

The effects of roasting on the allergenic properties of peanut proteins

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Background: Because of the widespread use of peanut products, peanut allergenicity is a major health concern in the United States. The effect or effects of thermal processing (roasting) on the allergenic properties of peanut proteins have rarely been addressed.

Objective: We sought to assess the biochemical effects of roasting on the allergenic properties of peanut proteins.

Methods: Competitive inhibition ELISA was used to compare the IgE-binding properties of roasted and raw peanut extracts. A well-characterized in vitro model was used to test whether the Maillard reaction contributes to the allergenic properties of peanut proteins. The allergic properties were measured by using ELISA, digestion by gastric secretions, and stability of the proteins to heat and degradation.

Results: Here we report that roasted peanuts from two different sources bound IgE from patients with peanut allergy at approximately 90-fold higher levels than the raw peanuts from the same peanut cultivars. The purified major allergens Ara h 1 and Ara h 2 were subjected to the Maillard reaction in vitro and compared with corresponding unreacted samples for allergenic properties. Ara h 1 and Ara h 2 bound higher levels of IgE and were more resistant to heat and digestion by gastrointestinal enzymes once they had undergone the Maillard reaction.

Conclusions: The data presented here indicate that thermal processing may play an important role in enhancing the allergenic properties of peanuts and that the protein modifications made by the Maillard reaction contribute to this effect. (*J Allergy Clin Immunol* 2000;106:763-8.)

Key words: Peanut allergy, IgE, Maillard reaction, roasting

An increase has been seen in the use of peanuts and peanut products in the past decade. Meanwhile, there has been a simultaneous rise in the development of cases of peanut hypersensitivity, as well as life-threatening reactions in individuals with peanut allergy.¹ Raw peanut extracts are used in the majority of the current studies and allergy tests, even though persons rarely ingest raw peanuts. Before this report, a limited number of studies have addressed the effect or effects of thermal processing

Abbreviations used

AGE:	Advanced glycation end product
CML:	Carboxymethyllysine
GS:	Gastric secretions
MRP:	Maillard reaction product
WPE:	Whole peanut extract

(ie, roasting) on the allergenic properties of peanut proteins. Thermal processing, such as roasting, curing, and various types of cooking, can cause multiple, nonenzymatic, biochemical reactions to occur in foods.² A major reaction that occurs during processing or browning of foods is known as the Maillard reaction,^{3,4} which is important in the development of flavor and color in peanuts, as well as many other processes of food technology. The modifications of the amino groups of proteins by means of reducing sugars leads to the formation of Schiff bases, which undergo rearrangement to form *Amadori products* (Fig 1). Subsequently, the Amadori products degrade into dicarbonyl intermediates. These intermediary compounds are more reactive than the parent sugars with respect to their ability to react with amino groups of proteins to form cross-links, stable end products called advanced Maillard reaction products (MRPs) or advanced glycation end products (AGEs). In addition to cross-linking, it is known that advanced Maillard reactions could lead to the loss or modification of amino acids, such as lysine (see carboxymethyllysine [CML] in Fig 1), malanoidin formation, and other non-cross-linking modifications to proteins that may have detrimental nutritional, physiologic, and toxicologic consequences.²

An array of studies have addressed the immunologic recognition and responses to the advanced MRPs (or AGEs). Protein products modified by the Maillard reaction have been shown to elicit an IgG response, which has been correlated by a number of studies to IgE production.⁵⁻⁸ AGEs have been shown to promote monocyte migration^{9,10} and cytokine production.¹¹ Although the effect of the AGEs in association with heightened immunogenicity, aging, and age-enhanced disease states, such as diabetic complications, atherosclerosis, hemodialysis-related amyloidosis, and Alzheimer's disease, have been investigated, few studies have been done to address the role of these products on the allergenic properties of ingested foods.¹²⁻¹⁴

In the current study roasted peanut extracts bound serum IgE from allergic individuals at significantly high-

