

Peanut Protein Allergens: The Effect of Roasting on Solubility and Allergenicity

Randall A. Kopper^a N. Joey Odum^a Moon Sen^c Ricki M. Helm^b
J. Steve Stanley^c A. Wesley Burks^d

^aChemistry Department, Hendrix College, Conway, Ark.; ^bDepartment of Microbiology/Immunology,

^cUniversity of Arkansas for Medical Sciences, Arkansas Children's Hospital Research Institute, Arkansas Children's Nutrition Center, Little Rock, Ark.; ^dDivision of Pediatric Allergy and Immunology, Department of Pediatrics, Duke University Medical Center, Durham, N.C., USA

Key Words

Peanut allergens · Enzyme stability, heat conditions

Abstract

Background: A contributing factor to food allergen stability is heat resistance. Peanut allergens in particular are resistant to heat, which results in their decreased solubility upon routine extraction and may have a profound influence on their continued presence in the digestive tract. Although there have been a number of studies characterizing soluble extracts of raw and roasted proteins, the relative solubility of the insoluble material following routine extraction for residual allergen characterization has not been investigated. The effects of various treatments on the re-solubilization and subsequent allergenicity of this insoluble peanut protein material are presented here. **Methods:** Various methods to resolubilize the insoluble protein material were used, including pH, proteases and glycosidases. Protease digestion of non-extractable peanut proteins with pepsin, chymotrypsin and trypsin was performed in appropriate buffers as previously optimized for peanut proteins. Glycosidase activity in the presence of protease inhibitors was performed at pH 2. Digested samples were then subjected to SDS-PAGE/Western blot analysis using serum IgE from peanut-sensitive individuals. **Results:** Progressive roasting of peanuts resulted in a significant decrease in protein solu-

bility. The acidic proteins were resolubilized moderately at high pH, with solubility decreasing as pH approached the pI of the protein. However, at pH 2 the solubility increased dramatically. More extensive resolubilization was observed with amylase treatment, presumably due to cleavage of glycoside of glycoproteins. The protein released into solution had a high IgE-binding capacity. While amylase was effective at resolubilizing this material, digestive tract proteases were not. **Conclusion:** The presence of these insolubilized peanut proteins provides a continuous source of major allergens to the gastrointestinal mucosal immune system.

Copyright © 2005 S. Karger AG, Basel

Introduction

The incidence of peanut allergy, especially among children, appears to have been increasing during recent years, probably due to an increase in the use of peanut products and their introduction into the diet at an earlier age [1–5]. Various studies over the last several years have examined the nature and location of the multiple allergens in peanuts [6, 7]. This study showed the allergenic portion of the peanut to be in the protein of the cotyledon and that it does not differ significantly between different varieties of peanut [8].

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2005 S. Karger AG, Basel

Accessible online at:
www.karger.com/iaa

Correspondence to: Ricki M. Helm, PhD
Department of Microbiology/Immunology, Arkansas Children's Nutrition Center
Slot # 512-20B, 1120 Marshall Street
Little Rock, AR 72202 (USA)
Tel. +1 501 364 3572, Fax +1 501 364 3161, E-Mail HelmRickiM@uams.edu

Peanuts and foods containing peanut proteins are rarely eaten raw. Peanuts are generally subjected to some form of heat treatment, usually roasting, before consumption. Roasting has been shown to reduce the solubility of peanut allergens, but the effect on their allergenicity, as determined by IgE-binding affinity, has been reported variously as unchanged [9] or as increased [10–12]. Heat treatment of purified Ara h 1 or the roasting of Ara h 1 within intact peanuts followed by its isolation and purification both resulted in similar denaturation of the protein molecule and a decrease in its solubility [9]. The fact that the Ara h 1 molecule underwent heat-induced denaturation while the IgE binding remained unaltered was interpreted as a demonstration that conformational IgE epitopes either play a minor role, or are not present, or are restricted to regions of the protein molecule that are not sensitive to heat denaturation [9].

