

Chapter 13

Strategies for the Modification and Evaluation of Allergenicity

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Abstract Allergic disease is a global public health problem with increasing attention being paid to the evaluation and modification of allergenicity. The major aim of this chapter is to provide an overview of the field of allergenicity evaluation and modification focusing on bioinformatics evaluation, in vitro evaluation, and in vivo evaluation, which are the most commonly used methods for allergenicity evaluation, followed by information on allergenicity modification, which is required to reduce the allergenicity of the allergen proteins. Site-directed mutagenesis and error-prone PCR, which are used to modify allergenicity, are elucidated and summarized in this chapter. The field of allergenicity evaluation and modification is expected to lay the foundation for the future safe application of allergen reagents.

Keywords Allergenicity · Evaluation · Site-directed mutagenesis · Animal model · Histamine

13.1 Introduction

With advances in society and changes in dietary habits, the incidence of allergic diseases has been continually increasing. Epidemiological surveys show that 20–30 % of the global population is affected by a variety of allergic diseases to

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different degrees (Furuta et al. 2005). Allergic diseases are chronic diseases caused by a wide range of factors including foods, dust mites, pollens, drugs, etc. (Geier et al. 2011; Scurlock and Burks 2004). Most allergic diseases are induced by closely related proteins or protein components. Therefore, bioinformatics and molecular biology methods used to evaluate the allergenicity of proteins and to reduce or even eliminate their allergenicity by site-directed mutagenesis have important practical significance and application value. The following are areas of particular importance for human health as it relates to allergic diseases:

1. Reducing the incidence of allergic disease

Evaluation of the allergenicity of proteins can help the allergic patient to reduce or completely avoid exposure to the sensitizing allergens and, thus, reduce the triggering of allergic diseases (Marini et al. 1996). Our meta-analysis demonstrated that allergen avoidance for newborns did not reduce the subsequent incidence of allergic diseases but did significantly reduce the incidence of asthma and wheezing in high-risk infants. However, previously sensitized patients who had reduced their exposure to known allergens did not subsequently show improvement in their lung functions. Hence, we argued that allergen avoidance may not always be successful in preventing allergic reactions (Wu et al. 2014).

2. Improving the safety and efficacy of allergen-specific immunotherapy

Allergen-specific immunotherapy is not only a very effective treatment in reducing allergic symptoms to a particular allergen (Bublin et al. 2010), but also provides a unique opportunity to prevent the development of allergic reactions to other potential allergens in the long-term course of the disease (Mobs et al. 2010). Traditional immunotherapeutic reagents are often crude extracts with an unknown composition. Because the use of this type of reagent could induce severe side effects and even life-threatening anaphylaxis due to their complex and unknown components, hypoallergenic allergen derivatives with reduced allergenicity have been recombinantly engineered to reduce side effects during allergen-specific immunotherapy (SIT).

3. Increasing the safety and efficacy of drugs in clinical applications

By evaluating and modifying the allergenicity of protein drugs (such as immunotoxins, etc.) early in their development, increases in the safety and security of the drug can be made well before any use in clinical applications, thus reducing the number of adverse effects and risks associated with drug development (Dean et al. 1994; Galbiati et al. 2010).

4. Promoting the safety of Genetically Modified (GM) food

In the field of agriculture, improvements to food crops focus on certain excellent agronomic traits, e.g., disease resistance, insect resistance, stress resistance, and improvement of the food quality. Some as yet unidentified transforming genes could be similar to allergen genes and the products of these transgenes could eventually enter into the human world or be consumed as food, so the safety of

transgenes is a growing public concern (Goodman and Tetteh 2011; Kuiper et al. 2001). In 1996, with the awareness that the 2S gene can increase protein content, the agriculture company Pioneer Hi Bred transformed the 2S gene into soybeans. However, immunological identification results showed that the 2S protein is an important allergen and that this transgenic soybean would likely cause allergic reactions (Nordlee et al. 1996; Saalbach et al. 1994) and, thus, this transgenic soybean or its products cannot be publicly sold for consumption. Therefore, evaluation and modification of the allergenicity of all transgenic genes in the GM process not only can avoid the potential allergenic problem, but also can improve the safety of GM foods.

The strategy for assessing protein allergenicity and the subsequent modification of protein allergenicity as discussed in this chapter is as follows: first, evaluate the allergenicity of the proteins using bioinformatics and/or experimental methods; second, predict the allergic epitopes and the modification sites through bioinformatics methods and obtain the mutant sequences that maintain the dimensional structure and biological activity but reduce the allergenicity; third, modify the allergen proteins through site-directed mutagenesis and re-evaluate their allergenicity.

13.2 Evaluation of Protein Allergenicity

Allergic disease is a global public health problem, with allergen detection and evaluation increasingly receiving much attention (Pawankar 2014). The allergenicity evaluation of the candidate proteins can be achieved by sequence alignment with allergen (Chrysostomou and Seker 2014; Kimber et al. 2003; Thomas et al. 2005; van Esch et al. 2011). Sequence alignment, using computer technology and allergen databases, identifies and compares the sequence identities and positivities between the target protein and the allergen, and then predicts the potential allergenicity of the protein (Gibson 2006). In vitro evaluation methods include enzyme-linked immunosorbent assay (ELISA) and histamine provocation tests (Thomas et al. 2004). In vivo evaluation methods include challenge tests in animal models (Aldemir et al. 2009; Ladics et al. 2010). Although there are many methods to evaluate protein allergenicity, there is not yet just one specific method that can effectively evaluate protein allergenicity. Therefore, development of a combination of evaluation methods is necessary to establish a fast, efficient, and accurate allergenicity evaluation procedure.

13.2.1 Bioinformatics Methods for Evaluation of Allergenicity

With the rapid increase in the identification of allergen protein sequences and related data, the application of bioinformatics to the field of allergy is becoming

more and more important (Brusic and Petrovsky 2003). Using bioinformatics methods, evaluation of the allergenicity of the target proteins can be quickly performed through identity predicting and cross-reactivity predicting (Aalberse 2007; Schein et al. 2007). This section will give a synopsis of the use of bioinformatics in allergenicity evaluation.

Allergen proteins always share some common characteristics, such as digestion resistance, resistance to acid/alkali, thermal stability, and certain specific amino acid sequences (Brusic and Petrovsky 2003). Allergen proteins have conserved structures and functions and, therefore, analysis of sequence similarities between target proteins and known allergens could be an efficient method for preliminary evaluation of the allergenicity of proteins.

