REVIEW ARTICLE

An Overview of Peanut Allergenicity and Enzymatic Treatment for Reducing It

Author

Jianmei Yu

Affiliation:

Department of Family and Consumer Sciences, North Carolina Agricultural and Technical State University, Greensboro, NC 27411 USA

Correspondence:

Jianmei Yu

Email: jyu@ncat.edu

Tel.: (+1) 336-285-4861

Fax: (+1) 336-334-7239

Abstract

Peanut allergy is one of the most severe food allergies. It represents a serious health issue and a food safety issue in the developed country. The peanut allergy is triggered by allergenic peanut proteins and 17 such proteins have been identified. Among these proteins, Ara h 1, Ara h 2, Ara h 3 and Ara h 6 are defined as major allergens because of their high contents in the peanut and their high allergenic potential. The eliciting dose of peanut allergy is low and the severity of allergic reaction increased with peanut protein dose. Due to the increasing applications of peanuts in food products, it is very difficult for people who are sensitive to completely avoid from exposure to peanut. Technologies that can reduce the allergenicity of peanuts will greatly contribute to the allergic safety of peanuts, reduce the severity of allergic reaction due to accidental exposure, and ease the stress of individuals who are allergic to peanuts. Although many methods have been proposed to reduce allergenicity or immunoreactivity of peanut and other food protein, enzymatic treatment is apromising because it is safer and practical although this method cannot completely desensitize peanut allergens. This review covered the characteristics of peanut allergy, methods of allergenicity evaluation, and effects of proteolytic hydrolysis on the allergenicity of peanut protein, peanut flour and peanut kernels.

Key words: Peanut allergy, Peanut allergens, Allergenicity evaluation, IgE-binding, Proteolytic enzymes, Enzymatic hydrolysis

1. Introduction

Although more than 170 foods have been reported to cause allergic reactions, milk, egg, peanut, tree nuts, wheat, soy, fish and crustacean shellfish are the eight major food allergens responsible for most of the serious food allergic reactions in the United States.[1]Peanut allergy is a growing public health problem. It is a medical issue and food safety issue. The prevalence of peanut allergy among children in the United States increased from 0.4% in 1999to approximately 2% among children in 2010 in a national survey [2, 3], similar to the results reported in a regional cohort.[4] Worldwide, peanut allergy is more prevalence in developed countries such as the Canada, United State, European countries, Australia. The incidence and severity of peanut allergy seems to be on the rise in recent years. In 2002, about 0.8% of young children and 0.6% of adults were reported to be allergic to peanuts in the United States and this rate increased to 1.4 % in 2008.[5, 6] In Canada, the percentage of children allergic to peanuts also increased from 1.3% in 2000-2002 to 1.6% in 2005-2007.[7] There is no cure about peanut allergy so far although early oral immunotherapy has been reported to be a safe and highly effective treatment.[8] Avoidance has been the best practice to protect peanut sensitive individuals from the risk of peanut allergy. To protect consumers, food companies in the US are mandatorily required to label any possible allergens in the prepacked food products according to Food Allergen Labeling and Consumer Protection Act of 2004.[9] However, It is very difficult for people who are sensitive to peanut to completely avoid from exposure to peanut because not everybody reads the label before consuming the food and because the wide applications of peanut or peanut-derived products in different types of foods as protein source and flavor enhancer.[10] Up to 75% of individuals with known peanut allergy experience reactions caused by accidental exposure.[11] This accounts for about 59% of the reported allergy related deaths.[12] The causes for this increase remain unclear underscoring the need to develop new methods to inactivate allergens before they cause allergic reactions.

2. Characteristics of Peanut Allergy

Peanut allergy is a typical IgE-mediate type I hypersensitivity.[13] The symptoms of an IgE-mediated disorder are typically related to the skin, gastrointestinal tract, and respiratory tract.[14] Most food allergies, such as allergies to milk, egg, wheat and soy, begin early in life and often resolve during their childhood, but allergies to peanut, tree nuts, fish, and shellfish usually persist, becoming a lifelong burden.[1] People who are allergic to peanuts seldom outgrow.[15] For example, 65% of children outgrow their wheat allergy by age12, 55% of children with egg allergy and 45% with milk allergy outgrew their symptoms by age 6-7, but only 16-22% outgrew their peanut allergy, 13% out outgrew their shellfish allergy.[15-18, 3] In addition, peanuts can cause a severe, potentially life-threatening allergic reaction (anaphylaxis). The allergic reactions can be unpredictable, and even very small amounts of peanut can cause one. Peanut allergy is one of the most common causes of food-induced anaphylaxis (FIA) in the United States, United Kingdom, and Australia and peanut and tree nuts are overwhelmingly and

disproportionately represented in case series of severe and fatal outcomes, severe allergic reaction, and visits to the emergency department for food anaphylaxis.[19]

3. Eliciting dose of peanut to cause objective allergic symptoms

Peanut allergy is triggered by allergenic proteins and peptides in peanuts.Accidental ingestion of a small amount of any peanut product can produce lethal allergic reactions among hypersensitive individuals.[12] The threshold dose inducing symptoms varies a great deal according to the individual and from study to study. The lowest observed adverse-effect level (LOAEL) determined by standardized double-blind placebo-controlled food challenges is usually used as eliciting dose.[20] It was reported that the minimum dose of peanut protein capable of eliciting an allergic reaction in highly sensitized individuals was 100µg for subjective symptom and 2mg for objective signs.[21] Studies in children under 5 years of age show that 10 mg of defatted peanuts caused subjective symptoms while 100 mg to 3g caused objective symptom. For the adults, the doses of defatted, roasted peanut flour that trigger subjective and objective symptoms were found to be 10mg and 300mg - 3g, respectively.[12] A study conducted in Demark with 487 patients (age 0.5-73.5 years) shows that the objective dose was 106.5 (59.7-190.6) mg roasted peanut, and adults showed more severe symptoms and signs than children, and peanut caused more severe reactions than the egg, milk and whole hazelnut.[22] However, a study conducted in Netherland found that objective eliciting doses (ED05) values for children and adults were comparable (2.86 mg peanut protein in adults and 6.38 mg in children).[23] Another study conducted in Ireland shows that a single administration of 1.5 mg of peanut protein elicited objective reactions in fewer than the predicted 5% of patients (average age 6.8 years) with peanut allergy and this dose of was suggested as safe single-dose for oral challenge.[24] A retrospective analysis of allergic reaction severities and minimal eliciting doses for peanut, milk, egg, and soy oral food challenges concluded that peanut allergic patients who experienced severe reactions had significantly higher minimum eliciting doses and threshold distribution doses than those who experienced mild and moderate reactions.[25] The determination of eliciting dose can help industry choose tests with a level of sensitivity capable of detecting food allergens hidden in industrial products and to specify protective measures for highly allergic individuals in order to prevent recurrent severe anaphylaxis.[26]