Peanut proteins from roasted peanuts were found to have significantly reduced solubility and when the insoluble portion was subjected to gel electrophoresis followed by Western blot analysis, the insoluble material appeared as a protein smear, which bound increased levels of human IgE antibodies [10]. The reduced solubility and higher IgE binding exhibited upon roasting was reported to be due to covalent modification of the allergens via the Maillard reaction [13]. Structural alterations of the allergens were proposed to be responsible for resistance to digestion by human gastric fluid and a 90-fold increase in the level of IgE binding.

However, all forms of heat processing of peanuts may not affect the allergens in a similar manner. It has recently been shown that frying or boiling appears to reduce the allergenicity of peanuts [14]. IgE binding to Ara h 1 and Ara h 2 was lower in fried and boiled peanuts in comparison with roasted preparations. This was correlated, at least in the case of Ara h 1, with a parallel reduction in the amount of soluble protein. In light of all of the factors affecting the solubility, digestibility and allergenicity of peanut protein allergens, an investigation to determine the relative stability of the extracted insoluble protein material was undertaken to place previous observations in perspective and provide a comprehensive explanation of this process.

Materials and Methods

Reagents

All reagents were of molecular biology grade purity. Enzymes were obtained from Sigma Chemical Company (St. Louis, Mo., USA: pepsin, cat. P-6887; trypsin, cat. T-8918; chymotrypsin, cat. C-4129;

alpha-amylase, cat. A-1278). Coomassie brilliant blue stain (cat. B-7920) was also purchased from Sigma.

Peanut and Flour Roasting

Florunner whole peanuts or dry defatted peanut flour derived from runner peanuts were roasted by heating in an uncovered conventional oven at 178°C for various times (up to 20 min). The peanut flour was defatted 3 times in a Soxhlet extractor using diethyl ether.

Insoluble Peanut Pellets

Whole raw or roasted peanuts were ground with a mortar and pestle. Raw or roasted peanut flour was used directly; 1.0 gram of this material was suspended in 50 ml of TE buffer (65 mM Tris, 1 mM EDTA, 200 mM NaCl, pH 8.3) and stirred for 30 min at room temperature. After centrifugation, the supernatant solution contained soluble peanut proteins (crude peanut extract, CPE). The insoluble material was washed and centrifuged three times with TE buffer. The resulting pellet was identified as the insoluble peanut pellet material.

Protease Digestions

CPE or insoluble peanut pellet was added to an enzyme solution with a concentration previously optimized for digestion of CPE (final concentrations: 10 µM pepsin, 0.25 µM chymotrypsin, or 0.10 µM trypsin). The reactions were carried out at 37°C in TE buffer at pH 8.3 for trypsin and chymotrypsin, or at pH 2.1 for pepsin.

Resolubilization of Insoluble Peanut Pellet Material at Different pH Values

Insoluble peanut pellet material was briefly washed and centrifuged 3 times with TE buffer of the appropriate pH to remove any soluble peanut proteins and insure that the pellet was at the correct pH. An aliquot of the insoluble pellet was then incubated in the same buffer for 30 min at 37°C with frequent vortexing to solubilize any extractable protein. The mixture was then centrifuged, and the supernatant liquid containing any soluble protein was analyzed by gel electrophoresis.

Resolubilization of Insoluble Peanut Pellet Material with Amylase

Insoluble peanut pellet material was briefly washed and centrifuged 3 times with TE buffer pH 2.1 to remove any soluble peanut proteins and insure that the pellet was at the correct pH. An aliquot of the insoluble pellet was then added to amylase solutions of 4 or 40 µM concentration and incubated for 30 min at 37°C. Then 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to selected reactions to inhibit the contaminant protease activity present in the commercial amylase preparation. The mixture was then centrifuged, and the supernatant liquid containing any soluble protein was analyzed by gel electrophoresis.

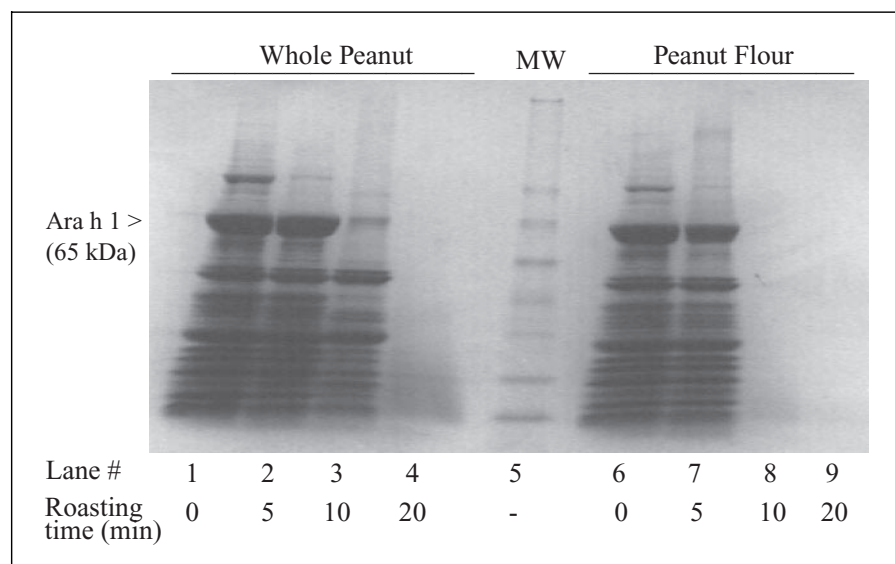
Periodate Oxidation of Insoluble Peanut Pellet Material with Sodium Periodate

Insoluble peanut pellet material was incubated in a solution containing 10 mM sodium periodate in 250 mM acetate buffer pH 4.5 for 1 h at 25°C in the dark with frequent mixing.

Gel Electrophoresis and Immunoblotting

Samples of reaction mixtures were mixed with 1/4 volume of SDS sample buffer (10% SDS, 40% glycerol, 600 mM dithiothreitol, and

Fig. 1. Effect of roasting on protein solubility in whole peanut and peanut flour. Soluble protein was extracted with TE buffer and analyzed by SDS-PAGE. Total protein extracted from roasted whole peanuts (lanes 1–4) and roasted peanut flour (lanes 6–9) were detected by Coomassie staining. Lane 5 contains protein MW standards.



bromophenol blue in 0.35 M Tris buffer pH 6.8), heated at 90 °C for 10 min, and electrophoresed on 4–20% gradient SDS-PAGE gels. Equal volumes of each sample (15 µl) were loaded to allow direct comparison of the relative amounts of protein present. Proteins were visualized with Coomassie brilliant blue staining. Western blotting was performed by electrophoretic transfer to nitrocellulose membrane using an Invitrogen XCell II blot module according to the manufacturer's instructions. After blocking in 2% BSA overnight at 4 °C, the membrane was probed with a 1:20 dilution of pooled blood serum from 16 peanut-allergic patients for 1 h at room temperature. IgE binding was detected using a 1:10 dilution of ¹²⁵I-labeled goat anti-human IgE secondary antibody (Diamed Inc., Windham, Me. USA; cat. ¹²⁵I, 4 µCi/5ml) for 2 h at room temperature. Autoradiography was performed at –80 °C.

Results

Effect of Roasting on Protein Solubility in Whole Peanut and Peanut Flour

Coomassie blue staining revealed the total extractable protein present in each sample, illustrating that roasting progressively reduced the solubility of peanut proteins (fig. 1). Protein solubility in peanut flour (lanes 6–9) was reduced more quickly than in whole peanuts (lanes 1–4), presumably due to its greater surface area. High molecular weight (MW) proteins, notably Ara h 1, are lost first, followed by smaller MW proteins. The loss of soluble protein from peanut flour was essentially complete in 10 min; however, this process required approximately 20 min for the intact peanuts.

Resolubilization of the Insoluble Roasted Peanut Pellet Material Using Three Proteases

The enzyme concentrations used were those previously optimized for digestion of soluble peanut proteins; 10 µM pepsin at pH 2.1, 0.25 µM chymotrypsin at pH 8.3, or 0.10 µM trypsin at pH 8.3. The results shown in figure 2 indicate that no additional protein was solubilized from the insoluble pellet upon treatment with pepsin (lanes 2–5), chymotrypsin (lanes 6–9), or trypsin (lanes 10–13). The pepsin concentration was much higher than the other enzymes used and was visible on the Coomassie-stained gel.

Resolubilization of the Insoluble Peanut Pellet Material at Different pHs

Periodate oxidation of the carbohydrates present and treatments of the pellets with a glycosidase (amylase) are shown in figure 3. Extraction of additional protein from the insoluble pellets was lowest at pH values near the isoelectric points of these primarily acidic proteins (pH 4 and 7), and greatest at pHs farther away (pH 2.1 and 8.3). The roasted peanut pellets contained more insoluble protein than the raw pellets and therefore yielded more protein when they were solubilized. Treatment of the pellets with sodium periodate (periodate oxidation) was not effective in freeing additional soluble protein (lanes 9 and 16). However, treatment with amylase was very effective in the liberation of soluble protein from the pellets (lanes 7, 8, 14, and 15), especially the roasted peanut pellets in lanes 14 and 15.

Fig. 2. Solubilization of the insoluble pellet using 3 proteases. The insoluble material formed upon roasting was incubated with 10 μ M pepsin at pH 2.1 (lanes 2–5), 0.25 μ M chymotrypsin at pH 8.3 (lanes 6–9), or 0.10 μ M trypsin at pH 8.3 (lanes 10–13) at the indicated times.

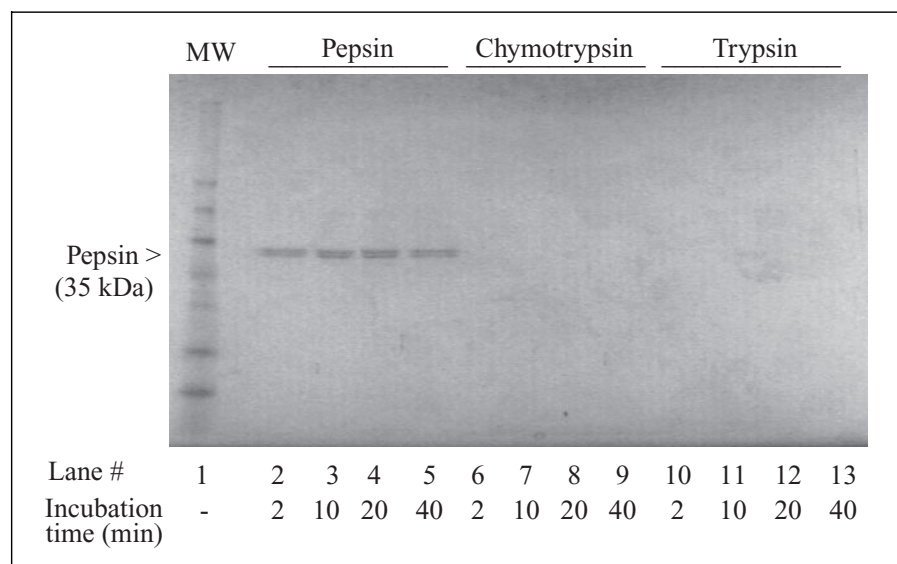
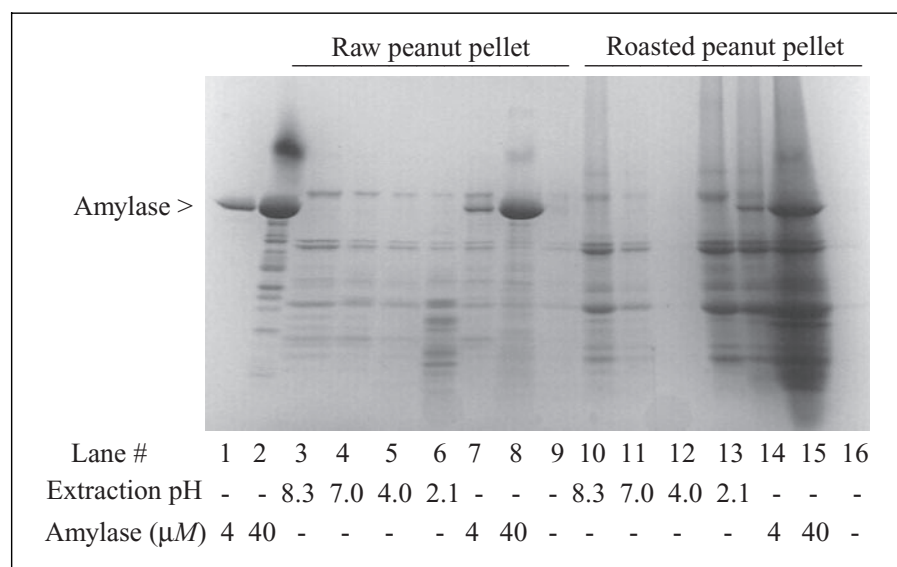


