RESEARCH ARTICLE

Digestion of peanut allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6: A comparative *in vitro* study and partial characterization of digestion-resistant peptides

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Scope: There are differences in stability to pepsin between the major allergens in peanut; however, data are from different reports using different digestion models. This study provides a comprehensive comparison of the digestibility of the major peanut allergens.
Methods and results: Peanut allergens Ara h 1, Ara h 2, Ara h 3 and Ara h 6 were incubated with pepsin to mimic the effect of gastric digestion. Samples were analyzed using SDS-PAGE. To further investigate resistance to digestion, Ara h 2 was additionally subjected to digestion with trypsin and residual peptides were characterized. Ara h 1 and Ara h 3 were rapidly hydrolyzed by pepsin. On the contrary, Ara h 2 and Ara h 6 were resistant to pepsin digestion, even at very high concentrations of pepsin. In fact, limited proteolysis could only be demonstrated by SDS-PAGE performed under reducing conditions, indicating an important role for the disulfide bridges in maintaining the quaternary structure of Ara h 2 and Ara h 6. Trypsin digestion of Ara h 2 similarly resulted in large residual peptides and these were identified.
Conclusion: Ara h 2 and Ara h 6 are considerably more stable towards digestion than Ara h 1 and Ara h 3.

Received: January 10, 2010
Revised: April 16, 2010
Accepted: May 7, 2010

Keywords:
Allergen / Digestion / Peanut / Pepsin / Trypsin

1 Introduction

Peanut allergy is both common and frequently severe [1–3]. Peanut allergens have been characterized to a great extent over the last decade, and various purification protocols have been published for some of the allergens. Figure 1 shows the SDS-PAGE pattern of a crude peanut extract (CPE), and assignment of the individual allergens included in this study. Major allergen Ara h 1 was described as a 63.5 kDa protein [4, 5] that occurs naturally in trimeric form of approximately 180 kDa through noncovalent interactions [6, 7]. The trimeric Ara h 1 structures often aggregate, forming multimers of up to 600–700 kDa [8, 9]. However, the aggregates and even the trimers are not stable in SDS. Thus only the monomer is shown in Fig. 1. The second identified major peanut allergen Ara h 2 migrates as a doublet at approximately 20 kDa [10]. This doublet consists of two isoforms that are nearly identical except for the insertion of the sequence DPYSPS in the higher molecular weight isoform [11]. Ara h 3 is a more complex allergen. After its initial identification as a 14 kDa protein [12], a full gene encoding a 60 kDa protein was successfully expressed [13]. Purification of Ara h 3 showed that in the peanut kernel Ara h 3 is present as a post-translationally proteolytically processed protein consisting of a triplet at approximately 42–45 kDa, a distinct band at approximately 25 kDa, and some less abundant peptide chains in the range of 12–18 kDa [14, 15]. Ara h 6 was recently purified by two independent groups [16, 17] with a molecular weight of approximately 15 kDa based on SDS-PAGE and 14 981 kDa as determined by mass spectroscopy [17]. Several minor peanut allergens, Ara h 4, 5, 7, 8 and 9 have also been described previously [18–21]. The cultivated peanut has been

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Abbreviations: CPE, crude peanut extract; **SGF,** simulated gastric fluid

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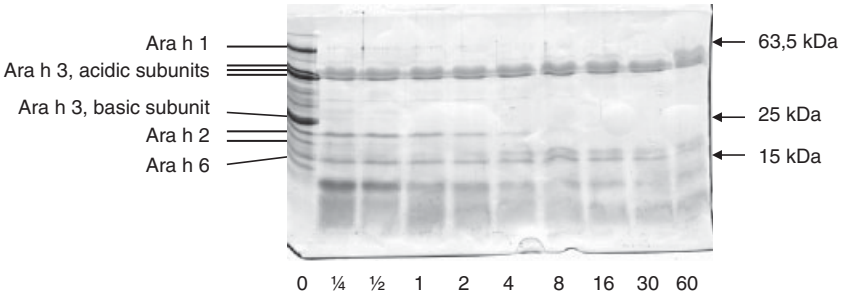

Figure 1. Characteristics and digestion of peanut extract. SDS-PAGE analysis under reducing conditions. Time course (minutes indicated at lower part). Right margin: molecular weight indication. Left margin: band assignment of individual allergens in the peanut extract.

described as an allotetraploid [22], which means that some of the sequence variants listed in databases for these peanut allergens may represent different genes and more than one sequence may occur within a single seed.

Initially, Ara h 1 was identified as the major peanut allergen [4, 23], and it was believed that Ara h 1 was a more important allergen than Ara h 2 [10]. However, the ability of Ara h 2 to release histamine from human basophils containing peanut-specific IgE [24–27] as well as the reactivity in skin test in peanut-allergic individuals is far more pronounced for Ara h 2 as compared with Ara h 1 [24]. For Ara h 6, IgE binding and histamine release from basophils are described to be similar to that of Ara h 2 [17]. The percentage of patients recognizing Ara h 3 is lower than that of patients recognizing Ara h 1 or Ara h 2 [18], and the potency of Ara h 3 to release histamine *in vitro* and *in vivo* is lower than that of Ara h 2 and Ara h 6, but in the range of that of Ara h 1 [24]. Some of the reported differences may be due to random differences in the human study populations, but the consensus seems to be that Ara h 2 is the dominant allergen in peanut and we sought to evaluate whether differences in relative stability to digestion in the digestive tract might explain the apparent trend in relative potency of the allergens.

Many of the major food allergens are comparatively resistant to digestion, and this general characteristic has been considered as a means to distinguish between potentially allergenic and nonallergenic proteins [16, 28–31]. Interestingly, digestion of a peanut extract with pepsin did not affect the IgE-binding properties, although substantial proteolytic breakdown was observed [32]. Digestion of peanut extracts consisting of various allergens and nonallergens results in a pool of peptides that is difficult to interpret [33]. Purified allergens can be used to circumvent this. Although some studies have been published on the digestion of individual peanut allergens [16, 30, 34], a direct comparison of the established major allergens from peanut has up to now never been made. We aimed to investigate the digestibility of peanut allergens, using a protocol applying pepsin, as recently proposed by the International Life Sciences Institute (ILSI) [29]. The purified allergens Ara h 1, Ara h 2, Ara h 3 and Ara h 6 were incubated under the same conditions, and the concentration of pepsin was varied to obtain more kinetic data. Furthermore, digestion-resistant peptides have been identified.

