

Insights into proteolytic processing of the major peanut allergen Ara h 2 by endogenous peanut proteases

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Abstract

BACKGROUND: The major peanut allergens are Ara h 1, Ara h 2 and Ara h 6. Proteolytic processing has been shown to be required for the maturation process of Ara h 6. The aim of this study was to examine whether Ara h 2 undergoes proteolytic processing and, if so, whether proteolytic processing influences its ability to bind human immunoglobulin E (IgE).

RESULTS: Ara h 2 isolated from peanut extract under conditions of protease inhibition revealed a single additional peak for its two known isoforms (Ara h 2.01 and Ara h 2.02), corresponding to a C-terminally truncated form lacking a dipeptide (RY). Ara h 2 isolated in the absence of protease inhibition, however, yielded two additional peaks, identified as C-terminally truncated forms lacking either a dipeptide (RY) or a single tyrosine residue. The IgE-binding capacity of the Ara h 2 truncated forms was not altered.

CONCLUSION: Ara h 2 undergoes proteolytic processing by peanut proteases that involves C-terminal removal of a dipeptide. Hence Ara h 2 isolated from peanut extract is a complex mixture of two isoforms expressed by different genes, Ara h 2.01 and Ara h 2.02, as well as truncated forms generated by the proteolytic processing of these isoforms.

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Keywords: Ara h 2; Ara h 6; proteolysis; IgE binding; food allergy

INTRODUCTION

Foods of diverse origin are described as potential inducers of allergic reactions in humans. Plant food allergens belong to various protein families, including the prolamin superfamily, the non-specific lipid transfer protein family, the cupin superfamily, the cysteine protease family, the α -amylase or trypsin inhibitor family, etc.^{1,2} Allergenic plant proteins function in plants as seed storage proteins, proteases or inhibitors of various proteolytic enzymes that protect plants from pathogens, but for some of them a precise function is still lacking. Biochemical characterisation of allergenic plant proteins has demonstrated that the major food allergens are, most frequently, glycoproteins ranging in size from 10 to 70 kDa and possessing an abundance of immunoglobulin E (IgE)-binding epitopes.² These glycoproteins induce allergic reactions by crosslinking of IgE molecules bound at the surface of effector cells such as mast cells or basophils. Moreover, proteins that are able to induce allergic sensitisation are absorbed through the gut epithelial barrier in an immunologically intact form³ or possess common structural features, e.g. the so-called cross-reactive epitopes, with the common respiratory allergens.²

Allergy to peanuts is the most common type of food allergy that can lead to a serious health outcome. Approximately 1% of the US population suffers from peanut allergy, and its prevalence is on the rise.⁴ Allergic reactions upon ingestion of minute quantities of peanuts are severe, because they can result in anaphylaxis and sometimes can even be fatal.⁵ Extensive

research on characterisation of peanut protein structure and identification of peanut allergens has been done and, so far, several proteins from peanuts have been characterised and identified as allergenic. Immunoblotting experiments have revealed that the major allergens recognised by the serum IgE from peanut-allergic patients are Ara h 1, Ara h 2 and, to a lesser extent, Ara h 3.^{6–8} Recently, emphasis has been placed on characterising Ara h 6 and Ara h 2, both members of the 2S albumin family. The 2S albumin family proteins are generally proteins of low molecular weight, and many have been classified as plant allergens. Their characteristic feature is a conserved 3D structure that includes at least four helical structures and relies on the highly conserved cysteine residues being organised in a specific pattern.⁹ Two

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isoforms of Ara h 2 expressed from different genes, Ara h 2.01 and Ara h 2.02, have been identified.¹⁰ Ara h 6 has been shown to be a very potent allergen, sharing epitopes with Ara h 2, with the immunological cross-reactivity between Ara h 2 and Ara h 6 relying on their biochemical similarity.¹¹ Ara h 6 undergoes posttranslational proteolytic processing that involves removal of a dipeptide (IR). Both the processed and the unprocessed isoform of Ara h 6 are present in peanuts, and it has been suggested that this kind of proteolytic processing is required for the maturation of Ara h 6.¹²

In the present study we isolated Ara h 2 from peanut extract under various conditions. We report that both Ara h 2 isoforms, Ara h 2.01 and Ara h 2.02, undergo proteolytic processing. Furthermore, these proteolytically processed Ara h 2 isoforms contribute, together with unprocessed Ara h 2 isoforms, to IgE binding with the IgE isolated from sera of patients allergic to peanuts.

