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Patient-tailored cloning of allergens by phage display: Peanut (*Arachis hypogaea*) profilin, a food allergen derived from a rare mRNA

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Abstract

A peanut cDNA phage surface display library was constructed and screened for the presence of IgE-binding proteins. We used a serum from a peanut-sensitized individual with a low specific IgE level to peanut extract and suffering from mild symptoms after peanut ingestion. A total of 10^{11} cDNA clones were screened by affinity selection towards serum IgE immobilized to solid-phase supports. After five rounds of selective enrichment, sequence determination of 25 inserts derived from different clones revealed presence of a single cDNA species. The cDNA-encoded gene product, formally termed Ara h 5, shows up to 80% amino acid sequence identity to the well-known plant allergen profilin, a 14 kD protein present only in low amount in peanut extracts. Immunoblot analysis of fifty sera from individuals sensitized to peanut showed that 16% had mounted a detectable IgE response to the newly identified peanut profilin. High-level expression as non-fusion protein in BL21 (DE3) was carried under control of the inducible T7 promoter. Peanut profilin was purified by affinity chromatography on poly-(L-proline)-Sepharose and yielded 30 mg l^{-1} culture of highly pure recombinant allergen. In spite of the high level of up to 80% amino acid identity to other plant profilins, inhibition experiments with recombinant profilins of peanut, cherry, pear, celery and birch revealed marked differences regarding their IgE-binding capacity.

Keywords: Food allergens; Phage display; *Arachis hypogaea*; Profilin

1. Introduction

Peanut allergy represents the most common cause of fatal and near fatal food-related anaphylaxis and affects a relevant number of individuals with a prevalence of about 1% in children [1]. Peanut allergic individuals may experience symptoms ranging from an oral allergy syndrome, mild urticaria, facial swelling, abdominal cramp to hypertension with anaphylactic shock. The relationship between clinical manifestation and the complex immune responses cannot be investigated without identifica-

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tion and characterization of the allergenic components of peanuts. The availability of highly pure single components would allow assessment of the relative contribution of each allergen to the potency of peanut extracts. This is an essential step to evaluate the relationship between severity of the clinical symptoms and molecular structures. Moreover, determination of patient-specific reactivity patterns against single peanut allergens opens up the possibility to generate patient-tailored immunotherapeuthic reagents according to the sensitization patterns. However, quantitative determination of specific IgE raised against single allergens in a patient's serum requires the availability of highly pure proteins. Recently, we isolated a panel of six peanut allergens by phage display technology [2]. In addition to the already known peanut allergens Ara h 1 [3] and Ara h 2 [4] we were able to identify three allergens with significant sequence similarities to seed storage proteins (Ara h 4, Ara h 6 and Ara h 7) and a fourth one (Ara h 5) corresponding to the well-known plant allergen profilin.

In this study we focus on the cloning procedure, expression and purification of the newly identified peanut profilin. IgE reactivity of sera to natural profilin is often difficult to detect because this protein is present in low amounts in crude allergenic extract [5]. Peanut extract represents a complex allergenic source containing many IgE-binding molecules derived from high and low abundance mRNA species [6,7]. The efficient handling of large cDNA libraries necessary for the identification of proteins encoded by rare cDNAs is facilitated by selective enrichment of clones expressing genes of interest. Classical screening of λ based cDNA expression libraries requires immobilization of the library to solid-phase. Obviously, immobilization of a library hampers selective enrichment of clones by specific gene-product/ligand interaction. Therefore, this is not the method of choice to isolate cDNAs present at low frequency in a library. For a patient-tailored identification of peanut allergens encoded by abundant as well as by rare transcripts, we applied a modified approach of the conventional phage display technology based on the pJuFo phagemid [8], which allows display of functional cDNA-expression products on filamentous phage surfaces [9–12]. The basic concept of linking the phenotype to its genetic information integrated into the phage genome, allows the survey of large libraries for the presence of specific clones using the discriminative power of affinity selection. As much as 10^{11} clones of a cDNA library kept in liquid phase can be screened on a single well of a microtiter plate coated with human IgE of sensitized individuals [8–10]. During successive rounds of phage growth and selection, specific enrichment of clones interacting with the solid-phase immobilized ligand can be achieved. Using serum IgE as ligand, only phages displaying allergens are retained on the surface and can be used, after washing and elution, to infect *E. coli* in order to amplify specific phages displaying IgE-binding proteins. cDNA phage surface display technology has been successfully applied to identify several novel genes with different physico-chemical properties ([2,13,14], for an overview see Ref. [15]) and, recently, combined with robotic-based high-throughput screening [15].

It has been shown that several plant profilins can be efficiently produced as native-like recombinant proteins in *E. coli* [16] and purified by affinity interaction with poly-L-proline (PLP, [17]). Thus, affinity chromatography with PLP-Sepharose was used to obtain highly pure recombinant peanut profilin and used to characterize the 14 kD actin-binding protein. The phylogenetically highly conserved 12- to 16-kD profilin family is considered to act as panallergen. Cross-reactivity has been demonstrated between profilins derived from several species of tree, grass and weed pollen as well as from many fruits and vegetables [18,19]. Therefore, profilins could partly account for IgE-mediated cross-reactive symptoms occurring in pollen and food allergy [20]. An IgE response to profilin derived from pollen could have an impact in evoking cross-reactive clinical symptoms after ingestion of profilin-containing foods. In this paper, the IgE-binding properties of the recombinant peanut profilin were tested and compared to the structurally closely related profilins from cherry (Pru a 4), pear (Pyr c 4), celery (Api g 4) and birch (Bet v 2) [21].

