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Research Article

Legumin allergens from peanuts and soybeans: Effects of denaturation and aggregation on allergenicity

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Legumin proteins Ara h 3 from peanuts and glycinin from soybeans are increasingly described as important allergens. The stability of an allergen's IgE binding capacity towards heating and digestion is considered an important characteristic for food allergens. We investigated the effects of heating and digestion on the IgE binding of Ara h 3 and glycinin. Both proteins are relatively stable to denaturation, having denaturation temperatures ranging from 70 to 92°C, depending on their quaternary structure and the ionic strength. Aggregates were formed upon heating, which were partly soluble for glycinin. Heating slightly decreased the pepsin digestion rate of both allergens. However, heating did not affect the IgE binding capacity of the hydrolyzates, as after only 10 min of hydrolysis no IgE binding could be detected any more in all samples. Peanut allergen Ara h 1, when digested under equal conditions, still showed IgE binding after 2 h of hydrolysis. Our results indicate that the IgE binding capacity of legumin allergens from peanuts and soybeans does not withstand peptic digestion. Consequently, these allergens are likely unable to sensitize *via* the gastro-intestinal tract and cause systemic food allergy symptoms. These proteins might thus be less important allergens than was previously assumed.

Keywords: Ara h 3 / Food allergy / Glycinin / Heat processing / Pepsin digestion

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1 Introduction

Soybeans and peanuts belong to the eight most significant allergenic foods. Peanut allergy has been well studied and often causes acute and severe reactions. The prevalence and characteristics of soybean allergy have been less well studied, but allergy to soybeans is assumed not to cause severe reactions [1]. Besides, not that many peanut allergic patients also suffer from clinically relevant allergies to soybeans [2, 3].

The most abundant protein in soybeans is glycinin, which belongs to the legumin protein family and comprises 25–35% of all proteins in soybeans [4]. Glycinin is described as a major allergen [5]. The homologous legumin protein in peanuts is denoted Ara h 3 and is highly abundant [6]. Ara h 3 has been designated both a major and a minor allergen, depending on the study population, as it was recognized by serum IgE of approximately 44, 53, and 77% of peanut-allergic patient populations, respectively [7–9].

Legumin proteins are approximately 60 kDa in molecular mass and consist of a basic (approximately 20 kDa) and an acidic polypeptide (approximately 40 kDa), linked together by a disulfide bridge. The acidic polypeptide of Ara h 3, in contrast to soy glycinin, is extensively post-translationally processed, yielding peptides with molecular masses between 13 and 45 kDa [6]. Legumin proteins associate *in planta* into hexamers, resulting in a molecular mass of ~360 kDa [10]. However, the quaternary structure of legumins after extraction is dependent on the ionic strength. At an intermediate ionic strength (I = 0.2 M) Ara h 3 is partially present as trimers [6], while glycinin shows the same behavior at a lower ionic strength (I = 0.03 M). The dissoci-

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Abbreviations: DSC, differential scanning calorimetry; **SEC,** size-exclusion chromatography

ation of glycinin induces an increase of nonstructured protein at secondary and tertiary level [11]. For Ara h 3 the effects of dissociation on the secondary and tertiary structure are not known.

For glycinin six genes have been identified, representing glycinin G1 – G5, and G7. For Ara h 3 also several genes are known (Swiss-Prot accession numbers O82580, Q9SQH7, and Q6IWG5). IgE binding to glycinin has been reported to occur to both the acidic [12 – 14] and the basic [5] polypeptides of glycinin. For Ara h 3 four IgE binding epitopes have been identified, all situated on the acidic polypeptide of the allergen [7]. In addition, IgE binding to the basic polypeptide of Ara h 3 has been reported [6, 14], indicating the presence of additional IgE binding epitopes.

Although clinically relevant crossreactivity between peanuts and soybeans is not very common, *in vitro* IgE crossreactivity between soybeans and peanuts has been reported several times [2, 15]. This could be caused by the sequence homology between soybean and peanut proteins. For example, two epitopes on the acidic chain of glycinin G1 are homologous to two epitopes on Ara h 3 [13], and one epitope on the basic polypeptide of glycinin G2 is highly conserved in both Ara h 3 and glycinin G1 [14].

Food allergens are believed to only be able to sensitize and exert systemic reactions after passing through the gastro-intestinal tract, as they can be absorbed when they have reached the intestinal mucosa. Thus, in order to cause systemic symptoms, protein fragments remaining after pepsin digestion should still be able to bind IgE. Therefore, a factor that is considered to be important for the allergenicity of food proteins is the resistance of their IgE binding capacity upon digestion in the gastro-intestinal tract. Another factor is the stability upon cooking and industrial processing, as most foods are being consumed after some kind of processing. Accordingly, information about the effects of heating and digestion on the IgE binding of food allergens is necessary to determine the allergenic potential of these proteins. Several food allergens have, therefore, been investigated for their stability to heat and digestion. For example, Ara h 1, one of the major allergens from peanuts, is still able to bind IgE after digestion [16].

