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Purification and characterization of natural Ara h 8, the Bet v 1 homologous allergen from peanut, provides a novel isoform

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Abstract

The peanut allergen Ara h 8 is an important allergen for birch pollen allergic patients because of the cross reactivity to its homologous Bet v 1. As the existence of Ara h 8 has been shown at the cDNA level so far (AY328088) and the allergen has indirectly been detected as natural protein, it was the aim of our study to identify natural Ara h 8 in peanut extract and to develop a purification strategy. This was achieved using a unique combination of purification steps, including optimized extraction conditions, size exclusion and ion exchange chromatography by treatment of the interfering contaminants with iodoacetic acid. A characterization of the protein by microsequencing showed discrepancies to the deduced amino acid sequence of AY328088. For this reason, we cloned and expressed a new Ara h 8 isoform from cDNA (EU046325). This IgE-reactive protein corresponds to the results of microsequencing, ESI-FTICR-MS and trypsin fingerprinting analysis of the authentic and purified nAra h 8. Apart from the ultimate use of recombinant allergens for diagnostic procedures, there is also a scientific need for the natural counterpart as it represents an excellent reference point by which to compare protein characteristics and to standardize diagnostic and therapeutic allergens.

Keywords: Ara h 8; peanut allergy; PR-10 protein; purification; recombinant expression; trypsin fingerprinting.

Introduction

Food allergy has emerged as a significant health problem. Peanut allergy is a major cause of food-induced fatal and near fatal anaphylactic reactions, and the incidence in children is increasing. Peanuts belong to the class of real food allergens which is able to sensitize patients by itself. Such allergenic sources are called class I food allergens in contrast to pollen-associated food allergens which are characterized by pollen-allergen-induced cross sensitizations and -reactions to food allergens (Breiteneder and Ebner, 2000). These allergenic sources are called class II allergens and typically elicit symptoms like the oral allergy syndrome (OAS) but occasionally also severe reactions. The birch pollen-related food allergy is an excellent example for class II allergens.

Birch pollen represents a predominant allergen source in Northern Europe. Bet v 1, the major birch pollen allergen, has although homolog allergens in hazel and alder, increasing the allergic reactions especially in springtime. A subgroup of birch pollen allergic patients reacts with an OAS to the Bet v 1 homologs in various fruits, vegetables, legumes and nuts. In contrast to this, severe anaphylactic reactions are observed to Gly m 4, the cross-reacting Bet v 1 homologous allergen from the legume soy (Kleine-Tebbe et al., 2002). The reason is not known, but it is assumed that there is a correlation between allergenicity and the ability to bind lipids (Mills et al., 2004). In the last years, a couple of Bet v 1 isoforms were identified, which show differences in their IgE-reactivity (Ferreira et al., 1996; Schenk et al., 2006). Even though there were various immunological reactive isoforms, some variants with reduced or completely lacking IgE-reactivity were detected.

The Bet v 1 homolog from peanut was identified as Ara h 8 (Mittag et al., 2004). This allergen might be the reason for the development of OAS in a subgroup of birch pollen allergic patients after peanut ingestion (Mittag et al., 2004). Severe reactions take place with Gly m 4 from soy have not been observed so far for Ara h 8. Ara h 8 was exclusively described as recombinant protein by application of DNA-technology. Peanut cDNA was amplified with a degenerated primer, designed on the basis of the Gly m 4 gene sequence (Kleber-Janke et al., 1999; Mittag et al., 2004). The protein concentration of this allergen in peanut extract is very low, since 80 to 90% of peanut proteins belong to the storage proteins of the 11 S glycinin, 7 S viciline or 2 S conglutin type (Boldt et al., 2005). Furthermore, the allergens with a molecular weight lower than 25 kDa are dominated by two isoforms of Ara h 2, by Ara h 6, by Ara h 6 fragments and by a 14 kDa fragment of Ara h 3 as determined by Western blotting.

The low concentration of Ara h 8 in peanut extract clearly emerges when trying to identify this allergen by specific patients' sera or rabbit antibodies by Western blotting under reducing conditions, because the corresponding protein band is very weak or even undetectable, respectively. The peanut proteins smaller than 25 kDa have therefore been enriched from peanut extract using size exclusion chromatography (SEC). Since some of the peanut allergens have similar pI values, the application of ion exchange chromatography (IEC)

will not result in fractions containing pure allergens. Apart from this disadvantage there is the general problem of IEC to isolate only one entity of the whole body of isoallergens. One idea to optimize separation conditions is to influence the extracting process by varying the pH value of the extraction buffer.

The aim of our study was to identify and to characterize natural Ara h 8 (nAra h 8) from peanut extract. A strategy for purification was developed and the characterization of the isolated protein was performed by N-terminal sequencing, mass spectroscopy and trypsin fingerprinting. These results of nAra h 8 were applied in order to identify the corresponding recombinant Ara h 8 (rAra h 8) isoallergen.

