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The major allergen of sesame seeds (*Sesamum indicum*) is a 2S albumin

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Abstract

Background: Allergic reactions induced by ingestion of foods containing sesame seeds are a well recognized cause of severe food-induced anaphylaxis. *Objective*: This study aimed to identify and characterize the clinically most important major allergen of sesame seeds. *Methods*: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and IgE immunoblotting were performed on sera of 10 patients selected for severe and documented allergic reaction after eating food containing sesame. The major allergen was purified by gel filtration and characterized by isoelectric point (p*I*), glycosylation and amino acid sequencing. *Results*: All the patients had positive IgE antibodies and skin prick tests (SPTs) to sesame. The major, clinically most important allergen was a protein with molecular mass of about 9000. It was not glycosylated, the amino acid sequence showed it was a 2S albumin with a p*I* of 7.3; the small and the large subunits, forming the whole protein, showed p*I* values of 6.5 and 6.0.

Keywords: Food allergens; Sesame seeds; Albumin

1. Introduction

Sesame seeds (*Sesamum indicum*) have long been used in several food products, and sesame oil is used in salad dressing in Oriental, Chinese and South American cuisines. More recently, foods containing sesame (breads, crackers, chips, vegetable burgers, oriental specialities) have been introduced to Western countries, where the oil has also been employed in the pharmaceutical industry as a vehicle of medications for intramuscular injection [1]. Sesame oil is also used in cosmetics, and in rare cases it can cause allergic contact eczema [2].

Economically, *Sesamum indicum* is the most important species in the *Sesamum* genus of the *Pedialaceae* family. In recent years, the annual worldwide production of sesame has amounted to around $2 \cdot 10^6$ tons. The seeds contain about 50 to 60% oil, which is mainly used for cooking and salad

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dressing. Sesame seeds are an extremely potent food allergic source; in sensitized subjects they may produce severe allergic reactions. Although symptoms such as urticaria, Quincke's oedema and asthma have been reported, it is important to stress that the majority of the cases are of anaphylactic shock [1,3–5]. More than half of the 25 patients reported in the literature suffered from anaphylaxis due to ingestion of sesame seeds. Sesame has also been described as an occupational sensitizer for bakers and other exposed workers [6].

The aim of our study was to identify and characterize the major allergens of sesame seeds by using the sera of patients with history of severe allergic reaction to sesame and specific IgE antibodies.

2. Materials and methods

2.1. In vivo methods

2.1.1. Subjects

Ten patients referred to the De Marchi Pediatric Clinic of Milan, to the Allergy Center of the 3rd Division of Internal Medicine of the University of Milan and to the Bizzozzero Division of the Niguarda Cá Granda Hospital of Milan were included in this study, on the basis of severe documented reactions induced by the ingestion of sesame seeds or foods containing sesame seeds.

The population was submitted to the skin prick test (SPT) with crude sesame seeds by the Prick+Prick method [7] and with a commercial extract (Lopharma), and to serum sesame seed specific IgE detection by the RAST/CAP system. No provocation tests were performed because of the severity of the reactions reported in patient's histories.

Ten healthy patients with no history of allergic reactions were selected as negative controls.

Sera of patients were collected and stored at −30°C until use.

2.2. In vitro methods

This study aimed to: (1) identify the major allergens of sesame seeds by electrophoretic methods; (2) purify them by high-performance liquid chromatography (HPLC); (3) characterize them by amino acid sequencing and isoelectrofocusing.

2.2.1. Identification of allergens

2.2.1.1. Extraction of allergens from sesame seeds. The extract was prepared in our laboratory as follows: sesame seeds were crushed in a mixer and diluted (1:5, w/v) in 0.1 *M* phosphate-buffered saline (pH 7.4) for 2 h at 4–8°C [8]. The extract was clarified by centrifugation and the supernatant was stored at −20°C. The protein concentration of the extract was 8.14 mg/ml, in line with findings reported by Lowry et al. [9].

2.2.1.2. Sodium dodecyl sulfate–polacrylamide gel electrophoresis (SDS–PAGE) separation and immunoblotting. Electrophoresis was carried out essentially as described by Neville [10] in a discontinuous buffer system in an SDS–polyacrylamide gel with a 6% stacking gel and a 7.5–20% separation gradient gel. The extracts were diluted 1:2 in sample buffer, containing tris(hydroxymethyl)aminomethane (Tris) adjusted to pH 6.1 with concentrated sulfuric acid, 2% SDS, 3% 2-mercaptoethanol, 5% glycerol, and 0.001% Bromophenol blue, and heated to 100°C for 5 min. The samples (0.16 mg/cm gel) were run at 6 mA/gel for 16 h in a Bio-Rad Protein IIxi vertical electrophoresis Slab Cell (Bio-Rad Labs., Richmond, CA, USA).