MATERIALS AND METHODS

Protein extraction

Raw peanuts were obtained from a local store and kept frozen at -18°C until use. Frozen peanuts were subsequently ground in a blender and defatted by lipid extraction with petroleum ether. The peanut powder obtained was air dried overnight. Proteins were extracted by suspending 10 g of peanut powder in 100 mL of 50 mmol L⁻¹ Tris and 0.2 mol L⁻¹ NaCl (pH 8.5) under protease-inhibitory conditions (1 mmol L⁻¹ phenylmethylsulfonyl fluoride, 1 mmol L⁻¹ ethylene diamine tetraacetic acid) or under conditions that did not inhibit the activity of proteases (50 mmol L⁻¹ Tris, 0.2 mol L⁻¹ NaCl, pH 8.5). Extracts were clarified by mixing with tetrachloromethane and centrifugation at $17\,000 \times g$ for 15 min. Clear extracts were aliquoted and stored at -18°C until further use.

Size exclusion chromatography

Proteins extracted from peanut in the presence or absence of protease inhibition were applied on a Superdex 200 XK 16/100 column in an ÄKTA Purifier 10 high-performance liquid chromatography (HPLC) system (Amersham Biosciences, Uppsala, Sweden) and eluted using appropriate buffers. The fractions obtained were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Those with a similar protein profile were pooled and concentrated by ultrafiltration.

Reverse phase chromatography (RPC)

Fractions containing peanut proteins in the mass range between 14 and 20 kDa were subjected to RPC on a Discovery BIO Wide Pore C5 column (10 cm $\times$ 4.6 cm) in a C5 reverse phase HPLC system (Supelco/Sigma-Aldrich, Taufkirchen, Germany). For elution, 1 mL L⁻¹ trifluoroacetic acid (TFA) and 1 mL L⁻¹ TFA in 900 mL L⁻¹ acetonitrile were used at 0.75 mL min⁻¹ in a linear gradient, with gradual increases from 0 to 100% over ten column volumes. Elution was monitored by measuring the absorbance at 280 and 215 nm.

Electrospray ionisation mass spectrometry (ESI-MS)

Fractions from the C5 RPC column were vacuum centrifuged at room temperature in an evaporator (Eppendorf, Hamburg, Germany) and dissolved in 1 mL L⁻¹ formic acid in 500 mL L⁻¹ acetonitrile to obtain a final concentration of 0.6 mg protein mL⁻¹. Protein mass measurements were carried out in an Agilent 6210

time-of-flight LC/MS system (Agilent Technologies, Santa Clara, CA, USA). Samples dissolved in a mobile phase were introduced via an Agilent 1200 HPLC system. Agilent MassHunter Workstation software was used for data acquisition and Agilent MassHunter Workstation software and Agilent Analyst QS were used for data analysis.

SDS-PAGE

SDS-PAGE was carried out using Hoefer Scientific Instruments apparatus Sypro Ruby (Amersham Biosciences, Uppsala, Sweden) and a discontinuous buffer system. Protein samples were mixed with loading buffer and boiled for 5 min at 95°C . Components were resolved on polyacrylamide gels (136.46 g L⁻¹ acrylamide, 3.74 g L⁻¹ bisacrylamide) under non-reducing conditions, along with a standard protein mixture for molecular weight reference (Fermentas, Vilnius, Lithuania). To visualise separated proteins, gels were stained with Coomassie Brilliant Blue R-250.

2D-PAGE

Initial separation was achieved by isoelectrofocusing in the first dimension (polyacrylamide gel (48.76 g L⁻¹ acrylamide, 1.34 g L⁻¹ bisacrylamide), pH 3.5–10 gradient of ampholytes) in a Multiphor II system (Pharmacia LKB, Uppsala, Sweden). After 30 min of incubation in equilibration buffer (62.5 mmol L⁻¹ Tris-HCl, pH 6.8, 50 mL L⁻¹ β -mercaptoethanol, 23 g L⁻¹ SDS, 100 mL L⁻¹ glycerol), the second dimension was run using polyacrylamide gel (136.46 g L⁻¹ acrylamide, 3.74 g L⁻¹ bisacrylamide).

