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Processing Can Alter the Properties of Peanut Extract Preparations

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As peanut allergy is an increasing public health risk, affecting over 1% of the United States and United Kingdom school children, it is important that methods and reagents for accurate diagnosis of food allergy and detection of allergenic foods are reliable and consistent. Given that most current experimental, diagnostic, and detection tests rely on the presence of soluble allergens in food extracts, we investigated the effects of thermal processing on the solubility and IgE binding of the major peanut allergens, Ara h 1 and Ara h 2. The soluble and insoluble fractions of peanuts that were boiled, fried, and roasted were subjected to electrophoresis and Western blot analysis using anti-Ara h 1 and anti-Ara h 2 antibodies and serum IgE from peanut allergic individuals. Overall protein solubility is reduced with processing and IgE binding increases in the insoluble fractions, due mostly to the increase in the amount of insoluble proteins, with increased time of heating in all processes tested. Therefore, it can be concluded that thermal processing of peanuts alters solubility, and the differences in protein solubility within various extract preparations may contribute to inconsistent skin prick test and immunoassay results, particularly when nonstandardized reagents are used.

KEYWORDS: Allergy; food allergy; peanut allergy; heating; thermal processing; diagnosis; diagnostic reagents

INTRODUCTION

In the past 5 years, peanut sensitization has tripled and reported peanut allergy in children has doubled in the United States and United Kingdom (*1*, *2*). Current estimates suggest that the prevalence of peanut allergy in children may be as high as 1.5% (*1*, *3*). Currently, there is no treatment for patients with peanut allergy, leaving avoidance as the only defense. According to the data released by the National Agricultural Statistics Service (NASS), the use of peanuts in confectionery is seemingly infinite and an increase in consumption of a majority of these products is seen from 1988 to 2008. With the wide number of applications for peanut and peanut products in processed foods, particularly in candy and confectionary products, and the potential for cross contact of intended peanut-free products with traces of peanuts, avoidance can be very difficult for allergic consumers. Consequently, peanut allergy is not only an increasing public health problem, but it also poses a challenge to the food industry and regulatory agencies in terms of food safety.

Reliable detection methods for food allergens are necessary to ensure accurate food labeling and to protect consumers suffering from food allergies. Currently, several different enzyme-linked immunosorbent assay (ELISA) methods are being used to detect either the common peanut allergens, such as Ara h 1 (Indoor Biotechnology and Tepnel) (*4*), or total peanut protein (r-Biopharm, Neogen) (*5*), all of which require that these proteins be extractable from a food source. Most of the commercial ELISA systems have been validated for detecting peanut in different food matrices. However, reports such as that by Poms et al. (*6*) demonstrate that the quantitative results obtained from these ELISA experiments may vary substantially for peanut and other foods (*7*), depending on the method used to process and or extract proteins from that food. For example, spike-and-recovery, a popular method used today, will demonstrate if an ELISA will work, but the effect of processing is not evaluated using this technique. However, use of naturally incurred standards, in which the allergenic food is incorporated into the food matrix of choice before the desired processing method and then processed within that matrix, would allow evaluation of the actual effect of processing on extraction, recovery, and detection efficiency of an ELISA method (*8*).

In food allergy diagnosis, once a clinical history consistent with food allergy has been identified, *in vivo* and *in vitro* tests are available to help confirm the diagnosis. The most popular *in vitro* analysis involves variations of an ELISA method, which is used to detect the presence of specific IgE in the serum of patients with suspected allergy to a particular food. ELISA analysis has higher sensitivity but reduced specificity when compared to the *in vivo*, routinely used skin prick test (SPT) method of diagnosis. There are, however, disagreements between the tests. For example, SPT

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may show a positive response, but the ELISA results will be negative or very mildly positive and less often *vice versa*. In such cases, one of the logical explanations for these discrepancies has been compositional differences in the extracts used for ELISA and SPT. This has been specifically shown for labile protein allergens, such as Mal d 1, in extracts (*9*) and/or cross-reactive carbohydrate determinants which may cause false positive results in ELISA-based immunoassay (*10*). IgE specific to cross-reactive carbohydrate determinants strongly affects the *in vitro* diagnosis of allergic disease (*10*). While people consume most foods in processed form, the food extracts used to standardize and develop these kits are often made from the unprocessed or raw form of a food. Also, the extractability of proteins in food is altered due to processing, and the differential solubility of individual proteins in various extract preparations may alter IgE binding in immunoassays, as well as influence SPT results. In the current work, the solubility, protein profiles, and IgE binding properties of peanut proteins, including Ara h 1 and Ara h 2, following different thermal processes are assessed.