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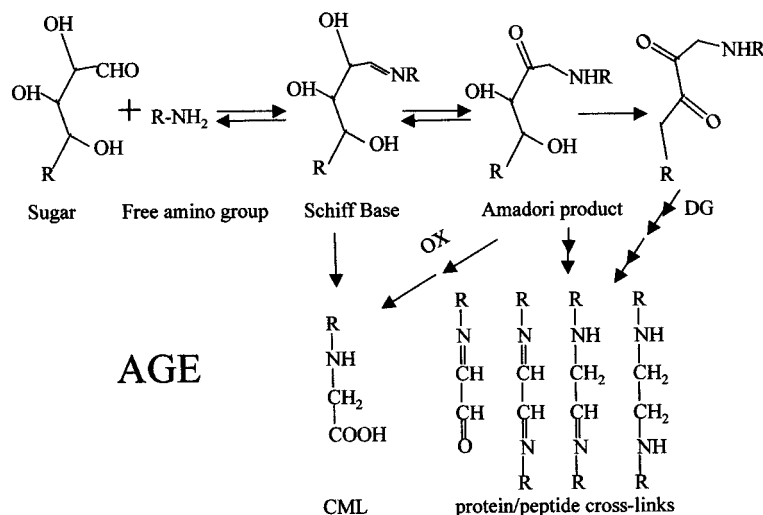


FIG 1. General reaction scheme for the Maillard reaction and the formation of the advanced glycation end products. The early stages of protein glycation leads to the formation of Amadori products from the Schiff base adduct. The Amadori product undergoes a dehydration reaction to form a glucosone (diketone), which is the proximate intermediate in the production of AGEs and protein cross-linking. An example of a glucosone, 4 deoxy glucosone (*DG*), is shown. The AGE (CML) is one of the predominant modifications found on proteins after the Maillard reaction.

TABLE I. Summary of inhibitory concentrations (IC₅₀) from the competitive ELISA experiments

	Raw ($\mu\text{g/mL}$)	Roast ($\mu\text{g/mL}$)	No sugar ($\mu\text{g/mL}$)	Glucose ($\mu\text{g/mL}$)	Galactose ($\mu\text{g/mL}$)	Xylose ($\mu\text{g/mL}$)	Mannose ($\mu\text{g/mL}$)
Flurunner	200	—	—	—	—	—	—
Sun Oleic	200	2.2	—	90.9	—	—	—
NC9	210	2.4	—	87.5	—	—	—
Ara h 1	—	—	17.4	4.0	3.2	5.4	4.0
Ara h 2	—	—	4.5	1.6	1.1	4.1	0.8
WPE	—	—	197	3.5	5.0	39	3.0

FI, Fold increase (see "Methods" section).

er levels than did raw peanut extracts. The contribution of the Maillard reaction to the increase in the allergenic properties of the major peanut allergens Ara h 1 and Ara h 2 was assessed. Because of the diversity of the biochemical modifications to proteins known to occur during roasting or browning of foods, the allergens were purified from raw peanuts and used in a well-documented,²⁻⁴ highly characterized, and isolated in vitro model system to determine whether the Maillard reaction alone affects the allergenic properties of these allergens.

METHODS

Maillard reactions

Purified Ara h 1 (1 mg/mL), Ara h 2 (0.8 mg/mL), or whole peanut extract (WPE; 6 mg/mL) was solubilized in PBS and incubated at 55°C in the presence of 0.2 mol/L fructose, glucose, arabinose, mannose, xylose, galactose, or dextrose for various amounts of time. Glucose, mannose, xylose, arabinose, and galactose were used because they are found in peanuts. Dextrose and fructose were

randomly chosen to test whether they could possibly reduce the allergenic properties of peanut proteins through the Maillard reaction.¹⁵ The progression of the reactions was monitored by using SDS-PAGE. Samples were collected after 10 days (representing the different stages of Maillard reaction progression caused by the rate of reaction with each sugar) and used in heat degradation, enzymatic digestion, or ELISA experiments. For measuring the degradation of the proteins in the presence or absence of sugars, we incubated the Maillard reactions at 55°C for 10 days followed by SDS-PAGE. Results of the heat degradation and enzymatic digestion reactions were visualized by Coomassie Brilliant Blue staining.

