DOI 10.1002/mnfr.200400028 Mol. Nutr. Food Res. 2004, *48*, 390–399

Isolation and characterization of natural Ara h 6: Evidence for a further peanut allergen with putative clinical relevance based on resistance to pepsin digestion and heat

Martin Suhr, Daniel Wicklein, Ute Lepp and Wolf-Meinhard Becker

Research Center Borstel, Borstel, Germany

Peanut allergy is a significant health problem because of its prevalence and the potential severity of the allergic reaction. The characterization of peanut allergens is crucial to the understanding of the mechanism of peanut allergy. Recently, we described cloning of the peanut allergen Ara h 6. The aim of this study was isolation and further characterization of nAra h 6. We purified nAra h 6 from crude peanut extract using gel filtration and anion exchange chromatography. The preparation was further characterized by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) with subsequent immunoblotting. Stability of nAra h 6 was studied by an *in vitro* digestibility assay as well as by resistance against thermal processing. Sequencing of nAra h 6 identified the *N*-terminal amino acid sequence as MRRERGRQGDSSS. Further results clearly demonstrated stability of nAra h 6 against pepsin digestion and heating. Immunoglobulin G (IgE) binding analysis and its biological activity shown by RBL 25/30-test of natural Ara h 6 supported the importance of this peanut allergen. Investigation of nAra h 6 revealed evidence for a further peanut allergen with putative clinical relevance based on resistance to pepsin digestion and heat.

Keywords: Ara h 6 / Digestion / Food allergy / Peanut allergens / Thermostability

Received: June 3, 2004; accepted: June 4, 2004

1 Introduction

Peanut allergy belongs to the group of immunoglobulin G (IgE)-mediated food allergies. It is characterized by a higher frequency of severe reactions than found in other food allergies. Fatal food-induced anaphylaxis affecting children as well as adults is often induced by exposure to peanut [1–5]. A recent survey in the USA suggests that 0.7% of children are allergic to peanuts [6]. In contrast to other food allergies such as to milk and egg allergy hypersensitivity to peanut is usually persistent. Only in 21.5% of the patients the peanut allergy is outgrown [7]. Due to its prevalence and the potential severity of allergic reactions identification and molecular characterization of peanut allergens is an essential step towards improving diagnosis and therapy.

Correspondence: Dr. Wolf-M. Becker, FZB, Parkallee 35, D-23845 Borstel, Germany
E-mail: wbecker@fz-borstel.de
Fax: +49-4537-188328

Abbreviations: IgG, immunoglobulin G; **nAra h 6**, natural Ara h 6 **rAra h 6**, recombinant Ara h 6; **RBL 25/30**, transfected rat basophilic leukemia cell line

Three major peanut allergens have been studied in detail. (i) Ara h 1 which belongs to the vicilin family is a 63.5 kDa glycoprotein recognized by more than 95% of patients with peanut hypersensitivity [8]. (ii) Ara h 2 with homology to the conglutin family of seed storage proteins was described as a glycoprotein which is composed of two closely migrating bands with a mean molecular mass of 17 kDa [9]. (iii) Ara h 3 was originally identified as a 14 kDa breakdown fragment and later classified as glycinin [10, 11]. However, based on IgE binding, various studies about serological characteristics of peanut allergy emphasize the diversity of peanut allergens [12–14]. These findings support the need for definite analysis of all allergenic components. Therefore, further peanut allergens were identified by phage display technology and their respective cDNA sequences were cloned in our laboratory. Clinical relevance was studied by IgE-binding after their recombinant expression in *Escherichia coli* using sera from peanut allergic individuals [15].

The identification of Ara h 4 which exhibits 56% amino acid identity to sequences of soybean glycinins revealed high amino acid identity (91.3%) to Ara h 3 indicating that both are members of the glycinin storage protein family. Nevertheless, both allergens were accepted separately by the IUIS Allergen Nomenclature Sub-Committee at point

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

of submission [16]. Ara h 5, a protein highly homologous to plant profilins, showed 13% IgE binding by use of sera from peanut allergic patients. Obviously, it only plays a minor role in peanut allergy and may explain sensitivity to pollen and food of some allergic individuals by cross-reactivity [17]. The allergens Ara h 6 and Ara h 7 have high similarity to proteins of the conglutin family related to the 2S superfamily of seed storage proteins. Ara h 6 and Ara h 7 show 59% and 35% amino acid sequence identity to Ara h 2, respectively. Despite these homologies we clearly found differences in IgE binding leading to the classification of Ara h 6 and Ara h 7 as allergens of intermediate frequency [15]. However, further analysis of these allergens is necessary for standardization and reliability of diagnostic extracts. Furthermore, understanding the biochemistry of these allergens may help to elucidate the mechanisms involved in the severity of peanut allergy.

Thus, the aim of this study was to characterize nAra h 6 after purification from crude peanut extract. We describe successful isolation of nAra h 6. Purification enabled definite identification by *N*-terminal sequencing. The results are important for further investigations concerning clinical relevance, improvement of diagnosis and development of immunotherapeutic approaches.

2 Materials and methods

2.1 Human sera

Patients sensitized to peanut were selected according to case history and radioallergosorbent tests. Sensitization was confirmed by Western blot analysis of IgE binding to peanut proteins and individual recombinant allergens. Sera were collected at the Medical Hospital Borstel after giving informed consent and used for detection of IgE binding to peanut proteins. Sera JG3, HF4, JB5, GK31, and SK26 were described previously [16].

2.2 Crude peanut extract

Peanuts (*Arachis hypogaea*, Virginia) were ground under liquid N_2 and further extracted in 0.1 M $(NH_4)_2CO_3$, pH 8 for 4 h at 4°C. After centrifugation the supernatant was filtered (0.2 µm pore size) and dialyzed. Samples were stored freeze-dried at −20°C until use. The protein content was determined according to the method of Bradford [18].

2.3 Chromatography

Chromatography techniques were carried out using a ÄKTApurifier HPLC system (Amersham Pharmacia, Freiburg, Germany). Peanut protein extract (1.2 mg) was dissolved in 50 mM K_2HPO_4/KH_2PO_4, 0.1 M NaCl (pH 7.5) and loaded onto a Superdex 200 column (Amersham Pharmacia). The gel filtration column was calibrated by separation of a protein standard (calibration kit; Amersham Pharmacia). Fractions containing Ara h 6 were pooled, dried by speed vacuum centrifugation and desalted on a HiTrap column (Amersham Pharmacia). Afterwards, Ara h 6 was resuspended in 20 mM Tris-HCl (pH 8) and loaded onto a MiniQ column (Amersham Pharmacia) to perform anion-exchange chromatography using 20 mM Tris-HCl (pH 8) and an increasing gradient up to 0.5 M NaCl as elution buffer.

