

Original article

Peanut allergen Ara h 3: Isolation from peanuts and biochemical characterization

Background: Peanut allergen Ara h 3 has been the subject of investigation for the last few years. The reported data strongly depend on recombinant Ara h 3, since a purification protocol for Ara h 3 from peanuts was not available.

Methods: Peanut allergen Ara h 3 (glycinin), was purified and its posttranslational processing was investigated. Its allergenic properties were determined by studying IgE binding characteristics of the purified protein.

Results: Ara h 3 consists of a series of polypeptides ranging from approximately 14 to 45 kDa that can be classified as acidic and basic subunits, similar to the subunit organization of soy glycinin. N-terminal sequences of the individual polypeptides were determined, and using the cDNA deduced amino-acid sequence, the organization into subunits was explained by revealing posttranslational processing of the different polypeptides. IgE-binding properties of Ara h 3 were investigated using direct elisa and Western blotting with sera from peanut-allergic individuals. The basic subunits, and to a lesser extent the acidic subunits, bind IgE and may act as allergenic peptides.

Conclusions: We conclude that peanut-derived Ara h 3, in contrast to earlier reported recombinant Ara h 3, resembles, to a large extent, the molecular organization typical for proteins from the glycinin family. Furthermore, posttranslational processing of Ara h 3 affects the IgE-binding properties and is therefore an essential subject of study for research on the allergenicity of Ara h 3.

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Peanut allergy is one of the most severe food allergies due to its persistency and life-threatening character (1). The prevalence of peanut allergy in the Western world has been estimated at from 1 in 10 000 up to 1 in 200 and seems to be increasing during the last decade (2). The nature of the allergenic compounds in peanuts has been studied extensively. Peanut-1 was the first allergen described, but its biochemical characterization was, however, limited (3). Ara h 1 was identified as a major peanut allergen (4, 5) of 63 kDa and has gained a considerable amount of attention in recent years (6–8). Ara h 2 was identified as a second important peanut allergen (5, 9, 10), with a molecular weight of 17–20 kDa. Ara h 3 was in the first instance identified by Eigenmann and co-workers as a 14 kDa peanut protein (11), but cloning its gene revealed a protein of approximately 60 kDa identified as peanut glycinin with IgE binding sites throughout the gene product (12). This suggests that

the gene product is present in the peanut in a processed form, similar to the situation for Ara h 1 (5). Meanwhile, Kleber-Janke et al. described an identical peanut allergen, and the official name given by the IUIS Allergen Nomenclature Sub-Committee was Ara h 4 (10). Ara h 3 and Ara h 4 are considered to be the same allergen, and are in this paper referred to as Ara h 3.

Molecular biology is a powerful tool in investigating the allergic nature of peanut proteins since most of the IgE epitope mapping has been performed with synthetic peptides based on the amino acid sequences found via their genes. However, a disadvantage of using fragments of recombinant allergens or synthetic peptides is that IgE binding depends on polypeptides that are not necessarily representing the native allergens as they are found in the peanut. Therefore, the physiological relevance of IgE epitopes identified in this way is not undisputed. Post-translational modifications such as proteolytic processing

have been reported for storage proteins from the *Leguminosae* and may affect IgE binding (5, 13, 14). The aim of this study was to purify Ara h 3 from peanuts and to investigate its biochemical characteristics and IgE-binding properties.

Materials and methods

Peanut protein extraction

Peanuts (*Arachis Hypogaea*) from the Runner cultivar (Cargill, Dawson, GA) were generously provided by Imko Nut Products (Doetinchem, The Netherlands) and were stored at 10°C until use. Peanuts were ground and protein extracts were made by mixing 25 g ground peanut with 250 ml 20 mM Tris buffer (pH 7.2). After 2 h stirring at room temperature, the aqueous fraction was collected by centrifugation (3000 g, at room temperature for 30 min). The aqueous phase was subsequently centrifuged (10 000 g at room temperature for 30 min) to remove residual traces of fat and insoluble particles. Protein concentrations were determined using Bradford analysis with BSA as a standard. Reducing SDS-PAGE from the extracts showed protein bands between 14 and approximately 100 kDa (not shown). Extracts (crude peanut extract, CPE) contained typically 26 mg/ml protein and were stored at -20°C.

