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Roasting and lipid binding provide allergenic and proteolytic stability to the peanut allergen Ara h 8

Abstract: Ara h 8 is the peanut allergen homologous to the birch pollen allergen Bet v 1. Because Bet v 1 has been shown to bind lipophilic ligands, the aim of this investigation was to determine the impact of lipid binding and roasting on the Ara h 8 structure and their influences on allergenicity. For the characterization of natural Ara h 8 (nAra h 8) from roasted and unroasted peanuts, circular dichroism spectroscopy, hydrophobic binding assay, immunohistochemistry, and immunoblot with sera of peanut allergic patients were performed and compared with results from recombinant Ara h 8 (rAra h 8) and Bet v 1. rAra h 8 displayed stronger hydrophobicity than rBet v 1. Patients' sera showed IgE reactivity with rAra h 8 and nAra h 8 from roasted peanuts, whereas fewer sera recognized nAra h 8 from unroasted peanuts. Simulated gastric digestion experiments demonstrated low proteolytic stability of rAra h 8, whereas the stability of nAra h 8 was increasingly higher in unroasted and roasted peanuts. The results demonstrate that IgE reactivity and thermal and proteolytic stability are reinforced in nAra h 8 after roasting, most likely due to Maillard reactions, lipid oxidations, and lipophilic associations. These aspects must be considered when estimating the allergenicity of Bet v 1-homologous proteins.

Keywords: Ara h 8; Bet v 1 homologue; gastric and pancreatic digestion; lipophilic allergens; peanut allergy.

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Abbreviations

ANS, 8-anilino-1-naphthalene-sulfonic acid; CD, circular dichroism; nAra h 8, natural Ara h 8; rAra h 8, recombinant Ara h 8.

Introduction

Peanut and peanut oil-containing products play a major role in food and cosmetic industry. Peanuts are offered on the market in rather different preparations, e.g., in China they are normally dried or boiled, whereas in the USA they are dry roasted or roasted in oil for variable intervals and at different temperatures (Maleki et al., 2000a; Beyer et al., 2001). Therefore, customers consume a mixture of differently processed peanuts (among others peanut butter) with considerably different consequences: whereas the prevalence of peanut allergy in China is 0.5% (Ho et al., 2012), it is over 2% in the USA (Gupta et al., 2011), which points to the influence of food processing on allergenicity. Peanut allergy is a very severe problem. About 1% of the Western civilization (Sicherer et al., 2003) is affected with increasing prevalence, especially the children in the USA and Europe (Du Toit et al., 2008). The symptoms are diverse, involving the whole system (gastrointestinal tract (e.g., vomiting, diarrhea), the respiratory tract (e.g., asthma), the skin (e.g., eczema), or the mouth and throat (e.g., oral allergy syndrome). In the majority of cases, the symptoms of peanut allergy are severe; some are even fatal. In fact, more than 60% of lethal anaphylactic reactions to food in the USA were ascribed to peanut (Bock et al., 2001).

Up to now, 12 peanut allergens have been identified and were assigned by the International Union of Immunological Societies Allergen Nomenclature

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Subcommittee. Most of them have been isolated from aqueous extracts. Some have already been identified as marker allergens for severe reactions, supporting the significance of a component-resolved diagnostic. Peanut contains about 50% of lipophilic components (peanut oil), with a fatty acid composition of 50% oleic acid, 30% linoleic acid, 10% palmitic acid, 3% stearic acid, and 1% arachidic acid (Özcan, 2010). Apart from consumption of peanut-containing food, people may also come into contact with peanut oil because of the widespread use in cosmetic formulas for adults and babies (e.g., crèmes, bathing oils, massage oils). In addition, peanut oil has been used as ingredient in some topical treatments for patients with atopic eczema (Zhai et al., 2003; Dohil et al., 2009). The role of peanut oil in the development of peanut allergy was controversially discussed (Lack et al., 2003; Strid et al., 2005). The lipid phase has so far been only partially characterized with regard to allergens. Although several studies (Olszewski et al., 1998; Ramazzotti et al., 2008) on the protein content in crude and refined peanut oil were performed, only a few lipophilic peanut allergens have been identified so far. This might be due to technical difficulties with the lipid matrix. Some peanut allergens are suspected to remain in cold-pressed and even in refined peanut oil. To our knowledge, two IgE-reactive proteins were detected in refined peanut oil until now: an 18-kDa protein assumed to be the major allergen Ara h 2 and a 14-kDa protein identified as an oleosin (Olszewski et al., 1998). Apart from the peanut oleosins (Ara h 10 and Ara h 11) and the lipid transfer protein Ara h 9, the Bet v 1-homologous protein Ara h 8 may be of high relevance due to probable associations with lipids.

Ara h 8 plays a major role in patients with birch pollen-associated peanut allergy (Mittag et al., 2004a). Usually, Bet v 1-homologous proteins belong to class II food allergens, which are thermally and proteolytically unstable and provoke only mild symptoms (Ebner et al., 1995). Although this allergen is normally found only in low concentration in peanut extract, when compared with the 80%–90% of peanut storage proteins (Riecken et al., 2008), its expression can be upregulated under stress and defense conditions (Fernandes et al., 2013).

In contrast to the usual Bet v 1-homologous allergens, Gly m 4 from soybean can cause severe anaphylactic reactions (Kleine-Tebbe et al., 2002). The reasons have not yet been fully elucidated.

The significance of Ara h 8 may lie in its structural similarity to Bet v 1: both allergens show ribonuclease activity (Bufe et al., 1996; Chadha and Das, 2006; personal communication) and the hydrophobic pocket binding

lipophilic ligands as demonstrated in the Bet v 1 molecule (Markovic-Housley et al., 2003; Mogensen et al., 2002; Mattila and Renkonen, 2009) may also exist in Ara h 8.

Mills et al. (2004) hypothesized that associations with lipophilic ligands play a role in allergenicity. Because peanut contains high amounts of lipids, we assumed a most likely association between Ara h 8 and lipids, which might even influence sensitization route and allergenicity. Sancho et al. (2011) investigated Bet v 1-homologous food allergens and observed a structural change and a stabilizing effect against proteolysis after incubation with phosphatidyl choline.

The isoform Ara h 8.0201 is detected in peanut, displaying a protein sequence identity of 43% to Bet v 1a, the major allergen of birch pollen (Riecken et al., 2008). Ara h 8.0201 was confirmed to be an isoform of natural Ara h 8 (nAra h 8) by mass determination and tryptic mass fingerprinting, where the peptides covered 94% of the sequence (Riecken et al., 2008).

Besides the influence of the association with lipophilic ligands, roasting might alter the allergenicity of proteins in peanut, which was already demonstrated for the peanut storage proteins Ara h 1, 2, and 6 (Maleki et al., 2000a,b; Mondoulet et al., 2005; Vissers et al., 2011).

The aim of this study was to examine the influence of lipids on the allergen Ara h 8 with regard to stability and allergenicity of this molecule in the light of the complex matrix effects increased by thermal processing of peanuts and to find an experimental access to this complex and difficult goal.

Results

Identification of nAra h 8 in roasted and unroasted peanuts

To localize nAra h 8 in peanut tissue, immunohistochemistry of roasted and unroasted peanut kernels was performed with an Ara h 8-cross-reactive anti-Gly m 4 antiserum.

After staining, immunoreactivity is distinguishable on the surface of hyaline and light blue particles in the peanut tissue (Figure 1). These structures were identified as starch granules using Lugol's solution. Whereas microscopic sections of unroasted peanut kernels show an intense staining of the hyaline particles inside the shrunken cytoplasm (Figure 1A), roasted peanuts reveal the structures of the disrupted cytoplasm and only a fainter immunostaining of the granules (Figure 1B). Figure 1C served as the negative control (without primary antibody).