MATERIALS AND METHODS

Apparatus. Slow-cooker (Acrockpot@) and CCD camera system (Fuji Photo Film Co., Ltd., Duluth, GA).

Reagents and Materials. Florunner peanuts, Georgia Green variety (a generous gift from Dr. Marshall Lamb of the USDA National Peanut Research Laboratory), vegetable oil (LouAna Pure Vegetable Oil, Ventura Foods, LLC., Opelousas, LA), dithiothreitol (DTT), sodium dodecyl sulfate (SDS), Criterion 26-well tris-glycine gels (Bio-Rad, Hercules, CA), 5% blotto (5% dry milk made in phosphate-buffered saline plus 0.05% Tween-20, PBST), Gel-Code Blue stain (Pierce, Rockford, IL), chicken anti-Ara h 1 (IgY, custom synthesized by Sigma Immunosys, The Woodlands, TX), chicken anti-Ara h 2 (IgY, custom synthesized by Sigma Immunosys The Woodlands, TX), horseradish peroxidase (HRP)-labeled anti-chicken IgY (Sigma Immunosys, The Woodlands, TX), HRP-conjugated anti-human IgE (Sigma Immunosys, The Woodlands, TX), and ECL substrate (Amersham Bioscience Corp., Piscataway, NJ). Serum collected from patients with documented peanut allergy was obtained from Dr. Wesley Burks of Arkansas and in compliance with the institutional review board of the University of Arkansas for Medical Sciences.

Thermal Processing of Peanuts. Shelled peanuts were (a) boiled (212 °F/100 °C) in water for 2.5, 5, 15, 30, and 45 min; (b) fried for 1, 2.5, and 5 min in pure vegetable oil that was stabilized at 320 °F (160 °C) in a slow-cooker; or (c) roasted in a dry roaster set at 320 °F (160 °C) and removed after 5, 10, 20, 30, and 50 min of roasting. The temperature 320 °F (160 °C) was chosen based on previously published literature (*6*, *11*, *12*) and was used for both the oil frying and roasting processes. A range of boiling, frying and, roasting times were used to observe the range of potential alterations over time. Three time points for boiling (5, 15, and 45 min) were used in the study and were chosen to represent time points similar to, above, and below those previously reported in the literature (*11*, *12*). Of the five roasting times, three specific time points were chosen to visually match the light, medium (most similar to commercially roasted), and dark color of the three fried sample time points for better comparison purposes.

Preparation and Solubilization of the Peanut Samples. Treated peanuts and raw, shelled peanuts were ground into a meal using a coffee grinder and partially defatted using petroleum ether extraction. The samples were solubilized by adding 50 mg of the peanut meal to 1.8 mL of phosphate-buffered saline (PBS) followed by sonication and centrifugation at 5500*g* for 15 min. The supernatant was removed and centrifuged two additional times to remove any particulate matter and fat remaining in the sample (referred to as the soluble fraction throughout this publication). The pelleted fractions after centrifugation (referred to as the insoluble fractions throughout this publication) of the exact same volume (250 μL) of each sample were solubilized by boiling for 5 min in standard electrophoresis sample buffer containing 1% SDS and 5 mM DTT (*13*). The samples in SDS-sample buffer were centrifuged at 5500*g* for 15 min. The pellets obtained following centrifugation of these samples were approximately 5−10% of the entire volume and, for the most part, contained larger pieces of peanut that remained insoluble. The supernatants in SDS-sample buffer (insoluble pellet) were removed and aliquoted and stored at −20 °C for future use.