The following polypeptides were used as low-molecular-mass markers (Pharmacia Biotech, Uppsala, Sweden): phosphorylase B (M_r 94 000), albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 100), α-lactalbumin (M_r 14 400).

The proteins were stained with a solution of Coomassie Brilliant Blue R-250 (Pharmacia-Upjohn, Uppsala, Sweden) and destained until the background was clear.

The separated proteins were electrophoretically transferred from the gel to a nitrocellulose membrane (pore size 0.45 μm; Amersham Pharmacia Biotech) using a Trans-Blot Cell from Bio-Rad at 0.45 A, 100 V, for 4 h at 4°C. The unoccupied protein binding sites in the nitrocellulose membrane were blocked by incubation in a solution of phosphate-buffered saline, pH 7.4 with 0.5% Tween 20 for 30 min at room

temperature. The nitrocellulose was then cut into strips, which were incubated overnight with each patient's serum diluted 1:4 in blocking solution. To reveal IgE binding to allergens, each strip was incubated for 6 h with ^{125}I-labeled anti-human IgE antiserum (Pharmacia) diluted 1:5 in blocking solution, followed by washing and exposure to X-ray film at −70°C for 5 days.

A protein of molecular mass about 9000 was identified as the absolute major allergen, as all the patients reacted to it; it was then purified.

2.2.2. Purification of major sesame allergen by HPLC

2.2.2.1. Gel filtration. The major sesame allergen was purified by gel-filtration chromatography: 200 μl of sample was separated on a Superdex 75 column, equilibrated and eluted with 1 *M* sodium chloride–0.035 *M* sodium phosphate buffer (pH 7.5), at a flow-rate of 0.4 ml/min. The absorbance was monitored at 280 nm. A calibration curve was prepared using proteins of known molecular masses: ribonuclease (M_r 13 700), chymotrypsinogen A (M_r 25 000), ovalbumin (M_r 43 000) and bovine serum albumin (M_r 67 000) (Pharmacia Biotech). The fractions corresponding to a protein of about M_r 9000 were pooled and concentrated by centrifugation with a Centricon 3 concentrator (Amicon, Beverly, MA, USA) with a an M_r cut-off of 3000; the resulting fraction was then analyzed by SDS–PAGE using the method described above. This fraction was tested by immunoblotting and found to have allergenic binding activity; it was thus decided to study it further.

2.2.3. Characterization of the purified major sesame allergen

2.2.3.1. Periodic Acid-Schiff (PAS) stain. To detect glycosylation of proteins, 15 μl of purified 9000, corresponding to a protein content of 9.1 μg/ml, was run on mini-gel, blotted onto a Problot membrane (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA) at 16 V constant for 60 min as described by Towbin and Gordon [11], except that methanol and SDS were omitted from the buffer. The membrane was then cut into two parts, a part was stained with Coomassie R-250 and the other with PAS-staining for glycoproteins. For PAS-staining, the membrane was fixed in 12% trichloroacetic acid for 1 h and tested with a 7% trichloroacetic acid solution containing 2% KIO_4; it was then maintained at 4°C overnight. The background was destained with methanol; glycoproteins appeared as purple bands. Milk whey proteins were used as controls.

2.2.3.2. Amino acid sequencing. Protein sequence analysis of the fraction obtained in gel filtration was done on a Perkin-Elmer Applied Biosystem 470A gas-phase sequencer equipped with a 120A phenylthiohydantoin-amino acid derivative analyzer. All chemicals were from Perkin-Elmer Applied Biosystems.

2.2.3.3. Isoelectrofocusing. The isoelectric point (p*I*) of the protein was determined by isoelectrofocusing on a Pharmacia-LKB Phast System Ready by using Phast Gel, with a pH gradient from 3 to 9. The gel was fixed and stained with Coomassie Brilliant Blue R-250 and run according to the manufacturer's instructions.

2.2.3.4. SDS–PAGE electrophoretic separation (8–25 gradient). A separation by SDS–PAGE was done on Phast Gel-Gradient 8–25 gels (Pharmacia-Upjohn) as described by Laemmli [12] to confirm the purity of the M_r 9000 protein purified by gel filtration.

The fraction was separated into three different bands; the sample was thus further processed.

2.2.3.5. Reduction and carboxamidomethylation. For sequence analysis the protein was modified by reduction in 6 *M* guanidinium chloride, 0.001 *M* EDTA, 0.1 *M* Tris–HCl, pH 8.3; 0.002 *M* dithiothreitol was added and the reduction proceeded under nitrogen, in the dark, at 37°C for 1 h.

