

Identification and partial characterization of multiple major allergens in peanut proteins

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Summary

Background Peanuts are a major cause of food allergies both in children as in adults which can induce an anaphylactic shock. The identification and characterization of peanut allergens could lead to more insight into the mechanism and contribute to the improvement of diagnostic tests and treatment for peanut allergy.

Objective In the present study, the peanut protein-specific immunoglobulin concentrations as well as their recognition of the various peanut proteins or protein subunits was determined in the plasma of peanut-allergic (PA) and non-allergic (NA) individuals. Moreover, two peanut allergens were characterized in more detail to confirm them as the earlier described Ara h1 and Ara h2.

Methods The presence of Ig-binding sites in peanut proteins was studied by immunoblotting assays whereas the concentrations of peanut-specific Ig was determined by ELISA.

Results Peanut proteins were found to contain multiple binding sites for immunoglobulins. Of these proteins, six were recognized by peanut-specific IgE present in more than 50% of the plasma samples of the PA group. Their molecular weights were ≈ 44 , 40, 33, 21, 20 and 18 kDa. The last three protein bands were recognized by peanut-specific IgE present in more than 70% of the PA plasma samples and were thought to contain Ara h2. This allergen as well as another protein that was thought to be Ara h1, which was not recognized by the majority of the patients' IgE-containing plasma samples, were isolated and the N terminal amino acid sequence was determined. Peanut protein-specific IgA, IgM, IgG and IgG-subclasses showed a more diverse recognition pattern of peanut protein in the PA group compared to the NA group. No differences were found in the plasma concentrations of peanut protein-specific immunoglobulins of the various classes between the PA and NA group.

Conclusions From the present study, we conclude that peanuts contain multiple allergens, of which six can be described as major allergens, Ara h2 included. In our population Ara h1 is not a major allergen. The recognition of peanut proteins by immunoglobulins is more diverse in PA individuals compared with NA individuals which, however, is not substantiated in the concentrations of peanut-specific immunoglobulins in plasma, other than IgE.

Keywords: food allergy, peanut allergy, peanut proteins, major allergens, IgE, immunoblotting, ELISA, Ara h1, Ara h 2, N-terminal amino acid sequence

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Introduction

The occurrence of food allergies is estimated between 1 and 2% of the western population [1,2], especially in young children [3]. Adverse reactions to foods involve the gastro-intestinal tract (nausea, vomiting and diarrhoea), the skin (hives and angioedema), and the circulatory system (hypotension and ultimately systemic anaphylaxis) [4].

Peanuts, along with milk and eggs, account for $\approx 80\%$ of adverse reactions to foods in patients with atopic dermatitis [5,6]. Fatal anaphylactic reactions induced by peanuts are not uncommon [7]. Allergic reactions to peanuts tend to persist for life, in contrast with foods like milk, eggs and soy. Of the positive food challenges with milk and egg in children younger than 3 years, 44% is negative when tested 1–7 years later whereas the majority was still positive in the response to peanuts [3].

Several food allergens inducing IgE-mediated disorders in humans, have been identified as in cod fish [8], shrimp [9–11] hen's egg [12,13], cow's milk [14,15], soybean [16] and peanut [17–22]. Peanut is an oil-legume containing about 44–56% of oil and 22–30% of protein [23]. The peanut protein can be divided into albumins, and the storage proteins arachin and conarachin which comprise about 87% of the total protein contents [24].

Sachs *et al.* described the first major allergen in peanuts, designated Peanut-I, which existed of two major bands with molecular weights of ≈ 20 –30 kDa [17]. Barnett *et al.* demonstrated IgE-binding to a Concanavaline A-reactive glycoprotein of ≈ 65 kDa, that the allergens of peanut are spread through both arachin and conarachin and that roasting does not seem to affect the allergenicity [18,19]. Burks *et al.* identified two major allergens, Ara h1 [20] and Ara h2 [21], with molecular weights of 63.5 and 17 kDa, respectively. Whether concanavaline A-reactive glyco-protein and Ara h1 or Peanut-1 and Ara h2 are the same proteins, is unknown.

The identification and characterization of allergens is essential for the understanding of the specific IgE mediated immune response [25]. Moreover, their identification could contribute to the improvement of diagnostic tests and treatment of peanut allergy. In a previous study we showed that the recognition of epitopes by Crude Peanut Extract (CPE)-specific T cell clones from a peanut allergic donor is diverse [26]. In this study, we investigated the recognition of peanut proteins by immunoglobulins in the plasma of peanut allergic patients (PA) compared with non-allergic (NA) individuals. To this aim, plasma samples from PA and NA subjects were analysed by immunoblotting and enzyme-linked immunosorbent assay (ELISA) for the presence of peanut specific IgA, IgE, IgG, IgM and IgG-subclasses antibodies. Furthermore, in order to confirm that the most recognized protein fraction was Ara h2, and

another protein the previously described major allergen Ara h1, the proteins were isolated and the N-terminal amino acid sequence was determined.