2 Materials and methods

2.1 Test proteins

CPE was prepared from ground peanut (*Arachis hypogaea*, variety: Runner) as described earlier [35]. Ara h 1, Ara h 2, Ara h 3 and Ara h 6 were purified as described earlier [14, 17, 36]. *N*-terminal sequencing was performed by Edman degradation, using bands excised from SDS-PAGE gels (SeCU, Utrecht, The Netherlands).

2.2 Proteases

Porcine pepsin was purchased from Sigma (St. Louis, MO, USA, ♯ P-6887). This product was chosen because it has the highest specific activity commercially available (3300 U/mg for this particular batch), and because other researchers investigating the digestibility behavior of potentially allergenic proteins use this product [29]. Trypsin from bovine pancreas (treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone to reduce the chymotrypsin activity) was obtained from Sigma (T-1426). The proteases were dissolved immediately before the digestion experiments and used within 15 min in order to prevent the possible loss of activity due to auto-digestion.

2.3 Pepsin digestion assay conditions

Tubes containing 1.52 mL of simulated gastric fluid (SGF) were prepared with the pH adjusted to 1.2 (0.063 N HCl, containing 35 mM NaCl and 4000 U pepsin). In a control experiment, the potential effect of adding pepsin and test proteins on the final pH was found to be negligible (less than 0.05 pH points). The SGF was prewarmed to 37°C for 5 min and 80 μL of 5 mg/mL test protein was added at time point $t = 0$. For CPE, due to a lower solubility, SGF was prepared at a higher concentration such that the addition of 400 μL of 1 mg/mL CPE resulted in the same final concentration of HCl, NaCl, pepsin and test protein. Pepsin:substrate protein ratio was 10 U pepsin:1 μg substrate protein. Starting with a pepsin-specific activity of 3300 U/mL and a substrate protein concentration of 250 μg/mL, 760 μg/mL

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pepsin was applied. Additionally, pepsin was diluted 10 or 100 fold with respect to the above calculation. Samples of 200 μL were collected at time points: 0.5, 2, 5, 10, 20, 30 and 60 min. Digestion was stopped at appropriate times by mixing with 70 μL of 200 mM $NaHCO_3$ (pH = 11.0) and 70 μL of five times concentrated electrophoresis buffer [37] containing 40% glycerol, 20% SDS, with or without 5% ß-mercaptoethanol, 0.33 M Tris (pH 6.8) and 0.05% bromophenol blue. The samples of 0.5, 1, 2 and 5 min were heated for 5 min at $>75^{\circ}C$ directly after taking the sample of time point 5 min. Samples of other time points were heated immediately after sampling. All samples were stored at –20°C until SDS-PAGE analysis.

The sample of time point $t = 0$ min was prepared by adding bicarbonate and Laemmli buffer and heating SGF prior to the addition of test protein. After adding the test protein, the samples were heated again to ensure full denaturation. Potential pepsin auto-digestion was tested by adding 80 μL of water to SGF and incubation of 60 min as described for test proteins. Protein stability at low pH was tested by preparing SGF without pepsin, and incubating the test protein for 60 min.

2.4 Trypsin digestion of Ara h 2

Lyophilized Ara h 2 was dissolved at 1 mg/mL in 65 mM Tris buffer pH 8.3 containing 1 mM EDTA, and mixed with trypsin such that a final concentration of 0.9 mg/mL Ara h 2 was reached. The final concentration of trypsin was adjusted to 7.2, 24 and 72 μg/mL. In total, 50 μL samples were taken at 5, 10, 20, 30, 40, 60 and 90 min and were immediately stopped by adding 1/5 volume of five times concentrated SDS-PAGE sample buffer (containing 40% glycerol, 20% SDS, 0.33 M Tris (pH 6.8) and 0.05% bromophenol blue) containing 1% DTT. To isolate the digestion-resistant peptides, digestion with 0.3 μM trypsin was stopped after 20 min by rapid removal of trypsin by means of anion exchange chromatography, followed by PMSF treatment (1 mM) in a boiling water bath for 30 min. Digestion-resistant peptides were further separated by size exclusion chromatography after reduction and alkylation of Cys residues as described previously for 2S albumin from Brazil nut [38].

2.5 SDS-PAGE

SDS-PAGE was performed essentially according to Laemmli [37] with the MiniProtean system (BioRad, Richmond, CA, USA) using manually prepared 15% polyacrylamide gels. A volume of 20 μL *per* sample, including Laemmli loading buffer, was loaded and electrophoresis was stopped just before the bromophenol blue-containing front reached the end of the gel. Gels were stained in 1% Coomassie Brilliant Blue R-250 (Sigma) in 50% methanol/20% acetic acid overnight. Subsequently, gels were washed with 50% methanol/20% acetic acid for 5 min and destained with 50% methanol/20% acetic acid for 30 min. After that, gels were further destained with 25% methanol/10% acetic acid for 2 h. The migration of Ara h 1, the basic subunit of Ara h 3 and Ara h 6 were used to indicate the molecular weights (63.5, 25 and 15 kDa, respectively) for gels shown in Figs. 1–5. For gels shown in Figs. 6 and 7, markers of 45, 30, 21 and 14 kDa were used.

