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Identification of a New Natural Ara h 6 Isoform and of Its Proteolytic Product as Major Allergens in Peanut

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Numerous food allergens of plant origin belong to the 2S albumin family, including peanut Ara h 2. In addition to Ara h 2, several other conglutins related to 2S albumins are present in peanut seeds. We evaluated the allergenicity of different peanut conglutins as compared with Ara h 2. Several conglutins were isolated from the kernel, i.e. Ara h 2, a new isoform of Ara h 6 and its derived product, which is likely to be naturally formed during seed processing. Enzyme allergosorbent tests performed on sera of peanut allergic patients showed that more than 94% of 47 analyzed patients had positive IgE responses to Ara h 6 isoform and to its degradation product. Skin prick tests with the new isoform of Ara h 6 led to a positive response in seven out of the eight tested patients. Both enzyme allergosorbent tests and skin prick tests showed that the reactivity of Ara h 6 was similar to, or even higher than, that of Ara h 2, suggesting that the present isoform of Ara h 6 is as allergenic as Ara h 2. In addition the IgE response to the plant processed (i.e., hydrolyzed) Ara h 6 new isoform is equivalent to the IgE response to the native isoform. The IgE immunoreactivity is mostly abrogated by chemical reduction and denaturation of Ara h 6 isoforms, which underlined the importance of tertiary structure in Ara h 6 immunoreactivity. These results, and particularly the high correlation between anti-Ara h 2 and anti-Ara h 6 IgE responses, emphasise the major role of 2S albumins in peanut allergenicity.

KEYWORDS: 2S Albumins; allergy; Ara h 6; IgE; peanut

INTRODUCTION

A majority of plant food allergens belongs to four protein families, the prolamin superfamily, the cupin superfamily, the profilins, and the homologues of the major birch pollen allergen (*1*). Biochemichal properties including thermal stability and resistance to proteolysis often characterize these allergens and are associated with structural features. Disulfide bridges and different post-translational modifications like phosphorylation, hydroxylation, or glycosylation contribute to their properties (*2*).

Since the identification of a major peanut allergen by Sachs et al. (*3*), many peanut allergens have been characterized, with most studies devoted to the three major ones, i.e. Ara h 1, Ara h 2, and Ara h 3 (*4*–*6*). Ara h 1 and Ara h 3 are members of the cupin superfamily and belong to mature 7S and 11S globulin families, respectively (*7*). Ara h 2, a member of the prolamin superfamily, can be related to 2S albumins (*8*). The 2S albumins correspond to proteins of low molecular weight, and their structure comprises the same motif of several disulfides bridges with cysteine residues in a specific pattern (*9*). Another characteristic feature of 2S albumins is a conserved 3D-domain including at least 4 α-helical structures. Most of the 2S albumins are observed as an association of two subunits that are separated after a degradation process. Numerous 2S albumins are known as major plant allergens (*10*), including Ara h 2 in peanut (*11*). In a previous study, we showed that an IgE response to Ara h 2 occurred in 77% of a French population of patients allergic to peanut (*12*).

The other 2S albumins that are present in peanut have been essentially produced by molecular biology techniques, and low IgE binding to recombinant Ara h 6 was reported (*8*). More recently, an isoform of Ara h 6 has been cloned in our laboratory. Its immunoreactivity was assessed by monoclonal antibody binding studies and compared with that of the natural form (*13*). We then demonstrated that the immunoreactivity of this recombinant Ara h 6 is highly dependent on correct refolding, further suggesting the interest of natural peanut proteins in assessing IgE binding to allergens.

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Here we purified different 2S albumins naturally present in the peanut kernel, and evaluated their allergenicity using in vitro and in vivo tests. Particularly we characterized a new isoform of Ara h 6. This protein appeared to be simultaneously present in the kernel in two forms (i) with its intact entire structure, (ii) as a heterodimeric protein resulting from a hydrolytic process which naturally occurs during seed maturation (called naturally or plant processed isoform).

MATERIALS AND METHODS

Allergic Population. The study population included 47 patients, mainly children (median age: 6 years) recruited at the Paediatric Allergy Clinic of the Hopital Necker-Enfants Malades (Paris, France). All the patients, 34 boys and 13 girls, had a confirmed peanut allergy based on an extensive history, physical examination, and objective manifestations observed after peanut ingestion. Numerous symptoms were reported involving skin, respiratory tract, gastrointestinal tract and cardiovascular system. Seventeen patients presented severe symptoms with anaphylactic shock, angio-oedema, or the involvement of three organ systems. Twenty-nine patients had mild–moderate reactions involving one or two organs. One patient (no. 2) had unconvincing manifestation of allergy to peanut.

