

Purification and immunoglobulin E-binding properties of peanut allergen Ara h 6: evidence for cross-reactivity with Ara h 2

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Summary

Background IgE-binding peanut proteins smaller than 15 kDa were previously identified as potential allergens in the majority of our peanut allergic population.

Objective To characterize the novel allergen in order to determine whether it was similar to one of the thus far identified recombinant peanut allergens (Ara h 1–7).

Methods An IgE-binding protein of <15 kDa was purified and identified via N-terminal sequencing. Its IgE-binding properties were investigated using immunoblotting, basophil degranulation, and skin prick testing. Possible cross-reacting epitopes with other peanut allergens were studied using IgE-immunoblotting inhibition.

Results The purified protein is a monomeric protein with a molecular weight of 14 981 Da as determined using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy. The amino acid sequence of the first 39 N-terminal residues is identical to that of Ara h 6, indicating that the allergen is Ara h 6. It is recognized by 20 out of 29 peanut-allergic patients on IgE-immunoblot, and its potent biological functionality is demonstrated by the degranulation of basophils, even at concentrations below 10 pg/mL, and by positive skin prick reactions. Ara h 6 has homology to Ara h 2, especially in the middle part and at the C-terminal part of the protein. Almost complete inhibition of IgE–Ara h 6 interaction with Ara h 2 demonstrates that at least part of the epitopes of Ara h 6 are cross-reactive with epitopes on Ara h 2.

Conclusions Peanut-derived Ara h 6 is a biologically active allergen recognized by the majority of our peanut-allergic patient population and can be considered a clinically relevant peanut allergen.

Keywords allergen, basophil, IgE, peanut, protein purification, skin prick test

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Introduction

Peanut allergy is one of the most severe food allergies because of its persistency and the often severe and sometimes life-threatening character [1]. The prevalence of peanut allergy in the Western world has been estimated at 1 in 10 000 up to 1 in 200 [2] and some reports indicated that it seems to have doubled in certain populations in the last 6 years [3–5]. The nature of the allergenic compounds in peanuts has been studied extensively. Peanut allergens Ara h 1, Ara h 2, and Ara h 3 have been purified [6–8] and characterized [8–13]. There is increasing evidence that Ara h 2 is the most often recognized allergen in peanuts [7, 13–16]. We have recently shown that a peanut protein that is not Ara h 1, Ara h 2, or Ara h 3 binds IgE from >60% of the patients from our peanut allergic population [16]. It can therefore be classified as an important major peanut allergen.

Besides Ara h 1, Ara h 2, and Ara h 3, three other IgE binding peanut proteins have been cloned, and their official names given by the IUIS Allergen Nomenclature Subcommittee are Ara h 5, Ara h 6, and Ara h 7 [13]. It is interesting to note that Ara h 6, and to a lesser extent, Ara h 7 are homologous to Ara h 2. Ara h 2, Ara h 6, and Ara h 7 all belong to the 2S albumin family, and have the characteristically conserved cysteine residues of this group of proteins [13]. Cloning of Ara h 5, Ara h 6, and Ara h 7 revealed proteins with molecular weights of 14, 14.5, and 15.8 kDa, respectively [8]. These are in the range of that of the molecular weight of the novel peanut allergen [16]. The aim of the present paper is to identify, purify, and perform functional tests of the novel peanut allergen, and to investigate the possible equivalence with previously cloned and expressed peanut allergens.

Materials and methods

Peanut protein extraction

Raw peanuts (*Arachis Hypogaea*) from the Runner cultivar (Cargill, Dawson, GA, USA) were purchased from Imko Nut Products (Doetinchem, the Netherlands) and were stored at

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10 °C until use. Fifty kilograms of peanuts were extracted three times with hexane. After extraction, the peanuts were dried and ground. The defatted peanut flour (1.3 kg) was extracted for 1 h while stirring in 13 L 50 mM Tris/HCl pH 8.2 at 4 °C. The solution was filtered using a kitchen sieve and cheese cloth. The filtrate is referred to as crude extract.

Fractionation by ammonium sulphate precipitation of the crude extract

Ammonium sulphate was added to the crude extract to attain a concentration of 40% saturation at 4 °C. The solution was centrifuged (45 min, 8000 g, at 4 °C). The cold supernatant was filtered over glass wool to remove fat particles. Ammonium sulphate was then added to a concentration of 80% saturation at 4 °C. The solution was centrifuged again (45 min, 10 000 g, at 4 °C). The pellet was then resuspended in 1.3 L 20 mM Tris/HCl pH 8.0 containing 1 mM EDTA. This preparation is referred to as concentrate.

