RESEARCH ARTICLE

Ara h 1 structure is retained after roasting and is important for enhanced binding to IgE

Jacqueline B. Nesbit[1], Barry K. Hurlburt[1], Catherine H. Schein[2,3], Hsiaopo Cheng[1], Hui Wei[4] and Soheila J. Maleki[1]

[1] U.S. Department of Agriculture, Agricultural Research Service, Southern Regional Research Center, New Orleans, LA, USA
[2] Sealy Center for Structural Biology and Molecular Biophysics, Department of Biochemistry and Molecular Biology, Galveston, TX, USA
[3] Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, USA
[4] Waters Corporation, Beverly, MA, USA

Scope: Ara h 1 from roasted peanut binds higher levels of serum immunoglobulin E than raw peanuts and this is likely due to the Maillard reaction. While Ara h 1 linear IgE epitopes have been mapped, the presence and importance of structural epitopes is not clear.

Methods and results: Mass spectrometry, immunoblot, ELISA, circular dichroism (CD), and structural analysis were used to compare structural and subsequent IgE-binding differences in Ara h 1 purified from raw (N) and roasted peanuts (R) and denatured Ara h 1 (D). Although N and R had similar CD spectra, the latter bound significantly more IgE. Decreased IgE binding was seen with the loss of secondary structure. This same IgE-binding pattern [R > N > D] was seen for the sera of ten peanut allergic patients. While the majority of linear epitopes are located on surface and structured regions of Ara h 1, our study shows that conformational epitopes of Ara h 1 bind better to IgE than linear epitopes.

Conclusion: Enhanced IgE binding to roasted Ara h 1 could be due to alterations such as chemical modifications to individual amino acids or increased epitope exposure. IgE binding is significantly reduced with loss of structure.

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1 Introduction

In order to prevent the development of or to improve diagnosis and treatments of food allergy, we must improve our knowledge of the fundamental allergen-immune system recognition and interaction. Of the peanut allergens characterized, Ara h 1 is one of the most recognized by patient sera [1]. Ara h 1 is a 63-kDa glycosylated, vicilin-like, and abundant seed storage protein [2, 3]. The homology-based molecular models based on other cupin super family of proteins, such as phaseolin [4, 5] or β-conglycinin [6], indicate that the Ara h 1 monomer consists of two beta-barrel core domains, similar to one another in structure with quite different sequences that are separated by an alpha helical linker region [7]. A recent crystal structure [8], which is used in this study, agrees well with these models. These monomers form homotrimers [5], and the degree of glycosylation is not known. The 23 linear immunoglobulin E (IgE)-binding epitopes identified by peptide dot spotting are evenly distributed along the linear sequence [5]. According to Shin et al. [5] and Barre et al. [6], the most exposed B cell epitopes occur at the C-terminal alpha helical region at the edges of the cupin region. These exposed epitopes share sequence and conformational similarity to other vicilin proteins in legumes [9]. It has been speculated that the linear IgE-binding epitopes of allergens are more important than the structural epitopes and perhaps contribute to longevity and severity of peanut or other food allergies in patients [10–12].

The reaction of reducing sugars with amino groups of amino acids (the browning process), known as the "Maillard reaction" was originally described by Maillard in 1912 [13]. Since then thousands of publications have documented this reaction during thermal processes (i.e. roasting and cooking) of foods and in human disease and aging [13–29]. The

Correspondence: Dr. Soheila J. Maleki, Research Chemist; USDA-ARS-SRRC, 1100 Robert E. Lee Boulevard; New Orleans, LA 70124
E-mail: soheila.maleki@ars.usda.gov
Fax: +1-504–286-4430

Abbreviations: CD, circular dichroism; **IgE**, immunoglobulin E

well-documented chemical modification of the amino acid side chains of proteins in foods during cooking begins with formation of a Shiff's base linkage of an aldehyde group of a reducing sugar with the alpha or epsilon amino groups of proteins, which then undergo Amadori rearrangement to form stable ketoamine derivatives. These derivatives are much more reactive than the parent sugars and chemically modify amino acid side chains (i.e. carboxymethyllysine or CML) and cross-link proteins. These final chemical modifications are known as advanced glycation end products or Maillard reaction products (MRP) [13–18, 20–22, 27, 30]. The effects of MRP on food allergy have been previously described and documented [19, 30–34] and while important in the development of flavor and color in peanuts as well as many other processes of food technology, may have detrimental nutritional, physiological, and toxicological consequences [20]. Our previous results have shown that purified Ara h 1 subjected to a simulated roasting model (which consists of inducing the Maillard reaction with purified Ara h 1) or within roasted peanuts is less soluble [35], forms covalently linked trimers, and has increased resistance to enzymatic digestion [34]. Some of the mechanisms that may contribute to the above characteristics and thus increased IgE-binding properties of roasted Ara h 1 are the structural (including denaturation, degradation, and oligomerization/aggregation) and chemical alterations caused by the Maillard reaction [34]. A study by Chassaigne et al. used mass spectroscopy to identify specific chemical modifications and compare the alterations in digestion patterns of the main peanut allergens in raw versus roasted peanut [29]. Other changes with roasting can include degradation of posttranslationally added glycosylation. In the current study, the contribution of conformational versus linear epitopes of Ara h 1 to IgE binding and therefore immune system recognition is compared. We also assess the contribution of structural versus roasting-induced alterations to IgE-binding properties of Ara h 1 purified from raw and roasted peanuts.

