Structural and Functional Alterations in Major Peanut Allergens Caused by Thermal Processing

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The majority of foods that we eat are subjected to some type of processing either at home or by the manufacturer. The biochemical reactions that occur in foods as a result of thermal processing can be both beneficial and harmful. Here, we briefly review the effects of thermal processing and some of the effects of the Maillard reaction on the allergenicity of food proteins. Specifically, we focus on the known effects of roasting on the allergenic properties of peanut proteins and the contribution of Maillard reaction products or advanced glycation end products to these observed effects. The most thorough understanding of the effects of thermal processing on allergenicity involves the peanut proteins. Thermal processing alters specific biophysical and immunological properties of peanut proteins, such as structure, function, solubility, digestibility, immunoglobulin E (IgE) binding, and T-cell responses. A better understanding of the effects of thermal processing-induced biochemical and immunological alterations is of utmost importance for proper risk assessment of existing and newly introduced proteins in the food source, as well as development of effective diagnostic tools and therapeutic treatments for food allergy.

To a large extent, the relationship between food antigenicity and processing is poorly understood because of the minimal number of studies in this area and the magnitude of processes that different foods undergo before reaching consumers. By "food processing" we mean any manipulation that a particular food undergoes from the time it is harvested until the time it reaches the consumer. Factors such as food composition, time, temperature, and conditions of harvest, storage, heating, and cooking can alter the properties of proteins in foods and render them either more or less antigenic. Thermal or nonthermal processing of foods can cause the proteins to become more or less antigenic and new allergens to be formed and must be a necessary part of assessing the allergenicity of existing and newly introduced foods.

Originally, it was thought that thermal processing would reduce allergenicity (1) because of the known disruptive effects of heat on most proteins. Interestingly, known cases of food allergy have either conferred with or differed from this observation. For example, one of the very first documented allergic patients was found to be allergic to proteins in cooked fish but not raw fish (2). Meanwhile, Urisu et al. (3) demonstrated that some patients who were allergic to raw, freeze-dried egg had no reaction to cooked egg white. Since then, studies have claimed that thermal processing or heating does not alter the allergenic properties of foods. However, one example (1) involved purified proteins that are not in the presence of sugars, lipids, oxidizing agents, or other proteins that normally exist in the natural environment of a protein or in a food matrix during processing. Malanin et al. (1) presented convincing evidence for the appearance of neoallergens in an allergic patient who reacted to pecan nuts that were either stored for 2 weeks or roasted but did not react to fresh pecans. Similar observations have been made concerning long-term storage of wheat flour extracts and pecan nuts (4), cooked versus uncooked shrimp (5), and heated soybean hulls and asthma (5) and enhanced skin reactivity of $\beta$-lactoglobulin subjected to the Maillard reaction (6). Few studies have shown a direct increase in allergenic response caused by thermal processing. As a result, it is mistakenly thought that because of the rarity of individuals who react specifically to processed but not unprocessed products, thermal processing is not significant enough to warrant concern. However, we support the idea that processing-induced alterations to existing proteins and formation of neoallergens in certain foods can increase the antigenicity of a particular food and the ability to sensitize susceptible individuals. Strong support for this notion comes from our work with peanut allergens.

Allergens and the Maillard Reaction

Food allergies that are immunoglobulin E (IgE)-mediated are classified as Type I. It is known that IgE-mediated mast cell degranulation is a strategy that evolved to eliminate respiratory or gastrointestinal pathogens. However, no reasons have been offered to explain why certain foods elicit...
immunological responses similar to those exhibited by multicellular metazoan parasites that reside in the respiratory and gastrointestinal systems, while other foods are hypoallergenic. Whereas many of the immunological and structural characteristics of food allergens have been explained (7), specific immunological and biophysical properties of the allergens that contribute to IgE antibody formation are not fully understood. Some of the immunological properties of allergenic proteins include their ability to bind serum IgE, elicit a positive prick skin test, stimulate T-cell proliferation, and cause histamine release from mast cells and basophils of sensitive individuals. Characteristic biochemical properties of allergens are that they are low-molecular-weight proteins or glycoproteins 5–100 kilodaltons (kDa), which are usually abundant in the food source and often stable to digestion by gastrointestinal enzymes (8). Functional similarities, such as protease activity, have also been associated with both food and inhaled allergens. Collectively, these properties seem to allow penetration of undigested allergenic fragments at the mucosal membrane, facilitating sensitization and the immediate symptoms observed in allergic patients. The majority of the immunological and biophysical properties of food allergens have been determined by using recombinant allergens or allergens in an unprocessed form, rather than in the processed form that they are in when ingested.

