

The 18 kDa peanut oleosin is a candidate allergen for IgE-mediated reactions to peanuts

Background: Peanut allergy is one of the five most frequent food allergies in children and in adults. Recently, we purified and evaluated the allergenicity of peanut oleosins, a family of small-sized proteins involved in the formation of peanut oil bodies.

Methods: Allergenicity of the purified native protein and of the recombinant protein was tested by Western blot and by IgE-RIA.

Results: We found IgE-binding with oleosin in 3 of 14 sera of patients who had suffered an allergic reaction to peanuts. Two sera reacted weakly against 16–18 kDa proteins corresponding to oleosin monomers, in Western blot. The main reacting bands had a molecular size estimated at ≈ 34 kDa, ≈ 50 kDa and ≈ 68 kDa and could therefore correspond to oleosin oligomers. IgE reactivity was higher in extracts from roasted peanuts. The same phenomenon occurred with crude soybean oil fraction, with two bands of 16.5 and 24 kDa corresponding to monomers, and two bands of 50 kDa and 76 kDa corresponding to dimers and trimers, respectively. The 18 kDa band was observed in the 3 Western blots of a membrane-enriched fraction of recombinant oleosin produced in the *Sf9*-baculovirus expression system (performed with the 3 patient sera).

Conclusions: We have characterized a new peanut allergen which belongs to the oleosins, a family of proteins involved in the formation of oil bodies. The protein may be involved in some of the allergic cross-reactions to peanuts and soybeans.

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Introduction

Adverse food reactions are defined as any aberrant reaction occurring after the ingestion of food and food additives. Non-toxic reactions may be the result of immune mechanisms, including allergy or nonimmune intolerance (1–4). Food allergy is more frequent in children than in adults and its prevalence varies among countries. Children with atopic disorders have a higher prevalence of food allergy (5). Peanut sensitivity is one of the five most frequent food allergies in children and in adults (5–7). In the USA, a recent survey estimated that it affects 1.3% of adults (8). Various allergens, such as peanut-1, Ara h1, Ara h2 and peanut agglutinin have been isolated and characterized (9–15). The responsibility of peanut oil has been demonstrated in several cases of severe adverse allergy reactions (16–19). Recently, we have isolated and characterized small-sized hydrophobic proteins in peanut oil which reacted with serum IgE of patients who had suffered an allergic reaction to peanuts (20). Other authors have also reported IgE-binding to proteins from soybean oil extract (21, 22). Peanut oil and peanut proteins are widely used in the food industry. The characterization of hydrophobic allergenic peanut protein is therefore of considerable interest.

Recently, we purified and evaluated the allergenicity of peanut oleosin (23), a small-sized protein family involved in the formation of peanut oil bodies (24). These proteins are present in seeds and pollen and are well conserved in plant evolution. The protein was purified and cDNA was cloned. Allergenicity of the purified native protein and of the recombinant protein was tested by Western blot and by IgE-RIA. We found IgE-binding with oleosin in the sera of 3 of the 14 patients who had experienced an allergic reaction to peanuts.

Materials and methods

Patients

Inclusion criteria of the 14 patients were the presence of atopic dermatitis and/or asthma, and/or urticaria, and/or bronchospasm. In all cases, peanuts were the allergen found responsible for the allergic reaction. All the patients were investigated by the Department of Allergy and Clinical Immunology of the University Hospital of Nancy, after informed consent, according to the criteria of Helsinki and to the recommendations of the ethical committee of our University Hospital Center. They underwent skin-prick tests for peanut seeds, peanut kernels, peanut oil extracts, and crude and refined oil protein extracts, as well as double-blind food-challenges with peanuts and peanut oil, and histamine release tests with freshly collected blood, as described (20).

Protein extracts

Plant materials Mature and immature (78 days after seedling) seeds of peanut (*Arachis hypogaea* L., c.v. Valencia, Landes, June–August 1997) were generously provided by the Department of Annual Crops of the CIRAD (Montpellier, France) and were stored at -20°C until use. Shelled cotyledons were freed from testae and embryonic axes, rinsed and then soaked in water for 1 h before use.