Purification of Ara h 3

Peanut allergens were purified by ion-exchange chromatography as described before for Ara h 1 (4, 5) and Ara h 2 (5, 9). Briefly, 60 ml peanut protein extract representing approximately 1.5 g peanut protein was applied on a 300 ml Source Q column (FPLC/AKTA protein purification system, Pharmacia, Uppsala, Sweden) previously equilibrated with 20 mM MOPS of pH 7.2 (loading buffer) at room temperature. The column was washed with loading buffer until the A_{280} of the effluent was less than 0.02. Proteins were eluted using a linear sodium chloride gradient in loading buffer (up to 1 M in 4000 ml at a flow rate of 100 ml/min). Fractions were collected and analyzed on SDS-PAGE. Fractions at 400–500 mM sodium chloride showed on SDS-PAGE a multiple band pattern that was identical in 10 consecutive fractions, indicating that the observed polypeptide chains were associated. These fractions were pooled and N-terminal sequencing of each individual band revealed that all polypeptides originated from Ara h 3 (see Table 1). Ara h 3 concentrations were determined by absorbance measurement (A_{280}

0.1% (1 mg/ml) = 0.6). Further purification steps were omitted in order to preserve the native character of Ara h 3.

Isolation of Ara h 3 subunits

Ara h 3 was concentrated to 25 mg/ml by centrifugation in a Centriprep microconcentrator (Millipore, 30 kDa cut-off membrane). Subsequently, the concentrated Ara h 3 solution was diluted in 8 M urea (final concentration 6 M) and disulfide bonds were reduced by dithiothreitol (DTT, end concentration 20 mM). The solution was buffered at pH 6.5 with 10 mM potassium phosphate buffer. After incubation at room temperature for 30 min., the unfolded reduced Ara h 3 was applied (5 mg) to a mini Q anion-exchange column (4.6 mM × 50 mM) equilibrated with 10 mM potassium phosphate buffer pH 6.5 containing 6 M urea. After loading and washing, the unbound protein was collected and the bound protein fraction was eluted with a linear salt gradient (15 ml, 0–1 M NaCl) in the same buffer and was also collected. The eluent was monitored at 280 nm. Collected fractions were separated by SDS-PAGE (12% acrylamide) and stained with Commassie Brilliant Blue (R-250).

Patient sera

Sera from 32 adult patients with a documented peanut allergy were collected. Each of these individuals had a positive skin prick test to peanuts and a convincing history of peanut allergy. Both non-allergic and allergic (but not peanut allergic) individuals were used as controls. Venous blood was withdrawn from the individuals and allowed to clot. Serum was collected by centrifugation and stored in aliquots at -20°C until use. The presence of IgE in the serum specific for peanut allergens was demonstrated by CAP FEIA (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden), and by SDS-PAGE in combination with immunoblotting. Patient sera that reacted with bands from Ara h 3 were used for further studies. All studies were approved by the Medical and Ethical Committee of the University Medical Centre Utrecht (Utrecht, the Netherlands) and patients gave written informed consent.

SDS-PAGE and immunoblotting

SDS-PAGE was performed essentially according to Laemmli (15) using a BioRad Mini Protean II system (BioRad, Hercules, CA) with 15% acrylamide gels (15 cm × 10 cm). Pre-stained molecular weight markers with molecular weights of 14.3, 21.5, 30, 46, 66, 97.4

Table 1. N-terminal sequences of Ara h 1, Ara h 2 and Ara h 3

Description	M_w of polypeptide (kDa)*	N-terminal sequence	Alignment (amino acid residue)	Identity	Reference
Ara h 1	63	R S P P G E R T G R	86–95	10/10	Burks et al. (6)
			1–10	9/10	Buschmann et al. (20)
			1–10	10/10	de Jong et al. (5)
Ara h 2	20	R Q Q W E L Q G G D R	1–11	11/11	Stanley et al. (28)
	17	R Q Q W E L	1–6	6/6	
Ara h 3	45	I S F R Q	1–5	5/5	Rabjohn et al. (12)
	42	I S F R Q Q	1–6	6/6	
	28	E E D Q	311–314	3/4	
	25	G I E E T I	326–331	6/6	
	16	R E F S	217–220	4/4	
	14	I S F R Q	1–5	5/5	
			1–5	5/5	Burks et al. (19)

* M_w were estimated based on SDS-PAGE results.

and 220 kDa were used as reference. Samples were mixed in a 1 : 1 ratio with 63 mM Tris buffer (pH 6.8) containing 1% (w/v) dithiotreitol (DTT), 2% (w/v) SDS, 0.01% (w/v) bromophenol blue and 20% (v/v) glycerol and were subsequently boiled for 5 min. Gels were stained with Coomassie Brilliant Blue R-250 and dissolved in a de-staining solution (10% HAc (v/v), 5% methanol (v/v) in water). After de-staining, gels were scanned with an ImageMaster DTS (Pharmacia, Uppsala, Sweden). To study the immuno-reactivity of the proteins, SDS-PAGE gels were prepared as described above and the separated proteins were transferred to polyvinylidene difluoride sheets (Immobilon-P, Millipore Corp. Bedford, MA) generally as described by Towbin (16). Membranes were blocked with 3% BSA in wash buffer (50 mM Tris, pH 7.5, containing 0.1% (w/v) BSA and 0.1% (w/v) Tween 20) for 1 h at room temperature. Patient serum was diluted 1/10 in wash buffer and applied on the membrane (overnight at room temperature). Bound IgE was detected using a commercial anti-human IgE conjugated to peroxidase (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) and a subsequent staining reaction for peroxidase activity using the ECL technique. Between each step, blot membranes were washed thoroughly with washing buffer five times. Blots were dried and scanned with an ImageMaster DTS (Pharmacia, Uppsala, Sweden). Non-specific binding of the anti-IgE antibody conjugate was measured in a similar blotting procedure omitting the incubation step with patient sera and appeared to be negligible.