Thermal processing, such as frying, roasting, curing, and various types of cooking, can cause multiple, nonenzymatic, biochemical reactions to occur in foods (9). One of the major reactions that occurs during cooking or browning of foods is the Maillard reaction (10, 11), which is important in the development of flavor and color in peanuts. The amino groups of proteins are modified by nonenzymatic condensation with reducing sugars, ketose, or aldehydes to form glycosylamines that can undergo rearrangement to form Amadori products. Subsequently, these Amadori products are degraded into dicarbonyl intermediates, which in turn are more reactive than the parent sugars and react with other amino groups to form cross-links or rearrange to form stable end products called advanced Maillard reaction products (MRPs) or advanced glycation end products (AGEs; 9). The glycoxidation products carboxymethyllysine (CML), carboxyethyllysine (CEL), and pentosidine are chemically characterized AGEs known to accumulate in proteins as people age. These AGEs accumulate with an accelerated rate in diabetes (12) and are thought to contribute to accelerated aging in this disease. It is known that in addition to cross-linking the Maillard reaction could lead to the loss or modification of amino acids such as lysine and cysteine and cause malondialdehyde formation and other non-cross-linking modifications to proteins that may have harmful nutritional, physiological, and toxicological consequences (12, 13).

**Immunological and Structural Alterations to Allergens Induced by Processing**

Some studies have addressed the immunological responses to advanced MRPs (or AGEs). The protein products modified by the Maillard reaction evoke an IgG response, which is correlated with IgE production (14–17). Recently, formation of circulating immune complexes between antibodies and AGE-modified proteins has been reported (18). For the first time, the nature of the immunogenic modifications that can elicit autoantibody formation against an AGE-modified protein was demonstrated by Virella et al. (19). These immune complexes have been implicated in the proinflammatory process leading to the development of coronary heart disease (20). AGEs also have been shown to promote monocyte migration (20, 21) and cytokine production (22). They are associated with heightened immunogenicity, aging, and age-enhanced disease states, such as diabetes, atherosclerosis, hemodialysis-related amyloidosis, and Alzheimer’s disease. However, few studies have addressed the role of these products on the allergenic properties of ingested foods (23–28).

Peanut allergy is becoming an increasingly serious health problem in first-world countries, such as the United States, Canada, and the United Kingdom. It is the most common cause of fatal anaphylaxis and of food-induced anaphylaxis (29, 30). Our initial studies revealed that roasted peanut extracts bound serum IgE from allergic individuals at significantly higher levels than did raw peanut extracts (28). To begin to understand the enhanced immunogenicity by roasted peanuts and the contribution of the Maillard reaction to IgE binding properties, we assessed susceptibility of the major peanut allergens to digestion by gastrointestinal fluid and their heat stability. As previously mentioned, a large number of biochemical modifications to proteins are known to occur during roasting/browning of foods. Therefore, purified allergens from raw peanuts were subjected to a well-documented (28) and isolated in vitro model system to determine if the Maillard reaction alone affects the allergenic properties of the allergens. In this simulated roasting model (SRM), proteins were incubated in the presence of sugars, heated over time, and assessed for allergenic properties. Following treatment in the SRM, the 2 major allergens, Ara h 1 and Ara h 2, from raw peanuts became more resistant to digestion with gastric fluid, and trypsin, less soluble, underwent structural modifications and bound higher levels of IgE (28). Deciphering the biophysical modifications to the allergens following the SRM and the implication of these changes on the immunological properties of peanut proteins was the next logical step.

In the SRM, intermolecular cross-links were formed between Ara h 1 monomers to generate covalently associated trimers and hexamers, and although higher-order structures were not observed for Ara h 2, it became highly resistant to digestive enzymes. Ara h 1 from raw peanuts forms stable trimeric complexes in solution at low concentrations (31), which is suggested to play a role in the allergenic properties of this protein (32). The formation of a trimeric complex allows the molecule some protection from protease digestion and denaturation, theoretically permitting passage of large fragments of Ara h 1, which contain several intact IgE binding sites, across the lumen of the small intestine, thus,
contributing to its allergenicity (32). The reversible association of Ara h 1 monomers, through hydrophobic interactions, hypothesized to be important in allergenicity (32), becomes irreversible because of the covalent cross-linking that occurs with thermal processing (27, 28). Therefore, Ara h 1 subjected to the SRM or purified from roasted peanuts becomes more resistant to digestive enzymes than previously determined for Ara h 1 from raw peanuts. In turn, this affects its overall ability to induce an allergic response.