Oil body purification and delipidation Oil bodies were routinely isolated by the classic 4-centrifugation-flotation steps on a sucrose cushion including the 2 M NaCl washing developed by Huang (24) as technically detailed earlier (23). Oil bodies were further subjected to a two-layer flotation by centrifugation, detergent washing with 0.1% (v/v) Tween 20, ionic elution with 2 M NaCl, treatment with a chaotropic agent (9 M urea) and integrity testing with hexane according to the recent method of Tzen et al. (25).

Each oil body preparation was delipidated with diethylether and chloroform-methanol (2:1, v/v) (23). Protein concentration in the dried extract and in all subsequent extracts was measured with the bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as a standard (Pierce Chemical Company, Rockford, IL, USA).

Purification of the 17 kDa peanut oleosin by preparative SDS-PAGE The 17 kDa oleosin was purified from classically extracted crude oil body proteins by two preparative SDS-PAGES using the Bio-Rad Model 491 Prep Cell (Hercules, CA, U.S.A.) with a continuous elution system. The first electrophoresis was carried out according to the Laemmli discontinuous buffer system. Proteins (10 mg) solubilized in 1-ml sample buffer were denatured at 100°C for 3 min and loaded onto a 7.0×3.7 cm ID polyacrylamide gel column (acrylamide-Bis, 37.5:1, Bio-Rad) consisting of a 12% (w/v) resolving gel topped with a 1-cm high 4% stacking gel. The migration was performed with a constant current of 40 mA (150–240 V) for 14 h. Starting from the bromophenol blue dye front, Tris-Glycine buffer was pumped through the elution chamber at a flow rate of 0.4 ml/min using a peristaltic P-3 pump and divided into 2-ml aliquots with a Frac-100 collector (Amersham Pharmacia Biotech, Uppsala, Sweden). Elution was monitored at 280 nm using a Gilson 117, UV detector (Middleton, WI, U.S.A.). Fractions corresponding to the first and main peak (retention time of 65–175 min) were analysed on 12.5% SDS-polyacrylamide slab gels with Coomassie blue staining. Those enriched with the 17 kDa protein were pooled and concentrated by ultrafiltration through a 5000 MWCO membrane using an Ultrafree-15 centrifugal device (Millipore Corporation, Bedford, MA, USA). The second electrophoresis was performed by transposing the Tris-Tricine SDS-PAGE conditions of Schägger and von Jagow (26) to a preparative scale. A denatured sample enriched with the 17 kDa protein (2.5 mg in 0.5 ml) was loaded onto a 7.5×2.8 cm ID polyacrylamide gel column (acrylamide-Bis, 32:1) consisting of a 16.5% resolving gel and a 0.5-cm high 4% stacking gel. A constant current of 50 mA (90–150 V) was applied for 20 h. Elution and fraction collection were the same as described above except for the effluent, which was 0.2 M Tris-HCl buffer (pH 8.9) like the anode buffer. The main peak (450–600 min retention time) was further analysed on 13% SDS-polyacrylamide slab gels with silver staining (27). Fractions containing the purified oleosin were pooled, concentrated and dialysed extensively for 48 h against 20 mM Tris-HCl buffer (pH 7.5) at 4°C .

A prerun at 30 mA for 30 min and sodium thioglycolate at a final concentration of 0.1 M in the cathode buffer reservoir were added to the two electrophoreses designed to purify protein for N-terminal sequencing.

RACE-PCR, cDNA sequencing and expression of the 17 kDa peanut oleosin in a *Sf9*-baculovirus system

Total RNAs from mature and immature peanut seeds were extracted according to the phenol-chloroform method of Haffner et al. (28) using ethanol and sodium acetate to precipitate RNA. Total RNAs from freshly cut mature leaves were isolated using guanidium thiocyanate followed by cesium chloride equilibrium ultracentrifugation (29).

Among 4 different N-terminally sequenced peptides, one cross-reacted with 2 soybean oleosin isoforms. This complete peptide was then selected to design a specific 30-mer long oligonucleotide in which most of the degeneracy was cleared up on the basis of codon usage in *Arachis hypogaea*. PCR amplifications were operated in a GeneAmp PCR system 2400 using 1.25 U of AmpliTaq DNA polymerase (PE Biosystems).