2D immunoblotting

Following 2D electrophoretic separation, proteins were transferred onto nitrocellulose membranes using an electroblotting system (Serva, Heidelberg, Germany). Efficient membrane transfer was verified by staining with 5 g L⁻¹ Ponceau S for 15 min. Nitrocellulose membranes were destained by washing with 30 mmol L⁻¹ Tris (pH 7.4) containing 9 g L⁻¹ NaCl and 1 g L⁻¹ Tween 20 (TTBS) and blocked with 10 g L⁻¹ human serum albumin (HSA; Octapharma AB, Stockholm, Sweden) in TTBS prior to probing with diluted sera. For immunoblotting experiments, sera from five persons, obtained from a local clinic, were pooled and used. All persons had previously reported clinical reactions to peanut (urticaria, rash, swollen tongue), were positive to peanut in skin prick testing and had IgE levels to peanut proteins higher than 0.5 kU L⁻¹. To examine the binding of IgE from the allergenic serum pool, membranes were probed with the serum pool diluted three times in TTBS. Bound IgE was detected using anti-human IgE antibodies (Sigma-Aldrich, Taufkirchen, Germany) labelled with alkaline phosphatase, diluted in TTBS/1 g L⁻¹ HSA in appropriate dilutions, as recommended by the manufacturer. Binding patterns were visualised with a substrate solution consisting of 1.5 mg of 5-bromo-4-chloro-3-indolyl phosphate and 3 mg of nitroblue tetrazolium in 10 mL of 100 mmol L⁻¹ Tris buffer containing 150 mmol L⁻¹ NaCl and 5 mmol L⁻¹ MgCl₂ (pH 9.6).

Genetic material and preparation of cDNA

Genomic DNA was extracted from peanuts using a DNeasy Plant kit (Qiagen, Valencia, CA, USA). RNA was extracted from peanuts using an RNeasy Plant kit (Qiagen). Extracted RNA was used as template for synthesis of cDNA using oligo-dT primer and a RevertAid First Strand cDNA Synthesis kit (Fermentas).

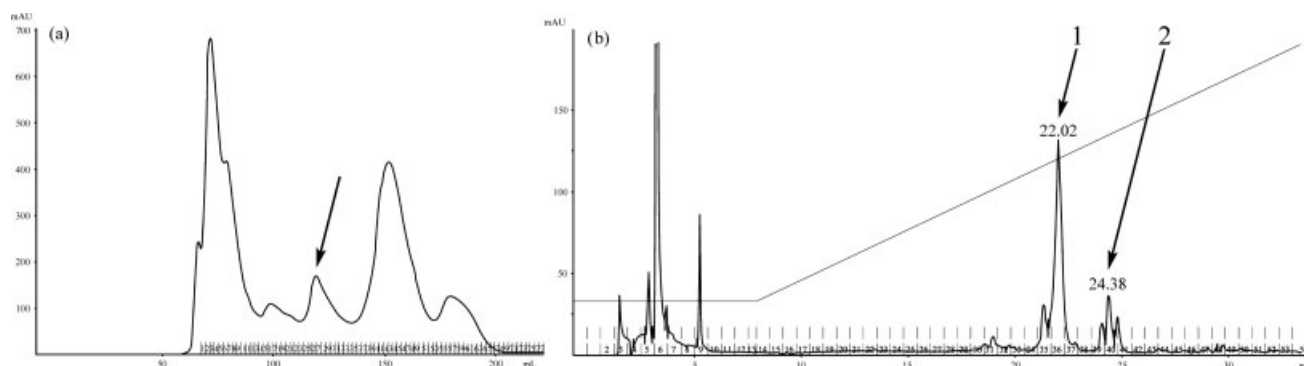


Figure 1. Isolation of Ara h 2 and Ara h 6 in absence of protease inhibition. (a) Elution profile of peanut extract applied on Superdex 200 column: arrow indicates peak containing both Ara h 2 and Ara h 6. (b) Elution profile of pooled fractions containing Ara h 2 and Ara h 6 on C5 RPC column: 1, Ara h 2 peak; 2, Ara h 6 peak.

Polymerase chain reaction (PCR) amplification of genes encoding Ara h 2 and Ara h 6

Specific primers for amplification of genes encoding Ara h 2 and Ara h 6 were created according to GenBank accession numbers AY117434 and EF609643 respectively. The gene encoding Ara h 2 was amplified using Pfu polymerase (Fermentas) with genomic DNA or cDNA as template in a volume of 25 μ L under the following conditions: 95 °C for 3 min; 30 cycles of 95 °C for 1 min, 56.5 °C for 75 s and 74 °C for 2 min; 72 °C for 10 min; held at 4 °C. The gene encoding Ara h 6 was amplified using TrueStart Taq polymerase (Fermentas) with both genomic DNA and cDNA as template in a volume of 25 μ L under the following conditions: 95 °C for 3 min; 30 cycles of 95 °C for 1 min, 55.5 °C for 75 s and 74 °C for 2 min; 72 °C for 10 min; held at 4 °C.