Reagents and Materials. Unless otherwise stated, all reagents were of analytical grade from Sigma (St Louis, MO). Buffers and reagents including antihuman IgE monoclonal antibody (mAb BS17) labelled with acetylcholinesterase were prepared as previously described (*12*).

Solid-phase enzyme immunoassays were performed in 96-well microtitre plates (Nunc, Roskilde, Denmark) using automatic Titertek microtitration equipment (washer, dispenser, and reader) from Labsystem (Helsinki, Finland).

Preparation of Whole Peanut Protein Extract (WPPE). Protein extraction was performed using commercial roasted Virginian peanuts. Ninety grams of peanut kernels were ground with a mortar and pestle and further homogenized using an ultra Turrax apparatus. The ground peanuts were defatted using cold ether, and the pellet was dried under nitrogen stream at room temperature. The dried pellet was then suspended in 20 mM phosphate pH 7.4 buffer including 1 M NaCl (10% w/v) and a protease inhibitor cocktail (4-(2-aminoethyl) benzenesulphonyl fluoride, leupeptin, pepstatin A, chymostatin, benzamidine). After stirring for 18 h at 4 °C, the suspension was centrifuged at 4000*g* for 20 min at 4 °C. The supernatant was collected whereas the pellet was resuspended in 4 M urea. After stirring for 4 h at room temperature, the soluble fraction was separated by centrifugation and the second supernatant was collected and pooled with the previous one. Pooled extracts were dialysed against 20 mM phosphate pH 7.4 buffer before storing at −80 °C.

Protein Purification. Dialysed extract was fractionated by precipitation using ammonium sulphate, which was added to 40% saturation. After centrifugation, the pellet was discarded and the supernatant was dialysed against 20 mM phosphate pH 7.4 buffer. After addition of 0.5 M NaCl, the dialysate was submitted to affinity chromatography using Con A Sepharose. The flow-through fraction was dialysed against 20 mM Tris pH 7.4 buffer. After addition of 4 M urea, the solution was applied to a Source 30Q anion exchange column (2.6*10 cm, Pharmacia, Uppsala, Sweden). Proteins were separated by a stepwise elution (0% of eluent B for 20 min and then steps of 5, 10, 20, 30, 100% of Eluent B for each 60 min). Buffer A was 40 mM Tris pH 7.4 containing 4 M urea and buffer B corresponded to buffer A with addition of 1 M NaCl. Fractions of 20 mL were collected and purification was then achieved by RP-HPLC using a 300 Å C4 Vydac column (250 × 22 mm)and an AKTA purifier system (Pharmacia). Elution was performed using a 90 min linear gradient from o to 60% of solvent B (B: acetonitrile/TFA 0.04%) in solvent A (A: H_2O/TFA 0.1%) at a flow rate of 12 mL/min and was monitored at 235 and 280 nm.

Protein Characterization. Purified fractions were collected and freeze-dried. They were characterized by MALDI-TOF mass spectrometry (MS) using a Voyager DE RP apparatus (PE Biosystems). Mass spectrometry analysis was performed with and without reducing pretreatment using solubilization in 50 mM carbonate buffer pH 8.5 and DTT (Dithiothreitol) addition. Reduced sample was diluted in 0.2% TFA (Trifluoroacetic acid) solution just before mass spectrometry analysis. Characterization was completed by amino acid composition and N-terminal sequence determination using the Edman method.

In addition, purified fractions were identified using proteomic analysis. Purified fractions were analysed by electrophoresis on polyacrylamide gels (15%). Protein bands detected by Coomassie blue staining were excised and in gel digested using trypsin after reduction and alkylation according to the protocol proposed by the EMBL Bioanalytical Research Group (www.narrador.embl-heidelberg.de/). Peptide mass pattern was characterized by MALDI-TOF mass spectrometry and used to identify the protein in databases using Profound and Protein Prospector engines.

Enzyme Immunoassays. Specific IgE were determined in allergic children using the enzyme allergosorbent test (EAST) previously described (*12*).

Briefly microtiter plates were coated by passive adsorption with whole peanut protein extract or with purified peanut proteins at a concentration of 5 μg/mL in 25 mM EDTA/50 mM carbonate buffer pH 9.3. After 24 h incubation, the plates were washed and bovine serum albumin was added as saturating agent to avoid nonspecific binding. Plates were washed just before use. 50 μL of serial dilutions of each serum were dispensed per well and incubated for 24 h at +4 °C. After extensive washing, 50 μL of antihuman IgE (i.e., BS17 clone) conjugated to acetylcholinesterase were added per well. Following an overnight reaction at 4 °C, plates were extensively washed and 200 μL of Ellman's medium used as enzyme–substrate were dispensed into each well. Resulting absorbance was measured at 414 nm (*12*).

