

Selective Cloning of Peanut Allergens, Including Profilin and 2S Albumins, by Phage Display Technology

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Key Words

cDNA cloning · Phage display · pJuFo · *Arachis hypogaea* · Recombinant allergens · IgE-binding proteins · 2S albumins · Profilin

Abstract

Background: Peanut kernels contain many allergens able to elicit IgE-mediated type 1 allergic reactions in sensitized individuals. Sera from sensitized patients recognize variable patterns of IgE-binding proteins. The identification of the IgE-binding proteins of peanut extract would facilitate improvement of diagnostic and immunotherapeutic approaches as well as development of sensitive test systems for the detection of hidden peanut allergens present as additives in various industrial food products and the investigation of their stability during processing of food products. **Methods:** We applied the pJuFo cloning system based on the phage surface display of functional cDNA expression products to clone cDNAs encoding peanut allergens. Sera (n = 40) of peanut-allergic individuals were selected according to case history, radioallergosorbent test and immunoblot analysis to demonstrate IgE binding towards the newly identified recombinant allergens. **Results:** In addition to the known allergens Ara h 1 and Ara h 2 we were able to identify four allergens with estimated molecular weights of 36, 16, 14.5 and 14 kDa. Three of

them formally termed Ara h 4, Ara h 6 and Ara h 7 show significant sequence similarities to the family of seed storage proteins and the fourth (Ara h 5) corresponds to the well-known plant allergen profilin. Immunoblotting of the six expressed recombinant allergens with 40 patients sera shows 14 individual recognition patterns and the following frequency of specific IgE binding: Ara h 1 was recognized by 65%, Ara h 2 by 85%, Ara h 4 by 53%, Ara h 5 by 13%, Ara h 6 by 38% and Ara h 7 by 43% of the selected sera. **Conclusions:** All of the selected peanut-positive sera can detect at least one of the six identified recombinant allergens which can be used to establish individual patients' reactivity profiles. A comparison of these profiles with the clinical data will possibly allow a further insight into the relationship between clinical severity of the symptoms and specific IgE levels towards the six peanut allergens.

Introduction

Peanut allergy is characterized by a higher frequency of severe and fatal reactions than found in other food allergies and represents a lifelong health problem often starting in early childhood. It represents the most common cause of fatal and near fatal food-related anaphylaxis and affects a relevant number of individuals with an incidence of around

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1% of the children [1–3]. Moreover, this particular food allergy is complicated by the common use of peanuts as a hidden additive in various foods [4, 5]. Hidden allergens are not obviously recognizable as such by allergic patients and therefore difficult to avoid.

In order to understand the relationship between clinical manifestation and the severe and complex immune responses, the allergenic components of peanuts need to be identified and fully characterized. Peanuts have a complex protein chemistry containing over 30 different proteins [6] and more than half of these are able to bind serum IgE from sensitized individuals [7]. Therefore, crude peanut extracts contain a mixture of nonallergenic and allergenic proteins not quantifiable at a single protein level. Consequently, the contribution of each single allergen to the severity of the anaphylactic reactions cannot be assessed and might account for the lack of relationship between the clinical severity of the symptoms and polyclonal peanut-specific IgE levels measured in single individuals [7]. The quantitative determination of specific IgE raised against a single allergen in serum requires the availability of highly pure protein [8]. The establishment of patients' reactivity profiles opens up new possibilities of immunotherapeutic approaches. Molecular biology and biotechnological methods allow, starting from the cloned complementary DNA (cDNAs), the production of allergenic proteins in virtually unlimited amounts [9, 10]. These technologies have been extensively used to clone, produce and characterize allergens from different sources as recently reviewed [10–12] and to characterize the two highly abundant allergenic glycoproteins Ara h 1 [13–15] and Ara h 2 [16, 17]: Ara h 1, a 66-kDa protein, belongs to the vicilin family of legume storage proteins [13] and Ara h 2, a 17.5-kDa protein, is a member of the conglutin family of seed storage proteins [17]. These two proteins are recognized by serum IgE from over 90% of peanut-sensitized patients and, according to the commonly accepted rules, considered as major allergens [11]. Recently a third peanut allergen (Ara h 3), supposedly corresponding to a family of seed storage proteins called glycinins, has been reported [18]. However, the availability of an extended panel of perfectly standardized recombinant allergens can substantially contribute to improving the development of sensitive methods for the detection of hidden allergens added to foods and to studying their stability.

