a mouse model by both preventive and therapeutic strategies (Fu et al. 2017a). Further results showed that Binf enhanced DCs maturation and the accumulation of CD103+ tolerogenic DCs in

intestine-associated lymphoid tissues, which subsequently lead to the differentiation of regulatory T cells for inhibiting Th2-biased responses. It has also been found that Binf regulated the changes of gut microbiota composition. Particularly, the increase in *Dorea* and decrease in *Ralstonia* were highly correlated with Th2/Treg ratio and may contribute to attenuating Tm-induced allergic responses.

In another study, anti-allergic lactic acid bacteria were screened using the human basophilic cell line KU812F (Fujii et al. 2014). As is known to all, IgE cross-linking with high-affinity receptors leads to Ca^{2+} influx and subsequent histamine release in KU812F cells. The abilities of lactic acid bacteria strains to inhibit the increase of Ca^{2+} level triggered by IgE cross-linking and to suppress histamine release induced by the increased Ca^{2+} level were examined. Among the strains tested, five lactic acid bacteria strains inhibited both the increase of Ca^{2+} and histamine release. Illumination of common structures and cell content of these lactic acid bacteria strains may provide a basic knowledge for the anti-allergic properties of foods and facilitate the development of anti-allergic foods.

In addition to lactic acid bacteria, some other probiotics has also been found possessing anti-allergic activities. *Clostridium butyricum*, a probiotic that has been used in several gastrointestinal diseases and is beneficial for gut and brain health, shows preventive and therapeutic effects on anaphylactic symptoms in food allergy mice caused by β -lactoglobulin (Zhang et al. 2017). The results showed that oral administration of *Clostridium butyricum* significantly attenuated intestinal anaphylaxis symptoms in the food allergy mice. Besides, secretory IgA and CD4+CD25+Foxp3+ Treg cells were enhanced by oral administration of *Clostridium butyricum*. Moreover, it also reversed the imbalance of Th1/Th2 and Th17/Treg. The research indicated that *Clostridium butyricum* could ameliorate β -lactoglobulin-induced intestinal anaphylaxis in mice and might serve as an additional or supplementary therapy for food allergy treatment.

7.3.3 Traditional Herbs for Food Allergy Treatment

As mentioned above, traditional herbal is a rich source for bioactive compound isolation. Traditional use of herbal medicines indicates substantial historical application. In quite a few developing countries, a large number of people rely on traditional herbal medicines in order to meet their healthcare needs. Although modern medicine has developed to high degree, herbal medicines have maintained their popularity for historical and cultural reasons. Chinese traditional medicine, Japanese traditional medicine, and Indian traditional medicine are representatives of the herbal medicines.

Some herbals or their extracts show anti-food allergy properties (Yamamoto et al. 2016; Lee et al. 2015). Skullcap (*Scutellaria baicalensis* Georgi) has been widely employed as a traditional herbal medicine to treat inflammation, allergy, and cancer. In recent years, its effects in treating food allergy have also been

explored (Shin et al. 2014). To investigate the effects of skullcap on food allergy, female BALB/c mice were sensitized with OVA and alum by intraperitoneal injection. After that (17 days), the mice were orally challenged with OVA every 3 days for a total of six times. To assess the preventive effects, the mice were orally administered with skullcap daily from day 17 to 34. Food allergy symptoms were assessed by the criteria involving diarrhea, anaphylactic response, and rectal temperature. Severe food allergy symptoms were observed in the control group. On the contrary, the group treated with skullcap had a significantly inhibited OVA-caused anaphylactic response and rectal temperature. Furthermore, the levels of OVA-specific IgE, Th17 cytokine (IL-17), and Th2-related cytokines (IL-4, IL-5, IL-10, and IL-13), which increase with food allergy, were significantly suppressed by skullcap administration. Therefore, the research demonstrates that skullcap treatment ameliorated OVA-induced food allergy symptoms via the regulation of systemic immune responses of Th cells. These results suggest that skullcap may serve as a potential agent for the prevention and treatment for food allergy.

It has been found that kakkonto, a traditional Japanese herbal medicine, inhibited the occurrence of allergic symptoms in a food allergy mouse model (Yamamoto et al. 2009). A recent research has been performed to investigate whether the herbal kakkonto could regulate T-cell activation and differentiation in the colon (Yamamoto et al. 2016). BALB/c mice were sensitized and then orally challenged with OVA. Subsequently, the food allergy mice were orally treated with kakkonto. Lamina propria cells from their colons were obtained and investigated. The results indicated that Kakkonto significantly decreased the proportion of CD69+ cells and the elevated mRNA expression of Th2-specific transcription factor GATA-3 in the lamina propria CD4+ T cells. This showed that kakkonto had an inhibitive effect on the activation and Th2 differentiation of lamina propria effector CD4+ T cells of the food allergy mouse colon. Moreover, kakkonto significantly elevated the proportion of Foxp3+CD4+ regulatory T cells in the lamina propria CD4+ T cells of the food allergy mouse colon. Likewise, the level of Foxp3-positive cells was markedly increased in the colonic mucosa of kakkonto-treated food allergy mice. The research indicated that the therapeutic action of kakkonto could be explained by the induction of Foxp3+CD4+CD25- regulatory T cells in the colon, which may be serve as a novel mechanism for the development of new anti-food allergy drugs or functional foods.

7.3.4 Other Developed Foods or Methods for Food Allergy Treatment

In recent years, the strategy for food allergy treatment has been changing, shifting from long-time avoidance of food allergen to the proactive allergen introduction starting from 4 months of age. A study has been performed to investigate the

efficacy and safety of peanut oral immunotherapy using CpG-coated poly (lactic-co-glycolic acid) nanoparticles containing peanut extract (CpG/PN-NPs) in a murine model of peanut allergy (Srivastava et al. 2016). C3H/HeJ mice of peanut allergic were rendered by means of oral sensitization using peanut and CT. Mice were grouped and subjected to a four-weekly gavage with CpG/PN-NPs, vehicle (PBS), nanoparticles alone, peanut alone, CpG nanoparticles, or peanut nanoparticles, respectively. After the therapy, mice were subjected to another five monthly oral peanut challenges. The results showed that mice of peanut allergy treated with CpG/PN-NPs but not vehicle or other treatment components were significantly protected from anaphylaxis to oral peanut challenges, as indicated by lower symptom scores, less change in body temperature, and a lower increase of plasma histamine levels. In addition, CpG/PN-NP treatment did not induce anaphylactic reactions. The treatment was in association with a prolonged and significant reduction in peanut-specific IgE/IgG1 level and an enhancement in peanut-specific IgG2a level. Compared to vehicle control mice, peanut recall responses in splenocyte cultures from mice that were treated with nanoparticle showed a significant decrease in the levels of TH2 cytokines (IL-4, IL-5, and IL-13) but an increase in the IFN- γ levels in cell supernatants. The preclinical findings demonstrated that peanut oral immunotherapy with CpG/PN-NPs might be a promising approach for peanut-specific immunotherapy in humans.

With the development of biotechnology, DNA vaccination has been considered as a promising strategy for the treatment of allergic diseases. However, currently, poor immunogenicity has limited its application. Researchers have tried to improve this approach. In the study performed by Zhu et al. (2016), BALB/c mice were intradermally injected with plasmid DNA encoding the peanut protein Ara h 2 (pAra h 2) or pAra h 2 which was pretreated with poly-L-lysine before or after sensitization with Ara h 2 protein. CD207+DCs and Treg cells in draining lymph nodes were investigated after the DNA immunization, as well as the production of cytokine in splenocytes. The results showed that pretreatment with pAra h 2 or poly-L-lysine-pAra h 2 contributes to lower levels of Ara h 2-specific IgG1, IgG2a, and IgE and mice in the poly-L-lysine-pAra h 2 group had a significantly lower level of antibodies than those in the pAra h 2 group, indicating that poly-L-lysine-pAra h 2 possessed stronger effects than pAra h 2. Increased numbers of CD207+ DCs and Treg cells were observed in the mice receiving intradermal injection with poly-L-lysine-pAra h 2. Moreover, splenocytes from poly-L-lysine-pAra h 2-treated mice secreted more IFN- γ and IL-10. This research indicated that modification of pAra h 2 with poly-L-lysine could improve its prophylactic and therapeutic effects in peanut-allergic reactions.

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Chapter 8

Food Allergy and the Microbiota: Implications for Probiotic Use in Regulating Allergic Responses



Abstract The gastrointestinal tract is colonized with an enormous number of microorganisms, which play a critical role in shaping the development and function of host innate as well as adaptive immune responses. Evidence from both epidemiologic investigations and experiments in animal models indicates that abnormalities in microbiota composition (dysbiosis) can influence the development of allergic disorders via effects on immune function. Correction of these abnormalities by the use of probiotics or prebiotics, therefore, could have therapeutic or preventive potential. Probiotics can have positive effects on health that contribute to nutrition, modulation of inflammatory and hypersensitivity responses, and prevention of intestinal infections. This chapter will review developments in our understanding of the gut microbiota and probiotics in relation to food allergies.

Food allergy, defined as an adverse immune response that occurs reproducibly on exposure to a given food, affects approximately 2.5% (ranging from 1% to 10%) of the general population in the world (Boyce et al. 2010; Prescott et al. 2013; Renz et al. 2018). Food allergies are most prevalent in the first few years of life. According to a global survey of changing patterns of food allergy burden in children, the prevalence of clinical food allergy in preschool children in developed countries and developing countries was as high as 10% and 7%, respectively (Prescott et al. 2013). Among American children (aged 0–17 years), the prevalence of food allergies increased from 3.4% in 1997–1999 to 5.1% in 2009–2011 (Jackson et al. 2013). Unfortunately, the cause of food allergy is still largely undefined. The combined effects of genetic/environmental/developmental factors may contribute to the observed global increase of prevalence of allergic disease. Based on current knowledge, genetic factors may predispose toward development of food allergy among selected individuals. There is building evidence that food allergy is more likely to occur in infants with a family history of atopic disease, especially in infants of atopic mothers (Tsai et al. 2009). It has been shown that a child born to a parent with peanut allergy has a sevenfold increase in the likelihood of developing the same allergy and a child born to a mother with asthma has a four times higher risk of acquiring the disease (Carroll et al. 2007; Cookson 2010; Hourihane et al. 1996).

