

Isolation and characterization of proteic allergens in refined peanut oil

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Summary

Allergic reactions to peanut oil are very much debated, even if the responsibility of peanut oil has been evoked in several cases of adverse reactions, including death related to severe asthma. The aim of the present study was to investigate the presence of allergenic proteins in peanut oil. Proteins were extracted from commercial refined peanut oil, with a relative content in the order of 0.1–0.2 µg per g of oil, and molecular sizes ranging from 14 up to 76 kDa in SDS-PAGE. Eight protein bands were systematically observed in crude, neutralized and refined oils, with a molecular mass ranging from ≈ 14 to 76 kDa, including one at 18 kDa which was identified by Western blot performed with serum from two allergic patients. The protein extract gave positive IgE-RIA with patient sera, positive *in vitro* leucocyte histamine release tests and positive skin-prick tests in allergic patients. The allergenic protein was purified by HPLC and [¹²⁵I] iodide-labelled. It had an isoelectric point at 4.5 in isoelectrofocusing. In conclusion, we have demonstrated the presence of allergenic proteins in crude and refined peanut oil. These proteins are the same size as two allergens previously described in peanut protein extracts.

Keywords: allergens, food allergy, peanut oil, peanut, proteins

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Introduction

Peanut is the second most frequent food involved in food-allergy in childhood [1–4]. Various allergens, such as peanut-1 [5], Ara h 1, Ara h 2 [6–10] and peanut agglutinin [11], have been isolated and characterized. Ara h 2 has also been partially sequenced [7]. The incidence of peanut allergy has been estimated at 13% among food allergies in Northern America [3]. Several cases of death related to anaphylaxis or to severe asthma have been reported after occasional intake of peanuts [1,12–15]. Peanut allergens are widely present in food products, and their ingestion is often accidental. The severity of food induced asthma, particularly in cases of hidden allergens, is likely to be underestimated [2]. This illustrates the necessity for knowing the

relative amount of peanut allergens in peanut products of the food industry.

The responsibility of peanut oil has been described in several cases of adverse allergic reactions, including death related to severe asthma [16–18]. In fact, allergic reactions to peanut oil are very much debated [19]. Some authors suggested that peanut oil did not contain any allergenic proteins using a double-blind provocation test by oil ingestion and RAST inhibition [19,20]. Controversely, Fries [14] in 1982, Moneret-Vautrin *et al.* [16] and Brown [17] in 1991 reported several cases of peanut oil allergy in children and in infants fed with adapted milks. IgE binding to another oil protein extract, namely soybean oil extract, has also been reported [21,22]. However, no food challenge was undertaken to investigate if soya bean oil can cause allergy. De Montis [23] and Hatahet [24] suggested two ways of sensitization, either by peanut oil used as a vitamin D excipient or by *in utero* sensitization. Furthermore, Hoffman *et al.* [25]

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Table 1. Clinical data and double-blind-food challenge in 11 patients where peanut was the allergen suspected (all have had atopic dermatitis and/or asthma and/or urticaria and/or bronchospasm)

Patients			Double-blind-food challenge		
No	Sex	Age (years)	Peanut oil +/- (mL)*	Other +/- (mL or g)*	Other clinical data
1	M	11.5	+ (5 mL)	+ Sunflower oil (5 mL)	Quincke oedema, reaction to maize
2	M	2	+ (5 mL)	–	Allergy to multiple allergens
3	M	3	– (16 mL)	–	Allergy to peas lentin
4	M	3	– (5 mL)	– Peanut seed (965 mg)	–
5	F	7.5	+ (5 mL)	–	Anaphylactic choc to Peanut
6	M	6	+ (5 mL)	+ Peanut seed (965 mg)	–
7	F	3	– (5 mL)	– Sunflower oil (5 mL)	Reaction after ingestion of biscuit
8	M	17	– (10 mL)	–	Anaphylactic choc in a restaurant, allergy to eggs
9	F	8	– (6 mL)	– Maise oil (6 mL)	–
10	F	5.5	– (15 mL)	+ Peanut seed (500 mg)	–
11	F	4.5	– (6 mL)	–	Urticaria after ingestion of biscuit, Quincke oedema

* Foot product quantity tested in the double-blind challenge.

recently observed the presence of specific IgE on immunoblots realized with cold pressed peanut oil extracts. However, no IgE binding was observed with extracts prepared with refined oils.

The presence of allergenic proteins in peanut oil was still to be proved, and this was the aim of the present study. Research for such a presence is of considerable interest since peanut oil is widely used in the food industry and in domestic foods. We have extracted and characterized by SDS-PAGE the proteins contained in peanut oil. Their allergenicity was tested by performing skin-prick tests, *in vitro* leucocyte histamine release, specific IgE assay, Western blotting and slot blotting.