3 Results and discussion

3.1 Pepsin digestion of CPE

In the first experiment, CPE was digested with pepsin according to the protocol of Thomas *et al.* [29]. This protocol was designed to investigate the comparative stability to pepsin of novel proteins. This protocol uses a high pepsin concentration, 760 μg pepsin and 250 μg substrate protein *per* milliliter. Taking into account the specific activity of the pepsin, the pepsin:substrate ratio is 10 U/μg, the same as Thomas *et al.* [29] used. Figure 1 shows the time course of the digestion as visualized by SDS-PAGE under reducing conditions. At time point 0, before adding pepsin, a characteristic peanut extract pattern was found. Arrows at the left margin indicate that the individual peanut allergens are of the expected molecular weights based on the previous descriptions (Section 1). By 0.25 min substantial proteolysis of the CPE was observed. Peptides in the molecular weight region of 10–25 kDa originate from this proteolysis, and some remained up to an incubation time of 30 min. Under reducing conditions (Fig. 1), clear bands at approximately 10 kDa were visible that correspond to a digestion-resistant fragment of Ara h 2 described by Sen *et al.* [30]. Because Ara h 2 is a minor constituent of the peanut [35, 39], more conclusive results cannot be obtained on individual peanut allergens using a mixture (whole extract) of peanut proteins. In contrast to the relative stability of the protein bands at approximately 20 kDa, the protein bands at higher molecular weights (63.5 kDa, Ara h 1 and 45 kDa, Ara h 3) disappeared rapidly. Note that the remaining band at approximately 40 kDa is pepsin and not a peanut protein. Because limitations exist in the interpretation of studies with CPEs, we repeated the digestion experiments with the purified individual allergens Ara h 1, Ara h 2, Ara h 3 and Ara h 6.

3.2 Pepsin digestion of individual, major peanut allergens

3.2.1 Ara h 1

Figure 1 shows that the Ara h 1 band from CPE disappeared quickly upon digestion with pepsin. Therefore, digestion of

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purified Ara h 1 was also conducted with lower concentrations of pepsin (Fig. 2). As expected, lowering the pepsin concentration resulted in a more gradual breakdown of peptides. When pepsin was applied in a 100 fold lower concentration as compared with the protocol described by Thomas *et al.* [29], resulting in 0.1 U of pepsin per microgram substrate, peptides between approximately 20 and 50 kDa appeared. The analysis on SDS-PAGE did not show differences between reduced and nonreduced samples, as expected based on the fact that Cys residues are not involved in intra or intermolecular disulfide bridges for Ara h 1. The trimeric [6] and the oligomeric [8] organization of Ara h 1 on the quaternary folding level is not supported by disulfide bridges and the denaturing conditions of SDS result in dissociation of these multimers. This explains why such multimers are not present on SDS-PAGE (Figs. 1 and 2). Eiwegger *et al.* [34] also investigated the pepsin-induced hydrolysis of Ara h 1 and applied a 20-lower concentration as compared with the protocol of Thomas *et al.* [29], comparable with the middle part of Fig. 2. Here, we found some peptides of approximately 5 and 10 kDa, stable for 2–8 min, in line with the data published by Eiwegger *et al.* [34] who showed a protein band at 10 kDa stable for up to 2 min, and a protein band at 6 kDa stable for up to 8 min. Another publication described the digestion of Ara h 1 with low-pepsin concentration, comparable to our lowest concentration [9]. Analysis was performed using size exclusion chromatography under denaturing conditions in order to exclude association of peptides by interactions that support the protein structure on the tertiary and quaternary folding levels. They found peptides of relatively high molecular weight, *e.g.* approximately half of the mass of the intact Ara h 1 monomer as judged on their chromatograms. This is consistent with our observations of Ara h 1 treated with the lowest concentration of pepsin (Fig. 2, lower part). Astwood *et al.* [28] investigated the digestibility of Ara h 1 using pepsin and applied immunoblotting with a monoclonal antibody to detect proteolytic breakdown products. The absence of any bands on their blot could be explained either by the fact that Ara h 1 was degraded rapidly, or by the loss of immuno-reactivity of Ara h 1 after only limited digestion [28]. Our experiments on the digestion of Ara h 1 confirm earlier studies that it is rapidly digested by the high concentrations of pepsin.

3.2.2 Ara h 3

A first hint of the relatively high digestibility of Ara h 3 is shown in Fig. 1 in which the bands of the acidic and basic subunits of Ara h 3 disappear quite rapidly from CPE. The band of pepsin which migrates at a similar molecular weight as the acidic subunits of Ara h 3 make it difficult to interpret the fate of this subunit. The digestion of purified Ara h 3 is shown in Fig. 3. Again, the pepsin band migrated at the same molecular weight level as Ara h 3, but under nonreducing conditions (Fig. 3B), the acidic and basic subunits were still associated by a disulfide bridge, giving rise to several bands at approximately 70 kDa [15]. Reducing conditions (Fig. 3A) showed dissociation of the subunits