The IgE binding capacity of soy glycinin is reported not to be affected by heating [17], while sequential hydrolysis by pepsin and chymotrypsin is reported to result in peptides of 20 kDa and smaller, with a 20 kDa peptide still being able to bind IgE [18]. The effects of heating and digestion on the IgE binding capacity of Ara h 3 have not been investigated yet. Our aim was to investigate these properties of Ara h3 and soy glycinin, and to compare the behavior of both allergens. As digestibility is dependent on the pepsin concentration and activity used, we also subjected unheated peanut allergen Ara h 1, belonging to the homologous vicilin protein family, to the same pepsin concentrations, in order to validate our results. The results provide insights into the contribution of Ara h 3 and glycinin to the systemic allergic symptoms of peanut allergy and soybean allergy, respectively.

2 Materials and methods

2.1 Materials

All chemicals were obtained from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, USA), unless stated otherwise. Peanuts of the Runner market type were generously provided by Imko Nut Products (Doetinchem, The Netherlands) and were stored at 4°C until use. Glycinin was purified from Hyland soybeans as described by Kuipers *et al.* [19]. (Oligomeric) Ara h 1 was purified from peanuts as described previously [20], with the exception that extraction was performed at pH 6.2. The ionic strengths of Ara h 1, Ara h 3, and glycinin ($\sim 4\,' 10^{-8}$ to $\sim 8 \times 10^{-8}$ M) in distilled water were arbitrary set at 0 M.

Plasma from five patients with allergy for peanuts (purchased from Plasmalab International, Everett, WA, USA) and serum from one patient allergic to peanuts were used in this study. Clinical characteristics consisted of a questionnaire filled in by the patients. All patients indicated that they suffered from peanut allergy. Besides, two patients clearly indicated that they suffered from allergic reactions upon eating soybean products (with symptoms like sore throat, itchy mouth, stomach, and intestinal upset). CAP-FEIA levels specific for peanuts were between 45 and >100 kU/L and for soybeans between 3 and 75 kU/L. Part of the plasma and serum samples were mixed in equal ratios, denoted Plasmapool, and used for IgE-immunoblotting and dotblotting experiments.

2.2 Ara h 3 purification

For the purification of Ara h 3, defatted peanuts were extracted in 50 mM Tris-HCl buffer, pH 8.2, at a meal solvent ratio of 1:10 w/v, during 1 h at room temperature under continuous stirring. Afterwards, the extract was sieved through cheese-cloth and subsequently centrifuged (25 min; $14\,000 \times g$; 4°C). The supernatant obtained was filtered over a 1.2 μm filter, and subsequently applied (13 mL applied *per* run) onto a 320 mL Superdex 200 XK 26/60 column (GE Healthcare, Uppsala, Sweden). The column was equilibrated and eluted with 50 mM Tris-HCl buffer, pH 8.2, at a flow rate of 4.3 mL/min, using an Äkta Purifier system (GE Healthcare) operated at room temperature. The eluate was monitored at 280 and 325 nm and fractions containing Ara h 3, as analyzed by SDS-PAGE, were collected and applied onto a 1.2 L Source Q Fineline column, using an Äkta Explorer system (GE Healthcare). The column was equilibrated with 50 mM Tris-HCl buffer, pH 8.2, and the flow rate used was 40 mL/min. After sample application and washing, a ten column volumes linear gradient from 0.1 to 1 M NaCl in 50 mM Tris-HCl buffer,

pH 8.2, was applied. The eluate was monitored at 280 nm and samples containing pure Ara h 3, as analyzed with SDS-PAGE, were collected and pooled. The pooled fractions were dialyzed in 10 000 MWCO dialysis tubings (Medicell, London, UK) against distilled water. Afterwards samples were lyophilized and stored at −20°C until use.

2.3 SDS-PAGE

Protein samples were analyzed using on a mini-protean II system (BioRad Laboratories, Hercules, CA, USA) according to the instructions of the supplier. Reducing conditions were obtained by adding β-mercaptoethanol to a final concentration of 10 mM and heating the samples for 5 min at 100°C. Tris-Tricine 16.5% Ready Gels (BioRad Laboratories) were used. Gels were stained according to the CBB procedure provided by the manufacturer. A polypeptide and a prestained protein molecular weight marker (article 161-0326 and 161-0373, respectively; BioRad Laboratories) were used for calibration.

2.4 Protein quantification

The nitrogen content of samples was determined using the combustion (Dumas) method on a NA 2100 Nitrogen and Protein Analyzer (CE instruments, Milan, Italy) using methionine as a standard. Protein conversion factors of 5.27 for Ara h 3 and 5.57 for soy glycinin were calculated from their amino acid sequences (Swiss-Prot accession number O82580 for Ara h 3 and accession numbers P04776, P04405, P11828, P02858, and P04347 for glycinin).