2. Experimental

2.1. Selection of sensitized patients

Three selected sera of patients with a well-defined

case history of peanut allergy and IgE antibodies raised against peanut profilin were used in this study. Serum 1 from a 41 years old individual (24.60 $kU_A\ l^{-1}$ specific IgE, RAST class 4) recognizing a single band in IgE-immunoblots with peanut extract was used to screen a peanut cDNA library displayed on phage surface. Sera 2 and 3 from two 26 and 27 years old individuals suffering from mild oral allergy symptoms after peanut ingestion (59.6 $kU_A\ l^{-1}$ and 80.7 $kU_A\ l^{-1}$, respectively, both RAST class 5) were used together with serum 1 as a serum pool for the immunoblot inhibition experiments. Both sera contain IgE against profilin and recognize a few additional bands in IgE-immunoblots with peanut extract. A fourth serum (>100 $kU_A\ l^{-1}$, RAST class 6) of a patient suffering from extremely severe reactions up to anaphylactic shock after ingestion of peanuts was used to show the extremely complex IgE response of highly sensitized individuals in immunoblots with peanut extract, whereas a serum from a nonallergic individual (serum N, RAST class 0) was used as a negative control.

2.2. RNA isolation, mRNA isolation, λ ZAP-cDNA library construction

Raw peanuts (*Arachis hypogaea*, Virginia) were obtained from Internut Handels GmbH (Hamburg, Germany). The peanuts were ground to a fine powder under liquid nitrogen with a coffee grinder. Total RNA of peanuts was isolated from 5 g powder according to a previously described method using guanidine-thiocyanate combined with a phenol/chloroform extraction in 2.5% (w/v) SDS containing extraction buffer [22].

Messenger RNAs were isolated from total RNA using the 'PolyATtract mRNA Isolation System IV' (Promega/Serva, Heidelberg, Germany). cDNA was synthesized using the 'ZAP-cDNA Synthesis Kit' (Stratagene, La Jolla, CA) and cloned unidirectionally into λ ZAPII with the 'Predigested λ ZAPII/*Eco*RI/CIAP-Cloning Kit' (Stratagene).

2.3. Generation and screening of a pJuFo phage surface display library

Phagemid preparation protocol and panning procedure are diagrammatically presented in Fig. 1.

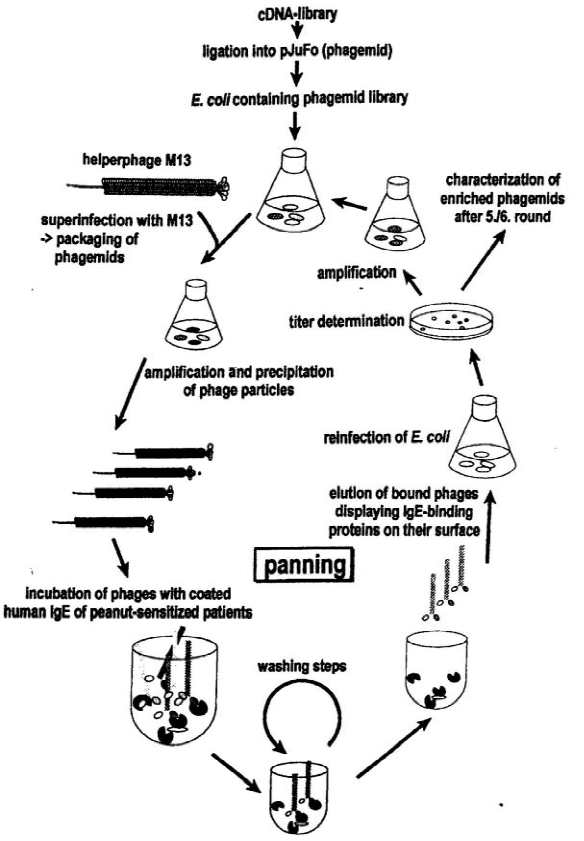

Fig. 1. Diagram of the application protocol of phagemid preparation and panning procedure.

Recombinant DNA techniques were performed according to standard methods [23]. Media were described in Sambrook et al. [23]. Inserts used for construction of a cDNA phage surface library were prepared by XbaI/KpnI restriction from in vivo excised pBluecscript phagemid DNA derived from the peanut λ Zap cDNA library [24]. The cDNA inserts (2 μg) were ligated into 1 μg of XbaI/KpnI restricted pJuFo phagemid [10] and the ligation mixture was transformed by electroporation into competent *E. coli* XL1-Blue cells (Stratagene). Three ml SOC medium were added and the culture shaken at 250 rpm for 1 h at 37°C. Then 10 ml SB medium were added and aliquots plated on ampicillin containing agar plates to determine the primary size of the library. The culture was shaken for 1 h, added to 100 ml SB medium containing antibiotics and further shaken for 1 h. To generate a cDNA phage surface

display library, 10^{12} plaque forming units helper phage VCMS13 (Stratagene) were added and incubation continued for 2 h at 37°C. Addition of 70 $\mu g\,ml^{-1}$ kanamycin occurred at this moment, followed by overnight incubation (37°C, 250 rpm). The supernatant was cleared by centrifugation and phage representing the amplified surface library recovered by PEG precipitation.

Phages displaying peanut allergens were selectively enriched from the library by consecutive rounds of phage growth and selection on microtiter plate wells coated with serum IgE from a peanut-sensitized individual. To obtain an allergen-specific IgE-coated ligand-surface, anti human IgE mAb TN-142 was coated to microtiter wells (Maxisorb, Nunc, Denmark). Free sites were blocked with blocking buffer (5% (w/v) skimmed milk powder in 1×PBS, pH 7.4) and 100 μl of serum was added to the well followed by incubation at 37°C for 2 h. After washing (with 0.5% (v/v) Tween-20 in 1×TBS, pH 7.4), about 10^{11} phagemid particles from the library were applied to a single well and incubated for 2 h at 37°C. The washing, elution (with 0.1 *M* HCl, pH 2.2, 0.1% (w/v) BSA), purification and re-infection procedure was repeated four times. Enrichment of phages adsorbed to the IgE-coated surfaces was monitored after each round of affinity selection by determination of the absolute number of phages eluted in proportion to the number of phages applied (enrichment factor). After the fifth panning round single phagemids were used to infect *E. coli* XL1-Blue cells in absence of helper phage in order to isolate the replicative form of phagemid DNA [25] for sequence determinations.

2.4. DNA sequence determinations and analysis

Phagemid sequencing was carried out following the dideoxynucleotide chain terminating method as described [26] 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 [27].