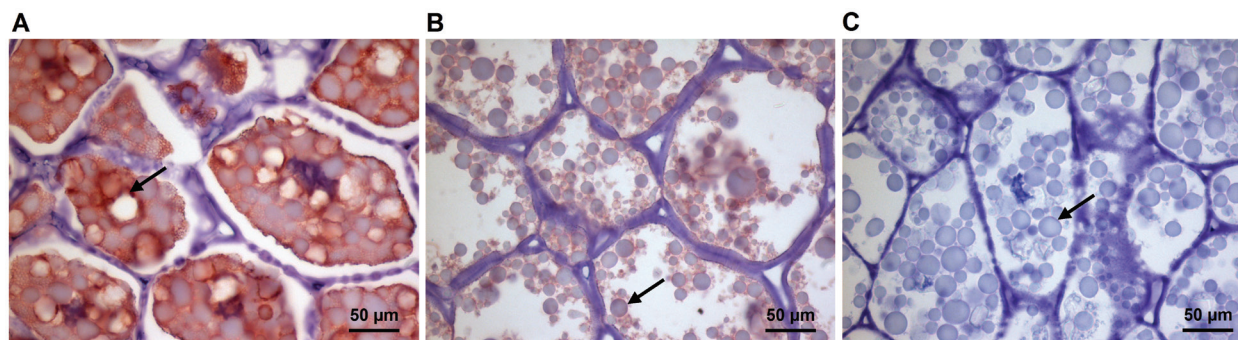


Figure 1 Immunohistochemistry for the identification of Ara h 8 in unroasted and roasted peanut kernels. Immunostaining was performed with anti-Gly m 4 antiserum. (A) Immunostaining of unroasted peanut, (B) immunostaining of roasted peanut, and (C) negative control staining of roasted peanut. The starch granules were identified using Lugol's solution and appear in these images as light blue particles. Arrows indicate the immunodetection of Ara h 8 on the surface of starch granules by the brown staining (original magnification 400×).

Isolation of Ara h 8 from lipophilic peanut extract

To isolate Ara h 8 from peanuts, aqueous extractions were examined at varying pH values (pH 2–12). Ara h 8 could only be obtained in minor amounts at pH 8. To improve Ara h 8 isolation, a lipophilic extraction was employed using chloroform and methanol at pH 11. The yields of nAra h 8 were about 20 and 8 μg from 1 g of unroasted and roasted peanut flour, respectively, as determined by densitometry. Both types of peanuts were extracted as described and separated by two-dimensional (2D) PAGE.

Figure 2 shows that the protein patterns of unroasted and roasted peanuts are considerably different. Both extracts contain the prominent protein components of 18 kDa at pIs of 5–7. Whereas the unroasted extract contains many protein spots of 5–60 kDa and in a wide pI range, the roasted extract shows only five protein spots of about 18 kDa and pI 4.5–6. Immunostaining with anti-Gly m 4 antiserum identified the 18-kDa proteins as Bet v

1-homologous proteins in peanut (data not shown). Thus, performing the lipophilic extraction under alkaline conditions, especially the 18-kDa proteins are enriched. These components were identified as Ara h 8 by N-terminal sequencing. However, the roasted peanut extract contains these proteins nearly without impurities. Because peanuts are usually consumed after roasting, the effort on further improvement of the isolation of Ara h 8 from unroasted peanuts was limited.

Immunological characterization

We developed a protocol for the isolation of nAra h 8 from peanuts by lipophilic extraction at pH 11 and purification by hydrophobic interaction chromatography.

As shown in Figure 3, we compared the two nAra h 8 samples (roasted and unroasted) with aqueous and lipophilic peanut extract as well as with the recombinant Ara h 8 (rAra h 8; isoform Ara h 8.0201) by immunoblotting

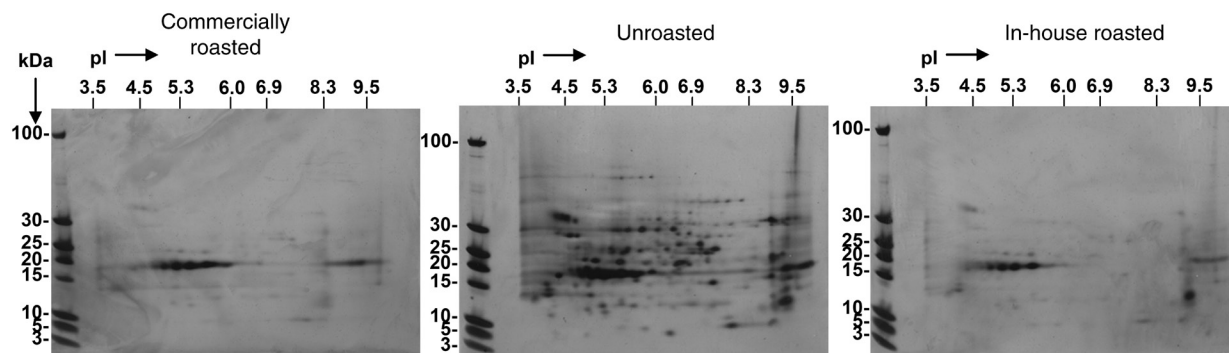


Figure 2 2D PAGE of lipophilic peanut extract: protein extraction from commercially available roasted and unroasted peanuts. Unroasted peanuts were roasted under defined conditions according to our in-house protocol. Proteins were silver stained.

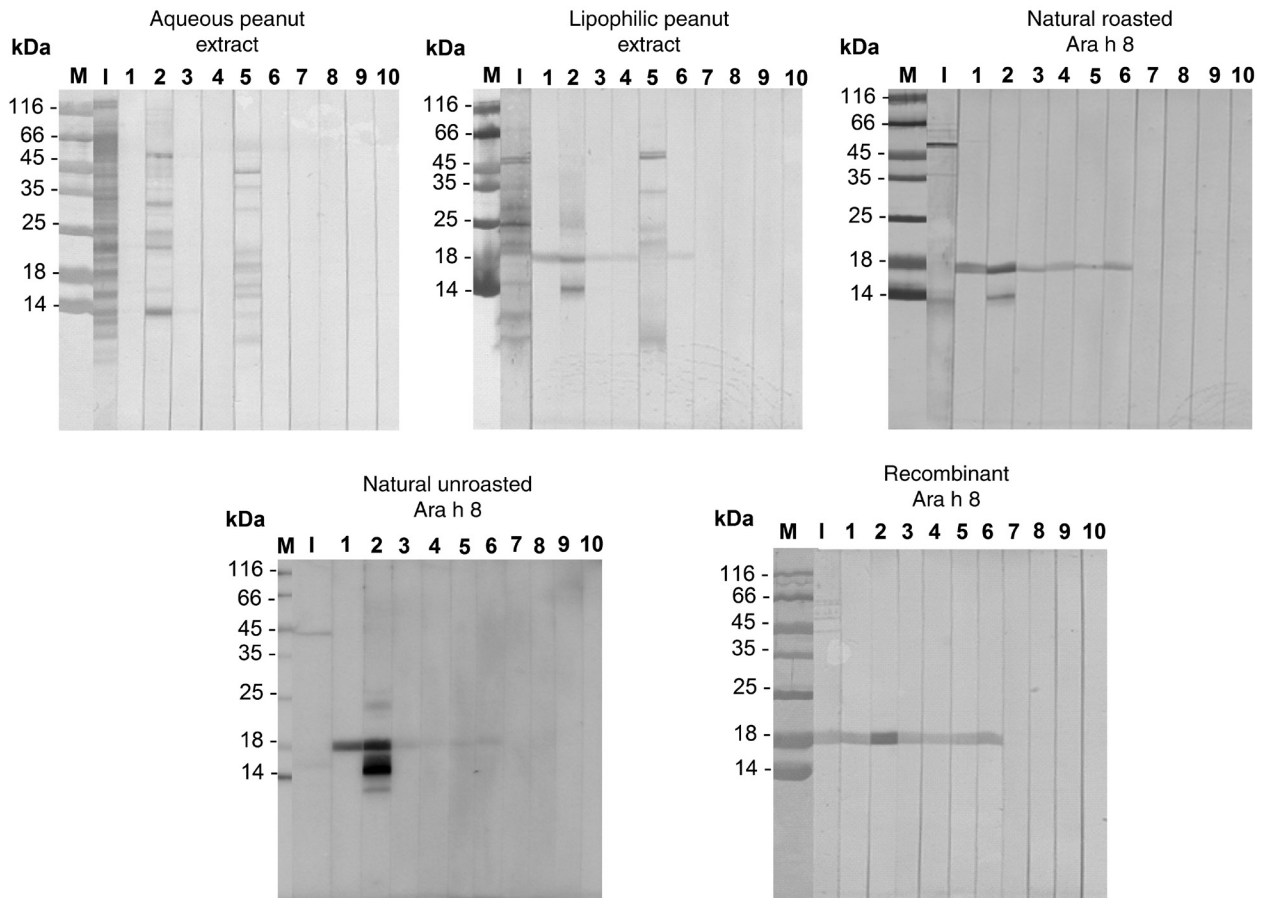


Figure 3 Identification of Ara h 8 by Western blotting.