SDS-PAGE and Western Blot Analysis. The exact same volume (20 uls) of protein in SDS-sample buffer, from each of the soluble and insoluble fractions, was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a tris-glycine gel. It is important to note that in order to determine the increase or decrease of protein levels in solution or the pellet the exact same volume was loaded per lane of SDS-PAGE and not same amount of protein. The SDS-PAGE was stained using Gel-Code Blue stain and photographed. The densitometric scan of the SDS-PAGE image was performed using Multi Gauge V (3.1) software associated with FUJIFILM luminescent image analyzer LAS-1000 (Fuji Photo Film Co., Ltd., Duluth, GA). For Western blotting, the proteins were transferred to a PVDF membrane. Membranes were preblocked for 1 h at room temperature (RT) in 5% blotto. Chicken anti-raw Ara h 1 (1:5000) and chicken anti-raw Ara h 2 (1:8000) (*14*) were diluted in 5% blotto and incubated with the PVDF membrane for 1 h at RT. Both antibodies have been shown to recognize both raw and thermally processed forms of these proteins. Serum IgE binding was performed in a similar way, with blocking at RT for 30 min in 2% blotto and then incubating with pooled sera from seven peanut allergic individuals diluted 1:30 and incubated with membranes for 2 h at RT. The HRP-labeled anti-chicken IgY (1:100 000) and HRP-conjugated anti-human IgE (1:10 000) were diluted in 2% blotto, and incubation time was 30 min at RT. Following secondary antibody incubation, membranes were washed extensively with PBS containing 0.05% Tween-20 (PBST) and incubated with ECL substrate according to the manufacturer's instructions. The chemiluminescence was then measured using a FUJIFILM luminescent image analyzer LAS-1000 (Fuji Photo Film Co., Ltd., Duluth, GA).

RESULTS

Thermal Processing and Solubility of Ara h 1 and Ara h 2. With the exception of a double blind placebo controlled food challenge (DBPCFC), the only available methods for detection and assessment of food allergens in research, clinic, and industry rely on soluble peanut proteins/allergens, and although SPT can be performed with the actual food, clinicians generally utilize solubilized, commercially prepared extracts for this procedure. For this reason, we chose to evaluate the solubility of peanut proteins, and the two major peanut allergens, Ara h 1 and Ara h 2, from peanuts that were boiled (lanes 3−8), fried (lanes 9−14), or roasted (lanes 15−20) for various periods of time by SDS-PAGE (**Figure 1**). In this figure, Ara h 1 (63 kDa), Ara h 2 doublet bands (19 and 21 kDa), and Ara h 3 (40 kDa) acidic subunits are indicated with arrows. In lane 1, Ara h 1 and Ara h 2 are in purified forms (*15*, *16*) as mobility controls. In lane 2, soluble extract from raw peanut is present. The proteins extracted into solution by buffer are considered the soluble portion (S) and indicated at the bottom of the first three lanes of each processing treatment. The pelleted portion of each sample, brought down by centrifugation following extraction of soluble material with buffer, is considered the insoluble part (I) of each sample and is indicated at the bottom of the last three lanes of each processing treatment. It is easy to see the reduction in the overall level of proteins following each heat treatment, particularly the Ara h 1 protein in the soluble fractions. A densitometric scan of the total protein levels in each lane is shown above the SDS-PAGE image in **Figure 1**. The *X*-axis of the densitometric scan corresponds to the lane numbers of the SDS-PAGE, and the *Y*-axis is the density of proteins in each lane measured and reported in arbitrary units (a.u.). The densitometric scan confirms what can be visualized on the SDS-PAGE, that the total level of protein in the soluble portion of the heat treated samples decreases with increased time of heating. The levels of Ara h 2 in each sample show much less visible change in comparison with Ara h 1, in either the soluble or the insoluble fractions. In addition, as the length of exposure to