Analysis of Ara h 2 and Ara h 6 genes

PCR products obtained by amplification of genomic DNA were ligated into the pET28b vector. Chemically competent cells of *Escherichia coli* strain DH5alpha were transformed with the vector carrying the desired gene and grown on antibiotic-selective agar plates. Control transformation was performed in parallel. Colonies resistant to antibiotic were analysed for copies of the Ara h 2 or Ara h 6 gene by colony PCR. Ara h 2 and Ara h 6 positive colonies used for sequencing of plasmids were selected randomly and grown in culture overnight. Genes were sequenced using T7 promoter and T7 terminator specific primers in a 3130 Genetic Analyser (Applied Biosystems, Carlsbad, CA, USA).

Molecular modelling of Ara h 2 isoforms

A BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) revealed that the sequence sharing the highest homology with Ara h 2 was Ara h 6. The reported 3D structure of Ara h 6 was used as template for Ara h 2 modelling by the multiple mapping method.¹³ Checking for energy minimisation of modelled structures was done by GROMOS 96 implementation in Swiss-Pdb Viewer.¹⁴ Structures of unprocessed and processed Ara h 2 isoforms were viewed and superimposed by Swiss-Pdb Viewer 4.01 (<http://www.expasy.org/spdbv/>). Analysis of Ramachandran plots of both structures showed no amino acid residues in restricted areas of the plot.

RESULTS

Isolation of Ara h 2 and Ara h 6 from raw peanut extract

Ara h 2 and Ara h 6 were isolated from raw peanuts in the presence or absence of protease inhibition, as outlined above. Following fractionation by gel filtration on a Superdex 200 column, a peak containing both Ara h 2 and Ara h 6 was detected in the elution profile of the peanut extract prepared under conditions that did not inhibit protease activity (Fig. 1(a)). An identical chromatographic profile was observed when the peanut extract was prepared under conditions of protease inhibition (data not shown). Pooled fractions containing Ara h 2 and Ara h 6 were subsequently applied on a C5 RPC column, and peaks for Ara h 2 and Ara h 6 were detected in the RPC elution profile (Fig. 1(b)). Similarly, under conditions of protease inhibition during isolation, Ara h 2 and Ara h 6 eluted at the same retention time (data not shown). Repeated preparation and fractionation of the peanut extract in the presence or absence of protease inhibition gave consistent results with identical chromatographic elution patterns.

SDS-PAGE of Ara h 2 and Ara h 6

Fractions from the isolation procedure under protease-inhibitory conditions were further analysed by SDS-PAGE and Coomassie staining. Pooled fractions as well as fractions containing purified Ara h 2 or Ara h 6 revealed the expected electrophoretic mobility for Ara h 2 and Ara h 6 (Fig. 2). Following staining, Ara h 2 appeared on the gel as a doublet, with two bands, representing the two known Ara h 2 isoforms Ara h 2.01 and Ara h 2.02, with approximate molecular weights of 16 and 18 kDa, while Ara h 6 appeared as a single band with an approximate molecular weight of 15 kDa. The electrophoretic profile remained the same regardless of whether reducing or non-reducing conditions were used for sample preparation.

Identification of isolated proteins by ESI-MS

Purified Ara h 6 and Ara h 2 were next subjected to ESI-MS analysis. Mass spectra of Ara h 6 isolated under non-inhibitory (Fig. 3(a)) and protease-inhibitory (Fig. 3(b)) conditions showed no difference between the two conditions. However, condition-specific differences were observed for Ara h 2 (Fig. 4). Ara h 2 purified in the absence of protease inhibition revealed the presence of two additional peaks for Ara h 2.01 and Ara h 2.02 isoforms (Fig. 4(a)). These isoforms were subsequently identified as truncated forms lacking a C-terminal amino acid tyrosine (Y) or a C-terminal dipeptide (RY) (Table 1). Under conditions of protease

