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Methods for Reducing Allergenicity of Peanuts and Peanut Derived Products - Their Efficacy, Feasibility and Safety

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Abstract

Peanut allergy, triggered by allergenic peanut proteins, is one of the most severe food allergies. The incidence and severity of peanut allergy seems to be on the rise in past a few decades. Although many kinds of immunotherapy have been proposed for treatment of peanut allergy, 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 ingestion, reduce the stress of individuals who are allergic to peanuts. Many methods have been proposed to reduce or modify peanut allergens to make peanut and peanut derived products less allergenic. These methods include genetic modification, gamma irradiation and pulsed-UV treatment, chemical modifications, enzymatic cross-linking and enzyme hydrolysis. The products produced by different methods may be used for immunotherapy, food products or food ingredient. This review summarized the mechanism and efficacy of each of these methods for allergenicity reduction, analyzed their feasibility and potential issues, and suggested future studies.

Keywords: Peanut allergens; Allergenicity; Genetic modification; Chemical modification; Physical treatment; Enzymatic modification

Abbreviations

CDC: Center for Diseases Control and Prevention; ciELISA: competitive inhibition ELISA; ELISA: Enzyme-Linked Immunosorbent Assay; FALCPA: Food Allergen Labeling and Consumer Protection Act; IgE: Immunoglobulin E; IgG: Immunoglobulin G; GA: Glutaraldehyde treatment; mRNA: messenger RNA; NHR: N-acetylhexosaminidase Release; POD: Peroxidase; PUV: Pulsed Ultraviolet; RA: Reduction/Alkylation treatment; RA-GA: Reduction/Acylation-Glutaraldehyde; RNAi: RNA interference; TGA: Transglutaminase; SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Introduction

Food allergy represents a specific type of food safety issue. Based on numerous studies, food allergy likely affects nearly 5% of adults and 8% of children, with growing evidence of an increase in prevalence [1]. The Food Allergen Labeling and Consumer Protection Act (FALCPA) of 2004 identifies eight foods or food groups as the major food allergens. They are milk, eggs, fish (e.g., bass, flounder, and cod), Crustacean shellfish (e.g., crab, lobster, shrimp), tree nuts (e.g., almonds, walnuts, pecans), peanuts, wheat, and soybeans [2].

Peanut allergy is a severe and lifelong type of food allergy 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 [3]. About 0.6-1.3% of US populations including 400,000 school age children are allergic to peanuts [4] which prevent them from enjoy this nutritious and delicious product. Studies in children under 5 years of age showed that 10 mg of defatted peanuts causes subjective symptoms while 100 mg to 3 g causes objective symptom. For the adults, the doses of defatted, roasted peanut flour that trigger subjective and objective symptoms were found to be 10 mg and 300 mg - 3 g, respectively [3]. 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 [5], and this rate increased to 1.4 % in 2008 [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]. The causes for this increase remain unclear underscoring the need to develop new methods to inactivate allergens before they cause allergic reactions, especially since up to 75% of individuals with known peanut allergy experience reactions caused by accidental exposure [8]. This accounts for about 59% of the reported allergy related deaths [3].

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 [9,10]. 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 kDa 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 [11]. 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 % [12].

More than 95% of peanut allergic individuals in the U.S. have specific IgE antibodies targeted at Ara h 1 and Ara h 2 [13]. Ara h 3 is a seed storage protein that is recognized by 45% of peanut-allergic

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individuals and also functions as a trypsin inhibitor. Ara h 4 is actually an isoform of Ara h 3. A homology search indicates 91.3% amino acid identity between Ara h 3 and Ara h 4, so Ara h 3 and Ara h 4 are considered to be the same allergens [14,15]. 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 [16-19]. Other allergens are considered minor due to the lower allergenic potency and low contents in the peanuts. However, monosentization to a single peanut allergen is relatively rare [20], and polysensitization of Ara h 2 and Ara h 1 and or Ara h 3 appeared to be predictive of more severe reactions [21,22].

Because peanuts are directly consumed and widely used in many food products as an ingredient, it is imperative to reduce the levels of these allergens in peanuts and peanut derivatives 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 clearly 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 modification of peanut proteins.