2.5. High-level expression and purification of recombinant profilins

The cDNA coding for the peanut profilin was subcloned into the high-level expression vector pSBET [28] to produce non-fusion proteins in *E. coli* BL21 (DE3) cells using the isopropyl-thio-β-galactoside (IPTG)-inducible T7 RNA Polymerase expression system [29]. Cultures were induced by addition of 0.4 m*M* IPTG at $OD_{600}=0.6$ and incubation continued for 3 h at 37°C. Cells were harvested by centrifugation, the pellets resuspended in lysis buffer (50 m*M* Tris–HCl, pH 7.5, 100 m*M* NaCl) and disrupted with a FRENCH® pressure cell disruption apparatus (SLM Aminco, Spectronic Unicam Instruments Inc., Cambridge, UK). Insoluble material was removed by centrifugation and recombinant profilin was purified from supernatants by PLP-Sepharose column chromatography [17]. The column was prepared by coupling 25 mg poly (L-proline) (ICN Biochemicals, Aurora) to 2 g of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden), washed with 10 volumes of PBS and equilibrated with four volumes of 2 $mol\,l^{-1}$ urea in PBS. Profilin was eluted with 6 $mol\,l^{-1}$ urea in PBS using an FPLC apparatus. Fractions containing pure peanut profilin were identified by SDS–PAGE/Western blot analysis and absolute protein yields determined according to Bradford [30]. Pooled fractions were dialyzed 24 h against water, lyophilized to reduce the volume and re-dissolved in PBS at a concentration of 1 $mg\,ml^{-1}$.

The cDNAs coding for the profilins of pear (Pyr c 4), cherry (Pru a 4) and celery (Api g 4) were subcloned into a modified form of the expression vector pET-30a (Novagen, Madison, WI, USA). Production as non-fusion proteins in *E. coli* BL21 (DE3) cells and purification procedures by PLP-Sepharose column chromatography were performed as described elsewhere [31]. The recombinant birch profilin Bet v 2 was purchased from BIOMAY, Linz, Austria.

2.6. SDS–PAGE and IgE-immunoblot analysis of recombinant profilins and peanut extracts

To visualize the patient's IgE-binding to recombinant profilins of cherry, pear, celery or birch profilin,

and to extract of raw and roasted peanuts (140°C, 40 min, roasted by Somaretec, Arlon, Belgium), 0.75 μg cm^{-1} recombinant profilins, 20 μg cm^{-1} extract of raw peanuts and 30 μg cm^{-1} extract of roasted peanuts were resuspended in reducing β-mercaptoethanol containing sample buffer (20 m*M* Tris, pH 6.7, 2 m*M* EDTA, 2% (w/v) SDS, 25% (v/v) glycerin, 3% β-mercaptoethanol, Bromphenolblue) and boiled for 5 min. After SDS–PAGE using 5% stacking gels and homogenous 15% separating gels [32], the proteins were visualized by staining with Coomassie Brilliant Blue R250 (Promega/Serva). For IgE immunoblot analysis separated proteins were transferred to nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany) using a semidry device [33]. After blocking, patient sera diluted 1:50 in 0.1 *M* Tris-buffered saline (pH 7.4) were 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-(AP)-conjugated anti human IgE (Allergopharma, Reinbek, Germany) as second antibody. Bands were visualized with nitroblue tetrazolium chloride (Merck) including 5-bromo-4-chloro-3-indolyl phosphate potassium (BCIP) salt (Promega/Serva). To detect natural profilin in blotted peanut extract, a rabbit anti ragweed-profilin serum at a dilution of 1:5000 was used. Bound antibody was detected with 1:10 000 diluted AP-conjugated goat anti rabbit IgG (H+L) (Dianova, Hamburg, Germany). The preparation of peanut protein extract was performed as previously described [34] and lyophilized samples were stored at −20°C.

2.7. IgE-immunoblot inhibition experiments

IgE inhibitions were performed with the newly identified recombinant peanut profilin Ara h 5 and with the profilins rPru a 4, rPyr c 4, rApi g 4 and rBet v 2 at concentrations of 100 ng, 1 μg, 5 μg, 10 μg, 20 μg, and of 50 μg, 100 μg, 200 μg for raw and roasted peanuts extracts. 0.1 *M* Tris-buffered saline, pH 7.4, served as negative control. Al final volume of 1 ml of a 1:50 diluted sample of a serum pool (serum 1, 2, 3, see above) was preincubated with an equal volume of solutions containing different concentrations of inhibitors for 2 h at RT with gentle shaking. Thereafter the preadsorbed sera were added to nitrocellulose blot strips containing 0.75 μg cm^{-1} electrophoretically separated rAra h 5, rPru a 4, rApi g 4, rPyr c 4 or rBet v 2. After washing, incubation was carried out under continuous shaking overnight at RT. Bound IgE was detected as described above.

3. Results

3.1. Construction of a λ-ZAP cDNA library and generation of a phage-displayed cDNA expression library of A. hypogaea

Although peanut allergics are sensitized to roasted peanuts, mRNA used for the synthesis of cDNA was isolated from total RNA derived from raw peanuts because only raw peanuts contain intact mRNA needed for the construction of cDNA libraries. We paid special attention to the synthesis of high quality of cDNA, which should contain most of the peanut's allergen repertoire. Therefore, we performed stringent quality controls of the isolated mRNA including in vitro translation, Northern blot hybridization and plaque screening with DIG-labeled Ara h 1 and Ara h 2 PCR products as hybridization probes [2]. These experiments confirmed a good quality of the isolated mRNA. In a first step, a λ-ZAP cDNA library with a primary size of 4×10^6 independent clones, 100% inserts and an average insert size of 1100 bp was constructed. Thereupon, we prepared a phage surface displayed cDNA library with a primary library size of 5×10^6 independent clones and an average insert length of 750 bp. A phagemid titer of 10^{12} colony-forming units ml^{-1} was obtained after amplification used for the subsequent panning procedure (Fig. 1).