Peanuts represent a complex allergenic source containing many IgE-binding molecules and classical screening of cDNA expression libraries might not be optimal to approach such complex systems [19, 20]. To isolate a panel of peanut allergens efficiently we have applied a recently described technology based on the phage surface display of

cDNA expression products [9, 19–24]. The major advantage of the cloning system termed pJuFo [21, 22] results from the physical linkage between gene products displayed on the phage coat and the genetic information required for their production [21–23]. The direct linkage of cDNA gene products via the phage surface to the DNA encoding the products itself allows screening of as much as 10^{11} single clones on a single well of a microtiter plate coated with a ligand. This semifluid screening approach which keeps the library in a fluid phase allows the specific enrichment of clones interacting with the solid-phase immobilized ligand by repeated rounds of clonal selection [20, 24]. The aim of this study was the isolation and characterization of IgE-binding proteins of *Arachis hypogaea*. In the present paper we report isolation and sequencing of a panel of cDNAs encoding six different peanut allergens. The frequency of specific IgE binding of the sera of 40 individual patients and the individual IgE reactivity pattern were studied to estimate the immunological relevance of these allergens.

Methods

RNA Isolation and Northern Analysis

Peanuts (*A. hypogaea*, Virginia) were obtained from Internut Handels GmbH (Hamburg, Germany). Total RNA of raw peanuts was isolated according to a previously described method [25] except 2.5% (w/v) sodium dodecyl sulfate (SDS) was added to the extraction buffer. The isolated RNA was quantified spectrophotometrically, fractionated on 1.1% (w/v) agarose gels containing 15% formaldehyde and 1×MOPS buffer (20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate, pH 7.2) and transferred onto positively charged nylon membranes (Hybond N⁺; Amersham, Braunschweig, Germany) by capillary blotting. After prehybridization of the membranes [25] hybridization was carried out with radiolabeled polymerase chain reaction (PCR)-amplified cDNA probes specific for Ara h 1 (1,400 bp) and Ara h 2 (630 bp). Radiolabeling was performed with a random-primed labeling kit (Amersham, Braunschweig, Germany).

mRNA Isolation, λ-ZAP cDNA Library Construction, Plaque and Immunoscreening

Messenger RNAs were isolated from total RNA derived from raw peanuts using the 'PolyATtract mRNA Isolation System IV' (Promega/Serva, Heidelberg, Germany). cDNA was synthesized using the 'ZAP-cDNA Synthesis Kit' (Stratagene, LaJolla, Calif., USA) and was cloned unidirectionally into a λ-ZAPII vector with the 'Predigested λ-ZAPII/EcoRI/CIAP-Cloning Kit' (Stratagene). In order to determine the frequency of the Ara h 1 and Ara h 2 messages within the constructed λ-ZAP cDNA library specific DNA probes were prepared by random-primed labeling with DIG-11-dUTP and used to screen 5×10^4 plaque-forming units (PFU) of the amplified cDNA library as described in the 'DIG System User's Guide for Filter Hybridization' (Boehringer, Mannheim, Germany). Positive plaques were detected with the chemiluminescent substrate for alkaline phosphatase 'CSPD' (Boehringer) and visualized by exposure to X-ray films.

Expression at protein levels was demonstrated by immunoscreening [26] of 5×10^4 plaques with monoclonal antibodies [27] raised against the Ara h 1 allergen.

Generation and Screening of a pJuFo Phage Surface Display Library

Inserts for the construction of a cDNA phage surface library were prepared from pBluescript phagemids excised *in vivo* from the peanut λ -ZAP cDNA by *XbaI/KpnI* restriction [22]. The ligation mixture consists of purified cDNA inserts and *XbaI/KpnI* restricted pJuFo at a ratio of 2:1 and was transformed by electroporation into electrocompetent *Escherichia coli* XL1-Blue cells (Stratagene, La Jolla, Calif., USA) to generate a phage surface display library by helper phage superinfection. Determination of the primary library size and further steps for the generation of a cDNA phage library construction were carried out as described [22, 23]. Phages displaying peanut allergens were selectively enriched from the library by consecutive rounds of phage growth and selection on microtiter plate wells [21] coated with captured serum IgE from peanut-sensitized individuals as ligand [19].