Fig. 3. Solubilization of the insoluble pellet at different pHs. The insoluble material remaining after the extraction of soluble protein from both raw (lanes 3–9) and roasted (lanes 10–16) peanut flour was incubated at pH 8.3 (lanes 3, 10), pH 7.0 (lanes 4, 11), 4.0 (lanes 5, 12) and 2.1 (lanes 6, 13). The pellets were incubated in the presence of amylase at concentrations of 4 (lanes 7, 14) or 40 μ M (lanes 8, 15). Lanes 1 and 2 contain only amylase at 4 and 40 μ M concentrations. Periodate oxidation results are shown in lanes 9 and 16.



Resolubilization of the Insoluble Peanut Pellet Material Using Amylase

The effect of amylase treatment is illustrated in figure 4. Crude peanut extract (lane 1), when incubated with 40 μ M amylase (lane 2), shows a loss of high MW protein bands and the formation of very low MW peptide fragments, demonstrating the presence of protease activity in the commercial amylase preparation. The addition of 1 mM PMSF, a protease inhibitor (lane 3), prevents the protein hydrolysis. Incubation of insoluble peanut pellet material from raw peanut in the presence or absence of amylase results in the release of very little soluble protein

(lanes 5–9) since most of the soluble protein had already been extracted. However, treatment of insoluble peanut pellet material from roasted peanut with amylase (lanes 11–14) liberates a large amount of soluble protein compared to the same treatment in the absence of amylase (lane 10). Higher amylase concentrations yielded more soluble protein (lanes 12 and 14 as compared with lanes 11 and 13). The presence of 1 mM PMSF in lanes 13 and 14 prevented proteolysis of the solubilized protein.

Fig. 4. Solubilization of the pellets using amylase. CPE (lane 1) treated with 40 μ M amylase in the absence (lane 2) and presence (lane 3) of PMSF. Lane 4 contains only 40 μ M amylase. The insoluble material remaining after the extraction of soluble protein from both raw (lanes 5–9) and roasted (lanes 10–14) peanut flour incubated in the absence of amylase (lanes 5, 10), in 4 μ M amylase without PMSF (lanes 6, 11), and with PMSF (lanes 8, 13). Insoluble material incubated in 40 μ M amylase without PMSF (lanes 7, 12), and with PMSF (lanes 9, 14).