Results

Strategies for purification of Ara h 8

The first step in the isolation of natural Ara h 8 was to optimize the extraction conditions. Buffers with different pH values were tested and the extracts were controlled by immunoblot analysis with mAb against Ara h 1, Ara h 3/4, polyclonal rabbit antisera against Ara h 2, Ara h 6, Gly m 4 and a serum of the peanut allergic patient JG3. The anti-Gly m 4 rabbit serum detected Ara h 8 in extraction samples, due to its cross-reactivity to Bet v 1 homologous proteins. Using the method of acidic extraction, with 0.1 M ammonium acetate at pH 5.0 as extraction buffer, resulted in an Ara h 8 (approx. 16 kDa) enriched extract with undetectable amounts of Ara h 3/4 (40 and 20 kDa) (Figure 1). Traces of Ara h 2 were present, detected only by patient serum (lane 8), but not by monospecific rabbit antiserum (lane 2). In addition, Ara h 1 (63 kDa) and Ara h 6 (15 kDa) were recognized by patient serum (lane 8) and specific detection antibodies (lane 5 and lane 3). The staining of the proteins on the membrane with India Ink showed no Ara h 8 corresponding band because of the low concentration of this protein in the peanut. To concentrate Ara h 8 and to separate it from larger proteins, like Ara h 1 and other proteins which are present in the acidic peanut extract, the method of SEC was used. By this way, proteins above 25 kDa were removed, whereas Ara h 8 and other smaller proteins were accumulated (Figure 2). A subsequent separation of the accumulated Ara h 8 by IEC was associated with a cross-contamination of Ara h 6, due to their similar pI of 5.0 (Figure 3a). Because Ara h 6 contains 8 cysteines which are involved in disulfide bond formations, an alkylation with iodoacetic acid was applied which caused an acidification of the protein. By this, Ara h 8 was separated from Ara h 6 using IEC as demonstrated by SDS-PAGE and immunoblotting of fraction B7 with rabbit sera against Ara h 6 and Gly m 4 (Figure 3b). Whereas no Ara h 6 protein band could be demonstrated, one intensive band was detected by using the anti Gly m 4 rabbit serum, indicating the presence of Ara h 8.

Further analysis of nAra h 8 was performed using Edman degradation. The 12 N-terminal amino acid residues were determined, but did not correspond with the deduced amino acid sequence of the published Ara h 8 (GenBank accession number AY328088; Ara h 8.0101)

(Figure 4). It differed in four amino acids and it was further confirmed, that the initial methionine was removed by *in vivo* processing.

Characterization of rAra h 8

To get more information about the Ara h 8 gene sequence, genomic DNA was analyzed using Ara h 8 specific primers. By this, we identified two exons and one intron in the DNA sequence (Figure 5). Furthermore, some discrepancies were observed when compared to the published DNA sequence, especially in the second exon. Because of the results of N-terminal sequencing and the discrepancies between the DNA sequences, RNA was isolated and cDNA clones were checked for the presence of an Ara h 8 full-length cDNA which correlated with the natural Ara h 8. The amino acid sequence of the mature protein deduced from these cDNA clones, termed Ara h 8.0201, is aligned with Ara h 8.0101 in Figure 4. There is a similarity of 51.3% between both sequences. A database search revealed a higher homology (85.1%) with the predicted amino acid sequence of the recently published AhPR10 (GenBank accession no. AY726607) (Chadha and Das, 2006) than to Ara h 8.0101 (Figure 4).

Our new Ara h 8 encoding cDNA was cloned into the plasmid pET32b and expressed in *E. coli* BL21(DE3). After lysis, approximately 80% of rAra h 8 was found in the supernatant. Purification was achieved by metal affinity chromatography. The yield of rAra h 8 was always >8.5 mg purified protein per litre culture medium. Immunoblot analysis of the recombinant protein showed IgE-reactivity with the five tested patients' sera (Figure 6), which were preselected with Ara h 8.0101. The reactivity was not influenced by the vector-specific thioredoxin- and S-tag, as controlled by a recombinant Ara h 8 construct, which was only fused to a C-terminal His-tag (data not shown).

ESI FT-MS and trypsin fingerprinting of nAra h 8

The structure of nAra h 8 was confirmed by high resolution ESI FT-ICR MS. The charge deconvoluted mass spectrum of positive ions (Figure 7) exhibited one prominent molecular ion at m/z 16 280.60 and, though with a lower intensity, another molecular ion at m/z 17 298.14. The base peak of the isotopic pattern of the first molecule (see inlet in Figure 7) is in excellent concordance with the calculated base peak of 16 280.17 Da of rAra h 8 (Ara h 8.0201), but not with the published Ara h 8 with the theoretical weight of 16 950.6 Da. In accordance with these findings, tryptic peptide mass finger printing of nAra h 8 yielded a coverage rate of 94% with the protein sequence of rAra h 8.0201 (Figure 8).