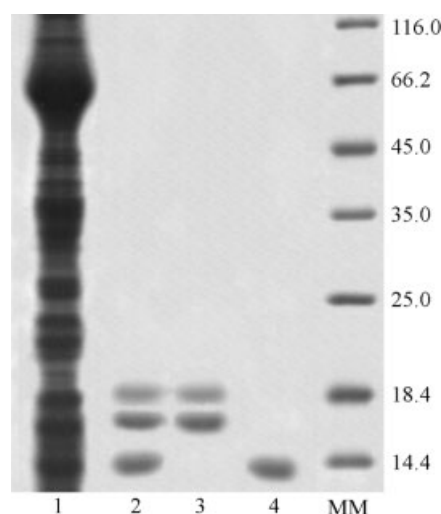


Figure 2. SDS-PAGE of Ara h 2 and Ara h 6 purification: lane 1, peanut extract; lane 2, pool after size exclusion chromatography containing Ara h 2 and Ara h 6; lane 3, purified Ara h 2; lane 4, purified Ara h 6; MM, molecular weight markers (kDa).

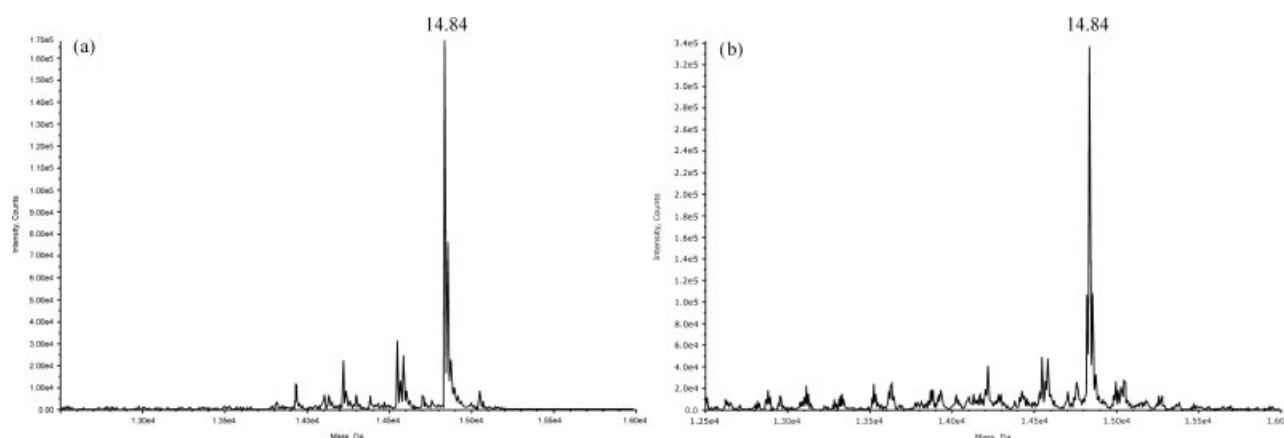


Figure 3. ESI mass spectra of isolated Ara h 6 in (a) absence and (b) presence of protease inhibition.

inhibition, however, only one additional peak was observed for Ara h 2.01 and Ara h 2.02 isoforms (Fig. 4(b)). This additional peak corresponded to a truncated form of Ara h 2.01 and Ara h 2.02 lacking a C-terminal dipeptide (RY) (Table 1). The mass obtained for the unprocessed Ara h 2.01 and Ara h 2.02 isoforms, as well as for Ara h 6, is in agreement with previous reports.^{10,12}

Analysis of Ara h 2 and Ara h 6 gene sequences

Specific primers for amplification of genes encoding Ara h 2 and Ara h 6 were made according to GenBank accession numbers AY117434 and EF609643 respectively, and genes corresponding to Ara h 2 and Ara h 6 were PCR amplified and sequenced. BLAST alignment of the identified gene sequence encoding Ara h 6 revealed 100% identity with the sequences already available under accession numbers EF609644.1 and EF609643.1,¹⁵ while alignment of the gene sequence encoding Ara h 2 showed that the cloned gene had 100% identity with the sequence available under accession number AY722689.¹⁶ and corresponding to the partial coding sequence of Ara h 2.

Molecular modelling of Ara h 2 isoforms

Subsequently, molecular modelling of the Ara 2.01 isoform using Ara h 6 as template with 57% identity was performed and revealed a

3D structure for Ara h 2.01 and its truncated form. The model of the tertiary structure of Ara h 2.01 revealed a predominantly α -helical secondary structure with all eight cysteine residues involved in the formation of disulfide bonds (Fig. 5). The structure consists of a core of four helices together with two smaller helices contributing to stabilisation of the core. Superimposing the modelled structures of Ara h 2.01 and its truncated form in Swiss-Pdb Viewer showed no noticeable differences in their 3D structure at the level of organised secondary structures. The only difference observed was in the region of the structure where the polypeptide backbone is randomly coiled, but not in the region involving the C-terminus (Fig. 5).

