

REGULAR ARTICLE

Analysis of the composition of an immunoglobulin E reactive high molecular weight protein complex of peanut extract containing Ara h 1 and Ara h 3/4

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Peanuts (*Arachis hypogaea*) contain some of the most potent food allergens. In recent years an increasing prevalence of peanut allergies both in children and adults has been observed in the USA and in Europe. *In vitro* identification and characterization of allergens including those from peanut have been frequently performed by Western blotting. However this method may alter the immunoglobulin E (IgE) antibody reactivity since the proteins are denatured by detergent treatment and/or reduction of disulfide bonds by reducing reagents and does not answer the question how peanut allergens interact with the human digestive apparatus and immune system. Size exclusion chromatography of peanut extract shows that approximately 90% of the total protein content is eluted as one peak in the exclusion volume with a molecular mass of over 200 kDa. The proteins of this fraction were analyzed by blue-native polyacrylamide gel electrophoresis (PAGE), immunoblotting, two-dimensional PAGE and Western blotting. A complex of Ara h 1 (Acc. no. P43237), Ara h 3/4 (AAM46958), Ara h 3 (AAC63045), Ara h 4 (AF086821), Gly 1 (AAG01363) and iso-Ara h 3 (AAT39430) was identified using patients' IgE and allergen-specific monoclonal antibodies; N-terminal sequencing and matrix-assisted laser desorption/ionisation-time of flight analysis verified these findings. A comparison of the peanut allergen sequences of Ara h 3/4, Ara h 3, Ara h 4 and peanut trypsin inhibitor (AF487543) and the proteins Gly 1 and iso-Ara h 3, not yet described as allergens, leads to the conclusion that these proteins are isoallergens of each other. It was shown that these isoallergens are post-translationally cleaved and held together by disulfide bonds in accordance to the 11S plant seed storage proteins signature.

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Abbreviations: BNP, blue native gel electrophoresis; Ig, immunoglobulin; LDS, lithium dodecyl sulfate

1 Introduction

Peanuts belong to the taxonomic family *Leguminosae* and originate from South America. Today peanuts are a major agricultural crop, cultivated worldwide, used for industrial production of oil and serve as a cheap protein source. American infants often consume peanut and peanut-containing

products early in life, 80% are exposed in the first year of life and 100% within the first two years. In the USA 0.6% of the population suffer from peanut allergy, in the United Kingdom peanut-allergic individuals account for 0.5% of the population [1]. Peanut allergy is especially dangerous due to severe symptoms such as anaphylactic reactions. The only “treatment” available to peanut-allergic individuals is avoidance [2]. Other forms of therapy such as specific immunotherapy are not yet possible due to frequent adverse reactions [3].

We know from previous work that the main portion of soluble peanut proteins are seed storage proteins, which are divided into protein families and superfamilies on the basis of their solubility in different solvents, sedimentation coefficients given in Svedberg units (S), structural and molecular characteristics [4]. Under native conditions they form heterodimers and large aggregates. It is essential to look at these characteristics of peanut allergens to understand the identification of newly described peanut allergens under native conditions which is the subject of this paper (Table 1).

As shown by Koppelman *et al.* [8] a peak in the exclusion volume of a Superdex 200 size exclusion chromatography (SEC) of peanut extract contains Ara h 3/4. The focus of this paper is to describe the molecular characteristics of peanut allergens of the high molecular weight complex under native conditions, because this complex interacts with the organism rather than separated individual allergens and, thus, complexed allergens may be the source of allergen sensitization.

Therefore, we investigated the protein complex of the SEC exclusion volume. To study this complex we combined the blue native gel electrophoresis (BNP) as a nondenaturing tool with immunoblot analysis to show the IgE reactivity of the components of the complex. Furthermore, SDS-PAGE, Western blotting, and N-terminal sequencing were performed as well to analyze the complex. In addition, the complex was investigated by 2-D PAGE as the combination of IPG- and SDS-PAGE followed by peptide mass fingerprinting (PMF) of the IgE reactive spots which lead to the identification of isoallergens. In parallel whole peanut extract was also analyzed to demonstrate the presence of the complex components in the extract.

2 Materials and methods

2.1 Peanut extract

Raw peanuts (Jumbo giants) were purchased from Seeberger (Ulm, Germany). For the production of peanut flour, peanut kernels were frozen for 4 h at -80°C and ground in a coffee grinder. Thirty grams of flour were extracted overnight in 250 ml of 0.1 M NH_4HCO_3 , pH 8.0. After decanting and centrifugation (15 min at 4°C , $10\,000 \times g$) the supernatant was filtered through cellulose acetate filters with pore sizes of 8.0, 0.8, 0.45 and 0.2 μm , respectively. Finally, the extract was dialyzed against *aqua bidest* overnight and lyophilized. The

Table 1. Characteristics of peanut allergens. Prolamins are important plant allergens abundant in seeds. They have a conserved backbone of cysteine residues and form disulfide bonds between alpha-helices, with flexible loops

Name	Peanut and soy allergens	Solubility in solvents	Structural characteristics	Molecular characteristics
Albumins (2S)	Conglutins Ara h 2 and Ara h 6, alpha-conglycinin [5]	Soluble in water	Heterodimeric proteins with 2 polypeptide chains (~ 3 kDa and ~ 9 kDa) linked by disulfide bonds; monomeric forms	Stable to enzymatic proteolysis, can bind lipids
Cupins have beta-sheet barrel-like structures, which gave the name from the Latin word <i>Cupa</i> . They are thermostable and form large variable aggregates held together with non-covalent interactions even after proteolytic digestion. High sequence homology.				
Name	Peanut and soy allergens	Solubility in solvents	Structural characteristics	Molecular characteristics
Vicilins (7S)	Ara h 1	Soluble in salt solutions	Likely to form dimers and highly stable trimers of 210 kDa	N-glycosylation with glycans of high-mannose type [6]. Resistant against denaturation [7]
Legumins (11S)	Ara h 3/4, soybean glycinin		Hexameric hetero-oligomeric proteins of 300–450 kDa with a mixture of monomeric structures of acidic (~ 40 kDa) and basic (~ 20 kDa) subunits linked together by disulfide bonds	Synthesis of a single polypeptide, subunits are cleaved post-translationally at conserved protease site (NGLEET) (www.ifr.ac.uk/PROTALL). They are rarely glycosylated and carry three variable regions with two IgE epitopes [5]

protein concentration was determined using the Bradford assay [9] with BSA as standard (Pierce reagent; Perbio Science, Bonn, Germany). The yields ranged from 8.0–10.0 mg/mL protein, representing a yield of 6.7–8.3% of the starting material.

2.2 Size exclusion chromatography

Peanut extract was separated by SEC using a calibrated Superdex 200 column (Amersham Biosciences, Freiburg, Germany). Calibration was done with a protein test mixture containing catalase (232 kDa), albumin (67 kDa) and chymotrypsinogen (25 kDa). For analytical chromatography an HPLC system (Äkta-Purifier; Amersham Biosciences) was used. One milligram of protein *per* run was applied onto the prepacked 10/300 GL column (length: 34 cm, width: 1.5 cm) with PBS (50 mM K_2HPO_4 and 0.5 M NaCl, pH 7.5). Eight 0.5 mL fractions (B9–B15) with a protein yield of 0.5–5 mg/mL in the exclusion volume were pooled, dialyzed and lyophilized. For preparative scale purification an FPLC system (Äkta-Prime; Amersham Biosciences) was used. *Per* run 28.5 mg protein in 3 mL ammonium hydrogen carbonate buffer (100 mM $(NH_4)HCO_3$, pH 8.0) containing a protease inhibitor cocktail (Complete; Roche, Mannheim, Germany) were applied onto the self-packed Superdex 200 column (gel bed: 20 cm long, 5 cm wide). The 5 mL fractions (B7–B10, B11–B13, B14–B15) in the protein peak of the exclusion volume were pooled, dialyzed, and lyophilized. For 2-D PAGE and native gel electrophoresis the fractions were desalted using a PD-10 column (Amersham Biosciences) according to manufacturer's instructions against *aqua bidest*.