Aqueous and lipophilic peanut extracts nAra h 8 and rAra h 8 were separated by SDS-PAGE and immunostained after blotting on PVDF membrane. nAra h 8 was only extractable under lipophilic conditions. M, molecular mass marker; I, India ink; 1, anti-Gly m 4; 2–6, patients' sera: no 1– no 5; 7, serum from a healthy individual; 8, anti-rabbit IgG; 9, anti-human IgE, 10, buffer control.

to determine immunoreactivity. By protein staining using India ink (a protein staining routinely used in blotting experiments), a number of protein bands are detectable in both extracts (Figure 3, lane I), but none showed a band of 18 kDa referring to Ara h 8. nAra h 8 from roasted and from unroasted peanuts was also not detectable by India ink but by Coomassie (data not shown). In contrast, rAra h 8 could be visualized by protein staining (lane I).

The identification of Ara h 8 was performed with the anti-Gly m 4 antiserum (lane I) and with patients' sera (lanes 2–6). The serum of a healthy individual served as negative control (lane 7). As further controls, sera of peanut allergic individuals with IgE reactivity to Ara h 1, 2, and 3, but not to Ara h 8, were used (data not shown). Although the aqueous extract reveals many protein bands, but no IgE reactivities of about 18 kDa, an immunoreactive protein of 18 kDa was clearly detectable in the lipophilic extract from roasted peanuts and in the purified nAra h 8 samples. All five patients' sera recognized nAra h 8

isolated from roasted peanuts, but only four were positive with nAra h 8 from unroasted peanuts, and the IgE reactivity was much weaker. Using rAra h 8.0201, all five sera were shown to be IgE-reactive.

Secondary structure analysis of Ara h 8

Further structural information about Ara h 8 was obtained from circular dichroism (CD) spectroscopy using nAra h 8 from unroasted peanuts, rAra h 8 and nBet v 1. Unfortunately, nAra h 8 from roasted peanuts could not be studied due to aggregation and turbidity of the solution.

CD measurements demonstrate that the spectra of rAra h 8 and nBet v 1 are similar in their secondary structure (Figure 4A). Using prediction programs 44% α -helical and 16% β -strand and 47% α -helix/14% β -strand were determined, respectively. The CD spectrum of nAra h 8 from unroasted peanuts shows clear differences in the far

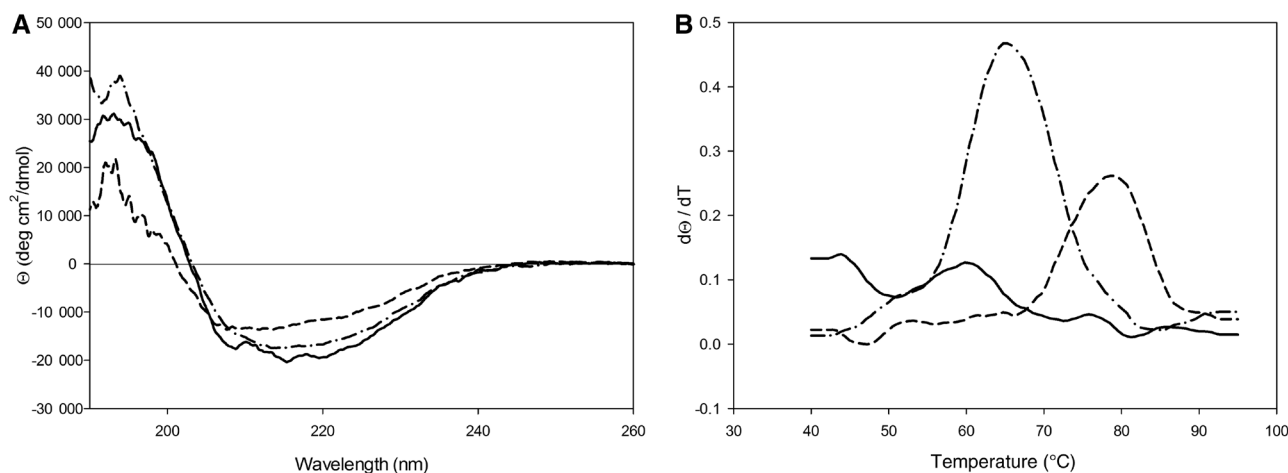


Figure 4 Characterization of Ara h 8 by CD spectroscopy.

(A) Far UV spectra of nAra h 8 isolated from unroasted peanuts (---), rAra h 8.0201 (---), and nBet v 1 (—). (B) Investigation of the thermal stability of the three samples at 210 nm. CD data were collected while increasing the temperature. Curve shows differentiated data ($d\Theta/dT$). The height of the maxima is proportional to the slope of the melting curve. All determinations were recorded in 10 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 7.5) with a protein concentration of 10 μM .

UV range (Figure 4A) and a lower calculated α -helical percentage (28% α -helix/15% β -strand). Because the preparations of nAra h 8 from roasted samples were turbid, CD spectroscopy was not possible.

To determine and compare the thermal stability of the three molecules, structural changes were determined in the thermal range of 40°C–95°C at 210 nm (minimum of the ellipticity). Whereas nAra h 8 shows the structural change at 78°C, the lipid-free allergen rAra h 8 and nBet v 1 showed maximal changes at 64°C and 60°C, respectively (Figure 4B). These results demonstrate that the thermal stability of nAra h 8 is much higher than that of rAra h 8.

Investigations on Ara h 8 for hydrophobic binding regions

To determine experimentally whether the molecular structure of Ara h 8 resembles Bet v 1 concerning hydrophobicity, 8-anilino-1-naphthalene-sulfonic acid (ANS) binding assays were performed with ANS and increasing concentrations of rAra h 8 and rBet v 1a, respectively. For these experiments, the two recombinant allergens were used to warrant that no lipid structures had already bound to the molecules. The CD spectra of nBet v 1 and rBet v 1a proved to be very similar (data not shown).

As shown in Figure 5, the ANS fluorescence signal increases with increasing amounts of rAra h 8, indicating a binding to hydrophobic regions. Corresponding studies with rBet v 1a displayed only 20% of the ANS fluorescence compared with rAra h 8 at a protein concentration of

80 μM . Lysozyme and the grass pollen allergen Phl p 6 served as control proteins and showed no fluorescence. Thus, rAra h 8 has a 5-fold higher capacity for binding the hydrophobic reagent ANS than rBet v 1a.

Analysis of the proteolytic stability of Ara h 8

To investigate the proteolytic stability of Ara h 8, *in vitro* digestion assays were performed with enzymatic surrogates. The treatment with Enzynorm f® and subsequently with Kreon® simulated the combined gastric and pancreatic

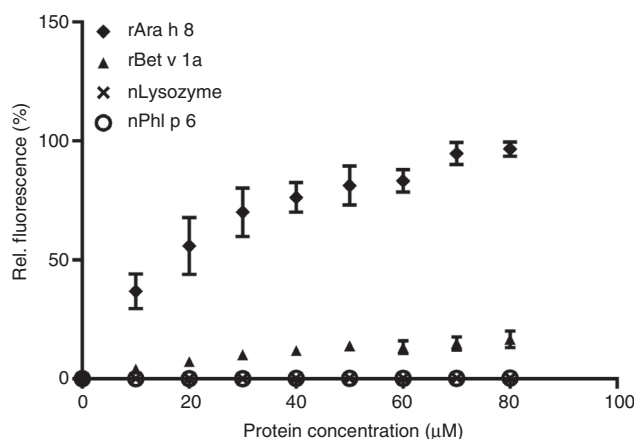


Figure 5 Investigation of hydrophobic binding regions of Ara h 8. Comparison of rAra h 8 with rBet v 1a in an ANS binding assay. Lysozyme and nPhl p 6 served as controls. Results were obtained from three independent experiments; mean $\pm$ SDs are shown.

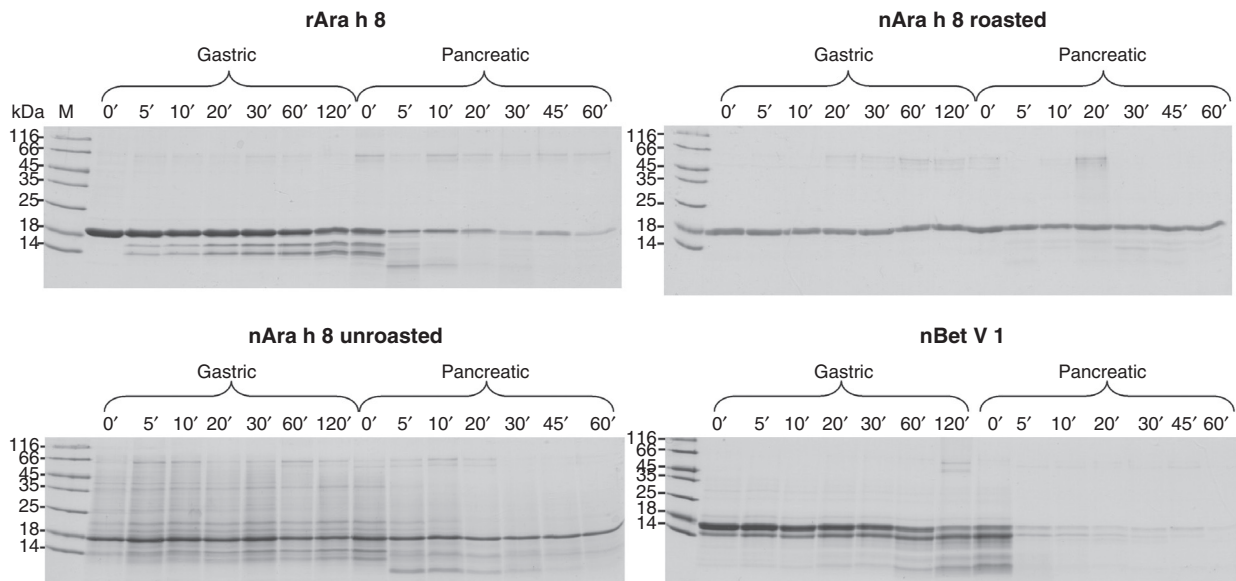


Figure 6 Comparison of rAra h 8 and nAra h 8 for their proteolytic resistance examined by SDS-PAGE.

The following Ara h 8 preparations were used: rAra h 8, nAra h 8 isolated from roasted and unroasted peanuts, nBet v 1. The samples were digested by the gastric and pancreatic surrogates Enzynorm f® (0.067 µg Enzynorm f® per 10 µg sample, pH 2, from 0 to 2 h at 37°C) and subsequently by Kreon® (0.57 µg Kreon® per 10 µg sample, pH 7, from 0 to 1 h at 37°C) and analyzed by SDS-PAGE with Coomassie staining.

digestion. As demonstrated in Figure 6, the treatment of rAra h 8 with Enzynorm f® generated two fragments of about 16 and 14 kDa (besides the undigested 18 kDa form), which remained stable to proteolysis even after incubation for 2 h. The following digestion with Kreon® led to a degradation of the fragments already after 5 min. After additional 20 min, the fragments were completely degraded, although some undegraded rAra h 8 is still detectable. Complementary investigations were performed with nAra h 8 isolated from roasted and unroasted peanuts. Interestingly, nAra h 8 from roasted peanuts showed no degradation during simulated digestion, whereas nAra h 8 from unroasted peanuts showed only a slight decrease in the intensity of the Ara h 8 band, with a few bands of fragments appearing, similar but less intensive than in rAra h 8. For control, nBet v 1 was treated accordingly and degraded in a time-dependent manner. After 20 min of pancreatic digestion, no protein was detectable anymore. These results demonstrate that nAra h 8 and rAra h 8 behave differently upon digestion: rAra h 8 degraded rapidly, whereas nAra h 8 showed a much higher proteolytic stability, especially the one isolated from roasted peanuts.

Discussion

In this study, we analyzed the influences of roasting and association with lipophilic ligands on structural properties

of the peanut allergen Ara h 8. The peanut matrix itself, with its high lipid content, was shown to alter the molecular characteristics considerably. Furthermore, it could be demonstrated that roasting modifies the Ara h 8 molecule and increases the IgE reactivity as well as thermal and proteolytic stability.

The difficulties in the isolation of Ara h 8 from peanut are well known (Riecken et al., 2008). We succeeded, however, when we performed a lipophilic chloroform/methanol extraction at alkaline pH. This extraction procedure already demonstrates the hydrophobic characteristics of Ara h 8. We isolated about 20 µg nAra h 8 per gram of unroasted peanut and only 8 µg per gram of roasted peanuts. The lower yield may be due to aggregation and/or degradation during the roasting process. However, the content of Ara h 8 in peanut is low, and lipophilic extraction is necessary to isolate the allergen in sufficient purity. Because allergen extracts for diagnostic and therapeutic purposes are usually isolated using aqueous buffers, allergens such as Ara h 8 are diminished or might get completely lost (as shown in Figure 3), especially when defatting procedures are performed before protein extraction.

The IgE reactivity of nAra h 8 was shown to be increased after roasting. This is in contrast to the instability of nBet v 1 and Bet v 1-homologous fruit allergens (Ebner et al., 1995) and suggests a stabilizing effect of Ara h 8 by the association with lipophilic ligands and/or by formation of neoepitopes due to roasting.

Besides IgE reactivity, we investigated the effect of lipid association on thermostability and digestibility of Ara h 8, which could have further influence on the allergenicity and the sensitization process. The melting temperature of nAra h 8 from unroasted peanuts (78°C) was considerably higher than those of rAra h 8 (64°C) and nBet v 1 (60°C). The increased thermostability of nAra h 8 might be caused by the lipid association. This might also be the reason for the decreased solubility and the tendency of nAra h 8 to aggregate.

An increase in allergenicity and structural stability by roasting has already been described (Maleki et al., 2000b, 2003; Mondoulet et al., 2005; Vissers et al., 2011) for class I (classical) food allergens of peanut, Ara h 1, Ara h 2, and Ara h 6, and was attributed to the Maillard reaction of reducing sugars and proteins. In the case of Ara h 8, such reactions are especially conceivable because of the proximity of Ara h 8 located on the surface of starch granules, which have been shown in grass pollen to be transmitters of allergens causing respiratory symptoms (Suphioglu et al., 1992). Hitherto, no increase in stability has been shown for class II food allergens, where the allergic reaction is due to cross-reactivity between food allergens and sensitizing pollen allergens. Although class II food allergens are usually thermally and proteolytically unstable (Ebner et al., 1995), severe anaphylactic reactions have been described for Gly m 4 (Kleine-Tebbe et al., 2002). The reason for the increased allergenicity has not yet been fully elucidated. However, peanut and soybean are much closer related to each other than to birch, and also, the sequence identity between Gly m 4 and Ara h 8.0201 is much higher (67%) than the identity of both allergens to Bet v 1a of 48% and 44%, respectively. Therefore, one might speculate that also Ara h 8 can cause severe allergic reactions.