4. Allergenic Peanut Proteins

Many studies have been conducted to characterize the specific proteins responsible for peanut allergy. So far, 17 allergenic proteins in the peanuts have been identified as shown in Table 1.[27-28] Among these proteins, Ara h 1, Ara h 2, Ara h 3 and Ara h 6 have been considered as major peanut allergens due to their high content in peanut or high allergenic potential. Ara h 1 is a 64 kD protein that comprises 12-16% of the total peanut protein. Ara h 2 (16-17 kDa) accounts for 5.9–9.3% of the total peanut protein.[29] It has been reported that all known peanut allergens comprise 85% of the total protein content of peanut while Ara h 1, Ara h 2, and

Ara h 3 together account for 75%.[30] Ara h 6 has been recently recognized as potent as Ara h 2. Ara h 6 shares 59% sequence identity with Ara h 2. Ara h 2 and Ara h 6 have similar immunoreactivity in chimeric IgE ELISA and are considered the most potent peanut allergens accounting for the majority of effector activity in peanut extracts.[31-35]

Research also shows that peanut allergy has different clinical and immunologic patterns in different geographical areas of the world. A study conducted in Netherland found that Ara h 1 is not a major allergen for the participants.[36] American patients frequently had IgE antibodies to rAra h 1 to 3 (56.7% to 90.0%) and often presented with severe symptoms; Spanish patients recognized 3 recombinant peanut allergens (rAra h 1 to 3) less frequently (16.0% to 42.0%), but were more sensitized to the lipid transfer protein rAra h 9 (60.0%); while Swedish patients detected rAra h 1 to 3 more frequently than Spanish patients (37.1% to 74.3%) and had the highest sensitization rate to the Bet v 1 homologue rAra h 8 (65.7%).[37]

Peanut proteins were found to contain multiple binding sites for immunoglobulins. These binding sites are called epitopes which contains different types and number of amino acids. Each peanut allergen contains many IgE-binding epitopes and so far the epitopes of Ara h1, Ara h 2, Ara h 3, Ara h 6 have identified by different researchers.[38] Twenty epitopes have been identified within the molecule of Ara h 1, 26 epitopes for Ara h 2, 7 epitopes for Ara h 6and 5 epitopes for Ara h 3.[39-44] The lengths of epitopes vary greatly and the smallest eiptope identified is 6 amino acids in length. The structural modification or breakdown of epitopes should contribute to reduced allergenicity of peanut proteins.

Table 1. Major peanut allergens, their biochemical name and molecular weight (MW) [27]

Allergen	**Biochemical name**	**MW(SDS-PAGE)**
Ara h 1	Cupin (Vicillin-type, 7S globulin)	64
Ara h 2	Conglutin (2S albumin)	17
Ara h 3	Cupin (Legumin-type, 11S globulin, Glycinin)	60, 37 (fragment)
Ara h 4	renamed to Ara h 3.02, number not available for future submissions	
Ara h 5	Profilin	15
Ara h 6	Conglutin (2S albumin)	15
Ara h 7	Conglutin (2S albumin)	15
Ara h 8	Pathogenesis-related protein, PR-10, Bet v 1 family member	17
Ara h 9	Nonspecific lipid-transfer protein type 1	9.8

Ara H 10	16 kDa oleosin	16 kDa
Ara h 11	14 kDa oleosin	14 kDa
Ara h 12	Defensin	8 kDa (reducing), 12 kDa (non-reducing), 5.184 kDa (mass)
Ara h 13	Defensin	8 kDa (reducing), 11 kDa (non-reducing), 5.472 kDa (mass)
Ara h 14	Oleosin	17.5 kDa
Ara h 15	Oleosin	17 kDa
Ara h 16	non-specific Lipid Transfer Protein 2	8.5 by SDS PAGE reducing
Ara h 17	non-specific Lipid Transfer Protein 1	11 kDa by SDS-PAGE reducing

5. Methods for evaluating the allergenicity of peanuts

Theoretically, any method that is used to diagnose peanut allergy can be used to test the allergenicity of peanuts or peanut derived product. However, some of the methods such as skin prick test and oral challenge are restricted to clinical research. There are both *in vitro* and *in vivo* methods which can provide important information about the relative allergenicity of a food product. The *in vitro* methods include IgE-testing, competitive inhibition ELISA, immune-blotting, and basophil activation methods.

Because peanut allergy is IgE-mediated immunoreactivity, the plasma and sera of patients allergic to peanut or animals sensitized with peanuts contain peanut specific IgE. Conventional IgE testing uses natural extracts containing a complex mixture of proteins, while allergen sIgE to component allergen tests for IgE binding to single allergens, allowing more precise profiling of the allergen-sIgE repertoire.[45] Western blot with individual or pooled sera containing peanut specific IgE can give detailed information about the allergenicity of specific proteins.[35, 46-48] The competitive inhibition ELISA (CiELISA) measures the ability of a protein extract to inhibit the IgE from binding to allergens using pooled plasma and can provide information about the relative allergenicity of several peanut extracts.[29, 49, 50]

IgE test using serum samples from individuals with peanut allergy history is an important adjunct tool inaccurate identification of causal food allergens.[51]The human's specific IgE antibody measurement has been useful in diagnosis with specific levels predictive of diseaseseverity in egg and peanut allergies.[52, 6] But in some unusual cases, a clinical reactivity may be absent despite presence of demonstrable foods specific IgE antibodies.

Component-resolved diagnostics (CRD) tests the specific IgE (sIgE) to specific allergens. This method can be used to identify major causal allergens of different individuals. Some recent studies found that Ara h 2 was most relevant in peanut allergy of children and adults, and both Ara h2 and Ara h 6 were best predictor of adult peanut allergy.[53-54] A systematic review found that the best combination of diagnostic accuracy measures was found for sIgE to Ara h 2, while the worst

diagnostic accuracy measures were found in general for sIgE to Ara h 8 and sIgE to Ara h 9. This finding was independent of geographical location. Compared to SPT and sIgE to peanut extract, sIgE to Ara h 2 was mainly superior in diagnosing peanut allergy in case of a positive test result.[23] Therefore, accurate quantification of Ara h 2 and Ara h 6can help predict the allergenicity of peanut or peanut derived product.

Basophil activation test (BAT) is a flow cytometry-based functional assay that uses live basophils in whole blood to detect the ability of IgE to mediate activation of basophils after stimulation with allergen. It measures the expression of activation markers such as CD63 or CD203c on the surface of basophils following stimulation with allergen.[55] BAT should be performed as early as possible after blood sample is withdrew, preferably within 4 hour because the expressions of CD63 and CD203C start decreasing after blood withdrawing.[56] BAT provide information about the cross-link capacity of allergenic proteins.[57] This method also effectively discriminates between allergy and tolerance in peanut-sensitized children, showing 97% accuracy, 95% positive predictive value, and 98% negative predictive value.[58] The findings of a recent study with 47 people severe allergy to peanuts, 22 subjects with peanut sensitization and 22 control subjects, all in the age range of 18–60 years, show that BAT-detected reactivity to peanut was significantly higher in patients who had a history of severe allergy to peanuts, as compared with patients who were sensitized to peanuts ($p < 0.001$), and the receiver operating curve (ROC) analysis showed that BAT had high sensitivity and specificity for predicting severe peanut allergy.[59] Despite the fact that the BAT has been validated for a wide-range of IgE-mediated conditions, there is still considerable variation in the performance of this test.[60] This method is interfered by several factors including population, study design, purity and concentration of allergens or extract, interval of blood collection and BAT performance procedure, etc. [58] It was suggested to limit BAT to subjects with a discrepancy between history and traditional tests, particularly, when total IgE were low.[61]