A PCR program was applied in RACE 3'. The 130-bp PCR product obtained was extracted from a 1% (w/v) agarose gel using the Qiaquick kit (Qiagen GmbH, Hilden, Germany) and subcloned into pGEM-T vector using *Escherichia coli* strain JM109 as the host (Promega Corporation, Madison, WI, USA). Double-strand DNA sequencing of the purified recombinant plasmid was achieved by the dideoxynucleotide chain termination method using T7 and SP6 universal primers and the ABI PRISM Dye Terminator kit as indicated by the manufacturer (PE Biosystems). Analyses were performed on the ABI 373 A DNA sequencer. From the derived DNA sequence, 3 overlapping oligonucleotides were synthesized and utilized as reverse primers in nested RACE 5'. The 600-bp fragment generated was gel-extracted, subcloned into pGEM-T vector and sequenced according to the procedures described above.

Each sequence was determined by 3 sequencing reactions on both DNA strands from different clones.

Oligo(dT)₁₅-primed cDNA produced after AMV reverse transcription (Boehringer Mannheim, Germany) of total RNAs from mature seeds were used for PCR amplification of the complete peanut oleosin open reading frame. Primers for PCR were designed to include a *Pst*I site and a vertebrate Kozak consensus sequence at the 5' end and a *Xba*I site at the 3' end of the coding sequence. The 560-bp PCR product was cloned into the *Eco*RI/*Pst*I restriction sites of pAcSG2 vector. The recombinant plasmid (1 µg) was used for translation by a *Sf9*-baculovirus expression system. The cells were infected with the recombinant virus (9.107 pfu/ml) in 75 cm² Petri plates (15.106 cells per plate). They were collected 72 h after infection and subjected to fractionation by a two-step centrifugation at 900g and 100 000g respectively. The 100 000g pellet and supernatant corresponded to the membrane-enriched fraction and to the cytosol respectively.

IgE-RIA

Ninety-six well plates were coated with 100 µl per well of either AraA (5 µg/ml), AraB (15 µg/ml) or purified peanut oleosins (10 µg/ml). After incubation overnight at 4°C , the plates were washed with sodium phosphate buffer (PBS), incubated 30 min at 37°C with a blocking solution of 0.5% (w/v) gelatine and washed again. Patient sera (100 µl/well) were diluted 1/10 in PBS containing 0.1% (v/v) Tween 20 and 0.5% (w/v) gelatine. The assay was performed in duplicate. After incubation for 2 h at 37°C , the plates were washed 5 times with PBS containing 0.1% Tween 20, incubated with a [¹²⁵I] anti-IgE tracer solution (Immunotech, Luminy, France) for 90 min at 37°C (≈ 15000 cpm/well) and washed again. Each well was removed and subjected to γ -counting for 1 min.

Immunoblots

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed using a Mini-protein II electrophoresis unit (Bio-Rad). The stacking and the separating gel contained 4% and 12% of acrylamide, respectively. Proteins were separated for 2 h at 125 V in

