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Short communication

Purification of allergenic proteins from peanut for preparation of the reactive solid phase of a specific IgE radioimmunoassay

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Abstract

Peanut is one of the most allergenic foods. Detection of specific IgE in the serum of allergic patients requires the purification of allergenic proteins. In the present work, proteins were recovered from peanut kernel after successive treatment in acetone and diethy ether. The proteins were dissolved in 0.05% TFA and analysed by RP-HPLC with a 0–100% gradient of methanol containing 0.05% TFA. The protein peaks were recovered and tested in SDS-PAGE. Eleven proteins were identified with a M_r ranging from 13 to 81. Western blotting was performed with sera from allergic patients. Allergenic proteins had a M_r of 15, 18, 19, 33, 41 and 67. By comparison, a protein fraction from peanut shell contained seven proteins with M_r ranging from 15 to 81. Only two proteins with M_r of 18 and 41 were detected in a Western blot. The protein fractions were coupled to epoxy-Sepharose and the gels were used as a solid reactive phase for detection by IgE-RIA of specific IgE from the serum of allergic patients.

1. Introduction

Peanuts are widely used in food products, such as peanut butter, powder, syrup, deflavored peanuts, and peanut flours [1–3]. New formulations are being developed to make use of peanut flour in bakery goods and snack foods. Allergy to peanut has been suggested to develop in 13% of infants who have been fed peanut protein formula since birth, or have been eating peanut products [4–7]. Allergy to peanut is one of the most common food allergies in childhood [8–15]. Several fatal cases have been published [16].

So far, there have been few reports on the allergenicity of peanut protein components. In this paper, we report the detection of allergenic proteins from peanut kernel and shell by Western blotting after partial purification by reversed-phase HPLC. The protein extracts have been used for the preparation of epoxy-Sepharose–protein affinity gels needed to perform a radioimmunoassay of specific IgE in the serum of allergic patients.

2. Experimental

2.1. Extraction of allergens from peanut kernels

Extraction was performed by previously published method with modifications [2]. Briefly, the kernels (100 g) were ground in a blender and

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placed in a 500-ml Erlenmeyer flask to be defatted. In the defatting procedure, 250 ml of acetone were mixed with the ground peanuts by stirring for 1 h at 4°C. The suspension was allowed to settle and the acetone was decanted and discarded. The kernel product was then mixed with 250 ml of diethyl ether and allowed to settle. The ether treatment was repeated five times. After the fifth ether extraction, the defatted kernel product was separated from the diethyl ether by aspiration and air-dried overnight. An aqueous extract of the defatted peanuts (peanut flour) was prepared by adding 300 ml of 0.1 *M* sodium bicarbonate (pH 8.0) and stirring for 20 h at 4°C. The extract was clarified by centrifugation for 30 min at 50 000 *g* at 2°C, filtered through Whatman paper no. 1 and stored at −5°C.

2.2. Extraction of allergens from peanut shells

The shells were ground in a blender mixed with 0/1 *M* sodium bicarbonate buffer (pH 8.0) and stirred for 20 h at 4°C. The extract was clarified by centrifugation for 30 min at 50 000 g at 2°C. The supernatant was removed and filtered through Whatman paper no. 1 and stored at −5°C.

2.3. Precipitation

Precipitation was performed as described previously [17]. Sodium deoxycholate (pH 8.5) was added to the kernel or shell extracts at a final concentration of 200 μg/ml. After 30 min at 4°C, a 20% solution of cold trichloroacetic acid (TCA) was added to 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 by suction. The sample was dissolved in 62.5 m*M* Tris-HCl (pH 6.8) containing 3% SDS in 0.5 *M* $NaHCO_3$ (final pH 8.8). The sample was dialysed overnight against 1 l of 6.25 m*M* 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 acid–triethylamine (90:5:5, v/v) and centrifuged. The supernatant was removed, washed with 1 ml of acetone and air-dried overnight.