Materials and methods

Subjects and plasma collection

The PA group consisted of 14 individuals with a clinically manifest peanut allergy as indicated by a double-blind, placebo-controlled peanut challenge, a positive skin-prick test (SPT) and a positive radio-allergosorbent test (RAST) scores to peanut proteins. The NA group consisted of 10 individuals with no history of allergy and with a negative RAST and SPT for seven common allergens (*alternaria*, birch, bird, cat, dog, grass and house dust mite) and peanut. Subjects' characteristics are summarized in Table 1.

Plasma was collected after centrifugation of heparinized venous blood at 300 g for 10 min at room temperature. The plasma was kept at -20°C until used.

Crude peanut protein (CPE) preparation

Peanut proteins were extracted from raw, unshelled peanuts of the Runner Jumbo 38/42 variety (van Zijl, Hilversum, the Netherlands) as described previously [27].

Table 1. Characteristics of the PA and NA subjects

	NA	PA
<i>n</i>	10	14
Mean age (years)	32.3	30.6
Sex (M/F)	2/8	3/11
Total IgE (IU/mL)	102 $\pm$ 148	2334 $\pm$ 4792
SPT ¹	negative	positive
RAST ¹	negative	positive
SPT ²	negative	positive
RAST ²	negative	positive
Clinical symptoms ³	AD (3), AE (2), Ana (2), C (1), Coll (1), D (5), Dt (1), GI (7), OAS (2), Prur (5), R (1)	
Peanut RAST score ⁴	1 (1), 2 (0), 3 (3), 4 (6), 5 (4)	

¹SPT/RAST for seven common allergens.

²SPT/RAST peanut specific.

³Clinical symptoms of PA patients after a double-blind, placebo-controlled peanut challenge at threshold dose with between brackets the number of patients. AD, flare-up of atopic dermatitis; AE, angioedema; Ana, anaphylactic shock; C, contact urticaria; Coll, collapse; Dt, dyspnoea; GI, gastro-intestinal symptoms; OAS, oral allergy syndrome; Prur, pruritus; R, rhinitis.

⁴Peanut-specific RAST score of PA patients with between brackets the number of patients.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed essentially according to Laemmli [28] using two types of prepacked gels 12% (two wells prep comb, BioRad Laboratories, Richmond, CA, USA) and 15% (10 wells, BioRad) Tris-HCl gels. Prior to loading, protein samples were reduced in extraction buffer, consisting of 1% (w/v) dithiothreitol (DTT), 63 mM Tris-HCl, 2% (w/v) sodium dodecyl sulfate (SDS), 0.01% (w/v) bromophenol blue, 20% (v/v) glycerol, pH 6.8, at 100 °C for 20 min. The 12% gel was loaded with 50 µL/well CPE (5 mg/mL) and the 15% gel with 10 µL/well. Pre-stained molecular weight markers (14.3–200 kDa, BioRad) were used as a reference. Electrophoresis was performed in 0.025 M Tris-HCl, 0.192 M Glycine and 0.1% SDS using a BioRad Mini Protean II system (15 min at 80 V, followed by 1 h at 160 V). If not used for immunoblotting, gels were stained with Coomassie brilliant blue R-250.

Immunoblotting

After separation of CPE by SDS-PAGE, proteins were transferred to polyvinylidene-difluoride membrane (PVDF-membrane, Immobilon-P Transfer membrane, Millipore Corporation, Bedford, MA, USA) essentially according to Towbin *et al.* [29]. Blotting was performed for 1 h at 100 V using a Mini Trans-Blot System and Power Supply BioRad. After blotting, remaining binding sites were blocked for 1.5 h with 3% milk powder (Protifar, Nutricia, the Netherlands) in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4). Subsequently the PVDF-membranes were cut in either two (for the 12% gel) or 10 (for the 15% gel) identical strips. The strips were incubated overnight with plasma 1:10 (v/v) in TBS buffer containing 1% milk powder for 15% gel blots; 1:25 (v/v) for 12% gel blots at room temperature on a rocking facility.