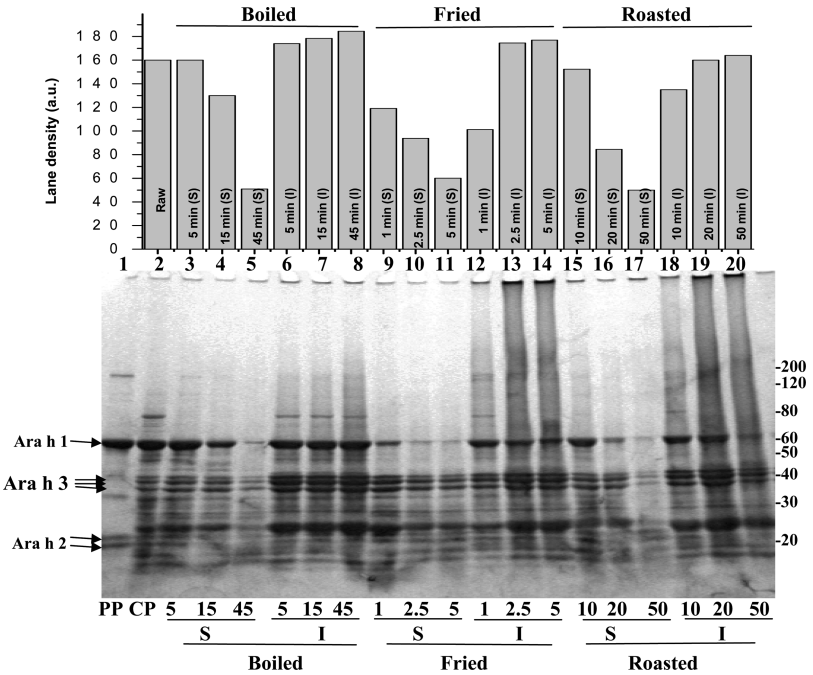

Figure 1. SDS-PAGE analysis of the effect of thermal processing on peanut proteins. Soluble (S) and insoluble (I) fractions of peanuts were assessed after boiling (lanes 3−8), frying (lanes 9−14), and roasting (lanes 15−20) for the indicated times (below each lane, in minutes). Lane 1 contains purified proteins (PP), indicated by arrows. Lane 2 contains crude protein (CP) extract from raw peanut. A densitometric scan of each individual lane (*X*-axis) was performed and is reported in the graph above the SDS-PAGE image in arbitrary units (a.u.) on the *Y*-axis.

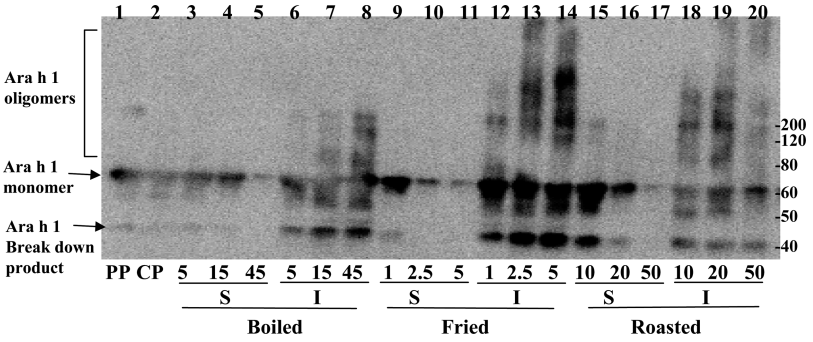

Figure 2. Western blot analysis with anti-Ara h 1 antibody. Soluble (S) and insoluble (I) fractions of peanuts were assessed for Ara h 1 profile after boiling (lanes 3−8), frying (lanes 9−14), or roasting (lanes 15−20) for the indicated times (below each lane, in minutes). Lane 1 contains purified Ara h 1 (PP) from raw peanut. Lane 2 contains crude protein (CP) extract from raw peanut.

heat increases, high-molecular-weight-smearing of the proteins in the lanes containing the insoluble fractions is seen.

Ara h 1 and Ara h 2 Following Thermal Processing. Western blot analysis using antibodies against Ara h 1 and Ara h 2 was used to confirm the results observed in SDS-PAGE, as well as to assess the relative levels, migration, and modifications to these major allergenic proteins. In **Figure 2**, specific anti-Ara h 1 antibody, shown here to recognize Ara h 1 in both raw and thermally processed samples, was used to identify Ara h 1 molecules in soluble and insoluble fractions of boiled, fried, and roasted peanut samples. The Ara h 1 monomer can be seen as a single band in lane 1, which contains pure Ara h 1, and in lane 2, which contains the soluble fraction of raw peanut. In the soluble portion of boiled, fried, and roasted samples, the relative Ara h 1 levels decrease with increased heat treatment. However, not only does the relative level of Ara h 1 increase in the insoluble fraction of each sample, it is also clear from specific bands and smears recognized by the antibody that Ara h 1 is present in the higher molecular weight aggregates or oligomers that were observed in **Figure 1**. The relative levels of the oligomeric forms of Ara h 1 are at much lower levels and molecular weights in the insoluble boiled samples than in the fried and roasted peanut samples.