Genetic Modification for Peanut Allergen Reduction

Genetic modification is a pre-harvest practice that can interfere or inhibit the synthesis of some allergenic protein during maturation of peanut. In the study of Chu and colleagues [23], RNA interference (RNAi) was used to degrade mRNA derived from the peanut protein genes to produce transgenic peanut lines with suppressed expression of Ara h 2 and Ara h 6 without affecting expression of Ara h 1 or Ara h 3, and no differences in seed weight or germination data were observed. RNAi was also used to produce transgenic peanut seeds that did not contain Ara h 2 and displayed decreased IgE-binding capacity [24-26]. Concerns and problems of genetic modification of peanuts are covered in the review of White and Colleagues [27]. These include (1) removal of Ara h 2 and Ara h 6, which are weak trypsin inhibitors, could increase the plant's susceptibility to fungal infection; (2) individual-seed analysis of the first transgenic generation revealed that often only one of the two seeds in a pod lacked Ara h 2. (3) Genetic modification of peanut affects all downstream stages, including agronomics, harvesting, handling, processing, utilization in food products, and most importantly, consumption. (4) Many targeted allergenic proteins are required for normal plant function; thus it would not be feasible to remove all allergenic proteins. (5) Opportunities for cross contamination are inevitable throughout production, including planting, harvesting, warehousing, processing, etc. (6) their removal could be detrimental to both peanut nutrition (amino acid composition and availability) and flavor. In addition, consumer concerns and acceptance of genetically modified food products are still issues because many people are afraid of the hidden long term harmful effect which is an obstacle for commercialization of this biotechnology.

Chemical Modification of Allergenic Peanut Proteins

Many chemical modification methods proposed to reduce the allergenic potential of food proteins were summarized by Stanic-Vucinic [28]. Chemical modifications include both covalent modifications and noncovalent modifications. In this review, only the methods used in the study of peanut and other legume proteins are discussed.

Covalent modifications of allergenic proteins

Among covalent modifications, Aacylation, reduction and alkylation, polymerization by glutaraldehyde were reported to be effective in reducing the allergenicity of pea and peanut proteins.

Acylation: Acylation of allergens by treatment with anhydrides, such as acetic or succinic acids, blocks positively charged amino groups on the protein molecule and the remaining free carboxyl groups of aspartic and glutamic acid residues make the net charge of the modified protein more negative. Szymkiewicz and Jędrychowski modified pea protein with acetic or succinic anhydride and found that not only the degree of acylation but also the type of anhydrides affected the extent of changes in the immunoreactivity of individual pea proteins. The greatest reductions in the immunoreactivity of albumins and legumin were observed during acylation with 0.2 g anhydrides (by 91-99% and 79-97% during succinylation and acetylation, respectively), while the immunoreactivity of vicilin fraction was reduced to 12% and 17%, respectively (when 1.0 g of anhydride was used) compared to the immunoreactivity of vicilin in native pea proteins [29]. They also found that the enzyme hydrolysis of acylation-modified pea proteins caused further significant reduction in the immunoreactivity of pea proteins [30]. The agents used in the acylation are considered as safe. The study was conducted with pea protein isolate. More studies are needed to discover how to apply this method to peas or peanuts, and to evaluate the sensory properties and storage stability of the products resulted from acylation.

Reduction and alkylation: Reduction and alkylation of peanut allergen isoforms Ara h 2 and Ara h 6 using non-food grade chemicals dithiothreitol and iodoacetamide was reported to effectively reduce IgE-binding potency of these two major peanut allergens by researchers in Netherlands and Serbia [31]. The study was conducted with purified Ara h 2 and Ara h 6 and how they can apply to food is unknown. Because dithiothreitol and iodoacetamide are not allowed to be in the food, how to remove the chemical residuals in the mixture and the safety of reduced and alkylated peanut proteins become big questions.

Reduction by sodium sulfite treatment: Sodium sulfite treatment effectively disrupted the structures of the cashew allergens, Ana o 2 and Ana o 3, in a temperature-dependent manner, and markedly lowered the binding of cashew proteins by rabbit IgG or IgE from cashew-allergic patients [32]. Although Sodium sulfite is a GRAS chemical allowed in certain food as preservative at a limited concentration sodium sulfite itself may cause allergic reaction to many people. According to International Chemical Safety Cards published on CDC website inhalation of this substance may cause asthma-like reactions and repeated or prolonged contact may cause skin sensitization [33]. As stated by the author, high concentration of sodium sulfite was needed to significantly reduce cashew allergenicity, this wouldcause off flavor to the products.