To obtain an allergen-specific IgE-coated surface, antihuman IgE mAb TN-142 (Novartis, Basel, Switzerland) was coated to microtiter wells (Maxisorb, Nunc). Free sites were blocked and the sera expressing high titers of IgE antibodies to peanuts were added to each well.

Selection of Sensitized Patients

Patients sensitized to peanut were selected according to case history, skin test reactivity to peanut extracts and radioallergosorbent tests (RAST). Only sera of patients with convincing clinical symptoms after peanuts ingestion, positive skin test reactions and positive RAST (>3) were included. A pooled serum (S1), supplied by Dr. Sally Kilburn of the Southampton General Hospital, Southampton, UK, was prepared with sera from a group of 34 individuals (17 female, 17 male) sensitized to peanuts with an average age of 11.4 years and an average total IgE of 1,000 kU/l.

Additionally, three individual sera (S2-4) were used to screen the phage surface display library: S2 was supplied by Dr. P. Thomas, Dermatologische Klinik und Poliklinik der Ludwig-Maximilians-Universität München, Germany. S2 is a serum of a 40-year-old health care worker with a documented latex allergy and with sensitization to peanut (24.60 kU_A/l peanut-specific IgE; A = Allergen specific antibodies) and grasses. S3 (RAST >100 kU_A/l) was supplied by Dr. U. Lepp, Klinik Borstel, Germany and S4 (RAST >100 kU_A/l) was supplied by Dr. Helbling, Bern, Switzerland. Additional sera used to determine the percentage of peanut-allergic patients that are sensitized to Ara h 1 to Ara h 7 (RAST >3) were provided by Dr. W.-H. Boehncke, Klinikum der Johann-Wolfgang-Goethe-Universität, Frankfurt/Main, Germany and by Dr. B. Niggemann, Zentrallabor Virchow-Klinikum, Berlin, Germany.

Isolation and Analysis of Phages Displaying IgE-Binding Proteins

After the sixth round of panning on wells coated either with the pooled serum S1 or one of the three individual sera S2-4, single phages were used to infect *E. coli* XL1-Blue cells in order to isolate the replicative form of phagemids [28]. Restriction analysis of 250 selected clones of each of the four independent panning experiments showed different restriction patterns. All clones with different insert sizes were used for Southern hybridization experiments (Qiabran, manufacturer's instruction, Qiagen, Hilden, Germany) with digoxy-

genin (DIG)-labeled Ara h 1 and Ara h 2 clones as probes. Focusing on new allergens, only DNA sequences of inserts that are not identical with Ara h 1 or Ara h 2 clones were determined.

Sequencing and DNA Sequence Analysis

Plasmid sequencing was carried out following the dideoxynucleotide chain-terminating method as described [29] with the 'Taq Dye Deoxy™ Terminator Cycle Sequencing Kit' (Applied Biosystems, Weiterstadt, Germany). Electrophoresis of dye-labeled fragments was performed in the ABI 373A DNA sequencer (Applied Biosystems). Homology searches were performed with BLAST and the Genetics Computer Group program FASTA [30].