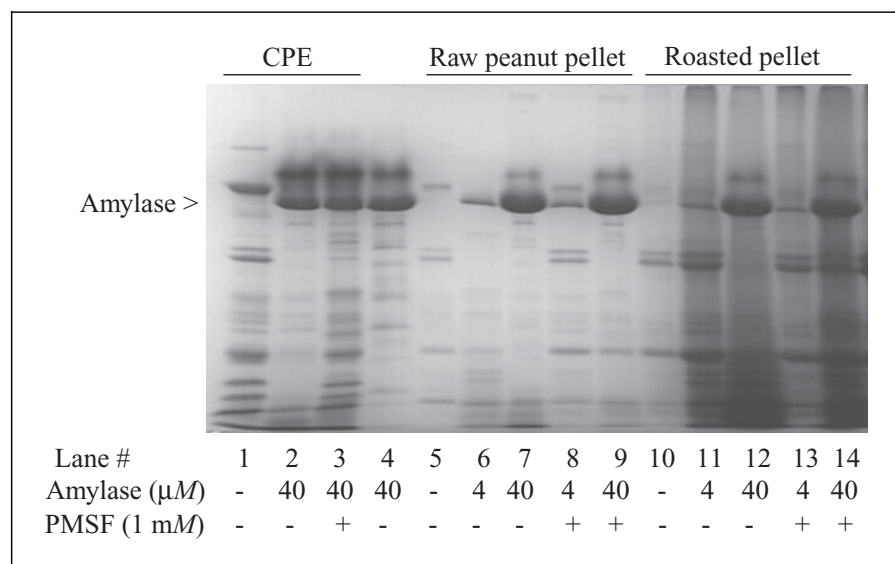
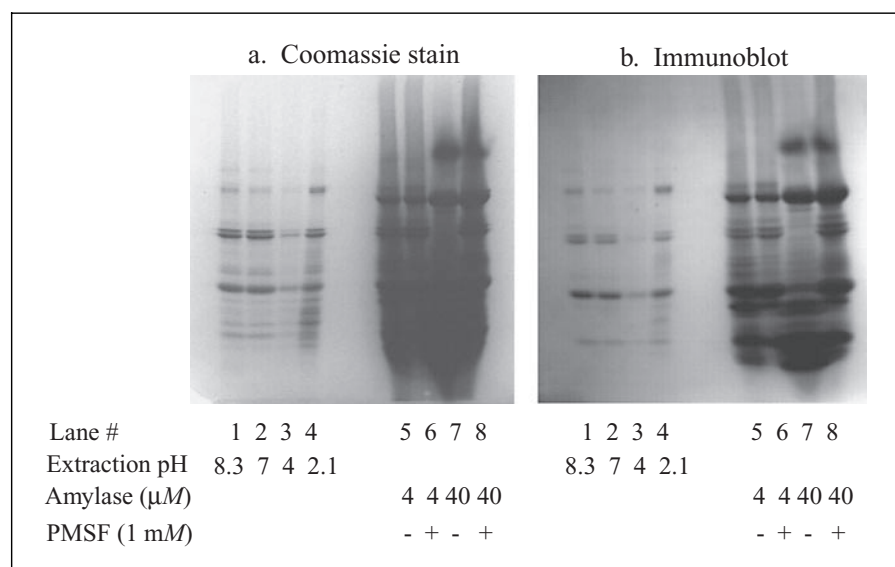


Fig. 5. IgE-binding capacity of solubilized protein from insoluble pellets. Solubilized protein liberated from roasted peanut pellets by extraction at four pH values (lanes 1–4) and by incubation with two different amylase concentrations both in the presence and absence of PMSF (lanes 5–8) analyzed by SDS-PAGE. Identical gels were Coomassie stained (a) or electrophoretically transferred to nitrocellulose membrane and probed with pooled patient blood sera from peanut allergic individuals (b).



Allergenicity of the Resolubilized Protein from Insoluble Peanut Pellet Material

The solubilized protein liberated by extraction at four pH values (lanes 1–4, fig. 5) and by incubation with amylase at two different concentrations both in the presence and absence of PMSF (lanes 5–8) was analyzed by SDS-PAGE. Identical gels were either Coomassie stained (fig. 5a) or electrophoretically transferred to nitrocellulose membrane and probed with pooled patient blood sera from peanut-allergic individuals (fig. 5b). Protein resolubilization is seen to be pH dependent as in figure 3, and the liberated protein binds peanut-specific IgE. The pro-

tein released from the pellet upon incubation with amylase increases with amylase concentration and demonstrates a high IgE-binding capacity. The presence of 1 mM PMSF in the amylase incubations prevented hydrolysis of the solubilized protein by contaminating proteases.