The most commonly used protein allergen databases are the International Union of Immunological Societies (IUIS) (King et al. 1994), Allergome (Mari and Scala 2006), Allergenica (<http://allergenica.gzhmu.edu.cn>), the Structural Database of Allergenic Proteins (SDAP) (Hileman et al. 2002; Ivanciuc et al. 2003), and Allergenonline (Richard et al. 2007). These databases use a local alignment strategy and are the commonly used sequence comparing methods (Do and Katoh 2008; Mount 2007). BLAST, with a fast search speed, is more effectively used on searches of large databases. FASTA searches are more sensitive to local matches, and therefore, FASTA is the preferred method to use when evaluating protein allergenicity.

In recent years, in combination with artificial neural network algorithms, a variety of software for allergen identification have been developed based on FASTA and BLAST sequence alignment. FAO/WHO, EVALLER, AlgPred (amino acid), AlgPred (dipeptide), AlgPred (ARPs BLAST), AllerHunter, and SORTALLER are the mostly common used allergen discrimination software (Martinez Barrio et al. 2007; Muh et al. 2009; Saha and Raghava 2006; Zhang et al. 2012). The sensitivity and specificity of these allergen discrimination softwares are shown in Table 13.1. The FAO/WHO is a method recommended by the UN Food and

Table 13.1 Comparison of different prediction methods

Methods	SE (%)	SP (%)	ACC (%)	MCC
FAO/WHO ^a	99.2	9.6	54.4	0.198
EVALLER ^b	86.6	99.0	92.8	0.863
AlgPred (amino acid) ^c	92.4	80.2	86.3	0.731
AlgPred (dipeptide) ^c	88.8	88.2	88.5	0.770
AlgPred (ARPs BLAST) ^c	81.8	98.0	89.9	0.809
AllerHunter ^d	82.2	99.2	90.7	0.826
SORTALLER ^e	98.6	98.4	98.5	0.970

Note: *SE* sensitivity; *SP* specificity; *ACC* accuracy; *MCC* Matthews correlation coefficients

^aMaking use of the script file in accordance with the FAO/WHO guidelines (2001)

^b<http://www.slv.se/en-gb/Group1/Food-Safety/e-Testing-of-protein-allergenicity/e-Test-allergenicity/>

^cwww.imtech.res.in/raghava/algpred/

^d<http://tiger.dbs.nus.edu.sg/AllerHunter/>

^e<http://sortaller.gzhmu.edu.cn/>

Agriculture Organization and the World Health Organization. This method has two criteria for identifying potential protein allergens: (1) the presence of at least six contiguous amino acids identical to a known allergen or (2) more than 35 % identity in the amino acid sequence of the expressed protein, using a window of 80 amino acids and a suitable gap penalty (using Clustal alignment programs or equivalent alignment programs) (Hviid 2007; Ladics 2008). Based on prior allergen identifying software, our lab has developed a new algorithm and bioinformatics evaluation software called SORTALLER (Zhang et al. 2012). SORTALLER uses a neural network algorithm with the advantages of having a high running speed and high accuracy as well as being more convenient, since multiple sequences can be evaluated at the same time. The sensitivity and specificity of SORTALLER are both more than 98 %, and it has now been linked to the Internet (<http://sortaller.gzhmu.edu.cn>) for public use. Regarding the specific principles, evaluating criterion, and application of these bioinformatics evaluating methods, please refer to Chap. 14 in this book.

13.2.2 Laboratory Methods for the Evaluation of Allergenicity

Although protein allergenicity can be quickly evaluated by bioinformatics methods, the accuracy of bioinformatics assessments depends on the allergen protein database and the corresponding algorithm and is affected by relatively high rates of false positives and false negatives (Thomas et al. 2005). In addition, current sequence similarity analyses mainly focus on comparing the primary amino acid sequence and predictions using conformational epitopes are relatively unreliable. Therefore, further evaluation of allergenicity using laboratory methods should also be done to obtain more accurate results.

Laboratory allergenicity evaluations are divided into serological and cytological methods (Thomas et al. 2009). Serological methods are mainly based on the binding capacity of the protein with specific serum immunoglobulin (IgE, IgG, etc.), while cytological methods depend on the production of inflammatory mediators after stimulation of the immunocyte with the protein (Thomas et al. 2009). In addition, these laboratory assessments are divided into in vitro evaluation and in vivo tests (Thomas et al. 2009). In vitro assessments include ELISA, mast cell and whole blood stimulation tests, etc. In vivo methods use the allergenic proteins in animal models or to directly stimulate the human body and then evaluate the allergenicity of the protein based on the presence or absence of allergic symptoms and/or the levels of specific IgE. In vivo allergenicity assessments include skin tests, provocation tests, and animal models. Provocation tests and skin tests use a small amount of allergen protein applied directly to humans followed by observation of symptoms to assess the potential allergenicity of the target protein. Animal models have been developed to establish a corresponding model for assessing the potential allergenicity of the target protein and to evaluate the allergenicity of the proteins

based on symptoms and production of antibody and cytokines. Rats, mice, and guinea pigs are the commonly used animals because they are similar to humans in their immune responding mechanisms. Furthermore, they have advantages such as that they are small and inexpensive and have short breeding cycles.

1. IgE binding assays for the evaluation of allergenicity

IgE is one of five immunoglobulin types in human serum and its concentration is normally very low, about 10–100 U/mL; however, patients with allergic or parasitic diseases have significantly higher levels. In 1966, Johansson (Sweden) and Ishizakas (Japan) first isolated IgE from ragweed allergic patients' serum and demonstrated the relationship between IgE and allergic diseases. IgE is mainly synthesized by B cells located in the mucosa-associated lymphatic tissue of respiratory and gastrointestinal tracts and is regulated by the levels of T_H1/T_H2 cytokines *in vivo*. It is widely accepted that an imbalance between T_H1/T_H2 cells leads to the development of allergic diseases. T_H2 cells synthesize high levels of interleukin 4 (IL-4), IL-5, and IL-13, which promote B cells to release allergen-specific IgE (Jeon et al. 2015). Detection of allergen-specific IgE in biological fluid is one of the methods of evaluation of proteins before and after modification. Common methods for detection of allergen-specific IgE are described below:

(a) Enzyme-Linked Immunosorbent Assay (ELISA)

Purified allergen is coated onto high binding plates to capture allergen-specific IgE in biological fluids. Among the most commonly used methods are the sandwich ELISA, capture ELISA, and competition ELISA. A sandwich ELISA measures allergens between two layers of antibodies (captured and detection antibodies). The allergen to be tested must contain at least two different antigenic sites capable of binding antibodies. The advantage of the sandwich ELISA is that the sample does not have to be purified before analysis, and the assay can be very sensitive (up to 2–5 times more sensitive than direct or indirect ELISAs).