Production and IgE Immunoblot Analysis of Recombinant Proteins

Inserts coding for so far unknown peanut allergens were subcloned into the high level expression vector pDS56/RBSII [31] to produce (His)₆-tagged fusion proteins in *E. coli* M15 cells as described [19]. The IgE-binding capacity of the recombinant (His)₆ fusion proteins was tested by Western blotting of whole cell lysates prepared from cultures of transformants by boiling pellets in reducing sample buffer [20 mM Tris, pH 6.7, 2 mM EDTA, 2% (w/v) SDS, 25% (v/v) glycerin, 3% (v/v) β -mercaptoethanol, Bromophenolblue]. After SDS-polyacrylamide gel electrophoresis (PAGE) using 5% stacking gels and homogenous 15% separating gels [32] separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany) using a semidry device [33]. After blocking patients' sera were diluted 1:50 in 0.1 M Tris-buffered saline (pH 7.4) and used as first antibody. The specific binding of the patients' IgE to the recombinant proteins was detected by incubating the membrane for 2 h with alkaline-phosphatase-conjugated antihuman IgE (Allergopharma, Reinbek, Germany) as second antibody and bands were visualized with nitroblue tetrazolium chloride (Merck) including 5-bromo-4-chloro-3-indolyl phosphate potassium salt (Serva, Heidelberg, Germany) [34].

To visualize the patients' IgE-binding reactivity against raw and roasted peanuts (160°C, 40 min, roasted by Sorematec, Arlon, Belgium), 10 μ g/cm peanut extract [27] were separated by SDS-PAGE (5% stacking gel, 12% separating gel) and incubated with patients' sera. Bound IgE antibodies were also detected with alkaline-phosphatase-conjugated anti-human IgE (Allergopharma, Reinbek, Germany).

Results

Construction and Screening of a Phage-Displayed cDNA Library

In order to prepare a cDNA library that represents genes coding for peanut proteins including all allergenic proteins, RNA as well as poly(A)⁺-RNA preparations isolated from raw peanuts were tested for transcript levels of the two major allergens Ara h 1 and Ara h 2 using radiolabeled PCR products of the two genes for hybridization of Northern blots. Although most patients are sensitized to roasted peanuts, we have to use raw peanuts as a source of mRNA because only raw peanuts contain intact mRNA and only in-

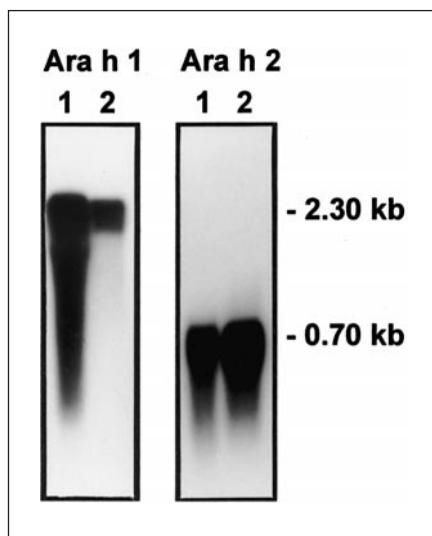


Fig. 1. Northern blot analyses of transcript levels of the two major peanut allergens Ara h 1 (66 kDa vicilin-like seed storage protein) and Ara h 2 (17.5 kDa conglutin-like storage protein). 25 μ g of total RNA (lane 1) or 0.5 μ g of Poly(A)⁺-selected mRNA (lane 2) from raw peanuts were hybridized with radiolabeled DNA probes specific for Ara h 1 or Ara h 2. The transcript sizes (kb) are indicated on the right side.

tact mRNA will lead to intact proteins on the phage surface. As shown in figure 1, Ara h 1 (2.3 kb) and Ara h 2 (0.7 kb) represent abundant transcripts within the isolated RNA and mRNA. Plaque screening of 10^4 PFU of the λ -ZAP cDNA library with a primary size of 4×10^6 independent clones, 100% inserts and an average insert size of 1,100 bp using DIG-labeled PCR products as hybridization probes yielded about 200 positive signals (2%) both for Ara h 1 and for Ara h 2, respectively. An immunoscreening of 5×10^4 plaques with mAbs raised against Ara h 1 [27] yielded 48 positive signals. After these quality controls of the λ -ZAP cDNA library a cDNA phage surface library was prepared. Titering of this library showed a primary library size of 5×10^6 independent clones with an average insert size of 750 bp and a phagemid titer of 10^{12} colony-forming units/ml after amplification.