Discussion

The present study describes for the first time the identification and purification of natural Ara h 8 and its corresponding DNA sequence (GenBank accession no. EU046325). The

detected protein differs significantly from the published isoform Ara h 8.0101 (Mittag et al., 2004) and was accepted from the IUIS (International Union of Immunological Societies) Allergen Nomenclature Sub-committee as Ara h 8.0201. Mittag and co-workers (2004) first identified, cloned and expressed Ara h 8.0101 in a cDNA library with a degenerated Gly m 4-deduced primer and subsequently verified it as a clinically relevant allergen. Interestingly, a cross-reactivity between Ara h 8 and Bet v 1 was shown by *in silico* investigation (Guarneri et al., 2005) and confirmed experimentally (Mittag et al., 2006). Although the advantages of recombinant allergens are highly praised and obvious, a purified natural Ara h 8 is urgently needed as a reference for recombinant proteins not only for identification of the authentic isoforms and the correct primary sequence, but also to have a model of comparison concerning three dimensional structure and localization of the conformational epitopes, including their immunological reactivity.

In our study, we achieved the purification of nAra h 8.0201 through the development of a three step purification protocol. The first step comprised the improvement of the extraction conditions. Using acidic conditions led to an accumulation of Ara h 8 in light of the fact that the content of other proteins is low (e.g. Ara h 2), or deleted (Ara h 3/4). The India Ink staining on the immunoblot analysis revealed, that even under optimised extraction conditions there was no Ara h 8 protein band detectable, but only in the immunological assay using the anti-Gly m 4 rabbit serum. The ‘unwanted’ proteins could be successfully separated by IEC, except for Ara h 6, due to the similarity of their pI. The pretreatment by alkylation with iodoacetic acid successfully eliminated Ara h 6 by the subsequently performed IEC. The reason for the excellent elimination result is that Ara h 6 contains 10 cysteine residues, which are involved in disulfide bridges; whereas Ara h 8 has none. Therefore, iodoacetic acid can only react with Ara h 6, causing a shift to acidic pI which finally allows Ara h 6 to be separated. The first analysis of the purified Ara h 8 protein, the characterization of the N-terminus, showed variations to the published Ara h 8 protein sequence. Our aim was to get the analogous nucleotide sequence of Ara h 8, which codes for the determined amino acid sequence of the N-terminus. The deduced amino acid sequence of our cDNA clones were compared with the results of Trypsin fingerprinting of nAra h 8. We found a cDNA which matched with these results, proving that we have found the natural Ara h 8 and its corresponding cDNA. On the other hand, we were not able to find and to identify the putative Ara h 8.0101 protein, only its gene sequence on gDNA level.

Our investigation of the Ara h 8 gDNA showed the presence of a functional intron (Figure 5). It has the typical characteristics of a plant intron: adenosine (A) and thymidine (T) richness (Goodall and Filipowicz, 1989; Csank et al., 1990; Ko et al., 1998) with a 5'-GT-3' splice junction at the 5'end and 5'-AG-3' at the 3'end (McCullough and Schuler, 1997). This has also been demonstrated for Ara h 1 (Viquez et al., 2003) and Ara h 3 (Viquez et al.,

2004). It is postulated that the presence of introns and exons in conjunction with the strength of a promoter is associated with higher expression of mRNA (Lorkovic et al., 2000). Ara h 8 belongs to the pathogenesis related proteins of PR-10 family; they are abundant in higher plants and play a potential role in plant defence in response to pathogens as well as various abiotic stress factors (van Loon and van Strien, 1999; Liu et al., 2003; Kav et al., 2004). The expression of the gene sequence is upregulated during that adaptation mechanism. It is likely that the Ara h 8 gene is associated with a strong promoter, which is regulated by both biotic interactions and abiotic factors.

The biological function of the PR-10 proteins is not known. Many studies suggest for example ribonuclease activity, as described in birch pollen (Bet v 1) (Bufe et al., 1996), ginseng (Moiseyev et al., 1994), *Capsicum annuum* (CaPR10) (Park et al., 2004), lupine roots (LaPR10) (Bantignies et al., 2000) and peanuts AhPR10 (Chadha and Das, 2006). Chadha and co-workers could confirm the assumption that the antifungal activity of AhPR10 is mediated by its RNase activity after internalisation of the protein. Ara h 8.0201 and AhPR10 are 85% identical; it is likely that Ara h 8 is also a RNase. Further aspects of the biological function are described in recent studies about ligand binding properties (Mogensen et al., 2002; Markovic-Housley et al., 2003), and a possible function as a membrane binding protein (Mogensen et al., 2007); both aspects having been demonstrated for Bet v 1. Mogensen et al. (2007) hypothesise that ligand binding can influence the membrane interaction, which allow the entry of a ligand to the cavity. They show a 3D structure of a complex consisting of Bet v 1 and two molecules of a plant steroid, assuming Bet v 1 to be a plant-steroid carrier. During our purification steps of nAra h 8 an untypical hydrophobic behavior was noticed, which could not be deduced from the structure. This provides evidence for an adduct formation with a steroid. The mass spectrometric analysis of purified Ara h 8 revealed in addition to the molecule mass of 16 281 Da a second molecule peak, which is 1163 Da higher – a second indication of the presence of a probable adduct. Peanut as an oil fruit contains an amount of about 40-50% lipids, mainly sterols and sterol ester. Therefore, it is very likely that Ara h 8 binds one or more of these molecules and operates as a sterol carrier.