However, genetic variance alone cannot explain the changing pattern of food allergy, and genetic mutations with clear relationship to allergy have been elusive. The mother not only transfers 50% of her genes to her baby, but she is also the exclusive environment during gestation and continues to be a major environmental factor while breastfeeding (Björkstén 2005). There is an increasing body of epidemiological and animal studies indicating that immunological and environmental factors are strong determinants of allergic disorders. Many studies suggest that abnormalities of the gut microbiota, a condition known as dysbiosis, can be induced by environmental and lifestyle factors and can influence immune function and the development of food allergy. Thus, a better understanding of how gut bacteria communities interact with the immune system will help open the way to novel preventive and treatment strategies for food allergy.

8.1 The Gut Mucosal Barrier

A major physiological function of the gastrointestinal tract is the digestion of food, absorption of nutrients and water, and elimination of unnecessary waste products. The intestine is also a major site of entry for many pathogens and antigens (Guarner and Malagelada 2003; Palmer et al. 2007). The gut mucosal barrier, constituted by the microbiota, the epithelium, and the immune cells that are resident in or recruited to the subepithelial mucosa, is able to distinguish innocuous antigens from pathogenic microorganisms and accordingly to elicit an appropriate response. This complicated system has evolved under two opposing evolutionary pressures: (1) providing the host with protective mechanisms against invasion by potential pathogens, such as bacteria, viruses, and protozoans, and (2) promoting immunological tolerance against the wide array of harmless environmental antigens, products of digestion, and the normal intestinal flora. Intestinal homeostasis is maintained under the control of the unique gut-associated lymphoid tissues (GALT), secretory IgA (SIgA), antimicrobial peptides (e.g., defensins), mucosal immune cells (e.g., Th1, Th2, Th17, and Treg cells), cytokines, chemokines, and commensal bacteria (Goto et al. 2016; Kiyono and Fukuyama 2004; Kurashima and Kiyono 2017). Failure to regulate precisely the mucosal immune system, or a breakdown of mucosal tolerance, may result in the development of an immune response to food protein (Lambrecht and Hammad 2014) or the induction of a destructive response directly against self-antigens (autoimmune disease). Food allergy is triggered by the ingestion of a dietary antigen via the gut mucosal membrane. There is now accumulating evidence that resident microbial communities play a key role in early host immunologic development and can modify risk for allergic disorders.

At the intestinal mucosal surface, foreign particles are taken up by specialized epithelial cells (M cells) and delivered to antigen-presenting cells (APCs), including primarily CD103^{high} dendritic cells (CD103^{high} DCs) and CX3CR1^{high} macrophages, located directly below the epithelium. Intestinal epithelial cells can also act as APC (Siddiqui and Powrie 2008; Sun et al. 2007). Processed antigens displayed on APCs are detected by T cells in the GALT, resulting in the induction and development of different effector

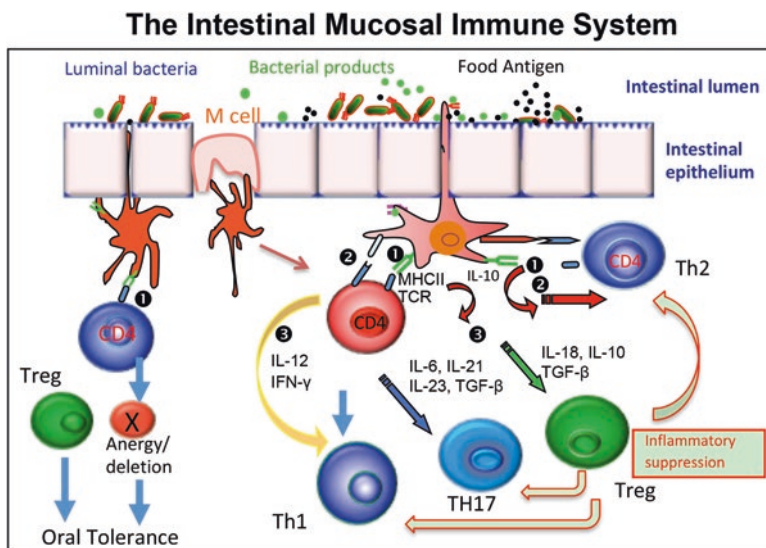


Fig. 8.1 The intestinal epithelium is a single cell layer that forms the interface between the external and the internal environments of a host and separates luminal antigens (bacteria, bacterial products, and food) of the gastrointestinal tract from the largest population of lymphocytes in the body. The intestinal mucosal immune system plays an important role in the induction and maintenance of tolerance to food and other innocuous luminal antigens and in the protection of the epithelial barrier against pathogenic invasion. This function of the intestinal epithelial mucosal system is tightly regulated and can be influenced by effective antigen presentation and by the cytokine microenvironment. The T-cell stimuli derived from DCs, therefore, include MHC II peptide (signal 1), the expression or absence of costimulatory molecules (signal 2), and the expression or absence of polarizing cytokines, which may provide an additional signal (signal 3). The induction of non-responsiveness (tolerance) is thought to be mediated by clonal deletion, clonal anergy, and the generation of regulatory T cells producing IL-10 and/or TGF- β . Failure to precisely regulate the mucosal immune system may result in the development of mucosal inflammation and an allergic response

T cells (Th1, Th2, and Th17) and Treg. Allergic diseases are characterized by an immunological response with Th2 cells producing IL-4, IL-5, and IL-13. Treg cells play a key function in the regulation of immune tolerance and allergic responses (Fig. 8.1).

Treg cells are composed of two different subpopulations: a subpopulation of Treg cells arises in the thymus and is known as CD4⁺FOXP3⁺ natural regulatory T (nTreg) cells (or thymus-derived regulatory T cells), which primarily mediate tolerance to self-antigens (Lio and Hsieh 2011); a second population of CD4⁺FOXP3⁺ Treg cells arises extrathymically in peripheral lymphoid tissues from a pool of naïve conventional CD4⁺FOXP3⁻ T cells after exposure to antigens (inducible Treg, iTreg). iTreg cells are particularly enriched in the gastrointestinal tract and in the lungs during chronic inflammation, with specificities directed against microbial antigens or environmental allergens (Curotto de Lafaille 2008a; Kretschmer et al. 2005; Mucida et al. 2005).

At the intestinal mucosa, CD103⁺CD11c⁺ DCs that express large abundance of TGF- β and retinoic acid, a vitamin A metabolite, facilitate the generation of iTreg cells

(Lathrop et al. 2011; Siddiqui and Powrie 2008; Sun et al. 2007). Both Foxp3⁺ nTreg and iTreg cell subsets play a key function in the maintenance of peripheral tolerance by suppressing reactivity to self-antigens and by containing the amplitude of immune responses to foreign antigens. The GALT, therefore, plays a unique role in determining whether tolerance or an immune response is induced. Immune tolerance is crucial for maintaining mucosal homeostasis given the tremendous number of foreign substances (such as food proteins) that APCs of the gastrointestinal mucosa encounter.

8.2 Intestinal Microbiome and Immune Function

8.2.1 Microbiome

The term “microbiome,” referring to the microorganisms that live on or inside another organism, is the sum of the microbes and their genomic elements in a particular environment and is an integral component of human physiology. The microbiome is thought to mediate health and disease (Lynch and Pedersen 2016). There are now numerous studies linking microbiota of the gastrointestinal tract, respiratory tract, and skin to allergic disorders (Fujimura 2016; Stein et al. 2016; Williams and Gallo 2015).

The gastrointestinal tract is colonized with an enormous number of microorganisms, including bacteria, fungi, and protozoa. There are more than 1000 different known bacterial species in the intestinal tract, with the major phyla being *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*. The number of bacteria is about the same as the number of human cells (Sender et al. 2016), and this community is extremely diverse (Ley et al. 2006). The microbiota plays a significant role in digestion and absorption of nutrients and protection against pathogen invasion. It is also a source of a large pool of microbial molecules, metabolites, and foreign antigens, many of which exert beneficial effects on the host, including development and maintenance of the gut epithelial barrier and the immune system (Riiser 2015). Moreover, the microbiome effectively adds a huge number of genes to the human genome, potentially increasing it up to 200 times (Turnbaugh et al. 2007). Microbiota composition can be impacted by a variety of factors including genes, age, environment, health, diet, antibiotic use, pathogen infection, psychosocial stress, etc. (Honda and Littman 2012). Epidemiologic data, as well as experiments in mice indicate that abnormalities in microbiota composition (dysbiosis), particularly early in life, can influence the development of allergic disorders via effects on immune function (Fujimura 2016; Ho and Bunyavanich 2018).

8.2.2 Microbiome and Immunologic Development

The host immune system has co-evolved with the microbiota populating the human body. The interaction between host and the acquired microbiota after birth plays a critical role in the development of host protective structures and innate immunity

(Salzman et al. 2007). It has long been appreciated that the gut microbiota has a critical role in the development of organized lymphoid structures and in the function of immune system cells. Germ-free (GF) mice display extensive deficiencies in secondary lymphoid structure development, in secretory IgA production and intraepithelial lymphocytes (Mazmanian et al. 2005; Talham et al. 1999), and in intestinal epithelial cell maturation (Hooper et al. 2001; Stappenbeck et al. 2002). The intestinal mucosa is the interface between a variety of immunogens from microbial and food origins present in the lumen of the gut and the underlying lymphoid tissues and immune cells. Among the various immune cells, the microbiota has been shown to be associated with the development of Th1, Th2, Th17, and Treg cells (Atarashi et al. 2011; Mazmanian et al. 2005). GF mice have defects in the spleen and mesenteric lymph nodes (MLN) and show reduced Treg cell induction and the absence of Th17 cells as well as Th1/Th2 imbalance, with a bias toward Th2 responses (Wu 2012). Intestinal commensals were also shown to be required for suppression of IgE and Th2 responses following antigen feeding (Sudo et al. 1997).