2 Materials and methods

2.1 Patient sera

Sera from peanut allergic individuals were collected after informed consent at the University of Arkansas for Medical Sciences (Little Rock, AR) and Tulane University (New Orleans, LA, IRB-approval number 09–00231) in accordance with the rules and regulations of the institutional review boards. While food challenge for research purposes was precluded in severely allergic patients, all those selected had early childhood onset and recurrent severe systemic allergic reactions to peanut resulting in emergency department visits as children and adults. There is little possibility of such patients "outgrowing" the allergy, indicating the involvement of relevant IgE epitopes. Specific IgE to peanut was measured by ImmunoCAP (Phadia, Uppsala, Sweden; Table 1).

2.2 Protein purification and treatments

Florunner peanuts were used either raw or roasted. The samples were obtained and roasted as previously described [35]. Ara h 1 was purified from both raw and roasted peanuts as

Table 1. Characteristics of the patient sera

Subject number	Gender	Age	Symptoms with peanut ingestion	SPT with peanut	ImmunoCAP IgE to peanut (KU/L)	Peanut proteins detected on WB
1	F	35	Anphx history[a)]	4+	21.5	Ara h 1, Ara h 2, Ara h 3, Ara h 6
2	F	36	SOB, wheezing and severe asthma after ingestion	3+	14.4	Ara h 1, Ara h 2
3	M	42	Anphx history[a)]	4+	23.1	Ara h 1, Ara h 2, Ara h 6
4	M	9	Vomiting, OP	>4+	39.3	Ara h 1, Ara h 2, Ara h 3, Ara h 6
5	F	9	Anphx history[a)]	2+	6.09	Ara h 1, Ara h 2, Ara h 3, Ara h 6
6	M	4	Urt, vomiting	>4+	8.05	Ara h 1, Ara h 2, Ara h 3, Ara h 6
7	F	6	Anphx history,[a)] severe AG after accidental ingestion	>4+	2.35	Ara h 1, Ara h 2, Ara h 3
8	M	12	SOB, wheezing and severe asthma after ingestion	3+	0.23	Ara h 1, Ara h 2, Ara h 3
9	F	7	AG of lips and tongue and SOB	2+	2.36	Ara h 1, Ara h 2, Ara h 3
10	M	20	Wheezing, AG, and Urt	4+	8.55	Ara h 1, Ara h 2, Ara h 3

KU/L, Kilo units/liter; anphx, anaphylaxis; WB, western blot; OP, oral; AG, angioedema (rapid swelling); Urt, uritcaria (hives); SOB, shortness of breath; SPT, skin prick test
a) Patient was not orally challenged

previously described [4, 5]. To generate a denatured Ara h 1, purified protein from raw peanuts was heated to 95°C for 10 min in Milli-Q water followed by centrifugation at 9400 × g for 5 min. The insoluble, precipitated protein pellet was dissolved in 0.1% TFA, 30% ACN. Immediately prior to use for circular dichorism (CD) and spot blot analysis, the sample was desalted using disposable gel filtration columns (G-25, PD-10, GE Healthcare, Piscataway, NJ) into Milli-Q water.

2.3 Circular dichroism (CD)

CD spectra of raw, roasted, and denatured, purified Ara h 1 were determined at a protein concentration of 0.1 mg/mL. The far UV (185–250 nm) CD spectra were obtained using a JASCO Model J-815 CD spectropolarimeter with a 1-cm path length with seven accumulations per sample. Background CD spectra of Milli-Q water or TFA/ACN were subtracted. The mean residue weight of 114.5 g/mole for Ara h 1 was used for calculating ellipticities. Secondary structural modes were estimated from ellipticities by multiple protein secondary structure prediction and calculation programs [36], and CDPro (data presented here http://lamar.colostate.edu/∼sreeram/CDPro/main.html) [37, 38].

2.4 Spot blot analysis

Different amounts of Ara h 1 purified from raw and roasted peanuts and denatured raw Ara h 1 in water were spotted onto polyvinylidene fluoride membranes and incubated with serum from the documented peanut allergic individuals (Table 1). The TFA/ACN in the partially denatured Ara h 1 would melt the membrane and was not used in this experiment. Ara h 1 amounts spotted on the membranes are the same as determined by protein concentration and ponceau S staining (J.T. Baker, Phillipsburg, NJ) [39] of membrane prior to IgE blots. Also, synthetic peptides of known Ara h 1 IgE epitopes were commercially synthesized on a SPOTS membrane (Sigma Chemical Company, St. Louis, MO). The membranes containing the synthetic peptides or Ara h 1 protein samples were probed with serum IgE as previously described [39].