(b) Western Blot (WB)

Western blot (WB) is a method that combines the advantages of electrophoresis with the immunological method of antigen–antibody binding. The samples tested can be crude extracts from cells or tissue or recombinant proteins that were separated through SDS-PAGE gel electrophoresis and then transferred to a NC or PVDF membrane. Specific antibodies are then used to detect the presence of the corresponding antigen. Non-specific antigen-binding sites can be blocked using agents such as bovine serum albumin (BSA) before adding the probe antibody. This method is now widely used in the study of gene expression at the protein level, the detection of antibody activity, the evaluation of allergenicity of recombinant allergenic proteins, and for screening of specific antibodies in patient serum. The disadvantage of WB is that it is a time-consuming operation and is, therefore, often combined with the ELISA method to improve efficiency.

(c) **The ImmunoCAP system**

The ImmunoCAP system was developed and is manufactured by the Swedish company Phadia and is an automatic detection system for the diagnosis of allergic diseases. This system measures total and specific IgE in human serum. It has been widely recognized worldwide and was named as “the gold standard in allergy detection” by numerous allergy experts because of its high specificity and sensitivity.

(d) **Radioallergosorbent Test (RAST)**

After Johansson’s group isolated IgE from allergic patients in 1967, they introduced the radioallergosorbent test (RAST) as a method to measure IgE in serum (Gleich and Jones 1975). The main steps include adding test serums and control serums to a microtiter plate that has been coated with purified allergen, followed by the addition of an isotope-labeled anti-IgE antibody. Bound radioactivity is detected and used to calculate the amount of specific IgE by a standard curve or judged as positive when the radioactivity of the specimen is higher than the healthy control by 3 times the standard deviation (3SD). There is a high correlation between RAST and skin testing and physicians are able to use both of these methods in the diagnosis and treatment of allergic disorders (Tandy et al. 1996). However, there are several disadvantages in the RAST method including costly reagents, time-consuming, risk of radioactive contamination/environmental pollution, and short expiration date of the radioisotopes, as well as the complications resulting from different kits using different reference serums that are inaccurate when compared with each other and that are affected by high concentrations of specific IgG in the serum.

2. Basophil/Mast cell activation tests for the evaluation of allergenicity

(a) **Whole Blood Histamine Release Assay (WBHR)**

Histamine is an important allergic inflammatory mediator, found pre-stored in the metachromatic granules within mast cells and basophils, and can be quickly released after stimulation. Mast cells reside in various tissues of human organs, while basophils are found in the blood (Jensen et al. 2014). The whole blood histamine release assay (WBHR) is an important screening tool for the detection of allergens or for evaluation of the allergenicity of allergens, and has a high correlation with specific IgE assays and skin prick testing (SPT). WBHR is not affected by high levels of total IgE or by the presence of anti-allergic drugs like β -agonists, steroids, or anti-histamines. When the patient’s skin is not suitable for SPT, or in the case of infants that cannot be tested by SPT, the WBHR is a simple method and a valuable diagnostic tool for screening allergens (Nolte et al. 1990). The required sample volume is small, only 3 mL of blood is needed for complete screening against the common allergens. The advantage of WBHR is not only in clinical testing, but also in scientific research in the evaluation of allergenic

proteins. All recombinant proteins, mutant proteins, and vaccines could be evaluated for their allergenicity using this method.

(b) Mast Cell Activation Test for the evaluation of allergenicity

Mast cells are secretory cells that resident in multiple tissues and organs and are best known for their involvement in many physiological and pathological processes, including allergic reactions, renovation of damaged tissue, chronic inflammation, tumor immunity, and others (Kinoshita et al. 1999). Mast cells play an important role in allergic and inflammatory reactions such as rhinitis, asthma, urticaria, and anaphylactic shock. Mast cells can be activated by re-exposure to allergens and release a series of immunoregulatory mediators including histamine, β -hexosaminidase, tryptase, chymase, vascular endothelial growth factor, and several cytokines and chemokines. These mediators initiate early- and late-phase inflammatory and allergic responses including increased vascular permeability, tissue swelling, bronchial contraction, leukocyte recruitment, glandular secretion, etc. Activation of mast cells (primary mast cells or mast cell lines) *in vitro* is an important method for the detection of allergies and for the assessment of the allergenicity of allergens with/without modification. Primary mast cells can be isolated from surgical tissue, obtained from tonsillectomy, circumcision, and resections of lung, colon, and other cancers. The characteristics of primary mast cells are their close proximity to the environment of the physiological condition but tissue-resident mast cells usually have large heterogeneity. Enzymatic digestion is the method frequently used for isolating tissue mast cells, often resulting in cells with low viability and less than desired purity. Mast cells can be derived from CD34⁺ stem cells isolated from bone marrow or umbilical cord blood by the addition of certain cytokines (Schmetzer et al. 2014) and these mature mast cells can be used in allergen stimulation assays. This method can overcome the problems resulting from the isolation of primary tissue-resident mast cells but it requires a lengthy production time and is costly (Holm et al. 2008). So in some regard, mast cell lines possess some advantages for use in *in vitro* activation tests for allergenicity assessment. Currently, the most commonly used mast cell lines include Laboratory of Allergic Diseases 2 (LAD2) (Kirshenbaum et al. 2014), Human Mast Cell-1 (HMC-1) (Xia et al. 2011), and Rat Basophil Leukemia (RBL-2H3) (Wan et al. 2014).

Mast cells are packed with 50–500 secretory granules, which account for over 40 % of its cell volume, and are “ready to go” immediately after cells are activated by an appropriate stimulus. The granules are pre-formed and newly synthesized in the Golgi, and, when activated, the secreted products are released extracellularly to cause inflammation. Many substances can activate mast cells and cause the degranulation reaction such as allergens, IgE, ionophore, compound 48/80, substance P, C5a, C3a, and so on. The mast cell stimulation assay is an important step in assessing the potential allergenicity of GM allergens by analyzing the inflammatory mediators in the mast cell supernatants and comparing the supernatants stimulated with wild-type (WT) allergen.

(c) Detection methods for various basophil/mast cell mediators

Histamine Detection

Histamine is derived from histidine decarboxylation, a reaction catalyzed by L-histidine decarboxylase. It is a type of biogenic amine with a small molecular weight and a short half-life. Histamine is found pre-formed in granules in mast cells and basophils. It can be released immediately after stimulation with allergens and causes bronchial smooth muscle contraction, increased mucus secretion, telangiectasia, increased vascular permeability, and provokes allergic diseases. Both IgE-mediated and non-IgE-mediated stimuli can cause histamine release. Mast cells can also be activated to release histamine via toll-like receptors (TLRs) but this depends on the cell phenotype (Meng et al. 2013). Quantification of histamine can be performed in several ways and many commercial ELISA kits are ready to use and accompanied by clear manufacturer's guidelines. Another method to detect histamine was previously described (see WBHR) and is dependent on glass fiber-coated plates and fluorometric detection using a Hisreader-501 device. Unfortunately, the short half-life and instability of histamine make it difficult to detect. Also, the complexity of the ELISA test for the quantitative analysis of histamine and the narrow detection range make these assays not ideal, and thus, other more sufficient tools are needed for the development of valid mast cell activation tests.