In conclusion, we have purified the natural Ara h 8, which is the basis for the production of recombinant proteins. Recombinant allergens are essential tools for the *in vitro* and *in vivo* diagnosis of peanut allergy. Diagnostic methods are presently based upon natural crude peanut extracts that on the one hand side contain a broad pattern of peanut proteins, IgE-reactive components and irrelevant contaminants. On the other hand, these extracts are not at all standardized, which means that the extracts have a large variability (Hefle et al., 1995; Akkerdaas et al., 2003). Using recombinant proteins allows the development of standardized products that are biochemically well-characterized and consistent. Component-resolved diagnostic and skin prick tests with recombinant peanut allergens seem to be a safe and

effective diagnostic tool (Skolnick et al., 2001; Astier et al., 2006). Furthermore, they form the basis for the development of hypoallergenic deletion variants, which may in future be used for specific immunotherapy.

Materials and methods

Sera and antibodies

Patients included in this study were either sensitised to peanut according to case history and/or positive tested by CAP-FEIA to rAra h 8 (Ara h 8.0101, Table 1). Sera of 4 patients (WE-J, MW-J, KH-J, and KU-J) were collected at the Department of Dermatology of the University of Heidelberg (Heidelberg, Germany), after the patients gave informed consent. The patients' sera KP28 and JG3 were collected at the Research Center Borstel (Borstel, Germany), and were specified elsewhere (Kleber-Janke et al., 1999; Suhr et al., 2004; Boldt et al., 2005).

The following monoclonal and polyclonal antibodies were used: rabbit anti-Ara h 2 (MAP3), rabbit anti-Ara h 6 (rAra h 6), rabbit anti-Gly m 4 (SAM 22), mAb anti-Ara h 1 (Pn-t), mAb anti-Ara h 3/4 (Pn-x), mAb anti-Ara h 3/4 (PEI-7B2) (Suhr et al., 2004; Boldt et al., 2005; Mittag et al., 2005).

Peanut extract

Thirty g of peanut flour were extracted overnight as described elsewhere (Boldt et al., 2005) in 250 ml of 0.1 M ammonium acetate, pH 5.0. The peanut extract was then dialyzed against *aqua bidest.* over night, lyophilized and stored at -20°C.

SDS-PAGE and immunoblotting

SDS-PAGE was performed according to Laemmli (1970) using the BioRad System (Bio-Rad, Hercules, CA, USA) with 12% or 15% acrylamide gels. Afterwards, gels were stained with Coomassie Brilliant Blue R-250.

For immunoblot analysis, the separated proteins were subsequently transferred to nitrocellulose (NC) (Schleicher & Schüll, Dassel, Germany) or polyvinylidenefluoride (PVDF) (Millipore, Schwalbach, Germany) membranes by semi-dry blotting for 30 min at 0.8 mA/cm² (Kyhse-Andersen, 1984). NC membranes were blocked for 30 min at room temperature in TTBS (100 mM Tris, 100 mM NaCl, 2.5 mM MgCl₂, 0.05% Tween 20, pH 7.4), PVDF membranes for 2 hours with TTBS containing 5% non-fat dry milk. Primary incubations of membranes with antibodies or patients' sera, diluted in TTBS, were carried out overnight. Bound antibodies were detected with an AP-conjugated secondary antibody for 2 hours. The staining reaction was performed using the NBT/BCIP System (Blake et al., 1984). Staining of proteins that had been blotted on PVDF membranes was performed with the India Ink (Winsor & Newton, London, UK) method (Hancock and Tsang, 1983). The membrane was

incubated for 2 hours in 1:1 000 India Ink diluted in TTBS.

Size exclusion chromatography (SEC)

Peanut extract was separated by SEC using a Superdex 200 column (Amersham Biosciences, Freiburg, Germany) on a HPLC system (ÄKTA Purifier, Amersham Biosciences) as described previously (Boldt et al., 2005). The Ara h 8 containing fractions, analyzed by SDS-PAGE and immunoblotting, were pooled and lyophilized.

S-Carboxymethylation of cysteine residues

For the S-carboxymethylation of cysteines, a reduction and alkylation was performed. For the reduction reaction, 1 mg of the lyophilized protein was resolved in 100 mM Tris pH 8.5 to get a final concentration of 1 mg/ml. Ten µl of 1 M DTT were added and the reduction was incubated for 1 h at 37°C. The following alkylation was performed by adding 20 µl 1 M iodoacetic acid (made up in 1 M NaOH) and incubated for 30 min at room temperature in the dark. The sample was dialyzed and lyophilized again.

Ion exchange chromatography (IEC)

The un- and pre-treated Ara h 8 was purified by anion exchange chromatography using Source 15Q column (Amersham Biosciences, Freiburg, Germany) on the HPLC system. The protein was applied to the column in 20 mM Tris, pH 8.0 and eluted in 20 mM Tris, 0.5 M NaCl, pH 8.0. The fractions were analyzed by SDS-PAGE and immunoblotting.

Protein sequencing

The protein spots were excised out from the gel and were transferred onto a methanol activated PVDF membrane (Millipore, Schwalbach, Germany) by passive adsorption as described by Reuter et al. (2005). The absorption was controlled by Coomassie staining and microsequencing was performed according to the Edman degradation method on a Procise protein sequencer (Applied Biosystems, Darmstadt, Germany) (Petersen et al., 1992).