Human IgE binding by Ara h 2 isoforms

In order to check the IgE-binding potency of the Ara h 2 truncated isoforms, a 2D western blot was performed. Ara h 2 isolated under protease-inhibitory conditions and analysed by 2D electrophoresis revealed two spots for Ara h 2.01 and Ara h 2.02 (Fig. 6(a)), which corresponded to the peaks previously identified by mass spectrometry (Fig. 4(b)). A 2D western blot probed with the sera from human subjects allergic to peanuts revealed the same two spots for Ara h 2.01 and Ara h 2.02 (Fig. 6(b)). No change in the

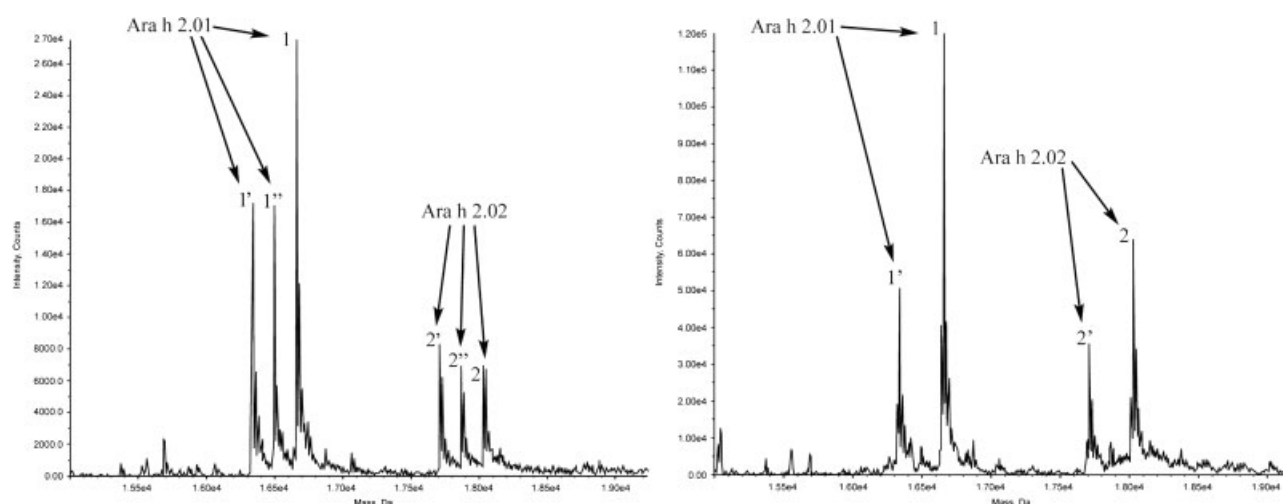


Figure 4. ESI mass spectra of isolated Ara h 2 in (a) absence and (b) presence of protease inhibition: 1, 1' and 1'', Ara h 2.01 isoforms; 2, 2' and 2'', Ara h 2.02 isoforms; single prime ('), isoforms lacking dipeptide RY; double prime (''), isoforms lacking Y.

Table 1. Mass of Ara h 2 isoforms obtained by ESI-MS

Peak	Molecular weight (Da)	Difference in mass (Da)	Removed amino acid	Isoform
1	16661.1409	–	–	Ara h 2.01
1'	16341.8126	319.3283	RY	Ara h 2.01
1''	16497.9138	163.2271	Y	Ara h 2.01
2	18033.5998	–	–	Ara h 2.02
2'	17714.3235	319.2763	RY	Ara h 2.02
2''	17870.3919	163.2079	Y	Ara h 2.02

1, 1' and 1'', Ara h 2.01 isoforms; 2, 2' and 2'', Ara h 2.02 isoforms; single prime ('), isoforms lacking dipeptide RY; double prime (''), isoforms lacking Y.

intensity of the spots was detected, however, in comparison with that observed after 2D electrophoresis (Fig. 6(b)).

DISCUSSION AND CONCLUSION

In the present study we isolated Ara h 2 in the absence and presence of protease inhibition to examine whether it undergoes proteolytic processing. We found that Ara h 2 underwent proteolysis by peanut proteases in a similar manner to Ara h 6 and that this proteolytic processing involved removal of a dipeptide from the C-terminus of both Ara h 2.01 and Ara h 2.02. Furthermore, we looked at the ability of these processed forms to bind human IgE and found that the IgE-binding capacity of Ara h 2.01 and Ara h 2.02 truncated forms was not altered. In conditions applied during the isolation of Ara h 2 that did not involve inhibition of naturally occurring proteases, further proteolysis by removal of a single tyrosine residue from the C-terminus of intact Ara h 2.01 and Ara h 2.02 also occurred.