Patients, material and methods

Patients

Inclusion criteria of the 11 patients were the presence of atopic dermatitis and/or asthma, and/or urticaria, and/or bronchospasm. In all cases, peanut was an allergen found responsible for the adverse allergic reaction (Tables 1 and 2).

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 Centre. They underwent skin-prick tests to peanut seed, peanut kernel and peanut oil extracts, to crude and refined oil protein extracts, double-blind food-challenge with peanut and peanut oil and

histamine release tests with freshly collected blood, as described [26].

Extraction of allergens from peanut oil

The extraction was performed with commercially available peanut oils from the European market. The trade marks of these refined oils were Ready, Imperial, Beffroi, Lesieur, Amphora and Homa.

Extraction was carried out as described previously [27–29]. Peanut oil (500 g) was mixed with 300 mL of petroleum ether. The mixture was then homogenized by stirring for 15 min at 4 °C. An aqueous extract was prepared by adding 100 mL of 0.1 M sodium bicarbonate (pH 8.0) and stirring for 48 h at 4 °C. The mixture was then centrifuged for 30 min at 50 000 *g* at 4 °C. 100 mL of 0.1 M sodium bicarbonate (pH 8.0) was again added to the organic layer, mixed for 30 min and centrifuged. The aqueous layers were pooled and stored at 4 °C.

The precipitation procedure was performed according to Mahuran *et al.* [28]. Sodium deoxycholate (20 mg/mL, pH 8.5) was added to the aqueous extract at a final concentration of 200 µg/mL. After 30 min stirring at 4 °C, a 20% (w/v) solution of cold trichloroacetic acid (TCA) was added at a final concentration of 6%. The mixture was allowed to stand on ice for 1 h and was then centrifuged at 50 000 *g* at 4 °C for 30 min. The supernatant was carefully removed from the small pellet by suction. The pellet was dissolved in 5 mL (max) of 62.5 mM Tris-HCl (pH 6.8) containing 3% SDS made 0.5 M in NaHCO₃ by adding solid NaHCO₃

Table 2. Cutaneous tests with peanut seed, peanut oil, peanut oil protein extract, double-blind-peanut oil challenge and CAP-RAST to peanut in 11 peanut allergic patients

Skin-prick test*										
Peanut					Peanut oil				Double-blind peanut oil challenge +/-	CAP-RAST to peanut (U/I)
Patient No	Native seed	Protein extract			Refined oil	Protein extract		Codein		
		Homemade 1 mg/ml	Stallergen™	Allerbio™		Crude oil 300 µg/ml	Refined oil 300 µg/ml			
1	+ (10,5)	- (1)	+ (6)	+ (4)	-	ND	ND	(2,5)	+	> 100
2	ND	ND	ND	ND	ND	+ (5)	+ (4)	(4)	+	70
3	+ (15)	+ (15)	+ (2,5)	+ (6)	-	- (1,5)	- (1)	(4)	-	> 100
4	+ (5)	+ (2,5)	+ (4)	+ (4)	-	ND	ND	(3)	-	> 100
5	+ (5,5)	+ (3)	+ (4)	ND	-	± (2)	± (2)	(5)	+	14.2
6	+ (3)	ND	ND	ND	-	ND	ND	ND	+	> 100
7	+ (6)	+ (3)	+ (3)	+ (4)	-	+ (2)	+ (2,5)	(2,5)	-	> 100
8	+ (13)	+ (5)	+ (5)	+ (7)	ND	+ (5)	+ (5)	(5)	-	> 100
9	+ (6,5)	+ (4,5)	+ (6)	+ (6)	-	+ (4)	+ (3)	(2,5)	-	> 100
10	+ (10)	+ (2)	+ (2,5)	+ (3)	-	ND	ND	(2,5)	-	3.2
11	+ (4)	- (1)	+ (5)	+ (4)	-	+ (1,5)	+ (3)	(2)	-	4.6

*The skin prick tests were performed and interpreted as described by Moneret-Vautrin [26]. Reactions were considered positive when the weal was > 3 mm or 50% of the codein positive control.

() Weal diameter (mm).

(final pH 8.8). The solution was dialysed overnight (4 °C) against 1 L of 6.25 mM Tris-HCl, pH 6.8, containing 0.3% SDS and then dried in a dessicator. The dried sample was washed with 1 mL of acetone, glacial acetic and triethylamine (90:5:5, v/v/v) and centrifuged. The supernatant was removed, the pellet washed again with 1 mL of acetone and air-dried overnight.

Protein content was determined with the Bicinchoninic acid as described by Smith *et al.* [30], using the Protein Reagent Assay (Pierce, Rockford, IL, USA). Bovine serum albumin (BSA) was used as standard protein.