2.5 Heating experiments

Ara h 3 and glycinin solubilized (2.5 mg/mL) in 35 mM sodium phosphate buffer pH 7.6, containing 0.1 M NaCl (I = 0.2 M), were heated at 100°C for 10 min. After heating, samples were cooled immediately on ice. Samples that were subjected to pepsin digestion afterwards were used directly, while for other investigations, samples were centrifuged at 22 000 × g for 5 min at 4°C, after which the supernatant was collected.

2.6 Differential scanning calorimetry (DSC)

DSC experiments were performed on a VP-DSC Microcalorimeter (MicroCal, Northampton, MA, USA). Thermograms were recorded from 20 to 120°C with a heating rate of 1°C/min. Experiments were conducted with protein samples solubilized (5 mg/mL) in 35 mM sodium phosphate buffer pH 7.6 (I = 0.03) with or without the addition of 0.1 M NaCl (I = 0.2 M).

2.7 Size-exclusion chromatography (SEC) under nonreducing and nondenaturing conditions

Protein samples (100 µL) were applied onto a Superose 6 10/300 column (GE Healthcare). The column was equilibrated and eluted with 10 mM sodium phosphate buffer, pH 7.6, with or without the addition of 0.1 M NaCl, at a flow rate of 0.5 mL/min using an Äkta Purifier system operated at room temperature. The eluate was monitored at 280 nm.

2.8 SEC under reducing and denaturing conditions

Guanidinium chloride (6 M) was added to protein samples. Next, samples were continuously stirred for 1 h at ambient temperature. Subsequently, DTT was added to a final concentration of 50 mM and samples were continously stirred for 1 h at room temperature. Afterwards, ACN and TFA were added to a final concentration of 30% v/v ACN, and 0.1% v/v TFA. After stirring for 1 h at room temperature, samples were centrifuged (10 min, 22 000 × g, 20°C). The supernatants (50 µL) were applied onto a Shodex Protein KW-802.5 column (8 × 300 mm, Showa Denko K. K., Kanagawa, Japan), using an Äkta Purifier system. The column was equilibrated and eluted with 30% v/v aqueous ACN containing 0.1% v/v TFA. The flow rate was 0.5 mL/min and the absorbance of the eluates was measured at 220 nm. The column was equilibrated using a low molecular weight gel filtration calibration kit (GE Healthcare), containing thyroglobulin, aldolase, and ribonuclease A. Furthermore, β-lactoglobulin, aprotinin, insulin chain B, angiotensin-I, and bradykinin (articles L0130, A6012, I6383, A9650, and B3259, respectively) were used for calibration of the column.

2.9 Digestion experiments

The pH of 2.5 mg/mL protein solutions in 35 mM sodium phosphate buffer pH 7.6, containing 0.1 M NaCl (I = 0.2 M), was adjusted to 2.0 with 2 M HCl. Next, samples were mixed 1:1 with 5 µg/mL pepsin (Sigma – Aldrich, article P6887) in 30 mM NaCl, of which the pH was adjusted to 2.0 with 1 M HCl. Samples were incubated at 37°C during different time intervals. The reaction was stopped by raising the pH to 7.0 – 7.2 with 2 M NaOH.

2.10 IgE-Immunoblotting and dotblotting

For IgE-immunoblotting proteins separated after SDS-PAGE were transferred to polyvinyldifluoride (PVDF) sheets (BioRad, article 162-0177) using standard tech-

niques. Samples for dotblotting (10 μL) were applied to the same PVDF sheets. Afterwards, the sheets were air-dried at 30°C. Membranes were blocked with 3% w/v BSA in TBS buffer (50 mM Tris-HCl pH 7.4, containing 150 mM NaCl) for 1 h at room temperature and subsequently incubated overnight at room temperature with 30 times diluted Plasmapool. After washing with washing buffer (TBS containing 0.5% w/v BSA and 0.1% w/v Tween-20) phosphatase-labeled anti-IgE was added (500 times diluted in washing buffer; Sigma – Aldrich, article A3076) and the membranes were incubated for 2 h at room temperature. After washing, membranes were stained using BCIP/NBT liquid substrate (Sigma – Aldrich, article B1911).

3 Results and discussion

Legumin proteins from soybeans and peanuts are increasingly reported as important food allergens [5, 7 – 9]. The withstanding of the IgE binding capacity of food allergens after (heating and) digestion appears to be a prerequisite for allergens to sensitize *via* the gastro-intestinal tract and to exert systemic allergic symptoms. We therefore investigated the effects of heating and pepsin digestion on the stability and IgE binding of Ara h 3 and glycinin, the major seed proteins from peanuts and soybeans, respectively.

3.1 Heat stability and quaternary structure of legumin proteins

Both Ara h 3 and glycinin were subjected to DSC analysis in order to determine the transition temperatures of these proteins. The data are given in Table 1. At an ionic strength of 0.2 M for glycinin a single transition was observed with a transition temperature of approximately 85°C. This transition temperature corresponds well with the earlier reported transition temperature of 86°C for glycinin under the same conditions [21]. For Ara h 3 two transitions were observed with temperatures of approximately 77 and 92°C. At a low ionic strength (I = 0.03 M) Ara h 3 also showed two transitions with transition temperatures comparable to those at I = 0.2 M. For glycinin at a low ionic strength a peak with a maximum around 78°C was observed with a shoulder peak with a maximum around 70°C, also pointing towards two transitions. All transitions were irreversible, as in second heating scans no transitions were observed (no further data shown).