Increased effort has been put into characterisation of the 2S albumins of peanuts. Among peanut allergens of the 2S albumin family, Ara h 2 and Ara h 6 are most potent at inducing degranulation in functional *in vitro* assays, as seen for example with rat basophilic leukaemia cells sensitised by serum IgE from a peanut-allergic patient.^{17,18} Molecular biology research on Ara h 6 has revealed that different variants of this allergen can be derived by single amino acid substitutions.¹⁵ Two isoforms of Ara h 6 have

been extensively characterised by Bernard *et al.*¹² Both isoforms originate from the same gene variant encoding Ara h 6, but one of them is the proteolytic product of the translated sequence. Further processing into mature form occurs by proteolysis at the ASA-MR cleavage site for Ara h 6 and the ASA-RQ cleavage site for Ara h 2.^{15,19} Bernard *et al.*¹² have shown that the maturation of Ara h 6 continues with the removal of dipeptide IR, which ultimately leads to Ara h 6 heterodimerisation. The mature heterodimer, a product of proteolytic processing, is thus present in peanut seeds along with the unprocessed form and shows no change in the IgE-binding capacity.¹²

Ara h 2 and Ara h 6 isolated under protease-inhibitory conditions were both shown to exist in the peanut extract as a mixture of multiple isoforms. However, for Ara h 2.01 and Ara h 2.02 isoforms the only proteolytic processing reported so far involved the removal of the signal peptide.²⁰ In this study we obtained similar results to those with Ara h 6 reported by Bernard *et al.*¹² showing that proteolytically processed isoforms of Ara h 2 lacking a C-terminal dipeptide are present in peanut extracts. The protease involved in the maturation of Ara h 6, because for both proteins the removed dipeptide contains one basic (R) and one bulky amino acid (I or Y), and cleavage of this dipeptide occurs at the C-terminal side of aspartic acid. Vacuolar enzymes involved in the maturation of seed storage proteins are known to cleave at the C-terminal side of aspartic acid^{19,20} and may be responsible for this kind of proteolytic processing of Ara h 6 and Ara h 2. Moreover, this kind of proteolytic processing cannot be observed by simple 1D electrophoresis owing to the very small change in molecular weight of the truncated forms in comparison with the unprocessed sequence. However, because removal of the dipeptide leads to elimination of a very basic amino acid (arginine) and changes the isoelectric point (pI) towards a more acidic pH, the shift in pI was sufficient to enable separation and visualisation of these forms by 2D electrophoresis.

Isolation condition-specific mass spectra were observed for Ara h 2 isoforms. Under conditions in which the activity of proteases present in peanut seeds was not inhibited, proteolysis at the C-terminus of Ara h 2, which involved removal of a single amino acid residue, was observed. These truncated Ara h 2.01 and Ara h 2.02 forms lacking one amino acid residue could not be identified by 1D



Figure 5. Model of 3D structure of Ara h 2.01: 1, C-terminus of full-length Ara h 2.01 (grey); 2, C-terminus of truncated Ara h 2.01 (black).