Discussion

It has been postulated that roasting crosslinks glycoproteins via the Maillard reaction [15, 16]. Based on this assumption, simple oxidation of the carbohydrate in-

volved in the protein crosslinking by periodate is insufficient to release soluble protein. This supports the theory that carbohydrate crosslinking between glycoproteins could be responsible for the reduced solubility of peanut proteins observed upon roasting. However, as also shown in figure 3, treatment of the insoluble peanut pellet material with amylase, a glycosidase, results in the liberation of soluble protein. Higher amylase concentrations solubilize more protein in a given time. Rapidly solubilized peanut allergens as those released by amylase activity in the mouth may be responsible for the immediate itching of the tongue and throat in peanut-sensitive individuals.

Amylase, even commercially available purified preparations, contains contaminating proteases. The contaminant protease activity in commercial amylase was demonstrated on soluble protein extracted from raw peanut flour. CPE treated with 40 μ M amylase was extensively degraded; however, the same treatment in the presence of 1 mM PMSF, a protease inhibitor, prevented degradation. If the protease activity is not inactivated, the soluble protein released by amylase's glycosidase activity is quickly hydrolyzed by protease action.

The resolubilized protein was shown to have a high IgE-binding capacity. The protein resolubilized by extended pH extraction and the larger amount liberated by incubation with amylase showed a high affinity for binding IgE, suggesting that intact protein may be reaching the small intestine in sufficient concentration to be a factor in sensitization and gastrointestinal allergic responses.

If the roasting process renders proteins insoluble at least in part by crosslinking of the glycoproteins through the Maillard reaction [13], cleavage of the glycoside linkages at acidic pH where the glycosidic bond is labile or by the action of glycosidases, should release large protein aggregates into soluble form. Extraction of the insoluble material at pH 2.1 released more soluble protein than at any other pH tested. This could also have a significant effect upon sensitization in the gut-associated lymphoid tissues because more peanut protein could be released by the combined enzymatic activity of amylase and pepsin in the stomach at an acidic pH of 2. The resulting solubilized protein should be fully capable of eliciting an allergic response in peanut-sensitive individuals and, potentially, of sensitization.

The structural changes experienced by peanut proteins upon heating may afford some degree of protection from gastric and intestinal digestion. Increased roasting times resulted in progressively reduced protein solubility. This is consistent with the observation by Koppelman et al. [9] in which Ara h 1 purified from heat-treated peanuts

exhibited secondary structure with increased folding and a decreased solubility. The fact that Ara h 1 exists as a trimeric complex [17] also supports the possibility of aggregation upon heating. Since we have demonstrated here that the insoluble peanut protein material can be brought back into solution under the appropriate conditions of pH or by glycolytic enzymes, allergenic material can be subsequently released in the stomach or small intestine. Whether the allergenic material is in the form of intact protein molecules or peptide fragments retaining IgE-binding activity as was shown for Ara h 2 [18], remains to be demonstrated.

The pH of the stomach during the ingestion of peanuts may be a significant factor in determining the allergenic potential of the peanut proteins. Peanuts eaten alone, especially on an empty stomach, will encounter a relatively acidic environment. Under these conditions, pepsin is optimally active and insoluble peanut protein material is most readily resolubilized and digested. A recent report on a murine allergy model indicates that the use of antacids for the treatment of dyspeptic disorders hinders peptic digestion by elevating gastric pH to approximately 5.0, where normally digestible dietary proteins remain intact and become potential food allergens [19]. Antacid medication was shown to impair gastric digestion with resultant promotion of IgE synthesis toward novel dietary proteins leading to food allergy. This finding supports the concept that insoluble peanut protein material, which survives initial gastric digestion, may be released at a later point resulting in sensitization or an allergic response. Although in vitro studies are valuable in assessing the biochemical characteristics of food allergens, more investigations into the in vivo responses are needed. In future studies, food allergens (peanuts and other food allergens) will be assessed for their presence in the gastrointestinal fluids in an animal under physiological conditions.

Acknowledgments

R.A.K. and N.J.O. were supported by an award from Research Corporation. AWB was supported by a grant from the National Institutes of Health.