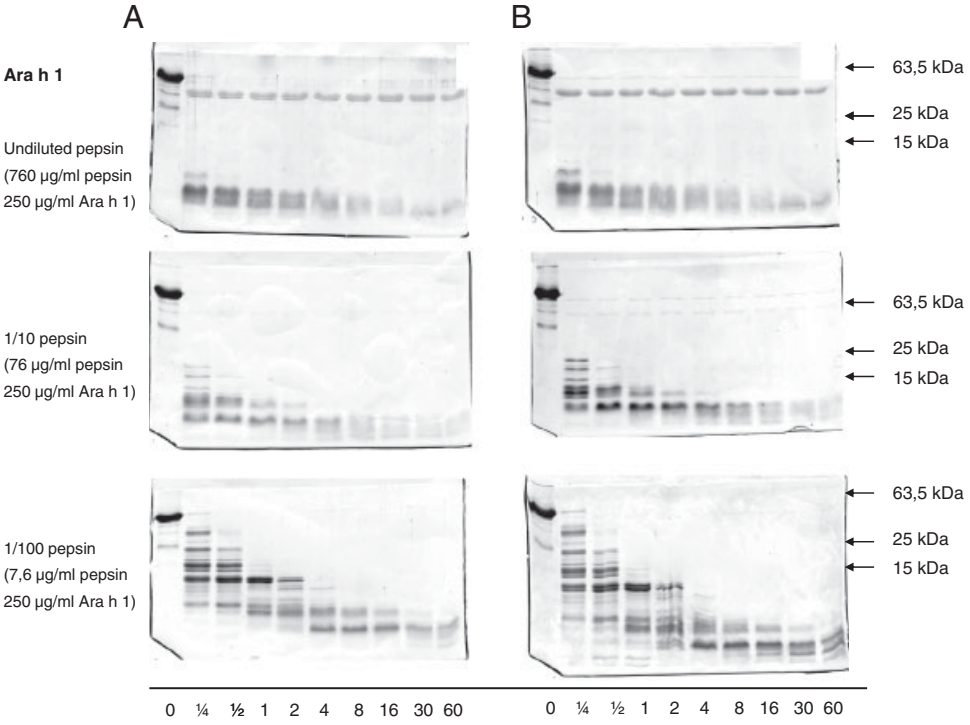

Figure 2. Digestion of Ara h 1. (A) SDS-PAGE under reducing conditions, (B) SDS-PAGE under nonreducing conditions. Incubation time (min) indicated at lower part. Right margin: molecular weight indication.

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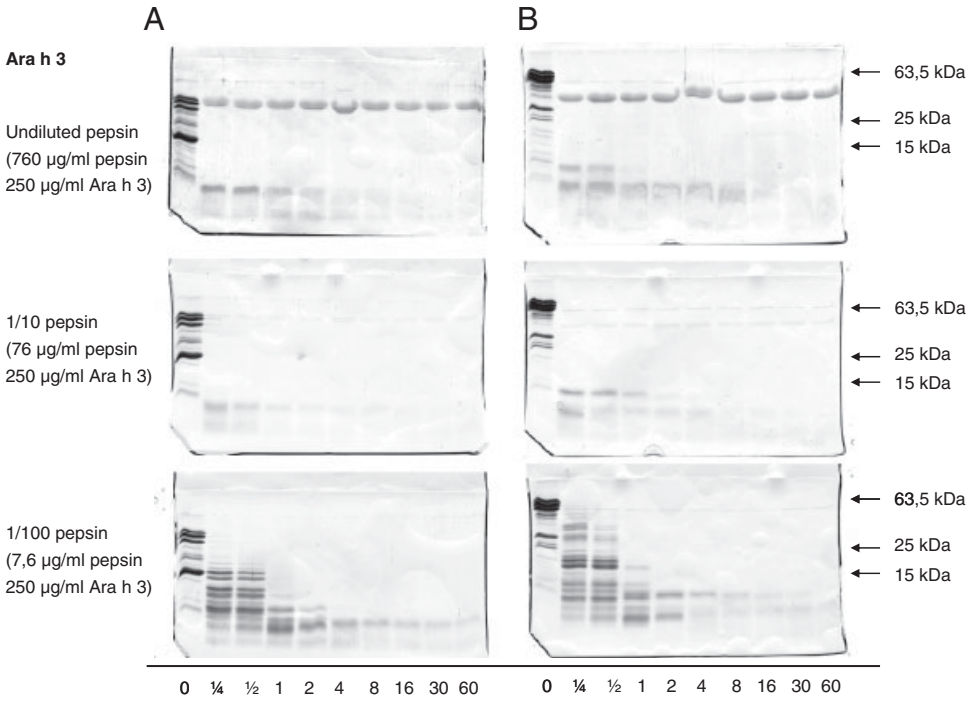

Figure 3. Digestion of Ara h 3. (A) SDS-PAGE under reducing conditions, (B) SDS-PAGE under nonreducing conditions. Incubation time (min) indicated at lower part. Right margin: molecular weight indication.

giving rise to the typical pattern for Ara h 3 with heterogeneity in the *N*-terminal acidic subunit [14, 15]. Pepsin digestion, as shown in Fig. 3, was rapid using the conditions applied by Thomas *et al.* [29] where all Ara h 3 were hydrolyzed after 0.25 min. The remaining peptide at approximately 10 kDa disappeared after 1–2 min. However, at the lowest concentration of pepsin (100-fold lower; 0.1 U of pepsin *per* microgram Ara h 3), some peptides of intermediate weight (10–30 kDa) remained for only 2–4 min. Little work has been reported by others on the digestion of Ara h 3. van Boxtel *et al.* [40] digested Ara h 3 with pepsin (pepsin:protein ratio = 1:500 w/w. They used the same pepsin as is used in this study (Sigma P-6887), with a similar specific activity. Therefore, 1:500 w/w corresponds to 0.07 U/µg Ara h 3, about a twofold lower ratio than our lowest concentration (lower part of Fig. 3). They analyzed by size exclusion chromatography, under reducing and denaturing conditions, allowing a comparison of molecular weight with SDS-PAGE (reducing conditions) analysis. After 10 min, the majority of the protein was found in the range of 7–14 kDa, and after 60 min the majority of the peptides were <7 kDa [40]. This remaining fraction may be explained by the lower pepsin concentration in the digest, in comparison to the pepsin concentrations of our experiments.