Other *In vitro* evaluation methods include bioinformatic analysis, serum screening, simulated gastric digestion, and heat stability as well as cell models, which may be widely used in studies to explore allergic pathways and mechanisms.[62]

In vivo evaluation methods include animal models and human clinical trials. Mouse and rat are small animalscommonly used to study food allergy, while pigs, dogs, and sheep are the main examples of large animal models that have been used for food allergy studies.[63] Among different mouse strains, C3H/HeJ and BALB/c more readily display Th2 responses than other common murine strains without developing oral tolerance.[64] Brown Norway (BN) rat is the strain most suitable for inducing specific IgE after oral sensitization.[65] However, there are reports that BALB/c mice can develop a robust IgE antibody response to peanuts without clinical reactivity.[66]

Skin prick test (SPT) using patients who are allergic to peanuts is the most common methods. SPT is an indirect measure of specific IgE bound to skin mast cells and it has been widely used for the diagnosis of IgE-

mediated allergic disease because the results are available quickly.[67] A positive SPT, with specificity < 100%, does not necessarily prove that the food is causal. In contrast, a negative SPT, with a negative predictive accuracy > 90%, suggests the absence of IgE-mediated allergic reactivity.[68] A recent study found that in children with anaphylaxis to peanut, basophil activation, peanut SPT and the ratio of peanut sIgE/total IgE were associated with reactivity threshold and LOAEL, but not with allergy reaction severity.[69] The golden standard method to diagnose if somebody is allergic to peanut or to test if a food item is allergenic to a specific population is oral food challenge (OFC). But OFC is dangerous because it can trigger severe reactions and must be administered in a specialized facility capable of dealing with anaphylactic shock.[70]

6. Current methods to reduce peanut allergenicity

Because peanuts are directly consumed and widely used in many food products as an ingredient, it is imperative to reduce the levels of allergenic proteins in peanuts and peanut derived products before they are mixed with other food ingredients in order to protect consumers from potential life threatening allergic reactions related to accidental peanut exposure in addition to labeling the presence of peanut. Many approaches have been studied to reduce allergenicity of peanuts and peanut protein extracts. These approaches include genetic, physical, chemical and enzymatic modifications of peanut proteins. Most of these approaches can be found in two reviews.[71-72] This review discusses enzymatic modifications of peanuts for allergenicity reduction.

Two types of enzymatic modifications were reported. One is cross-linking of allergenic proteins to bury the epitopes. Proteins can become cross-linked with other proteins or polysaccharides in the presence of peroxidase or transglutaminase because proteins contain tyrosine and glutamine residues.[49, 50] According to the amino acid sequences of identified epitopes of Ara h 1, Ara h 2, Ara h 3 and Ara h 6., only a few epitopes contain tyrosin and glutamine [43, 73], thus enzymatic cross-linking may not be an effective approach to reduce the allergenicity of peanut protein. Another type is proteolytic hydrolysis of allergenic proteins to breaks down proteins into fragments/peptides without or with reduced allergenicity. Following discussions will focus on enzymatic hydrolysis on peanut allergenicity.

7. Enzymatic Hydrolysis to Reduce Peanut Allergenicity

In fact, enzymes are known to have played an important part in food production since ancient times. At present, nearly all commercially prepared foods contain at least one ingredient that has been made with enzymes. Enzymatic hydrolysis has long been used in food processing for different purposes, for example, proteases including chymosin, papain, and other peptidases have been used for protein hydrolysis, milk clotting, low-allergenic infant-food formulation, enhanced digestibility and utilization, flavor improvement in milk and cheese, meat tenderizer, prevention of chill haze formation in brewing.[74] Protease preparations have been used by the food

industry to modify the functional properties (such as solubility, viscosity, gelling property, elasticity, cohesion, emulsification, foam stability and whipability of food proteins, to improve flavor and nutritional quality of food products, to produce bioactive peptides, and to produce hypoallergenic food ingredients.[75-78] Hypoallergenic infant formulas are produced from caseins or whey proteins by means of heat denaturation and enzymatic hydrolysis which are sometimes combined with ultrafiltration.[79-82] It was found that incubation of heated whey with Alcalase, flavourzyme or papain for 60 minutes completely degraded the antigenic proteins beta-lactoglobulin.[81] Similar results were obtained by Herranz and Colleagues who used Alcalase, Neutrase and bromelain to hydrolyze principal allergens from milk, β-casein and β-lactoglobulin.[82] Extensively hydrolyzed formulas (EHFs), derived from bovine casein or whey, are tolerated by approximately 95% of individuals with cow's milk allergic.[80]

7.1 Proteolytic enzymes

Proteolytic enzymes are also termed as peptidases, proteases or proteinases. In the United States, the enzymes used in food are under strict regulation of US Food and Drug Administration (FDA). Only those listed as General Recognized As Safe (GRAS) can be used for modification of food ingredients. The proteases on the GRAS list includes bromelain, papain, ficin, pepsin, trypsin, pancreatin, mixedprotease enzyme product derived from *Bacillus licheniformis*,rennet (animal derived) and chymosin preparation, Aminopeptidase enzyme preparation from *Lactococcuslactis* and urease enzyme preparation from *Lactobacillus fermentum*.[83] They are classified into endopeptidases and exopeptidases. The endopeptidase cleaves in the middle of protein molecule, while the exopeptidase acts near the end of polypeptide chains, for example, flavourzyme. Furthermore, exopeptidases are termed aminopeptidases if they act at the N-terminus, and carboxypeptidases are those acting on peptide bonds from the C-terminus.[78]

The specificity of a protease determines the position at which the enzyme will catalyze peptide bond hydrolysis. Some proteases are highly substrate specific, while some are less specific. For example, trypsin acts on lysine and arginine residues, while chymotrypsin acts on large hydrophobic residues such as tryptophan, tyrosine and phenylalanine.[84] Pepsin, an aspartic endopeptidase, is much less specific than serine endopeptidase trypsin and chymotrypsin.[85] The selection of proper protease is usually based on the purpose of hydrolysis. Pepsin is usually used for simulating gastric digestion, trypsin and chymotrypsin are used for simulating intestine digestion. Serine protease subtilisin produced by different strains of Bacillus bacteria has different specificity and catalytic efficiency.[86] Alcalase is a protease preparation dominated by subtilisin from *B. licheniformis*. It is a liquid preparation of serine endopeptidase that can hydrolyze most of peptide bonds with higher specificity for aromatic (Phe, Trp, and Tyr), acidic (Glu), sulfur-containing (Met), aliphatic (Leuand Ala), hydroxyl (Ser), and basic (Lys) amino acid residues.[87] Papain is a typical cysteine proteases from latex of unripe fruit of *Carica papaya*. This enzyme has exceptional stability to heat at neutral pH. Structurally, papain has seven subsites for

binding, and S1 subsite is relatively nonselective, but S2 subsite has strong preference for the hydrophorbic amino acid side chains of phe, Tyr, Val and Leu of substrates.[84] Compared with trypsin and chymotrypsin, Alcalase and papain are much less specific. Flavourzyme is a protease preparation from *Aspergillus oryzae*. Eight enzymes including two aminopeptidases, two dipeptidyl peptidases, three endopeptidases, and one α-amylase have been identified from Flavourzyme preparation obtained from one strain of *Aspergillusoryzae*.[88] This enzyme preparation has been widely used in food industry to improve flavors of animal and plant products.[89-91] The non-selective/specific or less specific proteases are anticipated to be more effective in reducing allergenicity of a protein molecule because they can cleave the larger peptide at many more positions than specific proteases, which may destroy more IgE-binding epitopes.