Mass spectrometric analysis

Electrospray Ionisation Fourier Transform Ion Cyclotron Mass Spectrometry (ESI FT-ICR MS) of nAra h 8 was performed in the positive ion mode using an APEX II-Instrument (Bruker Daltonics, Billerica, USA) equipped with a 7 Tesla actively shielded magnet and an Apollo II ion source. Mass spectra were acquired using standard experimental sequences as provided by the manufacturer. The reduced and alkylated protein was purified by Reversed-Phase Zip-Tip (Millipore, Billerica, USA). After lyophilisation the sample was dissolved in a 50:50:0.001 (v/v/v) mixture of methanol, water and acetic acid and sprayed with a flow rate of 2 µl/min. Capillary entrance voltage was set to 3.8 kV, capillary skimmer voltage to 120 V, and dry gas

temperature to 150°C. The mass spectrum was charge deconvoluted and mass numbers given refer to the neutral molecular masses.

Tryptic digest and MALDI-TOF-MS

The protein was separated by SDS-gel electrophoresis, excised, destained and digested overnight with sequence grade trypsin from bovine pancreas (Roche Diagnostics GmbH, Mannheim, Germany) as described in the literature (Shevchenko et al., 1996).

For MALDI-TOF analysis of the tryptic fragments were mixed 1:1 (v/v) with saturated matrix solution (purified α -Cyano-4-hydroxycinnamic acid, Bruker Daltonik) and analysed on a Reflex III (Bruker-Daltonics) in the reflector TOF configuration applying an acceleration voltage of 20 kV.

Characterization of the Ara h 8 gene sequence from gDNA

The purification of gDNA from approximately 50 mg peanut flour was performed using the DNeasy Plant Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Oligonucleotides were designed according to the published Ara h 8 sequence (GenBank accession no. AY328088) (forward: GATATCGGGCGTCTTCACTTTCG; reverse: GAATTCCCATATTGAGTAGGGTTGG). After amplification by PCR, the obtained fragment was ligated into pGEM-T cloning vector and verified by sequencing.

Characterization of the Ara h 8 gene sequence from mRNA

Total RNA was isolated of 30 mg of peanut flour using the SV Total RNA Isolation System (Promega, Mannheim, Germany). To generate cDNA, the First Strand cDNA Synthesis Kit (GE Healthcare, Munich, Germany) was used with the Not I d(T)₁₈-Primer (GE Healthcare) for the identification of the 3' gene sequence. Afterwards a PCR was performed with a primer recognising the NotI d(T)₁₈-Primer and a gene specific forward primer (GATATCGGGGTGTTTCACTTTTG). The PCR product was ligated into the pCR2.1-TOPO cloning vector (Invitrogen, Karlsruhe, Germany). After transformation into chemical competent *E. coli* DH5 α cells, positively tested transformants were checked by sequencing.

Heterologous expression of Ara h 8

For expression, the Ara h 8 gene sequence was amplified with sequence-specific primers according to the sequencing results (forward: GATATCGGGGTGTTTCACTTTTG; reverse: GAATTCCCGTTGGACAAAACATAAC). The PCR product was ligated in the EcoRI and EcoRV restriction sites into the pET32b expression vector (Novagen, Madison, WI, USA) and expressed in *E. coli* BL21(DE3) cells (Stratagene, La Jolla, CA, USA).

Purification of recombinant Ara h 8

The cell pellet was lysed under native conditions and the His-tagged protein was purified using

Characteristics of natural Ara h 8

Talon Metal Affinity resin (Clontech, Mountain View, CA, USA). The purified protein was dialysed against *aqua bidest.*, lyophilized and stored at -20°C.

Immunological characterisation of rAra h 8

IgE reactivity to the recombinant Ara h 8 preparations was investigated by immunoblotting experiments. The purified protein was separated by 15% SDS-PAGE (5 µg/cm) and blotted on PVDF membrane. Sera of five peanut allergic patients (WE-J, MW-J, KH-J, KU-J, and KP28), preselected by rAra h 8 (Ara h 8.0101) positive results in CAP-FEIA test (Table 1), were used for immunoblotting studies.

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Table 1 Clinical characteristics of 6 patient's sera used for immunoblot analysis.

Patient	Total IgE (kU/l)	rAra h 8		Peanut		Soy		Birch	
		(kU/l)	CAP	(kU/l)	CAP	(kU/l)	CAP	(kU/l)	CAP
WE-J	5.1	5.1	3	n.d.	n.d.	0	0	10.3	3
MW-J	637	45.9	4	2.0	2	0.4	1	>100	6
KH-J	296	9.2	3	0.7	1	0.6	1	66.3	5
KU-J	156	8.8	3	3.1	2	0.5	1	35.2	4
KP28	1696	>100	6	80.7	5	28.7	4	>100	6
JG3	3693	n.d.	n.d.	>100	6	12	3	9.2	3

The concentration of total serum IgE is given for each patient. The reactivity to rAra h 8, crude soy-, peanut-, and birch pollen extract is indicated in kU/l and the corresponding CAP classes.