2.5 Competitive inhibition ELISA

ELISA was carried out as previously described [39]. Briefly, purified native Ara h 1 (50 ng/well in 0.1 M $NaHCO_3$, pH 9.6) was used to coat a 96 well plate, blocked with 2% dry milk in phosphate-buffered saline +0.05% Tween 20 (PBST). Diluted raw or roasted, purified Ara h 1 (indicated in Fig. 4C) were mixed with equal volumes of serum (diluted 1:10), rotated at 4°C for 1 h and added to the coated plates for 1 h at 37°C. Plates were incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgE and developed using HRP substrate 3,3′,5,5′-tetramethylbenzidine (KPL, Gaithersburg, MD). The absorbance was read at 495 nm. The denatured Ara h 1 cannot be concentrated enough in aqueous solution in a soluble form for ELISA assay.

2.6 3D mapping of epitopes

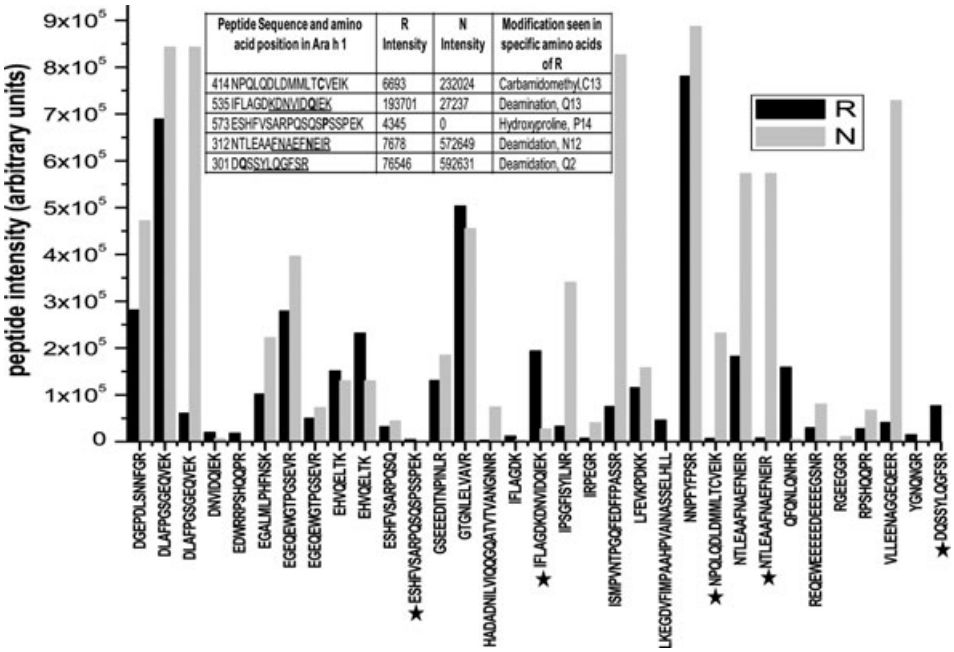
The ribbon model of residues 173–585 of Ara h 1 (using the template 3S7I.pdb for Ara h 1 crystal structure) was prepared with space filled IgE-binding sites with the MPACK suite and minimized with FANTOM [40–42], which is available in SDAP [5]. Surface exposure of residues in the IgE epitopes on the models were determined with GETAREA [43], using default water radius (1.4 Å) and a cutoff for exposure as 30% of total area of the side chain. Prosite was used to determine possible glycosylation sites; only one (NASS, residues 521–524) was identified with a high degree of surface exposure. Exposure of epitope residues deemed likely to be affected by glycosylation at this site were those with CB distances <10 Å to the CB residues of the NASS site.

2.7 LC-MS

For mass spectrometric analysis, in-solution digestion with trypsin was carried out according to Chassaigne et al. [38] with slight modifications as described in Water's application notes [44]. Briefly, a 4.2 μg/μL solution of Ara h 1 raw or roasted sample in 50 mM ammonium bicarbonate (pH 8.6) buffer was incubated with a 0.2% w/v solution of RapiGest SF and heated at 80°C for 15 min. Next 2.5 μL of DTT was added and the solution was heated to 60°C for 30 min. Following a brief cooling period, centrifugation, and addition of 2.5 μL of a 300 mM of iodoacetamide, the sample underwent a 30-min incubation in the dark. Trypsin (Promega, Sunnyvale, CA) was added at 50 ng/uL in ammonium bicarbonate and incubated overnight at 37°C. Ten microliters of a 5% TFA solution was then added and incubated for 90 min at 37°C. The digests were centrifuged and the supernatants were analyzed via tandem LC MS, using a nano AQUITY UPLC system and a quadropole-time of flight MS (XevoQTofMS) tandem mass spectrometer (Waters Corp., Beverly, MA). Chromatographic separation was accomplished using a nanoACQUITY BEH C18, 75mMX 150 mm analytical column at a 300 nL/mL flow rate. Mobile phase A used was 100% H_2O, 0.1% formic acid, and mobile phase B was 90% ACN, 10% H2O, and 0.1% formic acid. Data files were processed using Waters PLGS software for database search.