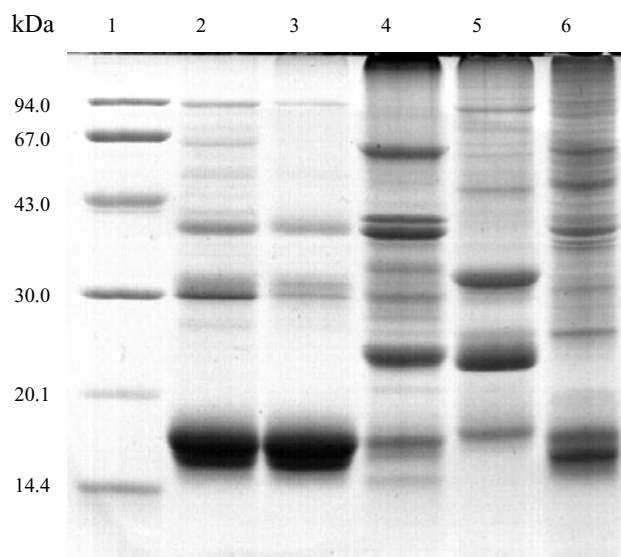


Figure 1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis with Coomassie blue staining of crude oil body fractions (COBF) isolated from various oil seeds. Lanes 1–6 (from left to right): low molecular mass standards (Pharmacia) (7.5 μ g); fresh peanuts (25 μ g); urea washed COBF from fresh peanuts (25 μ g); roasted peanuts (25 μ g); fresh soybeans (25 μ g); fresh maize (25 μ g). The main bands of 16–18 kDa proteins corresponded to oleosin monomeric isoforms. Some of the higher molecule-size bands corresponded to oligomers.

25 mM Tris-HCl (pH 8.3), 0.192 M glycine, 0.1% SDS (w/v). The molecular mass markers used were LMW (Amersham-Pharmacia): phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and lactalbumin (14.4 kDa). Protein bands were stained with Coomassie blue. Proteins were electrotransferred onto nitrocellulose membrane (0.2 μ m, Bio-Rad) in 25 mM Tris buffer containing 192 mM glycine and 20% (v/v) methanol (pH 8.3). The transfer was done using a Trans-blot semidry transfer cell (Bio-Rad) during 30 min at 15 V. The membrane was blocked by incubation in 20 mM Tris-HCl (pH 7.4), 0.15 mM NaCl (TBS), 3% (w/v) BSA for 2 h at room temperature and was then washed 2 times with gentle rocking in 20 mM Tris-HCl buffer containing 0.15 M NaCl (TBS) with 0.05% (v/v) Tween 20. Nitrocellulose was then probed overnight with the serum from the allergic patient (diluted 1:10 in TBS with 0.1% Tween 20). It was washed again 5 times and incubated for another night with ^{125} I-anti-IgE (Immunotech), diluted 1:10 in TBS with 1% (w/v) BSA. All the incubations were performed at 4°C. IgE binding was detected by autoradiography. The film (Hyperfilm-MP from Amersham-Pharmacia) was kept at -80°C and exposed for 3–15 days.

Results and discussion

Oil bodies were isolated from imbibed mature peanut seeds. The protein content of the derived extract was resolved by Tricine SDS-PAGE with Coomassie blue staining of the gel. After only one centrifugation-flotation step, the protein pattern was directly enriched with specific 15–18, 33–35 and 44 kDa bands. Three additional centrifugation-flotations, including a salt washing with 2 M NaCl, succeeded in removing most of the globulins (arachins and conarachins) and increased

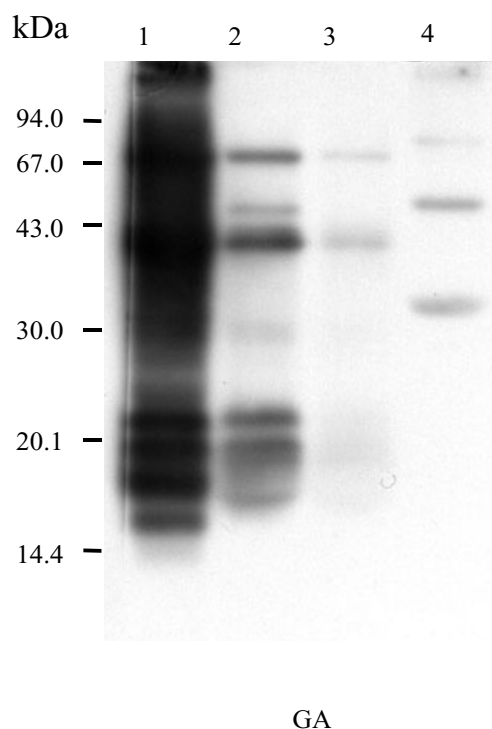


Figure 2. Western blot of crude oil body fractions (COBF) with serum of patient GA. Radioautography was performed after incubating the membrane with ^{125} I-iodinated anti-IgE. Lane 1: roasted peanuts; lane 2: fresh peanuts; lane 3: urea washed COBF from fresh peanuts; lane 4: fresh soybeans. IgE reacted against monomeric and oligomeric oleosin, when tested with roasted peanut extract, while they reacted mainly against oligomers with the other extracts.

the enrichment of the low- M_r proteins (Fig. 1). The 17 ± 0.3 kDa band was the most abundant and constituted 53% of the total oil body proteins.