Polymerization by glutaraldehyde: It is disclosed in the patent

of Koppelman and colleagues (2010) that modification of peanut conglutin (Ara h 2 and Ara h 6) with Glutaraldehyde (GA) results in neither a change of secondary structure nor insubstantial decrease of IgE binding (only 2-3 fold), but Reduction/Alkylation treatment (RA) changes secondary structures, whereas RA treatment followed by GA modification (RA-GA) results in a tertiary structure due to modification of Cys and Lys residues [34]. The IgE-ELISA and IgE blot demonstrate that treatment with RA-GA decreases IgE-binding up to a hundred fold and also induce a strongT cell response in T cell proliferation tests. These data demonstrate that all threemodifications lead to a reduction in IgE binding, with the strongest reduction observed afterboth reduction/alkylation and glutaraldehyde treatment. The *ex vivo* study demonstrates that RA-CPE (Crude Peanut Extract) and RA-GA Ara h 2/6 causes significantly lower (100-fold lower) maximum beta-N-acetylhexosaminidase Release (NHR) than native CPE and Ara h 2/6 [35]. Such chemically modified Ara h 2/6 has great potential as alternative candidate for safe immunotherapy. Further work is needed to demonstrate if this method is feasible for producing allergenicity reduced peanuts or peanut-derived products as foods for human consumption. In addition, GA is a toxic chemical which is unsuitable for food applications. Many adverse health effects on humans have been reported in association with biomedical uses of GA [36]. Therefore, the safety of RA-GA modified food protein needs to be studied.

Non-covalent modifications

The noncovalent modifications are the interactions of food components with food allergens resulting in insoluble complexes thus lowering the level of soluble allergens and reducing their allergenic properties. Also they can reduce digestibility of food allergens and consequently their allergenicity by hindering access of digestive enzymes. Phenolic compounds and phytic acid are known to form soluble and insoluble complexes with proteins [28]. Most of the studies about non-covalent modifications of peanut proteins were conducted by Chung and his colleagues in USDA-ARS.

Treatment of peanut extract with phytic acid formed complexes with the major peanut allergens (Ara h 1 and Ara h 2) with reduced the solubility in acidic and neutral conditions, thus a 6-fold reduction in IgE binding was observed after treatment with phytic acid by competitive inhibition ELISA using a pooled serum from peanut-allergic individuals [37]. The question here is that samples used for ciELISA are soluble portions which are lower in allergen concentration because allergens are precipitated by phytic acid treatment, and the reduction in IgE-binding should be the outcome of lower allergen concentration. Therefore, it will be very important to investigate whether the allergenicity or IgE-binding of peanut protein-phytic acid complex or the whole food matrix.

In another study of Chung & Champagne (2009), roasted peanut extracts and liquid peanut butter at a protein concentration of 5 mg/mL were each treated with a phenolic compound (caffeic, ferulic, and chlorogenic acids; each dissolved in dimethylformamide) at a final concentration of 50-100 mM for 60 min under constant stirring. After centrifugation at 8000 g for 10 min, the supernatants thus obtained were subjected to SDS-PAGE and ciELISA analysis. Results showed that addition of the phenolics precipitated most of the major peanut allergens, Ara h 1 and Ara h 2, and that complexation was irreversible. IgE binding was reduced approximately 10- to 16-fold [38]. Similar to the phytic acid treatment, the IgE-binding was tested using supernatant and the allergenicity of polyphenol-allergen complex was not reported. In addition, dimethylformamide is a toxic organic solvent [39]. How to remove the residual of dimethylformamide from final product needs to be considered. It is also important to explore a safer solvent to replace dimethylformamide to dissolve phenolic compounds.

In another study, Chung and Reed used tannic acid to reduced the allergenicity of peanut butter extract because tannic acid interacts with proteins to form complex which was resistant to gastric digestion (pH 2.0) and intestine digestion (pH 8.0), and epitopes on the allergens are covered during complex formation, making the epitopes inaccessible to antibodies and resulting in reduced allergenicity [40]. Since tannic acid interacts with both allergen and non-allergen peanut proteins, such treatment has two obvious deficiencies: first, peanut nutrition is reduced to a great extent, and second, intake of much indigestible food may cause stomach discomfort and thus greatly limit consumption of peanut products [41]. In addition, no evidence shows that this method is effective to peanut butter itself (not extract) and peanut kernels.