2.4. Reversed-phase HPLC

Peanut extracts were analysed by reversed-phase HPLC (Waters, Milford, MA, USA) using a two-pump gradient system. A LiChrosorb RP-18 column (244 × 4 mm I.D.) (Merck, Marburg, Germany) was eluted with a linear gradient of 0.05% TFA in water (v/v) as phase A and 0.05% TFA in methanol as phase B (v/v) at a constant flow-rate of 1.0 ml/min. The dried kernel or peanut shell extracts were dissolved in 200 μl of phase A. The collected fractions were aliquoted, dried in a dessicator overnight and analyzed by electrophoresis and Western blotting.

2.5. Protein determination

Protein concentrations of the extracts and fractions from reversed-phase HPLC were determined with the Bicinchoninic acid (BCA) Protein Reagent Assay (Pierce, Rockford, Il, USA) according to the manufacturer's instructions [18]. Bovine serum albumin (BSA) was used as protein standard.

2.6. Electrophoresis (SDS-PAGE) of shell and kernel extracts

Polyacrylamide gel electrophoresis of the shell and kernel aliquots in the presence of sodium dodecyl sulphate (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). Aliquots were resuspended in 10 μl of SDS-PAGE buffer–10 m*M* Tris-HCl (pH 8.0) containing 1 m*M* EDTA, 2.5% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue and heated 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 proteins were visualized using the silver staining technique described previously [19].

2.7. IgE radioimmunoassay

Sera were obtained from 2 patients allergic to peanut; G.A. was a 4-year-old male and C.I. a 18 year old female. Both patients were treated for asthma and atopic dermatitis. The diagnosis was established from the clinical report and positive skin prick tests. Control sera for controls were obtained from non-atopic individuals.

Solid-phase immobilization of allergens to epoxy-Sepharose

The solid-phase was prepared by coupling kernels or shell protein extracts to epoxy-actived Sepharose, according to the method described previously [20–22]. A 300-mg sample (dry weight) of epoxy-actived Sepharose (Pharmacia) was swollen in water (1:20, w/v) and washed successively with 200-fold quantity water, 0.5 *M* NaCl and 0.1 *M* sodium carbonate (pH 9.0) up to a final volume of 1 ml of epoxy-actived Sepharose suspension. A 2-mg quantity of kernel or shell protein extract was coupled to 1 ml of epoxy-actived Sepharose gel. The suspension was mixed by slow rotation overnight at 37°C. The gel was washed successively with 200-fold quantity water, 0.5 *M* NaCl and 0.02 *M* Tris-HCl (pH 7.4) containing 0.15 *M* NaCl, 1% Tween 20 (v/v) and 0.02% NaN_3 (w/v) in order to eliminate the remaining free kernel or shell protein extracts and to block the epoxirane groups.

Radioimmunoassay of specific IgE

A 50-μl volume of gel suspension was incubated with 50 μl of serum in duplicate at room temperature for 3 h. The gel was then washed 3 times with 2 ml of Tris-HCl buffer and 50 μl of ^{125}I-anti-IgE was added and incubated for 18 h. The reactive phase was washed again 3 times with Tris-HCl buffer. Quantitation of specific IgE corresponded to the percentage of γ-radioactivity of anti-IgE bound to the reactive phase (percentage > 2 is considered as positive). The capacity of IgE-RIA to diagnose allergenicity to peanut was evaluated as described previously [22] by estimating the ratio of the percentage uptake of ^{125}I-anti-IgE of allergen-peanut patient versus non atopic-individuals (ratio > 2 indicate the presence of specific IgE). Inhibition of the IgE-RIA was performed by the same protocol except that the patient serum was pre-incubated with peanut extract for 2 h at room temperature prior to its incubation with the reactive phase. The percentage inhibition was estimated from γ-counting of the solid phase with and without preincubation of patient serum with the extract. The percentage inhibition was calculated using the following formula: $\% = [(cpm_i - cpm_0/cpm_i] \cdot 100$ where cpm_0 and cpm_i represent respectively the radioactivity adsorbed to the solid phase without or with pre-incubation with the protein solution tested.