Table 1. Clinical data of 11 peanut allergic patients

Patient #		Age (years)	Symptoms[a)]		Cap	Total IgE (kU/L)	Specific IgE reactivity [16]
JG3	1	25	OAS, R, D, A, N, U	Shock	6	3693	rAra h 2, 4, 6, 7
GK31	2	12	OAS, U, A	Shock	6	393	rAra h 2, 4, 6, 7
KH37	3	34	OAS		5	166	
JB5	4	22	OAS		4	3961	rAra h 2, 4, 7
UM33	5	22	OAS, U, N		5	452	
J T	6	11	AD, A		6	1221	
AS	7	6	R, A, U		6	2198	
WZ	8	27	OAS		6	1969	
HJ	9	30	OAS, A	Shock	6	450	
SK26	10	25	OAS, D, R, N, A	Shock	5	438	rAra h 2, 4, 6, 7
HF4	11	36	OAS, A, U, N	Shock	5	329	rAra h 2, 4, 6, 7
LH	12	59	OAS, R → tree nuts		0	8.2	IgE control

a) OAS, oral allergy syndrome; R, rhinitis; A, asthma; N, neurodermatitis; U, urticaria; D, diarrhoea; AD, atopic dermatitis

2.4 Electrophoresis and immunoblotting

Discontinuous SDS-PAGE was performed according to the method of Laemmli [19] ready-to-use NuPAGE™ Bis-Tris gels (Invitrogen, Groningen, Netherlands) were applied for separation of low-molecular-mass proteins (<25 kDa) according to manufacturer's instructions. After electrophoresis proteins were either stained with Coomassie blue or for Western blot analysis transferred to nitrocellulose (NC) membranes (0.45 µm; Schleicher & Schuell, Dassel, Germany) by semidry blotting [20] at 0.8 mA/cm^2 for 30 min or to polyvinylidene difluoride (PVDF) membranes (Millipore, Eschborn, Germany) using the tank-blot system of the manufacturer. NC membranes were blocked by incubation in 0.1 M TBS (pH 7.4) containing 0.05% v/v Tween 20 (TBST) for 1 h under gentle agitation. Blocking of PVDF membranes was achieved by shaking in 5% non-fat dry milk/TBS. Afterwards, the membrane was incubated in an appropriate dilution of primary antibody. Human sera were diluted 1 : 10 in TBST. The Ara h 6 specific rabbit serum was applied in a dilution of 1 : 10 000 whereas the anti Ara

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

h 2 serum was diluted $1:1 \times 10^6$. Specific binding of human IgE or rabbit antibodies were detected using either a mouse antibody anti-human-IgE (1:2000; Allergopharma, Reinbek, Germany) or goat antibody anti-rabbit-IgG (1:5000; Dianova, Hamburg, Germany) conjugated with alkaline phosphatase (AP). Bound antibodies were visualized after three washes with TBST by incubation with NBT/BCIP as substrate in AP-Puffer (0.1 M TBS, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5).

2.5 Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

2-D PAGE was carried out according to the method of Görg *et al*. [21]. Isoelectric focusing in the first dimension was done using polyacrylamide (PAA) strips (4% PAA) with an immobilized pH gradient (3.6–9.3) containing 8 M urea and 10 mM dithiothreitol. A sample of 25 µg protein was applied and separated at 3000 V and 2 mA for 5 h. After subsequent equilibration steps in buffer I (6 M urea, 30% glycerol, 50 mM Tris-HCl, pH 8.8, 2% SDS, 0.06 M DTT) and buffer II (6 M urea, 30% glycerol, 50 mM Tris-HCl, pH 8.8, 2% SDS, 0.14 M iodoacetamide) SDS-PAGE was performed at 600 V and 50 mA using gradient gels (5–20% PAA). The p*I* and molecular mass were determined using an IEF test mixture (pH range 3–10; Serva, Heidelberg, Germany) and low-range SDS standard (Bio-Rad, München, Germany) as marker proteins, respectively. Thermoblotting was used to perform immunological tests.

2.6 *N*-Terminal sequencing

N-Terminal protein sequencing was performed by sequential Edman degradation. After separation by SDS-PAGE proteins were blotted onto a PVDF membrane. After staining with Coomassie blue relevant proteins were excised and subjected to protein sequencing employing a 476A sequencer (Applied Biosystems, Weiterstadt, Germany).

2.7 Recombinant expression of peanut allergens

For generation of polyclonal rabbit antisera, Ara h 6 and Ara h 2 were expressed in *Escherichia coli* BL21(DE3)-RIL and purified as his-tag fusion proteins as described previously [22].

2.8 Rabbit antisera

Chincilla bastard rabbits (Charles River Wiga, Sulzfeld, Germany) were immunized by intramuscular injections of 100 µg rAra h 6, 100 µg rAra h 2, and 100 µg Multiple Antigen Peptides of epitope 3 of Ara h 2 (MAP-3), respectively, using the TiterMax Adjuvant system (Sigma, Deisenhofen, Germany).

2.9 Digestibility and thermostability of nAra h 6

Purified nAra h 6 (0.4 mg) as well as 0.4 mg conalbumin (Sigma, Deisenhofen, Germany) were incubated with 0.04 mg pepsin-agarose (64.9 U/mg solid; Sigma) in 0.25 M formic acid puffer (pH 3) under gentle agitation. At different times (t = 0, 30, 60, 90, 120 min) pepsin-agarose was collected by centrifugation (3 min, $3180 \times g$). The supernatants were analyzed by SDS-PAGE. Thermostability of peanut proteins was studied by generating biscuits: peanut extract was heated in the presence of sugar, butter and wheat flour for different times (t = 15, 40 min) at 180°C to stimulate thermal food processing. Biscuits were extracted as described above for production of crude peanut protein extract.

2.10 Mediator release from transfected rat basophilic leukemia (RBL 25/30) cells

The mediator release assays followed a protocol established by Hoffmann *et al.* [23]. Briefly, RBL 30/25 cells expressing the α-chain of the human Fc-RI receptor were plated in 96-well cell culture plates (Nunc, Wiesbaden, Germany) at 1.5×10^5 cell per well. The cells were passively sensitized with IgE-containing human (JG3, PEI 163) sera and a serum form a nonallergic subject (PEI 231), respectively. After washing, the cells were stimulated with peanut extract or nAra h 6. The antigen-specific release was quantified by measuring ε-hexosaminidase activity and expressed as per cent of the total ε-hexosaminidase content that was obtained by lysing the cells with Triton-X100. For measurements of spontaneous release and possible nonspecific effects, naive RBL cells were incubated with Tyrode's buffer, cell culture medium, and antigen without specific antibodies, respectively.