3.2.3 Ara h 2

In contrast to Ara h 1 and Ara h 3, Ara h 2 was more stable (Fig. 4). Even at the high-pepsin concentration (10 U of pepsin *per* microgram of substrate, upper part Fig. 4), the protein band with the highest molecular weight remained intact for up to 4 min. This could only be observed when reducing conditions during the SDS-PAGE analysis were applied. Under nonreducing analysis conditions (Fig. 4B), virtually no proteolytic breakdown was observed. The necessity of reduction to visualize proteolysis demonstrates that intramolecular disulfide bonds keep the hydrolysis products together as a single molecule with a molecular mass similar to the native Ara h 2. Thomas *et al.* [29] used 10 U/µg of substrate to investigate the digestibility of Ara h 2 also. In contrast with our results, they describe a rapid disappearance of both the larger and the smaller isoforms of Ara h 2. In their discussion, they speculate that a trace of the reducing agent DTT that was used during the purification of their Ara h2 may have denatured the protein making it more susceptible for digestion by pepsin [29]. Using a similarly high-pepsin concentration (pepsin:protein ratio of 1:2 w/w), Sen *et al.* [30] investigated the digestion of Ara h 2 as well. They found a digestion-resistant peptide of 10 kDa, in line with our observations (Fig. 4A). The described peptide remained largely intact after subsequent digestion with trypsin/chymotrypsin [30]. In a recent article, Lehmann *et al.* [41] described the digestion of Ara h 2 with trypsin and chymotrypsin and found a peptide of similar size. In their analysis, reduction before SDS-PAGE was necessary to visualize hydrolysis, in line with the results of Sen *et al.* [30] and our results. It is likely that pepsin, as well as trypsin/chymotrypsin-induced hydrolysis, results in a similar stable peptide, with minor differences at the *N*-terminal and/or *C*-terminal part. This is explained by the fact that proteolysis is restricted by the Ara h 2 structure, rather than by the specificity of the applied proteases [30, 41]. Astwood *et al.* [28] had, some time ago,

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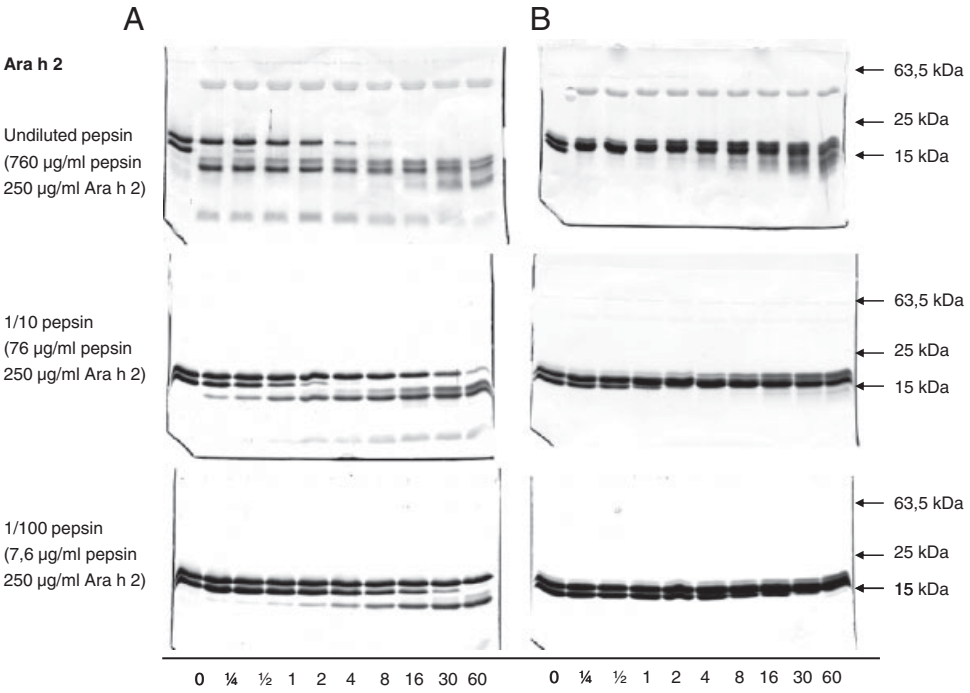

Figure 4. Digestion of Ara h 2. (A) SDS-PAGE under reducing conditions, (B) SDS-PAGE under nonreducing conditions. Incubation time (min) indicated at lower part. Right margin: molecular weight indication.

described the digestibility characteristics of Ara h 2. They showed that Ara h 2 is stable toward pepsin-induced hydrolysis, using a protocol similar to that of Thomas *et al.* [29]. However, intact Ara h 2 migrated on their SDS-PAGE as a single band of approximately 14 kDa, which is not in line with the current understanding of Ara h 2. Possibly, the protein was the other abundant 2S albumin, now known as Ara h 6 [16, 17]. When studied in more detail, the larger isoform of Ara h 2 (Fig. 4A) was more stable than the smaller one. This was also observed by Herman *et al.* [42], who digested purified Ara h 2 with pepsin. Furthermore, the lowest pepsin:allergen ratio results in one main breakdown product, whereas at higher ratios at least two or three distinct bands were visible. Probably, one peptide bond is cleaved relatively easily, whereas a few other peptide bonds require more rigorous pepsin digestion (*e.g.* a prolonged time or higher pepsin:allergen ratio).

3.2.4 Ara h 6

Ara h 6 (Fig. 5) showed a digestion pattern which is very similar to that of Ara h 2 (Fig. 4). More precisely, Ara h 6 disappeared with a rate somewhat faster than the larger isoform of Ara h 2, and somewhat slower than the smaller isoform of Ara h 2 (Fig. 4). Ara h 6 was substantially digested after only 1 min at the highest pepsin concentration. Lowering the pepsin concentration resulted in a more gradual breakdown, and with the lowest pepsin concentration, some Ara h 6 was intact after 30 min. Ara h 2 and Ara h 6 are both 2S albumins with a high degree of amino acid identity [16–18] and one could speculate that proteolysis would result in peptides of similar molecular weight. Digestion of Ara h 6 resulted, as visualized on SDS-PAGE under reducing conditions, in a stable peptide of approximately 10 kDa, similar as for Ara h 2, even when the highest pepsin concentration was applied. As for Ara h 2, the intramolecular disulfide bridges of Ara h 6 maintain the digestion fragments as a single molecule. Although the digestion of Ara h 6 was more rapid than that of (the larger isoform of) Ara h 2, a similar large peptide remained for the course of the experiment (1 h) even when the highest concentration of pepsin is applied. However, in contrast to Ara h 2, digestion of Ara h 6 at the lowest pepsin:allergen ratio resulted in at least two breakdown products, and showed (qualitatively) the same band pattern as digestion of Ara h 6 digested with the highest pepsin:allergen ratio.