A study led by North Carolina State University investigated the allergenicity of polyphenol-fortified peanut matrices prepared by complexing various polyphenol-rich plant juices and extracts with peanut flour [42]. They found that polyphenol-fortified peanut matrices reduced IgE binding to one or more peanut allergens (Ara h 1, Ara h 2, Ara h 3, and Ara h 6). Peanut protein-cranberry polyphenol fortified matrices triggered significantly less basophil degranulation than unmodified flour in an *ex vivo* assay using human blood and less mast cell degranulation when in an oral challenge test using peanut-allergic mice. Further study found that when digested with pepsin, the basic subunit of the peanut allergens Ara h 3 and Ara h 2 were more rapidly hydrolyzed in peanut protein-cranberry or green tea polyphenol complexes compared to uncomplexed peanut flour [43]. Peptides from peanut protein- cranberry polyphenol and peanut protein–green tea polyphenol complexes were substantially less immunoreactive (based on their capacity to bind to peanut-specific IgE from patient plasma) compared to peptides from uncomplexed peanut flour. These results suggest that peanut protein-polyphenol complexes may be less immunoreactive passing through the digestive tract *in vivo*, contributing to their attenuated allergenicity. Attenuated Total Reflectance determined by Fourier Transform Infrared Spectroscopy (ATR-FTIR) suggested changes in secondary protein structure. The reduced immunoreactivity by polyphenol fortification of peanut flour was likely due to changes in protein secondary structure or masking of epitopes [42]. This method is simple and safe because no chemicals are introduced in the process. The product is suggested by authors for potential applications in oral immunotherapy. If the product is to be used as food ingredient, the sensory attributes (such as color and flavor) and consumer acceptance tests have to be conducted.

Physical Treatment of Peanuts for Allergenicity Reduction

Traditional food processing methods

Many studies have revealed that dry roasting increase the

allergenicity of peanut proteins because roasting or extensive heating causes polypmerization of peanut allergens, Maillard reaction between reducing sugar and proteins, and reduction in the protein solubility peanuts [44-48]. However, some traditional food preparation/processing methods such as boiling and pressure cooking of peanut have reported to reduce the *in vitro* allergenicity of peanuts. The study of Beyer and colleagues found that compared with roasted peanuts, the relative amount of Ara h 1 was reduced in the oil fried and boiled peanut protein preparations, resulting in a significant reduction of IgE-binding intensity. Although statistically significant, the reduction of the allergen content and the allergenicity of peanuts by regular boiling and oil frying process are very limited because the target allergens are still visible on SAS-PAGE and immunoblot of protein extracts of boiled peanuts [49]. Similar results were reported by Mondoulet and colleagues that the IgE-binding capacity of whole peanut protein extracts prepared from boiled peanuts was 2-fold lower than that of the extracts prepared from raw and roasted peanuts, but no significant difference was observed between protein extracts from raw and roasted peanuts. It is noteworthy that the proteins present in the cooking water were also recognized by the IgE of peanut-allergic patients [50].

High pressure methods: The autoclave or high pressure cooking seems more effective than regular boiling. Autoclaving at extreme condition (2.56 atm, for 30 min) significantly decreased IgE-binding capacity of peanut allergens as demonstrated by *in vitro* and *in vivo* experiments [51]. The immunoreactivity of peanuts treated with 600 MPa and 800 MPa for 10 min was significantly lower ($P < 0.05$) than those of the control group by 69.2 ± 5.3% and 73.3 ± 1.9%, respectively. However, high pressure treatment at 800 MPa decreased total essential amino acid content as well as two nutritional indexes, the chemical score and the essential amino acid index, by 32.4 ± 2.1% and 31.1 ± 3.2%, respectively [52]. The loss of nutritional value of autoclave was not reported.