Experimental evidence suggests that defined microbial products can play a dominant role in the induction of distinct class of immune responses. Colonization of animals with the ubiquitous gut microorganism *Bacteroides fragilis* directs the development of the Th1-associated immune response (Mazmanian et al. 2005). Segmented filamentous bacteria (SFB) colonization of mice results in accumulation of Th17 cells and, to a lesser extent, in an increase in Th1 cells (Gaboriau-Routhiau et al. 2009; Ivanov et al. 2009). In other studies, colonization of gnotobiotic mice with a complex cocktail of 46 mouse *Clostridial* strains, belonging mainly to cluster IV and XIVa of the *Clostridium* genus, results in the expansion of colonic lamina propria and systemic Tregs (Atarashi et al. 2011) and many of these Tregs expression IL-10, which can enhance the anti-inflammatory branches of the adaptive immune system. Together, these results suggest that the development of Th cells can be controlled by the microbiota and that commensal bacteria have a general impact on immunity that reaches well beyond mucosal tissues. The dysregulation of Th-cell development by an altered microbiota may contribute to the pathogenesis of diseases (Su et al. 2018).

The molecular mechanisms responsible for the effects of the microbiota on T-cell development are not fully understood. They may involve a variety of pattern recognition receptors (PRRs) which are capable of detecting microbe-associated molecular patterns (MAMPs) including Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) (Hooper et al. 2012; Lavelle et al. 2010). For instance, it has been shown that PSA of *B. fragilis* can be specifically recognized by CD11c+ DCs, which migrate to lymphoid organs and signal Th1 lineage differentiation through the IL-12/Stat4 pathway (Mazmanian et al. 2005). Gram-negative bacterial peptidoglycans are sensed by NOD1 expressed on intestinal epithelial cells (Fritz et al. 2006). It is also known that sensing of commensal microbiota through the TLR-MyD88 signaling pathway triggers several responses that are critical for maintaining host-microbial homeostasis.

Bacterial lipopolysaccharide (LPS) (Vaishnavi et al. 2008) or flagellin (Kinnebrew et al. 2010) can induce production of RegIII γ , one of the best-characterized mucosal antimicrobial peptides, in a MyD88-dependent manner

(Cash et al. 2006). The flagellin signals are relayed through TLR5 expressed by CD103⁺CD11b⁺ DC in the lamina propria, stimulating production of IL-23 that, in turn, promotes the expression of IL-22 by innate lymphoid cells (Kinnebrew et al. 2012). These observations show the importance of intestinal microbiota in shaping the development and function of host innate as well as adaptive immune responses.

8.2.3 *Microbiome and Oral Tolerance*

The healthy mucosal immune system is inducing and sustaining the unresponsiveness status to dietary antigens—that is known as “oral tolerance” characterized by the depletion or anergy of reactive T cells (Schwartz 1990; Takahashi et al. 1995) and/or the induction of Tregs cells at mucosal sites (Curotto de Lafaille et al. 2008b; Josefowicz et al. 2012). Soluble protein antigens, including those derived from foods, typically induce antigen-specific peripheral nonresponsiveness or oral tolerance. The induction and maintenance of oral tolerance to food and other innocuous luminal Ags are central to the effective function of the intestinal mucosal immune system in guarding the epithelial barrier against pathogenic invasion. Disruption of the gut mucosal immune system can lead to failure to induce tolerance, allowing allergic sensitization and the development of food allergy. However, the exact mechanism by which oral tolerance is maintained, or lost, remains unclear.

The microbiota plays a fundamental role in the balance of the host immune system between activation and tolerance. Experimental evidence shows that oral tolerance could not be induced in GF mice and feeding of GF mice with LPS was sufficient to restore oral tolerance (Wannemuehler et al. 1982). Reconstitution of GF mice with *Bifidobacterium infantis*, a dominant commensal bacteria, also rescued oral tolerance in GF mice (Sudo et al. 1997). Oral tolerance is initiated by CD103⁺ DCs, which capture antigen in the lamina propria and migrate to the MLN, inducing naïve T cells to differentiate to Tregs, through a mechanism dependent on TGF- β and retinoic acid (Coombes et al. 2007; Esterhazy et al. 2016; Sun et al. 2007). Local environmental factors in the intestine play a key role in the maturation, maintenance, and phenotype of CD103⁺DCs. CD103⁺ DCs express the enzyme retinaldehyde dehydrogenase-2 (RALDH2) (encoded by *Aldh1a2*). This enzyme converts vitamin A to retinoic acid, which promotes the differentiation of naïve T cells into Treg cells (Coombes and Powrie 2008). Alteration of RALDH activity is associated with impaired oral tolerance (Hall et al. 2011). The phenotype of CD103⁺ DCs can be regulated by microbial signals. In response to these microbial signals, intestinal DCs and macrophages are activated, and macrophages produce IL-1 β , which leads to granulocyte macrophage colony-stimulating factor (GM-CSF) by innate lymphoid cell 3 (ILC3) and subsequently retinoic acid and IL-10 release by DCs and macrophages. Retinoic acid and IL-10 promote Treg accumulation and intestinal homeostasis (Mortha et al. 2014). Epithelial production of cytokines and other factors, such as IL-25, IL-33, TSLP, and GM-CSF, is critical in balancing tolerance versus Th2 responses by DCs.

These observations suggest that microbiota may regulate tolerance via different mechanisms, including the regulation of intestinal barrier integrity, enhanced IgA production, and induction of Tregs. For example, it has been shown that *Clostridia*-containing microbiota can enhance production of IL-22 by ILCs and CD4⁺ T cells, resulting in reduced intestinal barrier permeability to allergens (Stefka et al. 2014). Colonization of *Clostridia* and *Bacteroides* promotes induction of IgA, which reduces allergen transport throughout the epithelial barrier (Stefka et al. 2014).

8.3 Microbiome and Food Allergies

8.3.1 Clinical Evidence

A potential role for the microbiome in food allergy has been suggested by epidemiologic studies and animal models linking environmental factors (differential microbial exposure and colonization in early childhood) to the development of food allergy. Differential microbial exposure in early childhood is associated with altered susceptibility to allergic disorders. For instance, in a Norwegian birth cohort of 2803 infants, cesarean delivery was found to be associated with increased risk of egg allergy by age 2.5 years in children with atopic mothers (Eggesbø et al. 2003). Children born by cesarean delivery had decreased microbial diversity and delayed *Bacteroides* colonization during the first 2 years of life (Jakobsson et al. 2014). Further insights into the interactions between the environment, host microbiome, and food allergy have been provided by investigations that compared genetically similar populations between Eastern and Western Europe. Distinct gut microbiota has been detected in infants from Estonia (Eastern Europe), where the prevalence of atopy was low, and Sweden (Western Europe), where it was high (Sepp et al. 1997). Estonian infants have high counts of *Lactobacilli* and *Eubacteria*, whereas Swedish infants have increased numbers of *Clostridia* (Sepp et al. 1997). Moreover, antibiotic exposure during prenatal and early-life stages results in disrupted microbiota in the host with decreased commensal microbial diversity and correlates with increased risk for the development of atopic disorders (Murk et al. 2011; Raciborski et al. 2012; Sun et al. 2015). It has been suggested that early bacterial exposure may protect children from becoming hypersensitive to allergens. In an Australian birth cohort study, it was shown that infants having older siblings and pets (Berni Canani R) had decreased risk of egg allergy by age 1 year (Koplin et al. 2012). Exposure to furry pets has been shown to enrich the abundance of gut *Ruminococcus* and *Oscillospira* in infants (Tun et al. 2017). Similarly, childhood in rural environments and consumption of unpasteurized milk are associated with protection from allergic conditions (Mutius and Radon 2008; Waser et al. 2007). These observations support the idea that decreased exposure of children to microbial products and microbial infections, which elicit Th1 immune response during childhood, can contribute to the development of abnormal immune responses, increasing susceptibility to allergy, asthma, and autoimmune diseases (hygiene hypothesis) (Bashir et al. 2004; Cahenzli et al. 2013; Jakobsson et al. 2014; Koplin et al. 2012; Rodriguez et al. 2011; Tun et al. 2017).

8.3.2 *Animal Models Linking Microbiota and Food Allergies*

Various animal models have been utilized to elucidate the relationship between intestinal microbiota and food allergy. GF mice are more responsive than WT control mice to oral sensitization with cow's milk protein and ovalbumin (Cahenzli et al. 2013). Mice with antibiotic-induced altered microbiota become more susceptible to the induction of food allergy upon oral challenge compared to untreated mice (Bashir et al. 2004). Moreover, food allergy-prone mice with a gain of function in the IL-4 receptor α -chain carry a distinct microbiota signature that was virtually nonoverlapping with the changes in the microbiota of similarly sensitized WT mice that otherwise did not have disease. Enhanced bacterial families *Lachnospiraceae*, *Lactobacillaceae*, *Rikenellaceae*, and *Porphyromonadaceae* have been detected in these OVA-sensitized food allergy-prone mice (Noval Rivas et al. 2013). Significantly, the food allergy phenotype could be transmitted by transferring the gut microbiota from the food allergy-prone mice to WT mice (Noval Rivas et al. 2013). These observations suggest that disease-associated microbiota may have a pathogenic role in the development of food allergy.

In murine models of food allergy, it has been shown that certain bacterial taxa, such as *Clostridia*, exert protective effects for food allergy, as colonization of gnotobiotic mice with selected intestinal *Clostridia* protected against systemic IgE response to egg and peanut proteins (Atarashi et al. 2011; Stefka et al. 2014). Colonization of 17 strains of human microbiota-derived *Clostridia* attenuated disease severity in egg-induced allergic diarrhea (Atarashi et al. 2013). These bacterial strains promote Treg and induce IL-10 production (Atarashi et al. 2013). Colonization with probiotics (*Lactobacillus acidophilus*) early in life results in a significant upregulation of regulatory cytokine response (IL-10 and TGF- β) in the intestinal mucosa in mice (Chen et al. 2005). Fecal microbiota of healthy infant displays a large abundance of *Bifidobacterium* spp. and *Bacteroides* spp. Certain bacterial strains within the genera *Bifidobacterium* and *Bacteroides* may have protective roles as colonization of mice with healthy infant fecal bacteria rich in these genera resulted in reduced systemic allergic reaction to oral challenge with cow's milk protein (Rodriguez et al. 2012).