β -hexosaminidase Detection

β -hexosaminidase is one of the main mediators released by mast cells and basophils after stimulation. The release of β -hexosaminidase is considered as a common indicator of allergic disease. Reagents required for the β -hexosaminidase assay can be made in the laboratory except for the substrate 4-nitrophenyl N-acetyl- β -D-glucosaminidase (Huang et al. 2015a). This method is also commonly used to validate an IgE-mediated allergic response.

Tryptase Detection

Tryptase is the most abundant serine protease and has been considered as a marker for mast cell/basophil activation. The serum level of tryptase is normally less than 12 $\mu\text{g/L}$ and increases in anaphylactic reactions; however, negative results do not exclude the possibility of allergic disease. Up to now, the tryptase detection assay is not significantly used for screening food allergy. Mast cell tryptase is a tetrameric neutral serine protease with a molecular weight of 134 KDa and is composed of four monomers with a molecular weight of 32 KDa. Tryptase can be divided into two subtypes: α -tryptase and β -tryptase that share approximately 90 % sequence identity between each other. α -tryptase is mainly secreted by inactivated mast cells and presents as proenzyme in the blood, while β -tryptase is located in mast cell secretory granules and is released when mast cell activation occurs. β -tryptase is released from activated mast cells in parallel with histamine when systemic anaphylaxis occurs, its level peaking at 15–120 min with a half-life of 90–150 min, while histamine levels peak at 5 min with a half-life of 15–30 min. There is a longer window of detection time for β -tryptase than for histamine,

which is significant for clinical investigations. High concentration of β -tryptase can be found in biological fluids collected 1–6 h after a suspected allergic reaction (Payne and Kam 2004).

3. Animal Models for the evaluation of allergenicity

Considering the increasing number of people suffering from allergic diseases, the development of preclinical approaches to evaluate the potential allergenicity of proteins has become more and more urgent. In the past decades, we have seen the evolution of animal models that can accurately simulate the human body response. Animal models are a safe and effective *in vivo* experimental tool that can be used to evaluate the allergenicity of proteins by testing specific antibodies and cytokines in serum, bronchoalveolar lavage fluid (BALF), nasal irrigation fluid, and other biological fluids, as well as for determining the severity of the allergic reactions. By far the most commonly used animal models include those for asthma, AR, food allergy, and atopic dermatitis (AD), and the most commonly used species are guinea pig, mouse, and rat.

(a) Animal species used for the evaluation of allergenicity *in vivo*

Early in the 1990s, European and American scholars started to use animal models in food allergy studies. Animals such as guinea pigs, mice, and rats have been used for assessment of the allergenicity of foods. In a study of cow's milk allergy, guinea pig was the appropriate model using oral sensitization (Kitagawa et al. 1995). Guinea pigs are also a suitable species that can be used in experimental models for shrimp and ovalbumin allergy. Nearly 100 % of sensitized guinea pigs can be induced to develop intestinal symptoms of food allergy after challenge. Due to the high level of homology in gene expression between murine and human, mice have been commonly used in allergy research. Rats also have many advantages compared with other species, especially in toxicity testing (Penninks and Knippels 2001). Due to their organ functions highly similar to humans, as well as readily to operate, Chinese miniature pig models have also been used for food/protein allergenicity assessment (Huang et al. 2010, 2015b).

Guinea pigs are one of the most widely used experimental animals for the study of allergic asthma/AR. The advantages of using guinea pigs are that they can be easily sensitized, with a stronger reaction than other types of animals after allergen challenge, and they develop type I hypersensitivity (Sutovska et al. 2015). Stable high titers of specific IgG and IgE are easily induced and symptoms of both of immediate and delayed reaction are seen. Their pathology and asthma symptoms might be similar to humans, but their reaction to OVA shows large individual differences. After sensitization, a portion of guinea pigs develop anaphylactic shock and die after OVA challenge, whereas other guinea pigs may not appear to have any or only minor asthmatic reactions. In order to prevent this occurrence, antihistamine drugs, such as diphenhydramine and pyrilamine, are usually given before the stimulation in order to prevent the death of guinea pigs from overreactions. Guinea pigs are also suitable for developing food allergy models using oral sensitization, followed by re-exposure to the same food allergens either by

injection or taking orally. Outcomes include death or stress shock, indicating that guinea pigs become sensitized in a way similar to humans, thus, they can be a good species to use in animal models of milk allergy.

Mice are also often used to build classical models of asthma/AR and the commonly used strains are BALB/C, CBA/J, and C57BL/6. Owing to their small size and short breeding cycle, mice can save greatly on the supply of allergen and the cost of these experiments is relatively low for mouse models. In addition, with the emergence of a large number of related immunology and molecular biological reagents in recent years, and due to the more clearly understood immunologic and genetic background of mice, their use in animal models is widespread. Both BALB/c and C57BL/6 are the most commonly used species and are useful for allergic/infectious/transgenic animal models of asthma/AR, as well as for food allergy models. BALB/c mice are easily induced to produce airway hyperresponsiveness (AHR), easily sensitized with OVA, and produce high titers of IgE. It is not easy to induce AHR in C57BL/6 mice, but they are able to be sensitized to house dust mite (HDM) and, thus, can be used to create an allergic asthma model induced by HDM. Mice can be sensitized with allergens such as peanut lectin, ovalbumin (OVA), and BSA by intraperitoneal injection (i.p.), or orally sensitized with Cholera toxin (CT) as an adjuvant together with food allergens such as peanuts and milk, in which case the mice produce high levels of specific IgG1 and IgE. Al(OH)₃ as an adjuvant to OVA or other food allergen proteins can be used in food allergy models. Mice are also often used for the construction of transgenic animal models in studying skin allergy diseases.