For the detection of CPE-specific IgA, IgE, IgM, IgG and IgG-subclasses, the 12% gel blots were placed in a mini-protean II multi screen (BioRad) containing 20 lanes which were used to incubate the blot with various monoclonal antibodies. Peroxidase (PO)-conjugated rabbit-antihuman-IgA, -IgE, -IgM and -IgG (DAKO, Glostrup, Denmark) were used in a dilution of 1:1000 (v/v) in TBS buffer/1% milk powder with exception of IgG which was used at a 1:500 (v/v) dilution in TBS buffer/1% milk powder. To detect IgG-subclasses, mouse-antihuman-IgG1, -IgG2, -IgG3 and -IgG4 (The Central Laboratory for Blood Transfusion Services, Amsterdam, The Netherlands) were used at a 1:1000 (v/v) dilution in TBS buffer/1% milk powder. After incubation at 37 °C for 2 h the lanes of the block containing anti-IgG-subclasses antibodies, were washed and incubated for another hour with PO-conjugated rabbit-antimouse-IgG

antibodies (DAKO). Subsequently, the blots were washed thoroughly and developed for peroxidase activity using chloronaftol/DAB staining.

To determine in more detail the IgE binding in the plasma, the 15% gels were used. The blots were incubated and stained as described above.

Specific binding to CPE on immuno blot was analysed with a computer programme (Diversity One™ V1.1; IDF Inc., Huntington Station, NY, USA) with which the blots are scanned and stained proteins, indicating specific binding, were detected.

CPE-specific ELISA

A CPE-specific ELISA was performed using 96-well flat bottom plates (Maxisorp, NUNC, Roskilde, Denmark) coated with CPE (1 µg/mL, 100 µL/well) in 0.1 M sodium carbonate buffer, pH 9.6, overnight at 4 °C. Plasma was used in threefold dilutions with starting dilutions as follows: for IgA 1:10, for IgM 1:20, for IgG 1:200, for IgG1 1:20, for IgG2 1:20, for IgG3 1:10 and for IgG4 1:10. After incubation for 1 h at 37 °C with plasma, binding of CPE-specific immunoglobulins was detected with the above described antibodies. PO-conjugated antibodies directed against IgA, IgM and IgG were used at a dilution of 1:2000, while antibodies against the IgG-subclasses were diluted 1:1000. For the detection of the IgG-subclasses the secondary antibodies, rabbit-antimouse-IgG-PO conjugated (1:1000) was used. Bound PO-conjugated antibodies were detected using 3,3',5,5'-tetramethyl-benzidine (TMB) as substrate and absorbance was measured at 450 nm. One arbitrary unit was defined as the amount of CPE-specific antibodies present in 1 mL of an appropriate dilution of pooled plasma. This pooled plasma consisted of plasma of all subjects used in this study.

Purification of Ara h 1 and Ara h 2

All steps were performed at room temperature. Lyophilized CPE was dissolved in 20 mM TRIS-bis-propane, pH 7.2 (TBP) to a final concentration of 1 mg/mL. Undissolved particles were removed by centrifugation (3000 g, 15 min) and the solution was applied on a 8 mL Source Q column (1 × 10 cm) previously equilibrated with TBP. After washing the column with 80 mL of TBP, a linear gradient of 200 mL (0–1 M NaCl in TBP) was applied to elute the bound proteins (2 mL/min). Fractions of 4 mL were collected and analysed by SDS-PAGE using 8–25% gels essentially as described above. To remove traces of peanut lectin from Ara h2 (less than 1%), Ara h2 was dialysed against 50 mM NaAc, pH 5.0 and loaded on a 1 mL Source S column (0.5 × 5 cm) equilibrated with 50 mM NaAc, pH 5.0. After washing with 10 mL of 50 mM NaAc, pH 5.0

the column was eluted using a 25 mL linear gradient (0–500 mM NaCl in 50 mM NaAc, pH 5.0) with a flow velocity of 0.25 mL/min. Fractions of 0.5 mL were collected and analysed by SDS-PAGE. Ara h1 and Ara h2 were stored at -30°C until use. Purified Ara h1 and Ara h2 blotted on PVDF membranes and blot fragments were N-terminal sequenced using a gas-phase sequencer (performed at SECU, Utrecht, the Netherlands).

Statistical analysis

Statistical analysis was performed using a Students' *t*-test taking $P < 0.05$ as level of significance.