7.2 Proteolytic hydrolysis of purified peanut allergens

Pepsin and trypsin have been used to investigate the changes of some major peanut allergens during gastric and intestinal digestions, respectively. Western blot analysis of sera from 5 subjects with peanut allergy showed multiple IgE-reactive proteins in crude intact peanut extract that were eliminated after pepsin treatment of the peanut extract. In contrast, pepsin-digested peanut induced significant T-cell proliferation responses *in vitro* in PBMCs from 7 subjects with peanut allergy, albeit at lower levels than that induced by intact peanut.[92] *In vitro* digestion of Ara h 1 with pepsin and porcine gastric fluid resulted in virtually identical hydrolysis patterns as observed on SDS-PAGE.[93] However, at low pepsin concentration, it took very long time (3-20hr) to achieve significant reduction of major allergens (Ara h 1 and Ara h 2) and their allergenicity.[94] The study of Koppelman and colleagues found that Ara h 1 and Ara h 3 were rapidly hydrolyzed by pepsin, but Ara h 2 and Ara h 6 were resistant to pepsin digestion even at very high pepsin concentrations.[95] Trypsin digestion of Ara h 2 resulted in large residual peptides with immunoreactivity similar to Ara h 2. This study also shows that at higher protease concentration, these allergens degraded faster than at lower enzyme concentration although Ara h 2 and Ara h 6 could not be completely hydrolyzed.

7.3 Enzymatic hydrolysis of peanut protein isolate and peanut flour

Typically, proteolytic hydrolysis of peanut proteins are conducted with aqueous suspension of peanut protein isolate or peanut flour for better contact between protease and target molecules. In addition, most of commercially available peanut protein isolate and peanut flour are made from roasted peanuts, in which the enzyme inhibitors are destroyed and the proteins are denatured or partially denatured depending on the degree of roasting. One study found that hydrolysis of roasted peanut protein extract by Alcalase for 90 minutes or the sequential treatment of Alcalase and flavourzyme for 30 minutes resulted in 100% reduction in IgE reactivity and the results were confirmed by Western blot with sera from peanut allergic individuals; the residual allergenicity of protein extracts varied with treatment

conditions; but single enzyme treatment with Flavourzyme for 30 and 300 minutes caused an increase and a 65% decrease in IgE reactivity, respectively.[48] Another study showed that the sequential enzyme treatment by alcalase and flavourzyme of peanut protein extracts resulted in 91.8% reduced IgE-binding in vitro, and only 1 of the 7 peanut sensitive individual had positive reaction to the enzyme treated peanut protein extract in the skin prick test.[96] This study shows that sequential enzyme treatment by Alcalase and flavourzyme not only reduce the allergenicity of peanut protein, but also reduce the allergenicity of other legume proteins. Other studies of using Alcalase to reduce allergenicity of food products other than peanuts also reported that certain allergenicity retained after alcalase treatment.[97-98] These indicate that some fragments produced by alcalase hydrolysis of allergenic proteins may remain epitopes which are and some allergenic proteins/peptides are resistant to further hydrolysis.

Most of the studies regarding enzymatic hydrolysis of peanut proteins and peanut flour could not lead to completely desensitization of peanut allergens. In one study, hydrolysis of peanut flour by Alcalase (pH 8.0, 60 ° C), pepsin (pH 2.0, 37 ° C) or Flavourzyme (pH 7.0, 50 ° C) for 60 min reduced IgE binding capacity as evaluated by Western blotting and inhibition ELISA, but IgE cross-linking capacity determined by basophil activation tests of hydrolysates were comparable to that of nonhydrolyzed control.[57]. However, these researchers also found that Alcalase treatment and sequential treatment by Alcalase and Flavourzyme of peanut flour had minimum IgE-binding and about 50% T-cell stimulation compared to the nonhydrolyzed peanut flour extract.[99] Similar findings were obtained in a study of allergenicity of soybean protein isolate (SPI) hydrolyzed by Alcalase, trypsin, chymotrypsin, bromelain, or papain.[98] This study found that the allergenicity of hydrolysate depended on the type of protease used. While the IgE immunoblot results with individual soybean-allergic sera showed an overall reduction in IgE binding to proteins for the SPI samples hydrolyzed with Alcalase, papain, and trypsin compared to the heated SPI control, the bromelain- and chymotrypsin-digested samples showed comparable staining patterns, and comparable IgE binding with the heated SPI control (observed with seven out of eight soybean-allergic sera used in 1D-immunoblot). More research is needed to overcome the controversial issues.

7.4 Enzymatic hydrolysis of peanut kernels

Peanut belongs to legume and contains many antinutritional factors such as lectin, trypsin inhibitor, alpha-amylase inhibitor, phytic acid, and condensed tannin.[100, 101] A major peanut allergen, Ara h 2, is considered as a trypsin inhibitor.[46] The diffusion of enzyme through peanut kernel can be very difficult and slow. Therefore, enzymatic hydrolysis of proteins in peanut kernels is expected to be more difficult, but it has some advantages over the treatment of peanut protein isolate or peanut flour because treated kernels can be processed into any desired form.

It is well known that the basic mechanism by which enzymes catalyze chemical reactions begins with the binding of the substrate (or substrates) to the active site on the enzyme which causes changes in the distribution of

electrons in the chemical bonds of the substrate and ultimately causes the reactions that lead to the formation of products; the products are released from the enzyme surface to regenerate the enzyme for another reaction cycle.[102] According to this mechanism, it is hypothesized that when peanut kernels are immersed in enzyme solution, the protease initially binds to protein molecules on the surface of kernel to form first product, the regenerated/released enzyme then reaches the protein molecules near surface; by repeating this cycle, enzyme finally creates a path to reach the inner part of the kernels.[103] Some pretreatment such as blanching and ultrasound sonication may loosen the structure of peanut kernels and facilitate the diffusion of enzymes.

Our research group has been conducted research on the enzymatic treatment of peanut kernels for reducing the allergenicity of whole peanut. One of the studies was conducted using digestive proteases trypsin and alpha-chymotrysin to treat both raw and roasted peanut kernels at different enzyme concentrations for different time.[104] This study found that trypsin and chymotrypsin rapidly reduced Ara h 1 and Ara h 2 in roasted peanuts, but the treatment was less effective in the case of raw peanuts even at high enzyme concentration due to the presence of enzyme inhibitors.