3.2. Selective enrichment of a cDNA clone coding for an IgE-binding protein: profilin

Selective enrichment of phages adsorbed to surfaces coated with serum IgE from an individual monosensitized to peanut profilin was monitored by determination of the absolute number of phages eluted in proportion to the number of phages applied to each panning round. The enrichment factor of infectious phagemid particles increased with each round of panning from 5.7×10^{-7} after the second, to

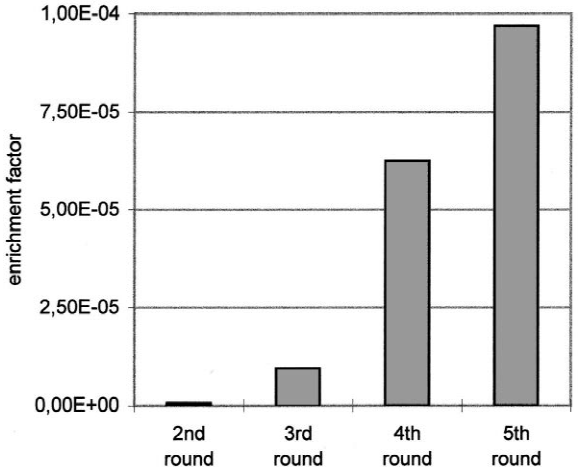

Fig. 2. Diagram of the enrichment of phages binding to serum IgE antibodies from individuals sensitized to *A. hypogaea* after 2, 3, 4 and 5 panning rounds: With each panning round 10^{11} phages displaying cDNA products from a peanut cDNA library were applied to a microtiter plate well coated with serum IgE. After adsorption and washing procedures, bound phages were eluted and used to infect *E. coli* for a further panning round of phage growth and selection. The enrichment factor is the yield (%) of phages from each panning round [=(number of phages eluted)×100/(number of phages applied)].

about 1×10^{-4} after the fifth round indicating a selective enrichment of phages displaying IgE-binding proteins (Fig. 2).

After five rounds of affinity selection, 25 clones carrying inserts ranging from 450 bp to 750 bp in length were investigated by sequence determination. DNA and amino acid sequence analysis and subsequent homology searches using Genbank, EMBL and SWISS-PROT databases revealed that all 25 selected clones carried cDNA inserts coding exclusively for a single protein with high sequence homology to the well-known plant allergen profilin. The coding region of the longest of the selected cDNA (743 bp) spans 396 nucleotides, predicting a protein of 131 amino acids with a calculated molecular mass of 14 kD. The deduced amino acid sequence of the allergen, named Ara h 5 (Acc. No. AAD55587) according to the recommendations of the Allergen Nomenclature Subcommittee [35] revealed a high degree of sequence identity to pear profilin Pyr c 4 (79%, Acc. No. AAD29410), cherry profilin Pru a 4 (74%, Acc. No. AAD29411), celery profilin Api g 4 (73%, Acc. No. AAD29409 [31]) and Bet v 2 (72%, Acc. No. AAA16522) (Fig. 3).

3.3. High-level expression and purification of recombinant profilin

For high level expression, the coding region of the Ara h 5 cDNA was subcloned into the expression vector pSBET [28]. The non-fusion peanut profilin was efficiently expressed in *E. coli* BL21(DE3) cells after three h of induction with IPTG as shown by the appearance of an additional 14 kD band in *E. coli* lysates separated with SDS–PAGE and stained with Coomassie Brilliant Blue (Fig. 4). Soluble profilin was purified by chromatography over a PLP-Sepharose affinity column. After elution with 6 mol l^{-1}

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                    *         20        *         40        *         60
Ara h 5   :  MSWQTYVDNHLLCEIEG---DHLSSAAILGQDGGVWAQSSHFPQFKPEEITAIMNDFAEPGSLAPTG  :  64
Pru a 4   :  MSWQAYVDDHLMCDIDG---NRLTAAAILGQDGSVWSQSATFPAFKPEEIAAILKDLDQPGTLAPTG  :  64
Api g 4   :  MSWQAYVDDHLMCEVEGNPGQTLTAAAIIGHDGSVWAQSSTFPQIKPEEIAGIMKDFDEPGHLAPTG  :  67
Pyr c 4   :  MSWQAYVDDHLMCDIDG---HHLTAAAILGHDGSVWAQSSTFPKFKPEEITAIMKDFDEPGSLAPTG  :  64
Bet v 2   :  MSWQTYVDEHLMCDIDG-QASNSLASAIVGHDGSVWAQSSSFPQFKPQEITGIMKDFEEPGHLAPTG  :  66

                  *         80        *        100        *        120        *
Ara h 5   :  LYLGGTKYMVIQGEPGAIIPGKKGPGGVTIEKTNQALIIGIYDKPMTPGQCNMIVERLGDYLIDTGL  :  131
Pru a 4   :  LFLGGTKYMVIQGEAGAVIRGKKGSGGITVKKTNQALIIGIYDEPLTPGQCNMIVERLGDYLIEQGL  :  131
Api g 4   :  LYLGGAKYMVIQGEPNAVIRGKKGSGGVTIKKTGQALVFGVYDEPVTPGQCNVIVERLGDYLIDQGL  :  134
Pyr c 4   :  LHLGGTKYMVIQGEGGAVIRGKKGSGGVTVKKTSQALVFGIYEEPLTPGQCNMIVERLGDYLIDQGL  :  131
Bet v 2   :  LHLGGIKYMVIQGEAGAVIRGKKGSGGITIKKTGQALVFGIYEEPVTPGQCNMVVERLGDYLIDQGL  :  133
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Fig. 3. Alignment of the deduced amino acid sequences of the peanut profilin Ara h 5 and four other plant profilins: Pru a 4 (cherry), Api g 4 (celery), Pyr c 4 (pear), Bet v 2 (birch). Identity between all five sequences is noted by dark grey shading, identity between only four of the five sequences is noted by light grey shading.