3.3 Trypsin digestion of Ara h 2

To mimic physiological digestion ultimately, *in vivo* experiments or dynamic digestion models [43] would be required. For this study, we choose to study the effect of trypsin on Ara h 2 without pretreatment of pepsin. It is clear from our experiments shown in Figs. 1 and 4 that Ara h 2 is rather stable toward digestion with pepsin and it is expected that part of Ara h 2 will reach the small intestine upon ingestion of peanuts. Additionally, as is known for larger amounts of food, the food bolus will pass the stomach before pepsin is exposed to the interior of the bolus, leaving at least part of the food proteins unaffected by pepsin [43]. Sen *et al.* [30] also performed digestion experiments with Ara h 2 using

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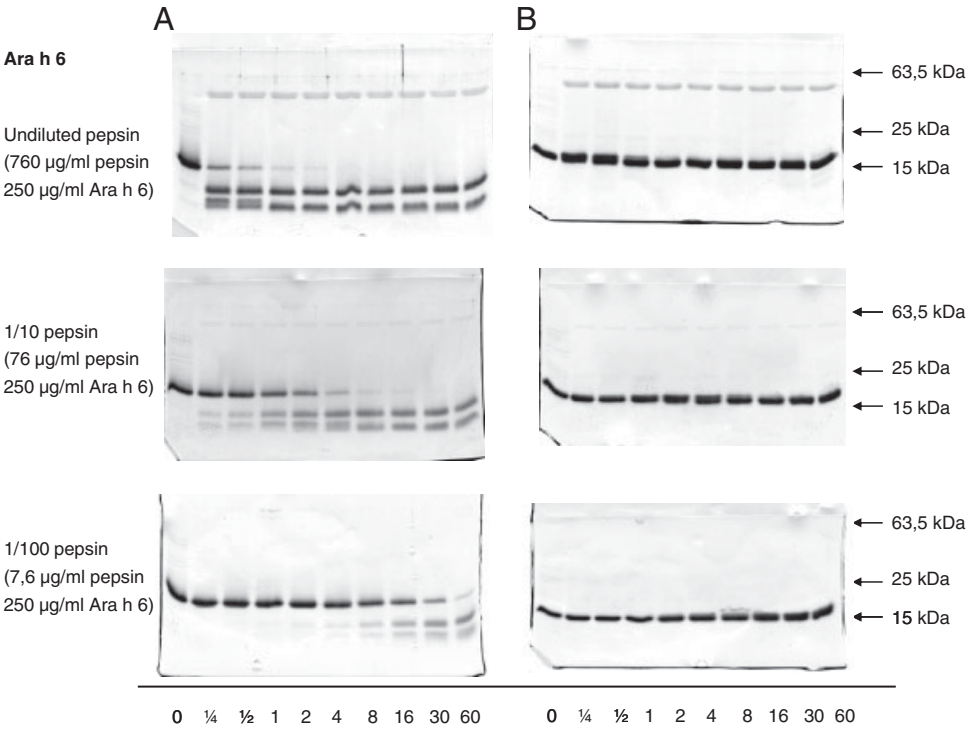

Figure 5. Digestion of Ara h 6. (A) SDS-PAGE under reducing conditions, (B) SDS-PAGE under nonreducing conditions. Incubation time (min) indicated at lower part. Right margin: molecular weight indication.

trypsin and found a peptide of Ara h 2 of similar size as we find upon digestion with pepsin. To confirm their observation, we performed trypsin digestion of Ara h 2 with several concentrations of trypsin (7.2, 24 and 72 µg/mL), with the middle concentration representing the conditions applied by Sen *et al.* [30]. Indeed, our results (Fig. 6) were fully consistent with those of Sen *et al.* [30]. Lehmann described a 9 kDa peptide after digestion of Ara h 2 with trypsin. They also described a 4 kDa peptide, but this peptide is hardly visible on their SDS-PAGE analysis. We do not observe a 4 kDa band in our experiment with trypsin. Interestingly, we do observe two bands in the molecular weight region of 9 and 4 kDa after digestion of Ara h 2 and Ara h 6 with pepsin. Of course, pepsin has a different specificity to that of trypsin/chymotrypsin, and the results cannot be extrapolated easily. However, there are many potential cleavage sites for both pepsin and tryspin throughout the sequence of Ara h 2 and Ara h 6 and only a few of them are cleaved in practice. This observation points in to the direction that the 2S albumin structure may be a more important factor than its primary sequence with regard to susceptibility of peptide bonds in 2S albumins from peanut.

3.4 Comparison of digestion of Ara h 1, Ara h 2, Ara h 3 and Ara h 6

The digestibility of allergens can be compared by following the disappearance of the intact protein bands on SDS-PAGE,

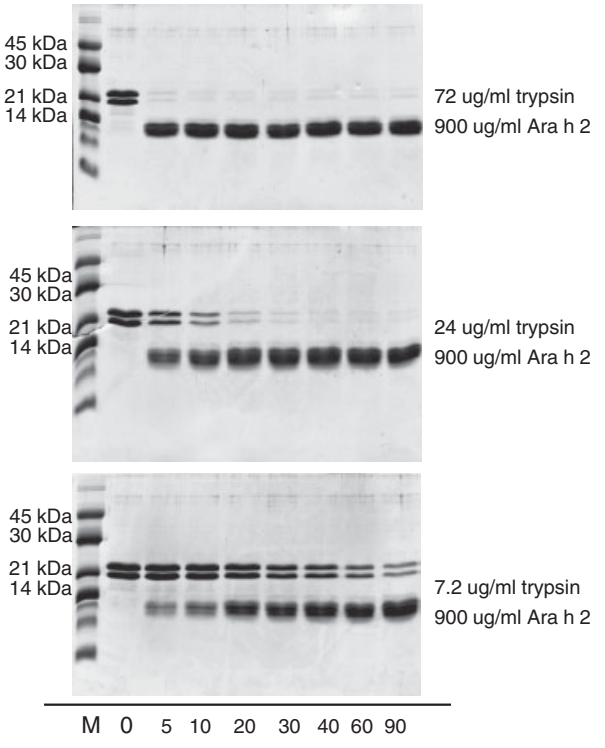

Figure 6. Digestion of Ara h 2 by trypsin. SDS-PAGE analysis under reducing conditions. Incubation time (min) indicated at lower part. Numbers at the right indicate the applied trypsin concentration. Left margin: molecular weight indication.