Immunoblot analysis

For the detection of IgE binding by high molecular proteins, immunoblot analysis was performed with serum IgE from a 10-person pool of individuals with peanut allergy.¹⁶⁻¹⁸ SDS-PAGE (12%) resolved proteins were transferred to a polyvinylidene fluoride membrane (Immobilon-P, 0.45- μm pore size; Millipore, Bedford, Mass) electrophoretically. The membranes were blocked in TRIS-buffered saline containing 0.5% Tween plus 1% BSA for 2 hours at room temperature. The membrane was then washed in

TRIS-buffered saline containing 0.5% Tween and incubated with a 1:10 dilution of pooled human sera for 1 hour. Detection of the bound IgE was accomplished by using alkaline phosphatase-labeled anti-human IgE secondary antibody (Kirkegaard & Perry Laboratory, Inc, Gaithersburg, Md). The immunoblot was incubated in the presence of the secondary antibody for 30 minutes, washed, and incubated with CDP-Star (BioRad Laboratories, Hercules, Calif), a chemiluminescent substrate for alkaline phosphatase according to the manufacturer's instructions and subsequently exposed to x-ray film.

Digestion reactions in gastrointestinal fluid

Gastric secretions (GS) were obtained from the stomach of a fasting individual. Purified Ara h 1, Ara h 2, and WPE samples from the Maillard reactions were incubated in the presence of a 1:10 dilution of GS in PBS (pH 2) for various times at 37°C. Aliquots were taken for SDS-PAGE analysis at 0, 5, 13, 30, and 180 minutes and overnight (approximately 15 hours). To confirm that the sugars do not interfere with the digestion reactions, the following controls were included. Samples of proteins were incubated in the presence and absence of the same concentrations of sugar as the Maillard reactions, with and without heating, and then subjected to digestion reactions.

Direct competitive ELISAs

A competition between free antigen (WPE or purified Ara h 1 or Ara h 2) and the corresponding antigen absorbed to the plate for binding to serum IgE was used. Ninety-six-well ELISA plates were coated with either 0.1 µg of raw WPEs from Florunner (FLO) cultivar or 0.5 µg of purified Ara h 1 or Ara h 2 in PBS (pH 9) overnight at 4°C. After 5 washes with PBS/0.5% Tween-20, plates were blocked with PBS/Tween-20 containing 3% BSA for 2 hours at room temperature. Plates were washed 3 times with PBS/Tween-20 and incubated at 37°C for 1 hour with a 1:25 dilution of pooled serum from allergic patients³⁻⁵ in the presence of increasing concentrations of competitor. Competitors (free antigen) consisted of increasing concentrations of either WPE, Ara h 1, or Ara h 2 from raw Florunner (control), rice proteins (negative control), samples from *in vitro* Maillard reactions with the various sugars, or roasted and raw WPEs from two different sources, Sun Oleic and NC9. The concentrations used for the competitor for WPEs ranged from 0.78 to 160 µg/mL and from 0.5 to 400 µg/mL for the purified proteins. Plates were then washed 5 times and incubated with an anti-human IgE antibody directly conjugated to alkaline phosphatase for 1 hour at room temperature. After incubation with the 1:3000 dilution of the secondary antibody, plates were washed 5 times. Color was developed by using nitrophenyl diamine diethanolamine (Sigma, St Louis, Mo) substrate, and the OD of each well was measured at 405 nm. A comparison was made on the basis of the amount of protein required to achieve 50% inhibition in comparison with the standard raw peanut extract or unmodified allergens from raw peanuts. Therefore the inhibitory concentration at 50% (IC_{50}) is defined as the concentration of free antigen required to inhibit IgE binding to coated wells by 50%. The percentage of inhibition reported here was calculated by using the following equation:

$$\frac{1}{(A_2 - A_1/A_0)} \times 100 =$$

% inhibition for any given concentration of inhibitor,

where A_0 is the absorbance in the absence inhibitor (maximum absorbance), A_2 is the absorbance at any given concentration, and A_1 is the average absorbance of control wells that contained no primary antibody (in this case serum IgE).