Results

Analysis of IgE binding to peanut proteins

To investigate which peanut proteins may play a role in peanut allergy, the IgE-binding to peanut proteins in plasma samples obtained from peanut allergic individuals was studied. Coomassie brilliant blue staining of the SDS-PAGE gel loaded with CPE demonstrated 14 bands in CPE with molecular weights ranging from about 10–70 kDa (Fig. 1). In general, immunoblotting demonstrated

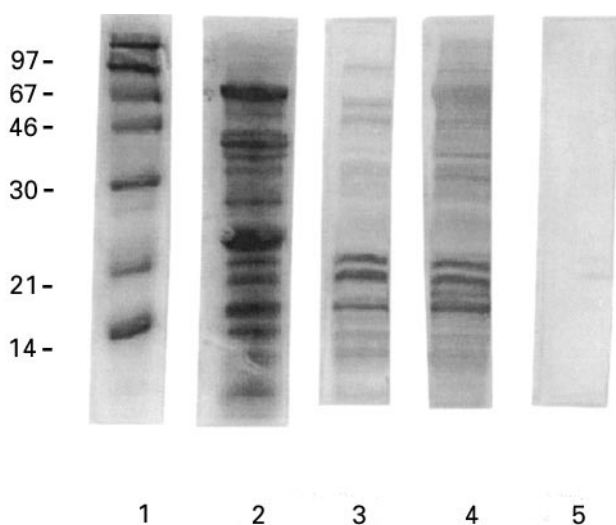


Fig. 1. Detection of CPE-specific IgE in plasma of the PA subjects. Lane 1: Coomassie brilliant blue staining of molecular weight markers. Lane 2: Coomassie brilliant blue staining of CPE (10 μL of a 5 mg/mL solution). Lane 3: Detection of CPE-specific IgE on immunoblot of CPE incubated overnight with individual plasma (1:10 v/v diluted) of donor R. Lane 4: Detection of CPE-specific IgE on immunoblot incubated overnight with pooled plasma of the PA group (1:10 v/v diluted). Lane 5: Control immunoblot which was not incubated with plasma, otherwise following the same procedure.

a similar protein pattern as Coomassie brilliant blue gel staining, although some additional protein bands could be discovered on immunoblots, mainly due to a higher sensitivity of the immunoblotting method. However, as is shown by incubating the immunoblot with an individual plasma (Lane 3) and pooled plasma of the PA group (Lane 4), not all proteins present in the CPE were recognized by patients' IgE. IgE-specific binding to the 14 peanut proteins visible on the SDS-PAGE gel was investigated by immunoblotting analysis using the individual plasmas of all 14 PA patients and non-allergic controls. No peanut-specific IgE-binding was detected using the plasma samples of non-allergic controls. In Fig. 2 the analysis of the IgE-binding is shown in percentages of reactive plasma samples of the PA individuals. IgE-binding occurred to almost all peanut proteins although the highest percentage of IgE-binding was found for six peanut protein bands that were designated protein band number 3, 4, 6, 10, 11 and 12. The estimated molecular weights of these proteins are ≈ 44 , 40, 33, 21, 20 and 18 kDa. The last three proteins were recognized by specific IgE in more than 70% of the investigated plasma samples of the PA group. Remarkably, one protein band of ≈ 23 kDa, which is clearly present on SDS-PAGE, did not bind any IgE. As the electrophoretic and immunoblotting analysis was carried out under reduced conditions, it is not possible to indicate if these proteins are actual individual proteins or subunits derived from higher molecular weight proteins. It was presumed that the proteins of ≈ 20 kDa

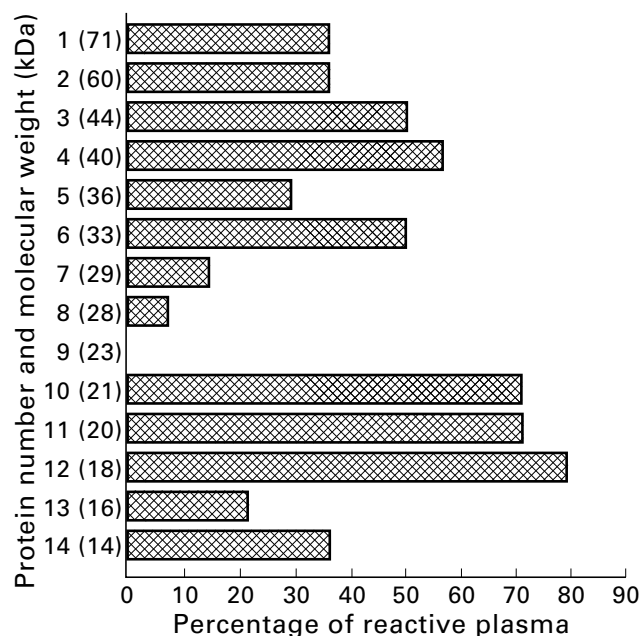


Fig. 2. Percentage of plasma samples of the PA individuals showing specific IgE binding to the different proteins of CPE.

which were recognized by more than 70% of the plasma samples were the previously described Ara h2. Therefore, these proteins have been isolated from CPE as well as Ara h1.