Rats have some advantages when developing allergic-related animal models. First, pure strains are widely available and vigorously reproduce. Second, their cost and related reagents are relatively inexpensive. Three, sufficient amount of specimen can be collected. Their late-phase reaction and IgE-mediated allergic reactions are similar to humans. After stimulation, the symptoms of the immediate-phase and late-phase reaction of airway hypersensitivity are easily observed. The time of occurrence of the late-phase reaction more closely resembles that of clinical patients. Rats are also a suitable animal model for investigating food allergy: their size is suitable for kinetic analysis of serum-specific antibodies on a single individual; they can be orally sensitized without adjuvant; and sensitized rats can show allergy symptoms after challenge similar to humans. BN rats are a high immunoglobulin (especially IgE) response strain and are widely used in models of food allergy, which can provide much useful information when evaluating any potential allergenicity of novel foods or proteins. BN rats have been used in food allergy models both with and without the use of adjuvant (Abril-Gil et al. 2015).

Animal models, such as those described using guinea pigs, mice, and rats, have been commonly used for the assessment of the allergenicity of proteins, but an ideal animal model has not yet been developed (Abril-Gil et al. 2015; Piacentini et al. 2003; Vinje et al. 2009). Thus, investigators should choose a suitable animal model according to their research purposes.

(b) Animal models for the evaluation of allergenicity

(i) Models of allergic rhinitis/asthma

Asthma is a serious respiratory disease threatening human health that can develop from rhinitis, and together they have been described as “one airway, one disease.” The morbidity of AR/asthma is rising, with characteristic recurrent and protracted symptoms that seriously affect the quality of life of patients as well as increasing financial burdens. Rhinitis/asthma is generally considered as chronic allergic airway inflammation and involves a variety of inflammatory cells, inflammatory mediators, and cytokines. Due to the complex processes leading to the occurrence and development of rhinitis/asthma, there are various restrictions in studying the pathogenesis of human disease in clinical practice and clinical trials (Rice et al. 2008). Prediction of the outcome of clinical treatment requires animal models that can simulate human disease. Rhinitis/asthma-related animal models are commonly used to explore the pathophysiology, etiology, pathogenesis, and treatment methods of allergic respiratory diseases, as well as to evaluate the allergenicity of aeroallergens such as pollen, mite, bacteria, animal fur, and so on. Different species of animals differ in their sensitivity to different allergens, and rodents, including guinea pigs, rats, and mice, are the most commonly used animals in building rhinitis/asthma-related animal models.

Although there are various limitations in using each type of animal as has been described, nevertheless, they play an important role in the study of AR/asthma and the evaluation of protein allergenicity. To validate the success of our animal models, we record and calculate the scores of sneezing, scratching, and other nasal symptoms after allergen challenge. After the models were successfully built, the animals are challenged with allergen. After 24–48 h, animals are then euthanized to collect specimens for various purposes: serum collection for detection of specific IgE and associated inflammatory factors, collection of nasal lavage fluid/BALF for counting the total number of nucleated cells and eosinophils, as well as testing of the supernatant for the detection of cytokines, collection of nasal mucosa tissue/lung tissue for observation of the infiltration of eosinophils and mast cells, and observation of thickening of bronchial smooth muscle by HE staining.

(ii) Models of food allergy

Food allergies are adverse to human health and the mechanism is still unclear. An animal model of food allergy is mainly used to assess the potential allergenicity of novel foods/proteins, especially GM foods (Boyce et al. 2011).

Clinical manifestations of food allergy differ widely between individuals; from abdominal discomfort, diarrhea, itchy skin, lips, and mucosal edema to life-threatening laryngeal edema and anaphylactic shock. Exploration of the mechanism of food allergy by taking advantage of animal models can not only overcome the difficulties caused by individual differences, but also avoid human trials that cannot be ethically conducted. After the models are developed and used, various tests can

be performed. For example, serum can be collected 3 h after allergen challenge for the detection of IgE, IgG1, IgG2a, IL-4, IL-10, IFN- γ , etc., and intestinal mucosa tissue can be collected for HE staining to observe changes of eosinophils and mast cells. Spleen can be used for the preparation of a single-cell suspension that can be stimulated with specific allergens, followed by the measurement of IL-4, IL-10, IFN- γ , and other cell cytokines in the supernatant.

(iii) Models of atopic dermatitis (AD)

AD is a chronic and relapsing, pruritic inflammatory skin disorder, with complex interactions between genetic and environmental factors playing an important role in its development (Bieber 2008). However, the precise pathogenesis of AD remains to be fully elucidated. The clinical manifestations of AD include extremely itchy skin, with scratching of the affected areas causing skin irritation, cracking, leaching of clear liquid, and even skin hardening, scaling, etc. A high incidence of AD occurs in infants and pre-school children. Dust mites and animal fur are important causes of AD. An animal model of AD is a very useful tool for evaluating the allergenicity of proteins with and without modification. BALB/c mice have often been used in animal models of AD induced by applying 2,4-dinitrochlorobenzene onto hairless dorsal skin.

13.3 Strategies for the Modification of Protein Allergenicity

After evaluation of protein allergenicity using bioinformatics and laboratory methods, allergenicity modification to reduce or even eliminate the allergenicity of the allergen proteins is necessary. Currently, the main strategy of protein allergenicity modification is to alter the coding sequences of the allergen proteins (Rabjohn et al. 2002). Certain steps are always involved in protein allergenicity modification. First, locating the high allergenicity sites on the allergen proteins using experimental and/or bioinformatics methods; second, predicting the specific amino acid sites that need to be modified using bioinformatics methods; and third, mutating the specific sites to reduce, or even completely eliminate, the allergenicity of the allergen proteins in order to ensure future security and safety in their application.

13.3.1 Epitope Prediction and Epitope Modification of Allergen Proteins

Allergen epitopes are the ultimate “culprit” in allergic reactions. Allergen epitopes can be either T cell epitopes or B cell epitopes depending on the different combinations of receptors. B cell epitopes can be linear epitopes or conformational

epitopes, while T cell epitopes are generally linear epitopes. Experimental methods and forecasting methods are the usual methods used for epitope mapping (El-Manzalawy and Honavar 2010; Friedl-Hajek et al. 1999; Malherbe 2009; Newell et al. 2013). The former methods have many advantages but also have many shortcomings, while the latter methods provide new epitope mapping strategies, with simple, convenient features, and are widely used in research and modification of allergen protein epitopes. For specific principles and methods about predicting T and B cell epitopes, please refer to Chap. 14 in this book. This section will describe the prediction and modification of allergen protein T cell epitopes.

1. Prediction of T cell epitopes using bioinformatics methods

In recent years, a number of T cell epitopes prediction methods have been developed, of which the machine learning method is widely used due to its high efficiency and accuracy. Using machine learning algorithms to predict allergen epitopes includes data collection and processing, model building, and parameter optimization. Some common T cell epitope prediction softwares based on these algorithms are NetMHCII2.2 (<http://www.cbs.dtu.dk/services/NetMHCII/>) (Nielsen et al. 2007), Rankpep (<http://bio.dfci.harvard.edu/Tools/rankpep.html>) (Reche et al. 2002), and ProPred-I (<http://www.imtech.res.in/raghava/propred1/>) (Singh and Raghava 2003). The NetMHCII2.2 server predicts the binding of random peptides to HLA-DR, HLA-DQ, HLA-DP, and mouse MHC class II alleles using artificial neuron networks, which has high accuracy, and is widely used for the prediction of T cell epitopes.

2. Using NetMHC-II for the prediction and modification of allergen protein T cell epitopes

- (a) Obtain the sequence of an allergen from NCBI and predict the affinity between the allergen and MHC-II molecules using the online software NetMHC-II (<http://www.cbs.dtu.dk/services/>). If the affinity score is above 0.5, then the epitope is predicted to be a dominant epitope and needs modification.
- (b) According to the basic principle of allergenicity modification, select eight amino acids (AA) before and after the definitive AA to carry out random AA replacement followed by analysis of the affinity of the newly modified allergen with MHC-II.

13.3.2 Strategies of Allergen Protein Modification Using Laboratory Methods

At present, the primary method for allergen modification is to change the coding sequence of the allergen protein, thereby reducing the allergenicity of the target protein. Site-directed mutagenesis technology and error-prone PCR are the most commonly used methods to modify the allergenicity of the allergen

(Hakkaart et al. 1998; Tsai et al. 2003). Site-directed mutagenesis can introduce aimed changes to DNA fragments (including base addition, base deletion, and base substitution) by overlap extension using PCR (Nohr and Kristiansen 2003). Site-directed mutagenesis is usually used to modify the allergenicity of allergen proteins through amino acid substitutions at targeted sites. Error-prone PCR is performed under artificially controlled conditions whereby changing the reaction conditions can increase the frequency of error-prone incorporation of nucleotides to achieve direct modification of the target protein (Pritchard et al. 2005). Error-prone PCR is usually used to modify allergen proteins in cases where their structures and high allergenicity sites are unknown. The allergenicity of the allergen proteins can be reduced or even eliminated using amino acid site-directed mutagenesis technology and error-prone PCR technology, thus laying a foundation for safe application of the allergen proteins.

1. Using site-directed mutagenesis to modify allergen proteins

(a) The principle of site-directed mutagenesis

Site-directed mutagenesis is a method that is used to make specific and intentional changes (usually advantageous variations) to the target-DNA sequence (which may be genomic or plasmid DNA) including base addition, base deletion, and base substitution (Nohr and Kristiansen 2003). Site-directed mutagenesis can quickly and efficiently improve the traits of target proteins and is the common method for modifying the allergenicity of allergen proteins. When site-directed mutagenesis is used to modify the allergenicity of the allergen proteins, an oligodeoxynucleotide, which contains the pre-designed site, is synthesized as a primer. Then, pairing the mutant primer with the DNA template, the oligodeoxynucleotide is elongated by DNA polymerase to complete the DNA replication. Subsequently, the double-stranded DNA, which contains one strand of WT and one strand of mutant, is transformed into the host cells and the mutant is screened using sequence technology.

(b) Methods of in vitro site-directed mutagenesis

Site-directed mutagenesis technology was developed in the 1980s after combining gene cloning technology and DNA chemical synthesis methods. At present, in vitro site-directed mutagenesis methods are divided into three main types: single-strand phage for site-directed mutagenesis, cassette mutagenesis, and PCR-mediated in vitro site-directed mutagenesis (Carter et al. 1985; Reikofski and Tao 1992; Wells et al. 1985). Single-stranded phage for site-directed mutagenesis uses a single-strand M13 DNA as a template for mutagenesis and an oligonucleotide containing the desired mutation as a primer for extension. Consequently, heteroduplex DNA forms, consisting of one parental non-mutated DNA strand and a mutated DNA strand, which is then transformed into competent bacteria, and the corresponding mutant is screened using DNA sequence technology. Cassette mutagenesis, unlike other methods, does not require primer extension using DNA polymerase. In this method, a segment of DNA that contains the mutation of interest is synthesized

and then inserted into a plasmid that is digested by restriction enzymes followed by subsequent ligation of the synthesized oligonucleotide containing the desired mutations to the plasmid. Single-stranded phage for site-directed mutagenesis needs to use a M13 phage vector and has low mutation efficiency, while cassette mutagenesis has close to 100 % mutant efficiency, but is limited by the availability of suitable restriction sites flanking the site that is to be mutated. The limitation of single-stranded phage for site-directed mutagenesis and cassette mutagenesis can be overcome using PCR-mediated in vitro site-directed mutagenesis, which combines PCR amplification technology and cloning transformation technology in a simple operation with high efficiency. PCR-mediated in vitro site-directed mutagenesis is always used to carry out site-directed mutation of proteins in vitro.

This section mainly focuses on the application of PCR-mediated site-directed mutagenesis in the modification of the allergenicity of allergen proteins.

PCR-Mediated In Vitro Site-Directed Mutagenesis

PCR-mediated site-directed mutagenesis is an in vitro method for creating specific mutations in a known gene. Exponential amplification by PCR generates a segment containing the desired mutation to be isolated from the original plasmid by electrophoresis, which can subsequently be inserted into the desired plasmid using recombinant DNA techniques. There are various methods to do this (Hemsley et al. 1989; Ho et al. 1989; Jeltsch and Lanio 2002; Landt et al. 1990; Weiner et al. 1994). Here, we describe a general strategy of PCR-mediated site-directed mutagenesis in vitro. The basic principle of PCR-mediated site-directed mutagenesis is to place the mutation site toward one end of the segment whereby the primers are designed to generate the mutations. This method requires just a single PCR step, but still has an inherent shortcoming of requiring long oligonucleotide primers with an appropriate restriction site near the mutation site(s). Alternatively, adopt three or four primers, two of which could be non-mutagenic primers that cover two suitable restriction sites, and produce a segment that can be digested and ligated into the target plasmid. These methods need multiple PCR steps so that the final segment to be ligated can contain the required mutations.

Improvements to PCR-Mediated Site-Directed Mutagenesis

In order to acquire the mutated genes, conventional PCR-mediated point mutation always requires three or four primer pairs and multiple PCR amplification steps. However, the mutated sequence can be acquired from one pair of primers and one cycle of PCR amplification, as outlined in Fig. 13.1 (Carey et al. 2013). First, one pair of primers, which contains the desired mutant site and has partially overlapping regions, is required for synthesis. The PCR products with complementary sticky ends can be generated using the synthesized mutated oligonucleotides, annealing the PCR product, and transforming into *E. coli*, whereby the sequence with the desired mutations can be acquired after sequencing picked colonies.

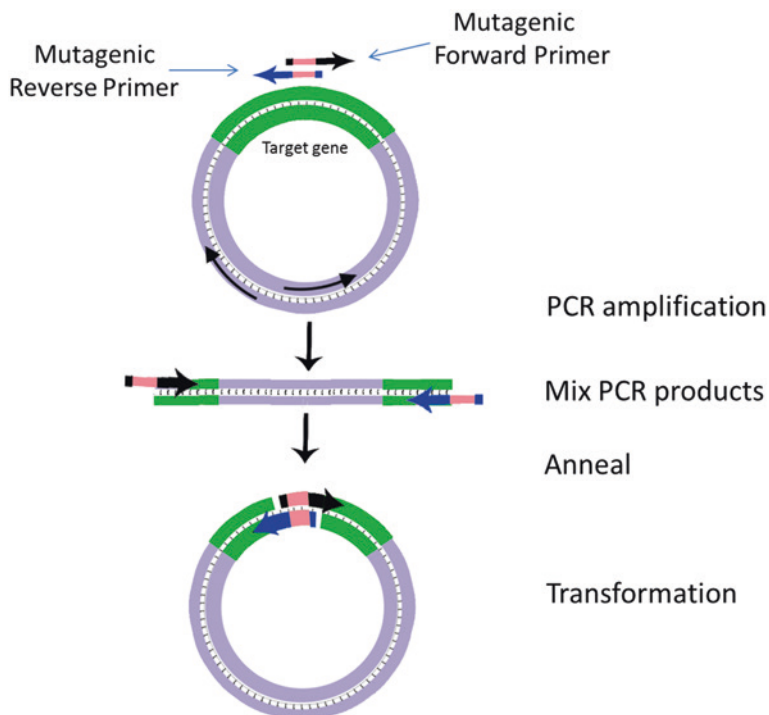


Fig. 13.1 The improvement of PCR-mediated site-directed mutagenesis (Carey et al. 2013)

The Application of PCR-Mediated In Vitro Site-Directed Mutagenesis in Protein Allergenicity Modification

PCR-mediated in vitro site-directed mutagenesis is a useful method for research on protein function such as activity sites, specificity, and allergenicity. In general, the following steps are always involved in allergenicity modification using PCR-mediated in vitro site-directed mutagenesis: (1) determining the specific sites to be modified; (2) designing mutant primers for PCR-mediated site-directed mutagenesis; (3) acquiring the mutated allergen sequence by amplifying the target allergen gene using the mutant primers; (4) annealing and transforming the mutant PCR products into host cells; and (5) verifying the mutant with the desired mutation by sequencing technology and evaluating the allergenicity of the mutant. In the past 10 years, many researchers have reported allergenicity modification using PCR-mediated in vitro site-directed mutagenesis. In summary, PCR-mediated site-directed mutagenesis is a simple, efficient, and cost-effective method for allergenicity modification. With the rapid development of hypoallergenic variants, PCR-mediated site-directed mutagenesis will become a powerful tool for the modification of allergenicity.

13.3.3 The Use of Error-Prone PCR in Protein Allergenicity Modification

1. The principle of error-prone PCR

Error-prone PCR is a method that can introduce random copying errors into any piece of DNA by utilizing an imperfect or sloppy reaction mixture (e.g., by adding Mn^{2+} or Mg^{2+} to the reaction mixture) (Pritchard et al. 2005). Generally, the replication of DNA by polymerase is extremely specific, with the difference in error-prone PCR being that the fidelity of the Taq DNA polymerase is modulated by alteration of the composition of the reaction buffer. When modifying allergen proteins using error-prone PCR, the coding sequence of the allergen proteins is used as a template and amplified under the error-prone conditions. The polymerase makes mismatches in the base pairing during DNA synthesis that generates the introduction of errors into the newly synthesized DNA strand. By carefully controlling the reaction conditions, the frequency of the mismatched nucleotide bases can be regulated. This method has been proven useful for the generation of random mutations during the expression and screening process in a mutagenesis step.

2. Reaction conditions for optimal error-prone PCR

- (a) Using Taq DNA polymerase: It is critical that a Taq polymerase be used as it has naturally high error rate and does not have proofreading ability. The proofreading of nucleotide sequences is a feature that is found in many commercially available DNA polymerases (Biles and Connolly 2004; Keohavong and Thilly 1989; Peterson 1988); however, use of a proofreading DNA polymerase in error-prone PCR will result in the automatic correction of the mis-pairing nucleotides and any mutations that were incorporated during the replication will be lost.
- (b) Increasing Mg^{2+} concentration: In general, magnesium ions are an essential cofactor for the DNA polymerase in PCR reaction systems and its concentration must be optimized for each PCR reaction system (Cadwell and Joyce 1994; Ling et al. 1991). Many components of the system bind magnesium ions, including dNTP, DNA polymerase, template, and primers. Because the activity of DNA polymerase requires the presence of free magnesium ions, the magnesium ion concentration in the reaction system must exceed the total dNTP concentration. Typically, in the presence of 0.8 mM total dNTPs, 1.5 mM Mg^{2+} needs to be added to the PCR reaction system leaving approximately 0.7 mM free Mg^{2+} for the DNA polymerase. When the concentration of Mg^{2+} is below normal concentrations, the less the specificity of the DNA polymerase and the higher the magnesium ion concentration, the lower the specificity of the DNA polymerase. In order to elevate the error rate in the replication process, a higher concentration of magnesium ion (about 7 mM) should be added to the error-prone PCR reaction system.

- (c) Adding Mn^{2+} : Generally, Mn^{2+} can also be added to the reaction system to promote the error rate (Lin-Goerke et al. 1997) in addition to increased Mg^{2+} . By varying the concentration of the Mn^{2+} added to the reaction system, the mutation rate can be varied from 1 to 5 nucleotides per 1 kb.
- (d) Using unbalanced dNTP concentrations: dNTPs can be added at either equal or unbalanced amounts. An unbalanced dNTPs mixture can increase the misincorporation of nucleotides into the newly synthesized DNA strand (Lin-Goerke et al. 1997). Several methods have been used, including increasing the concentration of dGTP, increasing both dCTP and dTTP, or increasing all but dATP. The amount of certain nucleotides can also be decreased. In our experiments, an unbalanced dNTPs mixture that includes 0.2 mM each of dATP and dGTP and 1 mM each of dCTP and dTTP is suitable.
- (e) Adding other substances to promote the error rate: Addition of alcohol and some triphosphate derivatives of nucleotides interfere with amplification and promote the rate of incorporation of mismatched bases (Fromant et al. 1995; Nishiya and Imanaka 1994; Shafikhani et al. 1997).
- (f) Increasing the number of amplification cycles: The amplification step of PCR is commonly 35 cycles, and increasing the number of amplification cycles also promotes the incorporation of mismatched bases (Pritchard et al. 2005).

3. The error-prone PCR procedure for allergen modification

The method of error-prone PCR is similar to conventional PCR cloning of a target except that a much larger pool of transformants is needed to increase the diversity of the library. In general, the following steps are always involved in allergenicity modification using error-prone PCR (Hanson-Manful and Patrick 2013; Pritchard et al. 2005):

- (a) Acquiring the randomly mutated allergen sequences by amplifying the target allergen gene under error-prone conditions;
- (b) Digesting the PCR product with suitable restriction endonucleases;
- (c) Ligating the digested PCR product into a suitable plasmid with DNA ligase;
- (d) Transforming the ligated products into a host cell to acquire a mutant library;
- (e) Screening mutants with reduced allergenicity by bioinformatics and experimental methods.

4. The superiorities and characteristics of error-prone PCR in allergen modification

Error-prone PCR has proved useful in directed modification of protein function *in vitro*, such as enzyme substrate specificity, enzyme activity, protein thermostability, and allergenicity (Huang et al. 2008; Liao et al. 2012; Stephens et al. 2009; Yu et al. 2009). A significant advantage for using error-prone PCR to modify the protein allergenicity is that it does not require detailed information on the allergen's structure or the precise prediction sites for amino acid substitution. Error-prone PCR involves producing enormous numbers of mutants of allergen mutant genes

in order to modify the allergenicity of the allergen proteins. The success of allergenicity modification depends on the efficiency in creating mutagenesis libraries and screening them for the mutants with reduced allergenicity. The variety of the library is a decisive factor and a large number of techniques, such as increasing the Mg^{2+} concentration, adding Mn^{2+} , etc., have been developed to attain high-quality mutagenesis libraries (see above). The screening of the mutant libraries is also a crucial factor for successful allergenicity modification using error-prone PCR. Many high-throughput screening methods, such as allergen protein chips, dot-blot array, etc., have been developed for subsequent allergenicity screening. For more information on allergenicity screening after error-prone PCR, please refer to Chap. 8 in this book.

13.4 The Re-evaluation and Validation of the Allergenicity of Allergen Proteins After Modification

The allergenicity of the modified allergens must be re-evaluated and validated by laboratory methods. WBHRs, Mast Cell Challenge Assays, IgE Binding Assays, and animal models are the most common methods for evaluating the allergenicity of the allergen proteins. The first three methods, which evaluate the allergenicity of the allergen proteins in vitro, mainly assess the potential allergenicity from the perspective of serology and cytology. The last method, which assesses the allergenicity of the proteins by establishing allergic disease models followed by analysis of allergic symptoms and measurement of the specific IgE levels, is usually the allergenicity evaluation method used in vivo. Various allergic disease models have been established, such as animal models of asthma, AR, AD, and food allergic diseases, to evaluate the allergenicity of allergen proteins. When using animal models to evaluate the allergenicity of the target proteins, allergenicity strength is estimated according to changes of the specific antibodies, allergic disease-related cytokines and the symptoms of allergic reactions. Rats, mice, and guinea pigs are the common animals used for animal models for allergic diseases as they have similar immunologic response mechanisms as humans, can provide a wealth of related immunological information, and have a small size, short breeding cycle, and low cost. Therefore, using animal models to evaluate the allergenicity of the proteins has great advantages.

The products of error-prone PCR are a mixture of multiple mutants. Evaluation of the allergenicity of each mutant using laboratory experiments is difficult to implement, so preliminarily evaluation of the allergenicity of the mutants acquired from error-prone PCR is accomplished using bioinformatics methods such as homology comparing, cross reaction analyzing, and antigenic reactivity, in order to identify the mutants with reduced allergenicity that retain their original three-dimensional structure and biological activity. Unfortunately, screening of mutants using only bioinformatics evaluation methods cannot confidently identify those

with completely reduced allergenicity and more stringent experimental evaluation methods are needed to assess the mutants.

At this time, there are no standardized methods for protein allergenicity assessment; however, evaluation of the allergenicity of the proteins can be achieved using existing technologies and methods. Although each method has its advantages and disadvantages, the allergenicity of the proteins can be evaluated effectively by combining a variety of evaluation methods.

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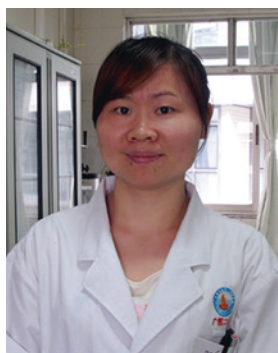
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Prof. Ailin Tao earned his doctorate degree from the State Key Laboratory of Crop Genetic Improvement of Huazhong Agricultural University in 2002, followed by a postdoctoral training at Postdoctoral Station of Basic Medicine in Shantou University Medical College, majoring in allergen proteins. His

most recent research has been on allergy bioinformatics, allergy and clinical immunology, and disease models such as allergic asthma, allergic rhinitis (AR), infection, and inflammation induced by allergy, inflammatory, and protracted diseases caused by antigens or superantigens. He has gained experience in the field of allergology including the mechanisms of immune tolerance, allergy triggering factors and chronic inflammation pathways, and allergenicity evaluation and modification for food and drugs. He proposed some new concepts including "Representative Major Allergens,"

“Allergenicity Attenuation” of immunotoxin and allergens, “broad-spectrum immunomodulator” as well as the theoretical hypothesis of “Balanced Stimulation by Whole Antigens.” Prof. Tao’s laboratory focuses on the diagnosis of allergic disease and the medical evaluation of food and drug allergenicity and its modification. Prof. Tao has now constructed a system for the prediction, quantitative assessment, and simultaneous modification of epitope allergenicity, which has been applied to more than 20 allergens, and he also developed a bioinformatics software program for allergen epitope prediction, SORTALLER (<http://sortaller.gzhmu.edu.cn>), which performed significantly better than other existing software, reaching a perfect balance of high specificity (98.4 %) and sensitivity (98.6 %) for discriminating allergenic proteins from several independent datasets of protein sequences of diverse sources. Furthermore, this program has a Matthews correlation coefficient as high as 0.970, a fast running speed, and can rapidly predict a set of amino acid sequences with a single click. The software has been frequently used by researchers from many institutions in China and over 30 countries worldwide, thus becoming the number one allergen epitope prediction software program. Prof. Tao has set up an allergen database ALLERGENIA (<http://ALLERGENIA.gzhmu.edu.cn>) that has several advantages over other databases such as a wide selection of non-redundant allergens, excellent stringency and accuracy, and friendly and usable analytical functions.