3 Results

3.1 Identification of nAra h 6 in crude peanut extract

Phage display technology enabled identification and cloning of Ara h 6. (Genbank Acc. No. AF092846). This protein was characterized as allergen by IgE binding after recombinant expression in *E. coli*. [15]. According to the deduced molecular mass of 14.5 kDa for Ara h 6 we improved elec-

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

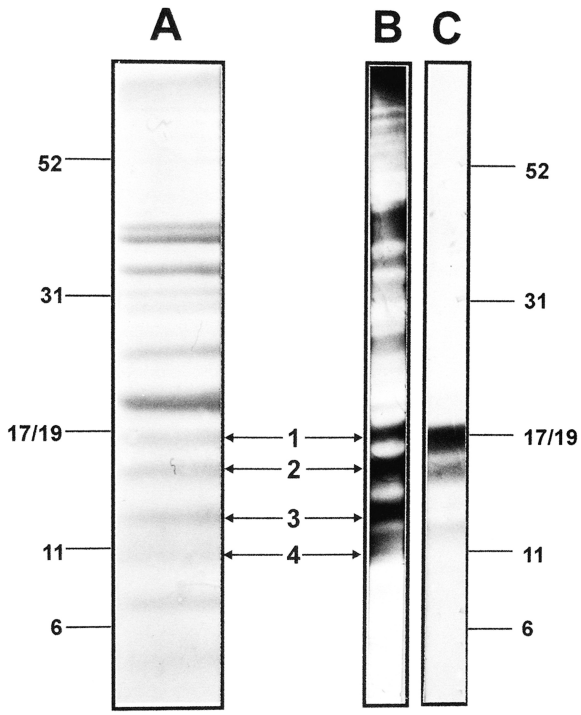

Figure 1. Identification of IgE binding peanut proteins. Crude peanut extract was separated using the NuPAGE™ Bis-Tris gel system. After Western blotting onto a PVDF membrane, proteins were either stained with Coomassie blue (A) or used for immunochemical detection applying serum JG 3 of a peanut allergic patient (B) or the specific rabbit antiserum raised against rAra h 2 (C). Molecular sizes of marker proteins are indicated (kDa). Four proteins (samples 1–4) subjected to N-terminal sequencing are marked by an arrowhead.

Table 2. Results of *N*-terminal sequencing of different IgE-binding peanut proteins

Sample	*N*-Terminal sequence	Peanut protein
1	RQQxELQGDR	Ara h 2
2	RQQxELQG	Ara h 2
3	MEDERGxQGDxS/MERERGxQGDS	Ara h 6
4	ISFRQ	Ara h 3/Ara h 4

x: amino acid not clearly identified

trophoretic resolution of low-molecular-mass proteins by using NuPAGE™ Bis-Tris gels for separation of peanut proteins. After transfer onto a PVDF membrane the proteins were either stained with Coomassie blue (Fig. 1 A) or used for immunochemical detection (Figs. 1 B, C). Several peanut proteins were detected by IgE-binding using serum from patient JG3 (Fig. 1 B). According to available data binding of IgE to Ara h 1 at about 63.5 kDa [8] and to Ara h 2 consisting of two isoforms with a mean molecular mass of 17 kDa could be identified [9]. Further reactivity is probably due to IgE binding to Ara h 3 and its fragments. Because of the expected molecular mass of Ara h 6 four IgE binding proteins (samples 1–4; Fig. 1) in the range between 10 and 20 kDa were subjected to *N*-terminal sequencing. The results are summarized in Table 2. The sequences of sample 1 and 2 showed identity to the *N*-terminus of Ara h 2 indicating identification of the closely migrating isoforms. This was confirmed by immunochemical detection using the Ara h 2 specific rabbit serum (Fig. 1 C). The *N*-terminal sequence of sample 3 with an apparent molecular mass of about 15 kDa revealed high similarity with sequence of Ara h 6. Furthermore, Edman degradation of another IgE binding protein (sample 4) identified a fragment of Ara h 3 whose *N*-terminal sequence corresponds to a 14 kDa fragment [10].

3.2 Purification of nAra h 6

After identification of Ara h 6 in crude peanut extract the allergen was purified by chromatography. Peanut proteins were separated according to their molecular masses by use of a Superdex 200 column. After gel filtration fractions were analyzed by SDS-PAGE. Western blot analysis using the specific rabbit serum detected Ara h 6 in fractions C12–C14 (Fig. 2 B). Protein staining with Coomassie blue identified Ara h 6 as one of the major protein components of these fractions (Fig. 2 A). Calibration of the column revealed that Ara h 6 could be detected in fractions corresponding to the expected molecular mass range. Furthermore, these fractions contained the isoforms of Ara h 2 as detected by Western blot analysis using the Ara h 2-specific serum (Fig. 2 C). A weak cross-reactivity of the Ara h 6-specific serum with Ara h 2 and *vice versa* could be observed (Fig. 2 B, C). This cross-reactivity is probably due to homology between both protein sequences. The proteins showed an apparently higher molecular mass in SDS-PAGE according to Laemmli than observed by application of the NuPAGE™-Tris-Bis gels (see Fig. 1). We have no explanation for this apparent difference in mobility. Nevertheless, immunochemical detection as well as IgE-binding confirmed the identity of the allergens investigated.

Fractions C12–C14 were pooled and loaded onto an anion-exchange MiniQ column for further purification of natural Ara h 6. Protein was eluted by applying an increasing salt gradient up to 0.5 M NaCl. As shown in Fig. 3 A, the A_{280} profile detected several protein peaks. Ara h 6 was identified in a number of fractions (B7–B15) by Coomassie blue staining (Fig. 3 B) as well as by Western blot analysis (Fig. 3 C), however clearly separated from Ara h 2 as detected by the use of Ara h 2-specific rabbit antibodies (data not shown). Retarded elution of Ara h 6 corresponding to at least two different peaks in the A_{280} profile indicates existence of isoforms harboring different elution properties. This was confirmed by results of 2-D PAGE using a sample from the gel filtration pool (C12–C14) containing enriched

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

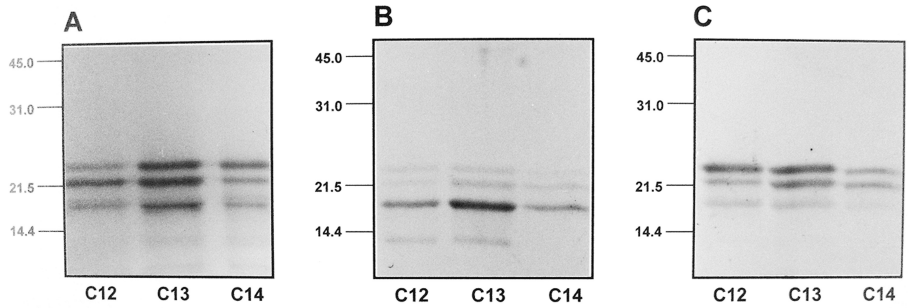

Figure 2. Isolation of nAra h 6 by gelfiltration of peanut proteins. Peanut proteins were separated performing gel filtration by the use of a Superdex 200 column. Fractions were analyzed by 15% SDS-PAGE according to Laemmli. The relevant protein fractions C12–C14 containing Ara h 6 are shown. Proteins were either stained with Coomassie blue (A) or analyzed applying the specific rabbit antiserum raised against rAra h 6 (B) or rAra h 2 (C), respectively. Molecular sizes of marker proteins are indicated (kDa).