Based on the observations from various animal models, some potential mechanistic insights have been proposed. (1) Intestinal microbiota plays a key role in modulating Th2 immunity. This is evidenced by the observations showing that both GF- and antibiotic-treated mice have exacerbated Th2 response and elevated IgE levels (Cahenzli et al. 2013; Hill et al. 2012). In the absence of microbial signals, there is increased expression of IL-33, a Th2-promoting cytokine, in the intestinal epithelium (Ohnmacht et al. 2015). Overexpression of epithelial-derived cytokines IL-25 or IL-33 drives allergic responses to dietary antigens by skewing the immune environment in the intestine from tolerogenic to pro-allergic (Chu et al. 2013; Lee et al. 2016). TLR-deficient mice display Th2-biased response and increased susceptibility to food allergy (Bashir et al. 2004). Microbiota induces ILC3 via IL-6 and IL-23 and thereby suppresses the Th2 responses (Ohnmacht et al. 2015). (2)

Microbiota has been found to influence the regulatory mechanism of host immune system. As discussed above, microbial colonization induces Ror γ ⁺ Treg cells (Ohnmacht et al. 2015). In the absence of these Ror γ ⁺ Treg cells, Th2-associated pathology is enhanced (Ohnmacht et al. 2015). (3) Furthermore, microbiota may affect food allergy susceptibility by promoting intestinal barrier function, reducing allergen uptake. Colonization of GF mice with *Clostridia* restored the fecal IgA production (Stefka et al. 2014). *Bacteroides uniformis* colonization partially restored IgA level (Stefka et al. 2014). Microbial colonization of GF mice with *Clostridia* induces IL-22 production by ILCs and CD4⁺ T cells (Stefka et al. 2014). IL-22 can protect intestinal epithelial barrier by promoting mucous secretion by goblet cells (Sabat et al. 2014) and reduce intestinal permeability to dietary allergens. Despite considerable progress, further work is needed to fully understand how precisely the intestinal microbiota regulates host responses to food allergens.

Increasing evidence supports the idea that certain commensal bacterial species are protective and enhance gut barrier function, while the presence of other bacteria can increase inflammation. Although it is unknown if a particular microbiota species promotes food allergy, some species such as *Lactobacillus*, *Clostridia*, *Bifidobacterium*, and *Bacteroides* have been associated with suppression of food allergy in mouse studies (Atarashi et al. 2011, 2013; Rodriguez et al. 2011). A better understanding of host-microbial relationships in food allergy and identification and functional evaluation of protective commensal microbes will inform strategies for the prevention and treatment of food allergy.

8.3.3 Microbiota Metabolites

Metabolites from intestinal microbiota are key determinants of host-microbe mutualism and, consequently, the health or disease of the intestinal tract. The major metabolites produced by bacteria in the gut are short-chain fatty acids (SCFAs) (Thorburn et al. 2014). SCFAs have received much attention for their immunoregulatory capacity. Diet is a critical determinant for gut bacterial ecology, diversity, and function. There is evidence showing that high-fiber feeding in mice resulted in improved oral tolerance and protected from food allergy by enhancing retinal dehydrogenase activity in CD103⁺ DCs, which have been implicated in gut homeostasis and Treg cell biology (Tan et al. 2016). Dietary fiber is fermented in the colon by anaerobic bacteria into SCFAs, mainly acetate, butyrate, and propionate. SCFAs have been intensively studied in the past decade. SCFAs can regulate host immune responses as signaling molecules through activation of G-protein-coupled receptor (GPRs), including GPR41, GPR43, and GPR109A, and as epigenome modifiers through their histone deacetylase (HDAC) inhibition activity (Basson et al. 2016; Licciardi et al. 2010). These GPRs are all expressed in intestinal epithelial cells, in addition to the differential expression in various sites and cell types (Ohira et al. 2017). The evidence highlights the broad role for SCFAs including modification of immune functions (Corrêa-Oliveira et al. 2016). It has been shown that SCFAs

increase Tregs in the intestinal mucosa via GPR43 (Garrett et al. 2007) or T-cell intrinsic epigenetic upregulation of the *Foxp3* gene (Furusawa et al. 2013). New evidence showed that dietary fiber and bacterial SCFA enhance oral tolerance and protect mice against food allergy (peanut allergy) (Tan et al. 2016). This study further showed that high-fiber diet-mediated protection against food allergy relies on vitamin A metabolism and promotes IgA responses. Mice lacking GPR43 or GPR109A, receptors for SCFAs, showed exacerbated food allergy and fewer CD103⁺ DCs (Tan et al. 2016), suggesting that GPR43 and GPR109a are required for dietary-fiber-mediated protection against food allergy. These studies, therefore, suggest that dietary elements regulate numerous protective pathways in the gastrointestinal tract, necessary for immune nonresponsiveness to food antigens. Unhealthy diets, such as those high in fat and low in fiber, may be associated with changes in gut bacterial ecology, epithelial integrity, and Treg cell development, contributing to compromised oral tolerance and increased susceptibility to food allergies.

8.4 Gut Microbiota as a Potential Therapeutic Target for the Treatment of Food Allergies

Increasing evidence supports the idea that certain commensal bacterial species are protective and enhance gut barrier function, while the presence of other bacteria can increase inflammation. As discussed above, the abundance, diversity, and metabolic activity of gut microbiota play important roles in the maintenance of homeostasis in a healthy state. Several factors associated with dysbiosis may influence food allergy, such as the mode of delivery in childbirth, low versus rich fiber diet, breastfeeding, and/or early-life antibiotic exposure, all of which affect bacterial load and diversity. This suggests that allergic phenotypes are associated with alterations in the intestinal microbiome and that this dysbiosis may drive the allergic response. Since abnormalities in the microbiota have been linked to the development of food allergy, correction of these abnormalities by the use of probiotics or prebiotics could have therapeutic or preventive benefits.

Probiotics are “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (<http://www.fao.org>). Probiotic bacteria usually belong mainly to *Lactobacillus* and *Bifidobacterium* groups, in particular *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* GG (LGG), and *Bifidobacterium bifidus*, which include different strains. Some common probiotics, such as *Saccharomyces boulardii*, are yeasts (Iannitti and Palmieri 2010). Probiotics are able to recolonize and restore microflora symbiosis in the intestinal tract and may influence the functionality of DCs, NK cells, monocytes, macrophages, and, to a lesser extent, B cells. Due to their ability to adhere to intestinal epithelial cells and to modulate and stabilize the composition of gut microflora, probiotic bacteria have been tested in preventing and/or treating allergic disorders. In a murine model of food allergy, *Lactococcus lactis* NCC 2287 oral administration for 7 weeks in

sensitized mice reduced allergic manifestation (Zuercher et al. 2012). Oral administration of a probiotic mixture (VSL#3) containing eight different strains has been shown to be effective in redirecting allergic Th2 response toward Th1 responses and in the protection against food allergen-induced allergic response in a mouse model of food allergy (Schiavi et al. 2011). A study also evaluated the effect of probiotics as an adjuvant to oral immunotherapy in children with peanut allergy. Co-administration of *L. rhamnosus* CGMCC 1.3724 and peanuts led to sustained desensitization and reduced peanut-specific IgE levels (Tang et al. 2015). However, evidence for therapeutic and preventive effects of probiotics on food allergy in human subjects is still sparse. Administration of LGG-supplemented casein formulas in infants with IgE-mediated cow's milk allergy led to enrichment of butyrate-producing taxa accelerating tolerance acquisition and reducing allergic response (Berni Canani et al. 2017). New studies are needed in order to clarify the functions and the utility of probiotics in food allergies. Translational studies have begun to explore the potential role for microbe modulating therapeutics for diseases such as food allergy. A better understanding of the complex and dynamic nature of host-microbial interactions and identification and functional characterization of healthy microbial communities (and microbial metabolites) that reinforce tolerance and barrier function in the intestine will allow for better targeted novel microbiota-based strategies, i.e., ones based on altering microbiota composition with pre-, pro-, and synbiotics for the prevention and treatment of the immunological abnormalities associated with dysbiosis, such as food allergies.

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Chapter 9

Risk Assessment and Control Management of Food Allergens



Abstract With the improvement of people's living standards, the labeling management of allergens in food is increasingly valued by people of all countries. However, compared with the United States, the European Union, and several other countries and organizations, most third world countries still have a large gap in the management level of allergen labels in food. This chapter summarizes the management of food allergens labeling, the revision process of labeling instructions, and the management of cross-contamination by major countries and organizations around the world. In addition, we compare the status of food allergens labeling management in the EU and China as a typical representative of third world countries and summarize the shortcomings in the Chinese management of food allergens, mainly including imperfect regulations and standards for food allergen labeling, lack of risk assessment of food allergens, no cross-contamination regulations, and low consumer awareness on food allergens.

9.1 Current Status of Various Countries Food Safety Regulation

9.1.1 *Global Specific Food Safety Regulations*

In 1948, the Charter of World Health Organization (WHO) detailed the WHO's special responsibilities related to food safety, which include assisting governments in strengthening health services related to food safety; promoting improved nutrition, sanitation, and environmental hygiene; developing international standards for food; and assisting in promoting food safety among consumers (Rosini and Daniele 2002).

The food safety and food strategy proposed by the United States in the food section of Chapter 21 of Title 9 of the United States Code stipulate the Food Safety Commission should consult with the Minister of Transportation, the Minister of Finance, relevant federal agencies, the food industry, consumer and producer groups, scientific organizations, and countries to develop an educational strategy for crisis communication and response to terrorist food threats to determine the methods

of techniques and procedures for food processing, production facilities, and transportation (West_Publishing 2015).