Controls were made using 20 sera from nonallergic patients, and one serum from a patient with cow's milk allergy.

The limit of detection, corresponding to the mean background value plus three standard deviations, was 0.1 International Unit (IU) of IgE per mL.

Skin Prick Tests. Skin prick tests (SPTs) using either whole peanut protein extract or purified and characterized fractions diluted to 50 μg/mL in 50% glycerol, were performed as previously described (*12*). Eight patients (i.e., 34, 41, 42, 43, 44, 45, 46, and 47) of the studied population presenting skin, gastrointestinal and/or respiratory symptoms underwent skin prick testing.

RESULTS

Protein Purification and Characterization. After successive steps of precipitation and affinity chromatography to discard the majority of glycinin polymers and Ara h 1 (*14*), peanut protein extract was fractionated using ion exchange chromatography (**Figure 1**). Based on protein quantity and SDS page analysis (data not shown), two peaks eluting at 0.1 M (Fraction I) and 0.2 M NaCl (Fraction II), respectively, were further studied.

RP-HPLC of fraction I showed a heterogeneous profile (**Figure 2**). MS analysis demonstrated that the peak eluted at a retention time (RT) of 47.3 min included two proteins of 16.67 and 18.05 kDa molecular weight (MW) corresponding to the isoforms of Ara h 2 we have previously reported (*14*). The peak at RT 53.2 min was characterised by a single molecular weight of 14.84 kDa (**Figure 3**).

RP-HPLC pattern of fraction II (**Figure 4**) was not different from that observed for fraction I. However, the protein with a 52.3 min RT had a molecular weight of 14.58 kDa (**Figure 5**). Other fractions containing various proteins with molecular weights ranging from 14.28 to 14.90 kDa were not further studied.

MALDI TOF analysis performed after reducing treatment revealed no significant change for the 14.84 kDa protein, whereas the 14.58 kDa protein disappeared and led to two different peaks at 9.14 and 5.44 kDa (**Figure 5**), suggesting

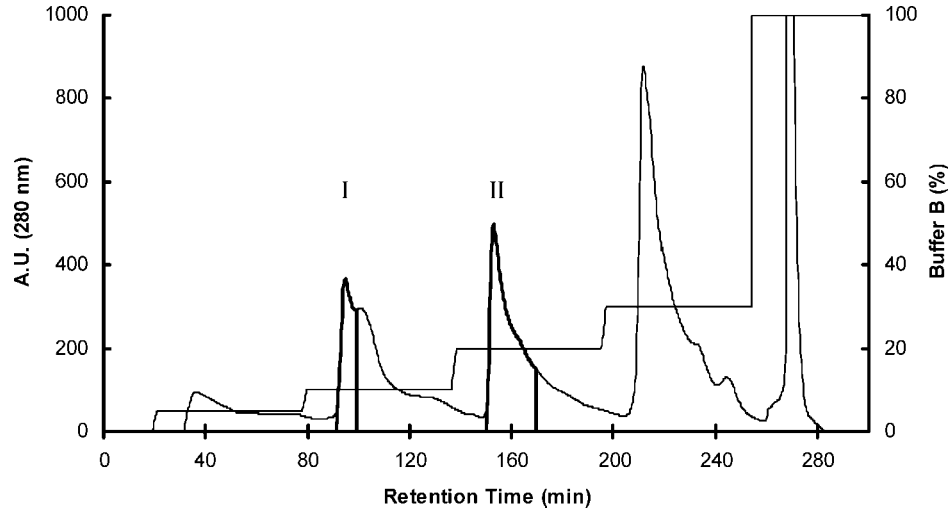


Figure 1. Anion exchange of peanut protein extract obtained after precipitation and affinity chromatography. The collected fraction eluted at 0.1 M (I) and 0.2 M (II) NaCl correspond to the framed area.

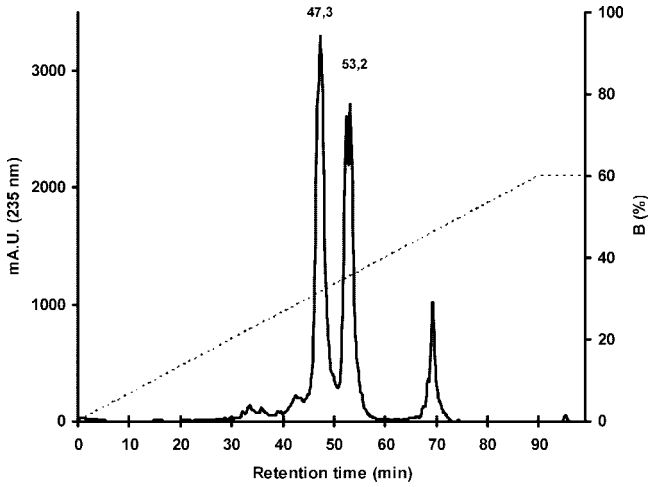


Figure 2. RP-HPLC chromatogram of fraction I. Peak of 47.3 min RT corresponds to Ara h 2.