Electrophoresis (SDS-PAGE) of oil extracts

Electrophoresis of the protein extracts in the presence of (SDS-PAGE) was performed with a 8–25% polyacrylamide gradient gel, in 0.20 M Tris-HCl (pH 7.5), 0.20 M Tricine, 0.55% SDS using the automated Phast System (Pharmacia, Uppsala, Sweden) [31,32]. Aliquots (10–20 µg) were resuspended in 10 µL of 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 2.5% (w/v) SDS, 5% (v/v) β-mercaptoethanol and 0.1% (w/v) bromophenol blue and heated for 3 min at 100 °C before loading. The following polypeptides were used as molecular mass markers: 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). The migration conditions were 250 V, 10 mA, 3 W, 15 °C for 65 Vh. The proteins were visualized using the silver staining technique derived from the method of Heukeshoven [32] described by Pharmacia for Phast System™.

Western blot

The peanut extracts were run on SDS-PAGE as described above. The proteins were electrotransferred onto nitrocellulose by blotting in 25 mM Tris-HCl buffer containing 192 mM glycine, 20% v/v methanol (pH 8.3).

Ponceau red staining of the membrane and immunodetection were performed as described by Wieths *et al.* [33]. Unoccupied protein-binding sites were blocked by incubation with 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl and 0.3% v/v Tween 20.

After washing six times (total 30 min) with 20 mM Tris HCl (pH 7.4) containing 0.15 M NaCl and 0.05% Tween 20, the nitrocellulose was incubated with the serum from allergic patients (diluted 1:5 with 0.1% Tween 20 in 20 mM Tris- HCl (pH 7.4), 0.15 M NaCl) for 3 h at room temperature, washed again and incubated for 3 h with ¹²⁵I-anti-IgE (obtained from Immunotech, Marseille, France). The tracer solution was diluted with 0.3% Tween 20 in the previous Tris-HCl buffer to obtain 30 000 cpm/cm². Incubations were

performed at room temperature under gentle rocking. IgE binding was detected by autoradiography on Kodak X-OMATTM film. The film was exposed at -70°C for 10 days.

Slot-blot immunoblotting

AmphoraTM and LesieurTM oil protein extracts were filtered onto nitrocellulose by using a Bio-DOT SF microfiltration apparatus (BIORAD, Hercules, CA, USA). Extracts were applied to the slots in 20 mM Tris-HCl (pH 7.4) and a house vacuum was run until all slots were clear of liquid. Ponceau red staining, unoccupied protein-binding sites blocking and immunoblotting were performed as described above with little modifications. The positive and negative sera were diluted 1:10. Strips were washed five times for 10 min only after first and second incubations. The second incubation was performed an additional 24 h with ^{125}I -labelled anti-human IgE diluted 1:10. The film was exposed at -70°C for 4 days.

Immunoblot inhibition was performed by using 1 mL of the serum from patient 2 (dilution 1:10) in Tris-Tween 0.5% buffer, which was submitted to extensive incubation with 30 or 9 μg of oil protein extracts for 48 h at room temperature. Serum was then incubated with nitrocellulose strips onto which 0.5 or 5 μg each oil protein extract had been slotted. Immunoblotting was carried out as described above.

Leucocyte histamine release (LHR)

In vitro leucocyte histamine release tests (LHR) were performed with heparinized blood as described recently [34].

Solid phase immobilization of allergens to epoxy-Sepharose and radioimmuno-assays of specific IgE

The solid phase was prepared by coupling oil protein extract to epoxy-activated Sepharose 6B, in sodium carbonate buffer 0.1 M pH 9.2, as described recently [35].

Epoxy-activated Sepharose 6B (Pharmacia, Uppsala, Sweden) (0.65 g) was expanded in water (1:10, w/v) and washed 200-fold successively with water and with 0.5 M NaCl and with 0.1 M sodium carbonate (pH 9.0). The suspension was mixed with 0.5 mg of allergen by slow rotation overnight at 37°C . The gel was then washed 200-fold using successively water, 0.5 M NaCl and 0.02 M Tris-HCl (pH 7.4) containing 0.15 M NaCl and 0.02% NaN_3 (w/v), in order to eliminate the remaining free oil protein extract and to block the epoxirane groups.

Gel suspension (50 μL) was incubated with 50 μL of serum in duplicate at room temperature for 3 h. The gel was then washed three times with 2 mL of Tris HCl 0.02 M pH 7.4 containing 0.15 M NaCl, 0.02% NaN_3 (w/v) and 1% Tween 20 (v/v), and 50 μL ^{125}I -anti-IgE was added and incubated for 18 h at room temperature. The reactive phase

was washed again three times with Tris-HCl buffer. Quantification of specific IgE corresponded to the percentage of γ -radioactivity of anti-IgE bound to the reactive phase. IgE-RIA was considered positive when the uptake of ^{125}I -anti-IgE to the solid phase incubated with patient sera was at least twofold higher than that obtained with control sera (from a non-allergic subject).