Figure legends

Figure 1 Immunoblot of crude peanut extract (pH 5.0).

12% SDS-PAGE, 15 µg peanut extract/cm, reduced conditions, PVDF membrane.

Lane 1: India Ink, lane 2: rabbit anti-Ara h 2 (MAP3), lane 3: rabbit anti-Ara h 6, lane 4: rabbit anti-Gly m 4 (SAM 22), lane 5: anti-Ara h 1 mAb (Pn-t), lane 6: anti-Ara h 3/4 mAb (Pn-x), lane 7: anti-Ara h 3/4 mAb (PEI-7B2), lane 8: patient serum JG3, lane 9: negative control anti-rabbit IgG, lane 10: negative control anti-mouse IgG+IgM, lane 11: negative control anti-human IgE.

Figure 2 Chromatogram and SDS-PAGE of SEC with acidic peanut extract.

(A) Chromatogram of SEC using a Superdex 200 column, monitored at 280 nm wavelength.

(B) Different pooled fractions were analyzed by 12% SDS-PAGE, Coomassie Blue stained.

The fractions in pool 1 and 2 included mainly the allergen Ara h 1 and further proteins >25 kDa, pool 3 proteins <25 kDa (Ara h 2, Ara h 6, and Ara h 8).

Figure 3 IEC of peanut extract below 25 kDa: chromatogram and SDS-PAGE of selected fractions.

(A) Chromatogram of IEC with proteins <25 kDa without previous acetylation, monitored at 280 nm wavelength. Different selected fractions were analysed by 15% SDS-PAGE.

The Ara h 8 containing fractions are associated with a cross-contamination of Ara h 6. (B) Chromatogram of IEC with proteins <25 kDa after previous acetylation, monitored at 280 nm wavelength. The fractions A15-B7 were analyzed by 15% SDS-PAGE. Immunoblotting analysis of the fraction B7, which contains the highest amount of isolated Ara h 8, does not show any cross-contamination with Ara h 6 (lane 1: rabbit anti-Gly m 4 serum; lane 2: rabbit anti-Ara h 6 serum).

Figure 4 Protein sequence alignment of Ara h 8.0101, Ara h 8.0201 and Ah PR10.

The proteins were deduced from cDNA, Ara h 8.0101 by Mittag et al (2004), Ara h 8.0201 in the present study and Ah PR10 by Chadha and Das (2006). Similar residues in all sequences are highlighted in black, matches in two of three sequences in grey. Amino acids numbering is indicated on the top of the alignment. The result of N-terminal sequencing is specified in bold and underlined letters above.

Figure 5 Analysis of the genomic DNA using Ara h 8.0101 specific primers: nucleotide acid and deduced amino acid sequence of the genomic clone of Ara h 8.

Upper case letters indicate exons sequence and lowercase letters the intron. The deduced amino acid sequence is above the corresponding DNA sequence, bold amino acids mark the differences to Ara h 8.0101.

Figure 6 Immunoblot analysis of recombinant Ara h 8 with patients' sera.

15% SDS-PAGE, 5 µg rAra h 8/cm, reduced conditions, PVDF membrane. Lane 1: India Ink, lane 2-6: Patient sera (WE-J, MW-J, KH-J, KU-J, KP28), lane 7: rabbit anti-Gly m 4, lane 8: anti-his antibody, lane 9: negative control anti-human IgE, lane 10: negative control anti-rabbit IgG, lane 11: negative control anti-mouse IgG.