Liquid chromatography

The nearly clear concentrate was filtered using a G3 glass filter and the filter was washed with 80 mL of 20 mM Tris/HCl pH 8.0 containing 1 mM EDTA and 1380 mL clear filtrate was obtained. A fraction of 230 mL was applied on a Sephadex (Pharmacia, Uppsala, Sweden) G75 column (7200 mL column volume, diameter 20 cm, height 23 cm) and eluted with 20 mM Tris, pH 8.0 at 100 mL/min. Fractions containing the target protein (3200–5700 mL) were immediately further processed using anion exchange chromatography. All steps until anion exchange chromatography were performed at 4 °C. The enriched fractions of the 12 Sephadex G75 runs were combined, warmed up to 25 °C and applied to a 3600 mL Source 15Q (Pharmacia) column (diameter 20 cm, height 12 cm) previously equilibrated with 20 mM Tris, pH 8.0 (loading buffer). After washing with loading buffer, the column was eluted with a 40 L salt gradient of 0–0.25 M NaCl in loading buffer at a flow of 100 mL/min. Fractions of 400 mL were collected and analysed for Ara h 6 content and purity. Purified Ara h 6 was stored in small portions at –20 °C. Ara h 2 was purified as described previously [8]. All buffers used were filtered through 0.45 µm Durapore membranes (Millipore, Bedford, MA, USA).

Protein measurement

Concentrations of purified Ara h 2 and Ara h 6 were determined by measuring the A_{280} . A_{280} (1 mg/mL) for Ara h 2 = 0.59 and A_{280} (1 mg/mL) for Ara h 6 = 0.21 as obtained using the Web-based program ProtParam from SwissProt (<http://www.expasy.org/tools/protparam.html>, accession codes Q8GV20 and Q9SQG5, respectively). Protein concentrations in extracts were measured with Bradford analysis (BioRad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard.

N-terminal sequencing

A sample of purified Ara h 6 (1 mg, approximately 70 nmol) was used for N-terminal sequencing using Edman degradation with an automated protein sequencer (Applied Biosys-

tems, performed at SeCU, Utrecht, the Netherlands). The first 39 amino acids were determined and compared with the published N-terminal sequence of cloned peanut allergens published in the SwissProt database.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry

All MS measurements were performed on a Bruker BIFLEX III MALDI-TOF mass spectrometer (Bruker Instruments, Bremen, Germany) equipped and operated according to standard procedures. The matrix used was a saturated solution of trans-3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, Sigma-Aldrich Chemical Co, St Louis, MO, USA) in 0.1% trifluoroacetic acid/acetonitrile (2/1,v/v). Before being mixed with matrix, the samples were purified by zip tips C18 (Millipore) using the procedure recommended by the manufacturer. The sample (one part) and matrix solution (two parts) were mixed and spotted onto the MALDI target plate. Spectra were acquired in positive ion mode and the signal was accumulated and averaged over 100–200 shots.

Patients

A selection of 29 adult patients with a documented peanut allergy was made based on the IgE-recognition of low molecular weight peanut proteins [16]. Each of these individuals had a positive skin prick test (SPT) to peanuts and a convincing history of peanut allergy. Peanut allergy was confirmed by double-blind placebo-controlled food challenges. Within this group of 29 patients, a selection of 23 patients that recognize Ara h 2 and/or other proteins in the low molecular weight range (10–20 kDa) were selected for further investigation. Both non-atopic and atopic (but not peanut allergic) individuals were used as controls. Venous blood was withdrawn from the individuals and allowed to clot. Serum was collected by centrifugation and stored in aliquots at –20 °C until use. The presence of IgE in the serum specific for peanut allergens was demonstrated by CAP-FEIA (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden), and by SDS-PAGE in combination with immunoblotting. All studies were approved by the Medical and Ethical Committee of the University Medical Center Utrecht (Utrecht, the Netherlands) and patients gave written consent.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting

SDS-PAGE and immunoblotting was performed using a BioRad Mini Protean II system (BioRad Laboratories) with 15% acrylamide gels (15 × 10 cm) as described previously for peanut allergens [16]. In case of inhibition experiments, increasing concentrations of Ara h 2 or Ara h 6 (1–100 µg/mL) were added to patient sera 1 h prior to applying on the blot membrane (room temperature). Bound IgE was detected using a commercial goat anti-human IgE conjugated to horseradish peroxidase (DPC, Los Angeles, CA, USA) with subsequent staining for peroxidase activity using the ECL technique. Non-specific binding of the anti-IgE antibody conjugate was measured in a similar blotting procedure with normal human serum instead of patient sera and appeared to be negligible.