During the roasting process, lipid oxidations with various biochemical reactions occur (Chung et al., 2002; Liu et al., 2011) aside from the Maillard reactions. Peanut is an oil plant with a lipid content of about 50% (Özcan, 2010). Changes due to roasting become obvious by comparing roasted and unroasted peanuts concerning smell, taste, and oiliness. Therefore, both types of reactions, Maillard reactions and lipid oxidations, must be considered.

The association with lipophilic ligands can even alter the structure and characteristics of Ara h 8 at room temperature. We determined the different secondary structures of nAra h 8 and rAra h 8, whereas rAra h 8 was similar to nBet v 1. The capability of Ara h 8 to bind lipids was demonstrated by ANS binding assay. This experiment (Figure 5) confirmed the hydrophobic binding domains in the

molecular structure of rAra h 8 with much stronger fluorescence signals than determined for rBet v 1a (Mogensen et al., 2002). Mogensen et al. (2002) demonstrated hydrophobic domains in rBet v 1a (Bet v 1.2801) with binding affinities to fatty acids (especially oleic acid and stearic acid) and plant sterols (e.g., dehydroergosterol). 2D NMR data confirmed that ANS was bound in the hydrophobic cavity of rBet v 1a (Mogensen et al., 2002).

Unfortunately, the concentration and purity of nAra h 8 needed were not achievable in the context of this article to perform similar experiments in order to demonstrate whether lipophilic ligands are bound in nAra h 8 as well.

In peanuts, the majority of the fatty acids are stored as triacylglycerols (Carrin and Carelli, 2010). Despite the esterification with glycerol, binding of one or more fatty acid chains seems possible as concluded from the 3D structure of triacylglycerols. We performed the experiments with the recombinant molecules, so that an interference with lipids already bound to the nAra h 8 could be ruled out. Preliminary results from the defatting of nAra h 8 and addition of lipids to rAra h 8 (summarized by Rennert et al., 2012) suggested a direct influence of lipids with the Ara h 8 molecule, but further experiments and controls showed problems in validity. Defatting by organic solvents, e.g., ether, did not show constant results, probably due to uncompleted defatting. The addition of lipids to the rAra h 8 was problematic because it could not be differentiated, whether the lipids just attached to the protein or whether they were incorporated into a hydrophobic cavity.

Experiments were performed with gastric and pancreatic surrogates to simulate gastrointestinal digestion; therefore, we did not just use pepsin. Lipid-free rAra h 8 was completely degraded in this setting. In contrast, nAra h 8 from roasted peanuts remained stable, whereas nAra h 8 from unroasted peanuts showed a retarded digestibility. Thus, the molecular stability increased from nBet v 1 < rAra h 8 < nAra h 8 isolated from unroasted peanuts < nAra h 8 from roasted peanuts. With regard to the results, the Ara h 8-lipophilic interaction seems to diminish the gastric and pancreatic digestibility of the Bet v 1-homologous peanut allergen Ara h 8.

Bet v 1-homologous allergens from fruits (e.g., Mal d 1 from apple) and vegetables (e.g., Api g 1 from celery) usually do not resist digestive enzymes in the gastrointestinal tract (Schimek et al., 2005). However, our results showed that nAra h 8 differs from nBet v 1 by its resistance to proteolytic enzymes. An increase in proteolytic stability was also observed by Sancho et al. (2011) after addition of phosphatidylcholine to Bet v 1-homologous food allergens. The association with lipophilic molecules might cause more severe allergic reactions than the oral allergy syndrome.

Our data concur with the recent observation of systemic allergic reactions induced by rAra h 8 (Mittag et al., 2004a) and Gly m 4 (Mittag et al., 2004b; Kosma et al., 2010). Furthermore, investigations with antacids indicated an impact of reduced gastric allergen digestion on the sensitization process (Pali-Schöll et al., 2010). According to our results concerning the proteolytic resistance of nAra h 8 and the investigations of Pali-Schöll et al. (2010), the role of the Ara h 8 stability in the sensitization process of peanut allergy may be considered as an important one: *via* the inhalant and/or the gastrointestinal sensitization route.

Our findings also demonstrate that class II food allergens that are usually associated with mild clinical symptoms can be stabilized by association with lipophilic ligands. However, greater structural effects are observed after roasting, when Maillard reactions and lipid oxidations have taken place.

We hypothesize that lipophilic association is also possible for other Bet v 1-homologous proteins in food with high lipid content, such as hazelnut and almond. This raises the question, whether such interactions in general might enhance allergic reactions, thus confirming a direct influence of the molecular structure on the allergenicity.

The specific interaction of nAra h 8 with lipids is also an interesting result in the context of a transcutaneous sensitization route for peanut allergens. Because we isolated the allergen from the lipid phase of peanut extracts and confirmed a nAra h 8-lipophilic association, it seems very unlikely that commercial peanut oil preparations are free from Ara h 8. With regard to other lipid-associated allergens, e.g., Ara h 9 (lipid transfer protein) or Ara h 10 and Ara h 11 (oleosins), it is possible that peanut oil-containing ointments could contain those allergens. Therefore, the investigation of further allergen-lipid associations is mandatory for future studies to confirm sensitization routes and the influence of the molecular structure on allergenicity.

Materials and methods

Peanut extracts

Aqueous peanut extraction was performed as described previously (Boldt et al., 2005) with modifications. Briefly, roasted peanuts ('Seeberger Riesen'; Seeberger, Ulm, Germany) were peeled and ground, and 5 g of peanut flour was dispersed in 40 ml of 0.1 M ammonium bicarbonate (pH 8) for 1 h at 25°C. After centrifugation (11000 g, 30 min), the extract was separated on an SDS-PAGE gel (15% acrylamide) and Coomassie stained or used for immunoblotting.

Lipophilic peanut extraction was performed according to D'Andréa et al. (2007) with modifications. Peanut flour (5 g) was dispersed in 40 ml of 50 mM sodium carbonate buffer (pH 11) for 1 h

at 25°C. Subsequently, chloroform/methanol (v/v=5/4) was added, and the mixture was centrifuged (11000 g, 20 min, 4°C) to remove the solid material. Afterward, the organic solvent was removed from the aqueous phase by rotary evaporation (37°C, 15 min), and the extract was dialyzed with double-distilled water overnight at room temperature.

The roasting of peanuts was performed according to the description of the American Peanut Shellers Association (www.peanut-shellers.org). Unroasted peanuts were incubated for 30 min at 150°C in a preheated oven. Extraction was performed as described above.

Allergens

nAra h 8 was obtained after the purification of the lipophilic extract from roasted and unroasted peanuts ('Seeberger Riesen'; Seeberger) by FPLC. Hydrophobic interaction chromatography (Phenyl Sepharose High Performance; GE Healthcare, Freiburg, Germany) was performed directly after extraction using 1 M ammonium sulfate. nAra h 8 was eluted with double-distilled water and further purified by gel filtration (Superdex 75; GE Healthcare) using 200 mM ammonium hydrogen carbonate (pH 8) for 90 min with a flow rate of 0.5 ml/min.

rAra h 8 (Ara h 8.0201; EF436550 (Riecken et al., 2008) and Bet v 1a (X15877) were cloned into a pET23b vector (Novagen, Darmstadt, Germany), expressed as C-terminal His-tagged proteins in *Escherichia coli* BL21 (DE3) and purified by immobilized metal affinity chromatography under native conditions with Ni-NTA agarose according to the manufacturer's description (Qiagen, Venlo, Netherlands).