The core objective of the European Commission's food safety policy is to ensure that the food industry (the largest manufacturing and employment sector in Europe) protects high levels of human health. The European Food Safety Authority independently completes risk assessment and risk management to ensure that food in Europe is safe (General_Accounting 2001). Therefore, food safety requires legislation to lay the minimum health requirements, local governments should make food companies meet the norms, and food companies need to follow the Hazard Analysis Critical Control Point (HACCP) guidelines and implement food safety programs (Bronowicka et al. 2006).

According to the Japanese Food Safety Law, all relevant factors in the circulation of food from production to sale may have an impact on the safety of food. Therefore, food safety must be taken to ensure the safety of all aspects of food circulation. The main body of responsibility includes national, local public bodies, food-related practitioners, and even consumers who play an active role in ensuring food safety (Takaya 2009).

Food Standards Australia New Zealand (FSANZ) has developed the Australian New Zealand Food Standards Code, which discloses that the purpose of food safety labeling is to ensure that only safe and suitable food is sold in Australia and New Zealand and requires food business operators to ensure that food meets all the requirements of food safety standards (Winger 2003). In addition, food companies that provide food services to special populations such as patients, elderly people who need care, and vulnerable people are required to process food in accordance with a certified and reviewed food safety plan (Liu et al. 2015).

9.1.2 Research Status of Food Safety Regulation in China

The third world includes more than 130 developing countries in Asia, Africa, Latin America, Oceania, and other regions, accounting for more than 70% of the world's land area and total population. Among them, China has become one of the typical representatives due to its rapid development in recent years. Even so, China started late in the formulation of food safety regulations; therefore, there is still abundant room for improvement in the formulation of relevant regulations. Article 150 of the newly revised China Food Safety Law provides a detailed description of the concept of food safety and food safety work for the first time. In that law, it is stated that food safety means that the food is nontoxic and harmless, meets the nutritional requirements that should be present, and does not cause any acute, subacute, or chronic harm to human health. And food safety work should base on prevention, risk management, full-process control, and social co-governance and establish a scientific and strict supervision and management system (Li et al. 2015).

9.2 Food Allergen Labels and Its Management Specifications

9.2.1 Current Status of International Food Allergen Label Research

In a study of existing problems in the implementation of food allergen labeling, after conducting a series of surveys of Canadian consumers, Sheth et al. believe that in order to increase consumer confidence in domestic food safety and reduce the chance of accidental intake of allergens, it is necessary to clarify the food allergen label and improve the education level of consumers (Sakellariou et al. 2010). In addition, Sakellariou believes that allergens management not only be based on general guidelines and descriptions, but also a clear explanation is needed. Studies have shown that the exact, restricted use, and possibly the words for each food allergen clause are easy misleading to the consumer (Sakellariou et al. 2010). Gendel (2012) considers food allergy to be a globally important public health issue. The regulatory risk strategy for allergic consumers focuses on providing information on the presence of food allergens through identification statements (Gendel 2012). Katrina et al. (2014) argue that the government's focus on food labeling has become the management of food allergens. Mandatory food allergen labeling has the effect of increasing consumer food safety levels, but most food producers' voluntary labels (called prophylactic allergen labels) have been used in a wide range of food packaging (Allen et al. 2014). Even so, Wang believes that in many foods, sensitizing ingredients are actually a small part. In addition, different countries and regions have different degrees of response to food sensitization due to different geographical factors and living habits. Therefore, isolating food allergens from the source is one of the best strategies to prevent food allergies (Wang et al. 2014b).

9.2.2 Research Status of Chinese Food Allergen Labels

The management of food allergens in China started late. The management standard related to the food allergens was the "Essential Food Safety Food Sensitive Markings for the Olympic Games" issued during the Beijing Olympic Games in 2008 but was abolished after the end of the Olympic Games (Guo et al. 2010). Chinese scholar believes that consumers cannot understand the food allergens contained in food by observing the appearance of food. Especially when coupled with the development of modern food technology, more and more types of foods increase the difficulty for consumers to identify food allergens (Simons et al. 2005). In order to prevent consumers from being able to identify food allergens and allergies after consumption, it is necessary to label the food allergens on the food packaging. Through food allergen labels, patient can be simplest and most effective to avoid preventing food allergies (Soares Weiser et al. 2014).

9.3 The Management of Food Allergen Labels in Developed Countries: Taking the EU as an Example

9.3.1 Management Organization of EU Food Allergen Labeling

The EU has a corresponding regulatory agency for food allergens, that is, the European Commission and the European Food Safety Authority (EFSA). EFSA aims to protect the interests of consumers, reflect the sensitization of consumers to the assessment report, and feed back to the European Commission. The European Commission will use this as a basis for legislation to better protect the health and safety of EU consumers. The European Commission is responsible for the legislation on allergen labeling in food. After the food allergen labels regulations were drafted, the subsidiary department of health and consumer protection was responsible for the promulgation and management of the regulations. They will supervise the implementation of food sensitization regulations in various regions and monitor the labeling of food allergens in the food chain (Gendel 2012).

EFSA is an independent organization from the European Commission that is primarily responsible for direct dialogue with consumers on food safety issues and for establishing a network of food hygiene and research institutions between member states. EFSA's affiliates related to food sensitization are special diet, nutrition, and allergy science groups that conduct scientific assessments of various allergens and submit the results to the European Commission as a basis for revision of relevant regulations (Wang et al. 2014b; Zou et al. 2016).

The European Commission and EFSA complement each other in managing food allergens. In 2003, the European Commission issued Directive 2003/89/EC. The most important change was the addition of Appendix IIIa to Directive 2000/13/EC, which lists the common 12 types of allergens and pointed out that “the dynamic and scientific management of Appendix IIIa will be implemented on the basis of science” (The European Parliament and of the Council 2003). In this regard, the European Commission encourages food producers and other scientific research institutions to conduct scientific research and submit applications for exempting certain food ingredient labeling regulations. At the same time, a special scientific group of dietary, nutritional, and allergy specialists was commissioned to conduct a comprehensive scientific assessment of these allergen components. The special diet, nutrition, and allergy professional science team reexamined 12 existing allergens and increased the sensitization evaluation of lupin and mollusks from 2004 to 2007. This led to the European Commission's five amendments to Directive 2000/13/EC, including the inclusion of lupins and mollusks in the list of allergens to be marked and the addition of material exemptions from mandatory labeling (Boyce et al. 2011; Fenton 2011; Simons et al. 2012).

9.3.2 The Formation Process of Food Allergen Labeling Regulations in EU

The label management of allergens in EU food is a part of food label management. Among which, the European Parliament and the Council of Europe amending Directive 2000/13/EC on the Uniform Food Labelling, Description and Advocacy of Member States is the basis for legislation on allergen labeling in food. Allergen-related regulations such as Directives 2003/89/EC, 2005/26/EC, and 2007/68/EC are modified based on the risk assessment of the Special Dietary, Nutritional and Allergy Science Panel on Directive 2000/13/EC.

Directive 2000/13/EC and its revised Directive are the only regulation in the EU that deals with the management of allergen labels in food before 2000 (Høst et al. 2000). But the directive 2000/13/EC does not regulate the identification of allergens in food (The_European_Parliament_and_the_Council 2000). In order to protect the safety of allergic patients, in 2003, the European Parliament and the Council issued Directive 2003/89/EC, for the first time, the sensitizing ingredients in food must be marked, and creatively added Appendix IIIa, which lists 12 categories of ingredients and substances that must be identified that may cause food allergies and intolerance.

Not all ingredients or products derived from food allergens are sensitizing. For example, some sensitizing ingredients may be modified during processing or undergo protein breakdown to lose sensitization (Gendel 2012). In this regard, the European Commission encourages food companies and research institutes to carry out relevant research to submit applications for exemption from labeling on certain food ingredients that do not cause adverse reactions in susceptible populations, and these applications were researched and demonstrated by the special dietary, nutrition, and allergy science team of EFSA. In 2004, EFSA conducted a scientific assessment of 27 applications for exemptions for 34 ingredients or products and finally gave a result of assessment, although whether it is possible or not possible to identify a product containing certain ingredients that may cause adverse reactions to sensitive individuals was not conclusive (Sakellariou et al. 2010; The_European_Parliament_and_of_the_Council 2005a). Therefore, the European Commission issued the Directive 2005/26/EC in 2005, and a list of temporary exemptions for food ingredients has been added to Appendix IIIa of Directive 2000/13/EC, and eight categories of material exemptions that are temporarily exempt from mandatory labeling are given (The_European_Parliament_and_of_the_Council 2005a).

On October 3, 2005, the European Commission issued Directive 2005/63/EC to increase the use of fish gelatin for the production of carotenoids as exempt substances, further enriching the list of temporary exemptions in Appendix IIIa of Directive 2000/13/EC (The_European_Parliament_and_of_the_Council 2005b). In the same year, EFSA issued the “Assessment Opinion on Lupin Labeling” and pointed out that lupin can directly cause allergic reactions and can have high-risk cross-contamination with peanuts (European_Food_Safety_Authority 2005). In 2006, EFSA proposed in the “Assessment of the Labeling of Molluscs” that the

allergen in mollusks is tropomyosin, which is consistent with the allergenic protein in crustaceans, so there is a phenomenon of cross-sensitization (European_Food_Safety_Authority 2007). In December of the same year, the European Commission issued Directive 2006/142/EC based on the above two assessments, adding lupins and mollusks to the list of Appendix IIIa of Directive 2000/13/EC (The_European_Parliament_and_of_the_Council 2006).

Since the Directive 2005/26/EC has a temporary exemption list, it is valid until November 25, 2007 (Sakellariou et al. 2010). In order to ensure the continuity of the regulations, the European Commission issued Directive 2007/68/EC and revised the Directive 2000/13/EC for the fifth time. The new sensitization list specifies the exemption provisions and provides for the permanent exemption of labeled labels for five categories of substances (The_European_Parliament_and_of_the_Council 2007).

At present, Directive 2000/13/EC (Revised 2007) is the latest and most complete food sensitization labeling regulation in the European Union. It is based on the scientific evaluation and the results of many revisions and integrations of the old regulations, which can protect the interests of consumers and meet the needs of relevant personnel in the food industry. The formation process of the EU food allergen labeling regulations is shown in Table 9.1.