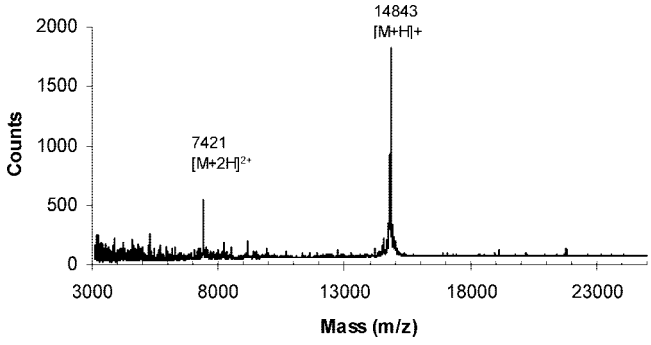


Figure 3. MALDI-TOF mass pattern of RP-HPLC fraction of 53.2 min RT.

that the purified entity corresponds to two polypeptides linked by disulfide bridges.

The N-terminal amino acid sequence analysis of 16.67 and 18.05 kDa MW proteins present in the RP-HPLC fraction eluted at 47.3 min RT revealed an unique sequence (i.e., RQQWELQGDR) matching the Ara h 2 sequence and confirming the identity of this purified entity.

The sequence analysis of the thirteen N-terminal amino acids (i.e., MRRERGRQGDSSS) of the 14.84 kDa protein demonstrated 100% identity with six mature forms of conglutins (i.e.,

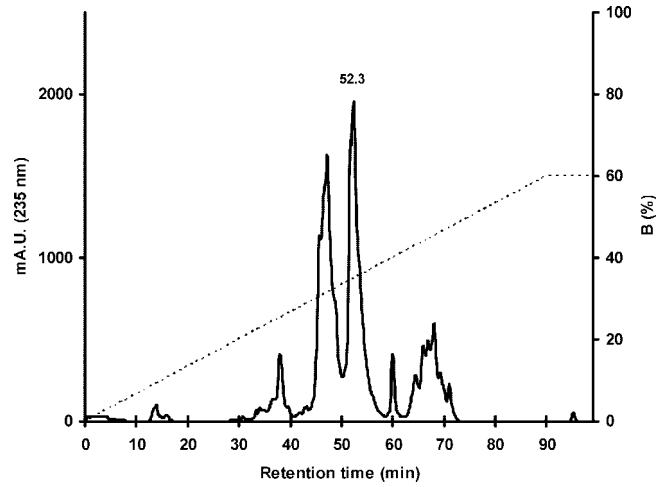


Figure 4. RP-HPLC chromatogram of fraction II.

after removal of the signal peptide) referenced in GenBank database with the following accession numbers AF092846, AY871100, AY848699, AY849314, AY722690, and AF366561.

However, the MW of our protein was higher than the mass deduced from the sequence of the recombinant protein originally described as Ara h 6 accession number AF092846 (*8*), calculated after removal of the signal peptide. Moreover, the amino acid composition of the mature form of this protein revealed fewer arginyl residues (10.5 versus 13.1%) and more glycinyl residues (7.3 versus 4.9%) than in our 14.84 kDa MW protein. This resulted in a tryptic peptide mass pattern quite different for both proteins. **Figure 6** shows the MS pattern obtained after tryptic hydrolysis of the 14.84 kDa protein. Identification of the peptides thus generated indicated a conglutin (Genbank accession number AF366561) that matched with more than 55% of the sequence. In fact, as shown in **Figure 7**, several sequences corresponding to Ara h 6 related conglutins have been referenced. With the exeption of the protein AF092846, most are similar with only few amino acid substitutions. Because of this high sequence homology, the tryptic peptide mass patterns of those proteins are very close. As an example, the only difference between proteins AF366561 and AY849314 is observed in the tryptic peptides CCDELDQMENTER (theoretical mass at 1698.624 taking into account the modifications of cysteines) for AF366561 and CCDELNEMENTQR (theoretical mass at 1697.644) for AY849314. Whereas our 14.84 kDa protein can be clearly