3 Results

Digested peptides from raw and roasted Ara h 1 are different and chemical modifications can be observed: Ara h 1 purified from raw (N) and roasted (R) peanut was digested by trypsin and the resultant peptides were separated by LC and the eluted



Peptide Sequence and amino acid position in Ara h 1	R Intensity	N Intensity	Modification seen in specific amino acids of R
414 NPQLQDLDMMLTCVEIK	6693	232024	CarbamidomethyLC13
535 IFLAGDKDNVIDQIEK	193701	27237	Deamination, Q13
573 ESHFVSARPQSQSPSSPEK	4345	0	Hydroxyproline, P14
312 NTLEAAFNAEFNEIR	7678	572649	Deamidation, N12
301 DQSSYLQGFSR	76546	592631	Deamidation, Q2

Figure 1. The mass spectroscopic analysis of peptide frequency in the tryptic digest of purified N and R. The relative peptide intensity for both R (black bars) and N (gray bars) is shown on the y-axis and the sequence of the unmodified peptides is shown on the x-axis. The inset table shows peptides for which the modified versions were found in R and black stars indicate these same peptides on the x-axis of the graph. The known IgE epitopes are underlined and the modified residues are in bold.

peptides were subjected to Q-TOF MS. A comparison of the relative distribution of peptides between raw and roasted peanuts is given for Ara h 1 in Fig. 1. Relative peptide intensities were calculated by normalizing the intensity of each peptide against the sum intensity of all identified peptides. As reported by Chassigne et al., the relative distribution of peptides changed as a function of roasting. For example, in Fig. 1 the ISMPVNTPGQFEDFFPASSR peptide, which was relatively intense in N was significantly lower in R. Peptides that were either not found in R or found to be modified in R are shown in the inset table of Fig. 1.

Ara h 1 purified from raw and roasted peanuts has similar secondary structure content: Far-UV CD (185–260 nm) was used to compare the secondary structure content of Ara h 1 purified from raw (N) and roasted (R) peanuts (Fig. 2). The CD spectra show that the secondary structure of N and R are very similar in that both structures exhibit minimums at ~208 and ~222 nm and a crossover near 200, typical characteristics of proteins with either separate $\alpha + \beta$ region or combined α/β regions. However, the large 208:222 ratio is indicative of a structure with separate α and β regions, which corroborates the Ara h 1 structure [8]. Further analysis of the CD spectra of N and R (Fig. 2A inset table) indicate that R contains very similar secondary structure to N (Fig. 2A) and the values obtained are valid according to normalized root mean square deviation values (NRMSD) that are used for internal software control and confirmed by other programs used to interpret protein CD spectra, such as K2D.

Temperature interval analysis and denaturation of purified, raw Ara h 1: To investigate the contribution of linear epitopes versus conformational epitopes in IgE binding, we denatured pure Ara h 1 in solution, which is very different from roasting within a low moisture and compact environment of a peanut. Initially, we performed temperature interval analysis on Ara h 1 purified from raw peanuts (Fig. 2B) to determine the denaturation temperature. The intensity of the far-UV CD spectra increased with temperature, but because the shape of the spectra did not change, it indicates that Ara h 1 unfolds and precipitates, which causes a decrease in Ara h 1 concentration in solution. Ara h 1 becomes partially denatured at 45°C and with increases in temperature above 45°C Ara h 1 begins to precipitate, eventually becoming irreversibly denatured due to exposure of the hydrophobic core.

When Ara h 1 is heated to 95°C and then cooled to 25°C, the protein cannot be resolubilized in aqueous solution. Also, white precipitates were observed in the sample at 95°C. The denatured Ara h 1 (D) was dissolved in SDS sample buffer and subjected to SDS-PAGE along with N and R (Fig. 2C).

Denaturation and resolubilazion of native Ara h 1: Purified N was denatured and precipitated by in-solution heat treatment. The precipitate was suspended in a mixture of organic solvents (ACN/TFA) and CD spectra were obtained at each step. When treated as such and desalted into water, Ara h 1 loses ~30% of its alpha helical content and unordered content increases by similar levels, which is referred to as denatured in Fig. 3. However, if the same resolubilized Ara h 1 is not desalted into water, the spectrum of Ara h 1 contains significantly less α-helical content than native or denatured and a threefold increase in β-sheet content as previously reported for these conditions [45]. The percentages for each secondary structure are shown in Fig. 3 (inset table) and valid according to NRMSD.