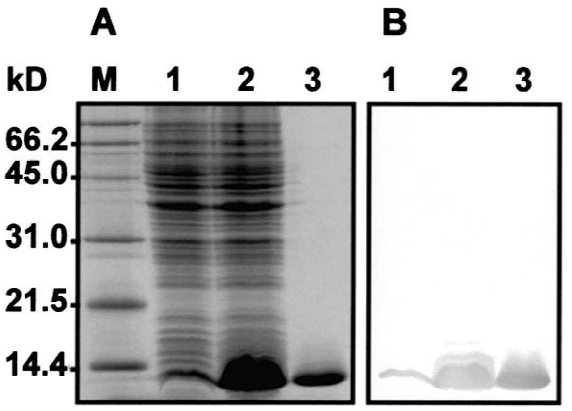

Fig. 4. Purification of recombinant peanut profilin expressed in *E. coli*. (A) Coomassie Brilliant Blue-stained proteins separated by a 15% SDS–PAGE, (B) Western blotting of A; Lane M: molecular mass markers, lane 1: total cell lysate from *E. coli* BL21 (DE3) containing pSBET with Ara h 5 insert before IPTG induction, lane 2: see lane 1 but 3 h after IPTG induction, lane 3: 2 μg PLP-Sepharose purified recombinant peanut profilin.

urea, a single IgE-reactive band with the expected molecular mass was detected in Coomassie Blue-stained SDS–PAGE and subsequent immunoblot analysis (Fig. 4). The yield of the purified recombinant peanut profilin recovered after purification was between 30 and 35 mg per liter of bacterial culture. No appreciable increase in yields was obtained by induction at higher cell densities, other IPTG concentrations or longer fermentation times.

3.4. IgE reactivity of sera from peanut allergic patients to peanut extract and recombinant profilins

Three sera from individuals suffering from mild allergic symptoms after peanut ingestion containing IgE against Ara h 5 were tested for IgE reactivity to extract of raw and roasted peanuts and for IgE cross-reactivity to different recombinant plant profilins. Serum 1 recognized only one band with a molecular mass of 14 kD in blotted raw extract and a double band with similar molecular masses in blotted extract of roasted peanuts, probably resulting from degradation during the heating process. The bands correspond to profilin as deduced from their reactivity with a rabbit anti ragweed-profilin serum (Fig. 5). It is noticeable that the India Ink staining of the blot did not show any relevant protein bands in this molecular mass region (Fig. 5), confirming the low abundance of profilin in peanut extracts. In addition to the 14 kD profilin band, serum 2 and serum 3 recognized a few higher molecular mass allergens in blotted extracts. The fourth serum of an individual suffering from severe symptoms up to anaphylactic shock after peanuts ingestion showed a complex IgE reactivity pattern in Western blot analyses of raw and roasted extracts, recognizing up to 30 protein bands of different molecular mass. However, the serum did not recognize the 14 kD profilin band. Roasting of the peanuts (140°C, 40 min) did not change significantly the IgE-specific reactivity pattern except a few small alterations probably due to protein instability (Fig. 5A, lane 4). Comparison of the IgE reactivity patterns to peanut extracts in Fig. 5A exemplifies sensitization to variable peanut allergen patterns in patients suffering from different clinical symptoms. Serum 1, 2 and 3 showed clear IgE-binding to all of the tested recombinant profilins (Fig. 5B). Although the IgE-binding of serum 1 to the recombinant allergens was weaker compared to the other two sera, it was clearly detectable in contrast to the complete lack of IgE reactivity of serum from a non allergic control individual (Fig. 5B). It is noticeable that the recombinant profilins of peanut, cherry, pear, celery and birch with a similar molecular masses as deduced from the amino acid sequences, showed different apparent molecular masses ranging from 13.5 kD to about 17 kD in Western blot analysis (Fig. 5B), probably reflecting conformational differences of these five profilins.

3.5. Inhibition experiments with peanut extract and recombinant profilins as inhibitors

Immunoblot inhibition experiments with both, peanut extracts and purified recombinant profilins, were carried out to demonstrate the presence of shared cross-reactive IgE-binding epitopes. Preincubation of the serum pool with 200 μg of raw peanuts extract inhibited IgE-binding to blotted peanut profilin much stronger than the concentration dependent decrease in IgE-binding observed using roasted peanut extract (Fig. 6A). Amounts of 5 μg peanut profilin (Ara h 5) or 5 μg cherry profilin (Pru a 4) in the preincubation solution were able to completely