Natural Bet v 1 was isolated from birch pollen (Allergon, Ängelholm, Sweden) according to Ferreira et al. (1996) with some modifications. Five grams of birch pollen extract was diluted in 20 mM Tris (pH 8) for 30 min. After centrifugation and concentration by filtration (Amicon, exclusion limit, 10 kDa; Sigma-Aldrich, Taufkirchen, Germany), the extract was separated from pigments on a Q-Sepharose column (GE Healthcare). The effluent contained Bet v 1 and was isolated using a C8 reverse phase column (HyperClone 5 µm MOS; Phenomenex, Darmstadt, Germany) with a gradient of 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in 90% isopropanol (solvent B) for 120 min. Collected fractions were tested for Bet v 1 and were pooled accordingly.

Protein concentrations were determined from Coomassie-stained gels by densitometry (ChemIDoc and Image Lab 4.1 software; Bio-Rad Laboratories GmbH, Munich, Germany).

Sera

Five peanut-allergic patients were included in this study after having given informed consent. All experienced allergic rhinoconjunctivitis due to birch pollen and showed symptoms after ingestion of peanuts. For details on the clinical phenotype, see Table 1. Specific serum IgE was determined by ImmunoCAP® [CAP-FEIA (fluoreszymeimmunoassay); Thermo Fisher Scientific, Freiburg, Germany]. The patients who knew to be allergic to peanut refused to have oral challenge tests with foods. The serum of a nonallergic, nonsensitized healthy individual was used as negative control. The study was approved by the respective local ethics committee from the University of Lübeck (approval numbers 10-126 and 12-220). After the peanut-allergic patients had been informed in writing about the aim of the study and

Table 1 Clinical data of the Ara h 8-allergic patients.

Patient number	Gender	Age (years)	CAP-FEIA (kU/l)		Symptoms
			Bet v 1	Ara h 8	
1	M	30	>100	>100	Itching of mouth and ears, swelling of throat after ingestion of fresh peanuts
2	F	34	20.4	17.5	Itching of throat, difficulties in breathing after ingestion of fresh peanuts
3	F	71	35.9	16.3	Oral allergy syndrome and seasonal rhinoconjunctivitis
4	F	36	72.0	10.6	Oral allergy syndrome and seasonal rhinoconjunctivitis
5	F	21	81.9	17.5	Oral allergy syndrome and seasonal rhinoconjunctivitis

the purpose of the serum sample collection (patient's information), each patient signed a written consent.

An anti-Gly m 4 antiserum raised in rabbits was proven to be cross-reactive with Ara h 8 (Riecken et al., 2008) and therefore used to detect the homologous Ara h 8 allergen in this article.

Immunohistochemistry

Immunohistochemistry was performed using formalin-fixed and paraffin-embedded samples from roasted and unroasted peanut kernels. After deparaffinization of 1- μ m sections, heat-induced antigen retrieval was performed by microwave treatment in 0.01 M sodium citrate buffer (pH 6.0) for 30 min. Endogenous peroxidase was blocked by incubation in 3% H₂O₂ for 10 min at ambient temperature. As primary antibody, we used rabbit anti-Gly m 4 antiserum diluted 1:100 in antibody diluent (pH 7) containing 1% boric acid (Zytomed Systems, Berlin, Germany) and incubated for 60 min at room temperature (Abdullah et al., 2009). Negative controls were included under omission of the primary antibodies. Detection was performed using a horseradish peroxidase polymer system (Zytochem Plus HRP Polymer System; Zytomed Systems) diluted 1:2 in antibody diluent for 20 min at ambient temperature with aminoethylcarbazole as chromogen (5 min). Counterstaining was achieved by Mayer's hemalum. The sections were covered and photographed using a Zeiss Axiostar plus microscope and a Leica DFC 320 camera. Images were processed using Fixfoto software (Koopmann, Wrestedt, Germany).

ANS binding assay

Investigations of the rAra h 8 structure to determine hydrophobic regions were performed using the fluorescent probe ANS (Sigma, St. Louis, MO, USA). The dye shows no fluorescence in polar environment but emits fluorescence signals when binding to the hydrophobic regions of proteins.

ANS was dissolved in 1 ml DMSO and diluted to 100 ml with double-distilled water to obtain a 1-mM stock solution. The experiments were performed with modifications according to Mogensen et al. (2002) with 10 μ M ANS and protein concentrations of 1–80 μ M in 50 mM phosphate buffer (pH 7) at 25°C. After 5 min of incubation in the dark, ANS fluorescence was measured using the fluorescence ELISA reader TECAN infinite M 200 (excitation, 380 nm; emission, 545 nm) (Tecan Group AG, Männedorf, Switzerland). Experiments were performed in three independent sets. Lysozyme (Merck, Darmstadt, Germany) and nPhl p 6 (Petersen et al., 1995) served as hydrophilic controls for unspecific binding of ANS. According to Rajashankar

et al. (2002), the 3D structure of Phl p 6 consists of four α -helices and does not form a hydrophobic pocket as found in the Bet v 1 molecule.

CD measurements

Samples were adjusted to a concentration of 10 μ M. Far-UV CD spectra (190–260 nm) were recorded in a 1-mm quartz cuvette on a Jasco J-715 A spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) equipped with a temperature controller at 20°C. Prediction of secondary structure (α -helix and β -strand) was performed using the DichroCalc and K2D3 programs (Louis-Jeune et al., 2012). To determine the changes in the secondary structure as a function of temperature (the melting temperature was determined as the main maximum of the differentiated curve $d\theta/dT$), the samples were heated by increasing the temperature linearly, starting from 40°C to 95°C, with a rate of 60°C/h monitored at 210 nm.

SDS-PAGE and Western blotting

SDS-PAGE was performed according to Laemmli (1970) using the Peqlab system (Peqlab Biotechnologie GmbH, Erlangen, Germany) with 15% polyacrylamide gels. The gels were stained with 0.2% Coomassie Brilliant Blue G-250.

For immunoblot analysis, the separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Schwalbach, Germany) by semi-dry blotting for 30 min at 0.8 mA/cm², as described previously (Kyhse-Andersen, 1984). The membranes were blocked for 2 h with TTBS (100 mM Tris, 100 mM NaCl, 2.5 mM MgCl₂, 0.05% v/v Tween 20, pH 7.4) containing 5% w/v nonfat dry milk. Primary antibodies were diluted in TTBS (rabbit anti-Gly m 4, 1:50000) (Riecken et al., 2008)] and patients' sera (1:10) and incubated overnight. Bound antibodies were detected by alkaline phosphatase-conjugated secondary antibodies, mouse anti-human IgE (Pharmingen, 1:1000) or goat anti-rabbit IgG (Jackson Immuno Research, West Grove, PA, USA; 1:5000) diluted in TTBS for 2 h. Immunostaining was performed using chromogen/substrate NBT/BCIP (Blake et al., 1984). For protein staining on PVDF membranes, India ink (Winsor and Newton, London, UK) was applied at a dilution of 1:1000 in TTBS for 2 h (Hancock and Tsang, 1983).

2D PAGE blotting and protein sequencing

Two-dimensional electrophoresis was performed as previously described with slight modifications (Görg et al., 2004). Briefly,

immobilized pH gradient strips (Novex IPG Zoom Strips; Invitrogen, Groningen, Netherlands) with a pH range of 3–10 were used for the separation in the first dimension (isoelectric focusing). Lipophilic peanut extract, 100 µg, was loaded onto the gel strips, and the separation was performed at 2 mA and 2 W with increasing voltage at 200 V for 20 min, 450 V for 15 min, 750 V for 15 min, and 2000 V for 1 h. SDS-PAGE was carried out at 200 V and 30 mA in the second dimension using gradient gels (Tris-Glycine ZOOM® gels, 4%–20%; Invitrogen). Molecular masses and pIs were determined by comparison with PageRuler Unstained Low Range Protein Ladder® (3.4–100 kDa) or Unstained Protein Molecular Weight Marker® (14–116 kDa) (Thermo Scientific) and IEF Marker 3–10, Liquid Mix (Serva, Heidelberg, Germany). Afterward, the gels were silver stained (Heukeshoven and Dernick, 1988). N-terminal microsequencing was performed as described (Petersen et al., 2006). Proteins were transferred by semi-dry blotting onto PVDF membrane using 10 mM *N*-cyclohexyl-3-aminopropanesulfonic acid with 10% methanol (pH 11.0) as transfer buffer. After washing the membrane with double-distilled water, it was stained by 0.1% Coomassie (Serva) in 50% methanol, destained in 50% methanol, and air-dried. Protein spots were excised, and protein sequencing was performed using a Procise protein sequencer with online PTH amino acid analyser (PE Biosystems, Weiterstadt, Germany).