S-Carboxamidomethylation was performed with 0.1 *M* iodoacetamide under the same conditions as for the reduction of the protein.

2.2.3.6. Reversed-phase chromatography. The reduced and carboxamidomethylated protein was purified by reversed-phase HPLC on a C_8 column (15 cm×4.6 mm, Vydac 208TP5415, Hesperia, CA,

USA), in 0.1% trifluoroacetic acid (TFA) and eluted with a linear gradient of 0–100%, 70% acetonitrile (ACN) in 0.1% TFA for 40 min at a flow-rate of 0.7 ml/min.

The chromatographic profile showed two large fractions, known as fraction 20.084 and fraction 21.929 for their elution time, plus other smaller fractions. Protein sequencing of fraction 20.084 was performed by the method described above. Fraction 21.929 presented a blocked NH_2-terminal sequence, thus we had to process it further by trypsin digestion.

2.2.3.7. Trypsin digestion of fraction 21.929. The second fraction of the reversed-phase HPLC C_8 separation was dried under nitrogen and dissolved in 170 μl of *N*-ethylmorpholine-acetate, pH 8.3; the reaction was performed with 2 μg of trypsin (Promega, Madison, WI, USA) in 15 μl of 50 m*M* acetic acid. Tryptic peptides were separated by reversed-phase HPLC on a C_{18} column (25 cm×4.6 mm, Vydac), in 0.1% TFA with a two-step linear gradient of 0–40% ACN over 1 h and 40–70% ACN for 10 min, at a flow-rate of 0.7 ml/min. Protein sequence analysis of fraction 2 was performed as described above.

3. Results

3.1. Subjects

The 10 patients (seven boys, aged 4–11 years, and three women, aged 23–36 years) in this study had severe systemic reactions to sesame seeds; in particular, four of them had urticaria–angioedema, three had laryngeal oedema, one had hypotension and gastrointestinal symptoms, one had severe asthma, and one anaphylactic shock. Almost all of them had very high levels of sesame specific IgE antibodies (Abs) and highly positive SPTs with both fresh seeds and commercial extracts. Table 1 lists the demographic data and the results of the ImmunoCAP assays for sesame on the sera of the 10 patients.

3.2. Identification of major allergens

SDS–PAGE of sesame proteins showed different components with apparent molecular masses ranging from ≅9000 to 83 000.

Fig. 1 gives the IgE immunoblot of sesame extract for each of the 10 patients, showing IgE binding to proteins with molecular mass ≅9000 (10 patients, 100%), 30 000 (eight patients, 80%), 14 400 (seven patients, 70%), 38 700 and 43 000 (six patients, 60%), 18 000 and 53 000 (five patients, 50%). Thus the M_r 9000 allergen was not only a major sesame seed allergen but also the most important one, since all the patients with systemic symptoms caused by ingestion of sesame seeds reacted to it.

3.3. Purification of M_r 9000 major sesame allergen by HPLC

Fig. 2A shows the chromatographic profile obtained from the gel-filtration column. The fractions corresponding to different runs of the M_r 9000

Table 1
Demographic characteristics and specific IgE antibody titers (in KU/l) to sesame of the 10 patients admitted to the in vitro part of the study

Patient	Sex	Age (years)	SPT	CAP system	Symptoms
1	M	7	++++	48.9	Glottis oedema
2	M	11	++++	68.7	Urticaria–angioedema
3	M	11	+++	8.0	Urticaria–angioedema
4	M	6	++	5.41	Urticaria–angioedema
5	M	4	++	0.749	Shock
6	F	23	+++	18.5	Glottis oedema
7	M	6	++++	>100	Asthma
8	M	9	++++	12.7	Urticaria–angioedema
9	F	36	+++	0.8	Glottis oedema
10	F	24	++	5.85	Gastrointestinal

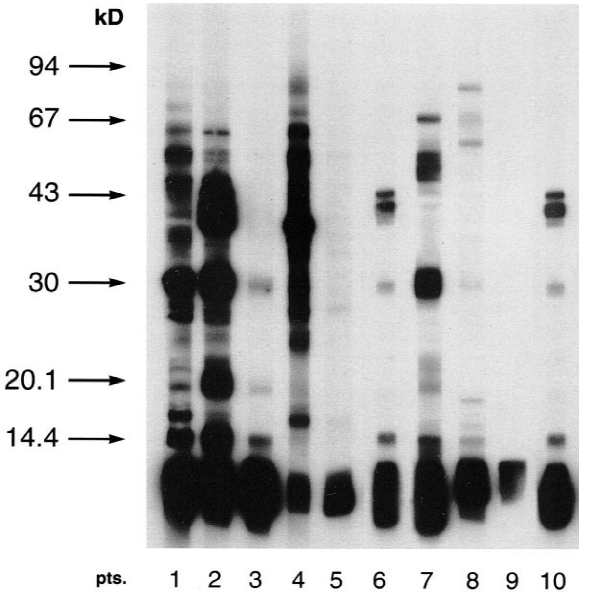

Fig. 1. IgE immunoblots of sesame extract with the sera from 10 sesame allergic patients. The M_r values of the markers are reported on the right.

protein were collected, pooled and concentrated to a final protein concentration of 0.85 mg/ml.