Inhibition of the IgE-RIA was performed by the same protocol, except that 50 μL of the patient's serum was preincubated with 1.6 μg of protein extract in 50 μL distilled water for 1 h at room temperature prior to its incubation with the reactive phase. Inhibition percentage was estimated by γ -counting of Sepharose with and without preincubation of the patient's serum with the protein extract, using the following formula:

$$I = \frac{\text{cpmo} - \text{cpmi}}{\text{cpmo}} \times 100$$

where cpmo and cpmi represented, respectively, the radioactivity adsorbed to the solid phase without and with preincubation with the protein extract.

Purification of allergenic proteins

The protein extract from refined oil was run in reverse phase — high performance liquid chromatography, using a two-pump gradient system (Waters, Milford, MA, USA). A LiChrosorb RP-18 column (244 $\times$ 4 mm I.D., Merck, Marburg, Germany) was eluted with a A/B linear gradient of 0.05% TFA in water (v/v) as phase A and of 0.05% TFA in methanol as phase B (v/v), at a constant flow-rate of 0.5 mL/min. The collected fractions were aliquoted, then dried in a dessicator overnight.

Isoelectrofocusing

The purified protein was iodinated using the method of Markwell *et al.* [36]. The iodinated protein was run in Superose 12TM gel filtration as described [37]. The peak corresponding to the iodinated protein was collected and studied by isoelectric focusing, in a 110 mL LKB column, type 8101 (LKB, Bromma, Sweden) packed with carrier ampholytes of pH ranging from 3.0 to 9.0 (Pharmacia, Uppsala, Sweden) at a final concentration of 1% in a 0–50% (w/v) sucrose gradient containing 5 M urea.

Results

Characterization of peanut oil protein extract

The bicinchoninic protein assay revealed 3.4 μg of proteins for 1 g of crude oil and 0.2 μg of proteins for 1 g of neutralized oil (Table 3). The commercial peanut oils tested had a protein concentration in the order of 0.1–0.2 μg per g

Table 3. Protein concentration of crude and neutralized peanut oils and of five commercially available refined peanut oils

Peanut oil	Protein concentration* (ng/g)
Crude [#]	3 400
Neutralized [§]	200
Impériale [†]	130
Reddy [†]	150
Homa [†]	160
Beffroi [†]	100
Lesieur [†]	200

* Proteins were extracted as described under method section. The concentration was determined by the bicinchoninic method from Smith *et al.* [30].

[#] Crude oil was obtained from cold-pressed peanuts.

[§] Neutralized oil was crude oil which was successively 'demucilinated' and neutralized.

[†] Trade mark from peanut oils available in the European community.

of oil. The industrial refining process therefore eliminated about 94% of the proteins contained in crude oil.

SDS-PAGE analysis of the extracts obtained from the different oils revealed 11 proteins bands in crude oil extract with molecular sizes ranging from 14 up to 76 kDa (Fig. 1). Eight protein bands were observed in neutralized and in refined oil extracts, with molecular sizes ranging from 14 up to 76 kDa. The 14, 15, 18, 24, 46, 61, 67 and 76 kDa proteins were present in the three extracts of crude, neutralized and refined oils. In all cases, the 14, 46, 61 and 76 kDa proteins were the most intensive bands of the three electrophoretic patterns, whereas the 18 kDa protein gave a very weak band in neutralized and refined oil extracts.

Allergenicity of the protein extract

The allergenicity of peanut oil and the protein extracts was studied in a group of 11 patients. All of these subjects had a positive CAP-RAST to peanut and positive prick test to peanut. The double-blind challenge were positive in four out of 11 cases (Table 2). Skin-prick tests were negative in all cases with native peanut oil. The skin-prick tests were positive in five out of seven cases in the protein extracts from refined oil. The same results were obtained with protein extract from crude oil (Table 2).

The *in vitro* leucocyte histamine release (LHR) was tested with either native refined oil casein micelles or protein extracts (Table 4). Out of the 11 patients tested, nine had a positive LHR with oil casein micelles and eight patients had a positive LHR with protein extract. Seven patients had