Anti-Ara h 2 specific Western blot analysis in **Figure 3A** (soluble Ara h 2 fractions) and **B** (insoluble Ara h 2 fractions) shows that soluble Ara h 2 is reduced by heat treatment, particularly in the roasted and boiled samples, but to a much lesser extent than that seen with Ara h 1, with the exception of the peanut sample boiled for 45 min. In this boiled peanut sample, the Ara h 2 protein is almost completely insoluble and barely detectable with the anti-raw Ara h 2 antibody in the soluble fraction (**Figure 3A**, lane 4). The anti-raw Ara h 2 antibody recognizes the Ara h 2 in the various forms of processed peanut and in both the supernatant and in the pellet fractions as well. Furthermore, although some faint bands are seen at higher molecular weights, it does not seem likely that Ara h 2 is as extensively involved in higher complexes or aggregates with itself or other molecules as seen with Ara h 1.

IgE Binding to Soluble and Insoluble Fractions of Thermally Treated Peanuts. Previously, it has been shown that IgE binding to roasted peanut proteins is 90% greater than that to raw peanut

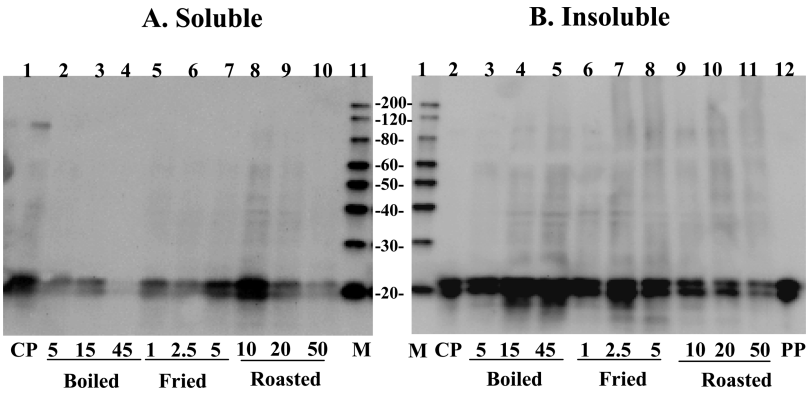

Figure 3. Western blot analysis with anti-Ara h 2 antibody. The Ara h 2 profile was assessed in soluble (**A**) fractions of boiled (lanes 2−4), fried (lanes 5−7), or roasted (lanes 8−10) peanuts and in the insoluble (**B**) fractions of boiled (lanes 3−5), fried (lanes 6−8), or roasted (lanes 9−11) peanuts. Crude protein extract (CP), purified protein (PP), and molecular weight marker (M) are shown in each panel.

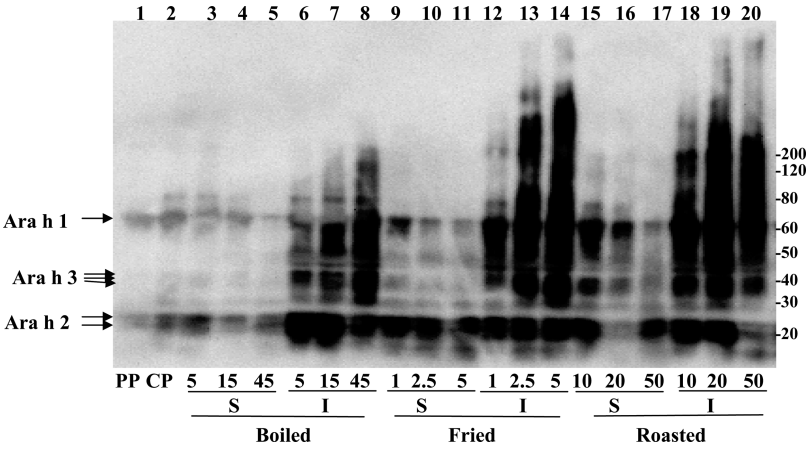

Figure 4. IgE binding to soluble and insoluble fractions of thermally treated peanuts. Pooled serum IgE binding to soluble (S) and insoluble (I) fractions of peanut samples assessed after boiling (lanes 3−8), frying (lanes 9−14), or roasting (lanes 15−20) for the indicated times (below each lane, in minutes). Lane 1 contains purified proteins (PP) as indicated by arrows. Crude peanut (CP) extract from raw peanut is in lane 2.