The two transitions observed for Ara h 3 and glycinin point towards the proteins being present in two different forms, with different transition temperatures. It has been reported that purified Ara h 3 occurs mostly as hexamers, but also partly as trimers at I = 0.2 M [6], while glycinin under these conditions occurs solely as hexamers [11]. SEC analysis confirmed this, as can be seen in Fig. 1: Ara h 3 at I = 0.2 M occurs partly as trimers and partly as hexamers, while glycinin occurs mainly in its hexameric form, and partly as aggregates with a high molecular weight, the latter probably being induced during lyophilization [6]. SEC analysis at a lower ionic strength caused a shift in the elution volumes of legumin hexamers and legumin trimers as a result of differences in the ionic strength. Taking this into account, it was clearly observed that at a low ionic strength (I = 0.03 M) relatively more trimeric Ara h 3 was present compared to the high ionic strength. Besides, at a low ionic strength glycinin also partly occurred in a trimeric form, which corresponds with literature data [11]. Thus, the transitions observed for both proteins are consistent with their dissociation behavior. Trimeric glycinin denatures at a lower temperature than hexameric glycinin [21]. Considering the homology between Ara h 3 and glycinin it seems obvious that the lowest denaturation temperature measured for Ara h 3 is the denaturation temperature of the trimeric form of the protein, while the highest transition temperature is the denaturation temperature of the protein in its hexameric form.

Table 1. Transition temperatures (°C) of Ara h 3 and glycinin trimers and hexamers at pH 7.6, with SDs, at different ionic strengths

Ionic strength	Ara h 3		Glycinin	
	Transition of trimers	Transition of hexamers	Transition of trimers	Transition of hexamers
0.03	78.3 ± 0.2	90.7 ± 0.5	~70.5[a)]	77.7 ± 0.1
0.2	76.6 ± 0.1	92.3 ± 0.1	–	85.2 ± 0.4

a) No SD could be calculated, because the transition peak was a shoulder peak.

From these results it can be concluded that Ara h 3 differs from glycinin in that at higher ionic strengths this protein also partly occurs as trimers. Besides, Ara h 3 hexamers and trimers denature at higher temperatures than glycinin hexamers and trimers, indicating that Ara h 3 is more stable to heat processing than glycinin.

Upon subjecting Ara h 3 and glycinin to heating above their transition temperatures and analyzing the soluble part on SEC (Fig. 2) it appeared that Ara h 3 solely formed insoluble aggregates, as no peaks were observed in the chromatogram. Dumas analysis confirmed the absence of protein in the supernatant. The heated glycinin showed a peak eluting just after the void volume of the column, which indicates the formation of soluble aggregates. Furthermore, a peak eluting after elution volumes higher than 15 mL was observed, which indicates the presence of dissociated polypeptides. These observations agree with the work of Mori *et al*. [22], who describe the formation of soluble aggregates upon heating and the dissociation of polypeptides from the glycinin complex, with the basic polypeptides precipitating upon heating. In our experiment part of the protein had indeed become insoluble after heating: Dumas analyses showed that 45% of the protein turned insoluble. This insol-

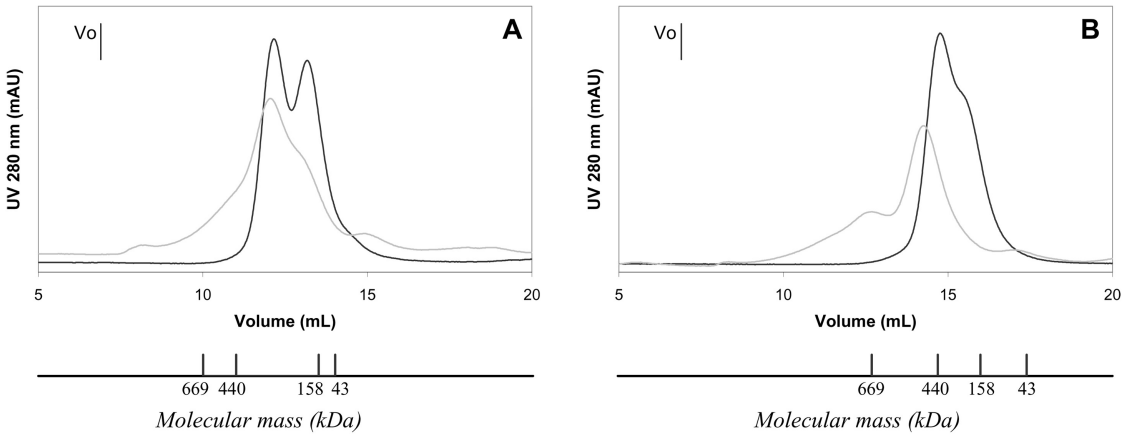

Figure 1. Size exclusion chromatograms of Ara h 3 (black lines) and glycinin (gray lines) at (A) $I = 0.03$ M and (B) $I = 0.2$ M. Indicated is the void volume of the column (Vo).