2.8. Western blot

Fractions form peanut shell or kernel were run on 8–25% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) as described above. The proteins were transferred onto nitrocellulose by blotting using the protocol described by Towbin *et al.* [23]. Unoccupied protein-binding sites were blocked by incubation overnight with 20 m*M* Tris-HCl (pH 7.4) containing 0.5 *M* NaCl and 3% gelatin. After washing three times with 0.05% Tween 20 in TBS, the nitrocellulose was successively incubated with serum from peanut-allergic patient (diluted 1:10 with 0.1% gelatine, 0.02% sodium azide in TBS) for 3 h at room temperature, washed and incubated for an additional 3 h with ^{125}I-anti-IgE (diluted 1:10 with 1% gelatine, 0.05% Tween 20 in TBS). Incubations were performed under gentle rocking. IgE binding was detected by autoradiography on Kodak X-Omat film. The film was exposed at −80°C for 5 days.

3. Results

The protein concentration of kernel and shell extracts was estimated at 39 and 0.97 mg/g, respectively. The kernel extract aliquots gave

four peaks with retention times of 23, 24, 25 and 26 min (Fig. 1A) in C_{18}RP-HPLC. By comparison, aliquots of shell extracts gave two major protein peaks with retention times of 23 and 26 min (Fig. 1B). The reproducibility of HPLC was studied by comparing the run-to-run retention times of these two peaks in 6 runs. The coefficients of variation were estimated at 1.3 and 0.7%, respectively, for the 23-min and the 26-min peak. SDS-PAGE analysis of kernel and shell extracts showed eleven proteins in the kernel extract with M_r ranging form 13 to 81 and five proteins in the shell extract with M_r ranging from 14 to 81 (Fig. 2). The protein fractions eluted from HPLC were analysed by SDS-PAGE (Fig. 3) and Western blotting (Fig. 4). SDS-PAGE of kernel and shell eluates showed respectively 11 bands with M_r ranging from 13 to 81 and 7 bands with M_r ranging from 15 to 81. Western blotting was performed with a pool of sera from peanut-allergic patients. It showed 6 allergenic proteins in kernel samples, with an M_r of 15, 18, 28, 34, 41 and 67, respectively. Two allergenic proteins were detected by Western blotting in shell samples with an M_r of 18 and 41, respectively. Therefore, the two peanut products (shell and kernel) had virtually two identical allergenic proteins, with an M_r of 18 and 41.