Selective Enrichment and Characterization of Human IgE-Binding Clones

The peanut cDNA phage surface display library was screened for the presence of IgE-binding gene products with one pooled serum (S1) and three individual sera (S2–4) from peanut-sensitized individuals. Western blot analyses of raw or roasted peanut extracts with S1–4

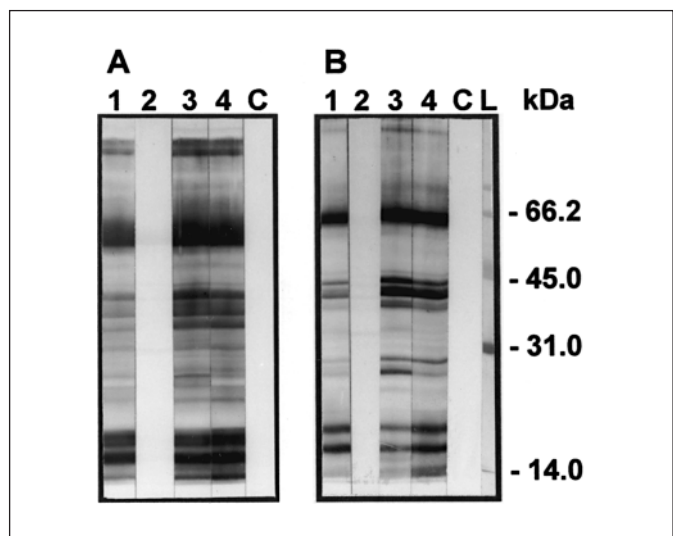


Fig. 2. Comparison of the IgE reactivity patterns of different sera, a serum pool S1 (lane 1) and three individual sera S2–4 (lanes 2–4) of peanut-sensitized individuals towards peanut extract (**A** = raw peanuts; **B** = roasted peanuts, 160°C, 40 min), separated on a 12% SDS-PAGE and immunoblotted S1, S3 and S4 were diluted 1:50, S2 was diluted 1:10. Lane C shows the lack of IgE reactivity of serum from a nonallergic individual as negative control. Lane L indicates the molecular weight markers (kDa).

showed IgE reactivity with up to 18 protein bands of different sizes (fig. 2) with the exception of the individual serum S2 that shows no distinct IgE binding. All in all, hopefully these four sera bind the majority of all potential IgE-binding peanut proteins. Roasting of the peanuts (160°C, 40 min) did not change the IgE-specific binding of the peanut-positive sera significantly. Differences of intensity are possibly due to a higher protein concentration of the raw peanut extract blotted on the membrane (fig. 2).

Selective enrichment of phages adsorbed to surfaces coated with captured IgE from S1–4 was monitored by determination of the absolute number of phages eluted in proportion to the number of phages applied. The enrichment factor of infectious phagemids increased with each round of panning from 4.3×10^{-6} after the first round to 2.6×10^{-3} after the fifth round indicating a selective enrichment of IgE-binding phages [19, 22]. After six rounds of affinity selection 100 of the 250 restricted clones of each of the four panning experiments carrying inserts ranging from 450 to 1,700 bp in size were further investigated by Southern hybridization with DIG-labeled Ara h 1 and Ara h 2 PCR products as probes. Sequence determination of the non-Ara h 1 and non-Ara h 2 clones of the four different screening experiments with the sera S1–4 revealed four inserts coding

Table 1. Characteristics of six different IgE-binding clones enriched by six rounds of affinity selection with IgE from peanut-allergic individuals

	Allergens ^a					
	Ara h 1	Ara h 2	Ara h 4	Ara h 5	Ara h 6	Ara h 7
Clone No.	1	2	3	4	5	6
GenBank Acc. No.			AF086821	AF059616	AF092846	AF091737
Insert length ^b , bp	1,680	719	1,170	743	627	637
ORF	2–1579 ^c	2–242 ^c	1–948 ^c	17–412	16–390	1–408 ^c
MW ^b , kDa	60.3	17.3	35.9	14.0	14.5	15.8
aa residues ^b	525	146	315	131	124	135
Isoelectric point ^b	5.4	5.7	5.5	4.6	5.2	5.6
aa identity/similarity, %	100/100	100/100	56/70	83/89	39/72	39/68
Protein family	Vicilin	Conglutin	Glycinin	Profilin	Conglutin	Conglutin
Organism	<i>A. hypogaea</i>	<i>A. hypogaea</i>	<i>G. max</i>	<i>G. max</i>	<i>L. angustifolius</i>	<i>L. angustifolius</i>
Genbank Acc. No.	L34402	L77197	X15123	AJ223982	X53523	X53523
Reference No.	15	16	36			

MW = Molecular weight; aa = amino acid.