Nordlee et al. (26) also reported that roasted peanuts bind IgE at higher levels than raw peanuts and that the IgE recognition sites in roasted peanuts differ from those of raw peanuts. They hypothesized that this may result because heat treatment increases the allergenicity of peanut proteins by increasing the availability of allergenic binding sites on the proteins that were previously unexposed. Our findings correspond with their findings and imply that, in addition to exposing previously unavailable sites, the covalent modification of the proteins during the roasting process may create novel IgE binding sites and enhance other allergenic properties, such as resistance to heat, degradation, and digestion by gastric secretions.

To test the validity of the SRM experiments, we roasted whole peanuts for various lengths of time and compared for solubility, IgE binding, and induction of T-cell proliferation (27). In addition, the biophysical and immunological properties of Ara h 1 and Ara h 2 purified from medium roast and raw peanuts were compared (27). Whole peanut extracts from the various roasted peanut samples showed decreasing solubility and T-cell proliferation, expansive structural changes to the proteins, and heightened IgE binding with increased roasting time. In addition, cross-linked Ara h 1 monomers, trimers, and hexamers, as well as Ara h 2 proteins were found in purified roasted protein fractions, as seen in the SRM. Antibodies against some AGE by-products, such as anti-CML, and hydroxynonenol (HNE), were used to determine if any of these specific modifications contribute to the increase in IgE binding (27). The level of CML modifications of the allergens correlated with the increase in IgE binding. We concluded, based on these findings, that thermal processing events can drastically alter the biophysical and immunological properties of proteins.

**Structure and Function of Ara h 2**

Some of the roasting-induced, biophysical mechanisms for enhanced allergenic properties of the major peanut allergens Ara h 1 and Ara h 2 have been discussed above. In short, both allergens bound higher levels of IgE, and the increase in IgE binding was correlated with increased CML modifications on the surface of the protein (33), specifically in the case of Ara h 2 and Ara h 1 trimer. Also, Ara h 1 was intermolecularly cross-linked to form highly stable trimers, and Ara h 2 was thought to form intramolecular cross-links as a result of roasting, without forming higher-order structures (27, 28). These modifications also stabilized these proteins against digestive enzymes. To explore the possible mechanisms for the enhanced allergenic properties of roasted Ara h 2, a homology search of the publicly available protein data bank (PDB) was performed to determine putative structures and/or functions for Ara h 2 (33). This search focused on protein domain homology rather than primary amino acid sequence. Ara h 2 was found to have protein domain homology to trypsin/alpha amylase inhibitors such as the Ragi seed bifunctional proteinase inhibitor (RBI) and to plant lipid transfer proteins (LTP). We tested the trypsin inhibitory activity of Ara h 2 from raw and roasted peanuts and examined the relationship of the structure, function, and allergenic properties of this major peanut allergen. Raw Ara h 2 acted as a weak trypsin inhibitor, and there was approximately 4-fold increase in this activity after roasting (33). In addition to being more resistant to trypsin digestion itself, Ara h 2 protected Ara h 1, a second major peanut allergen, from degradation by trypsin (33). This protective characteristic was enhanced with the use of Ara h 2 purified from roasted peanuts.

The circular dichroism (CD) spectra of Ara h 2 from raw peanuts in untreated, partially reduced, fully reduced, urea-treated, and roasted forms suggest that the predominant stabilizing factors in Ara h 2 structure are disulfide bonds and that Ara h 2 is primarily composed of alpha-helices and random coils/loops and very little, if any, beta sheets. The CD spectra demonstrate a very slight difference in the secondary structure of native, untreated Ara h 2, urea-treated, partially reduced and roasted Ara h 2. Most differences between the native, partially reduced and the roasted Ara h 2 were seen in the lower wavelength range (185–200 nm), which reflects a decrease in alpha-helices in favor of beta-strands and random-coil or loop formation. However, a significant alteration was observed in the secondary structure of the native Ara h 2 that was treated with 50mM dithiothreitol (fully reduced Ara h 2) in comparison to the other spectra.