proteins (*17*); therefore, we investigated the IgE binding to both soluble and insoluble fractions of boiled, fried, and roasted peanuts (**Figure 4**). The IgE binding to Ara h 1 in both fried and roasted soluble portions decreases (**Figure 4**) as do the levels of Ara h 1 (**Figures 1** and **2**) with increased heating time. In the soluble fractions (S) of boiled (45 min) and roasted (dark roast, 50 min) peanuts, even though it seems like a reduction in the level of extractable Ara h 2 (**Figures 1** and **3A**), IgE binding to Ara h 2 is not different in the boiled sample and is higher in the dark roast peanut than in the other soluble samples (**Figure 4**). IgE binding to Ara h 2 in the insoluble fraction of each sample is higher than that in the soluble samples in all cases, with the exception of Ara h 2 in the dark roasted peanut sample where reduced IgE binding is seen. The decreased levels may be due to oligomerization and or degradation over lengthy, high temperature, thermal processing. Changes in IgE binding to Ara h 2 in the soluble and insoluble fractions is consistent with the changes in the levels of the proteins in these fractions.

IgE binding to Ara h 1 is consistent with protein levels and anti-Ara h 1 antibody binding in **Figures 1** and **2**. There is a significant increase in IgE binding to the insoluble fractions with a simultaneous decrease in IgE binding to soluble fractions consistent with the changes in the levels of protein in these fractions. As seen in SDS-PAGE (**Figure 1**), larger complexes form in the insoluble fractions with increasing heat and IgE binds to these high molecular weight aggregates.

IgE binding to both Ara h 1 and, to a much lesser extent, Ara h 2 decreases in the soluble fractions as the exposure times increase. The decrease in Ara h 2 is mostly seen in the case of boiled samples. This reflects that there is less Ara h 1 and potentially less Ara h 2 in the soluble fractions with increased time of heating. However, the opposite is observed in the insoluble fractions. As exposure time to heat increases, binding of IgE to the insoluble fractions also increases, primarily due to the increase in the protein levels in these samples. It is also possible that whatever modifications are occurring in the samples may be recognized and bound by IgE. Based on the molecular weight of 36−40 KDa (indicated by arrow in **Figures 1** and **4**) of SDS-PAGE and IgE binding bands, Ara h 3 is also one of the proteins that becomes less soluble with heat treatment and shows increased IgE binding in the insoluble pellet as the levels increase in these fractions.

DISCUSSION

For diagnosis of severe food allergy, the clinician often relies on SPT or ELISA-based methods to assess the presence of food-specific IgE. Industry and research laboratories also use various forms of immunoassays, often in the form of kits to detect allergenic food on surfaces or within other foods. All of these methods may not only depend on using differently prepared food extracts; they rely on the extractable form of a particular allergenic food, often purchased from manufacturers. Because discrepancies are seen in results of clinical and laboratory tests, we investigated the alterations in the characteristics of peanut proteins subjected to different thermal processing conditions, within the context of peanuts.

It has previously been shown that roasted peanut proteins in general as well as Ara h 1 and Ara h 2 bind higher levels of IgE than raw peanuts (*17−19*). Here it is shown that thermal processing of peanut alters the solubility of the proteins and causes structural and chemical modifications that may, in part, contribute to the previously reported increase in IgE binding. Overall, peanut proteins become less soluble, and large molecular weight smears are apparent with increased time of heating in all of the insoluble fractions, indicating aggregation of proteins via covalent modifications other than disulfide linkages. Higher molecular weight aggregates must be covalently linked, because proteins held together with noncovalent interactions such as hydrophobic, electrostatic, or disulfide bonds would dissociate into monomeric components following boiling in SDS-sample dye containing dithiothreitol (a disulfide bond disruptor) and SDS prior to SDS-PAGE in denaturing buffer and Western blot analysis.

On the basis of previous studies (*17−19*), it is known that the Maillard reaction, which involves the interaction of reducing sugars with free amino groups of proteins and is enhanced with heat treatment, can cause specific chemical modifications to proteins, such as carboxymethyllysine (*18*), and cause inter- and intramolecular covalent cross-linking or degradation of bonds, such as disulfide linkages (*17−19*). It has specifically been shown that purified Ara h 1 forms covalently cross-linked higher molecular weight aggregates when heated in the presence of reducing sugars (*17*). Accordingly, by SDS-PAGE analysis in **Figure 1**, we see that although proteins become much less soluble over time with boiling, as well as with other treatments, there is much less aggregate formation due to chemical cross-linking in the insoluble fractions of the boiled samples, presumably due to the presence of water, which inhibits or decreases the Maillard reaction. Previous data (*17*) and the reduction of aggregate formation in the presence of water during thermal treatment indicate that the Maillard reaction may play a significant role in the aggregation of proteins in fried and roasted peanuts.