ELISA techniques

Elisa techniques were based on an earlier report of our group on Ara h 1-IgE interaction (8). Ninety-six-well plates were coated with 10 µg/ml Ara h 3 using PBS as coating buffer. Subsequently, wells were blocked with 1% (w/v) BSA (in PBS containing 0.1% (w/v) Tween 20, blocking buffer) to diminish the nonspecific binding. Patient sera were diluted in blocking buffer and serum dilutions were incubated on the coated wells for 2 h at 37°C. Bound IgE was detected using an anti-human IgE antibody conjugated to horse radish peroxidase (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) and was expressed as A₄₉₀. Between each step, plates were washed five times with PBS containing 0.1% Tween 20. Nonspecific binding was studied using serum from a nonallergic individuals as well as from an allergic (but not peanut allergic) individual, and appeared to be negligible. Uncoated plates incubated with peanut-allergic patient serum did not bind IgE either, indicating that nonspecific binding of IgE to plastic is also negligible.

Size exclusion chromatography

Protein solutions (1 mg/ml) were dialyzed against the elution buffer (35 mM potassium phosphate buffer pH 7.6 containing 100 mM potassium chloride, I is 0.2 M) and applied to a Superdex 200 column (3.2 mM × 300 mm) running on a SMART microLC system (Pharmacia) at 50 µl/min. The eluent was monitored at 280 nm. The column was calibrated using thyroglobulin (669 kDa), ferritin (440 kDa), catalase (242 kDa), aldolase (158 kDa) and serum albumin (67 kDa).

Differential scanning calorimetry

DSC thermograms were recorded on a micro DSC III (Setaram, Caluire, France) using 0.9 ml vessels. Ara h 3 (0.9 ml, 2 mg/ml in 35 mM potassium phosphate buffer pH 7.6 containing 0.1 M KCl, I = 0.2) was heated from 20 to 115°C at a heating rate of 1 K/min and subsequently cooled to 20°C at the same rate. The transition

temperature is defined as the temperature at the minimum/maximum heat flow of the endothermic/exothermic transition.

Results

Purification of Ara h 3

Peanut allergens Ara h 1 and Ara h 2 were purified according to our earlier published method using anion-exchange chromatography (5). With this step, Ara h 3 eluted at a somewhat higher conductivity than Ara h 1. Figure 1 shows the chromatogram of the Source Q

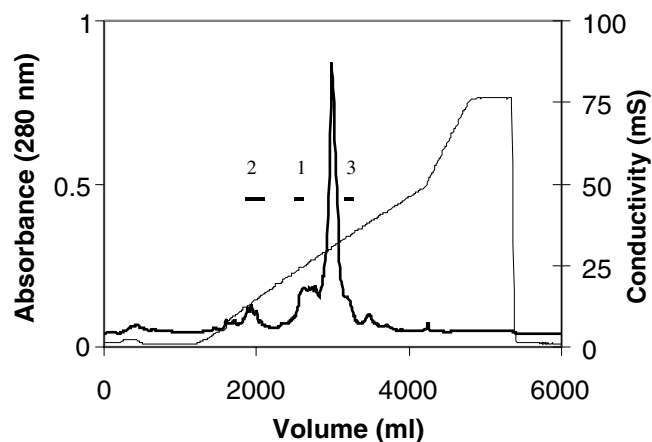


Figure 1. Elution pattern of peanut allergens by anion-exchange chromatography. Thick line: Absorbance (left Y-axis); thin line: conductivity (right Y-axis). Bars represent pools.