In Table I, the IC_{50} for IgE binding by the modified WPE or purified allergens divided by the IC_{50} for IgE binding by the corresponding unmodified samples was used to determine the fold

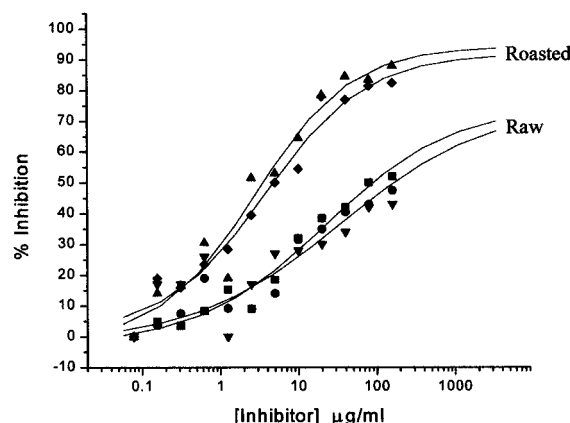


FIG 2. IgE-binding properties of roasted versus raw peanuts. Increasing concentration of raw peanut extracts from brand 1 (raw, circles; roasted, triangles) and brand 2 (raw, inverted triangles; roasted, diamonds) were used to compete for IgE binding with the raw Florunner WPEs that coats the wells. Increasing concentrations of the raw Florunner extract (squares) were used as free antigen to compete with itself for IgE binding as a standard for comparison.

increase in competition. Protein concentrations were determined by using the Biorad protein assay reagent kit (BioRad Laboratories).

RESULTS

Comparison of the IgE-binding properties of raw peanut and roasted peanut extracts from two different varieties

Raw and roasted peanut extracts from two different sources were used in direct competitive ELISAs to determine whether roasting results in increased IgE binding. The binding curves for competitive inhibition are shown in Fig 2. The summary of the concentrations of competitor sample (free antigen) required to inhibit IgE binding to coated wells by 50% (IC_{50}) is shown in rows 1 to 3 of Table I for both raw and roasted forms of each market variety (Sun Oleic and NC9, respectively) tested. The data in Fig 2 demonstrate that the roasted forms of the two peanut varieties compete at approximately 90-fold higher level for binding to serum IgE of allergic individuals. Also, although the raw WPEs demonstrate IgE-binding profiles similar to each other, the slopes of the binding curves differ significantly from those of the roasted extracts.

Effects of the Maillard reaction on IgE-binding properties of peanut allergens

WPEs and purified Ara h 1 and Ara h 2 from raw Florunner were subjected to the Maillard reaction *in vitro*, and competitive inhibition ELISA was used to compare the IgE-binding properties of these modified samples with the corresponding unmodified samples. The summary of the IC_{50} values are shown in Table I (rows 4-6) for reactions with the sugars tested. The sug-