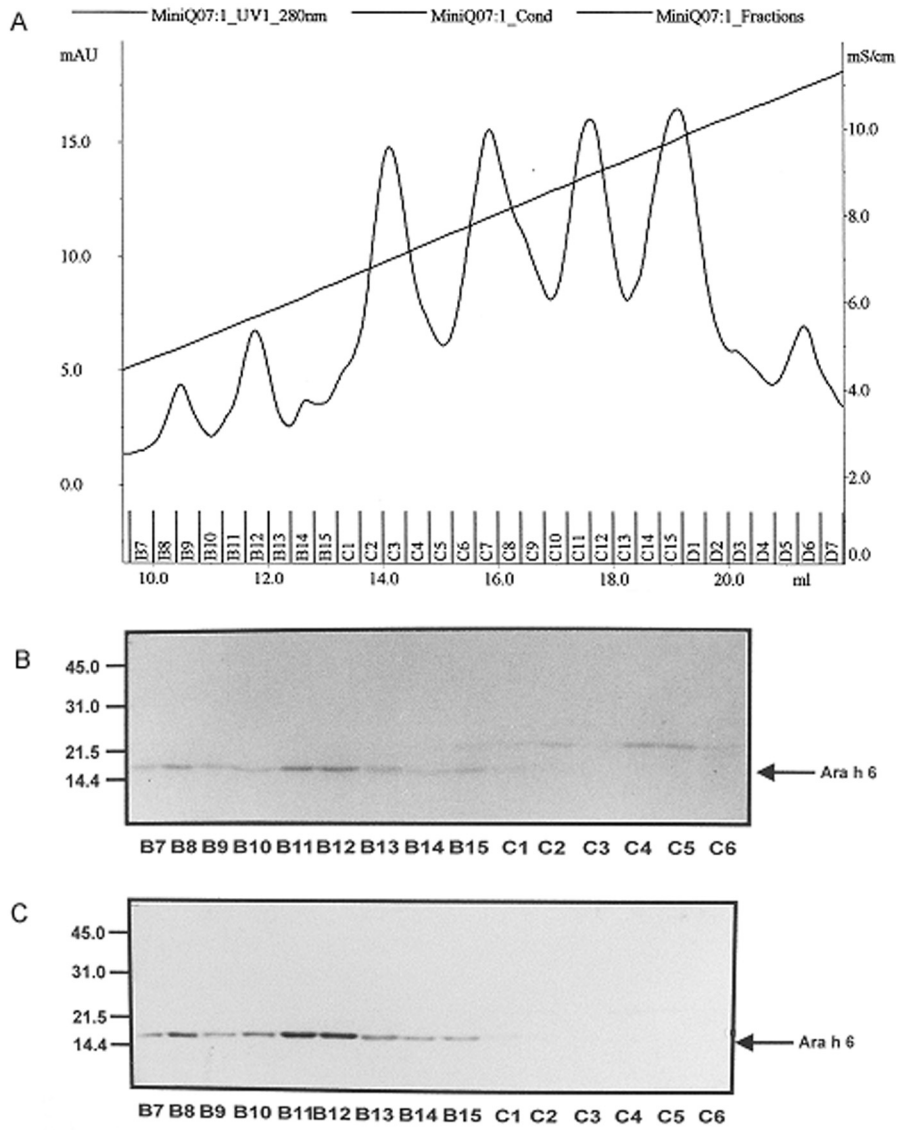

Figure 3. Purification of nAra h 6. After gel filtration enriched Ara h 6 was further purified by anion-exchange chromatography. The elution profile is illustrated (A). Protein peaks were detected by measuring $A_{280\,nm}$ (mAU: milli absorption units). Numbering of the fractions and increasing conductivity (in mS/cm) are indicated. Fractions B7–C6 were analyzed by 15% SDS-PAGE according to Laemmli. Proteins were either stained with Coomassie blue (B) or analyzed by the use of the Ara h 6-specific antiserum (C). Molecular sizes of marker proteins are indicated (kDa).

Ara h 6. 25 µg protein was analyzed by 2-D PAGE and stained with Coomassie blue (Fig. 4A) or used for Western blot analysis applying the Ara h 6 specific antiserum (Fig. 4B). Immunodetection identified at least three distinct protein spots corresponding to isoforms of Ara h 6 with different p*I* values in the range between p*I* 5–6. This is in accordance with the calculated p*I* of 5 derived from the translated cDNA sequence.

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

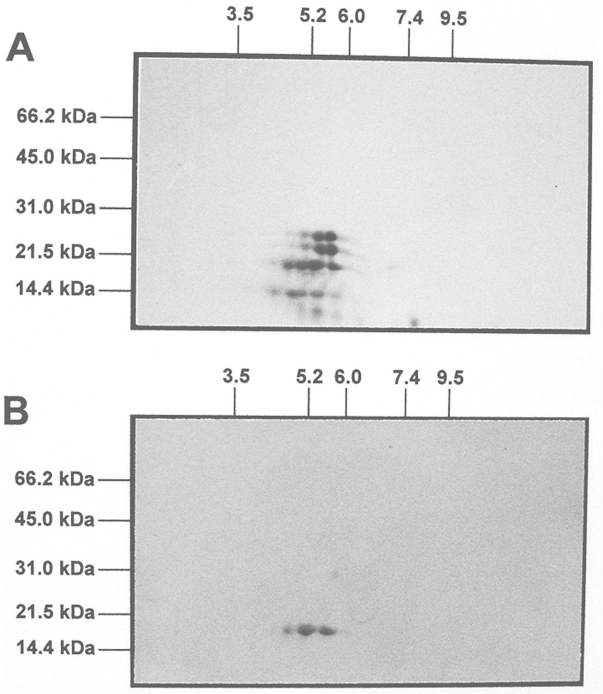

Figure 4. 2-D PAGE of nAra h 6. 25 μg of nAra h 6 enriched by gel filtration were analyzed by 2-D PAGE and stained with Coomassie blue (A) or, after Western blotting, used for immunochemical detection of nAra h 6 (B) applying the specific rabbit antiserum. Molecular sizes of marker proteins are indicated (kDa).

3.3 *N*-Terminal sequencing of purified nAra h 6

Natural Ara h 6 purified by anion-exchange chromatography was separated by SDS-PAGE, blotted onto a PVDF membrane, and stained with Coomassie blue. Sequencing by Edman degradation identified the *N*-terminal amino acid sequence as MRRERGRQGDSSS. This sequence revealed 100% identity to the aa sequence of Ara h 6 deduced from the cDNA clone starting with Met_6. Whereas *N*-terminal sequencing of Ara h 6 separated by SDS-PAGE of crude peanut extract revealed only partial homologues sequences (Table 1), Edman degradation of purified Ara h 6 definitely identified the authentic *N*-terminus.

