

The major peanut allergen, Ara h 2, functions as a trypsin inhibitor, and roasting enhances this function

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Background: The widespread use of peanut products, the severity of the symptoms, and its persistence in afflicted individuals has made peanut allergy a major health concern in western countries such as the United States, United Kingdom, and Canada. In a previous study, the authors showed that the allergenic properties of peanut proteins are enhanced as a result of thermal processing.

Objective: The purpose of this investigation was to determine whether any specific functions are associated with the major peanut allergen, Ara h 2, and whether the functionality of this protein is influenced by processing. An assay was developed and used to assess structure/function changes in Ara h 2 induced by roasting and the effect of these alterations on the allergenic properties of this major peanut allergen.

Methods: A protein domain homology search was used to determine possible functions for Ara h 2. One of the putative functions (protease inhibition) was tested by means of appropriate enzyme assays and protein gel electrophoresis. Circular dichroism was used to compare the structural properties of Ara h 2 purified from raw and roasted peanuts.

Results: Ara h 2 purified from peanuts is homologous to and functions as a trypsin inhibitor. Roasting caused a 3.6-fold increase in trypsin inhibitory activity. Functional and structural comparison of the Ara h 2 purified from roasted peanuts to native and reduced Ara h 2 from raw peanuts revealed that the roasted Ara h 2 mimics the behavior of native Ara h 2 in a partially reduced form.

Conclusions: The data indicate that thermal processing might play an important role in enhancing the allergenic properties of peanuts. Not only has it previously been shown to affect the structural and allergic properties of peanut proteins but also, for the first time, the functional characteristics of an allergen. These structural and functional alterations are likely to influence the allergenicity of peanuts. (*J Allergy Clin Immunol* 2003;112:190-5.)

Key words: Food allergy, peanut allergy, IgE, Maillard reaction, roasting, AGE

Abbreviations used

CD: Circular dichroism
DTT: Dithiothreitol

Approximately 7 million Americans have food allergies. Allergic reactions to foods are responsible for up to 200 deaths each year in the United States.¹ Reports indicate that peanut allergies cause most of the annual emergency department admissions from food allergies and approximately 63% to 67% of deaths² caused by anaphylaxis. According to another study, the most obvious rise in food allergy referrals has been seen in the case of peanut and nut allergy.³ This observation might be due to the large increase in the use of peanut products at an early age⁴ and heightened awareness of food allergies in the past decade.

Previously, we addressed the effects of thermal processing on some of the allergenic properties of peanut proteins.^{5,6} Thermal processing such as roasting, curing, and various types of cooking can cause multiple nonenzymatic, biochemical reactions to occur in food.⁷ One of the predominant reactions that occurs during thermal processing of foods is known as the Maillard reaction,^{8,9} which is important in the development of flavor and color. In addition to protein cross-linking, it is known that advanced Maillard reaction products, also known as advanced glycation end-products, could lead to the loss or modification of amino acids such as lysine and free sulfhydryl groups.¹⁰ Most alterations to protein structure are due to the heat-induced interactions of sugar components with amino acids to form compounds such as carboxymethyl lysine,¹¹ malanoidin, and other non-cross-linking modifications to proteins that might have detrimental nutritional, physiologic, and toxicologic consequences.⁸ Although the effect of the advanced glycation end-products 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,¹²⁻¹⁶ few studies have addressed the role of these products on the allergenic properties of ingested foods.¹⁷⁻²⁰

Some of the roasting-induced biophysical mechanisms for enhanced allergenic properties of the major peanut allergens, Ara h 1 and Ara h 2, were previously explored.⁵ Both allergens bound higher levels of IgE, and

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the increase in IgE binding was correlated with increased carboxymethyl lysine modifications on the surface of the protein.⁶ In these studies,^{5,6} Ara h 1 was found to be intermolecularly cross-linked to form highly stable trimers, and Ara h 2 was thought to form intramolecular cross-links because of roasting, without forming higher order structures. These modifications were also shown to stabilize these proteins against digestive enzymes.

Here, possible mechanisms for the enhanced allergenic properties of roasted Ara h 2 were examined. A homology search of the publicly available protein data bank was performed to determine putative functions for Ara h 2. This search was focused on protein domain homology rather than the primary amino acid sequence of Ara h 2 through use of a net-based search engine (<http://us.expasy.org> and <http://www.ncbi.nlm.nih.gov/Structure/MMDB/mmdbs.shtml>). The goal of this study was to develop a functional assay for Ara h 2 and to compare the functional and structural differences of this major allergen purified from raw and roasted peanuts. In addition, the relationship of altered structure to the function of Ara h 2, which might directly influence the protein's allergenicity, was explored. Optimally, if a certain form of processing can enhance allergenic characteristics of a food, it is possible that other processing events can be identified to reduce these allergenic properties.