Basophil degranulation

Basophil activation was analysed by the increased binding of antibodies directed against CD63 after degranulation according to the method of Knol et al. [17]. Basophil activation was performed with peripheral blood mononuclear cells (PBMC) from non-peanut allergic donors sensitized with a serum pool from peanut-allergic patients, as described previously [16]. After activation for 45 min at 37 °C, cells were washed twice in ice-cold phosphate buffered saline supplemented with 2% (v/v) fetal calf serum (FACS-buffer) and kept on ice. Subsequently, cells were incubated with PE-conjugated anti-CD63 (CLB-gran/12, 435; Central Laboratory of the Blood transfusion service, Amsterdam, the Netherlands) antibodies and FITC-conjugated goat-anti-human IgE (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) for 30 min on ice followed by duplicate washes. Degranulation was analysed by flow cytometric analysis on a FACScan (Becton Dickinson, San Jose, CA, USA). In brief, basophils in the PBMC suspension were selected on basis of their forward and side scatter characteristics and anti-IgE binding [18]. Degranulation of basophils was quantitated as the basophils that showed more anti-CD63 binding than the isotype control and was depicted as percentage positive cells. Previously it was shown that the percentage positive cells are strongly correlated to the percentage histamine release [17].

Skin prick testing

SPTs were performed according to Dreborg and Frew [19] using purified Ara h 2 and purified Ara h 6 at increasing concentrations (0.01, 0.1, 1, 10, and 100 µg/mL) and a commercial peanut extract (ALK-ABELLÓ, Nieuwegein, the Netherlands) on the flexor aspect of the forearm in four adult peanut allergic patients. Histamine dihydrochloride (10 mg/mL) was used as positive control, and saline was used as a negative control (ALK-ABELLÓ). Prior to the skin tests, patients discontinued antihistamines for 1 week, and did not use corticosteroids on the day of skin prick testing. The exact SPT reactivity was measured after 15 min by copying the marked weal reaction with transparent adhesive tape onto a record sheet. The weal areas were quantified by means of computer scanning [20].

Results

Purification and identification of Ara h 6

The purification protocol starts with an ammonium sulphate precipitation followed by gel filtration chromatography. This step is based on our experience that Ara h 1 and Ara h 3 behave as multimeric proteins under native conditions, eluting in the void volume of the gel permeation column. In Fig. 1, the chromatogram is shown and the bar represents the pooled fractions that were used for further purification. The main peak at approximately 3 L is a mixture of Ara h 1 and Ara h 3, both eluting in the void volume of the column. The peak at approximately 8 L is caused by low molecular weight polyphenols and does not contain protein (not shown). The pooled fractions were applied to an anion exchange column and eluted with a salt gradient. Figure 2 shows the elution profile, and the arrow indicates where the target protein elutes from the column. The pure fraction contained approximately

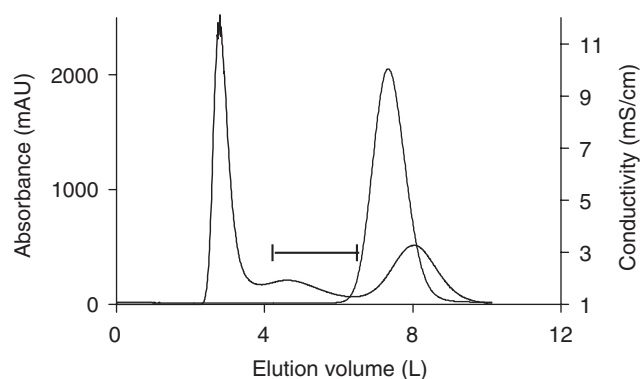


Fig. 1. Size exclusion chromatography of peanut proteins. A Sephadex G75 column was loaded with the concentrated peanut protein solution obtained the 40–70% ammonium sulphate precipitation. Thick line, Absorbance (left Y-axis); thin line, conductivity (right Y-axis). Bar represent pool used for further purification.

800 mg protein. Figure 3 shows the SDS-PAGE analysis of several fractions during the purification. The purity of the target protein (lane 4) is estimated to be >95%. The N-terminal amino acid sequence of the purified protein is given in Table 1, together with sequences that are highly homologous to the found sequence. The first 39 amino acids were analysed; subsequent amino acids gave peaks that were too low for proper assignment. The data clearly demonstrates that the isolated protein is Ara h 6, and that it has stretches homologous to Ara h 2 in the sequenced N-terminal part of the protein. At cycle 14 and 26 no increase in signal was observed. This is indicative for Cys residues or a glycosylated or phosphorylated amino acid. The molecular weight of Ara h 6 as determined using MALDI-TOF mass spectroscopy was 14981 Da (data not shown).