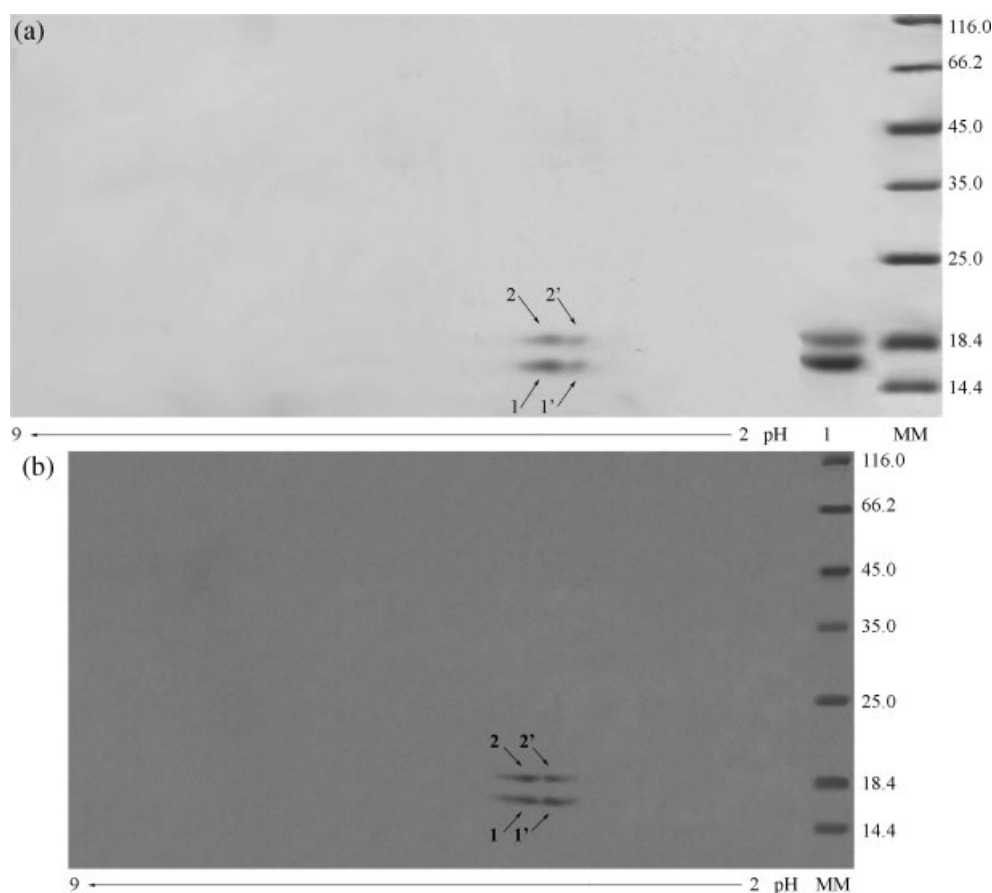


Figure 6. Analysis of Ara h 2 isolated under protease-inhibitory conditions, showing (a) 2D electrophoresis and (b) western blot of same sample probed with human sera: MM, molecular weight markers (kDa); 1 and 1', Ara h 2.01 isoforms; 2 and 2', Ara h 2.02 isoforms.

or 2D electrophoresis owing to very slight changes in molecular weight (163.22 Da). Instead, the observed removal of a single amino acid residue from Ara h 2.01 and Ara h 2.02 could only be detected by the method of mass spectrometry. This type of C-terminal proteolysis of Ara h 6 has not been observed under the same conditions of isolation, suggesting that Ara h 2 is likely more prone to proteolytic degradation than Ara h 6. Furthermore, weaker susceptibility of Ara h 6 to proteolysis may possibly explain recent results showing Ara h 6 to be a more potent allergen than Ara h 2.¹⁷

The PCR-amplified gene sequences of Ara h 2 and Ara h 6 obtained in this study were in agreement with those already available and published on GenBank gene sequences and were used for modelling the tertiary structure. The proteolytic processing of Ara h 2.01 and Ara h 2.02 occurs at the C-terminus, but it likely does not have a dramatic influence on the protein structure, because no computed and defined secondary structures have been observed in this region. The 3D model of the tertiary structure of Ara h 2 consists of a compact core in which all IgE-binding epitopes reside, and, so far, no IgE-binding epitopes have been identified at the C-terminus. Hence it has been assumed that proteolytic processing does not influence the IgE-binding capacity of Ara h 2.^{21–23} Indeed, the C-terminal proteolytic processing of Ara h 2.01 and Ara h 2.02 isoforms did not alter the IgE-binding potency of the truncated forms, as demonstrated by 2D immunoblotting (Fig. 6), and these results are in agreement with the predicted behaviour of Ara h 2. Thus the truncated forms of Ara h 2.01 and Ara h 2.02 are potentially equally capable of eliciting an immunological response in peanut-sensitized patients.

In conclusion, we have identified two new Ara h 2 isoallergens in peanut extract. Ara h 2 undergoes C-terminal proteolytic processing by peanut proteases, leading to the production of two isoallergens with molecular weights of 16.34 and 17.71 kDa respectively, which are present alongside the previously reported isoforms of Ara h 2, i.e. Ara h 2.01 and Ara h 2.02, with respective molecular weights of 16.66 and 18.03 kDa. The unaltered IgE binding of these proteolytically processed forms suggests that the maturation of Ara h 2 does not alter the IgE-binding epitopes. These results provide additional insight into the molecular properties of peanut allergens, demonstrating that various proteins of the 2S family contribute and, together with their naturally processed forms, may be involved in peanut allergenicity.

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