References

- 1 Sicherer SH, Munoz-Furlong A, Burks AW, Sampson HA: Prevalence of peanut and tree nut allergy in the US determined by a random digit dial telephone survey. *J Allergy Clin Immunol* 1999;103:559–562.
- 2 Hefle SL, Nordlee JA, Taylor SL: Allergenic foods. *Crit Rev Food Sci Nutr* 1996;14:1269–1273.
- 3 Hourihane JO, Roberts SA, Warner JO: Resolution of peanut allergy: Case-control study. *Br Med J* 1998;316:1271–1275.
- 4 Bahna SL: Man shall not live by peanut alone! *Pediatrics* 1998;102:148–149.
- 5 Kmietowicz Z: Women warned to avoid peanuts during pregnancy and lactation. *Br Med J* 1998;316:1926.
- 6 Bush RK, Taylor SL, Nordlee JA: Peanut sensitivity. *Allergy Proc* 1989;10:261–264.
- 7 Gillespie DN, Nakajima S, Gleich GJ: Detection of allergy to nuts by the radioallergosorbent test. *J Allergy Clin Immunol* 1976;57:302–309.
- 8 Taylor SL, Busse WW, Sachs MI, Parker JL, Yunginger JW: Peanut oil is not allergenic to peanut-sensitive individuals. *J Allergy Clin Immunol* 1981;68:372–375.
- 9 Koppelman SJ, Bruijnzeel-Koomen CAFM, Hessing M, de Jongh HHJ: Heat-induced conformational changes of Ara h 1, a major peanut allergen, do not affect its allergenic properties. *J Biol Chem* 1999;274:4770–4777.
- 10 Maleki SJ, Chung S-Y, Champagne ET, Khali-fah RG: Allergic and biophysical properties of peanut proteins before and after roasting. *Food Allergy Intol* 2001;2:211–221.
- 11 Nordlee JA, Taylor SL, Jones RT, Yunginger JW: Allergenicity of various peanut products as determined by RAST inhibition. *J Allergy Clin Immunol* 1981;68:376–382.
- 12 Burks AW, Williams LW, Thresher W, Con-naughton C, Cockrell G, Helm RM: Allergenic-ity of peanut and soybean extracts altered by chemical or thermal denaturation in patients with atopic dermatitis and positive food chal-lenges. *J Allergy Clin Immunol* 1992;90:889–897.
- 13 Maleki SJ, Chung S-Y, Champagne ET, Rauf-man J-P: The effects of roasting on the aller-genic properties of peanut proteins. *J Allergy Clin Immunol* 2000;106:763–768.
- 14 Beyer K, Morrow E, Li X-M, Bardina L, Ban-non GA, Burks AW, Sampson HA: Effects of cooking methods on peanut allergenicity. *J Al-lergy Clin Immunol* 2001;107:1077–1081.
- 15 Maleki SJ, Viquez O, Jacks T, Dodo H, Cham-pagne ET, Chung SY, Landry SJ: The major peanut allergen, Ara h 2, functions as a trypsin inhibitor, and roasting enhances this function. *J Allergy Clin Immunol* 2003;112:190–195.
- 16 Chung SY, Butts CL, Maleki SJ, Champagne ET: Linking peanut allergenicity to the pro-cesses of maturation, curing, and roasting. *J Agric Food Chem* 2003;51:4273–4277.
- 17 Maleki SJ, Kopper RA, Shin DS, Park C-W, Compadre CM, Sampson H, Burks AW, Ban-non GA: Structure of the major peanut allergen Aha h 1 may protect IgE-binding epitopes from degradation. *J Immunol* 2000;164:5844–5849.
- 18 Sen M, Kopper R, Pons L, Abraham EC, Burks AW, Bannon GA: Protein structure plays a criti-cal role in peanut allergen stability and may determine immunodominant IgE-binding epi-topes. *J Immunol* 2002;169:882–887.
- 19 Untersmayr E, Scholl I, Swoboda I, Beil WJ, Forster-Waldl E, Walter F, Riemer A, Kraml G, Kinaciyan T, Spitzauer S, Boltz-Nitulescu G, Scheiner O, Jensen-Jarolim E: Antacid med-ication inhibits digestion of dietary proteins and causes food allergy: A fish allergy model in BALB/c mice. *J Allergy Clin Immunol* 2003;112:616–623.