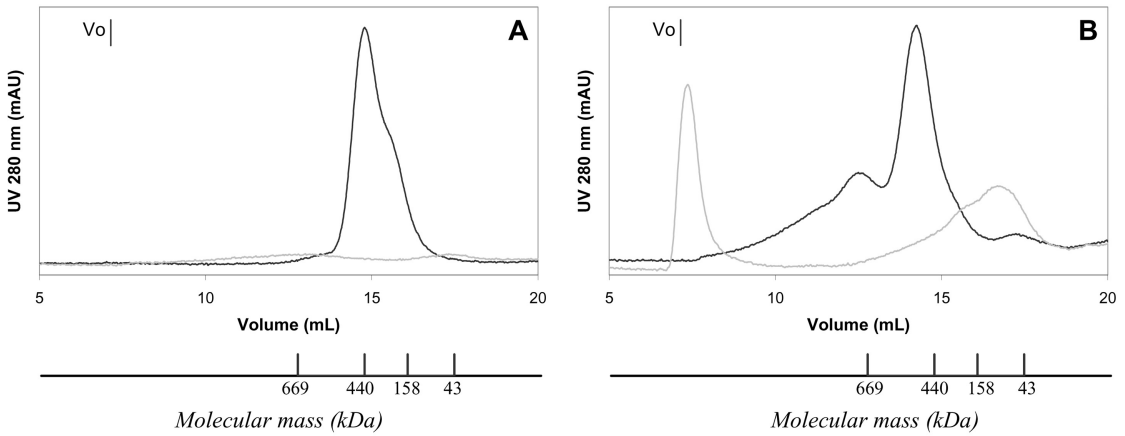

Figure 2. Size exclusion chromatograms of (A) Ara h 3 and (B) glycinin, before (black lines) and after (gray lines) heating at 100°C at 0.2 M. Indicated is the void volume of the column (Vo).

ubility is likely mainly caused by the insolubilization of the basic polypeptides upon heating [22].

Thus, despite their homology, Ara h 3 and glycinin differ in their aggregation behavior, as glycinin under the conditions applied formed (partly) soluble aggregates, while Ara h 3 only formed insoluble precipitates. Furthermore, the dissociation behavior of glycinin upon heating was not observed for Ara h 3.

3.2 Pepsin digestion

An important characteristic of food allergens is their stability to digestion, *i. e.*, the withstanding of IgE binding after pepsin digestion. Pepsin digestibility is often studied for food allergens under stomach conditions (pH 2.0 and 37°C). The ratios of pepsin to protein concentration that are commonly used in nutritional studies, as well as in studies concerning the effects of proteolytic digestion on protein allergenicity, generally range between 0.1 and 0.001 w/w [23]. We investigated the digestibility of Ara h 3 and glycinin before and after heating and used a pepsin to protein concentration ratio of 0.002 w/w. Peanut allergen Ara h 1 was digested under the same conditions, in order to verify if, under the conditions applied, this allergen would still be IgE reactive after digestion, as is described in literature [16].

The parental proteins and the hydrolyzates obtained upon peptic digestion were analyzed on SEC under denaturing and reducing conditions, as shown in Figs. 3–5. As it can be seen, at $t = 0$ the unheated and heated Ara h 3 and glycinin preparations showed comparable elution patterns, with two large peaks eluting directly after the void volume of the column. The peaks are comparable to the ones corresponding to the basic and acidic polypeptides of glycinin when analyzed on a similar column [24]. The small peak eluting at approximately 7.5 mL probably represents degradation products or smaller parts of the protein remaining after post-translational processing [6].

After only 10 min of pepsin hydrolysis for Ara h 3 almost all protein had been degraded into peptides and after 60 min of hydrolysis peptides with molecular masses lower