Roasting enhances, denaturation reduces IgE binding: Purified N, R, and D Ara h 1 were immediately spotted onto

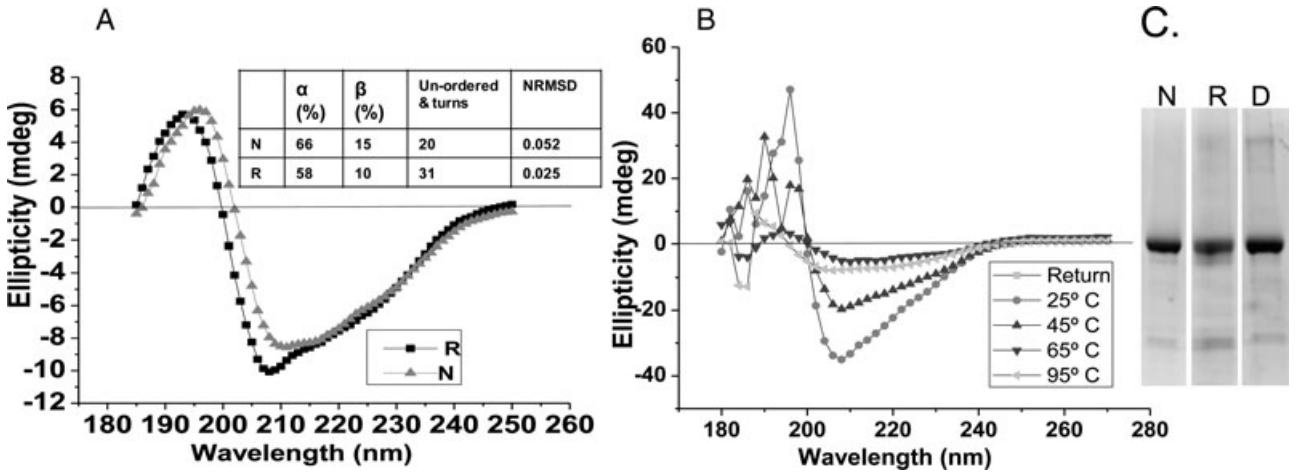


Figure 2. The secondary structure and denaturation of Ara h1 isolated from raw or roasted peanuts. (A) Far UV (185–260 nm) CD spectrum of Ara h1 protein purified from raw (■, black) or roasted peanuts (▲, gray). The inset table displays an estimation of Ara h1 secondary structure content. (B) CD spectra (190–250 nm) of Ara h1 purified from raw peanuts and exposed to temperatures ranging from 25 to 95°C. (C) SDS-PAGE of denatured raw Ara h1 (D) and Ara h 1 from raw (N) and roasted peanuts (R).

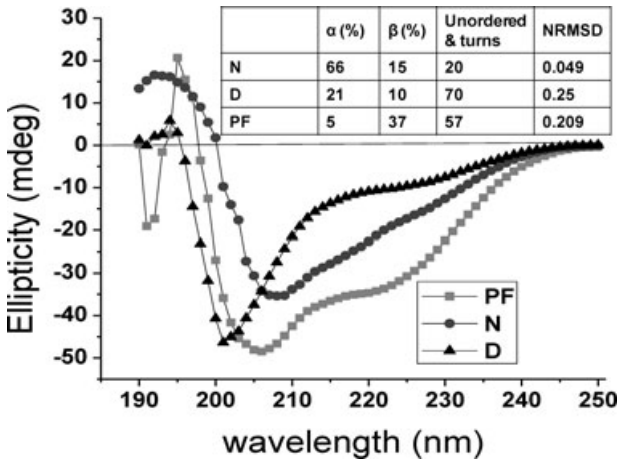


Figure 3. Denaturation Ara h1 purified from raw peanuts. (A) CD spectra of Ara h1 isolated from raw peanuts (N) [49], after denaturation by heat in solution, resolubilization in 30% ACN and 0.1% TFA (ACN/TFA) where protein is partially folded (PF), and desalting into water (Denatured, D) were compared. The inset table indicates the corresponding percentages of secondary protein structure.

membranes at various concentrations indicated in Fig. 4(A). Each membrane was incubated with serum from an individual peanut allergic patient. The patient number and associated characteristics are shown in Table 1. Western blots (not shown) of peanut allergen recognition for each patient sera was performed and findings reported in Table 1. The densitometric measurement of spots at 520 ng level is shown in Fig. 4(B). IgE of all peanut allergic patients tested bound stronger to R than N and weakest to D. Competitive inhibition ELISAs with R and N Ara h 1 (Fig. 4C) with sera from patients number 1 and 2 (Fig. 4A and Table 1) were performed to confirm data in spot blot and to show that the binding of Ara h 1 to the membrane was not affecting the IgE-binding intensity. The relative 50% inhibition for each is shown in the figure, and in each case, R inhibits IgE binding anywhere from 9-fold to >12-fold higher than N for the patients tested (Fig. 4C). D was used only for spot blots as it was not soluble, in aqueous solution, for the length of time and at high enough concentration necessary to give meaningful results in the competitive inhibition ELISA assay.