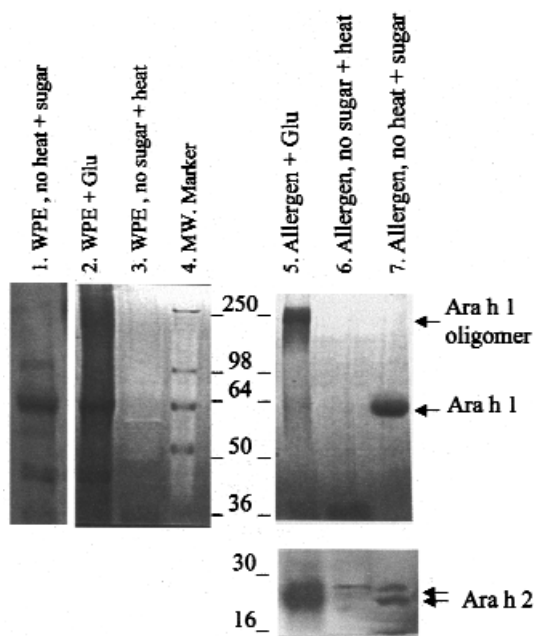


FIG 3. SDS-PAGE analysis of the modified peanut proteins that were subjected to the *in vitro* Maillard reaction. WPEs were heated in the presence of glucose (Glu; lane 2), the absence of sugars (lane 3), or unheated in the absence of sugar (lane 1, control) and subject to electrophoresis. Molecular weight standards are shown in lane 4. Ara h 1 and Ara h 2 were heated in the presence of glucose (lane 5), in the absence of sugars (lane 6), or unheated in the presence of sugars (lane 7, control) and subject to electrophoresis. Arrows indicate the location of Ara h 1 monomer and trimer and Ara h 2 doublet.

ars used in the reactions with the proteins are shown in the top row of the table, and the fold increase in the competition is in the column immediately to the right of the indicated sugar. For example, in the case of Ara h 1, there is an increase in the competition for IgE binding from 2.2-fold for the reaction of Ara h 1 with mannose to 5.4-fold for the reaction with xylose. WPEs and the purified allergens modified through the Maillard reaction demonstrate increased IgE-binding properties in comparison with their unmodified counterparts. Also, the difference in the slope of the IgE-binding curves (data not shown) for the unmodified samples and the modified samples seem to mimic the slopes of the raw versus roasted peanut curves in Fig 2, respectively.

Structural changes caused by the Maillard reaction result in the formation of proteins that are more stable to degradation

In Fig 3 the modifications made to the peanut proteins as a result of the Maillard reaction were assessed by using SDS-PAGE analysis. WPEs and purified Ara h 1 and Ara h 2 from raw Florunner were heated in the absence or presence of sugars, such as glucose, fructose, xylose, dextrose, arabinose, mannose, or galactose. In Fig 3 WPEs that have been heated in the absence (lane 3)

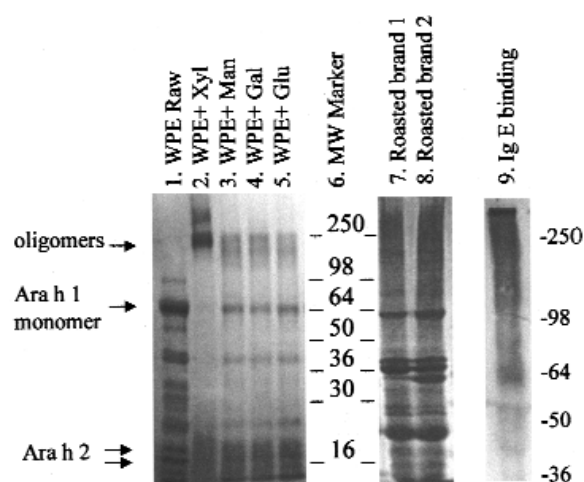


FIG 4. Comparison of WPEs from roasted peanuts with WPEs that have undergone the *in vitro* Maillard reaction by SDS-PAGE. WPEs were heated in the presence of xylose (Xyl; lane 2), mannose (Man; lane 3), galactose (Gal; lane 4), and glucose (Glu; lane 5) or absence of sugars (lane 1, control) and subjected to electrophoresis. Molecular weight standards are shown in lane 6. Lanes 7 and 8 contain the proteins from the two market varieties of roasted peanuts. In lane 9 proteins from roasted WPEs were transferred to nitrocellulose and probed with serum IgE from allergic individuals.