In addition, in order to ensure the health and safety of people with gluten intolerance, the European Commission established Regulation (EC) No 41/2009 in 2009. This directive regulates the ingredients and labels of foods available to people with gluten intolerance. Only foods with the gluten content of less than 20 mg/kg are allowed to be labeled “gluten free” on their label, and foods with the gluten content of less than 100 mg/kg can be labeled “micro-gluten-containing” on the label (The_European_Parliament_and_of_the_Council 2009).

In summary, the labeling regulations for allergens in EU foods are in the process of continuous revision. Since 2003, the European Commission has revised the Food Allergen Marking Directive 2000/13/EC six times in the evaluation of EFSA. Since the newly revised Directive 2000/13/EC is primarily for prepackaged food, there are no provisions for bulk food. In order to further accommodate the requirements of the European Union Regulation (EC) No 1169/2011 “Food Information for Consumers,” the EU Directive 2000/13/EC (2007 revised edition) was revised in December 2014, and the revised content covers the labeling requirements for bulk foods (The_European_Parliament_and_of_the_Council 2011).

9.3.3 Identification Management of Food Allergens in EU

Labeling of food allergens in the EU regulates and divides the source of food allergens into two types: one is an allergen that is intentionally added, such as adding allergen ingredients as food ingredients, and the other is an allergen that is inadvertently brought in, which mainly refers to allergens brought in by cross-contamination (The_European_Parliament_and_of_the_Council 2011).

Table 9.1 Revision process of EU labeling regulations for allergic foods

Release date	Directive	Directive title	Revised contents
March 2000	Directive 2000/13/EC	Amending Directive 2000/13/EC of the European Parliament and of the Council on the approximation of the laws of the member states relating to the labeling, presentation, and advertising of foodstuffs	This regulation ensures that consumers can obtain all information about the product manufacturer, storage methods, etc. through the label. This regulation prohibits food labels from indicating that foods can treat and cure human diseases
November 2003	Directive 2003/89/EC	Amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs	For the first time, food labels must be labeled with all food ingredients that may cause consumer allergies; Appendix IIIa of the Directive is added to clearly list 12 categories of ingredients and substances that must be identified that may cause food allergies and intolerance
March 2005	Directive 2005/26/EC	Establishing a list of food ingredients or substances provisionally excluded from Annex IIIa of Directive 2000/13/EC of the European Parliament and of the Council	The Directive sets out a list of food ingredients or substances that are temporarily excluded from Annex IIIa of the European Parliament and Council Directive 2000/13/EC, including food ingredients in gluten-containing cereals, eggs, fish, soy, milk, nuts, celery, and mustard
December 2006	Directive 2006/142/EC	Amending Annex IIIa of Directive 2000/13/EC of the European Parliament and of the Council listing the ingredients which must under all circumstances appear on the labeling of foodstuffs	The revised Directive requires member states to permit the sale of food that complies with the Directive and prohibits the sale of food that violates the Directive. Add lupins and mollusks to the list in Appendix IIIa
November 2007	Directive 2007/68/EC	Amending Directive 2007/68/EC amending Annex IIIa to Directive 2000/13/EC of the European Parliament and of the Council as regards certain food ingredients	Revised Directive 2000/13/EC. Adjusted the food ingredients in Appendix IIIa that may cause allergies, reenacted the list of allergen ingredients, added exemption provisions, which stipulated that five types of substances can be permanently exempted from labeling

(continued)

Table 9.1 (continued)

Release date	Directive	Directive title	Revised contents
May 2008	Directive 2008/5/EC	Concerning the compulsory indication on the labeling of certain foodstuffs of particulars other than those provided for in Directive 2000/13/EC of the European Parliament and of the Council	The directive specifies additional food labels that need to be mandatory to better inform consumers about the properties of the food. Eight categories of foods that require mandatory additional identification are listed. The label of the food to which the packaging gas is added must be marked with “protective gas in the package.” The addition of the sweetener needs to be marked “sweetener,” and the addition of sugar and sweetener requires labeling “sugar and sweetener”
May 2009	Directive 2007/68/EC	Amending Annex IIIa to Directive 2000/13/EC of the European Parliament and of the Council as regards certain food ingredients	This Directive amends the food ingredients in Annex IIIa of 2000/13/EC. The revised food ingredients mainly include gluten-containing cereals, crustaceans and their products, eggs and their products, fish and their products, peanuts and their products, mustard, milk and dairy products, nuts and celery and its products, mustard and its products, sesame and its products, sulfur dioxide or sulfate at a concentration of less than 10 mg/kg or L, lupin and its products, and mollusks and their products. The above food ingredients are highly susceptible to adverse reactions in certain populations and must be labeled on food packaging

9.3.3.1 Identification Management of Allergens in Food Ingredients

The labeling of allergens that are intentionally added to food as food ingredients is mainly based on the latest revision of Directive 2000/13/EC. The directive was repeatedly revised and improved under the joint promotion of EFSA, scientific research institutions, and food companies. The types of sensitizing substances to be marked, the types of exempted substances, and the methods of labeling were separately specified.

The EU requires mandatory labeling of food allergens in the original 14 categories, including (1) gluten-containing cereals (wheat, rye, barley, oats, spelt wheat, cammet buckwheat, or hybrids thereof) and products thereof; (2) crustaceans and their products; (3) eggs and their products; (4) fish and their products; (5) peanuts and their products; (6) soybeans and their products; (7) milk and its products

(including lactose); (8) nuts, namely, almonds, hazelnuts, walnuts, cashews, pecans, Brazil nuts, pistachio fruits, macadamia nuts, and Queensland nuts and their products; (9) celery and its products; (10) mustard and its products; (11) sesame and its products; (12) concentration (in terms of sulfur dioxide) of more than 10 mg/kg or 10 mg/mL of sulfur dioxide or sulfite; (13) lupin and its products; and (14) mollusks and their products.

Due to the low dosage of certain allergens and the weak sensitization, according to the assessment of the EFSA group, the European Commission has identified five categories of sensitizing substances that have been partially exempted from the label, as shown in Table 9.2.

The European Commission has a uniform regulation on the way in which food allergens are intentionally added. The following two methods are recommended: (1) Mark “Include xx allergens” after following the ingredients. (2) The name of the allergen species is clearly mentioned after the ingredient list.

9.3.3.2 Management of Food Allergens Caused by Cross-Contamination

Pretty low level of allergens can also cause severe allergic reactions, and the EU places great emphasis on the management of cross-contamination. In the actual production process, food A and food B may share a production line due to cost and other factors. If one of A or B is a food allergen, it is easy to remain in the

Table 9.2 List of the foods exempted from allergen labeling

Serial number	Sensitized species	A substance that is partially exempt
1	Gluten-containing cereals (wheat, rye, barley, oats, spelt, cammet or its hybrids) and their products	(1) Wheat-based glucose syrup, including dextrose. (2) Wheat-based maltodextrin. (3) Barley-based glucose syrup. (4) Cereals used to produce distilled or edible alcohol
2	Fish and its products	(1) Fish gelatin used as a carrier for vitamins or carotenoids. (2) Fish gelatin or gelatin for use as a clarifying agent in beer or wine
3	Soybean and its products	(1) Refined soybean oil. (2) Natural VE (E306), natural D- α -VE, natural D- α -VE ester, natural D- α -VE succinate extracted from soybean. (3) Vegetable oil derived from plant sterols and phytosterols extracted from soybean. (4) Plant stanol ester produced from camphorol sterol extracted from soybean
4	Milk and its products (including lactose)	(1) Whey for the production of distilled or edible alcohol. (2) Lactitol
5	Nuts, such as hazelnuts, walnuts, cashews, pecans, Brazil nuts, pistachio fruits, macadamia nuts and Queensland nuts, and their products	Nuts used to produce distilled or edible alcohol

production line, and it is difficult to completely eliminate it by simple procedures such as washing the production line. EU management of cross-contaminated food allergens includes the development of regulations to reduce allergen residues and label management to alert consumers.

As early as 1994, the European Union promulgated the “Special Requirements for the Production and Control of Allergen-Containing Food” to regulate the production process of allergen-containing food in the production line. Its member states have also introduced corresponding production control guidelines to reduce the impact of cross-contamination. In addition, in order to regulate the problem of allergen labeling caused by cross-contamination, Finland issued the “Guidelines for Allergen Warning Labels in Foods Affected by Cross-Contamination” in 2005 and creatively proposed a recommendation statement on allergens. Subsequently, the United Kingdom also issued the “Allergen Management and Consumer Information Guide” to guide consumers to pay attention to allergen information caused by cross-contamination. Table 9.3 listed the regulations on cross-contamination of food allergens in the EU and member states (Crevel and Cochrane 2014; Wei et al. 2011).

Table 9.3 Cross-contamination regulations for food allergens in the EU and its member countries

Date	Enacting institution	Name of laws and regulations	Content
1994	The Health and Consumer Protection Directorate General of the EU	Special requirements for the production and control of allergen-containing foods	It is pointed out that the control of allergens in production should include raw material control, production process control, postproduction products and label control, etc. and indicate how to control
2004	Food Safety Authority of Ireland (FSAI)	Draft guide to prevent cross-contamination of allergens	Describes areas where food production and gluten-free food handling are prone to cross-contamination, and details of preventive measures to eliminate or reduce allergen cross-contamination in gluten-free foods
2005	Finland food and beverage industry	Guidelines for allergen warning signs in foods affected by cross-contamination	Suggested statement about the allergen and flowchart of the production guide
2005	Food Association in France (SOPEXA)	Good practice guidelines for reducing the presence of accidental major allergens	Explain how the food industry should deal with allergen management issues in production
2006	British Food Standards Agency (FSA)	Allergen Management and Consumer Information Guide	Explains how to manage food allergens (especially caused by cross-contamination) and use appropriate recommended identifiers
2008	European Medicines Agency (EMA)	Guide to production and quality control of allergen-containing products	It is recommended to recommend allergen-containing foods to control raw materials, production, postproduction products, sealed packaging, etc. in the production process

The types of food allergens that need to be identified due to cross-contamination and the types of exempted substances are subject to the Directive 2000/13/EC (revised version).