Epitopes of Ara h 1: The IgE in sera from patients number 1 and 2 bound to previously defined, linear IgE epitopes of Ara h 1 [5], as shown by a SPOTs blotting (Fig. 5A). Both patients had similar epitope specificity, recognizing primarily the previously identified epitopes 4 and 9 and to a lesser extent 5, 6, 7 in the arginine-rich, N-terminal region, and epitopes 11,12,15,16, 17 in the cupin domains. All of the epitopes recognized by the patient sera have a relatively high degree of surface exposure according to the crystal structure (red amino acids, top of Fig. 5B) [8]. Three of the Ara h 1 epitopes (11, 15, 16) recognized by the patient sera are immunodominant. The epitopes cluster in the protein, epitopes 11 and 12 are segregated to a helical/loop area, while epitopes 15, 16, and 17 are near one another in the highly structured β-sheet region on the opposite cupin domain. Epitope 10, which was not detected by either of these patients, may be oriented toward the center of the Ara h 1 trimer in the intact protein (within the nut body).

We also mapped the only glycosylation site and found that it was in the middle of the epitope cluster formed by 15, 16, and 17. Residues at the center of all three of these epitopes (underlined in Fig. 5B) are within 10Å of this site, and thus their surface exposure might be affected by glycosylation (which may be altered by roasting).

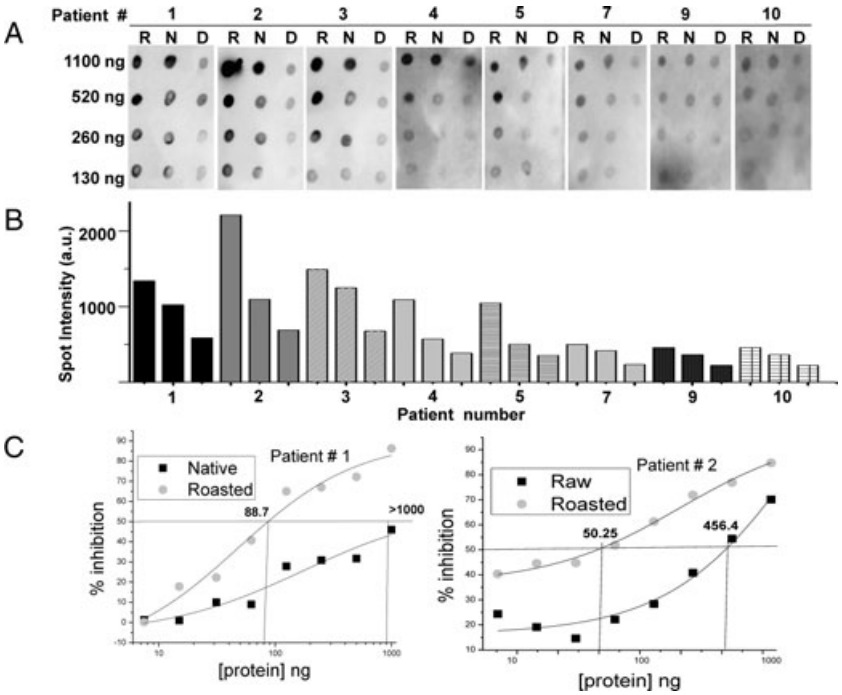


Figure 4. Structure and chemical modification of Ara h1 affect IgE binding. (A) Indicated amounts (left of immunoblots) of denatured (D), folded, raw Ara h 1 (N) and roasted Ara h 1 (R) were spotted onto membranes and incubated with sera from peanut allergic individuals. (B) Densitometric measurements of each of the three spots per patient sera at 520 ng reported in arbitrary units (a.u.). (C) Comparison of the competitive inhibition ELISA of Ara h1 from raw and roasted peanuts for two patients indicated in figure.

4 Discussion

Roasted peanut extracts bind serum IgE from peanut allergic patients at a 90-fold higher level than raw peanut extracts [34]. Chemical modifications that are incurred in the allergens, Ara h 1 and Ara h 2, due to the Maillard reaction which occurs during storage and heat treatment of food [14, 18–20, 22, 30, 31, 33] contributed to the higher IgE binding [34, 46]. In the current study, two questions were asked: (1) is enhanced IgE binding to Ara h 1 from roasted peanut mostly due to altering the protein structure, or to roasting-induced chemical modifications to the surface of the allergen; and (2) how important is the structure to IgE-binding properties of Ara h 1. Ara h 1 was purified from both raw and roasted peanuts, denatured, and digested and then assessed for changes in digestibility, secondary structure, stability, and IgE binding. This molecular structural study was performed to determine what a peanut allergic person's serum IgE encounters following ingestion of peanuts and not necessarily a direct or functional measure of allergenicity.