Gastric and pancreatic digestion

Enzynorm f® (Nordmark, Uetersen, Germany) and Kreon 40.000® (Solvay, Hannover, Germany) solutions were prepared as described

previously (Schimek et al., 2005) to simulate the natural conditions of digestion. Briefly, Enzynorm f® and Kreon® tablets were ground and dissolved in isotonic saline. The simulated digestion was performed at 37°C with a protein/enzyme ratio of 10 µg Ara h 8:0.067 µg Enzynorm f® [equivalent to 0.000046 Ph.Eur.E (European Pharmacopoeia unit)] in dry extract from porcine stomach mucosa at pH 2.0 for 2 h and with a protein/enzyme ratio of 10 µg Ara h 8:0.57 µg Kreon® (equivalent to 0.002287 Ph.Eur.E) at pH 7.0 for 1 h. Additionally, aliquots were drawn at different time points. Enzymatic degradation was stopped by increasing the pH to 12 with 1 M NaOH. The samples were adjusted to pH 7 and desalted (protein desalting spin columns; Thermo Scientific, Rockford, IL, USA) for SDS-PAGE and Coomassie staining.

Acknowledgments: Parts of this project were kindly supported by the Kanert Foundation for Allergy Research and by the Deutsche Forschungsgemeinschaft (SFB/TR22 Z01). The excellent technical support of Marisa Böttger, Markus Hartmann, Daniela Warneke, and Jasmin Tiebach is gratefully acknowledged.

Conflict of interest statement: The authors declare to have no financial/commercial conflicts of interest.

Received June 18, 2013; accepted September 13, 2013; previously published online September 20, 2013

References

- Abdullah, M., Schultz, H., Kähler, D., Branscheid, D., Dalhoff, K., Zabel, P., Vollmer, E., and Goldmann, T. (2009). Expression of the acute phase protein haptoglobin in human lung cancer and tumor-free lung tissues. *Pathol. Res. Pract.* 205, 639–647.
- Beyer, K., Morrow, E., Li, X.M., Bardina, L., Bannon, G.A., Burks, A.W., and Sampson, H.A. (2001). Effects of cooking methods on peanut allergenicity. *J. Allergy Clin. Immunol.* 107, 1077–1081.
- Blake, M.S., Johnston, K.H., Jone, G.J., and Gotschlich, E.C. (1984). A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Anal. Biochem.* 136, 175–179.
- Bock, S.A., Muñoz-Furlong, A., and Sampson, H.A. (2001). Fatalities due to anaphylactic reactions to foods. *J. Allergy Clin. Immunol.* 107, 191–193.
- Boldt, A., Fortunato, D., Conti, A., Petersen, A., Ballmer-Weber, B., Lepp, U., Reese, G., and Becker, W.M. (2005). Analysis of the composition of an immunoglobulin E reactive high molecular weight protein complex of peanut extract containing Ara h 1 and Ara h 3/4. *Proteomics* 5, 675–686.
- Bufe, A., Spangfort, M.D., Kahlert, H., Schlaak, M., and Becker, W.M. (1996). The major birch pollen allergen, Bet v 1, shows ribonuclease activity. *Planta* 199, 413–415.
- Carrin, M.E. and Carelli, A.A. (2010). Peanut oil: compositional data. *Eur. J. Lipid Sci. Technol.* 112, 697–707.
- Chadha, P. and Das, R.H. (2006). A pathogenesis related protein, AhPR10 from peanut: an insight of its mode of antifungal activity. *Planta* 225, 213–222.
- Chung, S.Y., Maleki, S., Champagne, E.T., Buhr, K.L., and Gorbet, D.W. (2002). High-oleic peanuts are not different from normal peanuts in allergenic properties. *J. Agric. Food Chem.* 50, 878–882.
- D'Andréa, S., Jolivet, P., Boulard, C., Larré, C., Froissard, M., and Chardot, T. (2007). Selective one-step extraction of *Arabidopsis thaliana* seed oleosins using organic solvents. *J. Agric. Food Chem.* 55, 10008–10015.
- Dohil, M.A., Alvarez-Connelly, E., and Eichenfield, L.F. (2009). Fluochinolone acetonide 0.01% in peanut oil: safety and efficacy data in the treatment of childhood atopic dermatitis in infants as young as 3 months of age. *Pediatr. Dermatol.* 26, 262–268.
- Du Toit, G., Katz, Y., Sasieni, P., Mesher, D., Maleki, S.J., Fisher, H.R., Fox, A.T., Turcanu, V., Amir, T., Zadik-Mnuhin, G., et al. (2008). Early consumption of peanuts in infancy is associated with a low prevalence of peanut allergy. *J. Allergy Clin. Immunol.* 122, 984–991.
- Ebner, C., Hirschwehr, R., Bauer, L., Breiteneder, H., Valenta, R., Ebner, H., Kraft, D., and Scheiner, O. (1995). Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). *J. Allergy Clin. Immunol.* 95, 962–969.
- Fernandes, H., Michalska, K., Sikorski, M., and Jaskolski M. (2013). Structural and functional aspects of PR-10 proteins. *FEBS J.* 280, 1169–1199.
- Ferreira, F., Hirtenlehner, K., Jilek, A., Godnik-Cvar, J., Breiteneder, H., Grimm, R., Hoffmann-Sommergruber, K., Scheiner, O., Kraft,