However, there is no uniform format for the EU, and each member country has a different form of labeling. The recommended methods of identification in the United Kingdom in the Allergen Management and Consumer Information Guide are as follows: (1) may contain xx allergens, (2) people who are not suitable for allergies to xx allergens, and (3) the production line of xx food is also used to produce xx allergens. No matter which kind of identification method, it has played a warning role for consumers and more powerfully guaranteed the food safety of consumers (Gojkovic et al. 2015).

9.3.3.3 Characteristic of Food Allergen Labels in EU

The EU's management of food allergen labels was characterized by a comprehensive food allergen labeling requirement based on the consideration of different eating habits in different member states, and some food allergens that most people tolerate were included in the pardon list. According to the data, food allergies are frequent in Europe; controlling the number of people and frequency of food allergy incident and cutting healthcare costs have become the ultimate goal of preventing food allergy in the EU. Since the beginning of 2000, the European Union has been paying attention to the labeling of food allergens, constantly updating the types of food allergens that need to be labeled, combining theory with practice, guiding practice with theory, and testing theory with practice. The European Commission's responsibility to update food allergen labels is included in the European Directive, which not only provides a basis for the updating of food allergen labels but also clarifies the European Commission's responsibility in food allergy prevention. To sum up, the requirements for food allergen labels in the EU were relatively strict, and policy support for food manufacturers labeling allergens on food packaging was provided.

9.4 Management of Food Allergen Labels in Other Countries and Regions

Different countries have different geography, culture, and eating habits. These differences have led to the differences in the sensitivity of people in different countries to different foods. These differences lead to the specific characteristics for the management of food allergies in different countries.

9.4.1 Regulation and Management of Food Allergen Labels in the United States

The food allergy situation in the US population published by Web MD (the largest medical health service website in the United States) is that the population of Americans suffering from food allergies is as high as 15%. In addition, a child with an allergic parent may have a 33% chance of developing an allergy, and a child with two allergic parents may have a 70% chance of developing an allergy. The number of emergency visits per year due to food allergies in the United States is 30,000 (Alldrick 2011; Baldus et al. 2008; Röder et al. 2010; Ross and Jackson 2013). These data provide important support for US food allergies management.

The Food and Drug Administration (FDA) issued the Food Allergen Labeling and Consumer Protection Act of 2004 on August 2, 2004, and the act regulates food labeling and related issues for food containing allergens. On October 5, 2005, several major food allergens were released and the contents of the list were explained. On June 18, 2015, the final guidance document was issued to help food manufacturers apply for the allergen exemption mark. If the requirements of the Act are violated, the food company and its managers who violate the regulations will be subject to either civil or criminal sanctions or even both, and the foods that do not meet the requirements will be detained. In addition, the FDA will require the food manufacturer to recall the product containing undeclared allergens.

This act is the first time that FDA has labeled food allergens in the form of legislation, making it easier for people to understand certain allergens in food. According to the analysis of known food allergy events, the reasons for the introduction of this bill in the United States are as follows: (1) 90% of the patients with allergic symptoms are caused by eating the eight major allergic foods, and there is no effective treatment for food allergies; (2) cases of food allergy increase year by year, and a group of people need emergency treatment or even died due to food allergies, and food allergy patients can only protect themselves by avoiding eating allergens; (3) the number of food incidents recalled due to disqualification of food allergen labeling has increased; (4) children with food allergies cannot accurately identify whether an allergen is present in the food or not.

The FDA has continuously explained and amended the contents of the bill. It can be seen that the US FDA was closely watched and deeply understood the food allergen labels based on reality. When food material may contain certain ingredients that cause allergic reactions and this ingredient will pose a threat to allergic people, the FDA will issue guidance documents in a timely manner to publish the situation.

In addition to this bill, the United States also has the provision of the *Codex Alimentarius*, the current good manufacturing practices, etc., which have different levels of regulation on food allergen labels, thereby forming a relatively complete institutional system. Therefore, food enterprises should produce according to regulations, and the government could play a regulatory role in the implementation of the system.

In the United States, FDA has established a national food allergen labeling regulation based on a large amount of relevant data on the premise of clearly understanding types of food that have easily caused allergic reactions in people. Confirmed by statistics of clinical history, American is more prone to food allergies, which is the reason why the US FDA imposes such detailed regulations on food allergen labeling and severe penalties for violations. However, currently the FDA only requires food manufacturers to mark eight major types of food allergens and their products. Therefore, some people think that the current policy only considers the majority of food allergens will produce adverse effects on the prevention of food allergies.

9.4.2 Regulation and Management of Food Allergen Labels in Japan

By integrating the contents of food labels in the Japanese Agriculture Standard, the Food Sanitation Law, and the Health Promotion Law, Japan has implemented the new Food Labeling Law from April 1, 2015, which stipulates the overall content of food labels. Moreover, the Cabinet Office Decree, “Food Labeling Standards,” promulgated on March 20, 2015, defines specific food labeling rules (Wei et al. 2011), and the food labeling content and Food Labeling Standards relate questions and answers issued by the Consumer Affairs Department supplement and explain the food labeling benchmark.

In the “Market on Allergic Substances in Foods” issued by the Consumers’ Office (Hall 2013), there are seven specific types of raw materials that cause obvious food allergy symptoms, high incidence of disease, and high cadmium: crab, wheat, buckwheat, eggs, milk, nut, and peanuts. In addition, 20 kinds of food were encouraged to be labeled, including abalone, squid, salmon roe, etc., among which sesame and cashew are first appeared in the list in 2014, and the scope and precautions of specific raw materials are explained in more detail in the annex. Determination of the list of allergens not only helps the food enterprises to implement the requirements of the food allergen label as required but also helps consumers to understand the regulations and ensure the smooth implementation of the regulations. In addition, the website of the Japan Consumers’ Office will publish the list of enterprises that violate the food allergen labeling regulations within 1 month. The publication includes the specific content of which company has missed what kind of labels and whether the specific raw materials are marked according to the regulations. The person in charge of the food enterprise that violates the regulations shall be subject to criminal or administrative penalties.

Most countries simply encourage consumers to play a role in food safety supervision without clarifying the role of consumers in food safety supervision. However, Japan’s food safety laws promote consumer participation and clarify consumer responsibilities (Wang et al. 2014a). Food safety is not only the responsibility of the state but also the responsibility of local public bodies, food-related practitioners,

and consumers; consumers can play a role different from the government and food companies in the supervision and thus report food safety issues by continuously deepening their understanding of food safety. Consumers should learn relevant food safety knowledge, deepen their understanding of food, and constantly strengthen their sense of ownership. Such regulations make consumers conducive to timely detection of food safety issues.

The provision of the Japanese food allergen label is formulated on the premise of reference to international standards and national conditions and therefore has certain forward-looking nature. The types or contents of food allergens that are mandatory and encouraged to be marked will be more perfect with social development. For example, the label of sesame and cashew nuts was encouraged by the Japanese Food and Health Law. They were not included in the food allergen requirements prior to this and were later included in the scope of encouraging labeling.

9.4.3 Regulation and Management of Food Allergen Labels in Australia and New Zealand

Australia and New Zealand's Codex Alimentarius Standard 1.2.3 provides for the specific description of food allergen labels. The content of standard mainly involves several types of common food materials, including grains, fish, milk solids, nuts, sulfites, cereals (wheat, barley, rye, oats, etc. and their products), crustaceans (and their products), and nuts (and their products). Even if the content of these food materials is low in food, the regulations clearly require manufacturers to specify content and categories of these food materials in detail. When the manufacturer was unable to determine if certain food ingredients in the product will cause an allergic reaction or the product is contaminated with certain allergenic ingredients from other ways, the manufacturer can voluntarily mark "may contain" (Silas et al. 2014).

The Australian government is concerned about the labeling of food allergens, and every year food is recalled for not indicated food allergens. There were 603 times of recalled food incidents between 2006 and 2015, of which 192 times were recalled because the manufacturer does not have a correct or clear label of food allergens, accounting for 31.84% of the total recalled food. As shown in Fig. 9.1, food allergens such as dairy products, soybeans, milk, gluten, eggs, tree nuts, nuts, almonds, sesame seeds, wheat, sulfates, and sulfur dioxide are all listed as reasons for recalled foods because the manufacturer does not clearly indicate the presence of these ingredients. In addition, nuts, dairy products, and wheat/gluten have become the most frequent type of recalled foods in Australia (Food_Standards_Australia_and_New_Zealand 2016). It can be seen that the Australian government's supervision of food allergens is especially detailed and stricter.

FSANZ also regulates the method of food allergen labels, which demands that food manufacturers, under the supervision of the government and consumers, have to standardize the labeling of food allergens. In addition, consumers can have a

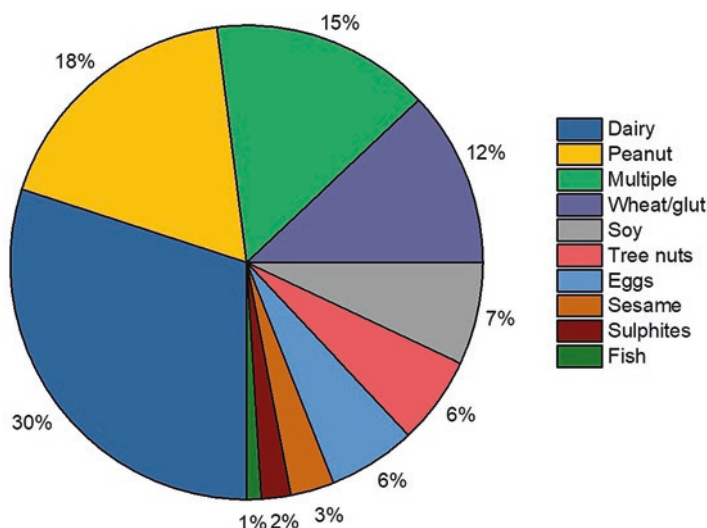


Fig. 9.1 Australia and New Zealand recalled unspecified proportion of food allergens between 2006 and 2015. Major types of food allergens include dairy, peanut, multiple, wheat, soy, nut, egg, sesame, sulfite, and fish

clearer understanding of food allergy through the government that provides more detailed and systematic publicity knowledge of food allergy, which provides a good guarantee for Australian to avoid foods and their products containing allergens.