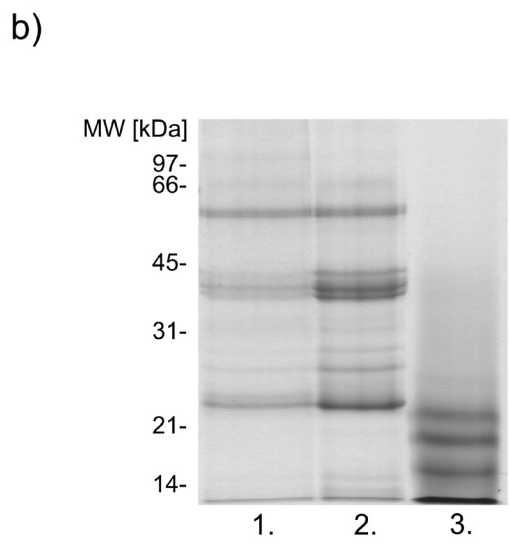
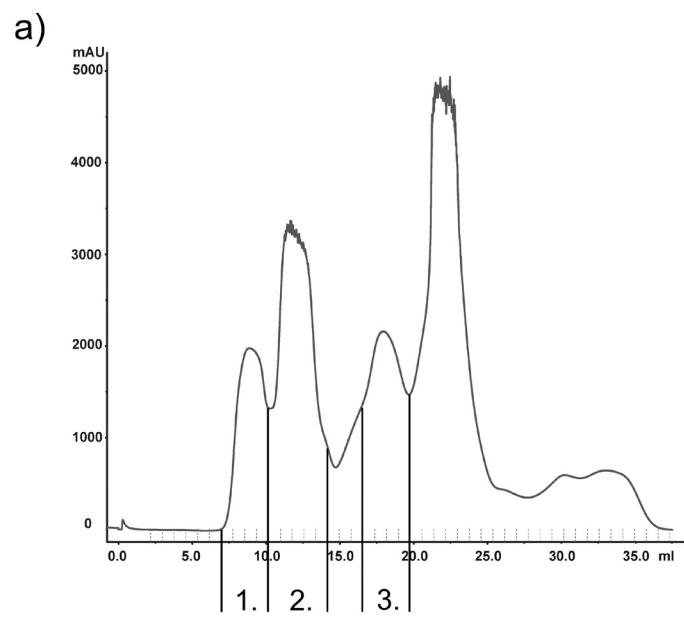
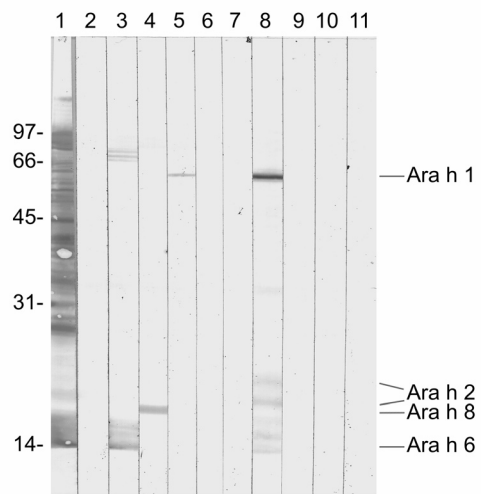
Figure 7 Mass spectrometric analysis of nAra h 8.

Overview of the ESI FT-ICR MS/MS spectrum and enlargement of the molecular ion region around 16 280 kDa.

Figure 8 Tryptic digestion of nAra h 8.

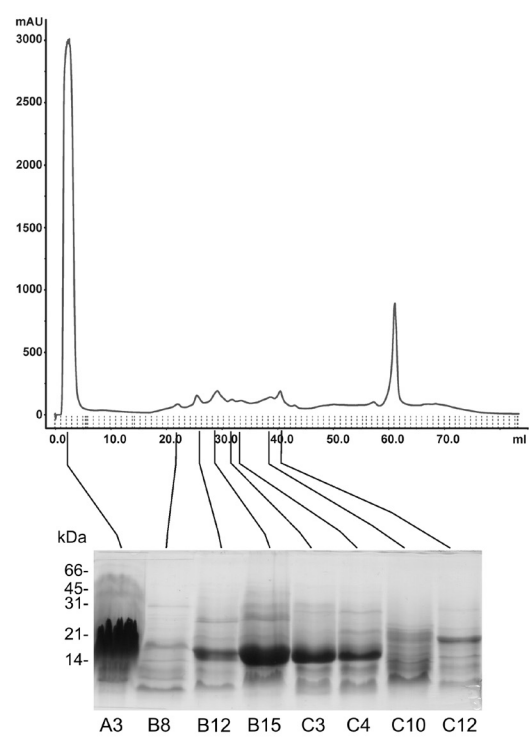
Protein sequence of Ara h 8.0201 covered with the identified fragments of tryptic digestion (grey marked regions) of nAra h 8. The black frame indicates the determined N-terminal sequence.

Characteristics of natural Ara h 8

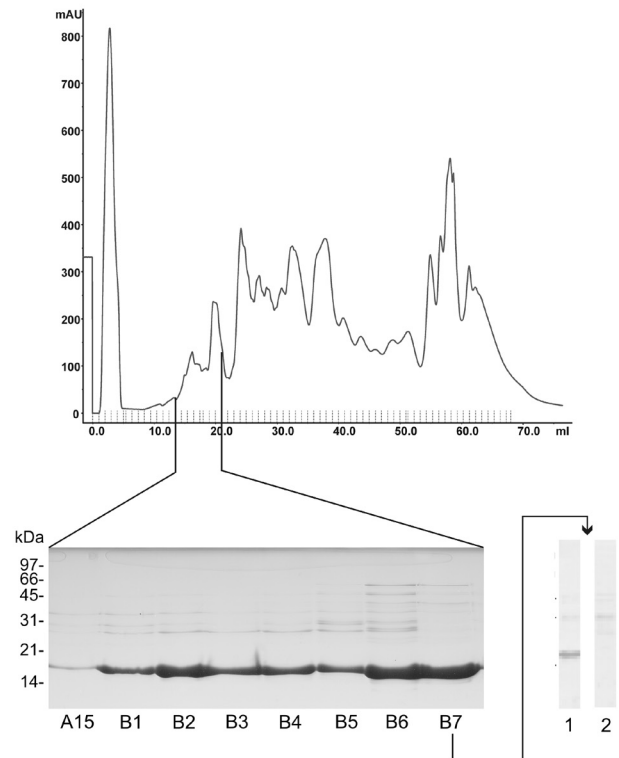


Characteristics of natural Ara h 8

a)