Immunoblotting analysis of peanut protein-specific immunoglobulin-binding

In order to compare peanut-specific immunoglobulin-binding in plasma samples obtained from peanut allergic individuals to non-allergic individuals, immunoblotting was performed with both individual and pooled plasma samples. Figure 3 shows the binding of IgA, IgE, IgM, IgG and IgG-subclasses to CPE on immunoblots after incubation with pooled plasma of the two groups. As expected, this figure shows that CPE contains multiple binding sites for the examined IgA, IgM, IgG and the IgG-subclasses. Moreover, CPE-specific IgE could exclusively be detected in the sera of PA subjects. Using these immunoblots incubated with

pooled plasma of the two groups, the diversity of Ig-binding proteins was determined. Except for IgM, the most diverse binding was found in the PA group as is shown in Fig. 4. The antibody binding to the proteins of 18–21 kDa is the most obvious in the plasma samples of PA patients. In the binding pattern of IgG1 no differences were observed between the groups.

Analysis of peanut protein-specific antibodies by ELISA

To appreciate the above described immunoblotting analysis in a more quantitative manner, ELISAs were developed for the measurement of CPE-specific immunoglobulins in the plasma of all subjects. No statistically significant difference was observed in the plasma levels of CPE-specific immunoglobulins (data not shown). However, CPE-specific IgG appeared to be elevated in the PA group, which was mostly due to elevated, but not statistically significant, levels of IgG4.

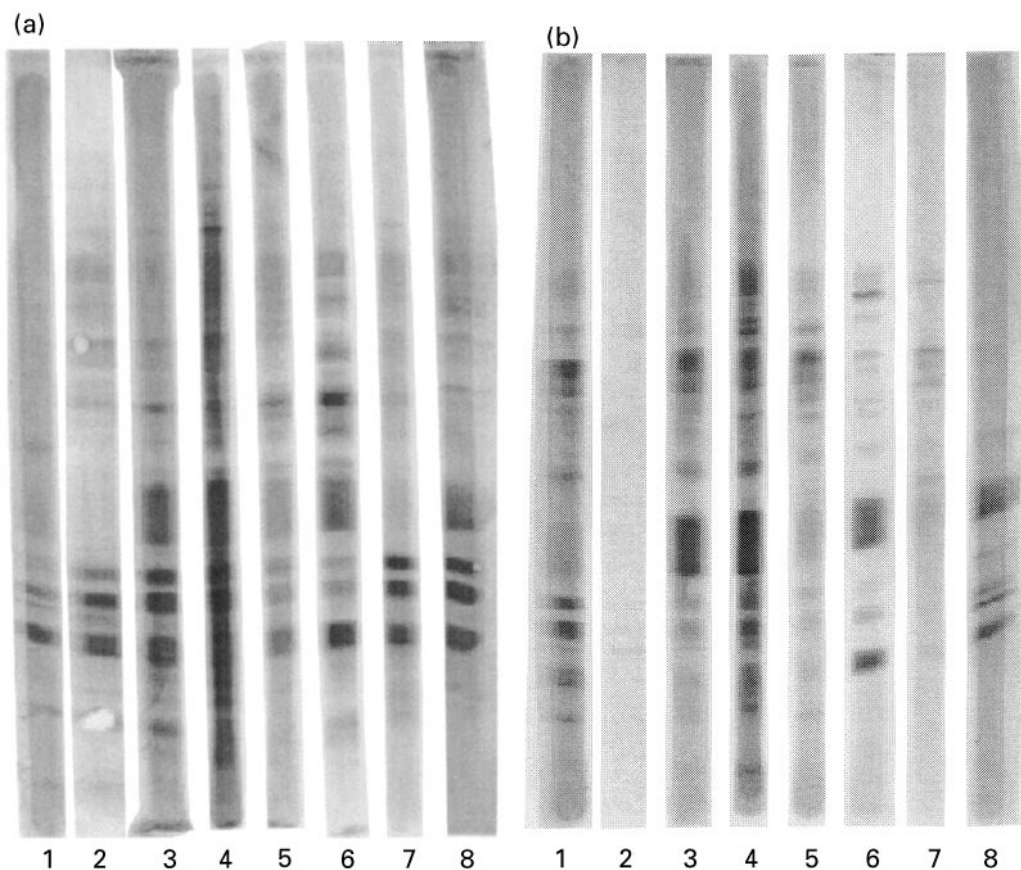


Fig. 3. Recognition of CPE-specific antibodies in pooled plasma from the PA (a) and NA (b) group. Lane 1: IgM; lane 2: IgE; lane 3: IgG; lane 4: IgG1; lane 5: IgG2; lane 6: IgG3; lane 7: IgG4 and lane 8: IgA. Western blots were incubated overnight with pooled plasma (1:25 v/v). Subsequently, CPE-specific antibodies were detected with antibodies against IgA, IgE, IgM, IgG and IgG-subclasses.