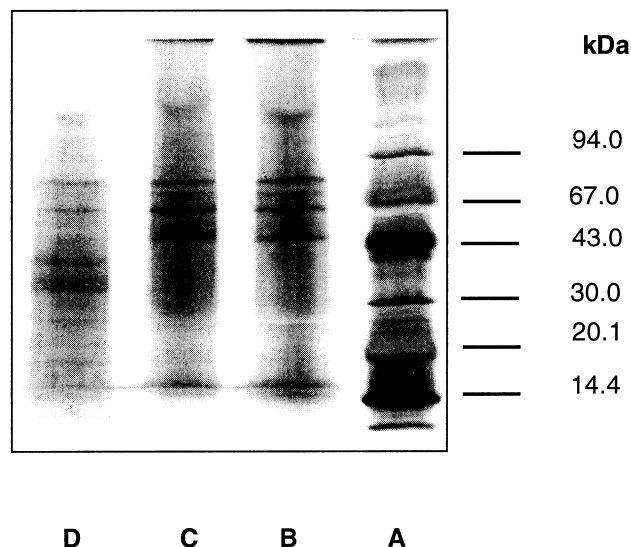


Fig. 1. Denaturing silver stained SDS/PAGE of protein extracts from crude, neutralized and refined peanut oils. Lane A corresponds to standard proteins (lactalbumin, soy bean trypsin inhibitor, carbonic anhydrase, ovalbumin, albumin and phosphorylase b). Their respective molecular sizes are indicated by dashes. Lane B: the extract from refined oil contained 8 proteins with respective molecular sizes at 76, 67, 61, 46, 24, 18, 15 and 14 kDa. Lane C: the extract from neutralized oil gave the same protein bands. Lane D: the extract from crude oil contained 11 proteins with respective molecular sizes at 76, 67, 61, 52, 46, 39, 32, 24, 18, 15 and 14 kDa.

a positive LHR with both oil casein micelles and protein extract, whereas only one patient had a negative LHR with both preparations.

Specific IgE radioimmunoassay was performed with the 11 patient sera, using protein extract of refined peanut oil covalently coupled to epoxy-Sepharose (Table 5). Uptake percentage of radio-labelled anti-IgE to the solid phase ranged from 7 to 34% for the positive RIA with a ratio patient/control from 2.5 to 22. We obtained only two negative assays, with a percentage uptake at 1.5 and 1.7%, respectively, and a ratio at 1. The inhibition percentage ranged from 9 to 52% in presence of soluble protein extract (Table 5).

A Western blot was performed from SDS-PAGE of refined oil protein extract. The proteins were blotted with serum from two patients ($n=6$ and $n=2$) and with radio-labelled anti-IgE. One band was identified in radioautography, with molecular size at about 18–20 kDa (Fig. 2). By contrast, the western blot performed with protein extract from peanut kernel gave seven bands with respective molecular masses at about 14, 18, 20, 30, 42, 62 and 78 kDa. The protein extract could inhibit IgE reactivity in immunoblots performed with two different protein amounts from protein extracts of two commercial refined oil (Fig. 3).

Table 4. *In vitro* leucocyte histamine release performed with blood from 11 patients allergic to peanut, incubated with increasing dilutions of either casein-oil micelles or refined oil protein extracts

Patient	Allergen preparation	Dilution of maximal histamine release	% of released histamine*	Interpretation (+/–)
1	Casein-oil	10^{-2}	17.0	+
	Oil protein extract	10^{-5}	25	+
2	Casein-oil	10^{-4}	20	+
	Oil protein extract	10^{-1}	45	+
3	Casein-oil	10^{-1}	7	+
	Oil protein extract	10^{-1}	3	–
4	Casein-oil	10^{-3}	6	+
	Oil protein extract	10^{-1}	15	+
5	Casein-oil	10^{-1}	2	–
	Oil protein extract	10^{-1}	0.5	–
6	Casein-oil	10^{-4}	9	+
	Oil protein extract	10^{-4}	18	+
7	Casein-oil	10^{-2}	56	+
	Oil protein extract	10^{-1}	2	–
8	Casein-oil	10^{-2}	33	+
	Oil protein extract	10^{-2}	50	+
9	Casein-oil	10^{-5}	15	+
	Oil protein extract	10^{-4}	26	+
10	Casein-oil	10^{-5}	1	–
	Oil protein extract	10^{-5}	10	+
11	Casein-oil	10^{-3}	25	+
	Oil protein extract	10^{-1}	51	+

* Histamine was determined by a radioisotope assay as described under methods. The release was considered positive when the percentage exceeded 5%, according to our previous evaluation of the method [34].

Purification of the allergenic protein

The protein extract from refined oil was run in RP-HPLC. The allergenic protein peak was identified by testing the inhibiting effect of the eluted fraction in an IgE-RIA performed with serum from patient GA. This peak was eluted at a retention time of 53 min corresponding to a mobile phase containing 100% of methanol (Fig. 4). This indicated that the protein was hydrophobic.

The isolated protein allergen was radiolabelled and analysed by column isoelectrofocusing. A single peak with a pI at 4.5 was obtained (Fig. 4).

Discussion

The aim of this study was to find out whether European commercial peanut oils contain allergenic proteins. The first part of our work consisted in proving the presence of

proteins in crude, neutralized and refined oils. The amount of protein in peanut oil is still a matter of controversy [27,38]. Our method of extraction enabled the extraction of 100–200 ng of protein per g of refined oil (Table 3). A similar amount of protein was obtained by Klurfeld [27]. Eight proteins, with molecular size ranging from 14 up to 76 kDa, could not be eliminated during the refining process (Fig. 1).