^a Nomenclature officially approved by the WHO/IUIS Allergen Nomenclature Sub-Committee [11].

^b Values calculated from the deduced amino acid sequences without His tag.

^c Partial coding sequences.

^d Also similar to 2S albumin of *C. maxima* (31%/59%), Genbank Acc. No. D16560.

^e Also similar to 2S albumin of *R. communis* (32%/61%), Genbank Acc. No. X54158 [39].

for so far unknown IgE-reactive peanut proteins (table 1). The open reading frames (ORF) of the cDNAs encoding the new allergens formally termed Ara h 4, Ara h 5, Ara h 6 and Ara h 7 were identified with start and stop codons [35]. The WHO/IUIS Allergen Nomenclature Sub-Committee [11] has officially approved the use of these numbers. Physicochemical and biological properties of the new allergens are reported in table 1. All three panning experiments with the three sera S1, 3 and 4 had the same result. They revealed six different allergens, whereas the panning with the serum S2 only revealed two different clones, Ara h 4 and Ara h 5.

The partial cDNA encoding Ara h 4 has a length of 1,170 bp with a coding region spanning 948 nucleotides, predicting a protein of 315 amino acids with a calculated molecular mass of 35.9 kDa. Homology searches using GenBank, EMBL and SWISS-PROT databases revealed a significant amino acid sequence similarity (70%) and identity (56%) to proteins of the glycinin family representing storage proteins exclusively synthesized in seed during embryogenesis [36]. The full length cDNA clone isolated by plaque screening has a length of 1,872 bp (ORF: 20–1609, 530 aa, 61.0 kDa).

Two of 100 clones derived from the screening with serum S1 and 19 of 20 clones from the screening with S2

carried inserts coding for Ara h 5, a protein highly homologous to plant profilins [37], showing up to 83% amino acid sequence identity and 89% similarity to sequences of this well-known allergen present in grasses, fruits and vegetables [38]. The coding region spans 396 nucleotides, predicting a protein of 131 amino acids with a calculated molecular mass of 14 kDa.

Clone 5 containing an insert of 627 bp with a coding region spanning 375 bp and clone 6 with a cDNA insert of 637 bp and an ORF of 408 bp predict proteins of 14.5 and 15.8 kDa, respectively. They revealed only a 35% amino acid sequence identity to each other showing that these two allergens are not isoforms. Both clones show similarities to sequences of proteins from the conglutin family related to the 2S superfamily of storage proteins, which is widespread in seeds of dicotyledonous plants. The proteins encoded by the clones 5 and 6, termed Ara h 6 and Ara h 7, respectively, show the highest homology (39% identity and 68–72% similarity) with the protein sequence of the precursor of the conglutin δ -protein of *Lupinus angustifolius*. Ara h 6 and Ara h 7 show 59 and 35% amino acid sequence identity to Ara h 2, respectively (fig. 3), which, in turn, belongs to the conglutin family of seed storage proteins [16] and also shows significant similarity to the 2S albumin precursor

Table 2. IgE reactivity of 40 individual sera to the six SDS-PAGE-separated recombinant peanut allergens and frequency of specific IgE binding by Western immunoblot analysis

	Allergen Ara h						Number of patients
	1	2	4	5	6	7	
+	+	+	+	+	+	+	10
+	+	+	+	+	+	+	1
+	+	+	+	+	+	+	4
+	+	+	+	+	+	+	1
+	+	+	+	+	+	+	3
+	+	+	+	+	+	+	2
+	+	+	+	+	+	+	1
+	+	+	+	+	+	+	1
+	+	+	+	+	+	+	2
+	+	+	+	+	+	+	2
+	+	+	+	+	+	+	1
+	+	+	+	+	+	+	4
+	+	+	+	+	+	+	7
+	+	+	+	+	+	+	1
IgE binding, %	65	85	53	13	38	43	

14 individual recognition patterns (table 2). For example 7 sera recognize only Ara h 2 and 10 sera detect nearly all of the six recombinant peanut allergens but not Ara h 5 (table 2). It is noticeable that there are several sera that recognize Ara h 2 and not Ara h 6 or Ara h 7. Therefore although there are significant sequence similarities between these three allergens (fig. 3) and although they all belong to the 2S albumin family of seed storage proteins, they might have a different IgE-binding potential.