3.4 IgE-binding analysis

Eleven clinically characterized patients' sera (Table 1, Section 2) were investigated in their IgE reactivity to peanut extract. All tested sera showed IgE binding to Ara h 2 and all but one (patient No. 5) to Ara h 6. Interestingly, the relative IgE-binding intensities against Ara h 2 and Ara h 6 of the tested sera do not correspond: For instance, patient No. 8 reacted strongly with Ara h 2 but only weakly with Ara h 6 in contrast to patient No. 6 showing weak IgE reactivity to Ara h 2 but stronger IgE binding to Ara h 6 compared to patient No. 8 (Fig. 5 A).

After purification of nAra h 6 from peanut extract we analyzed IgE binding using human sera which were previously

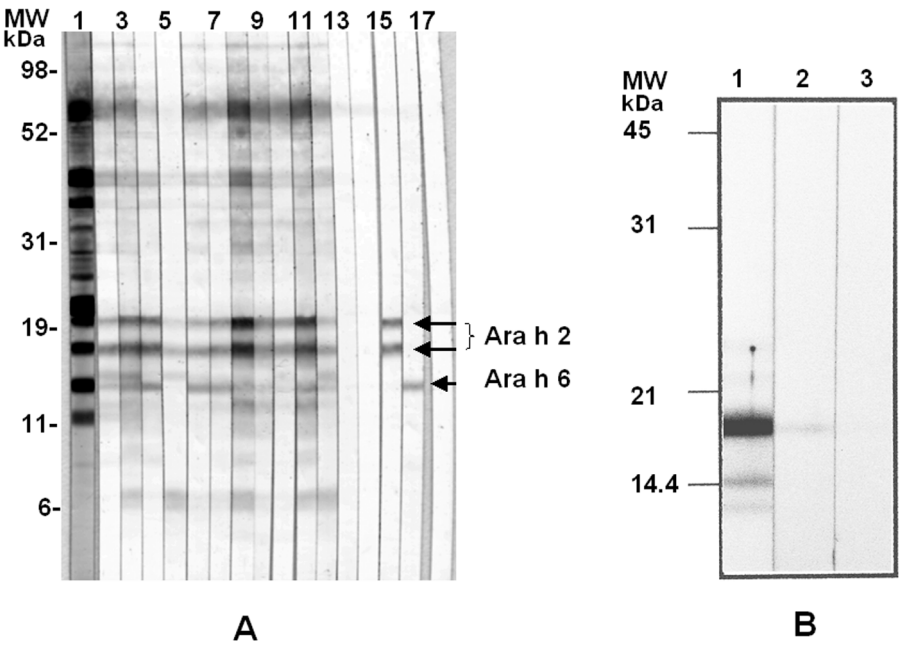

Figure 5. IgE-binding to peanut extract (A) and nAra h 6 (B). (A) Crude peanut extract (15 μg/cm) was separated using the NuPAGE™ Bis-Tris gel system. After Western blotting onto a PVDF membrane, proteins were either stained with India Ink (1) or used for immunochemical detection applying 11 sera of peanut allergic patients following the order of Table 1 (2–12); 2, JG3; 5, JB5; 12, HF4: 13, IgE control; 14, mouse anti-human IgE control; 15, rabbit anti-Ara h 2 (MAP-3); 16, rabbit anti-Ara h 6; 17, goat anti-rabbit IgG control. Molecular sizes of marker proteins are indicated (kDa). (B) The purified allergen was separated by 15% SDS-PAGE and transferred to a nitrocellulose membrane. IgE-binding was assessed by applying a 1:10 dilution of sera from different peanut-allergic patients (lane 1, JG3; lane 2, HF4; lane 3, JB5). A monoclonal anti-human-IgE antibody was applied for visualization of bound IgE. Molecular sizes of marker proteins are indicated (kDa).

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

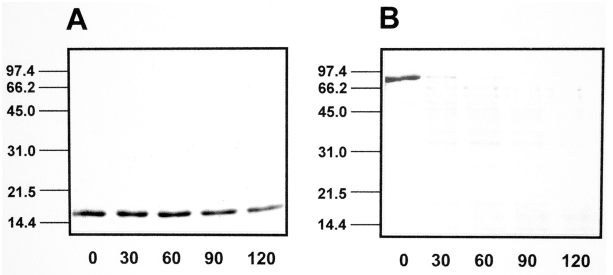

Figure 6. *In-vitro* digestibility using immobilized pepsin. Stability of nAra h 6 (A) and conalbumin (B) was studied by pepsin digestion, as described in Section 2. At different times (t = 30, 60, 90, 120 min) samples were withdrawn and analyzed by 12% SDS-PAGE with subsequent Coomassie blue staining. The respective controls (3 μg each lane) without addition of pepsin are shown (t = 0). Molecular sizes of marker proteins are indicated (kDa).

characterized according to their IgE binding to peanut allergens recombinantly expressed in *E. coli*. [16]. As expected from the previous study, IgE binding to nAra h 6 could not be detected using serum JB5 (Fig. 5B, lane 3) which showed likewise no IgE reactivity to rAra h 6. However, HF4 (lane 2) showed weak and JG3 (lane 1) strong IgE binding to nAra h 6 confirming identity of the purified allergen.

3.5 Stability of nAra h 6

It was proposed that the ability of food allergens to reach the intestinal mucosa is a prerequisite to allergenicity of food proteins. This necessarily implies stability against different conditions of the digestive tract. Therefore, allergens may be quite resistant to digestive enzymes and various forms of food processing. Firstly, purified Ara h 6 was incubated with agarose-immobilized pepsin. At different times samples were withdrawn and analyzed by SDS-PAGE with subsequent protein staining or Western blot analysis. Ara h 6 was clearly detected after 120 min of digestion indicating resistance against proteolysis (Fig. 6A). Used as appropriate control, stability of conalbumin which was known to be degraded rapidly using simulated gastric fluid (SGF) [32] was analyzed. As expected, conalbumin was quickly degraded and only detectable in the control sample without pepsin (Fig. 6B). After incubation with pepsin for 30 min several fragments became visible by Coomassie blue staining.

Next, we heated peanut extract in the presence of sugar, butter, and wheat flour as biscuit material for different times at 180°C to simulate a roasting process. Protein extracts of the biscuits were analyzed by SDS-PAGE. Stability of Ara h 6 and Ara h 2 was assessed by immunochemical detection using the specific rabbit antisera. To keep the analysis comparable equal amounts of protein were analyzed. As shown in Fig. 7A protein pattern as well as protein content changed after 15 min (lane 2) and 40 min (lane 3) of heating compared with the control sample without heating (lane 1). Western blot analysis using the specific rabbit sera showed that Ara h 6 (Fig. 7C) as well as Ara h 2 (Fig. 7B) could be detected after 15 (lane 2) and 40 min (lane 3) of heating indicating resistance against food processing. Furthermore, both proteins were detected by IgE-binding using serum from patient JG3 again indicating clinical relevance of these allergens (data not shown). As already described (Fig. 2), cross-reactivity of the sera are due to the homology between the sequences of Ara h 6 and Ara h 2. Nevertheless, both allergens could be clearly identified by Coomassie blue staining of the protein extract after 40 min. Taken together, the results demonstrate stability of Ara h 6 against pepsin digestion and heating indicating a resistant conformation that potentially could promote allergenicity of this protein.