For roasted peanuts, sequential treatment by trypsin and α-chymotrypsin demonstrated to be more effective in reducing Ara h 1 and Ara h 2 than single protease and ultrasound sonication seems improved the treatment efficiency.[105] The results of IgE-binding test conducted using competitive inhibition ELISA showed that the IgE-binding of roasted peanut extract from ultrasound pretreatment and sequential treatment by trypsin and alpha-chymotrypsin was about 40% lower compared to the untreated control. Non-specific protease Alcalase produced from *Bacillus licheniformis* was more effective in reducing peanut allergens and more complete degradation of Ara h 2 was achieved by alcalase than by trypsin. Under the best treatment conditions, the average reduction of allergenic potency of roasted peanuts was 50-60% in a human skin prick tests with 9 peanut allergic children.[106]

In our recent study, Alcalase, papain, Neutrase and bromelain were used to treat raw peanuts under the optimal pH andtemperature of each protease. The quantitative results obtained by a sandwich ELISAshow that the reductions of Ara h 1, 2, 3 and 6 in raw peanuts were 99–100%, 95–99%, 35–46% and 85–88%, respectively, depending on the enzyme used and enzyme concentration. Alcalase was the most effective peptidase in degrading Ara h 1, Ara h 2 and Ara h 6 of raw peanuts; papain was effective in reducing Ara h 1 and Ara h 2, but less effective than alcalase in reducing Ara h 6; bromelain was less effective in reducing Ara h 2 and Ara h6 compared with Alcalase and papain; and Neutrase was the least effective protease in reducing Ara h 2 and Ara h 6.[107]. SDS-PAGE did not show obvious presence of Ara h 3 in the extracts of protease hydrolyzed peanuts, but the sandwich ELISA detected significant quantity of Ara h 3. This suggests that the quantification method of Ara h 3 may need to be further validated.Western blots show that the IgE-bindings of both soluble and insoluble proteins of Alcalase and papain treated raw peanuts were the lowest

although not completely eliminated. It was also found that roasting of enzyme-treated peanuts significantly reduced the solubility of Ara h 3 and Ara h 6 ($P < 0.0001$), but did not significantly affect Ara h 2. The post-enzyme treatment roasting slightly enhanced IgE-binding of both soluble and insoluble portion of the peanuts.[103]

8. Conclusion

Overall, enzymatic hydrolysis can reduce the allergenicity of peanut proteins to different degrees depending on the type of enzymes and the degree of hydrolysis. Less specific proteases such as Alcalase and papain are generally more effective in hydrolyzing peanut proteins than specific protease, especially for raw peanuts. However, proteolytic hydrolysis cannot completely desensitize peanut due to the presence of resistant allergens. These studies also confirmed that Ara h 1 can be quickly and completely hydrolyzed by any of most of proteases tested, but Ara h 2 and Ara h 6 are more resistant to the enzymatic digestion even at higher enzyme concentration. Therefore, to achieve complete desensitization, enzymatic hydrolysis of peanut or peanut derived products may need to be combined with other techniques. However, the allergenicity of peanut protein or protein extract obtained from different methods have been evaluated by *in vitro* IgE-binding test, *ex vivo* basophile activation test or *in vivo* skin- prick-test. It is not very clear how well these tests represent the clinical reality. In addition, most of the allergenicity testing methods only tests the soluble portion of the peanut or peanut protein extracts, the allergenicity of insoluble portion is still unknown. Therefore, oral challenge study of enzymatically modified products should be conducted to evaluate the allergenicity of enzyme treated peanut products.

Acknowledgments

The author of this article would like to express my sincere gratitude to USDA-NIFA and Agricultural Research Program at North Carolina A&T State University for the support of my research and writing.

Author contributions

Jianmei Yu wrote this manuscript.

Conflicts of Interest

The authors declare no conflicts of interest

References

1. Food Allergy Research Education [www.fare.org]. Facts and Statistics. McLean, VA 2018. Available from: https://www.foodallergy.org/life-food-allergies/food-allergy-101/facts-and-statistics.
2. Sicherer SH, Muñoz-Furlong A., Burks AW, Sampson HA. Prevalence of peanut and tree nut allergy in the US determined by a random digit dial telephone survey. J Allergy Clin Immunol. 1999; 103: 559–562.
3. Gupta RS, Springston EE, Warrier MR, et al. (2011). The prevalence, severity, and distribution of childhood food allergy in the United States. Pediatrics. 2011; 128: 29-e17.
4. Bunyavanich S, Rifas-Shiman SL, Platts-Mills TA, Workman L, Sordillo JE, Gillman MW, et al. Peanut allergy prevalence among school-age children in a US cohort not selected for any disease. J Allergy Clin Immunol. 2014; 134 (3), 753–755.
5. Sampson HA. Update on food allergy. J Allergy Clin Immunol. 2004; 113: 805-819.
6. Sicherer SH, Sampson HA. Food allergy. J Allergy Clin Immunol. 2010; 125, S116-25.
7. Ben-Shoshan M, Kagan RS, Alizadehfar R, Joseph L, Turnbull E, Pierre YS Clarke AE. Is the prevalence of peanut allergy increasing? A 5-year follow-up study in children in Montreal. J Allergy Clin Immunol. 2009; 123: 783-8.
8. Vickery BP, Berglund JP, Burk CM, Fine JP, Kim EH, Kim JI et al. Early oral immunotherapy in peanut-allergic preschool children is safe and highly effective. J Allergy Clin Immunol. 2017; 139:173-81.
9. FDA, 2004. Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA). https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Allergens/ucm106187.htm.
10. Kline M. Manufacturing Foods with Peanut Ingredients (Abstract). In: Stalker HT and Wilson RF, Editors. Peanuts-genetics, processing, and utilization. San Diego: Academic Press and AOCS Press, 2016. p. 429-445.
11. Kagan RS, Joseph L, Dufresne C, Gray-Donald K, Turnbull E, Pierre Y, Clarke AE. Prevalence of peanut allergy in primary school in Montreal Canada. J Allergy Clin Immunol. 2003; 112:1223-8.
12. Sicherer SH, Sampson HA. Peanut allergy: Emerging concepts and approaches for an apparent epidemics. J Allergy Clin Immunol. 2007; 120: 492-503.
13. Al-Muhsen A, Clarke AE, Kagan RS. Peanut allergy: an overview. Canadian Med Assoc J. 2003; 168 (10)
14. Burks AW. Peanut allergy. Lancet 2008; 371: 1538–46
15. Collins SC. Outgrowing Food Allergies-Evidence Shows Multiple Factors Affect Outcome. Today's Dietitian. 2013; 15(5): 12.
16. Keet CA, Matsui EC, Dhillon G, Lenehan P, Paterakis M, Wood RA. The natural