The difficulties encountered while purifying oleosin isoforms are their close M_r and their strong hydrophobicity. The 17 and 16 kDa proteins are predominant in the peanut oil body extract and are tightly bound to the oil body since they remained associated after urea washing. These two proteins are therefore genuine oleosins which can presumably be classified as high and low- M_r isoforms according to Tzen et al. (30). The conserved central hydrophobic domain shared by all oleosins (approximately 70 residues) represents a weak epitope and induced no or nondetectable antibodies. In an aqueous environment as well as in the oil body (24), exposed epitopes are hydrophilic residues of the N- and C-terminal domains (31). Hydrophobic residues of the central stretch are buried within the core generated by the interacting proteins (32). The open reading frame of the oleosin gene encoded a polypeptide of 176 residues with a calculated molecular mass of 18.4 kDa and a theoretical pI s of 9.8. The relative mobility of the purified peanut oleosin subject to SDS-PAGE was consistent with a molecular mass of 17 kDa, which is 1.4 kDa smaller than the cDNA-deduced value. In

Coomassie-stained gels overloaded with the purified peanut protein and on the immunoblots, a band of approximately 33 kDa was detectable which probably represented homodimers of the 17 kDa isoform. Oleosin dimers have been previously described in rapeseed (32), maize (33) and additional higher-order oligomers in peanuts (23).

Of the 14 sera tested in AraA IgE-RIA, 3 were very positive, with a tracer uptake higher than 2000 cpm/well. Two other sera were weakly positive. These 5 sera were also positive when tested in AraB IgE-RIA. The sera of patients GA and RO were selected for performing Western blot analysis with crude oil body fractions from either fresh or roasted peanuts and from fresh soybeans. Both reacted weakly against 16–18 kDa proteins corresponding to oleosin monomers. The main bands identified in Western blot had molecular size estimated at ≈ 34 kDa, ≈ 50 kDa and ≈ 68 kDa and could therefore correspond to oleosin oligomers (Fig. 2). The same phenomenon occurred with crude soybean oil fraction, with two bands of 16.5 and 24 kDa which could correspond to monomers and two bands of 50 kDa and 76 kDa which could correspond to dimers and trimers, respectively (Fig. 2). The IgE binding was increased in immunoblots performed with extracts from roasted peanuts and soybeans, compared to those performed with extracts from fresh seeds. This phenomenon has already been observed with the peanut allergens Ara h1 and Ara h2 and may be explained in part by the Maillard reaction (34). The reactivity of IgE to both crude oil protein extracts from peanuts and soybeans suggested a cross-reactivity of oleosins. Other bands were also identified which could correspond to proteins associated with oleosin. In order to ascertain that the 18 kDa band was a peanut oleosin, we performed a Western blot analysis with the purified 18 kDa peanut oleosin and with a membrane enriched fraction of recombinant oleosin produced in the *Sf9*-baculovirus expression system, using the 3 sera which were positive in IgE-RIA. The sera of patients GA and HBJ gave a weak positive 18 kDa band in the immunoblot performed with the purified oleosin (Fig. 3). In contrast, the 18 kDa band of recombinant protein was detected with the 3 sera. Again, higher M_r bands were observed which could correspond to oligomers in the Western blot of proteins from the *Sf9* membrane fraction (Fig. 3).