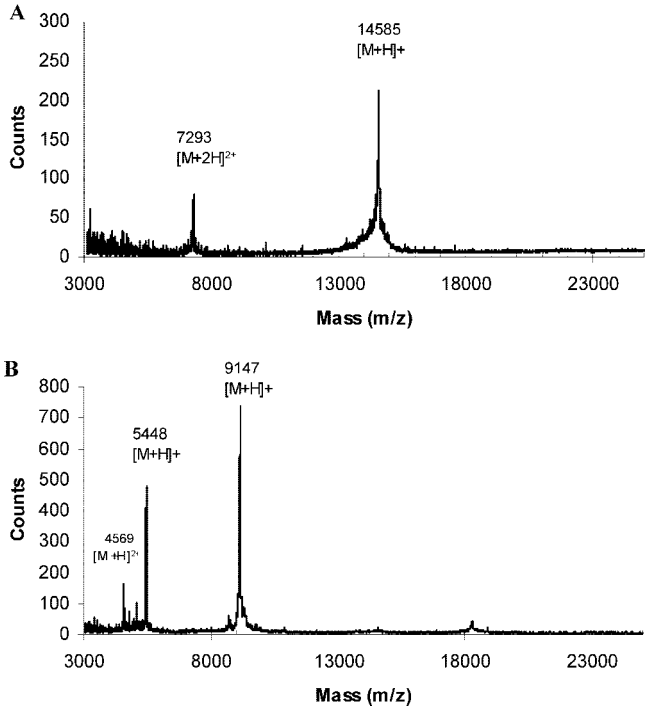
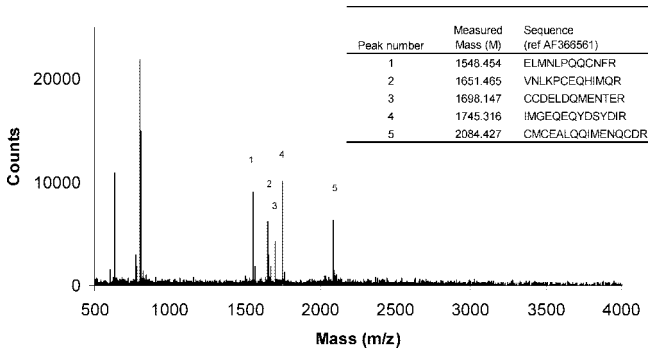


Figure 5. MALDI-TOF mass spectrometry patterns of RP-HPLC fraction with a retention time of 52.3 min. **A** in nonreducing conditions. **B** in reducing conditions.



Peak number	Measured Mass (M)	Sequence (ref AF366561)
1	1548.454	ELMNLPQQCNFR
2	1651.465	VNLKPCEQHIMQR
3	1698.147	CCDELDQMENTER
4	1745.316	IMGEQEQYDSYDIR
5	2084.427	CMCEALQQIMENQCDR

Figure 6. MALDI TOF MS pattern of tryptic digest from the 14.84 kDa MW protein. Identification of peptides using Profound software.

related to Ara h 6, it is difficult to distinguish whether it refers to the AF366561 or the AY849314 form.

The determination of the N-terminal aminoacid performed on the 14.58 kDa MW protein revealed 2 different amino acid residues (i.e., M and S). Further sequencing showed that one sequence matched the N-terminal part of the 14.84 kDa protein described above, while the other corresponded to an internal part of the protein (i.e., STRSSDQQ). The different results obtained including the occurrence of two N-terminal sequences confirm that the two polypeptides originated from the same protein and suggest that the 14.58 kDa protein corresponds to a naturally processed Ara h 6 isoform.

IgE Binding Studies. IgE binding studies were performed on purified Ara h 2, on the new Ara h 6 isoform and on its naturally processed derivative. For comparison IgE binding studies were also performed on whole peanut protein extract (WPPE).

Table 1 shows the huge variability of the IgE response of the patients to WPPE and to the purified allergens and the high frequency of positive response. More than 94% of the patients had IgE specific to each isolated protein.

Although the levels of specific IgE greatly differ depending upon the patients, it is noteworthy that IgE response to Ara h 2, Ara h 6 isoform, and plant processed Ara h 6 are correlated and vary in parallel to the response to WPPE. They are in the same order of magnitude for the majority of patients. However a few important differences were observed in patients 10 and 47 who presented an anti Ara h 2 IgE level twice higher than the anti Ara h 6 IgE levels. Conversely specific IgE levels of patients 5 and 6 were 2 times lower for Ara h 2 than for Ara h 6. Differences between specific IgE levels antinative Ara h 6 and antiplant processed Ara h 6 were generally weak and below 33% excepted for three patients who had an IgE response to the plant processed Ara h 6 that was 2-fold higher than to the native isoform. The mean and median values of the IgE responses to the native and the plant processed Ara h 6 isoform were at least as high as those to Ara h 2. It is noteworthy that the IgE response to WPPE is lower than the sum of the IgE responses to the purified allergens. This is due to the format ot the EAST where more allergens are available for IgE binding when pure allergens are directly adsorbed on the microplates than when a mixture of several proteins among which Ara h 2 and Ara h 6 are not the most abundant fractions is coated. This may also partly reflect an IgE cross reactivity of the three allergens.