history of wheat allergy. Ann Allergy Asthma Immunol. 2009; 102(5):410-415.
17. Skolnick HS, Conover-Walker MK, Koerner CB, Sampson HA, Burks W, Wood RA. The natural history of peanut allergy. J Allergy Clin Immunol. 2001; 107(2):367-374.
18. Peters RL, Allen KJ, Dharmage SC, Koplin JJ, Dang T, Tilbrook KP, et al. Natural history of peanut allergy and predictors of resolution in the first 4 years of life: A population-based assessment. J Allergy Clin Immunol. 2015; 135(5):1257-66.
19. Cianferoni A, and Muraro. Food-Induced Anaphylaxis. Immunol Allergy Clin North Am. 2012; 32(1): 165–195. doi:10.1016/j.iac.2011.10.002.
20. Sicherer SH, Morrow EH, Sampson HA. Dose-response in double-blind, placebo-controlled oral food challenges in children with atopic dermatitis. J Allergy Clin Immunol. 2000; 105(3):582-6.
21. Hourihane JB, Kilburn SA, Nordlee JA, Hefle SL, Taylor SL, Warner JO. Anevaluation of the sensitivity of subjects with peanut allergy to very low doses of peanut protein: a randomized, double-blind, placebo-controlled food challenge study. J Allergy Clin Immunol 1997; 100(5):596-600.
22. Eller E, Hansen TK, Bindslev-Jensen C. Clinical thresholds to egg, hazelnut, milk and peanut: results from a single-center study using standardized challenges. Ann Allergy Asthma Immunol. 2012; 108(5):332-6. doi: 10.1016/j.anai.2012.03.010.
23. Klemans RJ, Blom WM, van Erp FC, Masthoff LJ, Rubingh CM, van der Ent et al. Objective eliciting doses of peanut-allergic adults and children can be combined for risk assessment purposes. ClinExp Allergy. 2015; 45 (7):1237-44. doi: 10.1111/cea.12558.
24. Hourihane JO, Allen KJ, Shreffler WG, Dunngalvin G, Nordlee JA Zurzolo GA, et al. Peanut allergen threshold study (PATS): novel single-dose oral food challenge study to validate eliciting doses in children with peanut allergy. J Allergy ClinImmunol. 2017; 39(5):1583-1590. doi: 10.1016/j.jaci.2017.01.030.
25. Zhu J, Pouillot R, Kwegyir-Afful EK, Luccioli S, Gendel SM. A retrospective analysis of allergic reaction severities and minimal eliciting doses for peanut, milk, egg, and soy oral food challenges. Food ChemToxicol. 2015; 80: 92–100.
26. Moneret-Vautrin DA, Kanny G. Update on threshold doses of food allergens: implications for patients and the food industry. CurrOpin Allergy Clin Immunol. 2004; 4(3):215-9.
27. Becker WM, Jappe U. Peanut Allergens. Chem Immunol Allergy. 2014; 100: 256-267.
28. World Health Organization [www.who.org]. WHO/IUIS: Allergen Nomenclature Subcommittee [updated 2016 Oct 1; cited 2018 Feb 28]. Available from: http://www.allergen.org.
29. Koppelman SJ, Vlooswijk RA, Knippels LMJ, Hessing M, Knol EF, van Reijsen FC, et al. Quantification of major peanut allergens Ara h 1 and Ara h 2 in the peanut varieties Runner, Spanish, Virginia, and Valencia, bred in different parts of the world. Allergy. 2001; 56(2): 132–137.

30. Hebling CM, Ross MM, Callahan JH, McFarland MA. Size selective fractionation and visual mapping of allergen protein chemistry in *Arachis hypogaea*. J Proteome Res. 2012; 11(11):5384–95.
31. Koppelman SJ, Wensing M, Ertmann M, Knulst AC, Knol EF. Relevance of Ara h 1, Ara h 2 and Ara h 3 in peanut allergic patients, as determined by immunoglobulin E Western blotting, basophile-histamine release and intracutaneous testing: Ara h 2 is the most important peanut allergen. ClinExp Allergy. 2004; 34(4): 583–590.
32. Chen X, Zhuang Y, Wang Q, Moutsoglou D, Ruiz G, Ren SE, et al. Analysis of the effector activity of Ara h 2 and Ara h 6 by selective depletion from a crude peanut extract. J Immunol Methods. 2011; 372(1-2):65-70.
33. Chen X, Wang Q, El-Mezayen R, Zhuang Y, Dreskin SC. Ara h 2 and Ara h 6 have similar allergenic activity and are substantially redundant. Int Arch Allergy Immunol. 2012; 160(3):251-8.
34. Kulis M, Chen X, Lew J, Wang Q, Patel OP, Zhuang Y, et al. (2012). The 2S albumin allergens of Arachishypogaea, Ara h 2 and Ara h 6, are the major elicitors of anaphylaxis and can effectively desensitize peanut-allergic mice. Clin Exp Allergy. 42(2): 326–336.
35. Koid, A, Chapman M, Hamilton R, van Ree R, Versteeg S, Dreskin SC, et al. Ara h 6 complements ara h 2 as an important marker for ige reactivity to peanut. J Agric Food Chem. 2014; 62 (1): 206–213.
36. de Jong EC, Van Zijverden M, Spanhaak S, Koppelman SJ, Pellegrom H, Penninks AH. Identification and partial characterization of multiple major allergens in peanut proteins. ClinExp Allergy. 1998; 28(6):743-51.
37. Vereda A, van Hage M, Ahlstedt S, Ibañez MD, Cuesta-Herranz J, van Odijk J, et al. Peanut allergy: Clinical and immunologic differences among patients from 3 different geographic regions. J Allergy Clin Immunol. 2011; 127(3):603-7. doi: 10.1016/j.jaci.2010.09.010.
38. Matsuo H, YokoojiT, Taogoshi T. Common food allergens and their IgE-binding epitopes. Allergol Int. 2015; 64: 332-343.
39. Burks AW, Shin D, Cockrell G, Stanley JS, Helm RM, Bannon GA. Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicilin protein and a major allergen in peanut hypersensitivity. Eur J Biochem. 1997; 245 (2): 334e9.
40. Flinterman AE, Knol EF, Lencer DA, Bardina L, den Hartog Jager CF, Lin J, et al. Peanut epitopes for IgE and IgG4 in peanut-sensitized children in relation to severity of peanut allergy. J Allergy Clin Immunol 2008; 121:737e43.
41. Stanley JS, King N, Burks AW, Huang SK, Sampson H, Cockrell G, et al. Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h 2. Arch Biochem Biophys. 1997; 342:244e53.
42. Shreffler WG, Lencer DA, Bardina L, Sampson HA. IgE and IgG4 epitope mapping by microarray immunoassay reveals the diversity of immune response

to the peanut allergen, Ara h 2. J Allergy Clin Immunol 2005; 116:893e9.