2.3 Blue native PAGE

Blue native PAGE (BNP) was performed according to Eubel *et al.* [10] with some modifications. 4–20% Tris-glycine zoom gels (Invitrogen, Karlsruhe, Germany) in combination with Laemmli [11] electrode buffer (50 mM Tris, 384 mM glycine) without detergent such as SDS or lithium dodecyl sulfate (LDS) were used to separate protein bands. The proteins were negatively charged by adding Coomassie G-250 containing sample buffer (8.125% glycerol, 141 mM Tris base, 106 mM Tris-HCl, 0.4 mM EDTA, 0.02% Coomassie G-250 and 0.0001% phenol red) and thus migrated according to their molecular weight. The gels can carry a protein load of up to 1 mg of protein *per* cm. The proteins were transferred onto NC membrane with a pore size of 0.45 μ m (Schleicher & Schüll, Dassel, Germany) using the thermoblot technique [12]. This method uses heat and pressure to transfer proteins via capillary blot onto the membrane. The gel, placed onto a piece of Parafilm M® (Roth, Karlsruhe, Germany) was transferred onto the heating block (50°C). The equilibrated membrane was layered onto the gel followed by two thin filter papers (GB002; Schleicher & Schuell) soaked in blotting buffer (25 mM Tris-HCl, 5% methanol, pH 10.4) and two dry thick filter papers (GB003; Schleicher & Schuell), a small

stack of paper towels and a glass plate. Finally, a 3 kg weight was placed on the glass plate. Every 3 min, membrane, filter papers and paper towels were replaced. Up to four replica blots *per* gel were prepared with negligible changes in the protein concentration on each blot. After blotting, the gel still contained protein and was Coomassie-stained. The working solution was made up from solution A containing 2.5 g $CuSO_4$ in 325 mL H_2O , and solution B containing 0.25 Coomassie Brilliant Blue R-250 and 0.25 g Croceine Scarlet (Serva, Heidelberg, Germany) dissolved in 125 mL isopropanol and 50 mL glacial acetic acid. The destaining solution was made up of 180 mL isopropanol + 90 mL glacial acetic acid *per* 1 L. The destained gels were stored in glycerol buffer (20 mL glycerol 87%, 70 mL glacial acetic acid filled up to 1 L) [13].

2.4 2-D native/reducing and native/non reducing LDS-PAGE

Lanes cut out from a BNP gel were incubated with lithium dodecyl sulfate (LDS) containing sample buffer (8.125% glycerol, 141 mM Tris base, 106 mM Tris-HCl, 73.5 mM LDS, 0.4 mM EDTA, 0.02% Coomassie G-250 and 0.0001% phenol red) under agitation for 1 h under non-reducing or reducing conditions (final concentration of DTT: 0.125 M) and transferred into the wells of a 10% or 12% Bis-Tris gel (Invitrogen). 3-(*N*-morpholino) propane sulfonic acid-containing running buffer (MOPS), (50 mM MOPS, 50 mM Tris base, 3.47 mM SDS, 1.03 mM EDTA, pH 7.7) was used. After electrophoresis at 50–100 V, thermoblotting and Coomassie staining of the gel were performed.

2.5 2-D PAGE

For the first dimension the proteins were separated by IEF on a precast polyacrylamide gel strip with an IPG of 4–7 or 3–10 (Invitrogen). The IPG strip was rehydrated in sample buffer (8 M urea, 2% CHAPS, 0.5% carrier ampholytes (pH 4–7 or pH 3–10), 0.002% bromophenol blue and 20 mM DTT) containing 100 μ g of sample protein. The IEF conditions for the Zoom® IPGRunner system (Invitrogen) were as follows: 200 V for 20 min, 450 V for 15 min, 750 V for 15 min, 1 h at 2000 V and 1 mA. Determination of the *pI* and the molecular mass of the proteins was carried out using an IEF marker, pH 3–10 (cat. 39211; Serva) and Multi Mark prestained standard (Invitrogen). After 15 min of equilibration each in LDS sample buffer containing 0.5 M DTT or 125 mM iodoacetamide, the strip was transferred to the well. For the second dimension 4–20% Tris-glycine zoom gels (NuPage) were used with MES, (0.05 M MES, 0.05 M Tris base, 3.47 mM SDS, 20.5 mM EDTA, pH 7.3). The gel run was performed according to the manufacturer's instruction for the Zoom IPG Runner System at 50–100 V. After performing the second dimension the proteins were transferred *via* thermoblotting onto NC membranes and the gels were stained with Coomassie (see Section 2.4).

2.6 Immunoblotting

After protein transfer the blots were blocked for 30 min in TBS-T (100 mM Tris, 100 mM NaCl, 2.5 mM MgCl₂, pH 7.4 plus 0.05% Tween-20) and dried. Afterwards the protein spots or bands were detected using India ink protein staining (1:1000) [14], mAbs and patients' sera diluted in TBS-T. TBS-T was also used for all washing steps. For first antibody incubation the blots were rinsed with TBS-T and incubated overnight at room temperature. After three washing steps the blots were incubated in secondary antibody solution for 2 h. After three washing steps the antibody binding was visualized using the chromogen-substrate mixture for alkaline phosphatase consisting of 0.0033% w/v nitro blue tetrazolium chloride and 0.017% w/v 5-bromo-4-chloro-indolyl-phosphate potassium salt (Serva) in TBS-AP (100 mM Tris, 100 mM NaCl, 5 mM MgCl₂, pH 9.5) for approximately 5 min at 37°C [15].

2.7 Antibodies and patients' sera

Two sera of peanut-allergic patients JG3 (1:100) and CAT (1:100) were used. JG3, a German peanut-allergic patient with a history of peanut exposure-associated anaphylaxis, was not orally challenged; she had a high concentration of peanut-specific IgE (>100 kU/L = UniCAP class 6). CAT (Switzerland) tested positive for peanut by double-blind placebo-controlled food challenge (DBPCFC) and suffered under provocation from flush, urticaria, nausea, conjunctivitis and cough; the amount of peanut-specific IgE was high (57.5 kU/L, UniCAP class 5). Serum of an allergic patient with positive skin prick test to grass pollen (UniCAP class 2) and house dust mite (UniCAP class 4) (data not shown) and without allergies to peanut was used as negative control. This patient had a concentration of 601 kU/L of total IgE but no IgE against peanuts. For the detection of human IgE reactivity the monoclonal mouse antihuman IgE-AP-labelled (1:2000; Allergopharma, Reinbek, Germany) was used. For the identification of Ara h 1 and Ara h 3/4 the mAbs Pn-t (1:20) and PEI-7B2 (1:100) were used, respectively. For detection goat antimouse IgG H&L-AP (1:5000 or 1:50 000, depending on the batch, both from Dianova, Hamburg, Germany) was applied. The mannose-specific lectin Con A (biotinylated, 1:1000; Sigma, Munich, Germany) that binds to N-glycans of the allergens, was used to detect glycoproteins. Streptavidin-AP (1:2000, Sigma) was used as detection reagent.