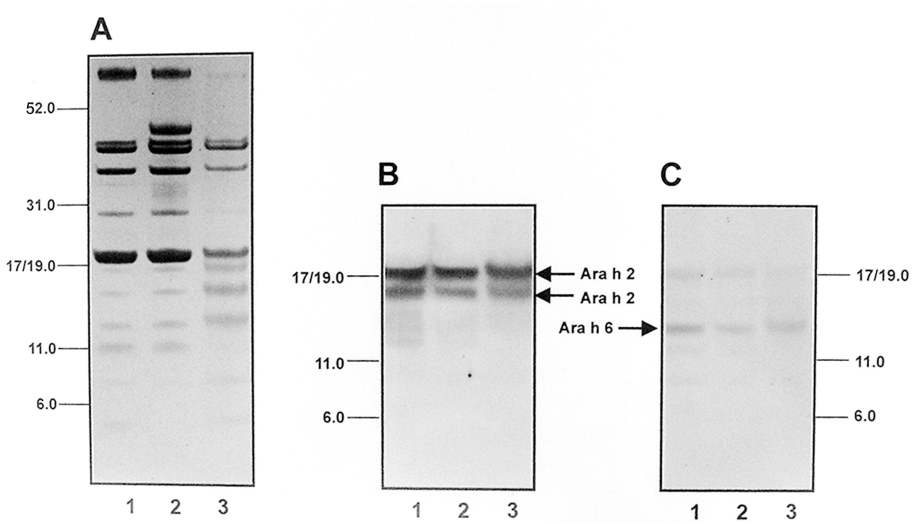

Figure 7. Resistance of peanut proteins against heating. Peanut extract was heated at 180°C as biscuit material. At different times, biscuits were extracted and the resulting protein extracts were analyzed by gel electrophoresis using the NuPAGE™ Bis-Tris gel system (15 μg each lane). Proteins were either stained with Coomassie blue (A) or, after Western blotting, used for immunochemical detection applying Ara h 2 (B) or the Ara h 6 (C) specific antibodies, respectively. Lane 1, control without heating; lane 2, after 15 min of heating; lane 3, after 40 min of heating. Molecular sizes of marker proteins are indicated (kDa).

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

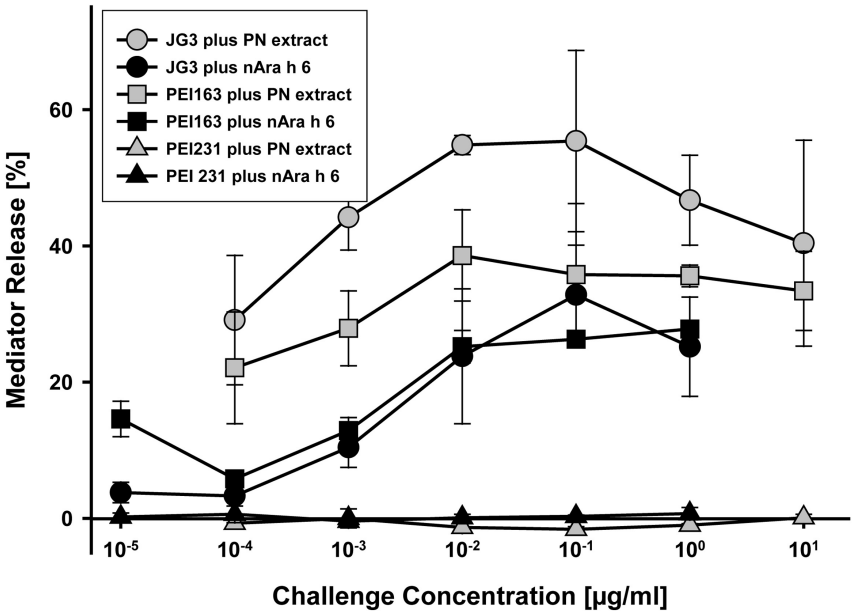

Figure 8. Mediator release from RBL 25/30. Cells sensitized with sera of peanut-allergic subjects (JG3, PEI 163) and a control subject (PEI 231). Sensitized cells were challenged with serial dilutions of peanut (PN) extract (10 µg/mL – 0.1 ng/mL) and purified natural Ara h 6 (1.0 µg/mL – 10 pg/mL).

3.6 RBL 25/30 cells

The mediator release experiments (Fig. 8) show allergenic activity of the purified natural Ara h 6. In comparison with the release induced by peanut extract, Ara h 6 accounts for up to 59.2% and 72.0% of the allergenic activity of the peanut extract.

4 Discussion

Because of the severity of allergic reactions to peanuts identification and molecular characterization of peanut allergens is important for a definite diagnosis. Based on IgE-binding several proteins have been identified suggesting a variety of allergenic proteins to be clinically relevant. In addition to the well-characterized major allergens Ara h 1, Ara h 2 and Ara h 3 further peanut allergens, namely Ara h 5, Ara h 6 and Ara h 7, were identified and cloned by phage display technology. As indispensable, but admittedly not sufficient, prerequisite of clinical relevance of the allergens their IgE-binding after recombinant expression in *E. coli* was shwon [15]. As member of the conglutin family Ara h 6 containing 10 cysteines in its primary structure came into focus. The aim of this study was further characterization of this allergen, especially identification of it in peanut extract as well as purification of natural Ara h 6. Because of the molecular mass of Ara h 6 deduced from the cDNA clone we used an optimized gel system which was shown to be superior to conventional electrophoretic methods for identifying low-molecular-mass hazelnut allergens [24]. IgE-binding identified several proteins in the expected molecular mass range. *N*-Terminal sequencing enabled identification of Ara h 6 which exhibited a molecular mass of 15 kDa as determined by protein gel electrophoresis. Previously, a 15 kDa peanut protein with strong association to severe allergic reactions against peanuts was described [13]. This protein was immunochemically detected by 54% of human sera but not further characterized by biochemical data. Whether this protein corresponds to Ara h 6 recognized by IgE from 38% of peanut allergic individuals in our study remains hence unclear [15]. Burks *et al.* [10] stated in their review that less than ten of the peanut proteins were likely to be clinically relevant. Proteins of less than 20 kDa were identified by *N*-terminal sequencing. The authors argued that each of these allergens was either a fragment or an isoform of Ara h 1, Ara h 2 or Ara h 3 suggesting that Ara h 6 recently identified in our laboratory may be an isoform of Ara h 2 because of 59% amino acid sequence identity. Despite these homologies we clearly found differences in the frequency of IgE-binding resulting in classification of Ara h 6 as minor allergen [15]. These findings could be confirmed by investigating more patients' sera in this study (Fig. 5 A). In contrast to Ara h 2 no consensus sequence for *N*-glycosylation (NQS/NQT) could be identified in the sequence of Ara h 6.