Anti-Ara h 1 and Ara h 2 Western blots demonstrate that Ara h 1 forms aggregates via covalent modification with other Ara h 1 molecules when within the context of peanuts and possibly with other proteins or protein fragments that are then highly insoluble in aqueous solutions. Much lower molecular weight and fewer Ara h 1 aggregates are observed in the insoluble boiled samples, likely due to decreased Maillard reaction-induced cross-linking in the presence of water. It was shown that while denaturation of most proteins at temperatures above 80 °C results in the loss of almost all secondary and tertiary structure, heating of purified Ara h 1 leads to a more structured secondary conformation of the protein, with an increased content of extended β-sheet structures leading to the formation of large protein complexes or aggregates (*15*). In the light of these studies, it would be interesting to compare structural changes in Ara h 1, purified from the context of roasted versus boiled and raw peanuts, and assess for correlations with IgE binding.

Meanwhile, in the SDS-PAGE (**Figure 1**) and anti-Ara h 2 Western blot analysis (**Figure 3**), it can be seen that Ara h 2 solubility appears slightly decreased, particularly visible with the boiled and roasted samples, with increased time of heating, but significantly less than that seen with Ara h 1. Also, the faint smears and bands at higher molecular weights in the fried and roasted and, to a lesser extent, the boiled samples indicate that Ara h 2 or fragments thereof may be engaged in higher order complexes.

Previous studies demonstrated that roasted peanuts exhibit increased IgE binding (*17*, *18*, *20*). Further studies demonstrated a correlation of advanced glycation end products (AGE) with increased IgE binding (*17*, *18*, *21*). This is also supported by Gruber et al. (*22*), who showed that thermal treatment of recombinant Ara h 2 in the presence of reactive carbohydrates induced a strong increase in the IgE binding activity, thus suggesting that chemical modifications might induce higher IgE binding. Here, relative IgE binding appears to be strongest with the insoluble portion of the longest heat treatment in all three boiled, fried, and roasted samples mostly due to the fact that there is more protein in these fractions. It is also possible that structural or chemical alterations that occur during heat treatment and cause the peanut proteins to become less soluble may contribute to the enhanced IgE binding reported in previous studies. IgE binds strongly to higher molecular weight bands and smears that appear in the insoluble fractions, potentially supporting the contribution of the Maillard reaction to enhanced IgE binding.

Beyer et al. compared boiled and fried peanuts to roasted peanuts and concluded, based on less IgE binding to Ara h 2 and Ara h 3 from fried and boiled peanuts, that frying and boiling produce less allergenic peanuts than roasting (*11*). While this may be true, in this study, IgE binding to Ara h 1 and Ara h 2 appears lower than that in the roasted samples in the soluble fraction with increased heating times. However, when the insoluble fraction of each sample is also considered along with the soluble fractions, fried peanuts do not seem to have significantly less IgE binding when compared to the roasted peanuts, while the boiled ones do. Also, if we compare the relative IgE binding (**Figure 4**) of the raw (lane 2) peanut to the soluble portion of each sample at the very lowest time of heating (lanes 3, 9, and 15), semiquantitatively it still appears like an overall increase in IgE binding to the heated samples, confirming previous observations (*17−19*, *21*, *22*). This observation seems logical, as the majority of people ingest cooked peanuts in one form or another and are more likely to develop IgE against the processed proteins.

The data here show that differences in the processing and preparation can drastically alter the overall protein solubility as well as the allergen and IgE binding profiles of a particular extract. These differences in commercial extract preparations may partially explain discrepancies in clinical and experimental test results, such as when a commercial food extract results in a negative SPT but use of the actual food presents a positive SPT in the same patient. Understanding the importance of the role an ingested form of a food, and the solubility and context or matrix of that food, plays in preparation of commercial extracts is a primary step in resolving disagreement in test results. Standardization of allergen extract preparations may benefit diagnosis and detection as well as appropriate food allergen labeling.

ABBREVIATIONS

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; SPT, skin prick test; DTT, dithoitheratol; IgE, immunoglobulin E.

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