Identification of Ara h 6 as a functional immunoglobulin E-binding protein

To prove that Ara h 6 purified from peanuts is indeed a functional allergenic protein, we examined Ara h 6-induced basophil degranulation. Basophils were stimulated with crude peanut extract (CPE) as well as the purified Ara h 2 and Ara h 6 proteins. As is shown in Fig. 4, Ara h 6 only induces basophil degranulation after sensitization of basophils with

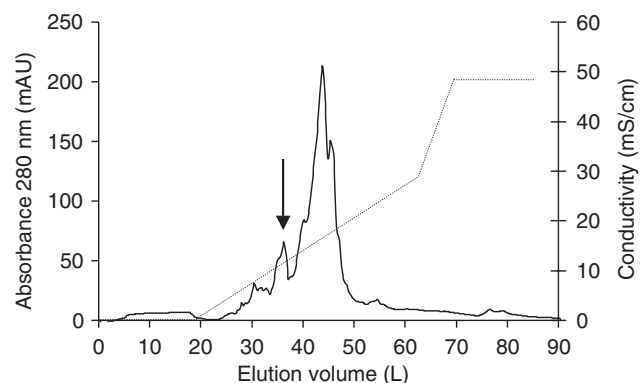


Fig. 2. Elution pattern of partially purified peanut proteins by anion exchange chromatography. A source Q column was loaded with the pooled fractions from the size exclusion chromatography column. Thick line, Absorbance (left Y-axis); thin line, conductivity (right Y-axis). The arrow indicates the fractions that contain the target protein.

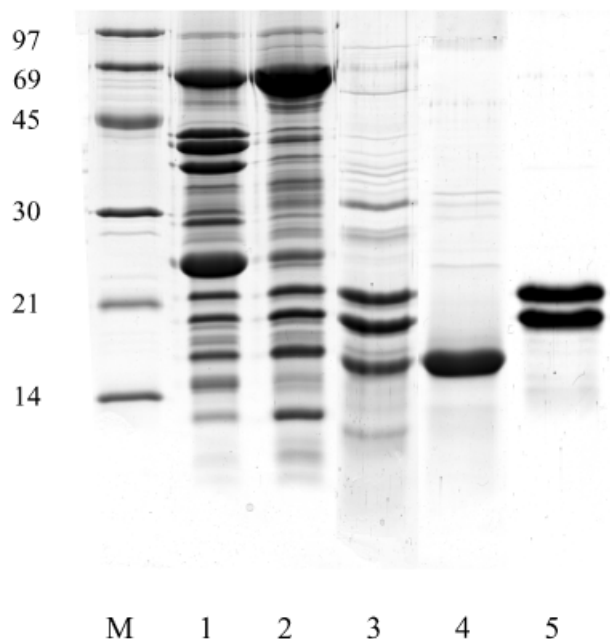


Fig. 3. SDS-PAGE patterns of peanut allergens. M, molecular weight markers (indicated at the left, kDa); 1, crude peanut extract; 2, ammonium sulphate pellet (40–70%); 3, partially purified peanut proteins after size exclusion chromatography; 4, purified target protein; 5, control sample of Ara h 2 [16]; M, molecular weight markers.

pooled serum from peanut allergic individuals. Degranulation was demonstrated at relatively low concentrations starting a 0.01 ng/mL. Furthermore, clear *in vivo* reactivity of Ara h 6 was demonstrated in SPTs in a subset of four peanut allergic patients. Concentrations of 1 µg/mL already gave positive reactions for both allergens, and increasing the concentrations of up to 10 and 100 µg/mL confirmed this reactivity. The average data for 100 µg/mL are shown in Fig. 5. Ara h 6 showed comparable reactivity to that of Ara h 2, suggesting a similar potency *in vivo*. A commercial peanut extract showed a positive SPT reaction in all patients. The response of both