Pulsed Ultraviolet (PUV) Method: Pulsed Ultraviolet (PUV) light is a non-thermal, high-peak power technology that consists of intense flashes of broad-spectrum white light with wavelengths from 200 nm (UV) to 1000 nm (near-infrared) region [53]. Each pulse may have up to 90000 times the intensity of sunlight at sea level, and may last only a few hundred millionths of a second, and thus a PUV light system can produce very high peak power pulsed light in a very short time. Therefore, PUV light has been successfully used as a novel technology to decontaminate food surfaces, water, wastewater, air and food contact surfaces [54,55]. The lethality of pulsed light treatment is related to Ultraviolet (UV) part of the spectrum which include photochemical and photothermal effect [55]. The study of using pulsed-UV to reduce food allergens is led by Dr. Wade Yang in University of Florida. The researchers used a Xenon Steripulse XL-3000 PUV light system consisting essentially of a pulsed UV lamp, a cooling blower, a treatment chamber, and the control module to treat peanut kernels and peanut butter. They found that 4 minutes treatment effectively reduced IgE-binding of peanut kernels, peanut extracts and peanut butter [56,57]. The advantage of this method is fast and can be used for different forms of peanuts. However, peanut is rich in unsaturated fat. Exposing peanuts to pulsed-UV or irradiation, may accelerate lipid oxidation which will result in rapid quality deterioration and harmful oxidation products. Another drawback of using pulsed-UV is that the solubility of peanut protein was significantly reduced after PUV treatment [56]. It will be extremely important to investigate the impact of PUV treatment on the storage stability and sensory properties of peanuts. In addition to pulsed UV treatment, gamma irradiation was also reported to reduce the IgG binding of whole peanut protein extract and Ara h 6 due to the loss of the α-helix structure [58]. However, the feasibility of using gamma irradiation for peanuts and peanut butter may be a problem as gamma irradiation is not allowed for high fat products.

Enzymatic Methods for Peanut Allergenicity Reduction

Enzymatic treatment is believed to be full of potential to produce allergen-free peanut. Unlike the methods described above, hydrolyzing allergenic proteins by enzymatic method using food grade proteases is a mild and safe approach to permanently destroy the food allergens. Two types of enzymatic treatments were reported. One is cross-linking of allergenic proteins to bury the epitopes. Another type of enzymatic treatment is proteolytic hydrolysis of allergenic proteins to breaks down proteins into fragments/peptides without or with reduced allergenicity.

Enzyme catalyzed protein cross-linking to reduce peanut allergenicity

Peroxidase (POD), also called tyrosinase, is a heme-containing enzyme catalyzing the oxidation of a variety of phenolic compounds. Proteins can become cross-linked with other proteins or polysaccharides in the presence of peroxidase because proteins contain tyrosine residues. Transglutaminase (TGA) catalyzes the formation of an isopeptide bond between a free amine group (e.g., protein- or peptide-bound lysine) and the acyl group at the end of the side chain of protein- or peptide-bound glutamine. Chung and colleagues treated peanut protein extract with POD and TGA and found that POD treatment of roasted peanut resulted in partial loss of Ara h 1 and Ara h 2 along with reduced IgE binding ability and formation of new polymers, but TGA treatment of roasted peanut protein extract had no effect on the content of Ara h 1 and Ara h 2 as well as IgE binding ability; both POD and TGA had no effect on the IgE binding ability of protein extract from raw peanut. The overall reduction of IgE-binding of POD treated peanuts was only about 20% [59]. The presence of caffeic acid enhanced the cross-linking of Ara h 1 and Ara h 2 catalyzed by POD [60]. Cross-link of peanut proteins induced by microbial tyrosinase from *Trichoderma reesei* and mushroom tyrosinase from *Agaricus bisporus* was reported to increased the bioavailability of major peanut allergen Ara h 2, but did not significantly change the allergenic or tolerizing properties of peanut [61]. Another study found that TGA treatment of either raw or roasted peanut protein extracts did not reduce the allergenicity of the extracts [62]. 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 [41,63]. Therefore, enzymatic cross-linking of peanut proteins is not an effective approach to reduce the immunoreactivity of peanut protein. It seems preserved molecular and immunological features of peanut allergens.

Proteolytic hydrolysis to reduce peanut allergenicity

Our previous study showed that treatment of roasted peanut kernels with digestive proteases trypsin and chymotrypsin

significantly reduced Ara h 1 and Ara h 2 in roasted peanuts, but the treatment was less effective in the case of raw peanuts [64]. This is largely because the presence of trypsin inhibitors in raw peanuts, and roasting of peanuts at high temperature destroyed the 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 [65]. These studies also show that Ara h 2 was more resistant to the enzymatic digestion. Although ultrasound pretreatment and sequential enzymatic treatment by trypsin and alpha-chymotrypsin improved the degradation of Ara h 2, the reduction of IgE binding of protein extract of treated peanuts was about 40%. 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 peanut was 50-60% in human skin prick tests [66].