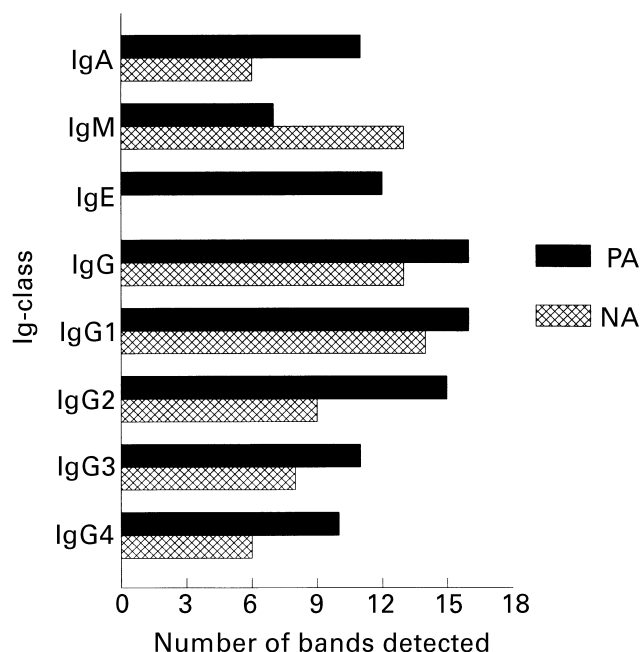


Fig. 4. Number of Ig-binding proteins detected using pooled plasma samples of the PA or NA group as estimated using the Diversity One computer programme.

CPE-specific IgE was not determined by ELISA because patients were selected based on a positive peanut-specific RAST.

Purification of Ara h1 and Ara h2

In order to characterize Ara h1 and Ara h2, these proteins have been isolated and the N-terminal amino acid sequence has been determined. Figure 5 (a, upper panel) shows the elution profile of the Source Q column. Ara h1 was found in fraction 33 and 34. Ara h1 migrated at ≈ 65 kDa and was more than 99% pure as judged by SDS-PAGE (Fig. 5b). The N-terminal sequence is shown in Table 2. Ara h2 was detected in fractions 16–19 and appeared to be a doublet at 14–21 kDa. Ara h2 contained $\approx 10\%$ peanut lectin (30 kDa) and another protein (25 kDa) and was therefore subjected to further purification using a Source S column. Figure 5(a, lower panel) shows the elution profile of Ara h2 from the Source S column. Ara h2 was found in fractions 20–26 and appeared to be pure in fractions 22–24 as judged by SDS-PAGE (Fig. 5b). The ratio of upper and lower band of Ara h2 differed slightly in fractions 22–24. Both the upper and lower band had the same N-terminal sequence (Table 2). Both Ara h1 and Ara h2 preparation were immunoreactive with patients' sera and had biological activity. This was tested by histamine release assay using

peripheral blood mononuclear cells from peanut allergic patients (data not shown).

Discussion

The present study shows that peanut contains multiple allergens. Six could be defined as major allergens based on their recognition by IgE present in more than 50% of the plasma samples of PA individuals. Moreover, three proteins or protein subunits with a molecular weight of ranging from 18 to 21 kDa were recognized by more than 70% of the peanut-specific IgE containing plasma of PA subjects in our study. Considering the molecular weights of these proteins (18–21 kDa), it was assumed that they contained the previously described doublet Ara h2 [21]. The other peanut allergen described by Burks *et al.* [20], Ara h1 was also assumed to be present in our CPE with a MW of ≈ 60 kDa. Both proteins which were thought to be Ara h1 and Ara h2 were isolated, sequenced and their identity confirmed. This will be discussed later.

Ara h1 and Ara h2 have been described as major allergens [20,21]. Our study confirms Ara h2 as a major allergen, however, Ara h1-specific IgE was present in $\approx 35\%$ of the plasma samples and therefore not considered to be a 'major allergen'. Moreover, the expression 'major allergen' could lead to some confusion. Burks *et al.* [20] based their identification of Ara h1 as a major allergen on a relative high concentration of Ara h1-specific IgE antibodies present in pooled serum of a number of patients rather than on frequencies in which Ara h1-specific antibodies occur in the individual sera. Three of the six identified major allergens have MW ranging from 18 to 21 kDa. Hefle *et al.* [22] described IgE-binding to six proteins with a MW range from 15 to 25 kDa which in addition showed skin test reactivity in 12 PA patients. This substantiates that this MW range contains at least three major allergens (Ara h2 included) which are also bioreactive [22]. In order to study these previously described allergens, Ara h1 and Ara h2 were purified using ionexchange chromatography developed with a linear salt gradient, resulting in a higher purity (Fig. 5b). Both Ara h1 and Ara h2 were subjected to N-terminal sequencing (Table 2). This is the first report of a N-terminal amino acid sequence of Ara h1 and Ara h2 purified from peanuts. Burks *et al.* [30] have cloned the Ara h2 gene and based on the cDNA, they have predicted the entire N-terminal amino acid sequence. Our N-terminal sequence of 25 amino acids was found with complete identity, in the sequence of Burks at position 86–111. This indicated that Ara h1 present in peanuts is a truncated form of the cDNA derived gene. Therefore, our data define the cleavage site for nascent Ara h1. Proteases involved in protein processing usually cleave their substrates after an arginine or lysine residue. Therefore, the fact that an