2.8 N-terminal sequencing

Proteins separated by both 1-D and 2-D gels were transferred onto methanol-activated PVDF membranes (Schleicher & Schuell) by tank electroblotting with transfer buffer (25 mM bicine, 25 mM Bis-Tris, 1.025 mM EDTA, 0.05 mM chlorobutanol and 20% methanol) according to the manu-

facturer's instructions (Invitrogen) and cut off from the membrane after Coomassie staining for PVDF membranes. Staining was for 10 min in Coomassie dye (0.06 mM Coomassie R-250 in 50% methanol) and the destaining for 7 min in destaining solution (50% methanol) [12]. Sequencing of IgE reactive spots and bands was done according to the Edman degradation method on a Procise sequencer (Applied Biosystems, Darmstadt, Germany).

2.9 Protein identification by mass spectrometric analysis

For in-gel reduction and digestion, spots corresponding to the IgE reactive proteins were cut out, reduced with 100 mM DTT (56°C, 1 h) and alkylated by adding iodoacetamide to a final concentration of 200 mM, and the samples were incubated in the dark at room temperature for 45 min. The proteins were then digested in-gel with trypsin (Promega, Mannheim, Germany) as described previously [16].

For MALDI-TOF MS, 0.5 µL of each peptide mixture from the in-gel digestion was applied to a target disk, and allowed to dry. Afterwards 0.5 µL of matrix solution (1% w/v CHCA in 30% acetonitrile, 0.1% TFA) was applied to the dried sample and allowed to dry under vacuum. Spectra of protein digests were obtained using a Reflex III MALDI-TOF spectrometer (Bruker, Bremen, Germany). MASCOT (<http://www.matrixscience.com/home.html>) software was used to analyze the MS spectra by the PMF method [17]. For the *de novo* sequencing experiments, the tryptic peptide mixture was analyzed before and after derivatization by addition of acetic anhydride, in an LCQ Thermo (San Jose, CA, USA) ion trap mass spectrometer fitted with a nano ESI source, as previously described [18].

3 Results

3.1 SEC of peanut extract

Size exclusion chromatography using a Superdex 200 column showed that approximately 90% of the proteins of the peanut extract had an apparent molecular mass of at least 200 kDa and eluted in the exclusion volume. The molecular masses of the peanut proteins were measured by calibrating the column with a protein mixture of known molecular mass first and using their retention times to control the following runs. To estimate how much protein is in the exclusion and inclusion volume of the column a mathematical integration of the peaks was performed. A black arrow in Fig. 1a indicates the peak, which contained 90% of total protein. After monitoring the runs by SDS-PAGE analysis, fractions B9-B15 were pooled according to their similar band patterns and used for the following characterization of the allergens.

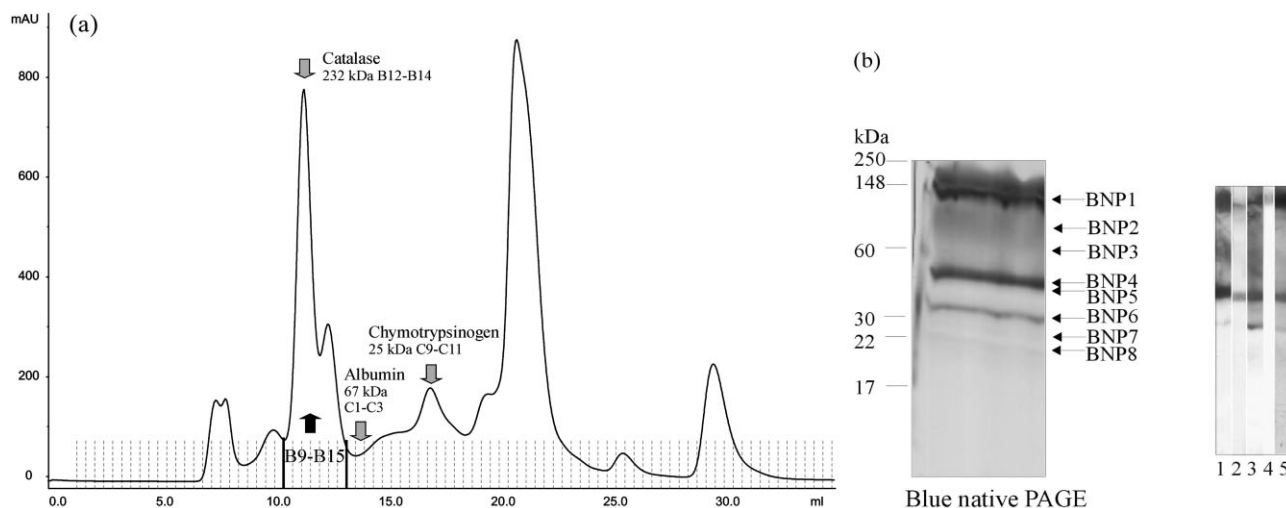


Figure 1. (a) Chromatogram of SEC of peanut extract using a calibrated Superdex-200 column monitored at the 280 nm wavelength. Black arrow indicates major peak of the exclusion volume containing Ara h 1 and Ara h 3/4 in the fractions B9-B15, which were pooled based on SDS-PAGE analysis. Grey arrows indicate molecular masses and fractions of proteins from the molecular weight calibration. There is no protein detectable in fraction C13 and higher. (b) BNP and immunoblot analysis of the fraction pool B9-B15. Left: Coomassie-stained BNP gel; right: immunoblot analysis; lane 1: protein staining with India ink, lane 2: mAb PEI-7B2 (anti Ara h 3/4), lane 3: IgE reactivity of patient serum JG3, lane 4: mAb Pn-t (anti Ara h 1), lane 5: lectin Con A.

3.2 Identification of peanut allergens by BNP

Blue native PAGE and immunoblotting of the fraction pool demonstrated the separation into eight bands with molecular masses between 22 and 150 kDa (Fig. 1b). Immunoblotting with patient serum JG3 showed that all bands are IgE reactive (lane 3) although bands BNP-7 and BNP-8 were only weakly stained by the total protein stain with India ink. Two mAbs showed that some of these bands contained Ara h 3/4 (lane 2, BNP-1(lower part) up to BNP-5) and Ara h 1 (lane 4, BNP-1 (upper part)). The mannose-specific lectin Con A (lane 5) detected the previously described glycosylation of Ara h 1 [6] and suggests a glycosylation of Ara h 3/4.

3.3 Characterization of peanut allergens by BNP/ LDS-PAGE and LDS/LDS-PAGE

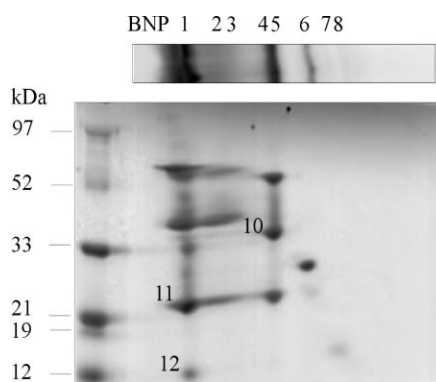
BNP followed by LDS-PAGE under reducing conditions of the high M_r bands BNP-1 to BNP-5 revealed separation into spots with lower M_r and similar protein pattern (Fig. 2a, 2b). The BNP bands BNP-6 to BNP-8 separated slightly differently than BNP-1 to BNP-5; these protein spots could not be identified by *N*-terminal sequencing because these proteins seemed to be blocked. The explanation for the fact that the high M_r bands separated by BNP are slightly blurred is that the ratios of the allergens to each other are variable in each BNP band. Both allergen-specific mAbs PEI-7B2 (anti Ara h 3) and Pn-t (anti Ara h 1) detected some of these proteins depending on the varying proportions of the allergens in each of the bands. The differing proportions were clearly demonstrated in BNP-1 (Fig. 1b, lanes 2 and 4).