To further analyse the specificity of the IgE response, native Ara h 6 isoform was reduced and carboxymethylated. Specific IgE levels to the native and the reduced Ara h 6 isoforms were compared using the first 40 sera of **Table 1**. We observed that 10 sera exhibited no significant IgE response to the reduced Ara h 6 isoform, whereas 19 other sera had a more than 4-fold lower IgE response to Ara h 6 after reduction. The last 11 sera had an IgE response to the reduced form between 26 and 60% of that to native Ara h 6.

Skin Prick Tests. Skin prick tests were performed on eight patients using whole peanut extract, Ara h 2, and native Ara h 6 isoform. Except for patient 44, all patients were positive to whole peanut protein extract, with a maximum wheal size ranging from 4 to 18 mm (**Table 2**). Seven patients reacted to native Ara h 6 isoform, while positive skin reactions to Ara h 2 were observed for all eight patients. Large wheal s were observed with native Ara h 6 isoform, whatever the age, sex, and symptoms of the patients. Wheal size observed with the Ara h2 and Ara h 6 isoforms was often closely related, but not directly correlated with specific IgE levels.

DISCUSSION

We have characterized two new Ara h 6 isoallergens in peanut. The isoallergen with a 14.84 kDa MW is a native form and it occurs together with its naturally processed isoform of 14.58 kDa MW in peanut. The plant processing is a proteolytic degradation that results in the loss of a dipeptide (i.e., IR) and the formation of two peptidic cleaved chains that remain associated by disulphide bonds. No other differences occur between the non processed and the processed isoform with regards to their amino acid sequence or post translational modifications.

Among the 2S albumins related proteins present in peanut, the allergenicity of the related 2S albumin Ara h 2 has been extensively described. Two other conglutins, i.e. Ara h 6 and Ara h 7, have been cloned (*8*). Since this report, five conglutins with structure close to Ara h 6 but differing by a few amino acid substitutions have been characterized by molecular biology. Recently, a natural protein identified as Ara h 6 by mass spectrometry and N-terminal amino acid sequencing has been

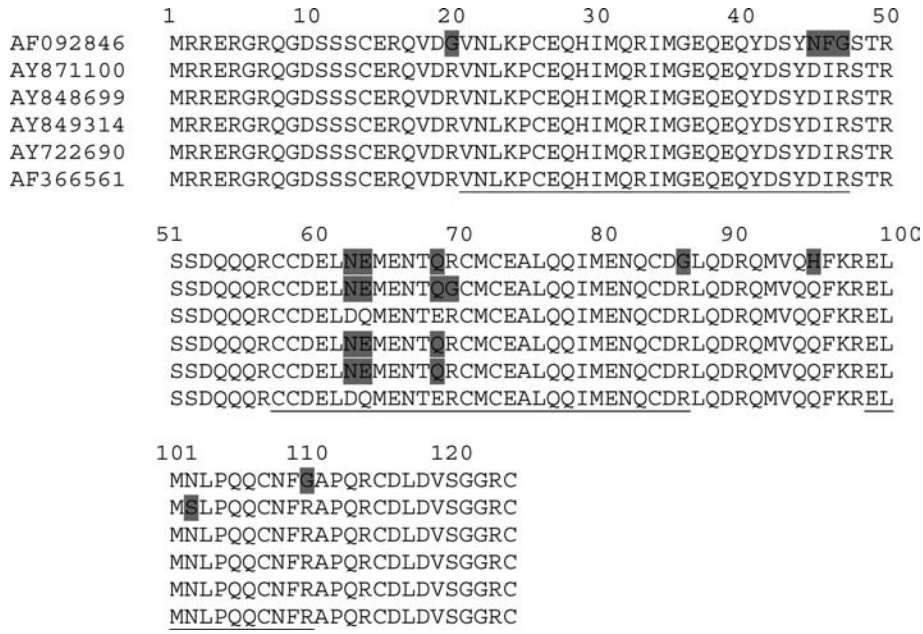


Figure 7. Sequence alignment of different isoforms of Ara h 6. Substituted amino acid residues are in shaded areas. Sequences corresponding to peptides identified after tryptic hydrolysis and mass spectrometry analysis (Figure 6) are underlined.