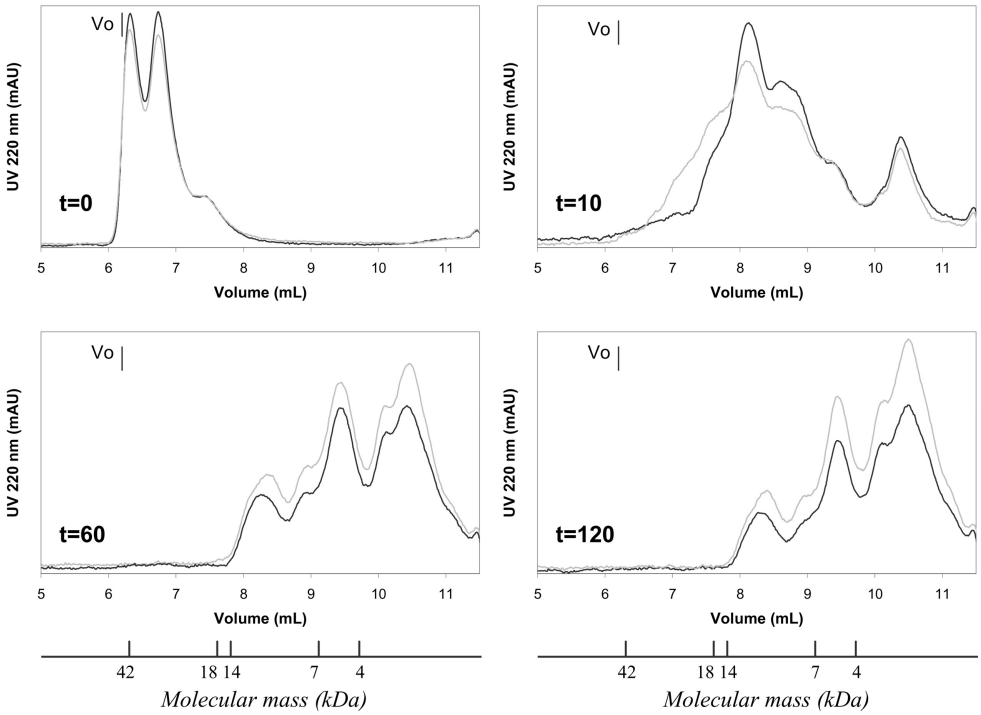

Figure 3. Size exclusion chromatograms under reducing and denaturing conditions of Ara h 3 samples after pepsin hydrolysis during different time intervals. Black lines: unheated Ara h 3; gray lines: heated Ara h 3. Indicated is the void volume (Vo) of the column and the hydrolysis time (in minutes).

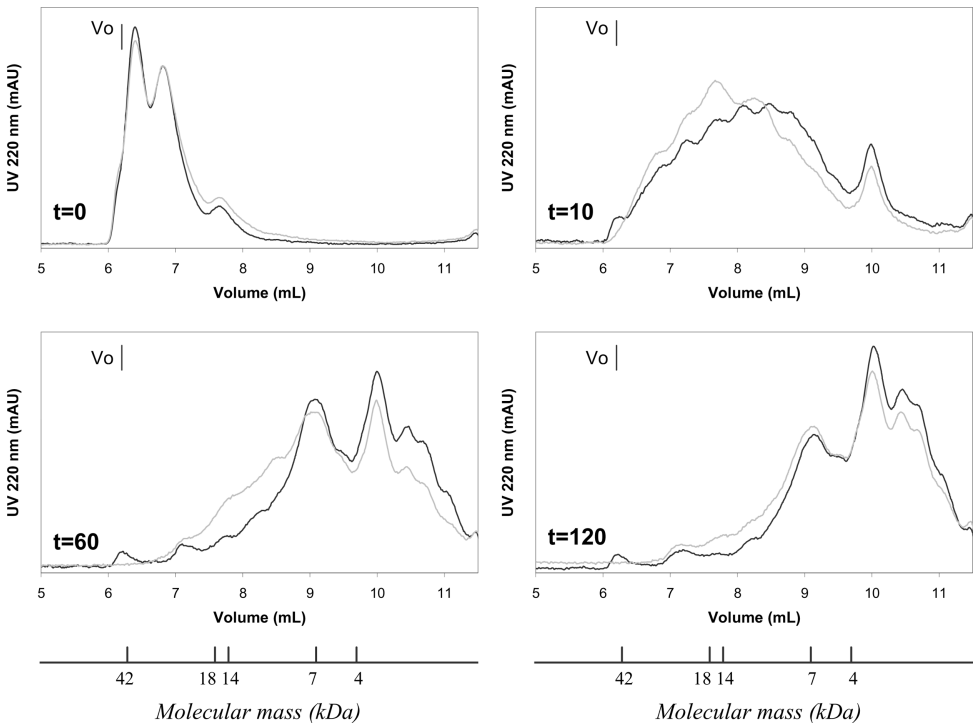

Figure 4. SEC chromatograms under reducing and denaturing conditions of glycinin samples after pepsin hydrolysis during different time intervals. Black lines: unheated glycinin; gray lines: heated glycinin. Indicated is the void volume (Vo) of the column and the hydrolysis time (in minutes).