- D., Breitenbach, M., et al. (1996). Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. *J. Exp. Med.* **183**, 599–609.
- Görg, A., Weiss, W., and Dunn, M.J. (2004). Current two-dimensional electrophoresis technology for proteomics. *Proteomics* **4**, 3665–3685.
- Gupta, R.S., Springston, E.E., Warrier, M.R., Smith, B., Kumar, R., Pongracic, J., and Holl, J.L. (2011). The prevalence, severity, and distribution of childhood food allergy in the United States. *Pediatrics* **128**, e9–17.
- Hancock, K. and Tsang, V.C. (1983). India ink staining of proteins on nitrocellulose paper. *Anal. Biochem.* **133**, 157–162.
- Heukeshoven, J. and Dernick, R. (1988). Improved silver staining procedure for fast staining in PhastSystem Development Unit. I. Staining of sodium dodecyl sulfate gels. *Electrophoresis* **9**, 28–32.
- Ho, M.H., Lee, S.L., Wong, W.H., Ip, P., and Lau, Y.L. (2012). Prevalence of self-reported food allergy in Hong Kong children and teens – a population survey. *Asian Pac. J. Allergy Immunol.* **30**, 275–284.
- Kleine-Tebbe, J., Wangorsch, A., Vogel, L., Crowell, D.N., Hausteiner, U.F., and Vieths, S. (2002). Severe oral allergy syndrome and anaphylactic reactions caused by a Bet v 1-related PR-10 protein in soybean, SAM 22. *J. Allergy Clin. Immunol.* **110**, 797–804.
- Kosma, P., Sjölander, S., Landgren, E., Borres, P., and Hedlin, G. (2010). Severe reactions after intake of soy drink in birch pollen-allergic children sensitized to Gly m 4. *Acta Paediatr.* **100**, 305–307.
- Kyhse-Andersen, J. (1984). Electrophoretic transfer of proteins from polyacrylamide to nitrocellulose: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods* **10**, 203–209.
- Lack, G., Fox, D., Northstone, K., and Golding, J. (2003). Factors associated with the development of peanut allergy in childhood. *N. Engl. J. Med.* **348**, 977–985.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Liu, X., Jin, Q., Liu, Y., Huang, J., Wang, X., Mao, W., and Wang, S. (2011). Changes in volatile compounds of peanut oil during the roasting process for production of aromatic roasted peanut oil. *J. Food Sci.* **76**, C404–C412.
- Louis-Jeune, C., Andrade-Navarro, M.A., and Perez-Iratxeta, C. (2012). Prediction of protein secondary structure from circular dichroism using theoretically derived spectra. *Proteins* **80**, 374–381.
- Maleki, S.J., Chung, S.Y., Champagne, E.T., and Raufman, J.P. (2000a). The effects of roasting on the allergenic properties of peanut proteins. *J. Allergy Clin. Immunol.* **106**, 763–768.
- Maleki, S.J., Kopper, R.A., Shin, D.S., Park, C.W., Compadre, C.M., Sampson, H., Burks, A.W., and Bannon, G.A. (2000b). Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. *J. Immunol.* **164**, 5844–5849.
- Maleki, S.J., Viquez, O., Jacks, T., Dodo, H., Champagne, E.T., Chung, S.Y., and Landry, S.J. (2003). The major peanut allergen, Ara h 2, functions as a trypsin inhibitor, and roasting enhances this function. *J. Allergy Clin. Immunol.* **112**, 190–195.
- Markovic-Housley, Z., Degano, M., and Lamba, D. (2003). Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. *J. Mol. Biol.* **325**, 123–133.
- Mattila, K. and Renkonen, R. (2009). Modelling of Bet v 1 binding to lipids. *Scand. J. Immunol.* **70**, 116–124.
- Mills, E.N., Jenkins, J.A., Alcocer, M.J., and Shewry, P.R. (2004). Structural, biological, and evolutionary relationships of plant food allergens sensitizing via the gastrointestinal tract. *Crit. Rev. Food Sci. Nutr.* **44**, 379–407.
- Mittag, D., Akkeraas, J., Ballmer-Weber, B.K., Vogel, L., Wensing, M., Becker, W.M., Koppelman, S.J., Knulst, A.C., Helbling, A., Hefle, S.L., et al. (2004a). Ara h 8, a Bet v 1-homologous allergen from peanut, is a major allergen in patients with combined birch pollen and peanut allergy. *J. Allergy Clin. Immunol.* **114**, 1410–1417.
- Mittag, D., Vieths, S., Vogel, L., Becker, W.M., Rihs, H.P., Helbling, A., Wüthrich, B., and Ballmer-Weber, B.K. (2004b). Soybean allergy in patients allergic to birch pollen: clinical investigation and molecular characterization of allergens. *J. Allergy Clin. Immunol.* **113**, 148–154.
- Mogensen, J.E., Wimmer, R., Larsen, J.N., Spangfort, M.D., and Otzen, D.E. (2002). The major birch allergen Bet v 1 shows affinity for a broad spectrum of physiological ligands. *J. Biol. Chem.* **277**, 23684–23692.
- Mondoulet, L., Paty, E., Drumare, M.F., Ah-Leung, S., Scheinmann, P., Willemot, R.M., Wal, J.M., and Bernard, H. (2005). Influence of thermal processing on the allergenicity of peanut proteins. *J. Agric. Food Chem.* **53**, 4547–4553.
- Olszewski, A., Pons, L., Moutéti, F., Aimone-Gastin, I., Kanny, G., Moneret-Vautrin, D.A., and Guéant, J.L. (1998). Isolation and characterization of proteic allergens in refined peanut oil. *Clin. Exp. Allergy* **28**, 850–859.
- Özcan, M.M. (2010). Some nutritional characteristics of kernel and oil of peanut (*Arachis hypogaea* L.). *J. Oleo Sci.* **59**, 1–5.
- Pali-Schöll, I., Herzog, R., Wallmann, J., Szalai, K., Brunner, R., Lukschal, A., Karagiannis, P., Diesner, S.C., and Jensen-Jarolim, E. (2010). Antacids and dietary supplements with an influence on the gastric pH increase the risk for food sensitization. *Clin. Exp. Allergy* **40**, 1091–1098.
- Petersen, A., Bufe, A., Schlaak, M., and Becker, W.M. (1995). Characterization of the allergen group VI in timothy grass pollen (Phl p 6). I. Immunological and biochemical studies. *Int. Arch. Allergy Immunol.* **108**, 49–54.
- Petersen, A., Dresselhaus, T., Grobe, K., and Becker, W.M. (2006). Proteome analysis of maize pollen for allergy-relevant components. *Proteomics* **6**, 6317–6325.
- Rajashankar, K., Bufe, A., Weber, W., Eschenburg, S., Lindner, B., and Betzel, C. (2002). Structure of the functional domain of the major grass-pollen allergen Phl p 5b. *Acta Crystallogr. D. Biol. Crystallogr.* **58**, 1175–1181.
- Ramazzotti, M., Mulinacci, N., Pazzagli, L., Moribondo, M., Manao, G., Vincieri, F.F., and Degl'Innocenti, D. (2008). Analytic investigations on protein content in refined seed oils: implications in food allergy. *Food Chem. Toxicol.* **46**, 3383–3388.
- Rennert, S., Krause, S., Becker, W.M., and Petersen, A. (2012). Lipids modify structure and digestibility of peanut allergen Ara h 8. In: *Environmental and Genetic Factors in Allergy and Clinical Immunology. Proceedings of the 27th Symposium of the Collegium Internationale Allergologicum (CIA)*, R. van Ree, J. Ring, and G. Marone, eds. (Pisa: Pacini Editore), pp. 241–243.
- Riecken, S., Lindner, B., Petersen, A., Jappe, U., and Becker, W.M. (2008). Purification and characterization of natural Ara h 8,

- the Bet v 1 homologous allergen from peanut, provides a novel isoform. *Biol. Chem.* **389**, 415–423.
- Sancho, A.I., Wangorsch, A., and Jensen, B.M. (2011). Responsiveness of the major birch allergen Bet v 1 scaffold to the gastric environment: impact on structure and allergenic activity. *Mol. Nutr. Food Res.* **55**, 1690–1699.
- Schimek, E.M., Zwölfer, B., Briza, P., Jahn-Schmid, B., Vogel, L., Vieths, S., Ebner, C., and Bohle, B. (2005). Gastrointestinal digestion of Bet v 1-homologous food allergens destroys their mediator-releasing, but not T cell-activating, capacity. *J. Allergy Clin. Immunol.* **116**, 1327–1333.
- Sicherer, S.H., Muñoz-Furlong, A., and Sampson, H.A. (2003). Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: a 5-year follow-up study. *J. Allergy Clin. Immunol.* **112**, 1203–1207.
- Strid, J., Hourihane, J., Kimbert, I., Callard, R., and Strobel, S. (2005). Epicutaneous exposure to peanut protein prevents oral tolerance and enhances allergic sensitization. *Clin. Exp. Allergy* **35**, 757–766.
- Suphioglu, C., Singh, M.B., Taylor, P., Bellomo, R., Holmes, P., Puy, R., and Knox, R.B. (1992). Mechanism of grass-pollen-induced asthma. *Lancet* **339**, 569–572.
- Vissers, Y.M., Blanc, F., Skov, P.S., Johnson, P.E., Rigby, N.M., Przybylski-Nicaise, L., Bernard, H., Wal, J.M., Ballmer-Weber, B., Zuidmeer-Jongejan, L., et al. (2011). Effect of heating and glycation on the allergenicity of 2S albumins (Ara h 2/6) from peanut. *PLoS One* **6**, e23998.
- Zhai, H., Ramirez, R.G., and Maibach, H.I. (2003). Hydrating effects of a corticoid oil formulation and its vehicle on human skin. *Skin Pharmacol. Appl. Skin Physiol.* **16**, 367–371.

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