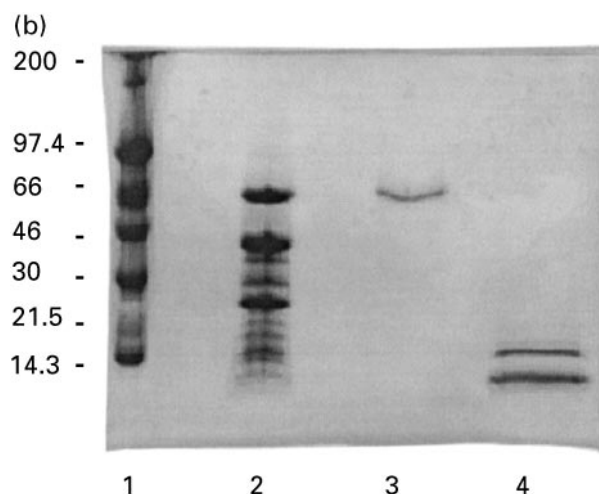
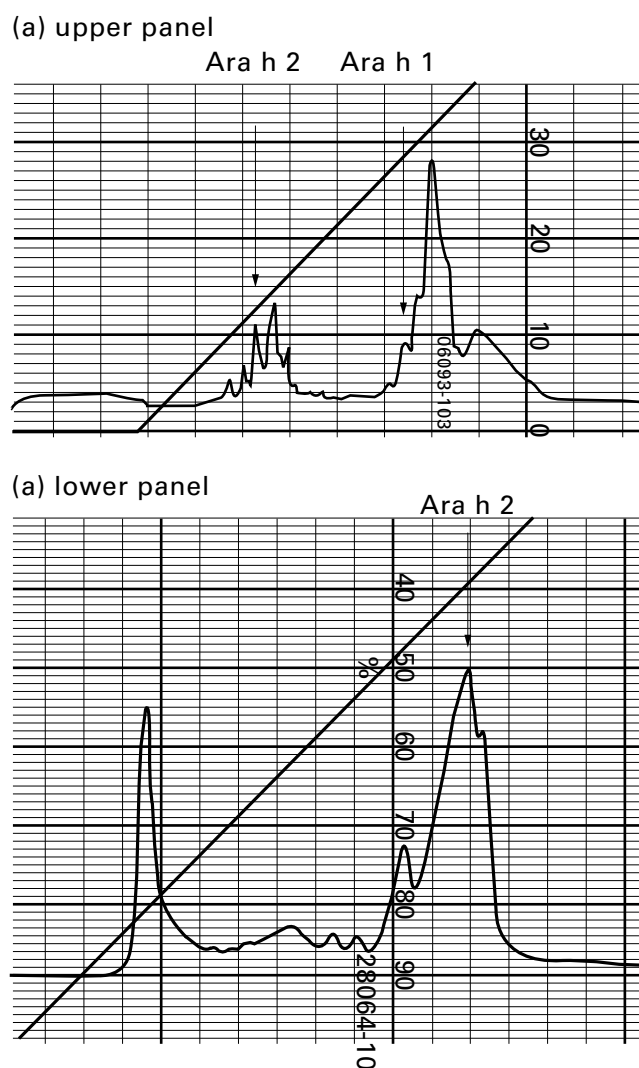


Fig. 5. (a) Purification of Ara h 1 and Ara h 2 using liquid chromatography. Upper panel: A 8 mL Source Q column was loaded with CPE, washed and developed with a linear salt gradient as described in methods. Arrows indicate the fractions containing Ara h 1 and Ara h 2. Lower panel: A 1 mL Source S column was loaded with the Ara h 2 containing fractions from the Source Q column, washed and developed with a linear salt gradient as described in methods. Arrows indicate the Ara h 2 containing fraction and peanut lectin containing fractions. (b) SDS-PAGE of Ara h 1 and Ara h 2 preparations. Lane 1: molecular weight marker; lane 2: CPE; lane 3: Ara h 1; lane 4: Ara h 2.