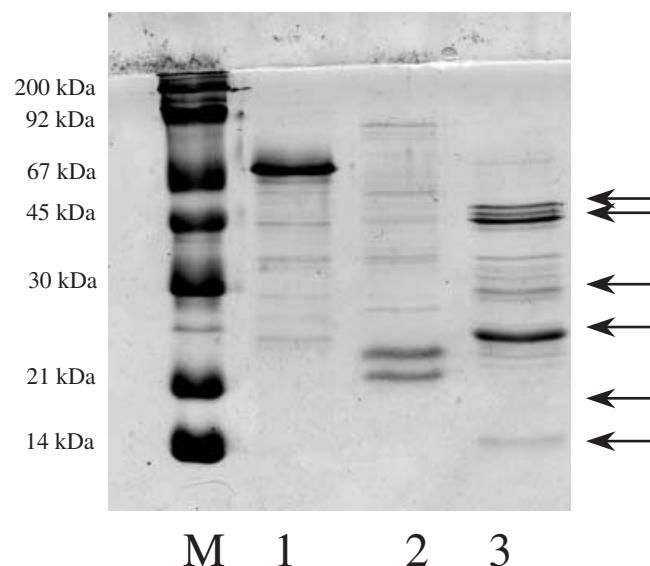


Figure 2. SDS-PAGE of peanut allergens. M: Molecular weight markers (indicated at the left); 1, pool 1; 2, pool 2; 3, pool 3. Arrows indicate the sequenced protein bands.

column with the bars indicating the pooled fractions where the different peanut allergens eluted. Figure 2 shows the SDS-PAGE of the different pooled fractions. Ara h 1 was found in pool 1 whereas pool 2 shows a doublet around 21 kDa, indicative for Ara h 2. Pool 3 contained a number of polypeptides and since the relative intensities of these bands were identical for 10 consecutive fractions, the polypeptides are likely associated to form one single hetero-multimeric protein. Pool 3 was subjected to a number of other purification steps under nonreducing conditions, i.e. cation-exchange chromatography, hydrophobic interaction chromatography and size exclusion chromatography, but none of these techniques resulted in a (further) purification of either polypeptide (not shown). Size exclusion chromatography indicated that the apparent molecular weight of the protein was around 400 kDa, suggesting a multimeric organization of the molecule. In order to assign the individual peptides, the protein bands were cut from a PVDF-blot membrane and their N-termini were sequenced. In some cases the amount of protein recovered in this way was insufficient for sequencing. The N-terminal sequences of six bands were determined and all appeared to be covered within the amino acid sequence translated from the DNA sequences of Ara h 3 published by others (10, 12) (for details see Table 1). The two largest polypeptides (45 and 42 kDa) as well as the 14 kDa polypeptide have an N-terminus identical to the recombinant gene-product described previously (10–12). The bands at 16, 25 and 28 kDa correspond to internal sequences starting at amino acids 217, 326 and 311, respectively. Taken together, these experiments demonstrate that Pool 3 contained a single protein with a hetero-multimeric structure.

Size exclusion chromatography

Because of the observed hetero-multimeric organization of Ara h 3 resulting in a M_w of approximately 400 kDa, we compared the size of Ara h 3 with soy glycinin and pea legumin, two other members of the glycinin family. Soy glycinin and pea legumin elute at an apparent MW of 430–440 kDa, indicating a hexameric 11S structure (Fig. 3). Ara h 3 eluted at a slightly higher elution volume corresponding to an apparent M_w of 360–380 kDa, which indicates an oligomeric structure very similar to glycinin and legumin: a hexameric 11S structure at neutral pH and an ionic strength of 0.2 M. The calculated molecular masses of the 11S storage proteins from legumes is 300–400 kDa (17), in line with the observed elution volumes. In the soy glycinin elution profile also a shoulder of aggregated glycinin is detected, which occurs upon freeze drying (C. Lakemond, personal communication). In the Ara h 3 preparation a small shoulder eluting at approximately 170 kDa can be observed possibly corresponding to the trimeric 7S form of the protein.

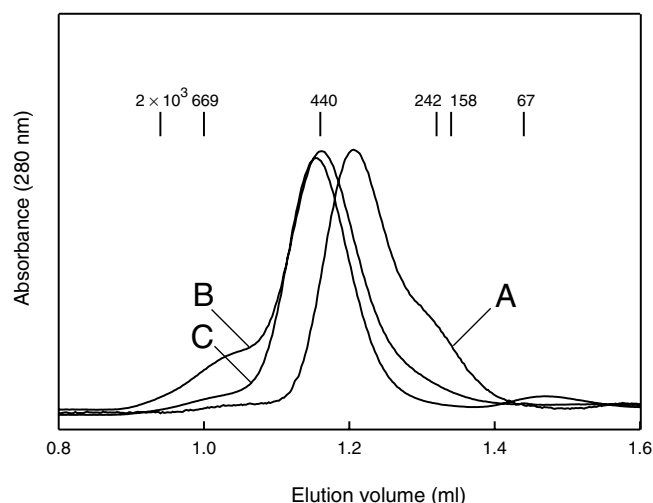


Figure 3. Size exclusion chromatography of glycinin molecules from different legumes. A, Ara h 3; B, soy glycinin; C, pea legumin. Numbers above indicate molecular weight markers (kDa).

Thermal unfolding behavior of Ara h 3

At pH 7.6 and ionic strength of 0.2 M the 11S form of Ara h 3 shows a thermal unfolding characterized by a T_m of 91°C. In addition to the endotherm an exothermic transition was also observed. This indicates aggregation following or coinciding with thermal denaturation. The thermal unfolding was irreversible.

IgE-binding tests

To prove that Ara h 3 purified from peanut is indeed an IgE binding protein, a direct IgE elisa was performed. Serum titers were assessed using elisa plates coated with Ara h 3. Figure 4 shows titration curves for patients 22, 35, 40, 44 and 48. Patients 22, 40 and 48 have high titers for Ara h 3 while patients 35 and 44 have lower titers. Control individuals (non-allergic and allergic but not peanut allergic) did not react in this assay ($A_{450} < 0.1$, not shown). Furthermore, plates without Ara h 3 coat did not bind any IgE from the peanut-allergic sera (data not shown). To further substantiate the IgE-binding properties of Ara h 3, and to investigate IgE-binding of the individual protein bands of Ara h 3, immunoblotting was performed. Figure 5 shows that patients 22 and 35 react intensely with Ara h 3, whereas patients 40, 44 and 48 show a moderate IgE-reactivity towards Ara h 3. Compared to the elisa, patients 40 and 48 react less intensively on immunoblot, whereas the IgE-binding for patient 35 is more pronounced in immunoblot. The Patients show a somewhat different specificity for the individual protein bands of Ara h 3. Patients 35, 40 and 48 react mainly with the N-terminal fragments (45, 42 and 14 kDa), whereas patients 22 and 44 show a more pronounced reactivity for

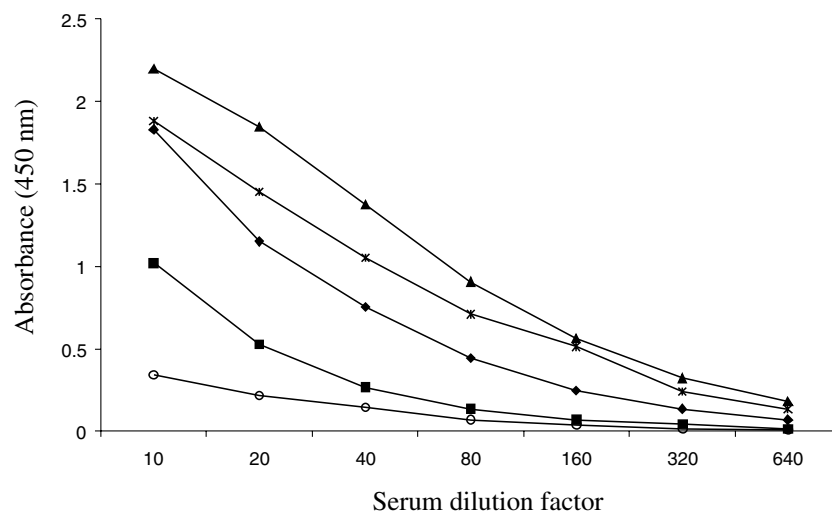


Figure 4. Direct IgE ELISA for Ara h 3 using individual sera from peanut-allergic patients. Triangles: Patient 40; crosses: Patient 48; diamonds: Patient 22; squares: Patient 35; circles: Patient 44.

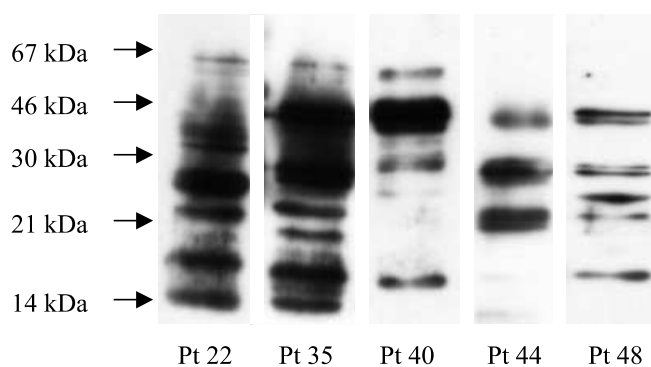


Figure 5. Western blot analysis. Western blot of Ara h 3 with individual patient sera, molecular weight markers are shown on the left.

the other fragments. It is interesting to note that patients 22, 35 and 40 also show some reactivity toward a 63 kDa protein band, probably a trace of Ara h 1 present in the Ara h 3 preparation as a minor contamination.