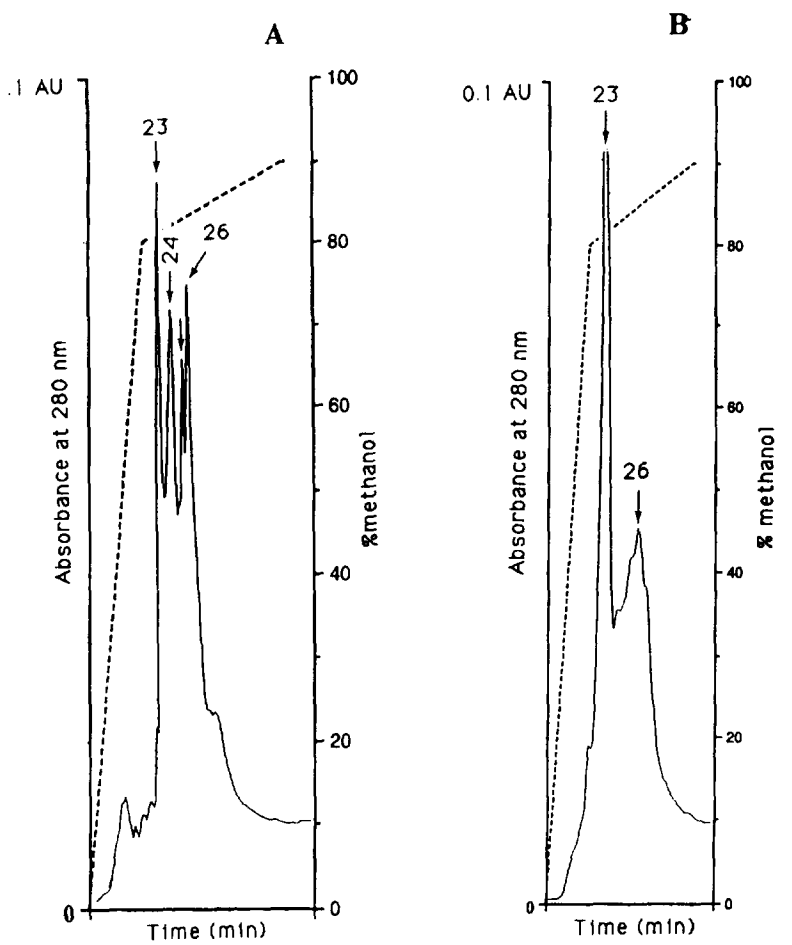

Fig. 1. Elution profile of kernel (left) and of shell protein extract (right) on reversed-phase HPLC. HPLC was performed with a 244 × 4 mm I.D. LiChrosorb RP-18 column eluted at 1.0 ml/min with a gradient of methanol and 0.05%trifluoroacetic acid (%B, ---). The retention times of the proteins are indicated at the top of the figure.

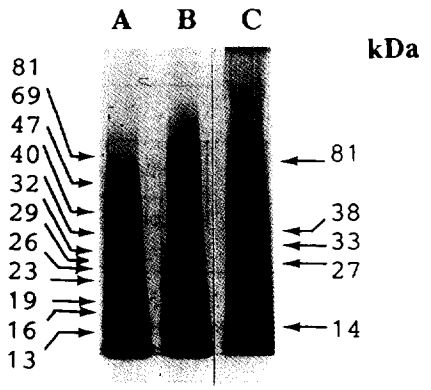

Fig. 2. SDS-PAGE analysis of kernel and shell extract in a 8–25% gradient gel. Protein bands were detected by silver staining. Lane A: shell extract (2.9 μg protein); lane B: standard proteins (phosphorylase b 94; albumin 67; ovalbumin 43; carbonic anhydrase 30; soybean trypsin inhibitor 20.1 and lactalbumin 14.4); lane C: kernel extract (2.4 μg protein).

Table 1 illustrates the results of the IgE-RIA. The IgE specific level varied from 42.8 to 52.6% for the RIA using solid-phase coupled to kernel proteins and from 3.3 to 4.4% when the solid-phase was coupled to shell proteins. The results were compared to Pharmacia peanut CAP (Table 1). RIA inhibition reached 91.8% with the kernel extract and only 21.7% with the shell extract.

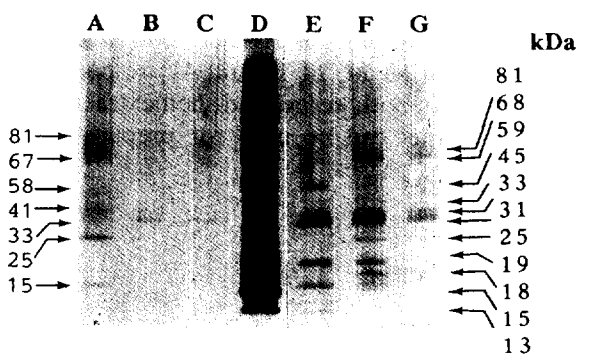

Fig. 3. SDS-PAGE analysis of kernel and shell fractions from RP-HPLC (see Fig. 1f). Electrophoresis was run in a 8–25% gradient and protein bands were detected by silver staining. Lane A, B, C correspond to the HPLC kernel fractions eluted with a retention time of 27, 26 and 25 min, respectively. Lane E, F, G correspond to the HPLC shell fractions eluted with a retention time of 25, 26 and 27 min. Estimation of M_r is indicated by arrows. Standard proteins (phosphorylase b 94; albumin 67; ovalbumin 43; carbonic anhydrase 30; soybean trypsin inhibitor 20.1 and lactalbumin 14.4) were run in lane D.

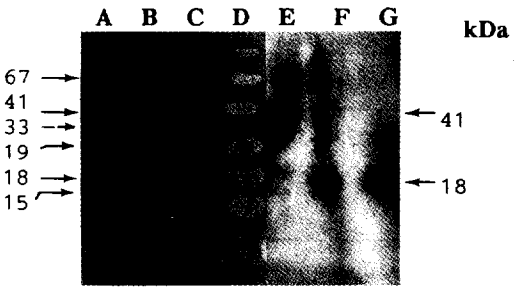

Fig. 4. Western blot analysis of kernel and shell fractions from HPLC. SDS-PAGE was performed as described in Fig. 3. Lane A, B, C correspond to the HPLC kernel fractions eluted with a retention time at 27, 26 and 25 min, respectively. Lane E, F, G correspond to the HPLC shell fractions eluted with a retention time at 25, 26 and 27 min. Estimation of M_r is indicated by arrows. Standard proteins (phosphorylase b 94; albumin 67; ovalbumin 43; carbonic anhydrase 30; soybean trypsin inhibitor 20.1 and lactalbumin 14.4) were run in lane D.