or presence of the glucose (lane 2) are shown. Proteins in raw WPE are normally seen as distinct, well-defined bands (lane 1), whereas, as a result of the Maillard reaction (lane 2), various cross-linking and non-cross-linking adducts are formed, causing the bands to appear as smears on SDS-PAGE. In addition, it is clear that when many of the proteins are heated in the absence of sugars they become degraded (lane 3) to a point where the majority of the bands are no longer visible by Coomassie staining at the indicated molecular weights (lane 4). It is possible that the protease or proteases present in the WPE preparation degrade the modified proteins less rapidly than the unmodified proteins. Similar results are seen with the purified allergens Ara h 1 and Ara h 2 (lanes 5-7). Ara h 1 becomes covalently cross-linked to form higher order structures, corresponding with the molecular weight of a trimer, when subjected to the Maillard reaction in the presence of various sugars (lane 5). In lane 7, where Ara h 1 and Ara h 2 have been heated in the absence of sugar, they are degraded over time. One of the major breakdown products of Ara h 1, previously noticed to be very stable (S. J. Maleki, 1998, unpublished observation), is observed as a band slightly below 36 Kd. Unmodified Ara h 2 normally appears as two distinct bands on SDS-PAGE (lane 7); however, when heated in the presence of the sugars, a smear is observed in each case, as seen in the reaction with glucose in lane 5. Although Ara h 2 has likely had various covalent modifications that cause the appearance of a smear on SDS-PAGE, higher order structures were not observed. The

modifications made to the proteins were shown to be AGEs or advanced MRPs by Western blot analysis with anti-CML and anti-AGE antibodies (data not shown).

Modifications seen in the *in vitro* Maillard reaction mimic structural modifications seen in roasted peanuts

In Fig 4 the migration patterns of proteins from raw and roasted peanuts are compared with peanut proteins from WPEs that had been subjected to the Maillard reaction *in vitro* by using SDS-PAGE. Proteins in raw WPEs are normally seen as distinct, well-defined bands (lane 1), whereas in the samples raw WPEs have undergone the Maillard reaction with the various sugars the bands appear as smears, higher order structures, or both (lanes 2-5). The Maillard reaction seems to occur at a higher rate in the presence of xylose than the other sugars (lane 2). The structural changes to the proteins in the isolated Maillard reactions are similar to the structural changes observed in the roasted peanuts (lanes 7 and 8).

Western blot analysis was used to determine whether the higher molecular weight smears observed in the roasted WPEs were recognized by serum IgE from allergic individuals (lane 9). The stacking gel is visible to demonstrate the large proteins found in this portion of the gel and the proteins that remain in the loading well because they are too large to enter the stacking gel. We believe that this is due to the cross-linking of proteins in roasted peanuts. The higher molecular weight proteins that appear as smears in roasted peanuts are recognized and bound by serum IgE of allergic individuals.

Peanut proteins become more resistant to digestive enzymes as a result of the structural modifications caused by the Maillard reaction

It is believed that one of the characteristics that makes a protein allergenic is its ability to resist digestion. The digestion pattern of Ara h 1 and Ara h 2 (data not shown) before and after they were subjected to the Maillard Reaction was examined. The proteins were incubated in the presence of GS for the indicated times and resolved by SDS-PAGE. Although there are some higher molecular weight (>30 kD) proteins present at 3 hours, after 15 hours, the majority of the unmodified Ara h 1 was digested into fragments below 20 kD (Fig 5, lanes 1-5). However, in the same time frame, fragments and smears with much higher molecular weights are seen in the digestion of the Ara h 1 sample that has been modified through the Maillard reaction (Fig 5, lanes 7-11). In the case of Ara h 2, where intramolecular, non-cross-linking, or both types of modifications seem to predominate as a result of the Maillard reaction (Fig 3), a significant amount of the protein remained intact after 15 hours of digestion with GS, whereas very little if any protein remained in the unreacted Ara h 2 after 3 hours of digestion (data not shown).