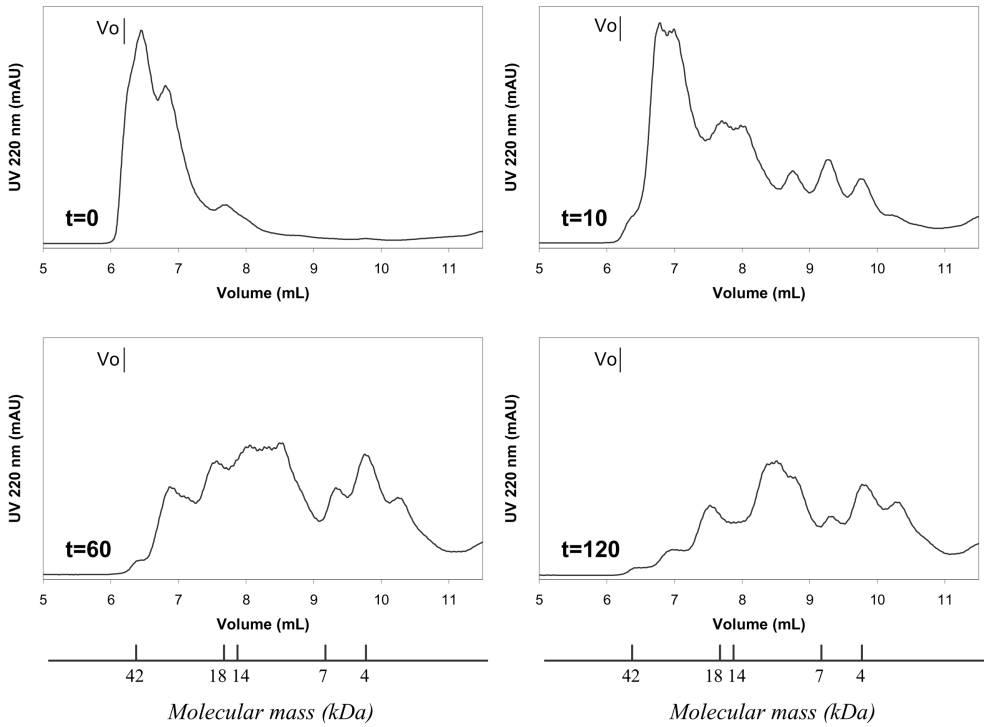

Figure 5. Size exclusion chromatograms chromatograms under reducing and denaturing conditions of unheated Ara h 1 samples after pepsin hydrolysis during different time intervals. Indicated is the void volume (Vo) of the column and the hydrolysis time (in minutes).

than approximately 9 kDa were obtained. The SEC pattern after 120 min of hydrolysis, which is an average gastric transit time [25], was similar to the SEC pattern after 60 min of hydrolysis, indicating that the peptides remaining after 60 min of hydrolysis were persistent.

After 10 min of hydrolysis heated Ara h 3 contained a larger proportion of relatively high molecular weight peptides than unheated Ara h 3. These results indicate that heating of Ara h 3 decreases the rate of pepsin hydrolysis. However, after hydrolysis times of 20 min and longer, no differences in degradation patterns could be observed any more (no further data shown), indicating that this effect was minor.

For glycinin after 10 min of digestion peptides with higher molecular masses were detected than for Ara h 3. After 2 h of hydrolysis peptides with molecular masses lower than approximately 25 kDa were present, with most peptides having masses of approximately 5 kDa or lower. After 10 min of hydrolysis different elution patterns were also observed for heated and unheated glycinin, with the pattern for heated glycinin showing relatively more high molecular weight peptides compared with the pattern for unheated glycinin. Thus, for glycinin it was also shown that heating decreased the rate of pepsin hydrolysis, as was also observed for Ara h 3. Moreover, for glycinin differences in peptide sizes were not only observed after 10 min of hydrolysis, but until up to 60 min of hydrolysis (no further data shown). This indicates that the effect of heating on the hydrolysis rate of pepsin is more pronounced for glycinin than for Ara h 3.

The digestion of the reference material, unheated Ara h 1, resulted in a slower degradation of the protein than the digestion of Ara h 3 and glycinin: After 10 min of hydrolysis a relatively large peak corresponding to the intact protein was still present. After prolonged (>10 min) digestion this peak further decreased, and peptides of lower molecular masses were formed. These peptides, in comparison with the peptides formed upon digestion of Ara h 3 and glycinin, had relatively high molecular masses, as they were approximately 28 kDa or lower. The digestion of Ara h 1 thus appeared to be slower than the digestion of Ara h 3 and glycinin. Besides, at similar conditions, the digestion of Ara h 1 resulted in peptides with higher molecular masses than the peptides obtained after hydrolysis of Ara h 3 and glycinin. The peptic digestion of Ara h 1 as described by Eiwegger *et al*. [26] resulted in a faster degradation of native Ara h 1 into peptides. However, the activity of pepsin they used (approximately 162 units *per* mg protein) was much higher than the pepsin activity that was used in the present study (approximately 6.5 units *per* mg protein).

3.3 IgE binding

Ara h 3 and glycinin and their hydrolyzates were investigated for their IgE binding by immunoblotting under reduc-