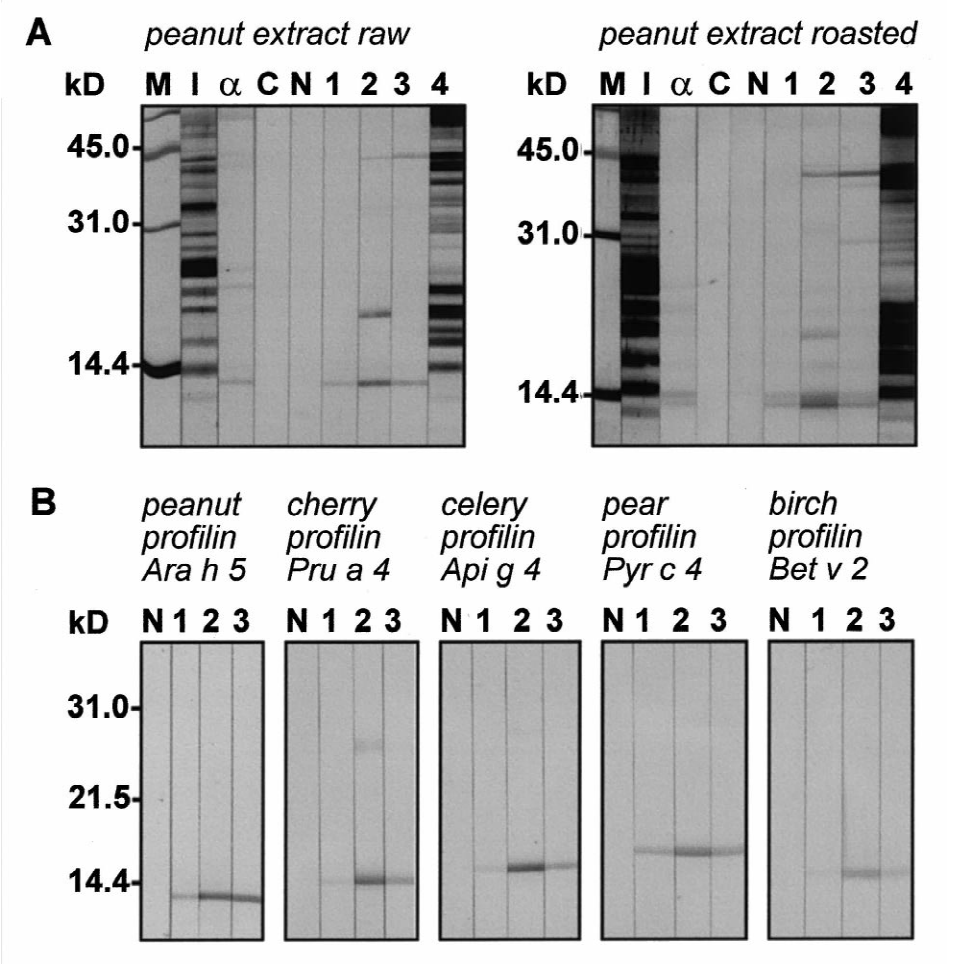

Fig. 5. IgE recognition of peanut extract and purified recombinant profilins by individual sera from patients sensitized to peanuts. (A) 20 μg cm^{-1} extract of raw, 30 μg cm^{-1} extract of roasted peanuts and (B) 0.75 μg cm^{-1} purified recombinant profilins separated by 15% SDS–PAGE and transferred onto nitrocellulose membrane were probed with 1:50 diluted sera from patients allergic to *A. hypogaea*, suffering from mild (lanes 1–3) or severe clinical reactions (lane 4) after peanut ingestion. Lane M: molecular mass marker, lane I: stained with India Ink, lane C: buffer control, lane N: probed with serum of a nonallergic individual, lane α: probed with anti ragweed-profilin serum.

inhibit the IgE-binding to the blotted recombinant peanut profilin, whereas 10 μg of the celery profilin (Api g 4) and 20 μg of the pear profilin (Pyr c 4) were required to show a detectable decrease in IgE-binding of the serum pool. Birch profilin (Bet v 2) even at high concentrations was unable to reduce IgE-binding to peanut profilin (Fig. 6A). In contrast, 1–5 μg of peanut profilin were sufficient to inhibit the IgE-binding of the serum pool to all of the five recombinant profilins tested (Fig. 6B).

4. Discussion

The identification of the peanut profilin as an IgE-binding component in peanuts is a good example for the selective power of the phage display technology. We screened a phage surface-displayed cDNA library derived from mRNA of raw peanuts with serum of a sensitized individual recognizing a single band in IgE Western blots of SDS–PAGE separated peanut extract. All 25 clones selected derived from

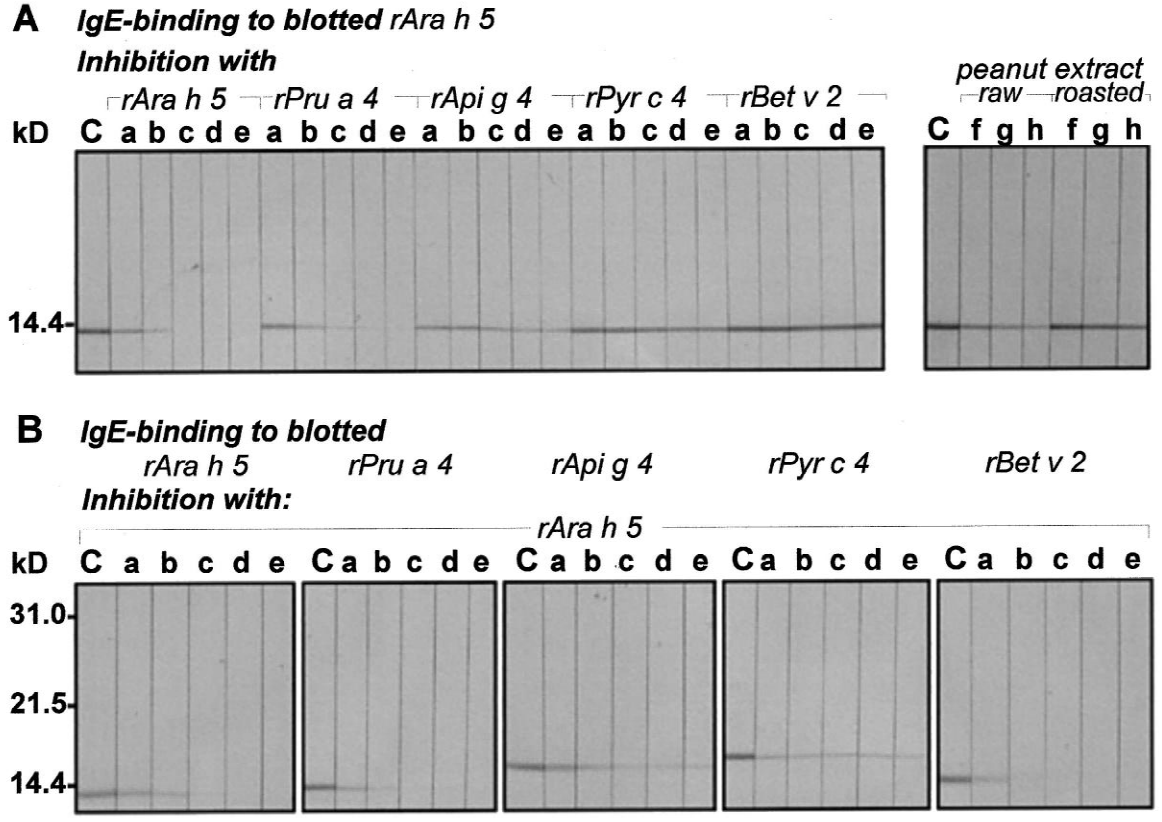

Fig. 6. (A) Inhibition studies with five different recombinant profilins and extract of raw or roasted peanuts by immunoblotting analysis. Immunoblotted purified recombinant peanut profilin Ara h 5 (0.75 μg cm^{-1}) was probed with a serum pool of three peanut-sensitized individuals (see serum 1–3, Fig. 5). Serum pool was preadsorbed with five purified recombinant profilins and with extract of raw as well as of roasted peanuts (140°C, 40 min) as inhibitors. (B) Inhibition studies with recombinant peanut profilin Ara h 5 by immunoblotting analysis. The five different recombinant profilins were immunoblotted and probed with the same serum pool used in A. Serum pool was preadsorbed with recombinant peanut profilin as inhibitor. Different inhibitor concentrations were used: Lane C: 0 μg ml^{-1}, lane a: 100 ng ml^{-1}, lane b: 1 μg ml^{-1}, lane c: 5 μg ml^{-1}, lane d: 10 μg ml^{-1}, lane e: 20 μg ml^{-1}, lane f: 50 μg ml^{-1}, lane g: 100 μg ml^{-1}, lane h: 200 μg ml^{-1}.