MS was used to determine change in the relative distributions and intensity of peptides derived from purified Ara h 1 as a function of roasting, which differs considerably from that of N. This change in relative peptide distribution is a clear indication that the protein is being modified during the roasting process. According to our findings and as suggested by Chassaigne et al., the lack of or significant reduction in the detection of predicted trypsin cleavage products from roasted Ara h 1 is most likely due to processing-induced modifications such as protein glycation [29]. Our findings and previous research [29, 44, 47] also lead to the identification of specific modifications to R as indicated in Fig. 1.

Our results, as well as our methodology, differ from two other studies. One [45], found no significant difference in IgE binding to Ara h 1 from raw or roasted peanut. These authors suggested that the heat-treated Ara h 1 was most likely denatured at high temperatures (80–110°C) and that since there was no differences in the IgE binding then there was no correlation between the native state of the protein and its IgE-binding affinity. We show here that at least some population of Ara h 1 molecules is not denatured even by the very high temperatures of roasting. A more recent study found that roasted Ara h 1 is completely denatured, but retains IgE-binding capacity equal to the raw Ara h 1 [47]. They also suggest that there was very little or no glycation found on roasted Ara h 1. However, it is also stated that a single Maillard reaction modification was measured, and that although the levels of frucotsamine content was low, it represents only one of thousands of potential Maillard adducts that could be formed during roasting. We note that the roasted Ara h 1 was purified in a different buffer, which contained potentially denaturing solvents such as ACN and TFA, than that used for isolation from raw peanut. This could influence the structure of roasted Ara h 1. Further, the buffers used for the CD measurements were not defined. In our CD experiments, the Ara h 1 preparations were suspended in water and therefore no curve-smoothing calculations or manipulations were performed, which is necessary when buffers that interfere with CD measurements are used.

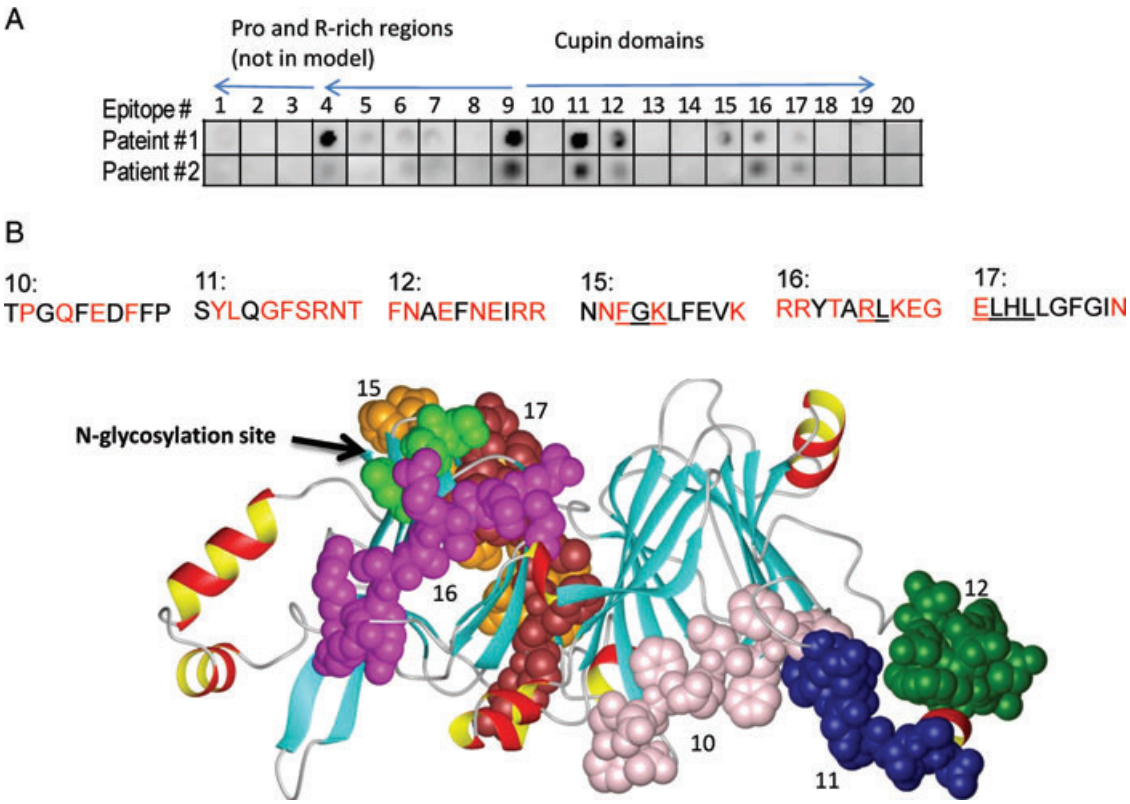


Figure 5. IgE-binding epitopes of Ara h1 and their 3D characterization. (A) Previously identified Ara h 1 epitopes were synthesized on SPOTs membranes and probed with the two patient sera indicated. (B) The structure of Ara h 1 (3S7I. pdb) is shown in ribbon format with the space filling epitopes shown in different colors and indicated with numbers of corresponding epitopes at the top of the panel. Side chains with >30% surface exposure according to GETAREA are highlighted in red in the amino acid sequence at the top of panel B. Residues in epitopes 15, 16, and 17 whose side chains are within 10 Å of the N-linked glycosylation site (NASS, light green in the protein structure, B) are underlined (top of B).