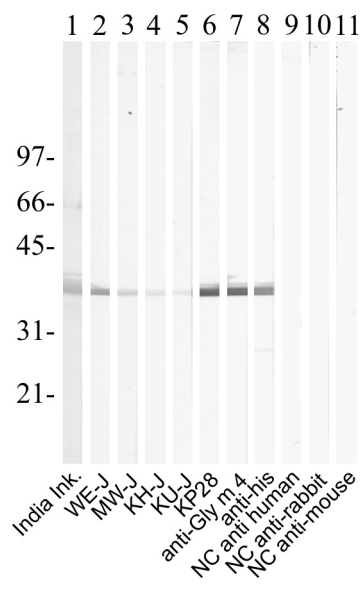
b)



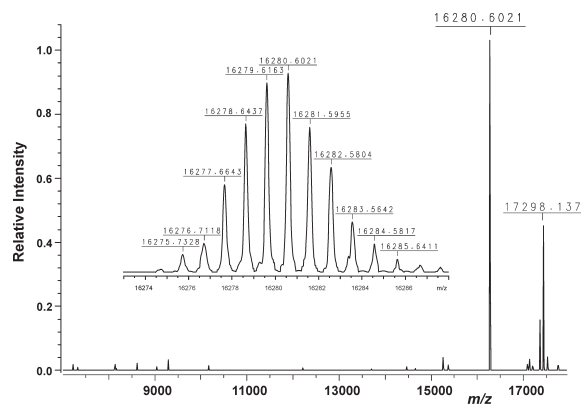
Characteristics of natural Ara h 8

M G V F T F E D E I T S T L P P A K L Y
 1 **ATGGGCGTCTTCACCTTCGAGGATGAAATCACCTCCACCCTGCCCCCTGCCAAGCTTTAC**
ATGCCGCAGAAGTGAAAGCTCCTACTTTAGTGGAGGTGGGACGGGGGACGGTTCGAAATG
 N A M K D A D S I T P K I I D D V K S V
 61 **AATGCTATGAAGGATGCCGATTCCATCACCCCTAAGATTATTGATGACGTCAAGAGTGTT**
TTACGATACTTCCTACGGCTAAGGTAGTGGGGATTCTAATAACTACTGCAGTTCTCACAA
 E I V E G N G G P G T I K K L T I V E _
 121 **GAAATCGTTGAGGGAAACGGTGGTCTGGAACCATCAAGAACTCACCATTGTCGAGG**_{gt}
CTTTAGCAACTCCCTTTGCCACCAGGACCTTGGTAGTTCTTTGAGTGGTAAACAGCTCC_{ca}
 _____Intron_____
 181 cagtaactattcaattcagtatacatatactatatgtgtgataattatgtgttgtaaa
 gtcattgataagttaagtcatatgtatgtatgatatacacactattaatacacaacattt
 _____D G E T K F I L H K V_____
 241 ttgtaataataataatgatatatatgcag**ATGGAGAAACCAAGTTTATCTTACACAAAGT**
 aacattattattattactatatatacgct**TACCTCTTTGGTTCAAATAGAATGTGTTTCA**
 E **A** I D E A N Y A Y N Y S V V G G V A L
 301 **GGAGGCAATAGATGAGGCTAACTATGCATACAACCTACAGCGTGGTTGGAGGAGTGGCGCT**
CCTCCGTTATCTACTCCGATTGATACGTATGTTGATGTCGCACCAACCTCCTCACCGCGA
 P P T A E K I T F E T K L V E G P N G G
 361 **GCCTCCACGGCGGAGAAGATAACATTTGAGACGAAGCTGGTAGAAGGACCCAACGGAGG**
CGGAGGGTGCCGCCTCTTCTATTGTAACTCTGCTTCGACCATCTTCCTGGGTTGCCTCC
 S I G K L **S V K F H S K G E A K P E E E**
 421 **ATCCATCGGGAAGCTGAGTGTGAAGTTCCACTCGAAAGGAGAAGCGAAGCCAGAGGAGGA**
TAGGTAGCCCTTCGACTCACACTTCAAGGTGAGCTTTCCTCTTCGCTTCGGTCTCCTCCT
D M K K G K A K G E A L F K A I E G Y V
 481 **AGACATGAAGAAGGGTAAGGCCAAGGGCGAAGCTCTCTTCAAGGCTATTGAGGGTTACGT**
TCTGTACTTCTTCCCATTCCGGTTCCCGCTTCGAGAGAAGTTCCGATAACTCCCAATGCA
 L A N P T Q Y
 441 **TTTGGCCAACCCTACTCAATAT**
AAACCGGTTGGGATGAGTTATA

Characteristics of natural Ara h 8



Characteristics of natural Ara h 8



Characteristics of natural Ara h 8

1 * 10 * 20 * 30 * 40 * 50
1 GVHTFEEESTSPVPPAKLFKATVVDGDELTPKLIPAIQSIEIVEGNGGPG
51 TVKKVTAVEDGKTSYVLHKIDAIDEATYTYDYTISGGTGFQEILEKVSFK
101 TKLEAADGGSKIKVSVTFHTKGDAPLPDEVHQDVKQKSQGIFKAIEGYVL
151 SN