4. Discussion

In previous studies the physicochemical properties of the major peanut allergens have been studied. Yu [24] described an M_r of 23 and 44 for subunits of arachin and an M_r of 67 for conarachin from the seeds. Arachin and conarachin are two major storage proteins of peanut. Fish et al. [25] identified a peanut agglutinin (lectin) in kernel that had a M_r of 29. Meier-Davis et al. [26] identified three major allergenic proteins that had respective M_r of 15, 20 and 66. Sachs et al. [27] identified a peanut allergen in kernel extract known as peanut-1 which contained two major bands with an M_r of 20 and 30, respectively. Barnett et al. [28] described a protein that had an M_r of 65 and a pI of 4.6. Burks et al. [29,30] identified two major allergenic bands, Ara hI and Ara hII. Ara hI had an M_r of 63.5 and a pI of 4.55. Ara hII was smaller and less acidic with an M_r of 17 and a pI of 5.2. Recently, Folgert et al. [31] identified an allergen with an M_r of 14. In our study, we have identified, by HPLC and Western blotting, 6

Table 1
Radioimmunoassay of specific IgE of peanut allergic patients using peanut protein extracts coupled to epoxy-Sepharose as reactive solid phase

Patient	^{125}I-Anti-IgE uptake to							
	Sepharose-Shell proteins			Sepharose-Kernel proteins			Pharmacia Peanut CAP	
	cpm	Uptake (%)	Inhibition (%)	cpm	Uptake (%)	Inhibition[a] (%)	cpm	Uptake (%)
C.I.	1451	4.4	21.7	14 206	42.8	91.8	15 553	37.1
G.A.	1087	3.3	–	17 448	52.6	75.1	9824	23.4
Control	538 ± 97	1.6 ± 0.3	0	311	1.3	0	709 ± 29	1.6 ± 0.06

[a] Inhibition of IgE adsorption to the reactive solid phase was performed by incubation the serum (50 μl) previously with 10 μg of soluble allergenic protein extract.

allergens in peanut kernel protein extracts with M_r values of 15, 18, 28, 34, 41 and 67. Therefore, it seems that our kernel protein extract contained most of the allergens previously described. Western blotting of the HPLC eluate from shell and kernel extracts showed two major allergens with an M_r of 18 and 41, respectively. The number of allergens found in kernel extract was much higher than in shell extract, with specific allergens having an M_r of 15, 28, 34 and 67. The allergenic proteins from both extracts were hydrophobic as they were eluted at the end of the methanol gradient. Shell and kernel protein extracts were coupled to solid-phase Sepharose to prepare a solid reactive phase to detect serum specific IgE in peanut-allergic patients. Comparison between the specific IgE uptake by a solid phase coupled with kernel and shell extracts showed that the relative amount of allergenic proteins in kernel extract was about 10-fold higher than that in shell extract (Table 1). This confirmed the data obtained by Western blotting. Moreover, a higher percentage of specific IgE uptake was obtained with the Sepharose–kernel protein than with Pharmacia solid phase (Table 1). This could indicate that the Pharmacia allergen extract contained only part of the allergens found in the Western blot.

Unfortunately, we were not able to confirm this hypothesis as the Pharmacia allergen preparation is only available a solid phase and not as a soluble extract. The IgE-RIA performed with solid-phase Sepharose has a within-run reproducibility with coefficients of variation ranging from 3.2 to 10.0% in our experience [22]. It is similar to that of Pharmacia RAST [22]. Our peanut protein Sepharose gel can thus be used for routine analysis of specific IgE in serum.

5. Conclusions

We have described an efficient procedure for the extraction of peanut allergenic proteins by solvent extraction and HPLC. Two allergenic proteins with an M_r of 18 and 41, respectively, were found in both kernel and shell extracts. The protein extract can be used for preparation of solid reactive phases.

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