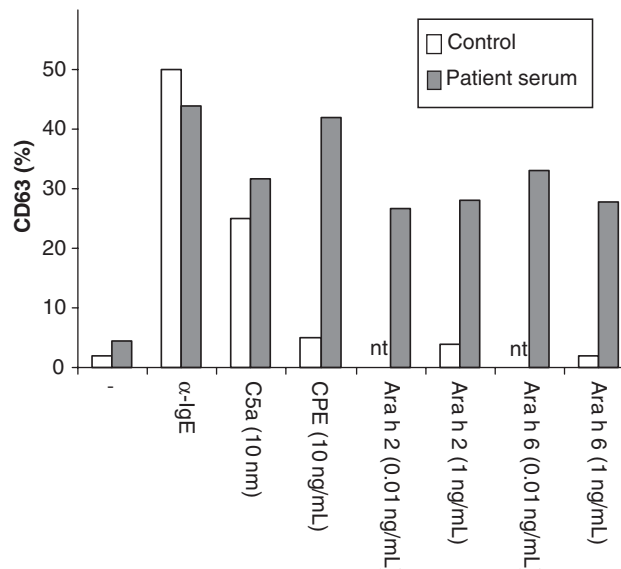


Fig. 4. Peanut allergen induced basophil degranulation. Degranulation of basophils loaded with patient's serum is induced by CPE, Ara h 2, and Ara h 6 in various concentrations, and controls anti-IgE and C5a (grey bars). Control basophils only degranulated with anti-IgE and C5a. Data shown is a representative experiment of four independent experiments. nt, not tested.

Ara h 6 and Ara h 2 is somewhat lower compared with the commercial peanut extract. This may be explained either by the fact that the commercial peanut extract contains other allergens as well (Ara h 1 and Ara h 3 for example) or by a higher concentration of protein.

Allergen recognition profiles

In order to investigate our peanut-allergic patients' specificity for the individual peanut allergens, IgE-immunoblots were prepared using individual patient serum. Figure 6 shows the IgE binding to CPE, purified Ara h 2, and purified Ara h 6 for the tested 23 patients. Some patients (40, 48, and 54) reacted

Table 1. N-terminal sequences of Ara h 2 and Ara h 6

Purified protein		MRERGRQGDSSS*ERQVDGVNLKP*EQHIMQRIMGEQE (stopped)
Ara h 6 [†]	1	AHASAMRRERGRQGDSSSCERQVDGVNLKPCEQHIMQRIMGEQEYDS-----
Ara h 2 [‡]	17	AHASA-RQQWELQGDRR-CQSQLERANLRPCEQHLMQKIQRDEDSYERDPYSPSPDPYSP
Identity		* *** * * ** ***** *
Ara h 6	49	--YNFGSTRSSDQQQRCCDELNEMENTQRCMCEALQQIMENQCDGLQDRQMVQHFKRELM
Ara h 2	75	SPYDRRGAGSSQHQRCCNELNEFENNQRCMCEALQQIMENQSDRLQGRQQEQQFKRELK
Identity		* ** * *** ***** ** ***** * ** * *****
Ara h 6	107	NLPQQCNFGAPQRCDDLVS
Ara h 2	135	NLPQQCGLRAPQRCDDLVS
Identity		***** ***** *

*Amino acids that could not be identified during sequence.

[†]Ara h 6: SwissPort accession number Q9SQG5.

[‡]Ara h 2: SwissPort accession number Q8GV20.

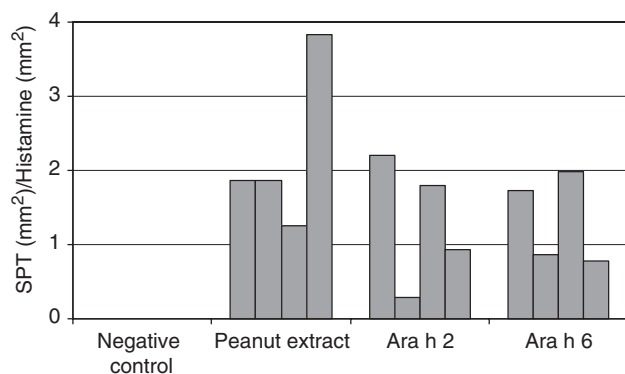


Fig. 5. Skin reactivity of CPE, Ara h 2, and Ara h 6. Commercial skin prick test extract for peanut (peanut extract), and the purified allergens Ara h 2 (100 µg/mL) and Ara h 6 (100 µg/mL) show significant skin reactivity. The bars represent data from four individual patients

intensely on the IgE-blot, resulting in exhaustion of the colour reaction, visible as a vague, lighter area within protein bands. The majority of the tested patients showed IgE binding to protein bands of approximately 15 kDa in CPE as well as to purified Ara h 6. In fact, only patient 34 and 47 reacted with Ara h 2 without any reaction towards Ara h 6. The serum of patient 47 exclusively reacts with Ara h 2. The serum of patient 34, however, reacts also with Ara h 1 (high molecular weight band in CPE lane), and with proteins of 10–15 kDa, possibly Ara h 5 or Ara h 7, while no reactivity is observed to purified Ara h 6.