METHODS

Trypsin activity assay

Trypsin-inhibitor activity was determined for optimal trypsin inhibition between 40% and 60% through use of a modification of the standard protocol described by Kakade et al²¹ in 1974. Reactions were performed in triplicate. The same amount of Ara h 2 samples (native, partially reduced, and roasted) was incubated in separate tubes with 0.5 mL of bovine pancreas trypsin (Sigma T4665) at 37°C for 30 minutes. Here, partially reduced Ara h 2 is referring to an Ara h 2 in which some, but not all, of its disulfide bridges are intact. Exactly 1.25 mL of prewarmed (37°C) substrate (*N*-benzoyl-L-arginine *p*-nitroanilide or BPNA; Sigma, St Louis, Mo) was added to the Ara h 2 samples and incubated at 37°C for 10 minutes. The reaction was stopped with 0.25 mL of acetic acid (30%), and the absorbance was measured at 410 nm against a blank. Trypsin inhibitor activity was determined by subtracting the absorbance reading of the trypsin enzyme from the absorbance reading of each peanut protein sample. Trypsin inhibitory activity was expressed as trypsin inhibitor activity per milligram of protein based on a standard curve prepared from incubation of different amounts of a known trypsin inhibitor (Sigma, St Louis, Mo) with trypsin. One trypsin unit is arbitrarily defined as an increase of 0.01 absorbance units at 410 nm.²¹

Digestion reactions with trypsin

Purified Ara h 1 from raw peanuts²² was mixed with either no Ara h 2, Ara h 2 purified from raw peanuts,²³ untreated (referred to as native), treated with 5 mmol/L dithiothreitol (DTT) overnight (referred to as partially reduced), or Ara h 2 purified from roasted peanuts²³ and incubated in the presence of 1 μ mol/L trypsin in PBS at pH 8.5 for various times at 37°C. The peanut samples were roasted for 20 minutes at 350°C. The concentration of the purified protein was determined according to Beer's law, through use of the absorbance or optical density at 280 nm and extinction coefficients

(ϵ , or molar absorptivity) of 18,380 mol/L⁻¹ for Ara h 2 and 40,390 mol/L⁻¹ for Ara h 1. Aliquots were taken for NuPAGE 4% to 12% BIS-TRIS SDS-PAGE (Invitrogen life technologies, Carlsbad, Calif) analysis at 2, 5, 10, 30 minutes, and 1 hour, 2 hours, and overnight (~15 hours). The gels were stained for 1 hour through use of GelCode Blue Stain Reagent according to manufacturer's instructions (Pierce, Rockford, Ill).

Secondary structure determination

Far UV (185-250 nm) circular dichroism (CD) spectra of Ara h 2 purified from raw and roasted peanuts were obtained. Specimens from raw peanuts were untreated (native), treated with 5 mmol/L or 50 mmol/L DTT overnight (partially reduced or reduced, respectively), or treated with 8 mol/L urea for 72 hours. Samples were desalted through use of disposable gel filtration columns (G-25, PD-10, Amersham Biosciences, Arlington, Ill) into Milli-Q water and immediately used in CD measurements. A CD spectrum of Milli-Q water was obtained for background purposes. Protein concentrations were 0.1 mg/mL, and spectra were obtained at room temperature with a JASCO Model J-710 spectropolarimeter. The mean residue weight of 117.4 g mole⁻¹ for Ara h 2 was used for calculating ellipticities. Secondary structural modes were estimated from ellipticities by multiple protein secondary structure prediction and calculation programs such as K2d through use of a Kohonen neural network with a 2-dimensional output layer,²⁴ Sspro,²⁵ PSIPRED (<http://bioinf.cs.ucl.ac.uk/cgi-bin/psipred>), APSSP2,²⁶ or PROFsec.^{27,28}