Ara h 2 is a monomeric protein containing 8 cysteine residues that can form 4 disulfide bonds. Partial reduction (reduction of some but not all disulfide bonds) of Ara h 2 is possible without significant impact on the secondary structure, but with a notable difference in function. However, a full reduction of disulfide bonds leads to significant alteration of structure and function. These findings suggest that more than one disulfide bond is formed in the Ara h 2 molecule. The alignment of the Ara h 2 sequence with the homologous bifunctional alpha-amylase/trypsin inhibitor sequence of RBI (PDB:1BIP; 34) suggests that all 8 cysteines may be involved in disulfide bond formation. If so, this explains the high stability of the Ara h 2 structure against denaturation by 8M urea or roasting and its resistance to digestion with trypsin. From the Ara h 2/RBI alignment, the trypsin-binding loop of Ara h 2 is likely located between residues 76 and 85. The homologous disulfide bridge according to RBI structure would be between cysteine 20 and 44 in RBI, which connects the 2 alpha-helices that encompass the trypsin-binding loop between them. Therefore, it is possible that disruption of any of these disulfide bonds in the partially
reduced Ara h 2 or loss of sulfhydryl group(s) in roasted Ara h 2 can lead to higher flexibility in the trypsin binding loop and possibly increased inhibitory activity. Loss of sulfhydryl groups is a documented result of the Maillard reaction (35, 36).

Our original estimate of regular secondary structure content (31% alpha, 10% beta) from CD agrees well with the content (33% alpha and 3% beta) from the 3-dimensional structure of the bifunctional proteinase inhibitor trypsin/α-amylase from Ragi seeds (PDB:1BIP, 34). Surprisingly, the results of another study concerning Ara h 2 structure using CD analysis yielded very different results (37). That study found that native Ara h 2, which was provided by our laboratory, consisted of 54% α-helix, 18.2% β-sheet, and 27.7% random coil configuration. It is possible that these discrepancies are due to the presence of phosphate-buffered saline and, importantly, DTT, in addition to other components in the Ara h 2 sample used for the CD scans (37). The presence of various chemicals can alter CD spectra; therefore, background subtraction, smoothing, and additional calculations are necessary, which can lead to the errors in estimation of the structural components. Regardless, both studies indicate that the disulfide bonds are major contributors to the stability of the Ara h 2. Our finding, based on our work, is that the redox state of the sulfhydryl groups influences the activity of Ara h 2 as a trypsin inhibitor.

**Discussion**

In summary, roasted peanut extracts were found to bind serum IgE from allergic individuals (28) at approximately 90-fold higher levels than raw peanuts, and the Maillard reaction was shown to contribute to the observed effect. In a more recent study (27), we demonstrated that roasted peanut extracts (as a whole) and Ara h 1 purified from roasted peanuts are much less soluble, whereas Ara h 2 solubility is less affected by roasting. Both allergens and whole peanut extracts from roasted peanuts had enhanced IgE binding in comparison to raw peanuts (27). Currently, we showed enhancement of an allergen-associated function caused by thermal processing (33). Although it is still quite possible, as previously thought, that the increase in intramolecular cross-links in Ara h 2 resulting from roasting contributes to protein stability and increases allergenic properties, it is also likely that enhanced trypsin-inhibitory activity plays a role in this observation. In addition to Ara h 1 intermolecular cross-linking, enhanced IgE binding, reduced solubility, and resistance to digestion caused by roasting, Ara h 2 becomes modified and provides additional protection against digestive enzymes. These findings suggest a possible mechanism for a synergistic enhancement of allergenic properties of different allergens in the same food or within a food matrix that can be further intensified by processing events such as roasting.

The Maillard reaction occurs naturally in animals with age and in a wide range of foods that have undergone diverse processing events. The latter are consumed by individuals on a daily basis. So, as suggested in a review by Davis et al. (38), virtually all foods would be allergenic if the Maillard reaction products alone formed complete IgE epitopes. Therefore, CML or other MRPs modifications of proteins alone cannot be responsible for the allergenicity of certain molecules by themselves, even though they enhance the allergenic properties of various foods discussed here (38). It may be that certain proteins that are more immunogenic (because of unique structure and/or amino acid sequence) become more likely to sensitize or elicit an immunological response from susceptible individuals as a result of added immunogenicity of the modifying glycation products.

Our ongoing studies indicate primarily that all future analyses of the allergenicity of foods should be performed on foods in the form in which they are ingested. In addition, it is highly important to analyze the allergic potential of genetically modified foods containing recombinant proteins for allergenic properties following the usual processing methods such as harvesting, storage, manufacturing, and cooking in addition to the analysis of primary sequence, structure, and physical properties of the purified protein.

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**References**