It was found that hydrolysis of roasted peanut protein extract by Alcalase for 90 min or the sequential treatment of alcalase and flavourzyme for 30 min resulted in 100% reduction in IgE reactivity [67]. These results were confirmed by Western blot with sera individuals with confirmed peanut allergy. None of the sera recognized any RP proteins after sequential endo- and exoprotease hydrolysis. Single enzyme treatment with Flavourzyme for caused an increase in IgE reactivity detected by ELISA, led to a 65% decrease in IgE reactivity when the treatment time was 300 min. The residual allergenicity of protein extracts made from alcalase treated roasted peanuts varied with treatment conditions. Other studies of using alcalase to reduce allergenicity of food products other than peanuts also reported that certain allergenicity retained after alcalase treatment [68,69]. This indicates that some fragments produced by alcalase hydrolysis of allergenic proteins may remain some allergenicity and peanuts thus produced are not safe to the hypersensitive individuals. More study is needed to overcome this issue. Kasera and colleagues (2015) investigated the effects of sequential enzyme treatment by alcalase and flavourzyme on the allergenicity of legume protein extracts. Results show that peanut protein extract hydrolyzed by alcalase and flavourzyme showed 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 [70]. 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.

The contradict results have also been reported. 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; however, IgE cross-linking capacity of hydrolyzed protein was retained as revealed by basophil activation tests, thus suggesting such hydrolysates are not hypoallergenic [71]. In another study, the allergenicity of Soybean Protein Isolates (SPI) hydrolyzed by Alcalase, trypsin, chymotrypsin, bromelain, or papain was evaluated by IgE immunoblots using eight soybean-allergic patient sera, and the biological relevance of IgE binding was evaluated by a functional assay using a humanized Rat Basophilic Leukemia (hRBL) cellline and serum from one subject [72]. Results indicated 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). However, the extracts of Alcalase, papain, and trypsin hydrolyzed SPI showed a similar or slightly reduced mediator release compared to the heated SPI control in the hRBL assay. The SPI treated with chymotrypsin and bromelain showed a higher mediator release compared to the heated SPI control. This study conclude that hydrolysis of soybean proteins by enzymes such as Alcalase, papain, trypsin, chymotrypsin, or bromelain did not remove IgE binding to all soy proteins for many soybean-allergicsubjects. More importantly, at least some individuals would likely still experience elicitation of food allergy when consuming SPI hydrolysates as demonstrated by basophil activation for atleast one subject of eight [72].

Overall, enzymatic hydrolysis can reduce the allergenicity of peanut proteins to different degree depending on the type of enzymes and the degree of hydrolysis, but it can not make peanuts hypoallergenic to all allergic individuals. However, comparing to other technologies and approaches aforementioned, enzyme hydrolysis has many advantages: (1) It is simple and does not need special equipment. It can be easily adopted by peanut processing manufacturers. (2) It can be used to treat peanut flour, peanut butter, peanut protein isolate/extract and peanut kernels. (3) It is safer compared to chemical modification because all materials used in the process are food grade. (4) It does not cause lipid oxidation. However, as described above, studies with skin prick tests demonstrated that enzyme hydrolyzed peanut protein extract had 50-91% lower allergenicity compared to the untreated samples [66-70], but the basophil activation tests showed limited or no reduction in allergenicity [71,72]. Therefore, oral challenge tests have to be conducted to get the most reliable results about the allergenicity of peanuts or peanut derived products. In addition, hydrolysis of peanuts and other legume by endopeptidase often produce bitter peptides, which may influence the flavor of hydrolyzed product [73-75]. Therefore, it is also important to study the sensory properties of enzyme hydrolyzed peanut products.

Conclusion

Each allergen reducing method discussed in this review can reduce the allergenicity of peanuts to certain level, but one single method can not solve the problem completely. Every allergen reducing method has its pros and cons. For both covalent and non-covalent chemical modifications of peanut proteins or peanut derived products for food use, it is extremely important to consider the safety of chemicals used for the modification, how to remove the chemical residues after the treatment and the impact of the chemical used on the sensory properties of product. The traditional physical methods such as boiling and high pressure treatments is less effective but is safe, while PUV method seems a more effective physical method but it also has higher potential to cause oxidation of peanuts. Enzymatic hydrolysis of peanut protein using proteolytic enzymes is considered as safe and effective method for reducing the allergenicity of peanuts and other legumes, but this method may not remove all IgE-binding

Table 1: Comparison of different methods for allergenicity reduction.