the phage screening carried cDNA inserts encoding the peanut allergen profilin. Plaque hybridization experiments of the peanut cDNA library with a DIG-labeled profilin-specific probe confirmed that profilin is a peanut allergen encoded by a rare mRNA species, since only 0.005% positive clones could be identified [2]. Thus, using this specific serum, profilin-displaying phages were enriched to virtually 100% after five rounds of panning. The results show that cDNA libraries displayed on phage surface are suitable for the selective isolation of patient-specific allergens even if the clones are underrepresented in the original library. This is especially important for cloning of allergens derived from rare transcripts, which, in terms of translation, yield low amounts of the gene products. Allergens like peanut profilin, which cannot be visualized by India ink staining even in overloaded gels, are unlikely to be accessible for N-terminal sequencing at protein level. PCR-based amplification of a gene is, however, dependent on sequence information for the design of gene product-specific primers. In contrast, the only requirement needed to clone genes by phage surface display is the availability of a

ligand able to interact with the displayed gene product [9]. The efficiency as well as the sensitivity of this technology was already demonstrated by several studies, describing the identification of allergens [2,8,13,14] and other genes from different sources [15].

Western blot analyses with peanut extract using sera of different peanut allergic individuals suffering from mild as well as from severe reactions after peanut ingestion showed significant individual differences concerning the IgE reactivity patterns ([2], Fig. 5A). Therefore, we hypothesize that the severity of clinical symptoms due to peanut ingestion could be related to the variable sensitization pattern of the single individuals to allergenic components present in peanuts. Preliminary investigations showed that patients suffering from severe allergic reactions to peanuts recognize a wide panel of peanut allergens in Western blot analyses, whereas individuals suffering from mild symptoms like oral allergy syndrome recognize exclusively peanut profilin [2]. Cloning and characterization of the whole allergen repertoire of peanut will allow to determine individual IgE reactivity profiles for each single allergic individual and to correlate these reactivity patterns with the clinical symptoms. This approach has been successfully applied to patients suffering from *Aspergillus fumigatus* allergy. In this case single component-based diagnosis of the allergic condition of patients suffering from different *A. fumigatus*-related pulmonary complications ended up in the discovery of disease-specific allergens [36]. The intracellular proteins rAsp f 4 and rAsp f 6 allow to discriminate with high sensitivity and specificity between asthma with *A. fumigatus*-sensitization and asthma with allergic broncho-pulmonary aspergillosis, both IgE-mediated diseases [36]. An analogous approach based on the whole peanut allergen panel applied to allergic patients suffering from different symptoms related to this food allergenic source may allow to associate the severity of the clinical symptoms with specific IgE reactivity patterns. Thus the dissection of the differential IgE response to the single allergenic components of peanut may enable the prediction of the severity of the symptoms to be expected from in vitro IgE-determinations.

Although the precise role played by profilins in food allergy remains to be determined, our results indicate that peanut profilin could be an important allergen for peanut-sensitized individuals suffering from mild symptoms. Eight of 50 arbitrarily selected peanut allergics with a well documented history of oral allergy symptoms showed IgE reactivity to the immunoblotted recombinant peanut profilin, but lacked to show IgE reactivity to the available recombinant allergens Ara h 1, 2, 3, 4, 6 and 7 [2,37]. The resulting provisional prevalence of sensitization of 16% indicates that peanut profilin is a minor peanut allergen [34], which shows, however, a high thermostability (Fig. 5). Sequence determination of the cloned cDNA revealed that peanut profilin is highly homologous to profilins cloned from a wide variety of sources such as grasses, weeds, trees and vegetables ([38–43], Fig. 3). Therefore, the ubiquitous 14 kD actin-binding protein family has been proposed to be one of the factors accounting for the observed cross-reactive reactions occurring between pollinosis and vegetable-food allergy [41,43–45]. The inhibition studies presented in this work clearly show that peanut profilin cross-react with profilin of cherry, pear and celery, whereas birch pollen profilin (Bet v 2) is not able to inhibit IgE-binding to peanut profilin. In spite of the high degree of sequence identity of 72% comparable to the sequence identity of cross-reactive profilins from other sources (Fig. 3), the lack of IgE reactivity to Bet v 2 of patients monosensitized to peanut profilin is likely to be related to differences in the three-dimensional structure of the proteins. However, the availability of the crystal structure of Bet v 2 [46] will allow to model the molecular structures of the different profilins to clarify this point.

Additional studies will be required to investigate the clinical relevance of the IgE cross-reactivity between peanut profilin and the profilins of cherry, celery and pear described in this work. However, the availability of highly pure recombinant profilins will allow to study the behavior of the proteins in skin test and histamine release experiments to answer questions about the role of profilins in the elicitation of cross-reactive symptoms related to food allergy.