isolated from crude peanut extract (*15, 16*). In the present study, we purified a natural protein matching the Ara h 6 sequence. The mature protein is formed after a cleavage at the ASA-MR site and the loss of the signal peptide with the C-terminal sequence ASA, as previously suggested for Ara h 2, Ara h 6, and Ara h 7 (*15, 17*). The molecular weight of 14843 Da is similar to the theoretical mass of the mature form of the conglutin described by Paik-Ro and referenced as GenBank accession number AF 366561 (*18*). However differences of about 100–400 Da are observed with the mass expected for the previously characterized recombinant or natural forms of Ara h 6 and particularly with the Ara h 6 described by Koppelman et al. (*16*). The amino acid composition also differs significantly, especially with regards to the proportion of Arginine and Glycine. As a consequence of the higher content of Arginine, the tryptic peptide map of our protein of 14.84 kDa MW is quite different from that expected for Ara h 6. These data thus confirm that this protein represents a new isoallergen of Ara h 6.

The comparison with the recombinant Ara h 6 called PSC33 produced using a synthetic gene and characterized by our Group showed a structural identity. No difference including post-translational modification was observed (*13*).

A recent study underlining that peanut is an allotetraploid with AABB genome constitution, showed the existence of three different copies of Ara h 6, one belonging to the A genome and the other two to the B genome (*17*).The occurrence of three cDNA sequences coding for at least two Ara h 6 sequences has been identified in *A. hypogeae* L. Translational differences between these sequences were found at positions 63, 64, and 69 of the mature protein, with an amino acid sequence DQE for one isoform and NEQ for an other one. Unfortunately, we could not discriminate whether the new isoform presently described belongs to genome A or B.

The diversity of isoforms seems to be enriched by the maturation process. Interestingly, one of the plant processed products from Ara h 6 identified in this paper corresponds to a 14.58 kDa polypeptide characterized by two subunits (5.44 and a 9.14 kDa) linked by disulphide bridges. Mass characterization of the fragments after reduction combined with N-terminal amino acid sequencing allowed us to identify fragments f(1–45) and f(48–124) of the 5.44 and 9.15 kDa peptides, respectively. This 14.58 kDa polypeptide thus resulted from Ara h 6 proteolysis and the loss of a dipeptide (i.e., IR) in position 46–47, the two parts remaining linked via disulphide bonds. It is unlikely that this processed protein could have been formed during roasting or extraction. In other studies, this processed form has also been systematically observed in raw peanut using different but carefully controlled conditions of extraction (i.e., buffers with various compositions, addition of multiple cocktails of protease inhibitors, extraction at 4 °C during two hours only) to avoid proteolysis (data not shown). Moreover, the maturation process and natural cleavages have been already found in glycinin from peanut and in 2S albumins from other plant species (*5, 19*). Indeed, 2S albumins are often described as heterodimers formed after processing, the two chains resulting from an enzymatic cleavage being linked by disulphide bonds (*19*). We have observed other products derived from Ara h 6 corresponding to the 9.14 kDa peptide associated with a fragment characterised as f(1–42) by mass spectrometry. Interestingly this last fragment presents the same C-terminal sequence as f(1–45). Indeed the sequence YD is repeated twice at positions 41–42 and 44–45. The results suggest the involvement of this sequence in the site of cleavage and a hydrolytic process involving a specific plant endoprotease. As an example, vacuolar enzymes responsible for maturation of seed storage proteins are known to cleave at the C-terminal side of aspartic acid (*20, 21*). Interestingly, the plant processed form of Ara h 6 coexists with the unprocessed form in peanut. This corroborates previous results suggesting the simultaneous occurrence of native Ara h 6 and corresponding breakdown fragments (*15*).

The presence of multiple native and/or naturally processed forms of Ara h 6 which may depend upon the cultivar and/or the stage of maturation of the seeds could greatly influence the immunoreactivity of Ara h 6. As an example, first studies using recombinant Ara h 6 led to its classification as a minor allergen, being recognized by less than 45% of allergic patients (*8*). More recently, Lehman et al. found that the IgE binding capacity of recombinant Ara h 6 was weaker than that of recombinant Ara

Table 1. Distribution of Human Ige Responses To Whole Peanut Protein Extract (WPPE) and Purified Allergens in 47 Sera from Patients Allergic To Peanut[a]