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or by following the existence and subsequent disappearance of peptides that originate from the intact protein bands, both provided that identical experimental conditions are applied. To our knowledge, this is the first study where the potential gastric digestibility of all of the major peanut allergens is investigated in one set of experiments. One study included both Ara h 1 and Ara h 2 [28], but as explained in the previous section of the digestiblityof Ara h 2, we doubt that the protein they considered Ara h 2 is indeed Ara h 2.

On examining the disappearance of the intact allergen bands, it was clear that Ara h 1 (Fig. 2), and both the acidic and basic subunits of Ara h 3 (Fig. 3) were digested rapidly when the conditions suggested by Thomas *et al.* [29] were applied. One could argue that the pepsin:protein ratio is comparatively high in the protocol for Thomas *et al.* [29]; however, it is accepted that such a ratio may represent stomach conditions [44]. Lowering the pepsin concentration by tenfold (middle gels in Figs. 2–5) also resulted in a rapid disappearance of both Ara h 1 and Ara h 3. Even at a 100-fold lower concentration of pepsin (bottom gels in Figs. 2–5), all intact protein bands of these allergens disappeared after less than 1 min. On the other hand, Ara h 2, in particular the larger isoform, and to a lesser extent Ara h 6, remained intact upon digestion for some time when using the highest pepsin concentration. On lowering the pepsin concentration by 10 and 100 fold, the intact protein bands remained for longer time periods. Ara h 1 and Ara h 3 disappear within 15 s (even at the lowest pepsin concentration), Ara h 2 and Ara h 6 remain for 30–60 min, indicating a difference in digestion kinetics of at least 100-fold. The larger isoform of Ara h 2 was most stable of all; it remains intact for several minutes at the highest concentration of pepsin, and for >60 min for the lowest concentration of pepsin, indicating that this allergen was digested at least 240 fold more slowly than Ara h 1 and Ara h 3.

When focusing on peptides that originate from the intact allergens, and the fate of these peptides, it was noticed immediately that the breakdown products of Ara h 3 (most abundant at the lowest concentration of pepsin, Fig. 3, lower gels) disappeared more quickly than those of the other peanut allergens. Even at this low concentration of pepsin, virtually all breakdown products disappeared after 4 min. For Ara h 1, under these conditions, breakdown products remained for the course of the experiment (60 min). However, when based on the comparative staining intensities, the breakdown products of the native Ara h 1 (after 60 min) appear to represent only a fraction of the originally present Ara h 1. On the contrary, for Ara h 6 and both isoforms of Ara h 2, peptides of approximately 10 kDa were generated, obviously more quickly when higher pepsin:allergen ratios were applied. These breakdown products, in their turn, remained for the course of the experiment, even at the highest pepsin:allergen ratio. Comparing the staining intensities of these breakdown products at 60 min with that of the native Ara h 2 and Ara h 6 appears to show that a major fraction of the original allergen is still present as peptides of approximately 10 kDa in line with the earlier work [30, 41, 42]. Next to the 10 kDa peptide, a peptide of 4 kDa was described as proteolytic breakdown product of peanut 2S albumin [41]. We saw such peptides of <10 kDa for Ara h 2, and such peptides were even more pronounced for Ara h 6. Investigating the SDS-PAGE patterns of the peptides that originate from digestion of the different peanut allergens, and their respective stability to further breakdown lead to the following overall picture: both Ara h 2 and Ara h 6 are degraded to large peptides that remain present during the course of the experiment, whereas for Ara h 3 and to a lesser extent Ara h 1, the emerging breakdown products are not stable.

3.5 Digestion-resistant peptides found in Ara h 2

In order to obtain a high yield of digestion-resistant peptide, the reaction product of the incubation with the highest concentration (at 20 min) was taken to investigate the digestion-resistant peptides. Purification of the reaction product showed that multiple peptides in the range of 10 kDa were formed after digestion with trypsin (Fig. 7). In fact, two distinct pools were identified, referred to as digestion-resistant peptide-1 (DRP-1) and digestion-resistant peptide-2 (DRP-2). The peptides were characterized by *N*-terminal sequencing and for two peptides, the *N*-terminus was the same as for the native protein

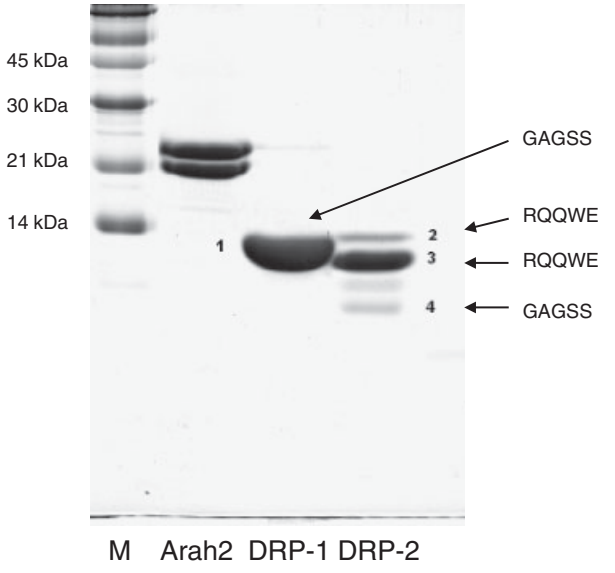

Figure 7. Purified reaction products of Ara h 2 digest. M: Marker proteins (indicated at left margin); Arah2 is the starting material; DRP-1 and DRP-2: digestion-resistant peptides of Ara h 2, isolated after digestion. Two pools could be separated. Amino acid sequences at the right indicate the *N*-terminal sequences of the protein bands.