Nonreducing LDS-PAGE followed by reducing LDS-PAGE (Fig. 2c) clearly show separation into smaller spots of some proteins. There is a similar diagonal distribution as shown in Fig. 2a, but it is pronounced because of the harsher conditions of the sample preparation which destroy intra- and intermolecular interactions.

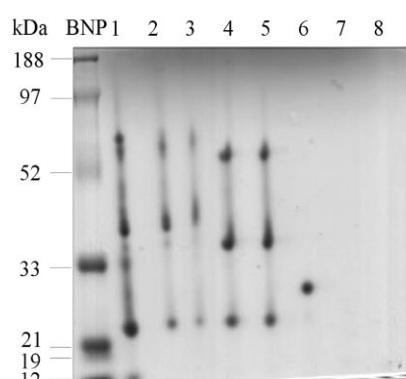
Figure 3 shows the reactivities of patient serum JG3 and the Ara h 3/4-specific mAb PEI-7B2 by immunoblot analysis. The SEC fraction pool was separated by BNP plus LDS-PAGE (Figs. 3a, 3b), and nonreducing LDS-PAGE plus reducing LDS-PAGE (Fig. 3c). PEI-7B2 clearly identifies Ara h 3/4 (Figs. 3a, 3b and 3c; always blot no. 1). In particular, Fig. 3b shows clearly that the mAb detected only the lower M_r part of BNP-1 which consisted of Ara h 3/4 but only a part of iso-Ara h 3 (BNP-4 and BNP-5) and no Ara h 1 (high M_r part of BNP-1) which were detected also by patient serum JG3 (Fig. 3a and 3b, blot no. 2). The mannose-specific lectin Con A identified the glycosylation of Ara h 1 (Fig. 3c, blot no. 2) and suggested a glycosylation of Ara h 3/4, which is far less intensive than that of Ara h 1.

3.4 *N*-terminal sequencing and MALDI-TOF

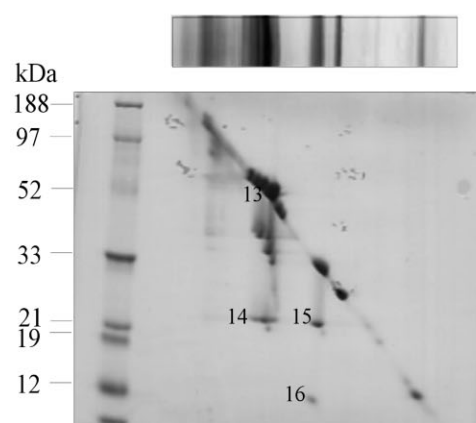
To confirm the LDS-PAGE and immunoblotting results, *N*-terminal sequencing was performed on spots shown in Figs. 2 and 4. IgE reactive bands under reducing SDS-PAGE conditions (Fig. 4a) and the PEI-7B2 and IgE reactive 2-D spots (Fig. 4b) of peanut extract were analyzed first. *N*-terminal sequencing of the six bands (Fig. 4a) resulted in amino acid sequences that are present in different regions of the peanut proteins Ara h 3, Ara h 4, Ara h 3/4, peanut trypsin inhibitor, and Gly 1 (Table 2).



(a) Blue native / reducing LDS-PAGE, whole strip



(b) Blue native / reducing LDS-PAGE, single cut out bands



(c) Nonreducing / reducing LDS-PAGE, whole strip

Figure 2. 2-DE analysis of the fraction pool B9-B15 (Coomassie stained gels). (a) 1. dimension: BNP; 2. dimension LDS-PAGE under reducing conditions. Numbers indicate the bands of the complete first dimension gel (BNP1 – BNP8) as described in Fig. 1b. Spot numbers 10–12 in the gel are analyzed further by *N*-terminal sequencing. (b) 1. dimension: BNP; 2. dimension LDS-PAGE under reducing conditions. The single bands BNP1 to BNP8 were cut out of the BNP gel and run separately. (c) 1. dimension: nonreducing LDS-PAGE; 2. dimension: reducing LDS-PAGE. Spot numbers 13–16 were analyzed by *N*-terminal sequencing (results summarized for 2a and 2c in Table 3).

To demonstrate identical proteins in the Superdex fraction pools the same spots and the 28 kDa spots were also analyzed (Fig. 4c). Figure 4d shows the IgE reactivity of these 28 kDa spots using the serum of patient CAT.

Using 2-D PAGE, the different proteins were separated in the first dimension according to their *p*I's and in the second dimension according to their *M*_rs. Table 3 gives a summary of the obtained sequences. The 2-D spots 1, 2, and 3 (Fig. 4b) of peanut extract (pH 3–10) were identical to spots 6, 7 and 5 (Fig. 4c) of the Superdex pool (pH 4–7), which demonstrates that the smaller pH range (pH 4–7) separates the isoforms far better from each other. Other peanut proteins such as Ara h 2 and Ara h 6 are visible in Fig. 4b in the molecular mass range below 30 kDa.

A unique match with GenBank database entries could only be found for the identified amino acid sequences of six spots. Three of these sequences were identified as iso-Ara h 3 (Table 3), a peanut protein not previously described. These three sequences contain the common sequence VTFRQGGEENE. The proteins differ in their *M*_r slightly from each other depending on the different gel types used (Fig. 4). Ten spots corresponded to more than one peanut protein because of their conserved sequence identities to each other. To substantiate these findings the corresponding spots were characterized by in-gel trypsin-digestion and following MS.

Figure 4c shows the white numbered spots that were analyzed by MS as well. Immunoblot analysis with the CAT patient serum (Fig. 4d) showed the IgE reactivity of the spots determined as *N*-terminal part of Gly 1 (spot 2) and Ara h 3/4 (spot 3) respectively (Table 4). The matching *C*-terminal parts for Gly 1 and for Ara h 3/4 are spot 6 and spot 5 respectively. The intense spots of 42 – 22 kDa were the *N*-terminal parts of the different isoforms while the smaller ones at approximately 28 kDa were *C*-terminal fragments of these proteins. The four spots at 28 kDa are IgE reactive, but the intensity of these IgE reactivities were, in comparison to the *N*-terminal parts, less intense. No IgE binding epitope has been described so far for the *C*-terminal parts. In this study IgE binding was detectable with the CAT patient serum. The white numbered spot 4, which belongs to the 28 kDa spots, could also be identified by *de novo* sequencing MS.