Until now, the question of whether peanut oils contain hidden allergens remained to be determined [39,40]. The second part of our work was then to determine if some of these oil proteins and native oil were allergenic or not. The protein extracts from refined oil gave a positive LHR in most cases. LHR were also performed with peanut oil casein micelle. The results were concordant with both preparations in most cases (Table 4). In fact, the micelles preparation was a product mimicking the milk formula. It may be expected that, if peanut oil contains residual proteins, the latter are

Table 5. Seric IgE specific to refined oil proteins determined by Sepharose-protein IgE-RIA

Patient	CAP-RAST to peanut (U/l)	IgE-RIA with oil protein-sepharose			% inhibition by oil protein extract
		% uptake*	P/C†	Interpretation	
1	> 100	9	6	+	0
2	70	14,5	9	+	9
3	> 100	24	15	+	39
4	> 100	18	12	+	44
5	14.2	10	6.5	+	ND
6	> 100	34	22	+	52
7	> 100	24	15.5	+	38
8	> 100	10,5	7	+	38
9	> 100	7	4.5	+	21
10	0.9	1,5	1	—	ND
11	4.6	1,7	1	—	ND
Controls <i>n</i> = 11	< 0.35	1.55 ± 0.07			

* % uptake corresponds to the ^{125} I-anti IgE bound to the solid reactive phase incubated with patient sera, as described under methods.

† The IgE-RIA was considered positive when the % uptake of the tracer was at least 2-fold higher with patient sera than with control sera.

adsorbed on the surface of casein micelles. The patients tested were not allergic to cow's milk proteins. This data therefore supported previous studies which suggested that milk formula prepared with peanut oil can lead to an allergic reaction [2,16,17]. Klurfeld *et al.* obtained proteins from vegetable oil which had a lectinic activity [27]. We cannot turn down the hypothesis of non-specific LHR, as lectin binding to basophile IgE can induce non-specific degranulation. However, the degranulation was not dose-dependent in our LHR series. We directly demonstrated the presence of specific IgE in the serum from our 11 patients, in order to confirm that positive LHR were the result of an immunospecific binding of peanut allergens to basophiles. We were able to find a positive IgE-RIA in nine of the 11 patients using peanut oil protein extract coupled to epoxy-Sepharose as a solid reactive phase. The specificity of the IgE binding to the reactive phase was assessed by a significant inhibition of the IgE-RIA, after incubating the serum with the corresponding soluble extract (Table 5). The allergenicity of our refined oil protein extracts was also validated by the positivity of the skin-prick test in five of the 11 patients (Table 2). However, the implication of these oil proteins in clinical allergic reaction is not completely established as we could not perform double-blind challenge with the extracts. Concerning native oil, four of the 11 peanut allergic patients react to native oil in double-blind challenge but all were negative in skin-prick tests with native oil. This pointed out the difference of sensibility of these two tests. Two previous studies

failed to detect any allergenic activity in oil, using the RAST inhibition technique, owing to technical difficulties related to the oily nature of the medium [20,40]. In fact, Hoffman [25] obtained a positive immunoblot with protein extracts from non refined peanut oil. It seems therefore that European oil, excepted British, is less safety for peanut allergic patient than American oil. The oil composition variations or the refining process might explain this difference in *in vivo* reactivity. This showed the need to investigate the allergenicity of protein extracts instead of that of oil phase.

The Western blot analysis of our refined oil protein extract enabled us to demonstrate which of the eight proteins from the protein extract reacted as allergens. One of the proteins with the lowest molecular weight, e.g. the 18 kDa protein reacted with the IgE of patients and was therefore considered as the allergens present in peanut oil. The 18 kDa protein had a molecular size and a pI close to the allergen Ara h 2 previously described by Burks *et al.* [7]. It was therefore very likely that it corresponded to the same protein. Unfortunately, we did not obtain a sufficient quantity of protein to carry out amino-sequencing, in order to confirm this hypothesis. A low molecular weight allergen with a molecular mass estimated at 14 kDa has recently been purified from crude peanut extract, using preparative electrophoresis and HPLC [42]. This allergen may correspond to the 14 kDa allergenic protein which we observed by Western blot on the peanut kernel protein extract (Fig. 2).