The frequency of recognition of the six different recombinant allergens analyzed by Western IgE immunoblotting was the following (table 2): Ara h 1 was detected by 65%, Ara h 2 by 85%, Ara h 4 by 53%, Ara h 5 by 13%, Ara h 6 by 38% and Ara h 7 by 43% of the 40 selected sera. These percentages indicate a considerable allergenic activity of these six peanut allergens.

Discussion

In this study we used a serum pool from 34 patients as well as 3 individual sera to enrich IgE-binding clones from the phage surface display library. In agreement with the IgE reactivity pattern of the pooled serum S1 and the 2 individual sera S3 and S4 compared to the individual serum S2 (fig. 2) the outcome of the screening with these 4 sera was quite different. Further analysis of 300 single clones derived

from the three panning experiments with S1, S3 and S4 revealed a high incidence of clones coding for Ara h 1 (20%), Ara h 2 (37%) and four repeatedly represented new sequences. The newly identified allergens termed Ara h 4, Ara h 5, Ara h 6 and Ara h 7 were characterized by determination of the nucleotide sequences and by showing their ability to bind serum IgE from peanut-sensitized patients (fig. 4, table 2). Three clones were identified as similar to the sequences of seed storage proteins that have been classified as water-soluble albumins or saline-soluble globulins. The cDNA sequence of Ara h 4 shows 56% amino acid identity to the sequences of the soybean glycinins [36], a family of storage proteins synthesized exclusively in seeds during embryogenesis. Glycinin-related genes become transcriptionally activated in a coordinated fashion early in embryogenesis and are repressed coordinately late in seed development. The full length Ara h 4 cDNA clone (1,872 bp) coding for a protein with a molecular weight of 61.0 kDa was identified by plaque screening of the constructed cDNA library. Interestingly, Burks et al. [18] identified the 14 kDa protein similar to a glycinin with soy-adsorbed sera to remove any cross-reacting antibodies. Quite recently Rabjohn et al. [40] published further information about the peanut allergen Ara h 3 and in addition to that the partial cDNA sequence is now accessible (Acc. No. AF093541). A homology search indicates 91.3% amino acid identity between Ara h 3 and Ara h 4, both members of the glycinin storage protein family. At the time of entry of the Ara h 4 sequence into the Genbank database and the approval of this allergen by the IUIS Allergen Nomenclature Sub-Committee [11] neither was the Committee informed of Ara h 3 nor was the Ara h 3 sequence accessible in a database. Therefore the glycinin allergen reported in the present paper was named Ara h 4 by the Committee.

Clones encoding Ara h 6 and Ara h 7 revealed ORF of 124 and 135 amino acids, respectively. These two allergens show a high sequence homology to proteins of the conglutin family derived from a variety of dicotyledonous plants including the precursor of the conglutin δ -protein of *L. angustifolius* and also the 2S albumin precursor of *C. maxima* and of *R. communis* [39], respectively. Recently, Teuber et al. [41] showed that the 2S albumin seed storage protein from walnut (*Juglans regia*) is a major food allergen and that 2S albumins can be viewed as universal allergens among seeds in which they occur although they are not necessarily cross-reactive [41]. They are typically heterodimeric proteins with large and small subunits which are encoded on one message as part of a large precursor polypeptide. After removal of the signal peptide, the polypeptide is cleaved, giving two subunits linked by disulfide bonds. A key char-