43. Otsu K, Guo R, Dreskin SC. Epitope analysis of Ara h 2 and Ara h 6: characteristic patterns of IgE-binding fingerprints among individuals with similar clinical histories. Clin Exp Allergy. 2015; 45:471e84.
44. Rabjohn P, Helm EM, Stanley JS, West CM, Sampson HA, Burks AW, et al. Molecular cloning and epitope analysis of the peanut allergen Ara h 3. J Clin Invest 1999; 103: 535e42.
45. Santos, AF and Brough HA. Making the most of In Vitro tests to diagnose food allergy. J Allergy Clin ImmunolPract. 2017; 5:237-4.
46. Maleki SJ, Viquez O, Jacks T, Dodo H, Champagne ET, Chung SY et al. The major peanut allergen, Ara h 2, functions as a trypsin inhibitor, and roasting enhances this function. J Allergy Clin Immunol. 2003; 112(1):190-5.
47. Schmitt DA1, Nesbit JB, Hurlburt BK, Cheng H, Maleki SJ. Processing can alter the properties of peanut extract preparations. J Agric Food Chem. 2010; 58(2):1138-43.
48. Cabanillas B, Pedrosa MM, Rodríguez J. Influence of enzymatic hydrolysis on the allergenicity of roasted peanut protein extract. Int Arch Allergy Immunol. 2012; 157, 41–50.
49. Chung SY, Maleki SJ, Champagne, ET. Allergenic properties of roasted peanut allergens may be reduced by peroxidase. J Agric Food Chem. 2004: 52: 4541-4545.
50. Chung SY, Kato Y, Champagne ET. Polyphenol oxidase/caffeic acid may reduce the allergenic properties of peanut allergens. J Sci Food Agric. 2005; 85: 2631–2637
51. Fleischer DM, Burks AW. Pitfalls in food allergy diagnosis: serum IgE testing. J Pediatr. 2015; 166(1): 8-10.
52. Sampson HA. Utility of food-specific IgE concentrations in predicting symptomatic food allergy. J Allergy ClinImmunol. 2001; 107(5):891-6.
53. Klemans RJ, Broekman HC, Knol EF, Bruijnzeel-Koomen CA, Otten HG, Pasmans SG, et al. Ara h 2 is the best predictor for peanut allergy in adults. J Allergy Clin Immunol Pract. 2013; 1(6): 632-638.
54. Klemans RJ, Knol EF, Bruijnzeel-Koomen CA, Knulst AC. The diagnostic accuracy of specific IgE to Ara h 6 in adults is as good as Ara h 2. Allergy. 2014; 69(8):1112-4. doi: 10.1111/all.12424.
55. Santos AF and Lack G. Basophil activation test: food challenge in a test tube or specialist research tool? Clin Transl Allergy. 2016; 6:10. https://doi.org/10.1186/s13601-016-0098-7
56. Sturm GJ, Kranzelbinder B, Sturm EM, Heinemann A, Groselj-Strele A, Aberer W. The basophil activation test in the diagnosis of allergy: technical issues and critical factors. Allergy. 2009; 64(9):1319-26.
57. Shi XL, Guo RS, White BL, Yancey A, Sanders TH, Davis JP, et al. Allergenic properties of enzymatically hydrolyzed peanut flour extracts. Int Arch Allergy Immunol. 2013; 162:123–130.
58. Santos AF, Douiri A, Becares N, et al. Basophil activation test discriminates

between allergy and tolerance in peanut-sensitized children. J Allergy Clin Immunol. 2014; 134(3):645–52.

59. Rentzos G, Lundberg V, Lundqvist C, et al. Use of a basophil activation test as a complementary diagnostic tool in the diagnosis of severe peanut allergy in adults. Clin Transl Allergy. 2015; 5:22.
60. McGowan EC, and Saini S. Update on the Performance and Application of Basophil Activation Tests. Curr Allergy Asthma Rep. 2013; 13(1): 101–109. doi:10.1007/s11882-012-0324-x.
61. Pignatti P, Yacoub M-R, Testoni C, et al. Evaluation of Basophil Activation Test in Suspected Food Hypersensitivity. Cytometry Part B. 2017; 92B: 279–285.
62. Huang K. Safety Assessment of Genetically Modified Foods. Singapore: Springer Nature Singapore Pte Ltd. 2017. p. 63-117.
63. Van Gramberg JL, de Veer MJ, O'Hehir RE, Meeusen ENT, Bischof RJ. Use of animal models to investigate major allergens associated with food allergy. J Allergy, 2013; Article ID 635695, 10 pages, http://dx.doi.org/10.1155/2013/635695.
64. Bailon E, Cueto-Sola M, Utrilla P. Rodriguez-Ruiz J, Garrido-Mesa N and Zarzuelo A, et al. A shorter and more specific oral sensitization-based experiment model of food allergy in mice. J Immunol Methods. 2012; 381(1-2): 41–49.
65. Kroghsbo S, Rigby NM, Johnson PE, Adel-Patient K, Bøgh KL, Salt LJ, et al. Assessment of the sensitizing potential of processed peanut proteins in brown norway rats: roasting does not enhance allergenicity. PLoS ONE. 2014: 9(5): e96475. doi:10.1371/journal.pone.0096475.
66. Morafo V, Srivastava K, Huang CK, Kleiner G, Lee SY, Sampson HA, Li AM. Genetic susceptibility to food allergy is linked to differential TH2-TH1 responses in C3H/HeJ and BALB/c mice. J Allergy Clin Immunol. 2003; 111(5):1122-8.
67. Khan FM, Ueno-Yamanouchi A, Serushago B, Bowen T4, Lyon AW, Lu C, Storek J, et al. Basophil activation test compared to skin prick test and fluorescence enzyme immunoassay for aeroallergen-specific immunoglobulin-E. Allergy, Asthma Clin Immunol. 2012, 8:1.
68. Dupont C. Food Allergy: Recent advances in pathophysiology and diagnosis. Ann Nutr Metab. 2011; 59(suppl 1): 8–18.
69. Reier-Nilsen T, Michelsen MM, Lødrup Carlsen KC, Carlsen KH, Mowinckel P, Nygaard UC, et al. Predicting reactivity threshold in children with anaphylaxis to peanut. Clin Exp Allergy. 2018; 48(4): 415-423. doi: 10.1111/cea.13078.
70. Rancé F1, Abbal M, Lauwers-Cancès V. Improved screening for peanut allergy by the combined use of skin prick tests and specific IgE assays. J Allergy Clin Immunol. 2002; 109(6):1027-33.
71. Stanić-Vučinić D and Veličković TC. Enzymatic and chemical modifications of food allergens. In: Pereira C, editor. Allergic Diseases – highlights in the clinic, mechanisms and treatment. InTech, 2012. p. 83-106. Available from www.Intechopen.com.
72. Yu J. Methods for reducing allergenicity of peanuts and peanut derived products -

their efficacy, feasibility and safety. Austin Food Sci. 2016; 1(3): 1012.