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References

[1] J.O.B. Hourihane, S.A. Kilburn, T.P. Dean, J.O. Warner, Clin. Exp. Allergy 27 (1997) 634.
[2] T. Kleber-Janke, R. Crameri, U. Appenzeller, M. Schlaak, W.-M. Becker, Int. Arch. Allergy Immunol. 119 (1999) 265.
[3] A.W. Burks, L.W. Williams, R.M. Helm, C. Connaughton, G. Cockrell, T. O'Brien, J. Allergy Clin. Immunol. 88 (1991) 172.
[4] A.W. Burks, L.W. Williams, C. Connaughton, G. Cockrell, T. O'Brien, R.M. Helm, J. Allergy Clin. Immunol. 90 (1992) 962.
[5] A. Vallverdu, J.A. Asturias, M.C. Arilla, N. Gomez-Bayon, A. Martinez, J. Martinez, R. Palacios, J. Allergy Clin. Immunol. 101 (1998) 363.
[6] C. Loza, J. Brostoff, Clin. Exp. Allergy 25 (1995) 493.
[7] T. Dean, Clin. Exp. Allergy 28 (1998) 7.
[8] R. Crameri, M. Suter, Gene 137 (1993) 69.
[9] R. Crameri, R. Jaussi, G. Menz, K. Blaser, Eur. J. Biochem. 226 (1994) 53.
[10] B. Schaefer (Ed.), Gene Cloning and Analysis: Current Innovations, Horizon Scientific Press, Wymondham, 1997, p. 29.
[11] B.K. Kay, J. Winter, J. McCafferty (Eds.), Phage Display of Peptides and Proteins, Academic Press, San Diego, 1996, p. 187.
[12] R. Crameri, S. Hemmann, K. Blaser, Adv. Exp. Med. Biol. 409 (1996) 103.
[13] R. Crameri, K. Blaser, Int. Arch. Allergy Immunol. 110 (1996) 41.
[14] M. Lindborg, C. Magnusson, A. Zagari, M. Schmidt, A. Scheynius, R. Crameri, P. Withley, J. Invest. Dermatol. 113 (1999) 156.
[15] R. Crameri, G. Walter, Comb. Chem. High Throughput Screen. 2 (1999) 63.
[16] J.A. Asturias, M.C. Arilla, N. Gomez-Bayon, A. Martinez, J. Martinez, R. Palacios, Allergol. Immunopathol. 25 (1997) 127.
[17] U. Lindberg, C. Schutt, E. Hellsten, A. Tjäder, T. Hult, Biochim. Biophys. Acta 967 (1988) 391.
[18] P. Vallier, C. Dechamp, R. Valenta, O. Vial, P. Deviller, Clin. Exp. Allergy 22 (1992) 774.
[19] T. Caballero, M. Martin-Esteban, J. Investig. Allergol. Clin. Immunol. 8 (1998) 6.
[20] R. van Ree, V. Voitenko, W.A. van Leeuwen, R.C. Aalberse, Int. Arch. Allergy Immunol. 98 (1992) 97.
[21] R. Valenta, M. Duchene, K. Pettenburger, C. Sillaber, P. Valent, P. Bettelheim, M. Breitenbach, H. Rumpold, D. Kraft, O. Scheiner, Science 253 (1991) 557.
[22] T. Kleber-Janke, K. Krupinska, Planta 203 (1997) 332.
[23] J. Sambrook, E.F. Fritsch, T. Maniatis (Eds.), Molecular Cloning, Cold Spring Harbor Laboratory Press, New York, 1989.
[24] J.M. Short, M.J. Fernandez, J.A. Sorge, W.D. Huse, Nucleic Acids Res. 15 (1988) 7583.
[25] G. Del Sal, G. Manfllioletti, C. Schneider, Nucleic Acids Res. 16 (1988) 9878.
[26] F. Sanger, S. Nickler, A.R. Coulson, Proc. Natl. Acad. Sci. USA 74 (1987) 5463.
[27] W.R. Pearson, D.J. Lipman, Proc. Natl. Acad. Sci. USA 85 (1988) 2444.
[28] P.M. Schenk, S. Baumann, R. Mattes, H.-H. Steinbiß, Biotechniques 13 (1995) 14.
[29] F.M. Studier, B.A. Moffatt, J. Mol. Biol. 189 (1986) 113.
[30] M. Bradford, Anal. Biochem. 72 (1976) 248.
[31] S. Scheurer, A. Wangorsch, D. Haustein, S. Vieths, Clin. Exp. Allergy 30 (2000) 962.
[32] U.K. Laemmli, Nature 227 (1970) 680.
[33] J. Khyse-Andersen, J. Biochem. Biophys. Methods 10 (1984) 203.
[34] W.-M. Becker, Int. Arch. Allergy Immunol. 113 (1997) 118.
[35] T.P. King, D. Hoffmann, H. Lowenstein, D.G. Marsh, T.A.E. Platts-Mills, W. Thomas, J. Allergy Clin. Immunol. 96 (1995) 5.
[36] R. Crameri, S. Hemmann, C. Ismail, G. Menz, K. Blaser, Int. Immunol. 10 (1998) 1211.
[37] P. Rabjohn, E.M. Helm, J.S. Stanley, C.M. West, H.A. Sampson, A.W. Burks, G.A. Bannon, J. Clin. Invest. 103 (1999) 535.
[38] A. Ledesma, R. Rodriguez, M. Villalba, Allergy 53 (1998) 520.
[39] R. Valenta, M. Duchene, S. Vrtala, P. Valent, C. Sillaber, F. Ferreira, M. Tejkl, R. Hirschwehr, C. Ebner, D. Kraft, Int. Arch. Allergy Immunol. 99 (1992) 271.
[40] R. van Ree, R.C. Aalberse, J. Clin. Immunoassay 16 (1993) 124.
[41] R. Valenta, M. Duchene, C. Ebner, P. Valent, C. Sillaber, P. Deviller, J. Exp. Med. 175 (1992) 377.
[42] E.A. Pastorello, C. Incorvia, V. Pravettoni, C. Ortolani, Clin. Rev. Allergy Immunol. 15 (1997) 415.
[43] R. Valenta, D. Kraft, J. Allergy Clin. Immunol. 97 (1996) 893.
[44] G. Pauli, F. de Blay, J.C. Bessot, A. Dietemann, ACI News 4/2 (1992) 43–47.
[45] A. Jankiewicz, H. Aulepp, W. Baltes, K.W. Bögl, L.I. Dehne, T. Zuberbier, S. Vieths, Int. Arch. Allergy Immunol. 111 (1996) 268.
[46] A.A. Fedorov, T. Ball, R. Valenta, S.C. Almo, Int. Arch. Allergy Immunol. 113 (1997) 109.