Cross-reactivity with Ara h 2

Based on the N-terminal sequence of Ara h 6, and the homology of Ara h 6 and Ara h 2 throughout the entire sequence, IgE directed towards Ara h 2 may cross-react with Ara h 6 and vice-versa. To investigate this possible IgE cross-reactivity between Ara h 6 and Ara h 2, inhibition of IgE was studied in an immunoblotting inhibition experiment. CPE, purified Ara h 6, and purified Ara h 2 were applied to SDS-PAGE gels, blotted and incubated with a pool of peanut-

allergic patient serum. This serum pool consisted of sera from patients that recognized both Ara h 2 as well as Ara h 6 on IgE-immunoblot without any reaction towards other peanut allergens. In Fig. 7, it can be seen that the CPE lane (left side from each blot), the Ara h 2 doublet, as well as Ara h 6 and another low molecular weight protein bind IgE potently. Purified Ara h 2 (middle lane of each blot) and purified Ara h 6 (right lane of each blot) also bind IgE as expected based on the results from the CPE lane. Inhibition of IgE binding with increasing amounts of purified Ara h 6 (Fig. 7b) results in a complete abolition of IgE binding to Ara h 6 even at a very low concentrations of 10 ng/mL. Under these conditions, inhibition of IgE binding to Ara h 2 is detectable, but it takes a 100-fold increase in Ara h 6 to inhibit the IgE–Ara h 2 interaction substantially. When Ara h 2 is present as an inhibitor (Fig. 7c), the IgE–Ara h 2 interaction is strongly reduced at 10 ng/mL, and completely abolished at 100 ng/mL, whereas the Ara h 6–IgE interaction remains. At a 10-fold higher concentration of Ara h 2, the Ara h 6–IgE interaction is inhibited almost completely. Increasing the Ara h 2 concentration up to 10 µg/mL results in a complete inhibition of Ara h 6–IgE interaction (not shown).

Discussion

Recent investigations of IgE binding profiles of peanut-allergic patients using isolated peanut allergens has provided new information on the relative importance of different peanut allergens. Ara h 1 and Ara h 2 were the two first identified peanut allergens, and both are recognized by the majority of patients in various peanut-allergic patient populations from different parts of the world [6, 7, 9, 13]. In a recent study, the relative importance of different peanut allergens was investigated using 32 well-characterized peanut allergic patients and a set of peanut allergens purified from peanuts [16]. It was found that the frequency of recognition of Ara h 2 was higher (81%) compared with the other major

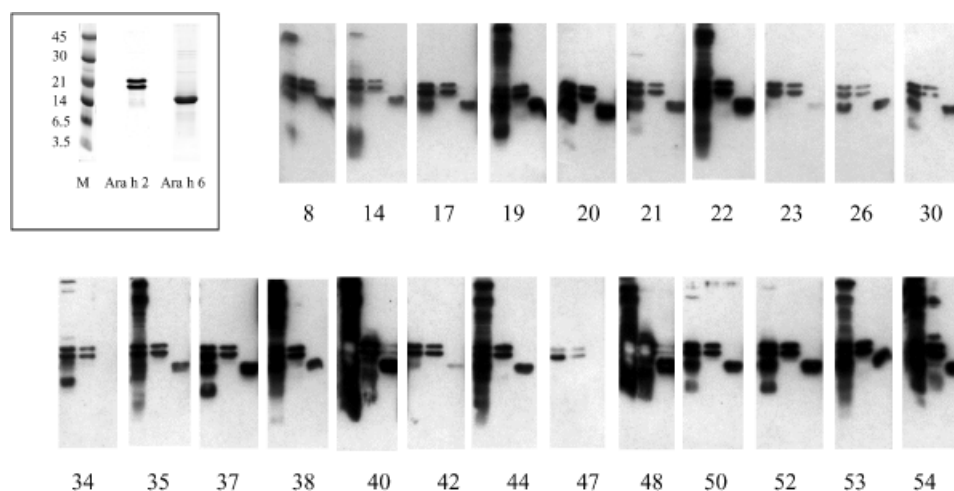


Fig. 6. IgE-reactivity of the individual patients towards CPE, Ara h 2, and Ara h 6. IgE-immunoblots of peanut allergens with individual peanut-allergic patient sera. The majority of patients recognizing Ara h 2 also react with Ara h 6. Patient number is indicated below each blot. The three lanes of each blot are: first (left) lane, crude peanut extract; second (middle) lane, purified Ara h 2; third (right) lane, purified Ara h 6. The insert in the upper left part shows a Coomassie-stained gel of marker proteins (M, indicated in left margin), Ara h 2 and Ara h 6.

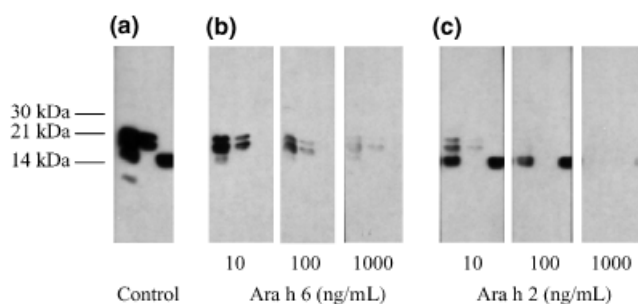


Fig. 7. IgE-Cross-reactivity between Ara h 2 and Ara h 6. Each IgE-immunoblot consists of three lanes; left, CPE; middle, purified Ara 2; right, purified Ara h 6. (a) control blot without inhibitor, (b) inhibition with increasing concentrations of Ara h 6, (c) inhibition with increasing concentrations of Ara h 2. Molecular weights are indicated in left margin.

allergen Ara h 1. Furthermore, the required concentration to trigger histamine release from basophils was lower for Ara h 2 compared with Ara h 1. The relevance of this latter observation was confirmed by SPT, demonstrating that 10- to 100-fold higher concentrations of Ara h 1 were needed to induce reactions comparable to that of Ara h 2 [16]. The fact that Ara h 2 is such a potent allergen may be explained by its stability. Ara h 2 belongs to the conglutin and 2S protein family [9], characterized by a compact structure, and held together by disulphide bonds. Ara h 2 is very stable towards breakdown by digestive proteases pepsin and trypsin [21, 22], and a stable peptide of 10 kDa remains after prolonged incubation with digestive enzymes [22]. This peptide contains several IgE-binding epitopes. Compact and stable proteins may be able to pass the stomach intact or only partially degraded [23, 24]. In fact, it was shown that peanut lectin, another small and stable peanut protein, enters the circulation in an intact form after ingestion of peanuts [25]. Recently it was shown that peanut allergens including those with molecular weights of 15–25 kDa (Ara h 2 and Ara h 6) may be present in the breast milk of lactating women [26]. It can be hypothesized that these allergens are responsible for the severe systemic reactions that are frequently observed in peanut allergic patients.

Besides Ara h 1 and Ara h 2, a number of other peanut allergens have been described, mainly via expression of cloned genes and IgE-binding studies [13, 27, 28]. One report describes the purification of Ara h 3 from peanuts [8], while for Ara h 5, Ara h 6, and Ara h 7, only recombinant forms have been described. In the present study, we purified a potent IgE-binding protein from peanuts that was described recently [16] and we identified it as Ara h 6 via its N-terminal sequence. We found a complete match of all 39 sequenced amino acids with Ara h 6 (SwissProt entry code Q9SQG5) that started at amino acid 6 of the published cDNA-translated amino acid sequence. The first five amino acids of the published Ara h 6 sequence probably represent a signal peptide that is cleaved off to form the mature protein. A similar situation was described for peanut-derived Ara h 1 by our group earlier [7]. The cDNA-derived sequences of Ara h 2 and Ara h 6 are homologous to a large extent [13] and Table 1 shows an alignment of both proteins based on sequences available in the SwissProt database. The experimentally obtained mass of 14981 Da is in agreement with the theoretical mass of 14491 Da as calculated from the

SwissProt sequence using the web-based program ProtParam (<http://www.expasy.org>).