RESULTS

Trypsin inhibition assay

A protein sequence homology search for Ara h 2 revealed that Ara h 2 has significant homology to trypsin inhibitors and bifunctional trypsin/ α -amylase inhibitors such as RBI from ragi seed. Higher similarity was found between Ara h 2 and other bifunctional trypsin/ α -amylase inhibitors, but the structures for many of these proteins have not been solved. On the basis of this observation, we wanted to determine whether Ara h 2 has trypsin inhibitory activity, and, if so, what happens to the activity after roasting. Ara h 2 was purified from raw and roasted peanuts through use of conventional chromatographic techniques and subjected to a trypsin inhibition assay. Trypsin inhibitory activity of Ara h 2 from raw peanuts in native or partially reduced form and Ara h 2 from roasted peanuts was determined according to the modified method of Kakade et al²¹ (Table I). The results in Table I demonstrate that Ara h 2 in a partially reduced (treated with 5 mmol/L DTT) form and Ara h 2 from roasted peanuts were approximately 3-fold more active as trypsin inhibitors than the native Ara h 2. Denatured Ara h 2 that was treated with 50 mmol/L DTT overnight had no detectable trypsin inhibitory activity.

Ara h 2 protects Ara h 1 from trypsin digestion

A second analysis was performed to confirm the previous trypsin inhibitory activity assays and to determine whether Ara h 2 would protect other allergens such as Ara h 1 from digestion. In Fig 1, Ara h 1 is incubated in the presence of trypsin only (no A2), or trypsin and

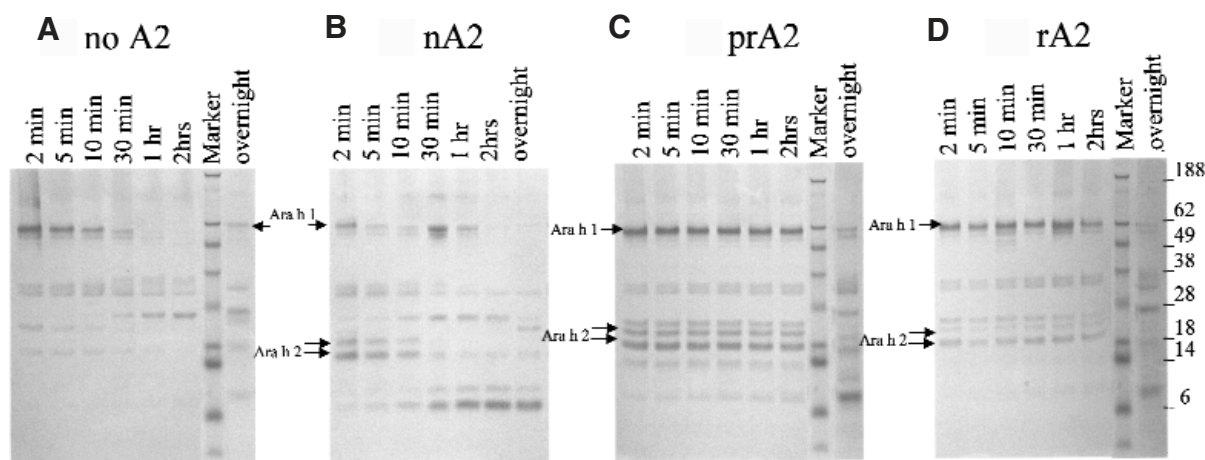


FIG 1. SDS-PAGE analysis of the digestion of Ara h 1 by trypsin in the absence and presence of Ara h 2 in raw, roasted, and partially reduced forms. Ara h 1 (indicated by arrow) is digested by trypsin for the designated amounts of time (2 minutes to overnight) while incubated in the absence (*no A2*, **A**) or presence of native (*nA2*, **B**), partially reduced (*prA2*, **C**), or roasted (*rA2*, **D**) Ara h 2. Ara h 2 migrating as a doublet (**B** to **D**) is indicated by arrows. The size of the molecular weight markers (Marker) is labeled on the left of **D**.

TABLE I. Measurement of the trypsin inhibitory activity of the raw, roasted, and partially reduced Ara h 2

Ara h 2 sample	[Ara h 2] $\mu\text{g}/\mu\text{L}$	Average ABS @ 410 nm	TIA	TIA/mL	TIA/mg sample	Fold increase in TIA over native Ara h 2
Native	0.90	1.170	20.6	206	229	—
Partially reduced	0.28	1.195	18.1	181	646	3.16
Roasted	0.20	1.220	15.6	156	780	3.59

TIA, trypsin inhibitory activity.