Although of gametophytic origin (haploid), pollen oleosins are closely related to tapetal oleosin-like proteins representing a sporophytic origin (diploid). This supports the hypothesis that all the genes encoding flower-specific oleosin-like proteins are expressed primarily or exclusively in the tapetum (35, 36). The homology of oleosins suggests a possible cross-reactivity between oleosins from seeds and pollens of various origins. It may also explain some of the patient cases

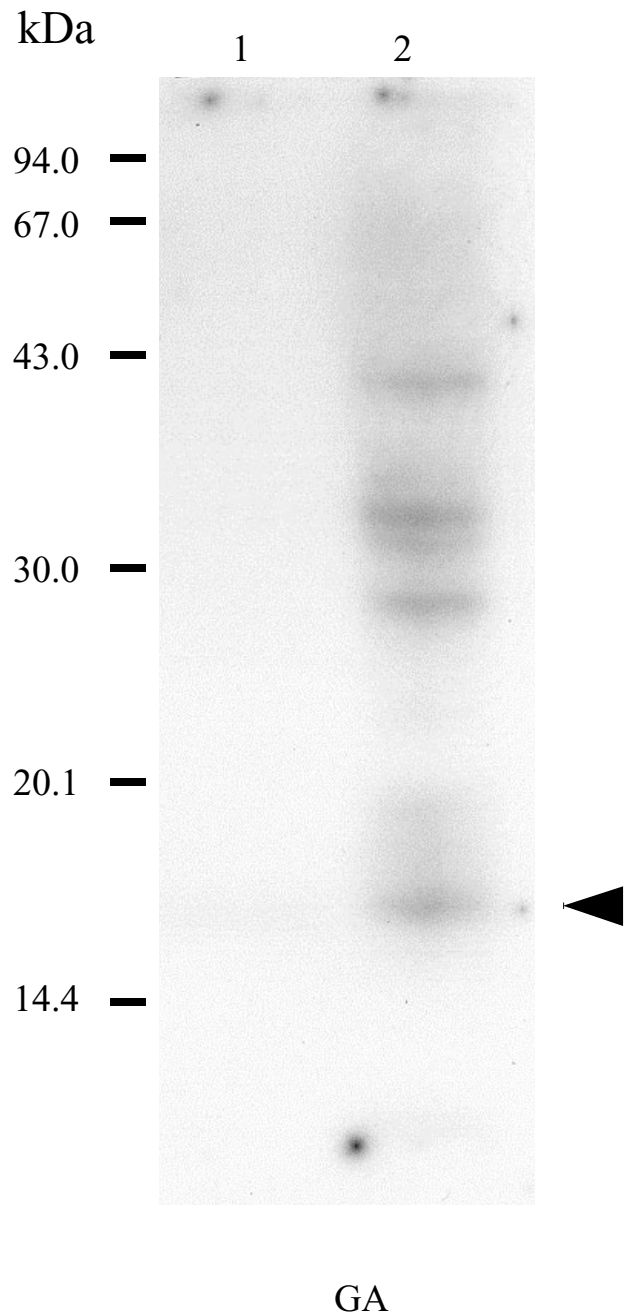


Figure 3. Western blot of purified oleosin and of recombinant oleosin with the serum of patient GA. Radioautography was performed after incubating the membrane with ^{125}I -iodinated anti-IgE. Lane 1: 18 kDa purified peanut oleosin; lane 2: membrane fraction of *Sf9* cells infected with the oleosin cDNA recombinant baculovirus. IgE reacted with both monomeric and oligomeric oleosins.

who have concomitant sensitivities to peanuts and soybeans (37). This should be investigated in further studies.

Allergic reactions to peanut oil protein are not frequent, considering that peanut oil is widely used in western countries. In our series of 14 patients who had suffered an allergic reaction to peanuts, only 3 were

clearly positive for peanut oleosin in IgE-RIA. Of these 3 patients, two underwent a labial provocation test with peanut oil protein extract, which was positive (20). This indicates that oleosins may be responsible for allergic reactions to peanut oil. Of the 3 domains of oleosin, the central core is the most hydrophobic and is not therefore a good epitope. The N- and C-terminal hydrophobic domains are better candidates. They have

homology with the corresponding oleosin domain from seeds and pollens and may be involved in cases of allergic patients reacting to various plant species (6, 8).

In conclusion, we have characterized a new peanut allergen which belongs to the oleosins, a family of proteins involved in the formation of oil bodies. The protein may be the cause of some of the allergic cross-reactions to peanuts and soybeans.

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