For further characterization we purified natural Ara h 6. Isolation by gel filtration revealed that this allergen is copurified with the isoforms of Ara h 2. Anion-exchange chromatography enabled us to clearly separate Ara h 6 from Ara h 2. Ara h 6 was detected by the use of a specific rabbit antiserum. Sequencing of by Edman degradation identified the *N*-terminal sequence as MRRERGRQGDSSS. This sequence is identical to the deduced aa sequence of Ara h 6. *N*-Terminal sequencing and immunological detection using the Ara h 6-specific rabbit serum identified breakdown fragments of Ara h 6. Whether these fragments naturally occur in peanut extract or develop during extraction and

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

purification is not clear yet and has to be investigated in further experiments. Fragmentation can be explained by genetic polymorphism which has been described for some plant seed storage proteins. This may result in variation of protein sequences and occurrence of isoforms which were clearly identified for Ara h 6 in this study. However, regardless of post-translational processing, IgE-binding characteristics to mature Ara h 6 are not concerned.

Recently, de Jong *et al.* [14] described the isolation and characterization of peanut allergens. The authors published results of *N*-terminal sequencing of isolated Ara h 2 although there was only a low degree of similarity to the aa sequence of Ara h 2 published by Burks [9]. Surprisingly, the sequence determined by de Jong *et al.* is identical to the predicted aa sequence of Ara h 6 [15]. Consequently, the results published by de Jong *et al.* characterizing Ara h 2 obviously refer to Ara h 6. However, we were not able to find two different isoforms with identical *N*-termini as would be expected from the data of that paper [14].

N-Terminal sequencing of the purified allergen confirmed identity of Ara h 6 starting with Met_6 of the sequence deduced from the cDNA (Genbank Acc. No. AF092846). A signal sequence could not be predicted, suggesting that a part of the signal peptide is missing. Probably, the five amino acids AHASA translated from the cDNA are part of a signal peptide, especially as alanine is present in the positions –3 and –1 relative to the signal peptide cleavage site. This corresponds to results from analysis of cleavage sites indicating a high probability for occurrence of ASA directly preceding the postulated cleavage site [25, 26]. Furthermore, the pattern identified in the sequence of Ara h 6 is identical to the sequence, recently identified for Ara h 2 by Viquez *et al.* [27]. The authors cloned full-length *ara h 2*. Analysis of the coding sequence revealed a 21 aa signal peptide resulting in cleavage within the sequence $AHASA_{21}$↓RQQWEL. Additionally, the same pattern can be found in the aa sequence of Ara h 7 (Genbank Acc. No. AF091737). According to the prediction cleavage of the signal peptide probably occurs in the motif $VASA_{21}$↓TRWDPD.

The allergens Ara h 2, Ara h 6 and 7 belong to the 2S albumins of plant seed storage proteins. These proteins represent a heterogeneous protein family sharing a common cysteine skeleton with at least eight conserved cysteine residues in identical positions [28]. Ara h 6 can be classified as a conglutin-homologue harboring ten cysteine residues in the primary sequence. This phenomenon is quite unusual compared to 2S albumins but not to other storage proteins in general. Although the number of cysteines is deviating, the conserved skeleton of eight cysteine residues is also present. This was also described for other seed storage proteins, *e.g.*, for conglutin-δ from lupine with nine cysteines in the primary sequence. The disulphide structure has been reported [29]. One cysteine is left unpaired again indicating the variant types of 2S albumins.

Food allergens and their epitopes may be quite resistant to digestive enzymes and various forms of technological processing. Limited proteolysis of food allergens might enhance mucosal transport and hence allergenicity [30]. After thermal processing, the peanut allergens Ara h 1 and Ara h 2 bound higher levels of IgE and were more resistant to digestion by gastrointestinal enzymes due to increased allergenicity of the so-called Maillard reaction products [31]. Therefore, stability of nAra h 6 against proteolysis and heat processing was investigated. Our results demonstrate that Ara h 6 is quite resistant to pepsin digestion as well as to heat treatment and apparently has similar properties as Ara h 2. It was previously shown that purified Ara h 2 is resistant to simulated gastric fluid (SGF) after 1 h of incubation [32]. Although constituents of the food matrix could influence stability, effects caused by food processing and preparation were not taken into consideration. Nevertheless, another study clearly described that digestion of peanut extract by gastric fluid did not significantly reduce IgE binding, whereas subsequent incubation with pancreatic enzymes resulted in only weak reduction of reactivity confirming stability of peanut proteins under conditions similar to those in the gastrointestinal tract [33]. Using SGF peanut proteins >30 kDa including Ara h 1 were degraded and several proteins <25 kDa seemed to be resistant to digestion, probably including Ara h 6. Our findings of stability of Ara h 6 are further supported by results of digestion experiments conducted by Sellers *et al*. [34] who described stability of a 14 kDa peanut protein against pepsin in SGF.

The mediator release experiments from Transfected Rat basophilic Leukemia (RBL 25/30) cells clearly showed allergenic activity of the purified natural Ara h 6 and support our view that Ara h 6 is a putative clinically relevant allergen. The results presented here indicate that Ara h 6 has unique properties supporting the difference between the proteins of the conglutin family of seed storage proteins. Up to now IgE-binding has been documented after recombinant expression of these allergens [15, 16]. Purification of nAra h 6 was confirmed by IgE-binding using defined patients' sera.

Summarized, in this paper we describe the positive identification of the peanut allergen Ara h 6 which exhibits characteristics of a food allergen regarding stability against digestion and thermal processing. Experiments are in progress in our laboratory to identify IgE-binding epitopes to further characterize this allergen and to analyze its relevance.

We thank J. Sarau and Stefani Fox for excellent technical assistance, L. Vogel for kindly providing the RBL 25/30 cells, G. Reese for performing the RBL-test (both from the Paul-Ehrlich-Institut, Langen, Germany), and Yasemin Darcan and Frauke Schocker for critical reading of the

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

manuscript. Our work was supported by the Deutsche Forschungsgemeinschaft Be 730/3-3,4.

5 References

[1] Hourihane, J. O., Bedwani, S. J., Dean, P., Warner, J. O., Randomised, double blind, crossover challenge study of allergenicity of peanut oils in subjects allergic to peanuts. *B.M.J.* 1997, *314*, 1084 – 1088.

[2] Hourihane, J. O., Kilburn, S. A., Dean, P., Warner, J. O., Clinical characteristics of peanut allergy. *Clin. Exp. Allergy* 1997, *27*, 634 – 639.