native (*nA2*), partially reduced (*prA2*), or roasted Ara h 2 (*rA2*) for the indicated times and subjected to SDS-PAGE analysis. It is clear that in the presence of the native Ara h 2 (Fig 1, **B**), Ara h 1 is only slightly protected from digestion with trypsin compared with Ara h 1 digested in the absence of Ara h 2 (Fig 1, **A**). Ara h 1 in the presence of either roasted or partially reduced Ara h 2, however, is highly protected against trypsin digestion (Fig 1, **C** and **D**). Even after overnight incubation of Ara h 1 in the presence of trypsin and partially reduced or roasted Ara h 2, some intact Ara h 1 can be seen. Denatured (or fully reduced) Ara h 2 does not protect Ara h 1 against trypsin digestion (data not shown). In fact, both Ara h 1 and denatured Ara h 2 are digested within 30 minutes of incubation with trypsin. Ara h 2 purified from roasted peanuts has an elevated absorbance compared with the same amount of Ara h 2 from raw peanuts, most likely because of the modifications to the protein caused by roasting. Therefore, it is notable that the amount of Ara h 2 in the roasted sample is significantly lower (Fig 1, **D**) than the amount of Ara h 2 in the other reactions (Fig 1, **B** and **C**). This observation would indicate that the roasted Ara h 2 is likely to be more active as a trypsin inhibitor than the partially reduced form.

CD analysis of the structural alterations that might contribute to the observed functional characteristics of the different Ara h 2 samples

The similarities and differences in the secondary structure of untreated Ara h 2 that was purified from raw peanuts (native), partially reduced Ara h 2, fully reduced Ara h 2, and Ara h 2 purified from roasted peanuts were compared through use of CD. The CD spectra in Fig 2 demonstrate a very slight difference in the secondary structure of untreated, urea-treated, partially reduced, and roasted Ara h 2. Most of the difference in the spectra of the native, partially reduced, and the roasted Ara h 2 is seen in the lower wavelength range (185–200 nm). The decrease in ellipticity in this region reflects a decrease in α -helices in favor of β -strands and random coil or loop formation. This effect seems to be more pronounced in the partially reduced Ara h 2 than in the roasted Ara h 2. A significant alteration is observed in the secondary structure of the native Ara h 2 that was treated with 50 mmol/L DTT (fully reduced Ara h 2) compared with the other spectra. The K2d program (see “Materials and Methods”) was used to determine the secondary structure

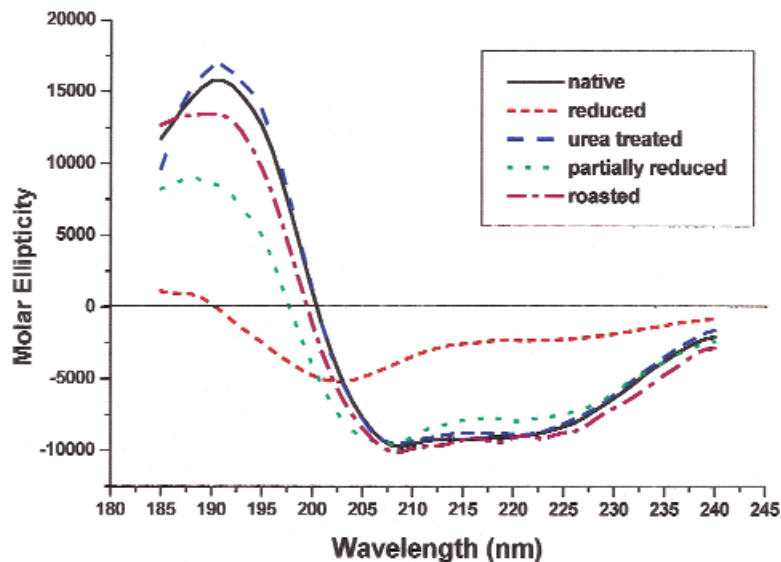


FIG 2. The secondary structure of roasted Ara h 2 is similar to the structure of native, partially reduced, and urea-treated Ara h 2. Circular dichroism spectra of Ara h 2 from roasted peanut and Ara h 2 from raw peanut in native, partially reduced, reduced, and urea-treated forms is shown above. The calculated molar ellipticity of the various forms of Ara h 2 (indicated in text box in the figure) is shown as a function of the wavelength (185 nm–240 nm).