N-terminal sequencing for the black numbered protein spot 3 (Fig. 4b) and spot 5 (Fig. 4c) resulted in the sequence containing VTFR present in iso-Ara h 3. The trypsin digestion of this spot resulted in fragments that showed structural similarities to the glycinin family. The location of spot 1 in the gel and the analysis of the surrounding spots clearly indicated that this spot near the white marked spot 2 and spot 3, is the *N*-terminal part of an isoform of the described allergens. This correlates with the results of *N*-terminal sequencing, which gave a 70–85% identity with the identified isoallergens of the complex. Spot 4 is the *C*-terminal part of this protein, which could clearly be identified by *N*-terminal sequencing (Table 3) and MALDI-TOF. This part of the pro-

Table 2. *N*-terminal sequences obtained and sequence alignment with the published peanut allergen sequences of Gly 1, Ara h 3/4, Ara h 4, Ara h 3, putative peanut trypsin inhibitor (pPN TI) and isoAra h 3. Sequences obtained by *N*-terminal sequencing (spots 1 to 7) are shaded in medium gray. IgE reactive epitopes as identified by Rabjohn *et al.* [19] are in bold and italicized and underlined. Dots represent identical amino acids and dashes represent gaps

	leader sequence	N-terminus of mature protein			
Gly1	-MIRGRLALSVCFCFLVLGASS	ISFRQQPEENACQFORLNAQRPDNRLESEGGYIETWNPNNQEFECAGVALSRLVLRNALRRPFYSNAPQEIFIQQGR			
Ara h 3	-----	-----I----- <u>LETWNPNNQEFECAG</u> -----			
Ara h 4	--MAKL.E..F.....	-----I-----			
Ara h 3/4	--MGKL.....	-----I-----			
isoAra h 3	---KL..L..V.....VT..GG..E.....	-----I-----Q.....T.....L..YV..S			
pPN TI	NYLHML.....	-----I-----			
Gly1	GYFGLIFFGCPSTYEPAQQGRRHQSRAPRRFEG--EDSQQQQQDQSHQKVRFRFDEGDLIAVPTGVALWMFNDHDTDVAVSLTDTNNNDNQLDQFPRR				
Ara h 3	RH.....HT...S...P...LQ--R-----H.....F.LY.....				
Ara h 4	Y...P...LQE--R-----H.N.....F.LY.....				
Ara h 3/4	P...Q--Q-----H.....F.Y.....				
isoAra h 3	E..Y...KPS...QVGQD.P-----H.....F.Y.E...T.T.S.SSIH.....				
pPN TI	P...Q--Q-----H.....F.Y.....				
Gly1	FNLAGNHEQEFLRYQQQSR---RRSLPSPSPSPQSPRQEERFSPRGQHSRRERAGQEEENEGGNIFSGFTPEFLAQAFQVDDRQIVQNLRGENESEEE				
Ara h 3	T.....QSR.....	 <u>GNIFSGFTPEFLQA</u> T.....			
Ara h 4	QSR.....H.R..R...R.....D.....E.....W.....				
Ara h 3/4	T...K..D.....G.....Q.....L.....D.Q				
isoAra h 3	.Y...Q.....QG-----R.--HY.QI.....VR.D.Q...S.....AQ...QH.....T.E.....R.Q				
pPN TI	P...Q--Q-----				
Gly1	GAIVTVKGGRLILSPDRKRG---ADEEEYDEDEYDEE---DRRRGSRGRNGNIEETICTASVKKNIGNRSEDIYNPQAGSLKTAND--LNL				
Ara h 3	 <u>VRGGLRILSPDRK</u> .R----- <u>DEDEYDEE</u> ---DRRRG.....A.....				
Ara h 4	R.....GT.....Q...H.Q---G.....G.....C.....G...H..D..RWFTQNCN---L				
Ara h 3/4	R.....RQYERP.....ERQQ.....S.....F.....ELQ...				
isoAra h 3	EEDSSRSPPSR..F...RSRPQQR---GKYDENR..YK.....S.....L..SSN.....RSV.E--D.				
Gly1	LILRWLGLSAEYGNLYRNALFVPHYNTNAHSIIYALRGRAHVQVDSNGNRVYDEELQEGHVLVVPQNFVAVAGKSQSDNFEYVAFKTDSDRPNIANFAGEN				
Ara h 3	P.....A.....R.....E.....S.....L.....				
Ara h 4	E.....S.....L.....				
Ara h 3/4	D..F.....E.....S.....L.....				
isoAra h 3	P..G.....QH.TI...M.....TL..T.VV..N.....A.A..E.Y..L.....S.....L.....				
Gly1	SIIDNLPPEEVVANSYGLPREQARQLKNNNPFFKFFVPPS-QQSLRAVA	AAG01363	5.48	60.45	
Ara h 3	.V.....Q.....P.....	AAC63045	5.68	58.35	
Ara h 4	.F.....F--P...	AF086821	5.48	61.01	
Ara h 3/4	.F.....E.....	AAM46958	5.52	61.74	
isoAra h 3	R.....FDH..M.E..	AAT39430	5.41	58.06	
pPN TI		AF487543	6.66	25.50	

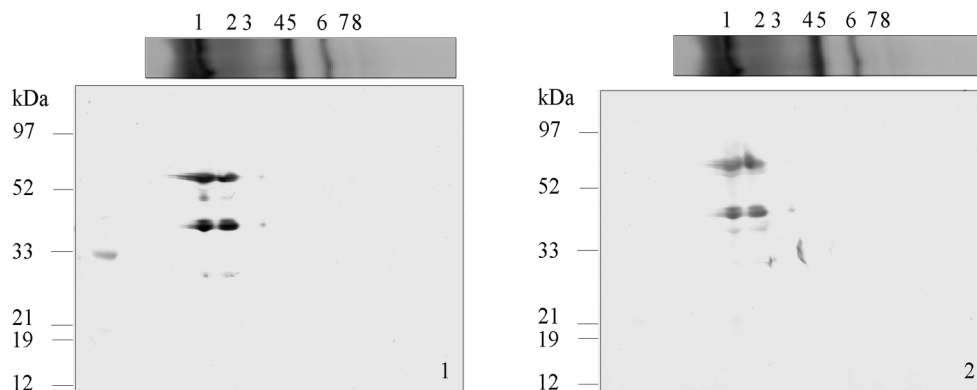
tein always started off with GIEETIC and showed no sequence differences for a stretch of seven amino acids. The previously described protease digestion site (NGLEET) (PROTALL; www.ifr.bbsrc.ac.uk/) seems to be the C-terminal start of all isoforms found in the high M_r complex with a conserved exchange from leucine to isoleucine. These amino acids have a similar chemical structure. This is in accordance with the 11S plant seed storage proteins signature (www.expasy.org).

Figure 2a gives an overview of the whole protein pattern after 2-D separation by BNP and LDS-PAGE. Some of these spots were analyzed further by *N*-terminal sequencing and confirmed the immunoblotting results already described. Spot 10 represented the *N*-terminal parts of iso-Ara h 3 and spot 12 those of Gly 1, Ara h 3, Ara h 4 and Ara h 3/4, respectively, whereas spot 11 represented the C-terminal part of these proteins. The 2-D separation by nonreducing LDS and reducing LDS-PAGE leads necessarily to nearly identical results. Spot 13 forms the *N*-terminus of iso-Ara h 3 and spot 16 of Gly 1, Ara h 3, Ara h 4 and Ara h 3/4, respectively, whereas spot 15 and spot 16 represents the C-terminal parts of these proteins.

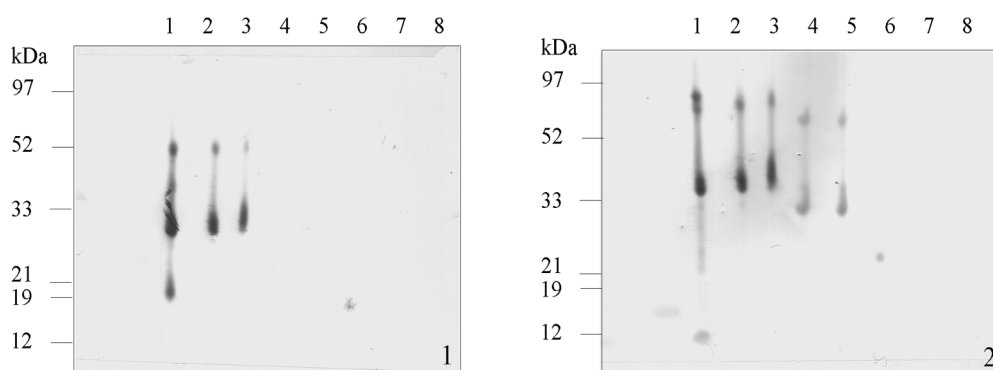
3.5 Sequence alignment

The alignment (Table 2) of amino acid sequences of Ara h 3/4, Ara h 3, Ara h 4, Gly 1 and iso-Ara h 3 showed high degrees of amino acid sequence identity, *e.g.*, 91.3% of Ara h 3 and Ara h 4. There was a similar high identity of 91.6% between Gly 1 and the sequence of the allergen Ara h 3/4. These results lead to the conclusion that these proteins are isoforms of each other, because two allergens are defined as isoforms of each other when the sequence identity of these proteins are at least 75%. Furthermore, the sequence of the putative peanut trypsin inhibitor showed an identity of 96.3% to the acidic subunit of Gly 1. This subunit corresponds to the *N*-terminal part of Gly 1, which is the acidic fragment of the whole protein.

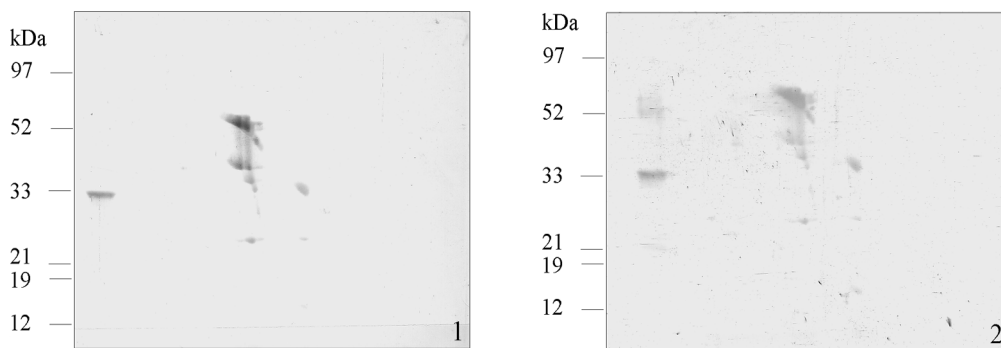
The alignment showed 19 completely conserved regions each ranging in length between 5 and 50 amino acid residues. Major IgE binding epitopes characterized by Rabjohn *et al.* [19] are also found in Gly 1, Ara h 4, Ara h 3/4 and the putative peanut trypsin inhibitor (pPN TI). Epitope 1 (Ara h 3 33–47) is present in all these proteins, whereas epitope 2 (Ara h 3 240–254) and epitope 4 (Ara h 3 303–317) are



(a) Blue native / reducing PAGE, whole strip, Western blots with PEI-7B2 (1) and JG3 (2)



(b) Blue native / reducing PAGE, single strips, Western blots with PEI-7B2 (1) and JG3 (2)



(c) Nonreducing / reducing SDS-PAGE, whole strip, Western blots with PEI-7B2 (1) and Con A (2)

Figure 3. 2-D immunological analysis of the fraction pool B9-B15. For separation conditions see corresponding gels in Fig. 2. The mAb PEI-7B2 was used for detection of Ara h 3/4 (3a-1, 3b-1, and 3c-1), the IgE reactivities of patient serum JG3 are shown (3a-2 and 3b-2), and glycoproteins were demonstrated by Con A-binding (3c-2).

present in Ara h 4, and epitope 3 (Ara h 3 279–293) is present in Ara h 3/4. Some sequence areas present a higher variability, for example in the leader sequence and the region before the protease digestion site (NGIEETIC) for about 50 amino acids.

4 Discussion

The most interesting finding of this study is the fact that the allergens Ara h 3/4 and Ara h 1 form complexes that contain Ara h 1 and Ara h 3/4 and its isoforms (Ara h 3, Ara h 4, iso-

Table 3. Origin of sequences obtained *N*-terminal sequencing of LDS-PAGE bands (LDS 1–LDS 6) or 2-D PAGE spots (spots 1–7 and 28 kDa spots). The expression '*N*-terminal' and '*C*-terminal' refers to the region of the respective peanut allergens that contain the particular sequence. The marked (*, **) sequence matches represent positions 134–142 and 133–141 of Ara h 4, indicating additional fragments that may be present in the complex found in the SEC exclusion volume

Sample and sample preparation	Spot or band no.	Molecular mass	Amino acid sequence	Gly 1	Ara h 3	Ara h 4	Ara h 3/4	iso-Ara h 3	pPN TI
Pooled fractions	10	~34 kDa	VTFRQ	–	–	–	–	<i>N</i> -terminal	–
B9-B15 BNP + red.	11	~21 kDa	GIEETIXTA	<i>C</i> -terminal	<i>C</i> -terminal	<i>C</i> -terminal	<i>C</i> -terminal	–	–
LDS-PAGE (Fig. 2a)	12	~11 kDa	ISFRQQPE	<i>N</i> -terminal	–	<i>N</i> -terminal	<i>N</i> -terminal	–	<i>N</i> -terminal
Pooled fractions	13	~60 kDa	VTFRQGGEEEN	–	–	–	–	<i>N</i> -terminal	–
B9-B15 nonred.	14	~21 kDa	GIEETIXTAS	<i>C</i> -terminal	<i>C</i> -terminal	–	<i>C</i> -terminal	–	–
LDS-PAGE + red.	15	~21 kDa	GIEETICTAS	<i>C</i> -terminal	<i>C</i> -terminal	–	<i>C</i> -terminal	–	–
LDS-PAGE (Fig. 2c)	16	~11 kDa	ISFRQQPEEN	<i>N</i> -terminal	–	<i>N</i> -terminal	<i>N</i> -terminal	–	<i>N</i> -terminal
Peanut extract	LDS1	~42 kDa	ISFRQQPXENA	<i>N</i> -terminal	–	<i>N</i> -terminal	<i>N</i> -terminal	–	<i>N</i> -terminal
LDS-PAGE (Fig. 4a)	LDS2	~36 kDa	ISFRQQPXENA	<i>N</i> -terminal	–	<i>N</i> -terminal	<i>N</i> -terminal	–	<i>N</i> -terminal
	LDS3	33 kDa	VTFRQGGEEENEXQF	–	–	–	–	<i>N</i> -terminal	–
	LDS4	21 kDa	GIEETIXTASVK	<i>C</i> -terminal	<i>C</i> -terminal	–	–	–	–
	LDS5	20 kDa	EDQSXQQDD	–	–	<i>N</i> -terminal*	–	–	–
	LDS6	12 kDa	ISFRQQPEENAXQF	<i>N</i> -terminal	–	<i>N</i> -terminal	<i>N</i> -terminal	–	<i>N</i> -terminal
Peanut extract	1	~36 kDa	ISFRQQPEE	<i>N</i> -terminal	–	<i>N</i> -terminal	<i>N</i> -terminal	–	<i>N</i> -terminal
IPG-PAGE +	2	42 kDa	ISFRQQPEE	<i>N</i> -terminal	–	<i>N</i> -terminal	<i>N</i> -terminal	–	<i>N</i> -terminal
SDS-PAGE (Fig. 4b)	3	~30 kDa	VTFRQGGEE NECQFQRLNAQ	–	–	–	–	<i>N</i> -terminal	–
	4	~28 kDa	EEDQSQQQQ	–	–	<i>N</i> -terminal**	–	–	–
Pooled fractions	5	~30 kDa	VTFRQGG	–	–	–	–	<i>N</i> -terminal	–
B9-B15 IPG-PAGE +	6	~36 kDa	ISFRQQP	<i>N</i> -terminal	–	<i>N</i> -terminal	<i>N</i> -terminal	–	<i>N</i> -terminal
SDS-PAGE (Fig. 4c)	7	~36 kDa	ISFRQQP	<i>N</i> -terminal	–	<i>N</i> -terminal	<i>N</i> -terminal	–	<i>N</i> -terminal
	28 kDa	28 kDa	GIEETI	<i>C</i> -terminal	<i>C</i> -terminal	<i>C</i> -terminal	<i>C</i> -terminal	<i>C</i> -terminal	–
	spots	28 kDa	GIEETI	<i>C</i> -terminal	<i>C</i> -terminal	<i>C</i> -terminal	<i>C</i> -terminal	<i>C</i> -terminal	–
		28 kDa	GIEETI	<i>C</i> -terminal	<i>C</i> -terminal	<i>C</i> -terminal	<i>C</i> -terminal	<i>C</i> -terminal	–

Ara h 3, Gly 1) in different ratios. These complexes have molecular masses of more than 100 kDa (Fig. 1b, Fig. 2b). This composition was demonstrated by BNP of the high molecular fraction obtained by SEC. BNP showed five slightly blurred bands ranging in size from 60–150 kDa, which indicates the heterogeneity of the complex composition. Most of these bands separated into almost identical separation patterns, even though the amount of the individual proteins varied.

Legumins form hexameric heteromeric complexes with molecular masses from 300–450 kDa; these complexes are held together by noncovalent bonds [20]. The BNP band with the highest molecular mass of 150 kDa (BNP-1) includes Ara h 3/4 with its isoforms and Ara h 1 (Fig. 2b), whereas the other BNP bands of approximately 100 kDa and 50–60 kDa seemed to be subsets of BNP-1. The bands with molecular masses of 30 kDa or less split into two and one spot, respectively. These spots were IgE reactive but could not be identified since they ran differently as compared to the other bands and were apparently *N*-terminally blocked.

Until now four sequences of the allergen Ara h 3/4 have been submitted to the GenBank database, named Ara h 3/4, Ara h 3, iso-Ara h 3 and Ara h 4. Another sequence desig-

nated as Gly 1, a glycinin-type seed storage protein that so far has not been described as IgE reactive, even though it contains amino acid sequences that were identified as IgE reactive epitopes of Ara h 3 [21]. Immunoblot analysis demonstrated IgE reactivity to the *N*-terminal part of Gly 1 with patient serum JG3 after SDS-PAGE and 2-DE. In contrast to JG3, the serum of patient CAT did detect the *C*-terminal parts of Gly 1 and the other allergens. Comparison of the amino acid sequences of Ara h 3/4, Ara h 3, Ara h 4, Gly 1 and iso-Ara h 3 (Table 2) showed a high degree of identity and the results of this paper give evidence that these proteins, including the putative peanut trypsin inhibitor, are Ara h 3/4 isoforms.

It is possible that the acidic part of these isoforms may have its own function as an inhibitor similar to Ara h 2 for example [22, 23]. PTMs occur during and after peanut maturation. Cleavage of the conserved protease digestion site always results in an acidic (*N*-terminal) fragment ending with an asparagine (N) and a basic (*C*-terminal) fragment starting with GIEETIC which is found in all identified isoforms. This seems to be a typical principle which is conserved in all 11S plant seed storage proteins.

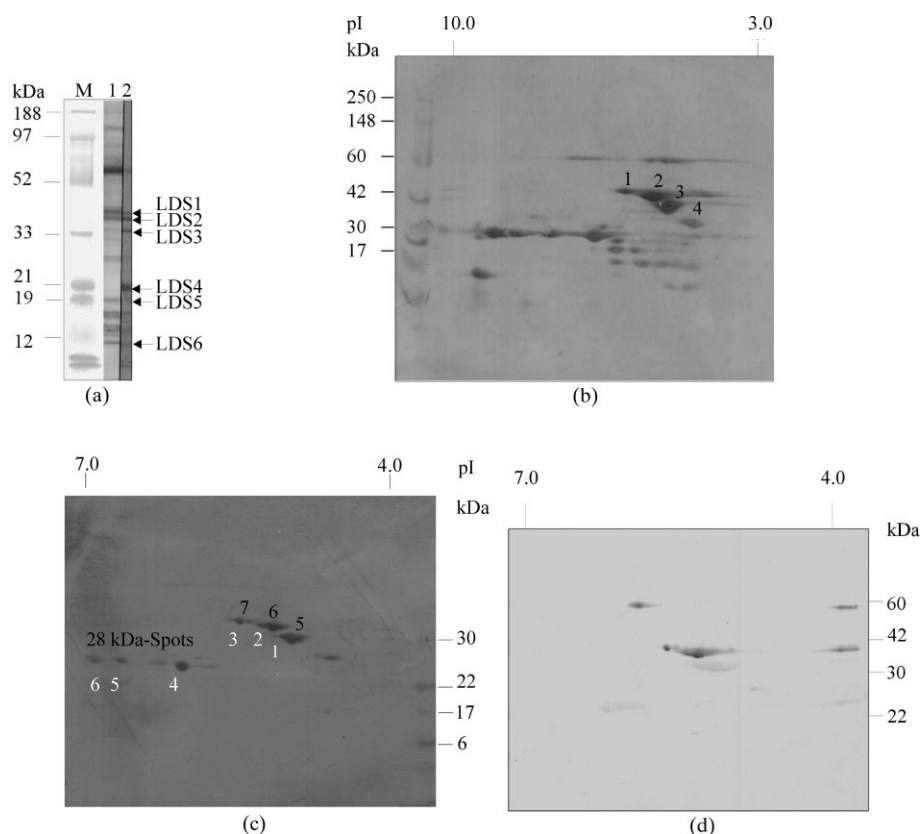


Figure 4. *N*-terminal sequencing of peanut extract and the fraction pool B9-B15. (a) Peanut extract separated by LDS-PAGE and blotted onto PVDF membrane. IgE reactivity (patient JG3, lane 1) and protein staining (Coomassie staining, lane 2) are shown. The IgE-reactive bands (LDS 1–6) were analyzed by *N*-terminal sequencing. (b) 2-D PAGE of peanut extract. Protein spots 1–4 were further characterized by *N*-terminal sequencing. (c) 2-D PAGE of fraction pool B9-B15. Protein spots were further analyzed by *N*-terminal sequencing from the PVDF membrane (numbered in black) and by MALDI-TOF analysis from the original gel (numbered in white). (d) Immunoblot with patient serum CAT to show the IgE reactivity of *N*- and C-terminal parts.

Interestingly the fourth identified IgE epitope [21] is not conserved in the iso-Ara h 3 sequence. This epitope is completely missing. There seems to be only a slight decrease of IgE reactivity of iso-Ara h 3 compared to the other proteins that carry all four IgE epitopes. The structure of this protein was deduced from a cDNA sequence but neither identified as protein nor as allergen in peanut extract. In contrast to the IgE reactivity, the mAb PEI-7B2 detected only a very small part of the protein spot (black spot 5, Fig. 4c), not the whole one and the intensity of antibody binding is very much reduced (data not shown).

SDS-PAGE of recombinant Ara h 3 always results in only one distinct protein band of 60 kDa [19, 24, 25]. The IgE reactive natural Ara h 3 may be detected at 14 kDa [26] under reducing conditions indicating PTMs that occur in the natural environment in peanuts and that *in vitro* heterologous

expression in *Escherichia coli* can not recreate these conditions [27–28]. SEC as a non-denaturing method is helpful to isolate the Ara h 3/4- and Ara h 1-containing complex from the peanut extract without destroying its natural state. BNP is an *in vitro* method to analyze the whole complex without destroying it. The principle for blue native PAGE is that Coomassie-blue charges proteins negatively; thus, they migrate in an electric field without influencing their quaternary protein structure dramatically.

The reason for the varying composition of the complexes may be the optimal storage of the complexes in the peanut kernel. In turn the varying composition leads to slightly different molecular weights. However, the different complexes contain the same subunits in different proportions as shown by the combination of BNP and SDS-PAGE under nonreducing or reducing conditions. The changing proportions of the components in the complex may explain why the protein bands in the native gel are not distinct but slightly blurred. It further explains why both antibodies specific for Ara h 1 (Pn-t) and Ara h 3/4 (PEI-7B2) recognize the same bands after separation under native conditions. When the structure changes slightly with its variable proportions, the detectable epitopes for the antibodies may vary in their arrangement and may sometimes not be accessible.

In 2-D PAGE these conformational problems may not be relevant because of denaturation due to the reducing and alkylating agents used for SDS-PAGE. This leads to a separation of the isoforms only according to their isoelectric points and their molecular weights. The antibodies are able to detect the now accessible epitopes of the isoforms on the blot. All spots are distinct because the secondary structure of the proteins is disturbed by SDS.

N-terminal sequencing of some of the 2-D spots at ~30 and 60 kDa and of one band (LDS-3) at 35 kDa on a reducing LDS gel gave rise to another new, previously unknown, amino acid sequence. This sequence VTFRQGGEE-NECQFQRLNAQ (iso-Ara h 3) shows high sequence identity (70–85%) to the sequences of Ara h 3/4 / Ara h 4 / Gly 1, and the putative peanut trypsin inhibitor but the sequences show a higher diversity within this group compared to other parts of these sequences. These six isoforms with their significantly high sequence identity belong to the family of seed

Table 4. Sequence coverage (shaded) obtained by MALDI-TOF analysis of 2-D PAGE spots after in-gel trypsin digestion. The spot numbers correspond to the spots in Fig. 4c. Some shaded areas represent consecutive peptides

N-terminal part of the protein:

```

1: VTFRQGGEENECQFQRLNAQRPDNRIESEGGYIETWNPNNQEFQCAGVALSRTLRLRNALRRPFYSNAPLEIYVQQGSGYFGLIFP
2: ISFRQQPEENACQFQRLNAQRPDNRIESEGGYIETWNPNNQEFECAGVALSRLVLRRNALRRPFYSNAPQEIFIQQGRGYFGLIFP
3: ISFRQQPEENACQFQRLNAQRPDNRIESEGGYIETWNP-NQEFECAGVALSRLVLRRNALRRPFYSNAPQEIFIQQGRGYFGLIFP

1: GCPSTYEPAQEGRRYQSQKPSRRFQVGQDDPSQQQQDSHQKVHRFDEGDLIAVPTGVAFWMYNDDEDTDVVTVTLSDTSSIHNQLD
2: GCPSTYEPAQQGRRHQSQRAPRRFEGEDQSQQQQDSHQKVRRFDEGDLIAVPTGVAFWMYNDHDTDVVAVSLT-DTNNNDNQD
3: GCPSTYEPAQQGRRHQSQRPPRRFQVGQDDPSQQQQDSHQKVHRFDEGDLIAVPTGVAFWMYNDHDTDVVAVSLT-DTNNNDNQD

1: QFPRRFYLAGNQEQEFLRYQQQGSR---PHY-----RQISPRVR---GDEQENEGSNIFSGFAQEFLQHAFO
2: QFPRRFNLAGNHEQEFLRYQQQ--SRRRSLPYSPYSPQSQPRQEERFSPRGQHSRRERAGQEENEEGGNIFSGFTPEFLAQAFQ
3: QFPRRFNLAGNHEQEFLRYQQQ--SRRRSLPYSP-SPQ-PKQEDREFSPRGQHRRERAGQEENEEGGNIFSGFTPEFLAQAFQ

1: VD-RQTVENLRGENEREEQGAIVTVKGGLRILSPDEEDESSRSPSRREEFDEDRSRPQQRKY-----DENRRGYKN
2: VDDRQIVQNLRGENESEEEGAIVTVKGGLRILSPDRKRGAD-----EEEEYDEDEYEYDEE-----DRRRGRGSRGRGN
3: VDDRQILQNLRGENESDEQGAIVTVKGGLRILSPDRKRRQYERPDEEEEYDEDEYEYDEEEERQQDRRRGRGSRGSGN

```

C-terminal part of the protein:

```

4: GIEETICSASVKKNIGRSSNPDIYNPQAGSLRSVN--ELDLPILGWLGLSAQHGTIYRNAMFVPHYTLNAHTIVVALNGRAHVQ
5: GIEETICTASFKKNIGRNRSPDIYNPQAGSLKTANELQLNLLILRWLGLSAEYGNLYRNALFVPHYNTNAHSIIYALRGRAHVQ
6: GIEETICTASVKKNIGRNRSPDIYNPQAGSLKTAN--DLNLLILRWLGLSAEYGNLYRNALFVPHYNTNAHSIIYALRGRAHVQ

4: VVDSNGNRVYDEELQEGHVLVVPQNFAVAAKAQSENEYLAFKTDSRPSIANLAGENSIIDNLPEEVVANSYRLPREQARQLKN
5: VVDSNGDRVDEELQEGHVLVVPQNFAVAGKSQSENFEYVAFKTDSRPSIANLAGENSFIDNLPEEVVANSYGLPREQARQLKN
6: VVDSNGNRVYDEELQEGHVLVVPQNFAVAGKSQSDNFEYVAFKTDSRPNIANFAGENSIIDNLPEEVVANSYGLPREQARQLKN

4: NNPFKFFVPPFDHQSMREVA
5: -NPFKFFVPPSEQNSLRAVA
6: NNPFKFFVPPSQQ-SLRAVA

```

spot 1 and spot 4	iso-Ara h 3	ACCESSION AAT39430
spot 2 and spot 6	Gly 1	ACCESSION AAG01363
spot 3 and spot 5	Ara h 3/4	ACCESSION AAM46958

storage proteins. As long as their function as storage protein and essential amino acids are not destroyed by cross-overs, deletions or inserts of amino acids there is no need for the plant to produce 100% identical proteins within this family. This investigation identified Gly 1 and iso-Ara h 3 for the first time as new isoforms of Ara h 3/4, which show IgE reactivity.

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