In this study, very little difference is seen in the secondary structure of N and R, yet there is a significant increase in IgE binding to R. Ara h 1 is not denatured by the high thermal treatment of processing, as is generally accepted, if roasted within the context of a peanut. These findings suggest that the roasting-induced chemical modifications [29, 34, 46] to the surface of the protein may play a more important role in IgE binding than enhanced linear epitope exposure due to protein unfolding, as previously speculated [12]. However, it is also possible that more epitopes are exposed for antibody binding without protein unfolding by degradation of bulky surface glycosylation. Glycosylation, for example, was found to inhibit IgE binding to beta-lactoglobulin [48]. There is a significant reduction in IgE binding following deliberate unfolding (or structural alteration) of Ara h 1, which suggests that conformational epitopes are present.

Some of the discrepancies are perhaps due to different methodologies and interpretations. Our study agrees with the previous study in that [35] R is much less soluble and extractible, but it is unclear whether this is due to high level cross-linking and aggregation of the Ara h 1 molecules in folded form or, as previously suggested, denaturation and aggregation [45]. R tends to be more stable to denaturation when heated in solution as seen in the much more stable minimum at 222 nM than seen with the N (data not shown). Also, when the insoluble N was resolubilized in ACN/TFA, an increase of β sheet structures from 12 to 57% was seen here confirming previous work [45], where they concluded that heat treatment increases the content of the β structures. However, our interpretation is that the Ara h 1 in ACN/TFA is not fully denatured, which might explain differences in IgE binding between the two studies. When the protein is desalted into water following ACN/TFA resolubilization, the majority of the secondary structure shifts to unordered loops and turns, which is what we consider denatured. These experiments were repeated and the CD spectra and spot blot analyses were reproducible.

In conclusion, we have shown that the IgE binding, measured by both solution-based and solid-phase methods, is significantly higher to R, as has been shown in our simulated roasting system (induced Maillard reaction) in the past [34]. Regarding differences seen in IgE-binding experiments, it is

highly possible that the patient pools from different countries have different binding specificities, which would explain the differences observed. It appears that a few of their patient sera IgE bound slightly higher to roasted Ara h 1 [47]. The low levels of glycation as they suggest is due to their measurement of a single specific glycation product, which may not be abundant [47]. There are literally hundreds of glycation products that result from the Maillard reaction, some that have yet to be defined. In their simulated Maillard reaction or glycated Ara h 1, they boiled the peanut protein, in solution, which primarily causes denaturation or as they reported a partial denaturation and significant reduction in IgE binding, which supports what is reported here. The authors go on to suggest that other chemical modifications due the Maillard reaction, such as lysinoalanin or other adducts may contribute to their observations and that processing-induced alteration of allergenic properties should be further investigated.

The cupin domain and epitopes are shown here because the majority of the linear IgE-binding epitopes in Ara h 1 occur at the loops and helices at the end of each cupin domain, which form a symmetrical dimer with very different sequences [5, 8]. Also, a significant portion of the Ara h 1 N terminus is unresolved in the crystal structure [8] and not present in homology models [5]. Both of our patients' sera recognized a group of epitopes that cluster near the only glycosylation site in the protein. Minimal change in secondary structure in R versus N, indicates that chemical modifications caused by the Maillard reaction [34, 46], as shown in Fig. 1, and other thermally induced chemical reactions contribute to enhanced IgE binding by R more than alterations in secondary structure. Also, the significant reduction of IgE binding to D in slot blot analysis suggests that the linear IgE-binding epitopes may play a lesser role in IgE binding to Ara h 1 than conformational ones at least for this set of patients who are unlikely to outgrow their peanut allergy. Considering the preference of IgE for binding to folded Ara h 1, it is quite likely that intact Ara h 1 or fragments thereof that maintain a secondary structure, escape the digestive process, are absorbed into the blood stream, and encountered by the immune system. These findings are important to designing peptide-directed vaccines and immunotherapies for peanut allergy, as the linear peptides may not compete for IgE binding with folded proteins. Also, these findings suggest that inducing unfolding of Ara h 1 and perhaps other peanut allergens by a food-processing method could result in a less allergenic product. In future studies we will attempt to identify more of the specific chemical modifications that occur as a result of the Maillard and other roasting induced reactions with the goal of identifying the ones that are recognized by serum IgE of peanut allergic patients.

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The authors have declared no conflict of interest.

5 References

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