of Ara h 2 in various forms by use of the calculated molar ellipticity values between 200 nm and 240 nm from the derived CD spectra of the various proteins. According to K2d, the native Ara h 2 consists of 31% α -helix, 10% β -strand, and 59% random coil and/or loops, and the same exact values were obtained for the Ara h 2 treated with urea for 72 hours. Partially reduced Ara h 2 consists of 25% α -helix, 17% β -strand, and 58% random coil and/or loops; roasted Ara h 2 is composed of 29% α -helix, 12% β -strand, and 59% random coil and/or loops. In addition, multiple programs (see “Materials and Methods” for Internet sites, and references 19–21) were used to predict the various secondary structure contributions to the observed spectra. All programs, without exception, indicated that Ara h 2 consists mainly of α -helix (30%–60%) and coils/loops (30%–60%) and contains little or no β -sheets (0%–16%). Ara h 2 from raw peanut was treated with 50 mmol/L DTT overnight and desalted into water to obtain CD spectra for fully reduced Ara h 2. As can be seen in the case of the fully reduced Ara h 2, most of the structured α -helix was lost (~5%–10% remaining), and only random coils and some β -sheet remain. Native Ara h 2 that was incubated in the presence of 8 mol/L urea maintained a secondary structure identical to the native Ara h 2 (as indicated previously), demonstrating that the structural integrity is intact and that the highly stable structure of Ara h 2 is predominantly stabilized through disulfide bonds.

DISCUSSION

In this study, the effects of roasting on the structure and function of a major peanut allergen were assessed. Ara h 2 was found to act as a weak trypsin inhibitor, and there

was approximately a 3.5-fold increase in this activity because of roasting. In addition to being more resistant to trypsin digestion itself, Ara h 2 was found to protect Ara h 1, a second major peanut allergen, from degradation by trypsin. This protective characteristic was enhanced in Ara h 2 purified from roasted peanuts. The CD spectra of Ara h 2 in various forms suggest that the predominant stabilizing factors in Ara h 2 structure are the disulfide bonds and that Ara h 2 is primarily composed of α -helices and random coils/loops.

Ara h 2 is a monomeric protein that contains 8 cysteine residues that are capable of forming 4 disulfide bonds. In this study, it is shown that a partial reduction (reduction of some, but not all, disulfide bonds) of Ara h 2 is possible without significant impact on the secondary structure but a notable difference in function. However, a full reduction of disulfide bonds leads to significant alteration of structure. These findings suggest that more than 1 disulfide bond is formed in the Ara h 2 molecule. The alignment of the Ara h 2 sequence with the homologous bifunctional α -amylase/trypsin inhibitor sequence of RBI from ragi seed²⁹ (PDB:1BIP²⁹) suggests that all 8 cysteines might be involved in disulfide bond formation (see Fig 3). If so, this would explain the high stability of Ara h 2 against 8 mol/L urea and roasting and its resistance to digestion with trypsin. If Ara h 2 and RBI are properly aligned, the trypsin binding loop of Ara h 2 is likely located between residues 76 and 85 (Fig 3). The closest disulfide bridge according to RBI structure is the disulfide bridge between cysteine 29 and 85 in RBI, both of which are absent in Ara h 2. The next closest disulfide bond and possibly more relevant would be between cysteine 20 and 44 in RBI, which connects the 2 α -helices that encompass the trypsin-binding loop between them.