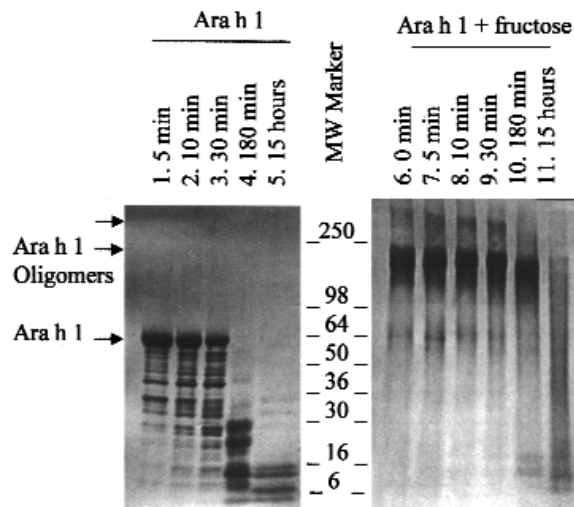


FIG 5. SDS-PAGE analysis of digested Ara h 1 with gastrointestinal fluid. Unmodified Ara h 1 was digested with gastrointestinal fluid for the indicated amounts of time (lanes 1-5). Ara h 1, modified through the Maillard reaction in the presence of fructose, was digested for the indicated amounts of times (lanes 6-11).

DISCUSSION

In this study the effects of roasting on the allergenic properties of peanuts was assessed. Roasted peanut extracts were found to bind serum IgE from allergic individuals at approximately 90-fold higher levels than raw peanuts, and the Maillard reaction was shown to contribute to the observed effect. Meanwhile, several studies have addressed the immunologic aspects of peanut allergenicity toward developing vaccines, immunotherapies,¹⁶⁻²¹ or both by using raw peanut extracts.

Few studies have addressed the allergenic properties of proteins as a result of food processing events, such as roasting or browning. Skin testing experiments have shown that products of the Maillard reaction in milk are associated with increased allergenicity.²²⁻²⁴ A recent study has demonstrated the reduction of allergenicity of soy proteins after a glycosylation reaction.¹⁵ In addition, even though a study by Ikeda et al⁸ has demonstrated an enhanced IgG recognition of several proteins conjugated to a Maillard reaction by products (CML) such as CML-BSA (CML conjugated to BSA) and CML-RNase, IgE binding by these products were not reported. Gillespie et al²⁵ claim that the IgE-binding properties of raw and roasted peanuts were equal.

Our study supports the findings of Nordlee et al²⁶ 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 these observations may be due to the fact that heat treatment increases the allergenicity of peanut proteins by increasing the availability of allergic binding sites on the proteins that were previously unexposed. Our study indicates that 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 GS, in addition to exposing previously unavailable sites.

Ara h 1 has been shown to form stable trimeric complexes in solutions at low concentrations, which is suggested to play a role in the allergenic properties of this protein.¹⁶ Using a molecular model of the trimer, a second study demonstrates that the trimer is stabilized through hydrophobic interactions and the location of the hydrophobic residues coincides with the IgE recognition sites.²⁷ The formation of a trimeric complex may afford the molecule some protection from protease digestion and denaturation, allowing passage of large fragments of Ara h 1 containing several intact IgE-binding sites across the lumen of the small intestine and therefore contributing to its allergenicity. In the current investigation Ara h 1 is cross-linked through the Maillard reaction to form covalently associated trimers and hexamers. The quaternary structure previously hypothesized to be important in allergenicity is now known to become covalently cross-linked because of thermal processing of peanuts and even more resistant to digestive enzymes than previously determined for unmodified Ara h 1 that was purified from raw peanut extracts.

In this study the increase seen in the IgE-binding properties of Ara h 1 and Ara h 2 after they were subjected to the Maillard reaction is not enough to account for the large increase seen in IgE binding by roasted peanut extracts. One possible explanation is that multiple biochemical reactions occur during thermal processing and the collective modifications to all peanut proteins can account for the increased IgE binding by roasted over the raw extracts. Other byproducts that may contribute to the higher levels of IgE binding by roasted extracts, such as lipid oxidation products, are currently under investigation by our laboratory. Inhibitors of certain biochemical reactions (ie, the Maillard reaction) that may be useful in decreasing the allergenic properties of roasted peanuts are also being analyzed.

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