acteristic of the sequences is the preservation of the cysteine residues [42]. This information corresponds to the sequence data (fig. 3). The mRNA of Ara h 7 contains 8.1% of the least used codons AGG/AGA in *E. coli* and additionally 11% of other rare codons read by minor tRNAs. An explanation for the two IgE-binding Ara h 7-specific expression products separated on the SDS-PAGE (fig. 4) might be an inefficient translation due to the so-called 'codon usage' of *E. coli* [43], as also stated by Burks et al. [15] in the case of the presence of truncated Ara h 1 translation products. Interestingly, Ara h 6 and Ara h 7 show high sequence similarities to Ara h 2, also homologous to the conglutin family of seed storage proteins as well as to the 2S albumin precursor protein of *G. max*. A comparison of the amino acid sequences of Ara h 2, Ara h 6 and Ara h 7 shows that the identity between these three allergens is less than 67% (fig. 3). According to the recommendations of the WHO/IUIS Allergen Nomenclature Sub-Committee [11] these three proteins cannot be considered as isoforms but as independent allergens as also confirmed by their independent and different IgE-specific binding data demonstrated by immunoblot experiments with the sera of 40 individual patients (table 2).

The fact that 19 of 20 clones from the S2-screening represent clones coding for Ara h 5, a protein highly homologous to various plant profilins [37], indicates a high amount of profilin-specific IgE in the serum S2 compared to the pooled serum S1 and the individual sera S3 and S4. Although the serum S2 was diluted 1:10 instead of 1:50, compared to the other sera its intensity of the IgE reactivity towards peanut extract shown by immunoblot is much lower and barely detectable (fig. 2). This indicates that profilin is not an abundant protein within the peanut seeds, which is also stressed by the fact that plaque hybridization of the cDNA library with a DIG-labeled profilin-specific probe identified only 0.005% positive clones. These results suggest that the selective enrichment of the IgE-specific clones depends on the absolute amount of the allergen-specific IgE present in the serum used for the panning experiments rather than on the frequency of the presence of a specific clone in the library indicating that the phage display technology is a very sensitive method. Profilin is a highly conserved ubiquitous 12- to 15-kDa actin-binding protein described to be a prominent cross-reactive allergen in birch pollen, grasses and wheat [38]. Therefore, there are discussions whether profilin is responsible for IgE cross-reactivities in about 20% of pollen- and food-allergic patients [44]. These cross-reactivities can be explained by the high degree of homology among different plant profilins reaching 70–80%. The peanut profilin shows 76% amino acid identity

to the *Phleum pratense* profilin and 83% identity to the soybean profilin (table 1). Therefore, an IgE response mounted against profilin present as aeroallergen might have an impact in evoking clinically complex allergic symptoms due to cross-reactive responses to profilins present in foods [45].

The frequency of the specific IgE binding of the sera of 40 patients versus these six recombinant allergens (table 2) leads to the conclusion that the allergenic potential of the four new allergens may also play an important role compared to the already known major allergens Ara h 1 and Ara h 2. First IgE-specific ELISA assays (data not shown) confirm the immunoblot analysis results. The fact that only 5 of 40 sera (13%) recognize Ara h 5 (table 2) leads to the conclusion that peanut profilin might play a minor allergenic role compared to the other five identified allergens.

As demonstrated in this study, the application of the pJuFo cDNA cloning system combined with a powerful screening procedure resulted in the identification of six peanut allergens ranging from 14 to 60 kDa and showing sequence similarities to known plant allergens. These peanut allergens were used for the establishment of patients' IgE reactivity profiles. All of the selected peanut-positive sera can detect at least one of the six recombinant peanut allergens and the patients show very individual recognition patterns. This makes it possible to investigate the contribution of each single allergen to the severity of the anaphylactic reactions. Identification and characterization of the major and minor peanut allergens and their immunodominant epitopes are important for further insight into the mechanism of the IgE-mediated reactions. This research may lead to the development of immunotherapeutic reagents containing patient-tailored combinations of these allergens. The availability of this panel of allergens will certainly facilitate the development of sensitive test systems for the detection of hidden allergens in various industrial food products. Heating of foods can reduce allergenicity or create neoallergens. At first glance, IgE immunoblot analysis indicates that the roasting of peanuts did not significantly change IgE-specific binding to allergic peanut components of the four different sera used for the panning experiments.

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