arginine residue at position 85 [30] precedes the cleavage site, underscores the hypothesis that the product from the gene as cloned by Burks needs to be processed to give mature Ara h 1 as it is found in peanuts. The propeptide of 85 amino acids may explain why Burks *et al.* have found a higher MW for the recombinant Ara h 1 [30], as compared to peanut-derived Ara h 1. The recombinant protein consists of 626 amino acids [30], corresponding to a molecular weight of 73 kDa. According to our N-terminal sequence, mature Ara h 1 counts 545 amino acids resulting in a molecular weight of ≈ 64 kDa. This molecular weight corresponds to

both our results on SDS-PAGE (Fig. 5b) and to the earlier published SDS-PAGE results [30].

Ara h 2 appears to be a doublet on SDS-PAGE and the chromatography profile of the last step shows a double peak as well. This is in accordance with the observations of others who have identified Ara h 2 as a major peanut allergen [21]. Since the bands of the doublet on SDS-PAGE have identical amino acid sequences (Table 2), and since patients' IgE recognizes both bands equally well, we believe that we deal with two proteins with a high degree of identity. Differences in electrophoretic and chromatographic behaviour may be

Table 2. Amino acid sequence of peanut allergens 1 and 2

Ara h1		R	S	P	P	G	E	R	T	R	G	R	Q	P	G	R	T	D	D	*	R	stopped																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																												
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explained by different post-translational modifications which may give rise to two distinct proteins sharing the same N-terminal amino acid sequence and IgE-binding characteristics. Burks *et al.* [21] have published the N-terminal sequence of Ara h2. This sequence was not complete since nine out of 31 amino acids were not identified. This hampered the comparison of our sequence with that of Burks. However, some homology was found and the homology may be raised if the lacking amino acids in the sequence published by Burks *et al.* [21] were known.

Other differences may be explained by the different peanut varieties used in Burks' and our study. The sequences of the upper and the lower band of Ara h2 of our preparation were identical and were not homologous with any other known protein as present in the Swiss database. Differences in the recognition pattern of peanut allergens observed in a number of studies, can be explained by various ways. Firstly, the preparation of peanut extracts have neither been standardized nor have the extracts been obtained from the same varieties of peanuts. This could lead to differences in protein composition of the extracts [31]. Also diverse food habits between populations and consumption of different peanut varieties as well as various genetic backgrounds could lead to differences in recognitions patterns of the peanut proteins by the investigated Dutch PA subjects.

In the plasma of both PA and NA subjects, IgA, IgG and IgM could also be detected against the six major allergens and to other proteins in CPE. However, analysing the number of immunoreactive peanut proteins in pooled serum of each group, the most diverse recognition pattern of the protein bands in all immunoglobulin-classes was found in the PA group. Especially the proteins of 18–21 kDa were recognized strongly by antibodies present in plasma samples of PA patients whereas the binding observed with plasma of non-allergic controls was less evident to these proteins. But no differences were found in the quantitative analysis (except IgE) of CPE-specific antibodies in the plasma of the two groups which could be due to the large variation which was observed especially in the levels of CPE-specific IgG4.

For several respiratory allergens a higher level of IgG, especially of the IgG4-subclass, has been detected in the sera of allergic individuals compared to non-allergic individuals [32,33] although this has been contradicted by others [34]. Also several studies investigated the role of food-specific IgG and IgG-subclass antibodies in food-allergic patients both in children [35–37] and adults [38–40] but also here the results were inconclusive. Our study also indicates that there are at least no systemic differences in peanut-specific IgA concentrations between PA and NA individuals. However, it cannot be excluded that differences in the gastro-intestinal tract are present. Remarkably, we did

find peanut-specific immunoglobulins in the plasma of peanut allergic patients at levels comparable to NA subjects. The majority of these patients have been on a peanut-free diet for more than 5 years. As immunoglobulins have a half-life time of ≈ 6 months, the concentration of peanut-specific immunoglobulins should be very low or at least diminished. The observed concentrations of peanut-specific immunoglobulins can only be explained by the unaware consumptions of minute amounts of peanut protein by the patients or could be due to crossreactivity.

This study shows that peanut contains multiple allergens. A total number of six protein or protein subunits were recognized by more than 50% of the specific IgE-containing plasma samples of PA patients and were therefore defined as major allergens. Also, PA individuals show a wide variety in recognition compared with NA individuals. This more diverse recognition pattern in PA patients did not lead to increased levels of peanut protein-specific immunoglobulins of the various investigated classes, except IgE.

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