Recombinant Ara h 6 was recognized by 38% of sera from the peanut-allergic population of Kleber-Janke et al. [13]. In our previous study we found IgE-reactivity towards the 15 kDa peanut protein, that is now identified as Ara h 6 in 20 from 32 patients (63%) [16]. This indicates that Ara h 6 is an important IgE-binding protein. Differences in percentage recognition may be explained by differences between recombinant vs. peanut-derived allergens, or differences between patient populations. Although recombinant peanut allergen may bind patient IgE, it was shown that post-translational processing of peanut allergens has important consequences for their molecular organization and the IgE-binding properties [7, 8], and this should be taken into account when dealing with recombinant peanut allergens. Another explanation of the observed differences in recognition of Ara h 6 may be found in the level of Ara h 6 in various cultivars of peanuts that are commonly used. However, the relative occurrence of the Ara h 1 and Ara h 2 protein in the different main peanut cultivars was about the same, approximately 13% and 7% of the total peanut protein content, respectively [14]. Re-evaluation of the SDS-PAGE gels from that study revealed that Ara h 6 is present in all test cultivars, at approximately the same concentration as Ara h 2, or somewhat lower. It should be noted, however, that assessment of the protein concentration based on dye binding as is the case for Coomassie-stained gels, is not a true quantitative method because of potential differences in dye binding properties between different proteins. Because Ara h 2 and Ara h 6 are similar proteins with similar amino acid compositions such method can be considered semi-quantitative. The SDS-PAGE band patterns indicate that Ara h 6 is a relevant peanut protein, and that it will be present in peanut-containing foods to a similar extent as Ara h 2. In the present study we show that the purified Ara h 6 binds IgE in immunoblotting (Fig. 6). This IgE binding is functional because basophils loaded with IgE from peanut allergic patients degranulate upon challenge with Ara h 6 (Fig. 5). Evidence for *in vivo* reactivity was obtained from SPTs, showing reactivity for Ara h 6 in well-documented peanut allergic patients. This indicates that Ara h 6 is a relevant peanut allergen.

When Ara h 6 was cloned, it was classified as a member of the conglutin protein family [13], with a high degree of homology to Ara h 2 and Ara h 7 [13]. IgE epitopes on Ara h 2 have been mapped [29] and these are mainly located at the N-terminal part of the protein (seven out of nine epitopes) while two epitopes are located in the middle part, and one other is found at the C-terminal part of the protein. Interestingly, the primary homology with Ara h 2 is in the middle- and C-terminal of Ara h 6, an area with only three IgE epitopes [29]. Two IgE epitopes that are mapped on Ara h 2 are not at all present on Ara h 6, because the latter protein lacks 14 amino acids in that specific area. Interestingly, recent investigations of Chatel et al. [30] showed that the light iso-form of Ara h 2 differ from each other in the same amino acid region. Compared with light iso-form of Ara h 2, the heavy iso-form is characterized by a 12 amino acid insertion that contains a third repeat of the major IgE binding epitope DPYSPS. This particular epitope is thus two times present in the heavy iso-form of Ara h 2, only once in the light iso-form

of Ara h 2 [30], and not at all in Ara h 6. On the other hand, four epitopes of Ara h 2 are found in Ara h 6 with a high degree of homology. We performed inhibition experiments with IgE-immunoblotting in order to assess to what extent IgE-binding to Ara h 6 was cross-reactive with Ara h 2 (Fig. 7). We found that IgE-binding to Ara h 6 is cross-reactive to Ara h 2, as Ara h 2 could inhibit the IgE–Ara h 6 interaction completely. The Ara h 2-IgE interaction could be inhibited by Ara h 6, but less pronounced. In fact, a small portion of Ara h 2 remained able to bind IgE even in the presence of high concentrations of Ara h 6, indicative for unique epitopes on Ara h 2. This is further supported by the observation that some patients (34 and 47) only recognize Ara h 2, without any reactivity towards Ara h 6 (Fig. 6). The explanation for the unique epitopes may be found in the missing amino acid sequence in Ara h 6 [13] corresponding to a portion of Ara h 2 where two epitopes are located [29].

Significant inhibition of IgE binding as observed on immunoblots started at concentrations of 10 ng/mL. Interestingly, this is low compared with the concentration of Ara h 1 needed to inhibit IgE–Ara h 1 interaction in immunoblot approaches (150 µg/mL, 15 000-fold more) [31] and ELISA (around 1 µg/mL, 100-fold more) [12]. These differences in inhibiting concentrations indicate that the affinity of the IgE–Ara h 2 interaction is high, resulting in a possible more potent allergenic character compared with other peanut allergens. A similar observation was made by van der Veen et al. [32] who showed that basophil–histamine release experiments require only low concentrations of Ara h 2 (<0.5 ng/mL) while higher concentrations of Ara h 1 are needed (9–40 ng/mL) [32]. In a recent study from our group we confirmed this observation [16]. Ultimately, oral food challenges with isolated peanut allergens are required to assess the *in vivo* potency of Ara h 2 and Ara h 6 on the one hand, and other peanut allergens like Ara h 1 on the other.

Conclusions

Our data show that the majority of peanut-allergic patients in our population recognize Ara h 6 *in vitro* and *in vivo*, indicating that it is another major peanut allergen. Moreover, Ara h 6 is a potent peanut allergen with IgE epitopes cross-reactive with Ara h 2.

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