Isolation of Ara h 3 subunits

Upon unfolding in 6 M urea and reduction of disulfide bonds by DTT the Ara h 3 multimer dissociates into subunits. In Fig. 6A, the elution profile of reduced Ara h 3 in 6 M urea from an anion-exchange column is shown. Two major fractions can be distinguished: a non-bound fraction (fraction 1) and a fraction eluting during the salt gradient; the latter fraction was collected in four different subfractions (fractions 2–5). In Fig. 6B, the SDS-PAGE electrophoretic pattern of Ara h 3 and fractions 1–5 from the anion-exchange chromatogram are shown. The

unbound fraction 1 consists predominantly of a 25 kDa polypeptide which corresponds to the basic subunit since its pK_a is higher than the pH during separation (pH 6.5) as the polypeptide does not bind to the anion-exchange column. The SDS-PAGE electrophoretic pattern of the bound fractions is dominated by 42 and 45 kDa polypeptides corresponding to the acidic subunits since their pK_a is below the pH during separation. A similar subunit structure was described earlier for soy glycinin, another member of the glycinin family (18). The theoretical pK_a of the basic subunit was calculated from the amino acid sequence published by Rabjohn (12) using the web-based program ProtParam (<http://www.expasy.org/tools/protparam.html>), the calculated pK_a of residues 326–509 is 8.0. Conversely, the calculated pK_a of the acidic subunit, residues 1–325, is 5.3. This is consistent with the ion-exchange behavior of the isolated subunits and their N-terminal sequences (see Table 1, especially the 25 kDa polypeptide with N-terminal sequence GIEETI). In addition to the major subunits we also observe the smaller proteolytic fragments shown in Table 1, indicating that Ara h 3 undergoes further proteolytic processing, which is not observed for soy glycinin.

Discussion

In the last decade, a number of peanut allergens have been identified and characterized and the purification of Ara h 1 and Ara h 2 from peanuts has been reported. Using molecular biology, their genes have been cloned, and recombinant Ara h 1 and Ara h 2 became available. Ara h 3 was identified as a 14 kDa peanut allergen (11) and its N-terminal sequence was determined by Edmann

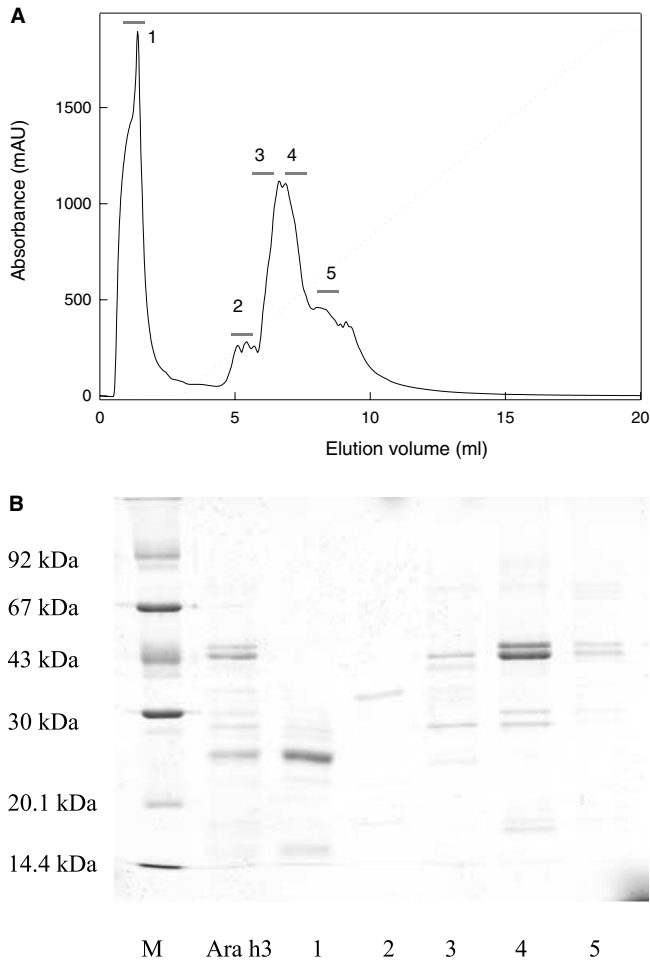


Figure 6. Separation of acidic and basic subunits of Ara h 3. Panel A: Ion-exchange chromatography of Ara h 3 under reducing and denaturing conditions. Line represents A_{280} ; dotted line represents salt gradient up to 1 M NaCl. Bars represent collected fractions (1, flow-through, unbound protein; 2–5, salt eluted fractions). Panel B: SDS-PAGE. M, molecular weight markers; Ara h 3; starting material before reduction, denaturation and chromatography; 1–5, collected fractions.