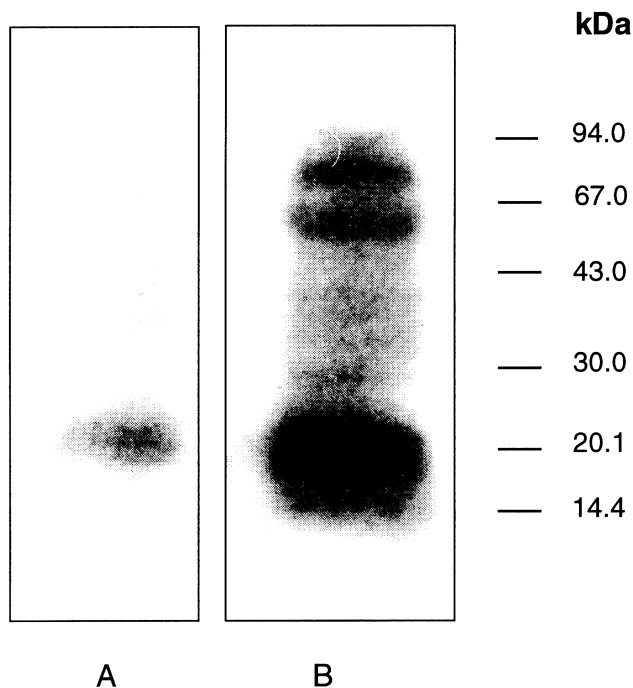


Fig. 2. Western blot of protein extracts from peanut kernel (lane A) and refined peanut oil (lane B). The extracts were run in SDS-PAGE, as shown in Fig. 1, electrotransferred on nitrocellulose sheet and blotted successively with serum from a patient allergic to peanut and with ^{125}I -labelled anti-IgE. After radioautography, one band was identified with the protein extract from refined oil, with molecular size at about 18 kDa. The same bands were identified with kernel extract, concurrently with bands corresponding to respective molecular sizes at 14, 20, 30, 42, 62 and 78 kDa. The same standard proteins as in Fig. 1 were used for estimating the molecular size of allergens. Their migration positions are indicated by dashes.

We recently showed, by Western blotting, the presence of a 18 kDa allergen in 2 protein extracts from peanut kernel and shell, respectively, using sera from allergic patients. This 18 kDa allergenic protein of kernel extract was found concurrently with several allergens of higher molecular size [29]. In contrast, only two allergens of 18 and 41 kDa were observed in the corresponding shell extract [29]. Only the 18 kDa peanut allergen was simultaneously present in both the shell and the kernel. This allergen may therefore correspond to Ara h 2 and to the 18 kDa allergens that we found in the peanut oil extract. In that case, this means that the 18 kDa allergen present in peanut oil originates from both the kernel and the shell. Recently, additional findings in two cases of patients allergic to peanut supported this conclusion as they had a positive IgE-RIA to kernel and shell peanut protein with a significant inhibition of both RIA in the presence of soluble protein extract from refined peanut oil [43]. Sensitization can therefore be provoked by a same allergen contained in inhalants and peanut food products.

From a theoretical point of view, one may be astonished

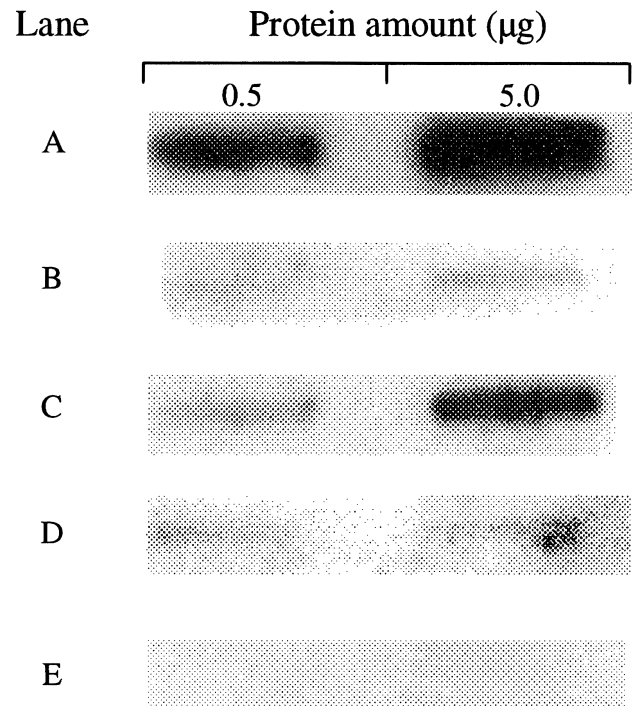


Fig. 3. Slot-blot immunoblots of peanut refined oil protein extracts. Two protein amounts 0.5 and 5 µg were tested. Lane A, LesieurTM oil extract vs. serum from patient 2 with peanut allergy. Lane B, same but serum from patient 2 was first incubated for 48 h with 9 µg of LesieurTM oil extract. Lane C, AmphoraTM oil extract vs. serum from patient 2 with peanut allergy. Lane D, same but serum from patient 2 was first incubated for 48 h with 30 µg of AmphoraTM oil extract. Lane E, extract vs control serum from a healthy subject without allergy.