	specific IgE response (IU ml^{-1})			
serum	WPPE	Ara h 2	Ara h 6 isoform	Degraded Ara h 6 isoform
1	0.2	0	0	0
2	0.9	0	0	0
3	0.7	1.0	0.5	0.6
4	0.9	0	0.5	0.5
5	4.4	0.6	4.3	3.9
6	1.8	1.5	3.1	2.7
7	2.0	1.2	2.3	2.6
8	4.4	4.3	3.2	4.7
9	4.3	3.1	4.4	5.2
10	3.5	4.8	1.8	3.5
11	4.6	3.5	2.9	5.1
12	17.0	16.8	11.2	11.8
13	15	7.6	5.6	7.2
14	19.0	10.3	15.2	18.2
15	24	20	21	26
16	24	21	26	26
17	59	40	46	55
18	150	104	90	121
19	37	24	25	27
20	92	54	41	44
21	74	50	76	72
22	156	208	163	163
23	97	93	59	70
24	139	89	87	96
25	173	89	158	164
26	341	111	153	167
27	218	179	185	204
28	166	87	108	120
29	333	172	175	177
30	253	238	195	216
31	320	255	244	259
32	474	219	245	258
33	438	335	376	383
34	447	198	218	225
35	984	763	702	842
36	501	451	442	401
37	1036	674	808	886
38	48	49	43	41
39	4.1	3.3	3.6	4.2
40	3.1	0.9	0.4	0.6
41	280	182	177	180
42	3.4	2.9	2.2	2.7
43	130	111	80	104
44	3.3	3.5	1.8	2.4
45	120	104	67	75
46	60	31	50	42
47	20	21.0	8.8	16.7
Mean	155.1	107.2	109.3	118
Median	59.0	40.0	43.0	42

[a] Specific IgE responses were determined using Enzyme Allergo Sorbent Test. Mean and median IgE responses for each allergen are shown at the bottom.

h 2 (*22*). In contrast, the present study demonstrates that natural Ara h 6 is as immunoreactive as natural Ara h 2, in IgE binding studies and in in vivo tests, e.g. skin prick test. These divergent results can be explained by the conformational difference between the natural and recombinant forms since the immunoreactivity of Ara h 6 is associated to its folding (*13*). The allergenicity of this natural Ara h 6 isoform purified from roasted peanut is in agreement with previous results from Koppelman et al. who showed that Ara h 2 and Ara h 6 extracted from raw peanut elicit comparable skin reactivity (*16*). Moreover, Suhr et al. underlined the allergenic activity of a natural Ara h 6 using mediator release experiments, and demonstrated that Ara h 6 is quite resistant to heat processing at 180 °C (*15*). The immunoreactive structure of Ara h 6 appears to be resistant to thermal

Table 2. Skin Prick Tests Reactions To Purified Peanut Allergens in Eight Patients

patient no.	age (year)/ sex	prick test (expressed in mm of maximum wheal size)		
		WPPE[a]	Ara h 2	Ara h 6 isoform
34	17/F	18	19	25
41	6/F	7	6	7
42	7/F	9	9	7
43	16/F	6	16	15
44	5/M	0	6	4
45	15/F	7	10	10
46	8/M	7	3	7
47	11/F	4	5	0

[a] WPPE, whole peanut protein extract.

treatment. As reported by Lehmann et al. (*22*), trypsin and chymotrypsin digestion of Ara h 6 led to stable proteolytic products composed of two fragments linked by disulphide bonds, and these protease-resistant cores still had allergenic properties. We confirmed these results with the new isoallergen of Ara h 6, which is naturally degraded by proteolysis. Indeed, the two subunits linked by disulphide bridges retained all the IgE reactivity observed for unprocessed Ara h 6. In contrast, a drastic decrease in IgE binding observed for chemically reduced and denatured Ara h 6 underlined the contribution of tertiary structure in Ara h 6 allergenicity. As previously observed (*13*), the recombinant isoform of Ara h 6 (called PSC33) contains five disulfide bridges. The four disulfide bridges of canonical 2S albumins were conserved and two additional cysteines were paired together. The different disulfide bridges contributed to the conformation of the Ara h 6 and consequently to its immunoreactivity. Reduction and S-carboxymethylation affect the totality of the disulfide bridges and do not allow to assess which disulphide bridge(s) may be particularly involved in the IgE binding capacity. Single modification of the different cysteines in the recombinant Ara h 6 isoform is in progress to further analyse the individual contribution of each disulfide bridge in the IgE reactivity of Ara h 6.

In conclusion, we have isolated and characterized a new isoallergen of Ara h 6, and observed that intact Ara h 6 coexists with a proteolytically processed isoform in peanut seed. Both the Ara h 6 isoform and its processed form that conserves a tertiary structure are equally able to bind IgE, suggesting that the immunoreactivity is not affected by the internal cleavage. Loss of IgE binding after Ara h 6 chemical reduction and denaturation confirmed the contribution of disulphide bridges to the IgE-immunoreactive structure. Moreover, the IgE response to each of these 2S albumins is similar to the IgE response to Ara h 2. In vivo tests confirmed the reactivity of the new Ara h 6 isoallergen. These results demonstrate that numerous proteins, isoforms and naturally plant processed products from the 2S albumin family may be implicated in peanut allergenicity.

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