Type of method	Mechanism	Advantage	Issues	References
Genetic Modification	Interfere or inhibit the synthesis of some allergenic protein during maturation of peanut	Suppress expression of specific allergens without affecting expression of other proteins	1. Increase the plant's susceptibility to fungal infection 2. Often only one of the two seeds in a pod lacked Ara h 2 3. May affects all downstream stages 4. May affect peanut nutrition and flavor	[23-27]
Chemical Modification				
Acylation	Block positively charged amino groups on the protein molecule and make the net charge of the modified protein more negative.	1. Provide important scientific information about the structural modification and allergenicity of peanut proteins. 2. May reduce IgE-binding effectively. 100 fold decrease in IgE-binding and also induce a strong T cell response in T cell proliferation tests in the case of RA-GA treatment.	1. Chemicals used for modification of peanut allergens are not food grade. 2. How to remove chemical residues after treatment is unknown. 3.Do not know if modified peanut proteins are safe to eat or not. 4. Chemicals used for modification of peanut allergens may affect nutritional and sensory quality of peanut and peanut derived products	[29-36]
Reduction	Reduction and alkylation (RA) of peanut allergen isoforms Ara h 2 and Ara h 6 using chemicals dithiothreitol, iodoacetamide, and sodium sulfite.			
Polymerization	RA treatment followed by glutaraldehyde (GA) modification (RA-GA) results in a tertiary structure due to modification of Cys and Lys residues.			
Non-covalent chemical modification	The interactions of food components (phytic acid and polyphenols) with food allergens resulting insoluble complexes thus lowering the level of soluble allergens and reducing their allergenic properties.	1. Chemicals used are commonly in food of plant origin. No unsafe chemicals are introduced in peanut flour 2. Can be easily applied to peanut flour and peanut protein isolate 3. The product is suggested by authors for potential applications in oral immunotherapy	1. Samples used for allergenicity test are lower in allergen concentration because allergens are precipitated by the treatment. 2. May affect sensory quality of the product 3. The complexation of polyphenol with protein may reduce the nutritional value of peanut product.	[28], [37-43]
Physical Modification				
Tradition food processing methods (Boiling, and oil frying)	Certain amount of allergen dissolved in boiling water. Structural change due to heat treatment during frying and boiling	1. The process is safe and easy. 2. no chemical is added	The reduction of allergenicity is very limited	[49-50]
High pressure treatment	Certain amount of allergen dissolved in cooking water. Structural change (partial denaturation) due to high pressure and high temperature.	1. No chemical is added 2. Significantly decreased IgE-binding capacity of peanut allergens	1. High pressure equipment is needed 2. Appearance of peanuts may be changed	[51-52]
Pulsed- UV treatment	A non-thermal food processing technique that involves discharge of high voltage electric pulses into the food product placed between two electrodes, which produce photochemical and photothermal effects.	1. Fast process (4-5 min) 2. Can be used for different forms of peanuts 3. No chemicals are introduced in the process	1. Expensive equipment needed 2. Using UV light radiation may cause lipid oxidation of peanuts and generate harmful free radicals in peanuts 3. Reduce solubility of Ara h 1 and its polymer	[53], [55-58]
Enzymatic Modification				
Enzyme catalyzed protein cross-linking	Proteins can become cross-linked with other proteins or polysaccharides in the presence of peroxidase (PO) or transglutaminase (TGA)	Simple and no toxic chemical is introduced in peanut flour or protein extract	Low efficiency: The overall reduction of IgE-binding of POD treated peanuts was only about 20%	[41], [59-63]
Enzymatic hydrolysis	Proteases break allergenic protein into peptides with smaller number of IgE epitopes.	1. Simple and high efficacy (40-100% reduction in IgE-binding, 50-85.7% reduction in SPT). 2. No unsafe chemicals are introduced to peanuts. Enzymes used are food grade. 3. Can be used for peanut kernels, peanut butter, flour and protein extract	May produce bitter peptides which make peanut or peanut derived product taste bitter.	[64-75]

epitopes for all peanut-allergic individual. However, the allergenicity of peanut protein or protein extract obtained from different methods has 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, these methods only tests the soluble portion of the peanut or peanut protein extracts, the allergenicity of insoluble portion is still unknown. Therefore, oral challenging study of the modified products should be conducted to ensure the safety of products. It is also important to evaluate the impacts of enzymatic modification on the nutritional and sensory quality of peanuts and peanut derived products to generate information about the feasibility of different methods for industry applications. A comparison of mechanism, efficacy and issues of different methods for peanut allergen reduction is given in (Table 1).

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