degradation after separation by SDS-PAGE (19). Recently, the gene for Ara h 3 was cloned, using degenerated cDNA probes based on the N-terminal sequence. This gene was expressed in a bacterial system as a 60 kDa IgE-binding protein (12). In an independent study, cDNA phage display techniques with IgE-binding as a positive selection criterion, resulted in a set of peanut allergens expressed as (His)6-tagged fusion proteins in *E. coli* (10). Both studies indicated a protein belonging to the glycinin family, and this protein is now designated as Ara h 3. It was remarkable that the molecular weights of both recombinant forms of Ara h 3 were 36 and 60 kDa, respectively (10, 12), whereas Ara h 3 as identified on immunoblots appeared to be 14 kDa (11, 19). In previous studies it has been speculated that these differences in

molecular weight were due to proteolytic cleavage (19). In this paper, we have isolated Ara h 3 from peanuts, and we provide its first biochemical and preliminary immun-chemical characterization.

Biochemical characterization of Ara h 3

Ara h 3 was purified as a hetero-multimeric protein with an apparent M_w of approximately 400 kDa. The subunits of which Ara h 3 is composed are associated under non-denaturing conditions and, based on their N-terminal sequences, all originate from one gene (10, 12). Their M_w varies from 14 to 45 kDa. Only after reduction and in the presence of chaotropic reagents, the subunits of Ara h 3 dissociate, and they can be fractionated using anion-exchange chromatography. The elution profile from such fractionation demonstrates the presence of acidic and basic subunits. The peptides of Ara h 3 identified in Table 1 have N-terminal sequences that align with the cDNA deduced amino acid sequence as published (10, 12). Furthermore, the 14, 42 and 45 kDa peptide have N-termini identical to the 14 kDa peanut allergen found by Eigenmann et al. (11) and Burks et al. (19). The fact that we find a number of 14–45 kDa peptides belonging to Ara h 3, whereas the predicted M_w of the gene product is around 60 kDa (10, 12) illustrates that peanut-derived Ara h 3 is proteolytically processed. This was earlier hypothesized by Burks et al. (19) who sought an explanation for the difference in molecular weight between Ara h 3 that was identified by Eigenmann et al. and the recombinant forms of Ara h 3. A similar observation was made earlier for another peanut allergen, Ara h 1, by Buschmann (20) and de Jong et al. (5), who showed that the N-terminus of peanut-derived Ara h 1 was found at position 85 of the amino acid sequence of recombinant Ara h 1. To exclude the possibility that the observed set of polypeptides was the result of proteolysis during the purification procedure, SDS-PAGE patterns of different stages of the purification procedure were compared. Fresh extracts of peanuts prepared in the presence of SDS, a condition that inhibits proteases, revealed the typical bands of Ara h 3 (14, 25, 42 and 45 kDa), and among others, a major band of 63 kDa (Ara h 1), and a doublet at 17–21 kDa (Ara h 2). The relative intensities of the Ara h 3 bands did not change as a function of the purification procedure (not shown). These data are in accordance with an earlier report on SDS-PAGE analysis of different peanut species (21), and indicate that the molecular organization of Ara h 3 is similar in different peanut species, including the extensive proteolytic processing.

Ara h 3 belongs to the glycinin storage protein family (10–12), and the presence of acidic and basic subunits can be explained according to the homology with soy glycinin, the best characterized protein of the glycinin family. Soy glycinin is composed of 30–36 kDa acidic and 20 kDa basic subunits (18) covalently linked by a

disulfide bond (22) into 55 kDa dimers of an acidic and a basic subunit. Three dimers form the 165 kDa trimeric 7S glycinin and two of these trimers fold into the characteristic 330 kDa hexameric 11S glycinin form (17, 23). Using the homology of the amino acid sequence of Ara h 3 to that of soy glycinin and the N-terminal sequences from Table 1 we expect that the peptide bond between N325 and G326 is proteolytically cleaved by an asparaginyl endopeptidase, analogous to soy glycinin. This gives rise to the major bands observed on SDS-PAGE: the 42–45 kDa acidic subunit and the 25 kDa basic subunit. Most likely the cysteines responsible for the disulfide bond covalently linking the acidic subunit (res 1–325) and the basic subunit (res 326–510) are cys 88 (acidic subunit) and cys 332 (basic subunit). Inspection of the Ara h 3 amino acid sequence revealed that the N-terminal 14 kDa proteolytic product (Table 1) is large enough to include cys 88 and is likely to be covalently bound to a basic subunit via a disulfide bond. The processing pattern of the 28 kDa peptide in Table 1 is more difficult to interpret. Alignment shows that the peptide starts at amino acid 311, which is near the acidic/basic subunit site of cleavage (325–326), indicating incomplete processing of the Ara h 3 preprotein. The calculated molecular mass of the peptide encompassing residues 311–510 is 23 kDa, which is in line with the observed mass of approximately 28 kDa, taking into account the accuracy of SDS-PAGE. On the other hand, the 16 kDa peptide starting at residue 217 corresponds to a molecular mass of 13 kDa when terminated at the acidic/basic cleavage point at amino acid 325, in line with the observed mass. In addition to the peptides identified in Table 1, minor components can also be distinguished indicating the presence of more proteolytic cleavage sites. Obviously, more work needs to be done in order to fully understand the posttranslational proteolysis of Ara h 3. It is clear, however, that the extent of proteolytic processing observed for Ara h 3 is higher than the single proteolytic cleavage site identified for glycinin. Additional experimental data that support the hypothesis that Ara h 3 adopts the structural organization of soy glycinin is obtained from size exclusion chromatography, which reveals an 11S behavior of Ara h 3. Furthermore, differential scanning calorimetry shows a considerable tertiary packing of Ara h 3, with a denaturation temperature of 91°C, comparable to the value reported for soy glycinin.