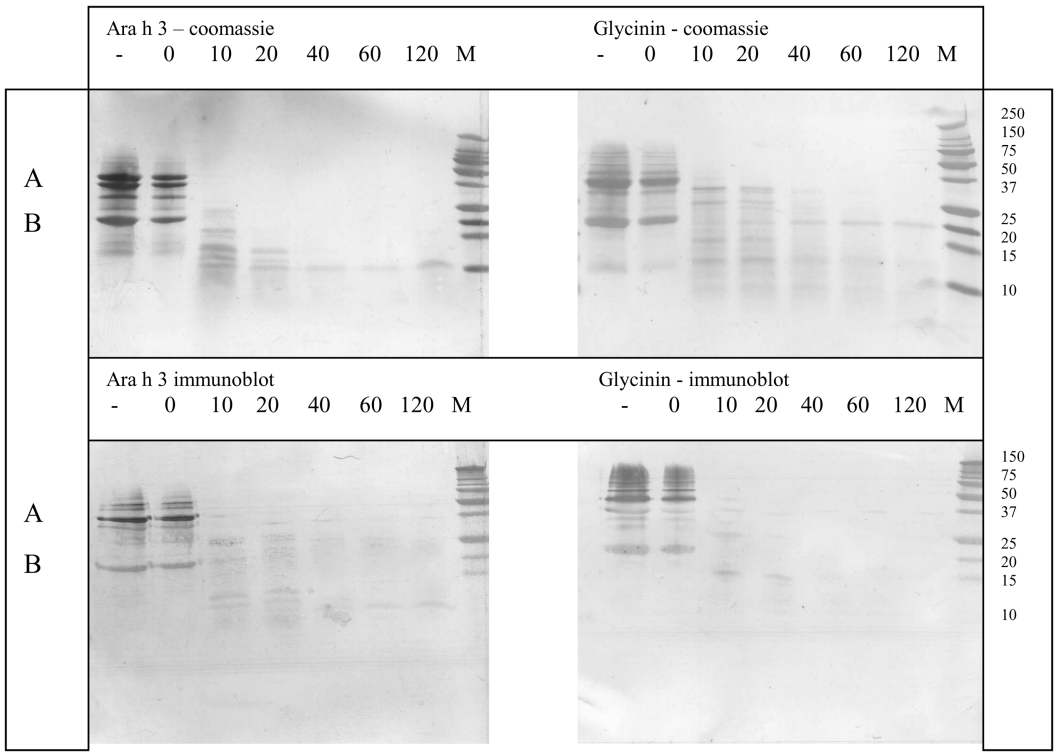

Figure 6. SDS-PAGE and IgE-immunoblots of Ara h 3 and glycinin. Indicated is the time of incubation of the samples in minutes (–, represents samples not subjected to stomach conditions). M = molecular weight marker, indicated right in kDa. Acidic (A) and basic (B) polypeptides are indicated left.

ing and denaturing conditions. The results for the unheated and heated parental proteins appeared to be similar, indicating that heating of Ara h 3 and glycinin did not cause changes in the IgE binding capacity of the allergens. Moreover, the results for the hydrolyzed heated and unheated samples were also comparable. Therefore, in Fig. 6 only the results for the heated samples are shown. As it can be seen, samples before subjecting to stomach conditions and samples being subjected to stomach conditions without pepsin incubation ($t = 0$) clearly showed IgE binding. As similar patterns were observed it can be concluded that the acidic pH of the stomach *per se* does not have an influence on the IgE binding of the legumin proteins. The IgE binding to both proteins was observed to both types of polypeptides, with the acidic polypeptide showing the most intense color, indicating the highest amount of IgE bound.

All Ara h 3 and glycinin samples being subjected to peptic hydrolysis did not to bind IgE, *i.e.*, a color similar to nonspecific binding was observed. This was confirmed by dotblot experiments (no further data shown). In contrast to this, all hydrolyzates of Ara h 1, when analyzed by dotblot, clearly showed IgE binding (no further data shown). Thus, according to our results, the IgE binding capacities of the legumin proteins Ara h 3 and glycinin are pepsin-labile. Although heating (slightly) decreased the pepsin digestion rate of both proteins, heating did not affect the IgE binding capacity of the hydrolyzates. These outcomes could imply that legumin allergens Ara h 3 and glycinin are not able to sensitize persons *via* the gastro-intestinal tract and, consequently, are not able to cause systemic food allergy symptoms.

The fast decline of IgE binding during pepsin hydrolysis is not common for known food allergens. As was shown in the present study, peanut allergen Ara h 1 was still able to bind IgE after pepsin digestion using equal pepsin concentrations. These results are in agreement with those reported in literature [16]. In addition, next to Ara h 1 peanut allergen Ara h 2 is also known to still be able to bind IgE after pepsin digestion [27]. These allergens, therefore, are more likely to cause systemic peanut allergic reactions than Ara h 3. In soybeans several other allergens have been identified. The digestibility of these allergens is not known. However, the absence of severe systemic allergic reactions to soybeans coincide with our results on the rapid hydrolysis and loss of IgE binding of glycinin.

4 Concluding remarks

In conclusion, in this study we have shown that the major seed storage proteins from peanuts and soybeans, legumin proteins Ara h 3, and glycinin, do not maintain their IgE

binding properties during digestion by pepsin. Heating the proteins prior to digestion only increased the pepsin stability of the proteins to a limited extent, but this did not have effects on the IgE binding of the remaining peptides. As the preservation of IgE binding capacity upon digestion is considered a prerequisite for a protein to sensitize *via* the gastro-intestinal tract and to exert systemic food allergic reactions, these legumin allergens could be not such important food allergens as was previously assumed.

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The authors have declared no conflict of interest.

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