The biggest feature of Australia and New Zealand food safety management is the food recall system. The food safety regulations on the management of food allergens were issued by the FSANZ, which stipulate that all food containing the specified food allergens, regardless of the proportion, must be labeled. Non-compliant foods and their products will be forcibly recalled upon discovery or report. It can be seen that Australia's management of food allergen labels has distinct feature from standard setting to recall system establishment.

9.4.4 Regulation and Management of Food Allergen Labels in Korea

In order to enable consumers to better understand the food information conveyed by food labels and promote the rapid development of the food industry, on April 8, 2015, the No. 2015-20 notice issued by the Korea Food and Drug Safety Agency revised the standard of food allergen labels. In the notice, not only the type of food material that required food allergen labeling was increased, but also the requirements for labeling methods were improved. Up to the present, in Korea, the categories of food material that needs to be marked as food allergen contain 18 types,

including egg, buckwheat, nut, crab, shrimp, sesame, pork, apple, peach, tomato, milk, sulfuric acid, sulfur dioxide, and newly added categories walnut, chicken, beef, jellyfish, and shellfish. The increase in the category of food allergens indicates that with the continuous development of food science and technology, more foods are recognized as allergens. Moreover, the types of food allergens in Korea have distinctive regional characteristics and are significantly different from those in Europe and the United States.

Different from other countries, in Korea, food allergen labels must be listed separately with clear font color (distinguished from the color of the food packaging). The amendment will be implemented from the date of publication, foods that have not yet been produced must be marked with food allergens as required, and foods that have already been produced are required to be fully labeled with food allergens before December 31, 2016 (Korea_Food_and_Drug_Safety_Agency 2015).

It can be seen that Korea was attaching importance to the prevention of food allergies, and all food materials that may cause food allergies to consumers are marked. In this way, consumers can understand the food allergens contained in the food by reading the food allergen label, so as to effectively avoid the intake of allergic food.

In addition, the Korea Food and Drug Safety Agency has revised the contents of food labeling standards several times a year. Besides, the government has set up some interactive units on the website of the Food and Drug Safety Agency, such as publicizing food information, setting up consumer forums, producing media publicity materials, setting up a reporting center, encouraging online policy participation, and providing channel to consumers to provide advice for government decision-making and report wrongdoing. Compared with other countries, Korean government frequently interacts with consumers by media, so they can listen to the public opinion and carry out reforms in a timely manner. As the carrier of information, the media has incomparable advantages in the dissemination of food information, enabling consumers to gain food knowledge by eyes and ears and work together to create a better food environment.

The latest regulations on the labeling of food allergens in Korea demand the food allergen labels to be clearly listed separately. In this way, it is convenient for consumers to understand the food allergen components, thereby achieving the purpose of effectively preventing from food allergy. Compared with other countries and regions, the types of food allergens that required to be labeled show a noticeable difference in Korean, such as the inclusion of tomatoes and peaches. After the introduction of the new food allergen label list, the government will give the relevant companies sufficient time to rectify the food allergen labeling in the packaging.

The Korean government brought a lot of convenience to people with food allergies by improved allergen laws and regulations and also provided food allergen information for consumers who do not know what kind of food they are allergic to. After the implementation of the new food allergen labeling regulations, South Korea has the largest number of food allergens that should be mandatory labeled.

9.5 Food Allergen Label Management System and Prospects: Taking China as an Example

9.5.1 *Development of Chinese Allergen Label Management*

At present, as an emerging force of third world countries, China has recognized the necessity of the management of food allergen labels, though the relevant system is still in its infancy, and the corresponding regulations and standards still need to be continuously established and improved. The earliest management standard related to food allergen labeling in China was the “Marketing of Food Safety Food Allergens in the Olympic Games” issued during the 2008 Beijing Olympic Games. The target audience of the standard is mainly the foreign consumers. The standard listed 12 kinds of food that are easy to cause food allergies and recommended labeling methods for food manufacturer. However, the standard was abolished after the end of the Olympic Games (Beijing_Municipal_Bureau_of_Quality_and_Technical_Supervision 2008; Li et al. 2009). In 2009, during the Guangzhou Asian Games, the “Asian Games Food Safety Food Allergen Labeling” was issued, which is basically similar to the content of the “Olympic Food Safety Food Allergen Labeling.” The standard is also automatically abolished after the Asian Games (Guangzhou_Quality_and_Technical_Supervision_Bureau 2009). In order to strengthen the management of food allergens, China issued the recommended national standard GB/T23779-2009 “Allergenic ingredients in prepackaged food.” The standard defines the concept of food allergens and lists eight kinds of food allergens that recommended to be labeled (China_National_Standardization_Administration 2009). In 2012, the national standard GB7718-2011, “General Rules for the Labeling of Food Safety National Prepackaged Foods,” was formally issued. The national standard officially included the food allergens into the scope of label management for the first time and recommended food manufacturers to label the eight kinds of food allergens listed in GB/T23779-2009. However, no specific regulations have been given for the identification and quantification of food allergens (Mohc_the_Minister_of_Health_of_the_People's_Republic_of_China 2011). In order to improve the country’s supervision of food safety, China has established a national food safety risk assessment expert committee in 2009 and a national food safety risk assessment center in 2011. But China’s risk assessment for food allergens is still required to be improved. At present, food risk assessment is carried out mainly on foodborne microorganisms, residues of agricultural and veterinary drugs, heavy metals and other pollutants, as well as illegal additives, but there is almost no risk assessment for food allergens (National_Center_for_Food_Safety_Risk_Assessment 2013).

9.5.2 The Enlightenment of National Food Allergen Label System to China

9.5.2.1 Improvement of Existing Sensitization Standards

There are about 160 types of currently known food allergens, and 90% of the food hypersensitivity incidents are caused by eggs, milk, fish, crustaceans, peanuts, soybeans, stone fruits, and wheat (Gendel et al. 2013; Villalba and Ruzicka 2014). These kinds of allergens were listed in GB7718-2011 “Food Safety National Standards for Prepackaged Food Labels” and called the “big eight” categories of food allergies. Although GB7718-2011 specifies the types of food allergens that should be marked, the rule is still nonmandatory, and the national standard was not given a specific guidance for labeling method. Therefore, China should introduce relevant standards as soon as possible to complete the method of food allergen labels. Certainly, the food sensitization situation in each country varies depending on regional differences and lifestyle habits. Hence, the dietary habits of residents should be considered in the establishment of the mandatory, recommended, and pardon list of food allergens.

9.5.2.2 Establishment of the Risk Assessment System

The EU currently has 14 types of allergens in the most common list of allergens. In addition, every revision of the European Commission’s list of allergens is based on scientific research by the Special Diet, Nutrition and Allergy Science Group. Similarly, China should give full play to the role of the Expert Committee on Food Safety Risk Assessment and train an expert group on allergen evaluation, nutrition, and allergy scientific team to evaluate food allergens (Grunert et al. 2010; Guthman 2010; Rothman et al. 2006; Vierk et al. 2007).

In addition, with the development of China’s economy, the dietary structure of consumers will also be changed, such as the increasing intake of meat foods, the increasing use of food additives, and the increasing frequency of genetically modified (GM) foods intake. Therefore, China should actively carry out relevant research works to conduct risk assessment on potential allergens and submit evaluation reports as the basis for revision of the regulations, so as to make the regulations fit the latest situation.

9.5.2.3 Strengthening Cross-Contamination Management

In 1994, the European Union Directorate for Health and Consumption promulgated the “Special Requirements for the Production and Control of Allergen-Containing Foods,” which included the regulation of raw materials, production processes,

products, and labels (Baldus et al. 2008). As a reference, China should also focus on controlling allergens from the source of production to reduce the residues of allergens in the production process. At present, China's food companies generally adopt HACCP, good manufacturing practice (GMP), sanitation standard operation procedure (SSOP), etc. as the standard for production operations, but there is no specific management norm for handling the cross-contamination of allergens, resulting in great difficulties in controlling allergen cross-contamination. Consequently, China should introduce relevant laws and regulations as soon as possible to make up for the gaps and so as to reduce the risk of sensitization caused by cross-contamination (Röder et al. 2010). On the other hand, the quantitative analysis and label method of food allergens introduced by cross-contamination are significantly different in various countries and absent in China; hence, the standard in line with China's national conditions should be regulated (Xie et al. 2011).

It should be noticed that the deliberate words should be used to label the cross-contamination allergens. The United Kingdom thinks that "may contain" was inaccurate and "may exist" should be used, while Canada believes that "may contain" are meaningless, and Chinese consumers are also less able to accept the label of "may contain." Article 48 of China's Food Safety Law was requiring that labels and instructions for food and food additives should be clear, obvious, and easily identifiable. In August 2011, the words "may contain" marked on the products sold by Unilever in China were questioned in the violation of that law. In this regard, China can improve relevant standards to perfect the recommended labels of cross-contamination allergens. Moreover, after using the standard for a period of time, the use of these recommended labels and the opinions of consumers on this label are investigated, and the feedback obtained can be used as the basis for the revision of the standard (Falci et al. 2001; Koeberl et al. 2017).

9.5.2.4 Strengthening Allergen Knowledge Education

At present, Chinese consumers do not know much about the types, management regulations, and identification methods of allergens in food; consequently, relevant government in China should improve consumer knowledge on food allergy by education. By this measure, it can not only enhance consumers' self-protection ability and ensure that consumers can avoid eating allergens by reading food labels but also helps to improve the effective interaction between consumers and relevant government departments. By understanding the laws, regulations, and standards of food allergy, the consumers can actively and effectively feedback relevant opinions, thus helping government departments to amend laws, regulations, and standards.

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