that refined oil contains trace amounts of proteins. Refined oil also contains amphipathic lipid traces such as phospholipids. These compounds can theoretically form inverted micelles in the oil phase, in which the hydrophobic proteic allergens can be included. A family of proteins named oleosins is known to interact with phospholipids and to participate in the formation, stability and storage of lipid bodies in soya bean, rape seeds, maize and nuts [44,45]. These proteins have a molecular size similar to that of the 14–18 kDa allergens of peanut oil [43]. We have recently hypothesized that these proteins may correspond to a new class of allergens [43]. It will therefore be of particular interest to compare the physicochemical properties and the aminoacid sequences of purified oleosins and of peanut oil allergens in order to confirm this hypothesis. Recently, Onaderra *et al.* showed that Sin a 1, a food mustard allergen of 14 kDa interacts with phospholipids vesicles [46]. This finding may support our hypothesis. This would also mean that the improvement of phospholipid elimination in the refining process could be a solution in order to decrease the relative protein content of refined oils.

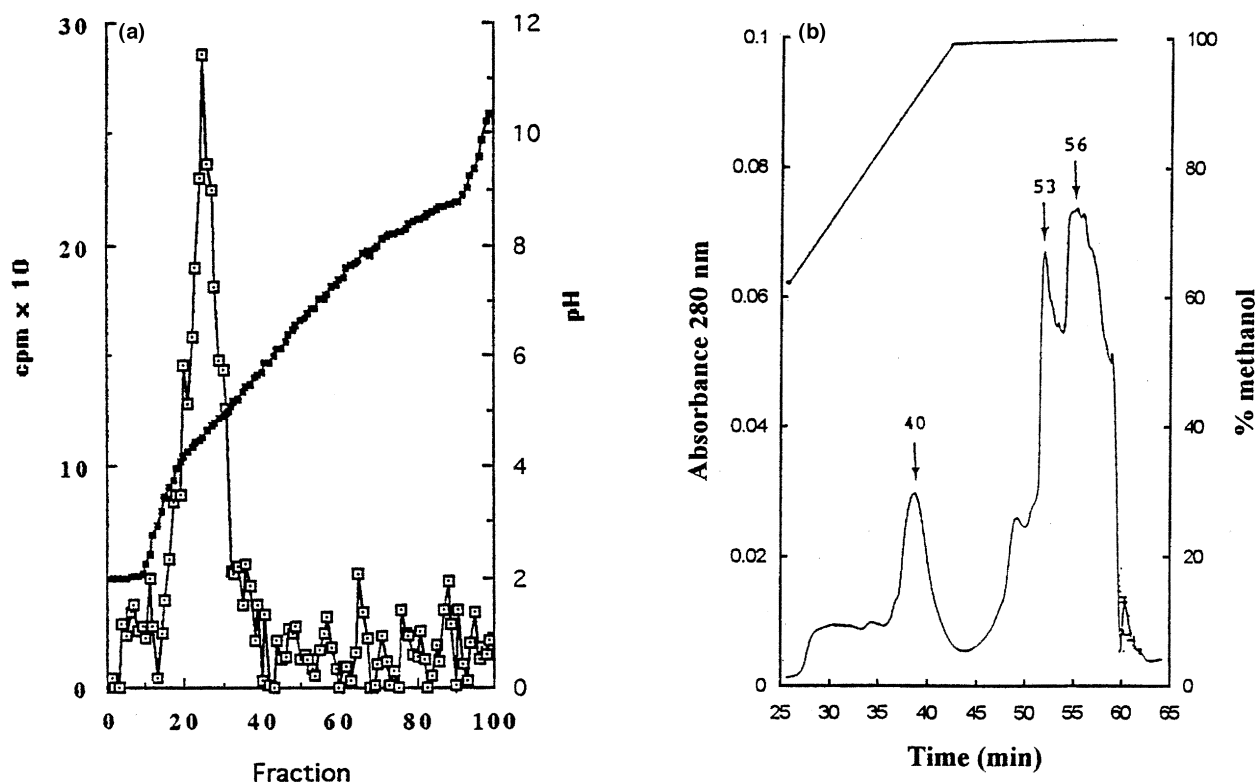


Fig. 4. Reverse phase HPLC and isoelectrofocusing of the purified allergen. Right: Elution profile (—) of protein extract from refined peanut oil on reversed phase HPLC. HPLC was performed with a Lichrosorb RP-18 column eluted at 0.5 mL/min with a gradient of methanol and 0.05% trifluoroacetic acid. Retention times are indicated by arrows. The allergenic fraction was detected by IgE-RIA inhibition as described under methods and was eluted at a retention time of 53 min. Left: Column isoelectrofocusing of the iodinated protein showed a single peak corresponding to an isoelectric point at 4.5.

In conclusion, we have demonstrated the presence of allergenic protein in refined peanut oils. This protein has the same molecular size as the one previously described in peanut protein extracts.

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