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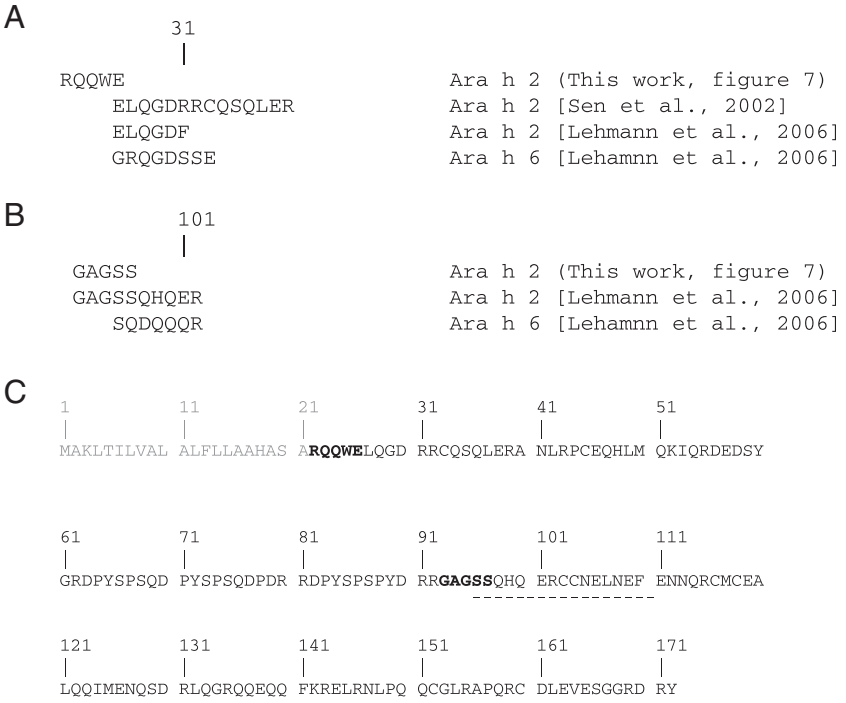

Figure 8. Sequence alignment of trypsin-resistant peptides of Ara h 2. (A) Peptides obtained from *N*-terminal peptides. (B) Peptides found originating from middle part. (C) Sequence of Ara h 2 (Swiss-Prot accession number Q6PSU2). Bold: identified sequences. Underlined area indicates cleavage sites that result in a 9–11 kDa *N*-terminal peptide.

(RQQWE, see Fig. 7). Earlier studies showed peptides with a similar molecular weight but a slightly shifted (three amino acids) *N*-terminus [30, 41], indicating proteolytic shortening of the *N*-terminus in other studies. Interestingly, Lehmann *et al.* [41] reported that this *N*-terminal fragment had a mass of about 5 kDa, while the predicted sequence was about 10 kDa, in line with the peptide reported by Sen *et al.* [30]. The difference was explained by the removal of amino acids in the *C*-terminal part of the peptide, but this should then have been extensive. Of the two *N*-terminal peptides we found, the one most abundant had a slightly lower molecular weight than the other. We also found an abundant peptide of approximately 10 kDa with an *N*-terminus corresponding to the middle part of Ara h 2 (GAGSS), suggesting that the *C*-terminal part of Ara h 2 is digestion resistant as well. This is in agreement with Lehmann *et al.* [41] who also reported a peptide with this *N*-terminus and with a similar molecular weight. However, Sen *et al.* [30] did not identify this peptide. Sequence data of the peptides found in this study are aligned with those of earlier reports and shown in Fig. 8. Using a molecular weight range of 9–11 kDa to describe the 10 kDa *N*-terminal peptide found by Sen *et al.* [30], the sequence underlined in Fig. 8B should indicate the cleavage site. Our data and those from Lehmann *et al.* [41] indicated an abundant peptide with the *N*-terminal sequence GAGSS. To explain this, cleavage should have taken place after the arginine residue preceding GAGSS, giving a molecular weight of 8.6 kDa for the *N*-terminal peptide. The deviation between 8.6 and 10 kDa can be explained by the imprecision of the analytical method (SDS-PAGE), but Sen *et al.* [30] hypothesized that the *C*-terminus of their *N*-terminal peptide was extended with about 20 amino acids, leaving no room for a peptide starting with GAGSS. Disulfide bridge mapping of 2S albumins of peanut allows both options to form a single molecule upon digestion that falls into two parts after reduction [45]. Taken together, our data are in agreement with the earlier study, but indicate a higher degree of heterogeneity of digestion-resistant peptides arising from Ara h 2. These peptides have sufficient length to suggest that they could both sensitize susceptible individuals enabling the development of hypersensitivity and subsequently elicit allergic reaction in peanut-allergic individuals.

4 Concluding remarks

By evaluating the potential gastric digestibility of purified major allergens from peanut in one series of experiments, it is concluded that Ara h 2 and Ara h 6 are far more stable to peptic digestion as compared with Ara h 1 and Ara h 3. This may explain the reason that why Ara h 2 and Ara h 6 allergens are more often recognized by IgE from the sera of peanut-allergic patients. Digestion-resistant peptides obtained after digestion of Ara h 2 with pepsin consist of a pool of relatively large peptides that may be able to elicit allergic reactions. This is not the case for Ara h 1 and Ara h 3 where peptides that originate from digestion are quickly broken down further. This improved understanding of the comparative gastric stability of the major peanut allergens, suggesting that immunotherapeutic strategies should perhaps be focused on Ara h 2 and Ara h 6.

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The authors thank André H. Penninks for critically reading the manuscript. This research was conducted with a contribution of the University of Nebraska Agricultural Research Division, supported in part by funds provided by the United States Department of Agriculture. Additional support was provided by the Food Allergy Research & Resource Program of the University of Nebraska. Mention of a trade name, proprietary products or company name is for the clarity of presentation and does not imply endorsement by the authors of the University of Nebraska.

The authors have declared no conflict of interest.

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