IgE binding of Ara h 3

The major IgE epitope as found in recombinant Ara h 3 at amino acid position 279–293 (12) is found in the large N-terminal fragments of 42 and 45 kDa. These bands represent an important part of Ara h 3 as judged by SDS-PAGE stained with Coomassie Brilliant Blue, and may therefore act as important IgE binding peptides of Ara h 3. The fact that a cleavage site is found in close proximity of this epitope, at amino acid position 311, may suggest that

this epitope is readily available for interaction with IgE. However, up to now there has been no information on the tertiary packing of Ara h 3 to support this hypothesis. It is furthermore interesting to note that the 14 kDa (N-terminal) peptide contains an IgE epitope (12), explaining why this peptide was recognized by serum from peanut allergic individuals (11). Since the IgE epitopes on Ara h 3 were mapped using recombinant protein and synthetic peptides it is valuable to investigate the peanut-derived Ara h 3 interactions with IgE from peanut allergic individuals.

From a Dutch population of 32 peanut allergic individuals, 12 patients (35%) clearly reacted with (peanut derived) Ara h 3 on immunoblot. This percentage is somewhat lower than described for recombinant Ara h 3 [44% (12) and 53% (10)]. This discrepancy may be explained by differences in patient population or by differences in biochemical characteristics of recombinant Ara h 3 compared to peanut-derived Ara h 3. The main IgE reactivity that we observed was directed towards the 45 and 42 kDa band (acidic subunits), whereas in other cases reactivity towards the 25 kDa band (basic subunit) was observed. This is in accordance with the epitope mapping of recombinant Ara h 3 because the 42 and 45 kDa peptides contain the major IgE epitope (279–293) whereas the C-terminal peptides of 25 and 28 kDa contain only a minority of IgE epitopes (12). IgE epitopes on soy glycinin have been localized by various investigators in the past. The acidic subunit of soy glycinin was found to be the main antigenic and allergenic part of soy glycinin, as demonstrated with IgE-immunoblotting by Pedersen et al. (24) and Herian et al. (25). The basic subunits of soy glycinin do not contain IgE binding sites (25). A recent paper of Beardslee et al. (26) shows that IgE epitopes on the acidic subunit of soy glycinin are significantly homologous to the epitopes described for recombinant Ara h 3 (12) located on the Ara h 3 acidic subunits (this work). However, another recent report on the IgE-binding of soy glycinin showed that the major IgE epitope is located on the basic subunit (27). Differences between these observations for soy glycinin are thought to originate from differences in patient serum (27). Taken together, there is evidence for IgE epitopes on both the acidic and basic soy glycinin subunits, with the acidic subunits being more often recognized by IgE. For peanut Ara h 3 this emphasizes that the 14 kDa polypeptide earlier identified as Ara h 3 (11) is not necessarily the only protein responsible for the allergenicity of Ara h 3. Our observations that for Ara h 3 both the acidic and to a lesser extent the basic subunits constitute IgE binding sites is in agreement with the reports on soy glycinin. Further research on the allergenicity of Ara h 3 using, for example, *in vitro* basophil-histamine release tests or *in vivo* skin prick test is necessary to investigate the relevance of the IgE-binding properties of the acidic and basic subunits for peanut allergic patients.

Conclusions

We conclude that Ara h 3 purified from peanuts is a posttranslationally processed IgE-binding protein consisting of basic and acidic subunits with molecular weights ranging from 14 to 45 kDa. The quaternary structure of Ara h 3 derived from peanuts resembles to a large extent that from glycinin proteins of the *Leguminosae* family. The posttranslational processing of Ara h 3 is an essential subject of study for research on the aller-

genicity of Ara h 3. The availability of a method to purify Ara h 3 from peanuts opens a new route for the in-depth characterization of this peanut antigen.

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