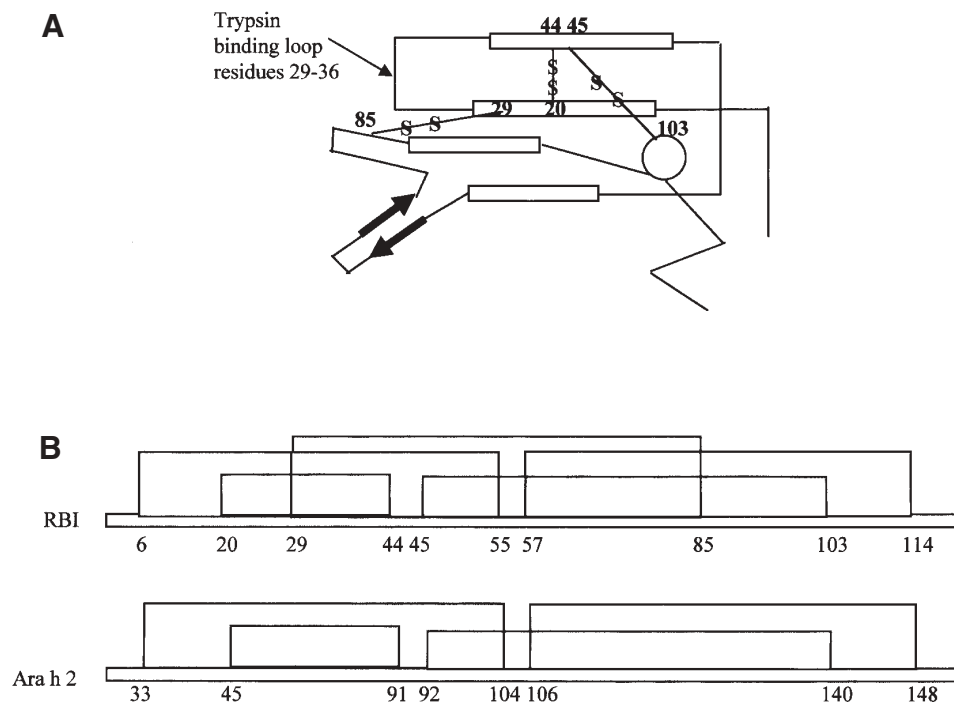


FIG 3. Possible model for the intramolecular disulfide bond formation within Ara h 2. **A**, Three of the disulfide bonds (shown as -S-S-) present in RBI²³ from ragi seed are shown above. Also, in **A**, the *thick opposing arrows* represent 2 short β -strands, and the *rectangles* represent α -helices in the structure of RBI. **B**, Schematic representation of the disulfide bond pattern of RBI and comparison with a possible pattern for disulfide bonds formed within the Ara h 2 protein on alignment of the 2 sequences and the cysteine residues. The numbers in both panels represent the position of a particular cysteine residue in the primary sequence of the indicated protein.

The homologous connection of cysteines in Ara 2 (Fig 3) would be between 45 and 91. Also, there is a second disulfide bond, which in RBI connects cysteine 45 in the same α -helix (discussed previously) with cysteine 103, located in a coil/loop region in RBI structure, with a homologous connection occurring in Ara h 2 between cysteines 92 and 140. Therefore, it is possible that disruption of either or both 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. It is important to note that loss of sulfhydryl groups is a documented result of the Maillard reaction.¹⁰

In our previous work,⁵ 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. In a recent study,⁶ 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 were shown to have enhanced IgE binding compared with raw peanuts.⁶ Currently, we have shown enhancement of an allergen-associated function caused by thermal processing. Although it is still quite possible, as previously

thought, that intramolecular cross-links caused by roasting contribute to protein stability and increase allergenic properties, it is likely that enhanced trypsin-inhibitory activity might also play a role in this observation.

Our estimate of regular secondary structure content (31% α , 10% β) from CD agrees well with the content (33% α and 3% β) from the 3-dimensional structure of the bifunctional proteinase inhibitor trypsin/ α -amylase inhibitor from ragi seeds, which was determined by nuclear magnetic resonance (PDB:1BIP²⁹). Our study is in disagreement with a recent study containing CD analysis of Ara h 2,²³ in which Bannon's group found that native Ara h 2, provided by our laboratory, consisted of 54% β -sheet, 18.2% α -helix, and 27.7% random coil configuration, whereas multiple calculations and predictions used to analyze the identical CD spectra in our study indicate that Ara h 2 is primarily composed of α -helices and coils. In the former study,²³ calculated molar ellipticity values are different in the 2 different scans of Ara h 2 at the same exact wavelength (250 nm).²³ In the scan from 190 to 250 nm, the molar ellipticity values are shown to be approximately 0 at 250 nm, whereas in the 250- to 320-nm scan, this value at 250 nm is approximately -17,000 for both the native and the reduced form of Ara h 2.²³ It is possible that these discrepancies are due to the presence of PBS and, importantly, DTT in

addition to other components in the Ara h 2 sample used for the CD scans.²³ The presence of various chemicals can alter CD spectra, and, therefore, background subtraction, smoothing, and additional calculations are necessary, which can lead to the erroneous results in some occasions. Our data indicate that the disulfide bonds are major contributors to the stability of the Ara h 2 and that the redox state of the sulfhydryl groups influences the activity of Ara h 2 as a trypsin inhibitor.

In summary, these findings suggest a possible mechanism for a synergistic enhancement of allergenic properties of different allergens in the same food that can be further intensified because of processing events such as roasting. In addition to Ara h 1 intermolecular cross-linking, enhanced IgE binding, reduced solubility, and resistance to digestion because of roasting, Ara h 2 becomes modified and provides it with additional protection against digestive enzymes. In future studies we plan to investigate other structure/function modifications such as α -amylase inhibitory activity and the effect(s) of thermal processing on these functions, which in turn can influence allergenicity.

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