[3] Hourihane, J. O., Roberts, S. A., Warner, J. O., Resolution of peanut allergy: case-control study. *B.M.J.* 1998, *316*, 1271 – 1275.

[4] Moneret-Vautrin, D. A., Rance, F., Kanny, G., Olsewski, A., *et al.*, Food allergy to peanuts in France-evaluation of 142 observations. *Clin. Exp. Allergy* 1998, *28*, 1113 – 1119.

[5] Pumphrey, R. S. H., Wilson, P. B., Faragher, E. B., Edwards, S. R., Specific immunoglobulin E to peanut, hazelnut and brazil nut in 731 patients: similar pattern found at all ages. *Clin. Exp. Allergy* 1999, *29*, 1256 – 1259.

[6] Sicherer, S. H., Munoz-Furlong, A., Burks, A. W., Sampson, H. A., Prevalence of peanut and tree nut allergy in the US determined by a random digit dial telephone study. *J. Allergy Clin. Immunol.* 1999, *103*, 559 – 562.

[7] Skolnick, H. S., Conover-Walker, M. K., Koerner, C. B., Sampson, H. A., *et al.*, The natural history of peanut allergy. *J. Allergy Clin. Immunol.* 2001, *107*, 367 – 374.

[8] Burks, W. A., Williams, L.W., Helm, R. M., Connaughton, C., Cockrell, G., Identification of a major peanut allergen, Ara h 1, in patients with atopic dermatitis and positive peanut challenges. *J. Allergy Clin. Immunol.* 1991, *88*, 172 – 179.

[9] Burks, W. A., Williams, L. W., Connaughton, C., Cockrell, G., *et al.*, Identification and characterization of a second major peanut allergen, Ara h 2, with use of sera of patients with atopic dermatitis and positive peanut challenges. *J. Allergy Clin. Immunol.* 1992, *90*, 962 – 969.

[10] Burks, W. A., Sampson, H. A., Bannon, G. A., Peanut allergens. *Allergy* 1998, *53*, 725 – 730.

[11] Rabjohn, P. R., Helm, E. M., Stanley, J. S., West, C. M., *et al.*, Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *J. Clin. Invest.* 1999, *103*, 535 – 542.

[12] Eigenmann, P. A., Burks, A. W., Bannon, G. A., Sampson, H. A., Identification of unique peanut and soy allergens in sera adsorbed with cross-reacting antibodies. *J. Allergy Clin. Immunol.* 1996, *98*, 969 – 978.

[13] Clarke, M. C. A., Kilburn, S. A., Hourihane, J. O., Dean, K. R., *et al.*, Serological characteristics of peanut allergy. *Clin. Exp. Allergy* 1998, *28*, 1251 – 1257.

[14] De Jong, E. C., Van Zijverden, M., Spanhaak, S., Koppelman, S. J., *et al.*, Identification and partial characterization of multiple major allergens in peanut proteins. *Clin. Exp. Allergy* 1998, *28*, 743 – 751.

[15] Kleber-Janke, T., Crameri, R., Appenzeller, U., Schlaak, M., Becker, W. M., Selective cloning of peanut allergens, including profilin and 2S albumins, by phage display technology. *Int. Arch. Allergy Immunol.* 1999, *119*, 265 – 274.

[16] Becker, W. M., Kleber-Janke, T., Lepp, U., Four novel recombinant peanut allergens: more information, more problems. *Int. Arch. Allergy Immunol*. 2001, *124*, 100 – 102.

[17] Kleber-Janke, T., Crameri, R., Scheurer, S., Vieths, S., Becker, W. M., Patient-tailored cloning of allergens by phage display: peanut (*Arachis hypogaea*) profilin, a food allergen derived from a rare mRNA. *J. Chromatogr. B* 2001, *756*, 295 – 305.

[18] Bradford, M. M., A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976, *72*, 248 – 254.

[19] Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, *227*, 680 – 685.

[20] Kyse-Andersen, J., Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *Biophys. Methods* 1984, *10*, 203 – 209.

[21] Görg, A., Postel, W., Günther, S., Two-dimensional electrophoresis: the current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 1988, *9*, 531 – 546.

[22] Kleber-Janke, T., Becker, W. M., Use of modified BL21(DE3) *Escherichia coli* cells for high level expression of recombinant peanut allergens affected by poor codon usage. *Prot. Expr. Purif.* 2000, *19*, 419 – 424.

[23] Hoffmann, A., Kaul, S., Fotisch, K., Danz, N., *et al.*, Mediator release assays based on human and murine IgE: potential and limitations in allergen standardization. *Arb. Paul Ehrlich Inst. Bundesamt Sera Impfstoffe Frankf. a. M.* 2003, *94*, 76 – 85.

[24] Schocker, F., Becker, W. M., Optimization of electrophoresis for the identification of low molecular mass allergens in hazelnuts. *J. Chromatogr. B* 2001, *756*, 105 – 111.

[25] Von Heijne, G., A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 1986, *14*, 4683 – 4690.

[26] Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Prot. Eng.* 1997, *10*, 1 – 6.

[27] Viquez, O. M., Summer, C. G., Dodo, H. W., Isolation and molecular characterization of the first genomic clone of a major peanut allergen, Ara h 2. *J. Allergy Clin. Immunol.* 2001, *107*, 713 – 717.

[28] Shewry, P. R., Napler, J. A., Tatham, A. S., Seed storage proteins: structures and biosynthesis. *Plant Cell* 1995, *7*, 945 – 956.

[29] Gayler, K. R., Kolivas, S., Macfarlane, A. J., Lilley, G. G., *et al.*, Biosynthesis, cDNA and amino acid sequences of a precursor of conglutin delta, a sulphur-rich protein from *Lupinus angustifolius*. *Plant Mol. Biol.* 1990, *15*, 879 – 893.

[30] Aalberse, R. C., Structural biology of allergens. *J. Allergy Clin. Immunol.* 2000, *106*, 228 – 238.

[31] Maleki, S. J., Chung, S. Y., Chamoagne, E. T., Raufman, J. P., The effects of roasting on the allergenic properties of peanut proteins. *J. Allergy Clin. Immunol.* 2000, *106*, 763 – 768.

[32] Astwood, J. D., Leach, J. N., Fuchs, R. L., Stability of food allergens to digestion *in vitro*. *Nature Biotechnol.* 1996, *14*, 1269 – 1273.

[33] Vieths, S., Reindl, J., Müller, U., Hoffmann, A., Haustein, D., Digestibility of peanut and hazelnut allergens investigated by a simple *in vitro* procedure. *Eur. Food Res. Technol.* 1999, *209*, 379 – 388.

[34] Sellers, C. L., Teuber, S. S., Buchanan, B. B., Chen, L., Peanut protein digestibility: a gastric and intestinal model. *J. Allergy Clin. Immunol.* 2000, *105*, S140 abstract No. 425.

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim