



Award Review

Analysis of Food Allergen Structures and Development of Foods for Allergic Patients

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Food allergy is one of the important health problems, and countermeasures are socially required. We have been undertaking studies on wheat allergens and their epitopes, and have developed a method for producing hypoallergenic wheat flour by enzymatic modification. The hypoallergenic products are now provided to patients. More noteworthy, by taking hypoallergenic cupcakes over a long period, more than half of patients are hyposensitized and become able to eat normal wheat products. This suggests that the hypoallergenic wheat flour can act as anti-allergenic via allergen-specific immunotolerance.

This series of studies was followed by expansive research on food allergy: analysis of epitopes of bovine serum albumin (the major beef allergen), isolation and identification of inhibitory peptides for allergen absorption at the intestine, evaluation of hesperetin as an inhibitor of degranulation of mast cells, and the development of PCR detection methods for verifying allergen labeling and for identifying hidden allergic ingredients in processed foods.

Key words: food allergy; epitope; hypoallergenic wheat flour; allergen permeation; Caco-2

The worldwide prevalence of allergic diseases such as asthma, atopic dermatitis, and allergic rhinitis has increased during the last two decades,¹⁾ and it is assumed that in Japan more than one-third of the population is now suffering from at least one of these diseases. Allergic diseases affect atopic individuals, who synthesize specific Immunoglobulins E (IgE) to environmental allergens. These allergens include grass and tree pollens, indoor allergens such as house dust mites and animal dander, and various foods. Hen's egg,²⁾ cow's milk,³⁾ wheat⁴⁾ and peanuts⁵⁾ are generally known allergens for food-allergic patients. Allergens are proteins characterized by their ability to induce a pathogenic IgE response in susceptible individuals, giving rise to asthma, atopic dermatitis, and rhinitis. Although the reasons that certain

individuals suffer particular hypersensitivities are unclear, there is evidence that both genetic and environmental factors influence susceptibility.⁶⁾ Analysis of genes contributing to allergic disorders has shown that susceptibility arises from complex multigenic interactions.⁷⁾ The dramatic recent increase in the prevalence of allergic sensitization provides evidence for additional roles of environmental factors in the pathogenesis of immune hypersensitivity.

It has been explained that allergic disease is induced by the following mechanisms: Exogenous antigens are processed by endosomal proteases to peptides in antigen presenting cells, and are presented to T cells by appropriate major histocompatibility complex (MHC) class II molecules present on the cell surface (Fig. 1, Step 2). CD4⁺ helper T cells are activated upon recognition of these peptide-MHC complexes by T cell receptor molecules, and they exert effector functions through various biological activities of secreted cytokines. Allergen-specific CD4⁺ T cells that produce Th2-type cytokines play a major role in the elicitation and progression of allergic diseases. The most important Th2 cytokine is interleukin-4 (IL-4), which drives IgE class switching by allergen-stimulated B cells (Fig. 1, Step 3). IgE binds with high affinity to IgE receptors (FcεRI) located on the surface of mast cells in tissues and basophils in the blood (Fig. 1, Step 4). Mast cells play a central role in inflammatory allergic reactions. Multivalent binding of antigens to receptor-bound IgE and subsequent aggregation of FcεRI provide the trigger for the activation of mast cells (Fig. 1, Step 5). FcεRI aggregation results in the release of inflammatory mediators from secretory granules, which contain preformed mediators such as histamine (Fig. 1, Step 6).

It is expected that food allergy can be prevented by inhibition of these steps. In this article, I will describe our studies on countermeasures to food allergy. Part of the current knowledge relating our studies will be also introduced.

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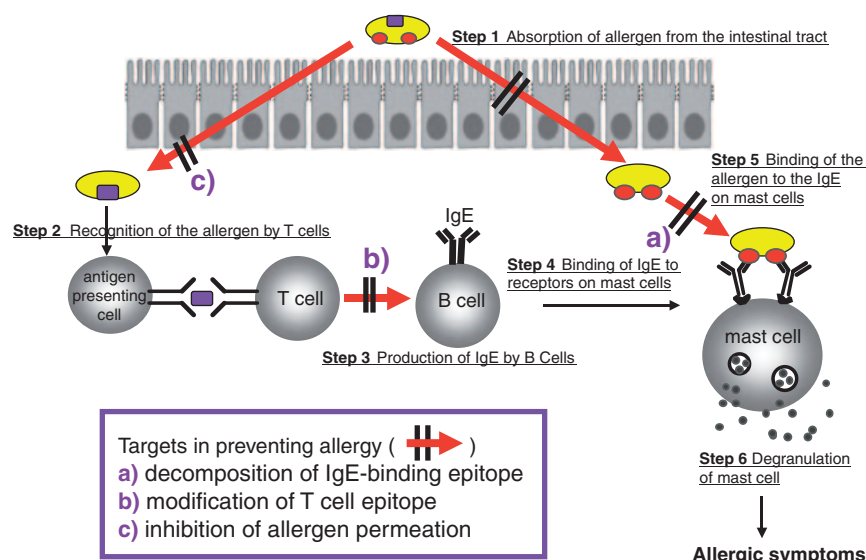


Fig. 1. Mechanisms of Allergic Reactions and Targets in Preventing Allergy.

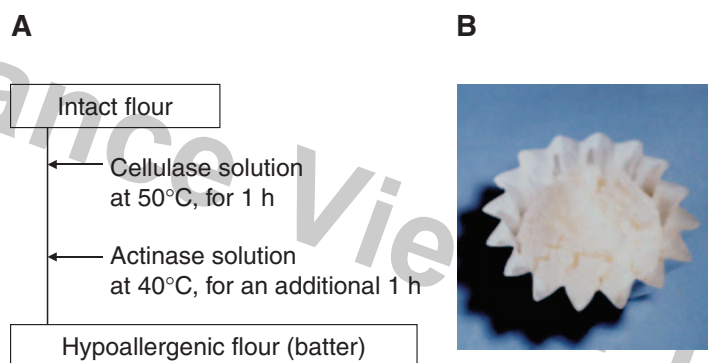


Fig. 2. Hypoallergenic Wheat Flour.

A, Method of producing hypoallergenic wheat flour.³⁷⁾ B, Photograph showing the hypoallergenic wheat product (cupcake)⁸⁾ which was clinically evaluated.⁴¹⁾

I. Identification of Major Wheat Allergens and Production of Hypoallergenic Wheat Flour

Wheat is one of the world's most important grains, especially for food, and a variety of flour- and wheat-derived products are consumed throughout the world, but hypersensitive responses to wheat have long been a public health problem since, in addition to cow's milk and eggs, wheat is one of the most common foods causing allergy in industrialized countries including Japan.

1. Symptoms of adverse reactions to wheat flour and responsible determinants

There are at least four kinds of symptoms of adverse reactions to wheat flour: celiac disease, baker's asthma, atopic dermatitis, and food-dependent exercise-induced anaphylaxis (FDEIA).⁸⁾ In these cases, certain wheat proteins induce abnormal allergic reactions in

sensitive individuals. Wheat proteins are classified according to their solubilities, water-soluble albumins, salt-soluble globulins, ethanol-soluble gliadins, and urea-, detergent-, or KOH-soluble glutenins.

Gluten-sensitive enteropathy is called celiac disease, and is caused by ingestion of the gliadin fraction.^{9,10)} The reported prevalence of this disease is between 1:300 and 1:1,000 in European countries.¹¹⁾ The amino acid sequences responsible for the disease have been characterized, and the minimum epitope structures were found to be Pro-Ser-Gln-Gln and Gln-Gln-Gln-Pro.¹⁰⁾ Although the disease is mediated by T lymphocyte-driven immunological activation in the gastrointestinal mucosa,¹¹⁾ the levels of total and wheat-specific IgE antibodies in celiac patients are usually not elevated. Therefore, in the general context of considering allergy synonymous with IgE-mediated hypersensitivity, celiac disease should not be classified as an allergic disorder.

The inhalation of wheat flour often causes asthma,¹²⁾ which is known as baker's asthma, a typical occupa-

Table 1. IgE-Binding Abilities of Low-Molecular Glutenin Peptides¹⁷⁾

Peptide	Relative ELISA value
(A)	
Ac-SQQQQPPF SQQQPPF SQQQQPPF SQQQPPF	1.0
Ac-SQQQQPPF SQQQPPF	1.1
Ac-SQQQQPPF	1.1
Ac-SQQQPPF	1.0
(B)	
Ac-GQQQPPF	1.1
Ac-SGQQPPF	nd*
Ac-SQQQPPF	0.8
Ac-SQQGPPF	1.0
Ac-SQQQPPF	nd
Ac-SQQQPGF	nd
Ac-SQQQPPG	0.9
(C)	
Ac-QQQPP	0.9
Ac-GQQPP	nd
Ac-QGQPP	0.7
Ac-QQGPP	1.0
Ac-QQQGP	nd
Ac-QQQPG	nd
QQQPP	0.6

*Not detectable

tional allergic disease that has been known since ancient Roman times. Extensive studies have identified certain proteins as allergens associated with asthma. Among these, alpha-amylase inhibitors (AIs) from the globulin fraction have been identified as major allergens.¹²⁾ The IgE-binding epitope structures of an AI (known as the 0.28 wheat AI) have been determined.¹³⁾

A third condition is skin inflammation, atopic dermatitis, which develops shortly after cereal-based products are ingested, usually resulting in eruption and itching. The wheat allergens associated with atopic dermatitis are heterogeneous and many attempts have been made internationally to identify the allergens, but little information was available on the molecular structures of the major allergens involved in atopic dermatitis when some groups, including ours, started to carry out systematic experiments.^{14–17)} Later, several groups identified wheat allergens.^{18–21)} The proteins responsible for wheat-induced atopic dermatitis are described below in section 2.

Another allergic condition is food-dependent exercise-induced anaphylaxis (FDEIA).²²⁾ FDEIA is a severe (life-threatening) form of allergy in which the ingestion of a specific food before physical exercise induces symptoms of anaphylaxis. Although various foods (including shellfish, celery, hazelnuts, peanuts, soy, peas, and bananas) have been associated with FDEIA, the most frequently reported cause of these reactions appears to be wheat.^{23,24)} Wheat-dependent, exercise-induced anaphylaxis is not as rare as previously thought, but is a rather poorly recognized disorder. Although the

mechanisms by which exercise provokes reactions in food- or wheat-dependent, exercise-induced anaphylaxis remain unclear, the gliadin fraction is thought to be an allergen.^{23,24)}

In the following sections, I focus on wheat allergens that cause atopic dermatitis.

2. Identification of wheat allergens and their epitopes

We evaluated the allergenicity of salt-soluble and salt-insoluble (gluten) fractions by means of enzyme-linked immunosorbent assay (ELISA) using serum from atopic patients allergic to wheat. Most patients were found to be sensitive to gluten.²⁵⁾ Hence, we first tried to identify a major epitope structure in gluten responsible for atopic dermatitis.

Gluten is insoluble in aqueous media. Hence, it was hydrolyzed with α -chymotrypsin to obtain soluble peptide fragments with allergenicity, since food allergens are often characterized by their high stability to digestive enzymes, their epitope structures remaining unchanged. The resulting hydrolytic reaction product was centrifuged, and the supernatant was submitted to gel filtration and reversed-phase HPLC. The allergenicity of the fractionated elute was evaluated by ELISA, and the primary structure of the peak with the highest allergenicity was determined.

The primary structure of the purified compound was a 30-mer peptide with the sequence (Ser-Gln-Gln-Gln-(Gln-)Pro-Pro-Phe)₄ (Table 1A). This allergenic peptide showed high sequence similarities (almost 90%) to low-molecular-weight glutenin precursors. Hence, we concluded that it originated from low-molecular-weight glutenin. Similarities of about 70% were also obtained between the sequence of the allergenic peptide and those of low-molecular-weight glutenin precursors from durum wheat. Surprisingly, a high degree of similarity (53.6%) was also found between the allergenic peptide and a *Saccharomyces cerevisiae* protein.²⁶⁾ Hence, it should be noted that wheat allergic patients are suspected also to be sensitive to the yeast using in bread making.

Repeat sequences in allergenic peptides such as (Ser-Gln-Gln-Gln-(Gln-)Pro-Pro-Phe)₄ might be favorable in crosslinking IgE antibodies and triggering the release of chemical mediators from mast cells in the body. Indeed, there are 44 and 25 Gln-Gln-Gln-Pro-Pro sequences in high-molecular-weight glutenin subunits x and y respectively (accession nos, CAC40686 and CAC40687). Like Gln-Gln-Gln-Pro-Pro repeat sequences, cod allergen (*Gad c 1*, allergen M) is known to contain three homologous IgE-binding tetrapeptides.²⁷⁾

Next, the peptides listed in Table 1 were synthesized according to the solid phase method. The N-terminal amino acid of each peptide was acetylated to mimic the conditions under which it exists in the intact form, and the allergenicity of each peptide was evaluated by ELISA.¹⁷⁾ As shown in Table 1A, (Ser-Gln-Gln-Gln-(Gln-)Pro-Pro-Phe)₄, (Ser-Gln-Gln-Gln-(Gln-)Pro-Pro-

Phe)₂, and Ser-Gln-Gln-Gln-(Gln-)Pro-Pro-Phe bound to IgE almost equally. There was no difference between the relative ELISA values of Ser-(Gln)₄-Pro-Pro-Phe and Ser-(Gln)₃-Pro-Pro-Phe. These data suggest that the Ser-Gln-Gln-Gln-Pro-Pro-Phe motif is involved in binding to IgE antibodies. To determine which amino acid residues in the motif are essential in binding to IgE, we attempted to replace each constituent amino acid residue in turn with Gly. When any of the asterisked amino acid residues in the sequence Ser-Gln*-Gln-Gln-Pro*-Pro*-Phe was replaced by Gly, the ELISA value dropped below the limit of detection (Table 1B). These amino acid residues are hence thought to be indispensable in binding to IgE. As Tables 1B and C also show, Gln-Gln-Gln-Pro-Pro, which lacks the N and C terminals of Ser-Gln-Gln-Gln-Pro-Pro-Phe, gave an ELISA value equal to that obtained with the full peptide.

We further examined to determine which amino acid residues in Gln-Gln-Gln-Pro-Pro are essential for binding to IgE (Table 1C), and found that the N-terminal glutamine residue and the two proline residues are essential. It was hence concluded that the IgE-binding epitope of the allergenic peptide comprises Gln-Xaa-Xaa-Pro-Pro, where Xaa are replaceable amino acid residues. Indeed inhibition ELISA assay showed that Ac-Gln-Gln-Gln-Pro-Pro binds to wheat-specific IgE in the serum of patients. To analyze the binding between Ac-Gln-Gln-Gln-Pro-Pro and the IgE antibody, we carried out the first NMR analysis of Ac-Gln-Gln-Gln-Pro-Pro.²⁸⁾ The data showed that the configurations of the amide bonds of the peptide backbone were all-*trans*.

As shown in Table 1C, the ELISA value obtained with Gln-Gly-Gln-Pro-Pro was lower by almost 30% than that obtained with Gln-Gln-Gln-Pro-Pro, and the value with non-acetylated Gln-Gln-Gln-Pro-Pro was almost half of that with acetylated Gln-Gln-Gln-Pro-Pro. Judging by these data, the second glutamine residue in Gln-Gln-Gln-Pro-Pro and acetylation of the N-terminal amino group are both advantageous in binding to IgE. Recombinant low-molecular-weight glutenins with many Gln-Gln-Gln-Pro-Pro motifs have been expressed in *Escherichia coli* by a pET vector system, and were confirmed to possess IgE-binding ability.²⁹⁾

As other allergens, thioredoxin,¹⁹⁾ lipid transfer protein,²⁰⁾ acyl-CoA oxidase,³⁰⁾ peroxidase,³¹⁾ and fructose-bisphosphate aldolase³²⁾ were identified.^{8,21)} In addition, the epitope structures of gliadin in patients with FDEIA²⁴⁾ and celiac disease³³⁾ have been recently reported.

3. Production of the hypoallergenic wheat flour and hypoallergenic products

Based on the above-described epitope structure, Gln-Xaa-Xaa-Pro-Pro, it was assumed that wheat flour becomes hypoallergenic by hydrolysis of peptide bonds near the essential proline residues of the epitope. Based on this idea, we screened for a food-usable enzyme

with high activity in hydrolyzing peptide bonds near the proline residues. The enzyme chosen should also be characterized by its low amylase activity, which is expected to minimize the development of sweetness, the deterioration of starch functionality, and volume loss in the product. In addition, amylase inhibitors cannot be used due to their allergenic potencies.^{12,20)} As a result of enzyme screening, bromelain and actinase showed high ability to hydrolyze peptide bonds near the proline residues with low amylase activities,^{34–37)} but some patients were also sensitive to bromelain itself.³⁸⁾ Hence, actinase was selected to produce hypoallergenic flour. Based on our preliminary ELISA data, actinase-treated flour retained a small degree of allergenicity, indicating the contribution of glycoproteins³⁹⁾ and/or mannoglucon.⁴⁰⁾ To decompose these carbohydrate allergens, we selected an enzyme with low amylase activity and the ability to reduce the remaining allergenicity. Cellulase was the best enzyme tested for this purpose, and it was used in the first step of the enzymatic process to avoid its inactivation by actinase (Fig. 2A). The reaction temperature was set at 50 °C, at which no gelatinization of starches occurred. Favorably, the numbers of microorganisms decreased at this step. A reaction time of 1 h and an enzyme/flour ratio of 0.5% were sufficient to decompose the carbohydrate allergens. The reaction product was cooled to 40 °C and then subjected to actinase treatment.

Actinase was used in the second step of the process to decompose proteinaceous allergens (Fig. 2A). It was dissolved in water, and the solution was mixed with the cellulase-reaction mixture. The subsequent reaction was carried out at 40 °C for 1 h, yielding a product that was hypoallergenic in most cases. Since hypoallergenic flour has poor functionality in food processing and must be processed into easy-to-eat foods, we have proposed methods to prepare certain food items, including bread, noodles, and cupcakes (Fig. 2B).

4. Clinical evaluation of the hypoallergenic flour

We investigated the safety and usefulness of a hypoallergenic cupcake in 15 children with atopic dermatitis and wheat allergy, who were treated at the Department of Pediatrics of Kansai Medical University.⁴¹⁾ All patients had a history of severe urticaria when cereal-based products were ingested. Among them, 13 patients showed no adverse response after consuming the hypoallergenic cupcake. The results of provocation with the hypoallergenic cupcake showed a positive immediate reaction (severe urticaria) in only two patients. Therefore, for most patients with wheat allergy, the hypoallergenic cupcake was judged to be safe.⁴¹⁾

Moreover, by taking the hypoallergenic cupcakes over a long period (more than 6 months), more than half patients were hyposensitized and became able to eat normal wheat products. Hence, we performed an open controlled study of hypoallergenic wheat oral immunotherapy in 20 children who had wheat allergy with a

history of immediate allergic reaction to wheat and were treated at the same hospital.⁴²⁾ Most of the children who took hypoallergenic wheat flour achieved the daily intake of normal wheat product, whereas only one-fifth of children did so in the control group. This suggests that the hypoallergenic wheat flour can act as an anti-allergenic *via* allergen-specific immunotolerance. Actually, we found that there are T-cell reactive peptides in the hypoallergenic flour (unpublished data). Professor S. Kaminogawa⁴³⁾ explained that three basic mechanisms are involved in oral tolerance: clonal anergy, active suppression, and clonal deletion. Further studies are necessary to clarify how T-cell reactive peptides in the hypoallergenic flour can induce immunotolerance.

II. Other Research Related to Food Allergy

Further research on the analysis of epitope structures was conducted, taking bovine serum albumin as a food allergen. In addition, we are trying to prevent food allergy by inhibiting allergen permeation in the intestine and by inhibiting degranulation of mast cells. In this section, the results of this research are described. Finally, I will briefly refer to our project on the development of a PCR detection method to verify allergen labeling and to identify hidden allergic ingredients in processed foods in daily life for people worried about food allergy.

1. Epitope peptides of bovine serum albumin, the major beef allergen, and their analog peptides for specific immunotherapy

In contrast with other allergens such as hen's egg,²⁾ cow's milk,³⁾ wheat,⁴⁾ and peanuts,⁵⁾ beef allergy was poorly recognized before the 1990s. Since then, papers^{44,45)} have appeared elucidating the nature, epidemiology, and symptoms of beef allergy in children allergic to cow's milk and children suffering from atopic dermatitis. It is now clear that beef allergy is not an infrequent occurrence, having an incidence between 3.3% and 6.5% among children with atopic dermatitis.⁴⁵⁾ The major beef allergen is bovine serum albumin (BSA).^{44,45)} Fiocchi *et al.*⁴⁴⁾ reported that all beef-allergic children tested reacted to BSA in a skin-prick test. Serum albumins are also known causes of cat- and dog-dander allergy,^{46,47)} but little detailed information about the epitope structure of BSA has been available.

Serum albumin is one of the most widely studied and applied proteins in biochemistry. It is also the most abundant protein in the circulatory system, accounting for 60% of total serum protein, with a concentration of approximately 40 mg/ml.^{48,49)} Serum albumins comprise about 580 amino acid residues and are characterized by a low content of tryptophan and a high content of cystine and charged amino acids, such as aspartic and glutamic acids, lysine, and arginine.⁴⁹⁾ The tertiary structure comprises three domains, I, II, and III (aa1–190, 191–382, and 383–581). BSA and human serum albumins

(HSA) display approximately 76% sequence homology and a repeating pattern of disulfides that is strictly conserved.^{49,50)} The fact that a major component of serum acts as an allergen is very surprising for the following reason: it is remarkable that albumins from animals, which are very similar in sequence, structure, and function to human serum albumin, are recognized by the human immune system as allergens instead of tolerance being induced.

Prior to our experiments, I hypothesized that BSA-specific antibodies and T cells react primarily with sequential epitopes in which the amino acid sequences differ greatly between bovine and human albumin. To test this hypothesis, 16 peptides corresponding to such regions were synthesized as candidate epitopes (Fig. 3).⁵⁰⁾ Among these, at least two regions, aa336–345 and aa451–459, bound to patient IgE antibodies. In more detailed experiments, EYAV (aa338–341) and LILNR (aa453–457) were found to be the cores of the IgE-binding epitopes. On the other hand, three regions, aa107–123, aa364–382, and aa451–459, were found to induce T cell proliferation in more than half of the patients tested. Of interest was that these three regions were also recognized by B cells.

Next, we generated hypoallergenic analog peptides of BSA that preserve T cell reactivity to retain the capacity to induce immunotolerance.⁵¹⁾ Substitution of the glutamic acid in the EYAV (aa338–341) and VMENFVAF (aa545–552) sequence to aspartic acid (E338D and E547D) led to a remarkable reduction in IgE-binding ability, but induced potent T cell proliferation. Moreover, in response to the substitutions, production of INF- γ markedly increased, as compared with that elicited by the native peptides. Hence, these analog peptides represent potential candidates for specific immunotherapy.⁵²⁾

2. Inhibition of allergen permeation at the intestine to prevent food allergy

The intestinal epithelium theoretically acts as a barrier restricting the permeation of macromolecules. However, a small proportion (10^{-3} to 10^{-4}) of dietary proteins can cross the epithelium to access and activate effector cells,⁴³⁾ and this is one of the risk factors in food allergy. In addition, it has been reported that antigen permeability through the intestinal tract was enhanced in allergic patients.^{53,54)} Hence, it is conceivable that enforcing the barrier function of the intestinal epithelium might help to prevent food allergy (Fig. 1, Step 1).

Based on this idea, we screened food components with inhibitory activity against allergen permeation in an *in vitro* model using the Caco-2 cell monolayer (Fig. 4A). Caco-2, a human colon adenocarcinoma cell line, has been used as a model to estimate the bioavailability of dietary components involving calcium ions,^{55,56)} amino acids and peptides,⁵⁷⁾ and polypeptides.⁵⁸⁾ Caco-2 cells differentiate under standard culture conditions to form confluent monolayers, and acquire

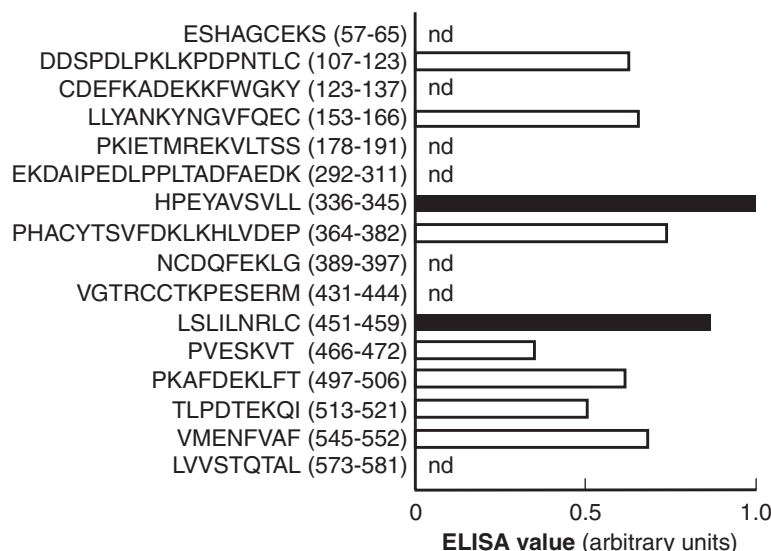


Fig. 3. IgE-Binding Abilities of BSA Peptides.⁵⁰⁾

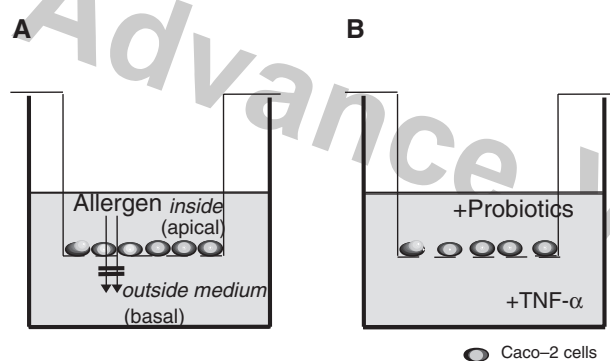


Fig. 4. Schematic Illustration of Evaluation Methods for Inhibitory Activity against Allergen Permeation (A) and for Recovery from Barrier Impairment (B).

many features of absorptive intestinal cells during culture.⁵⁹⁾ The cells spontaneously exhibit various enterocytic characteristics, including the expression of brush border enzymes, nutrient transporters, and the formation of intercellular tight junctions (TJ).⁶⁰⁾ We used Caco-2 cells as an intestinal model and ovalbumin (OVA) as a representative food allergen.

First we selected Edam cheese as the starting material to isolate compounds with such activity. Crude extract of Edam cheese was fractionated on a Sep-Pak-C₁₈ cartridge and reverse-phase HPLC, accompanied by activity measurement of each fraction. By ¹H-NMR and N-terminal amino acid analyses, the active compound was identified as Asn-Lys-Ile-His-Pro-Phe from aa47–52 of β -casein.⁶¹⁾ This hexapeptide showed remarkable inhibition of OVA permeation at concentrations of 10^{-5} M to 10^{-7} M.

Next we searched the peptides with the same activity from enzymatic hydrolysate of cheese (EHC). EHCs are generally produced by hydrolysis of cheese with

commercial proteases, and are used in the food in several ways, such as the sole source of cheese flavor in a product, to intensify an existing cheesy taste, or to give a specific cheese character to a more bland-tasting cheese product.⁶²⁾ Amino acid sequence analysis identified the active peptide from the EHC as GPIVLNPWDQ, a sequence identical to aa102–111 of α s₂-casein.⁶³⁾ This decapeptide significantly inhibited OVA permeation at a concentration of 10^{-6} M. It was also found that a pentapeptide half, NPWDQ, is essential to the inhibitory activity, since NPWDQ, but not GPIVL, had nearly the same inhibitory activity as GPIVLNPWDQ.⁶³⁾

The results suggest a novel point concerning the significance of lactation in preventing protein (allergen) permeation in the small intestine, since this kind of peptide would be produced from casein by proteases in the infant digestive tract, and its production might prevent allergen permeation. There exist at least two possible mechanisms by which GPIVLNPWDQ and NPWDQ could inhibit OVA permeation in Caco-2 cells: (i) inhibition of the endocytosis of OVA, and (ii) the enforcement of TJ functions. To assess changes in the expression of TJ-associated proteins, western blotting analyses were performed using anti-claudin-1, anti-occludin, and anti-ZO-1 antibodies. Of the three TJ-associated proteins, only the claudin-1 level was changed by NPWDQ peptide treatment of Caco-2 cells; that is, NPWDQ significantly increased claudin-1 expression (manuscript in preparation). Hence, it is presumed that casein-derived peptides, such as NPWDQ, inhibit allergen permeation at least in part by regulation of epithelial tight junction.

In addition, we found that the hypoallergenic wheat flour described above possessed potent inhibitory activity against OVA permeation, and that the amino acid sequence of the active compound was WSNS-

GNFVGGK, which was derived from the cellulase preparation used to produce the flour.⁶⁴⁾ According to a structure-activity relationship study, we also confirmed that Trp residues without a free carboxyl group are required for the inhibitory activity.⁶⁴⁾

Now I am undertaking research on inflammatory bowel disease (IBD) using a similar *in vitro* model (Fig. 4B). A breakdown of the epithelial barrier is implicated in this disease, in which tumor necrosis factor- α (TNF- α) has been found to play an important role.⁶⁵⁾ It is believed in clinical practice that probiotic bacteria have properties possibly effective in treating and preventing relapse of IBD,⁶⁶⁾ but the mechanisms by which probiotic/commensal bacteria regulate IBD and the compounds responsible for the activities have not been identified. We found that *Enterococcus hirae*, a mucosal bacterium, reversed TNF- α -induced impairment and IL-8 production in Caco-2 cells effectively.⁶⁷⁾ It has been determined that Toll-like receptor (TLR)-2 signaling is crucial for preserving the intestinal barrier functions by us⁶⁷⁾ and by other groups.⁶⁸⁾ This finding should contribute greatly to the treatment of IBD as well as food allergy.

3. Inhibition of degranulation of mast cells by hesperetin in *Citrus unshiu*

Degranulation of mast cells and subsequent release of inflammatory mediators such as histamine are the last step to occur in the development of allergic symptoms (Fig. 1, Step 6). Hence substances such as some kinds of flavonoids that can inhibit degranulation are expected to show anti-allergic activity.⁶⁹⁾ We selected *Citrus unshiu* and attempted to evaluate its activity. The fruits were squeezed for orange juice making by a standard method, and the residue was powdered. Fifty percent methanol extract of *Citrus unshiu* powder (MEC) showed potent inhibitory activity against histamine release from basophils of patients suffering from seasonal allergic rhinitis to cedar pollen.⁷⁰⁾ To examine this anti-allergic mechanism in detail, we proceeded to use rat basophilic leukemia RBL-2H3 cells. MEC significantly inhibited IgE-induced release of histamine and β -hexosaminidase from RBL-2H3 cells.⁷⁰⁾ Since MEC contained a variety of flavonoids, we selected nobiletin, hesperetin, and hesperidin (hesperetin glycoside formerly called vitamin P) as representative compounds, and further evaluated their inhibitory activities. Among the flavonoids tested, hesperetin was the most potent, on the while, hesperidin had far less, if any, inhibitory activity.⁷⁰⁾ The mechanism by which flavonoids inhibited degranulation process was then examined. Hesperetin and nobiletin suppressed phosphorylation of Akt-1, a direct downstream effector of phosphatidylinositol 3-kinase (PI3-K).⁷⁰⁾ Hence, it was assumed that these flavonoids suppress IgE-mediated stimulation of basophils through the PI3-K pathway.

Next we evaluated the effects of MEC on cytokine production in peripheral blood mononuclear cells

(PBMCs) obtained from patients with seasonal allergic rhinitis to cedar pollen.⁷¹⁾ The levels of cytokines, such as TNF- α , IFN- γ , IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IL-13, and GM-CSF, produced by pollen-stimulated PBMC were measured. We found that MEC suppressed pollen-induced TNF- α release and increased IFN- γ release from PBMCs. These results suggest that *Citrus unshiu* powder has an immunomodulatory effect *in vitro* and that proper use of it would be effective in managing seasonal allergic rhinitis to cedar pollen.

In using *Citrus unshiu* powder, it is important to take the bioavailability of hesperetin into consideration. Hence, the cell permeability of hesperetin and hesperidin was measured using Caco-2 monolayers.⁷²⁾ In the presence of a proton gradient, hesperetin permeated cells in the apical-to-basolateral direction more than 400-fold higher than that of hesperidin. Hesperetin was efficiently absorbed from the intestine; on the other hand, the transport of hesperidin was mainly *via* paracellular diffusion and was highly dependent on conversion to hesperetin *via* the hydrolytic action of microflora. In a previous article,⁷²⁾ we offer insight into the absorption characteristics of hesperetin, that is, into proton-coupled and energy-dependent polarized transport.

4. Detection method of the allergic ingredients in processed foods

Labeling of food allergens is required in many. In Japan, labeling is divided into two stages, mandatory and recommended, according to the number of cases of actual illness and the degree of seriousness.⁷³⁾ The Ministry of Health, Labor, and Welfare has made it mandatory to declare five food items (eggs, milk, wheat, buckwheat, and peanuts), and has announced its intention to declare another 20 items (abalone, squid, salmon roe, shrimp/prawn, oranges, crab, kiwi fruit, beef, tree nuts, salmon, mackerel, soybeans, chicken (poultry), pork, mushrooms, peaches, yams, apples, gelatin, and bananas).⁷³⁾ As mentioned above, beef, chicken, and pork are recommended to be separately labeled in Japan. Pork has also become one of the labeled foods among 11 items in Korea.

To verify allergen labeling of foods and in order to identify hidden allergens in processed foods, it is important to provide sensitive and specific detection methods. In general, these methods are based on the detection of species-specific proteins by enzyme-linked immunosorbent assay (ELISA) and of species-specific DNA molecules by polymerase chain reaction (PCR).⁷⁴⁾ PCR leads to *in vitro* amplification of specific target DNA sequences using the appropriate oligonucleotide primer pairs. This technique offers a high level of sensitivity, and an optimized PCR procedure amplifies the specific target sequence even in a very complex pool of genomic sequences.

We have developed a specific PCR method to detect porcine DNA in foods.⁷⁵⁾ The primer pair was designed to detect the gene encoding porcine cytochrome b for the

specific detection of pork with high sensitivity. The amplified DNA fragment (130 bp) was specifically detected from porcine DNA, while no amplification occurred with other species, such as cattle, chicken, sheep, and horse. When the PCR method developed was used to investigate commercial food products, porcine DNA was clearly detected in those containing pork in the list of ingredients. In addition, 100 ppb of pork in heated *gyoza* (pork and vegetable dumpling) was detected by this method. This method is specific and sensitive, making it applicable in detecting trace amounts of pork in processed foods.

We have also designed PCR primers based on chicken gene and have confirmed specific amplification of chicken from raw materials, certain kinds of model processed foods, and commercial food products.⁷⁶⁾ To examine the specificity and cross-reactivity of these primer sets, DNA from other edible meat species (quail, duck, turkey, pig, cattle and sheep) was tested. No DNA amplification was observed with DNA extracted from quail, duck, turkey, pig, cattle, or sheep.⁷⁶⁾ In addition, a rapid real-time PCR quantitative PCR detection method for pork, beef, chicken, horseflesh, and mutton in foods is now being developed.⁷⁷⁾

This line of project is useful not only in verifying allergen labeling and identifying hidden allergic ingredients in processed foods for daily life, but also in the detection of hidden and/or disguised meat mince in processed foods, which would verify ingredient labeling and gain consumers' trust. This is conducted as a national project of the Ministry of Health, Labor, and Welfare of Japan, with National Institute of Health Science, Itoham Foods, Inc., Nippon Meat Packers, Inc., and Marudai Food Co.

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