73. Zhou Y, Wang J, Yang X, Lin D, Gao Y, Su Y et al. Peanut allergy, allergen composition, and methods of reducing allergenicity. Int J Food Sci. 2013; 2013:1-8. http://dx.doi.org/10.1155/2013/909140.
74. Chandrasekaran M. Enzymes in Food and Beverage Processing. Boca Baton: CRC Press; 2015. p. 117-138.
75. Chiang W, Shih C, Chu Y. Functional properties of soy protein hydrolysate produced from a continuous membrane reactor system. Food Chem. 1999; 65: 189-194.
76. Tsumura K, Saitoa T, Tsugea K, Ashida H, Kugimiyaa W, Inouyeb K. Functional properties of soy protein hydrolysates obtained by selective proteolysis. LWT-Food Sci Technol. 2005; 38: 255-261.
77. Udenigwe CC and Aluko RE. Food Protein-Derived Bioactive Peptides: Production, Processing, and Potential Health Benefits. J Food Sci. 2012; 71(1): R11-R24.
78. Tavano OL. (2013). Protein hydrolysis using proteases: An important tool for food biotechnology. J Mol Catalysis B: Enzymatic. 2013; 90: 1– 11.
79. Besler M, Steinhart H and Paschke A. Stability of food allergens and allergenicity of processed foods. J Chromatogr B. 2001; 756: 207–228.
80. Bahna SL. Hypoallergenic formulas: optimal choices for treatment versus prevention (Review Article). Ann Allergy Asthma Immunol. 2008; 101: 453-459.
81. Kim SB, Seo IS, Khan MA, Ki KS, Lee WS, Lee HJ, Shin HS, Kim HS. Enzymatic hydrolysis of heated whey: iron-binding ability of peptides and antigenic protein fractions. J Dairy Sci. 2007; 90(9):4033-42.
82. Herranz I, Molina E, and LópezExpósito I. Use of food-grade enzymes to obtain milk proteins hydrolysates with hypoallergenic properties. Clin Transl Allergy. 2013; 3(Suppl 3): P8.
83. US Food and Drug Administration. Washington DC: Enzyme Preparations Used in Food (Partial List). [updated 2018 Jan 4, 2018; cited 2018 Mar 12]. Available from: https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/EnzymePreparations/default.htm.
84. Whitaker JR. Proteolytic enzymes. In: Whitake JR, Voragen AG, Wong DWS, editors. Handbook of Food Enzymology. New York: Marcel Dekker; 2003. p. 993-1018.
85. Ahn J, Cao M, Yu Y, and John R. Engen JR. Accessing the reproducibility and specificity of pepsin and other aspartic proteases. Biochim Biophys Acta. 2013; 1834(6): 1222–1229.
86. Wells JA, Cunningham BC, Graycart, Estell DA. Recruitment of substrate-specificity properties from one enzyme into a related one by protein engineering. Proc Natl Acad Sci USA. 1987; 84(15):5167-71.
87. Doucet D, Otter DE, Gauthier SF, Foegeding EA. Enzyme-induced gelation of extensively hydrolyzed whey proteins by alcalase: peptide identification and determination of enzyme specificity. J. Agric. Food Chem. 2003: 51, 6300-6308.

88. Merz M, Eisele T, Berends P, Appel D, Rabe S, Blank I, et al. Flavourzyme, an Enzyme Preparation with Industrial Relevance: Automated Nine-Step Purification and Partial Characterization of Eight Enzymes. J Agric Food Chem. 2015; 63: 5682−5693.
89. Nchienzia HA, Morawicki RO, Gadang VP. Enzymatic hydrolysis of poultry meal with endo- and exopeptidases. Poult Sci. 2010; 89: 2273–2280. doi: 10.3382/ps.2008-00558.
90. Qi H, Xu Z, Li Y, Ji X, Dong X and Yu C. Seafood flavourings characterization as prepared from the enzymatic hydrolysis of *Undaria pinnatifida* sporophyll by-product. Int J Food Prop. 2017; 20(12): 2867-2876, DOI: 10.1080/10942912.2016.1256302
91. Zhang W, Zhao F, Yang T, Liu S. Protease/peptidase (Flavourzyme) pretreatment on palm kernels improves the aroma of resultant palm kernel oil after kernel roasting. Eur J Lipid Sci Technol. 2017; 119 (8): DOI · 10.1002/ejlt.201600460
92. Hong SJ, Michael JG, Fehringer A, Leung DY. Pepsin-digested peanut contains T-cell epitopes but no IgE epitopes. J Allergy Clin Immunol. 1999; 104: 473-8.
93. Kopper RA, Odum NJ, Sen M, Helm RM, Stanley SJ, Burks WA. Peanut protein allergens: gastric digestion is carried out exclusively by pepsin. J Allergy Clin Immunol. 2004; 114(3): 614-8.
94. Maleki SJ, Schmitt DA, Galeano M, and Hurlburt BK. Comparison of the Digestibility of the Major Peanut Allergens in Thermally Processed Peanuts and in Pure Form. Foods. 2014; 3:290-303. doi:10.3390/foods3020290.
95. Koppelman SJ, Hefle SL, Taylor SL, de Jong GA. Digestion of peanut allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6: a comparative in vitro study and partial characterization of digestion-resistant peptides. Mol Nutr Food Res. 2010; 54(12): 1711-21. doi: 10.1002/mnfr.201000011
96. Kasera R, Singh AB, Lavasa S, Prasad KN, Arora N. Enzymatic hydrolysis: A method in alleviating legume allergenicity. Food Chem Toxicol. 2015; 76: 54–60.
97. Cabanillas B, Pedrosa MM, Rodríguez J, González A, Muzquiz M, Cuadrado C, et al. Effects of enzymatic hydrolysis on lentil allergenicity. Mol Nutr Food Res. 2010; 54, 1266–1272.
98. Panda R, Tetteh AO, Pramod SN, Goodman RE. Enzymatic hydrolysis does not reduce the biological reactivity of soybean proteins for all allergic subjects. J Agric Food Chem. 2015; 63: 9629−9639.
99. Shi X. Enzymatic Hydrolysis for Improving the Value of Peanut Flour Protein. Master's Thesis. Raleigh: North Carolina State University; 2012. p. 58-80. https://repository.lib.ncsu.edu/bitstream/handle/1840.16/7957/etd.pdf?sequence=2
100. Krupa U. Main nutritional and antinutritional compounds of bean seeds – a review. Pol J Food Nutr Sci. 2008, 58(2): 149-155.
101. de Medeiros AF, Rocha MGF, Serquiz AC, Machado RJA, Lima VCO, de Carvalho FMC, et al. Characterization of novel trypsin inhibitor in raw and toasted peanuts using a simple improved

isolation. Acta Chromatogr. 2017; DOI: 10.1556/1326.2017.00353

102. Whitaker JR. Enzyme-catalyzed reactions: experimental factors that affect rates. In: Whitake JR, Voragen AG, Wong DWS, editors. New York: Marcel Dekker; 2003. P. 31-48.
103. Mikiashvili N and Yu J. Changes in immunoreactivity of allergen-reduced peanuts due to post-enzyme treatment roasting. Food Chem. 2018; 256: 188-194.
104. Yu J, Ahmedna M, Goktepe I, Maleki SJ, Cheng H. (2011). Enzymatic treatment of peanut kernels to reduce allergen levels. Food Chem. 127; 1014-1022. doi:10.1016/j.foodchem.2011.01.074
105. Li H, Yu J, Ahmedna M, Goktepe I. Reduction of major peanut allergen Ara h 1 and Ara h 2 in roasted peanuts by ultrasound assisted enzymatic treatment. Food Chemistry, 2013, 141, 762-768. doi:10.1016/j.foodchem.2013.03.049.
106. Yu J, Hernandez M, Li H, Goktepe I, Robinette C, Auerbach A, et al. Allergenicity of roasted peanuts treated with a non-human digestive protease. Food Res Int. 2015; 69: 341-347. doi:10.1016/j.foodres.2015.01.007
107. Yu J and Mikiashvili N. Effectiveness of different proteases on reducing peanut allergen content in raw peanuts. Proceedings of 100th North Carolina Association of Family and Consumer Sciences Annual Conference; 2018 Mar 22-14; USA, Wilmington; 2018.