Fig. 2B shows the SDS–PAGE of total sesame seed extract and of the protein corresponding to the M_r 9000 band purified by HPLC gel filtration. The immunoblot of this fraction using the serum of patient No. 1 was highly positive.

3.4. Characterization of the purified major sesame allergen

3.4.1. PAS staining

PAS staining for glycosylation performed on the fraction after gel filtration ruled out any glycosylation.

3.4.2. Amino acid sequencing

The fraction after gel filtration gave a main N-terminal sequence: Pro–Ser–Gln–Gln–Asp corresponding to the small chain of castor bean 2S albumin (Accession No. P01089), and also some minor N-terminal sequences not identifiable.

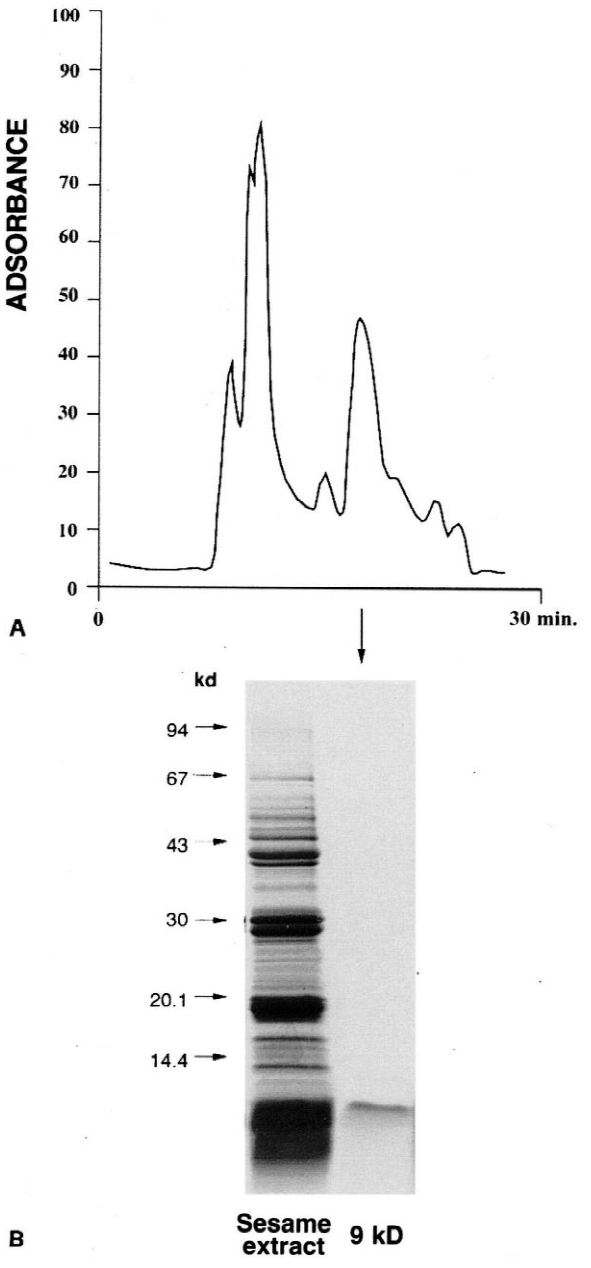

Fig. 2. Chromatographic profile of sesame seed extract separated on a Superdex 75 HPLC column by size-exclusion chromatography (top). SDS–PAGE of sesame extract (left lane) and of purified 2S albumin (right lane) stained with Coomassie Brilliant Blue (bottom).

3.4.3. SDS–PAGE separation (8–25 gradient gel)

This separation revealed three different bands, having the most relevant a molecular mass close to 9000 (data not shown).

3.4.4. Isoelectrofocusing

Isoelectrofocusing of the fraction collected after gel filtration gave three different p*I* values (7.3, 6.5 and 6.0), demonstrating the presence of three different proteins in this peak.

The band corresponding to p*I* value 7.3 was largely present; this result and N-terminal sequences obtained allowed to assume the presence of a unique protein partially degraded in two chains forming the whole protein.

3.4.5. Reversed-phase HPLC

Fig. 3 shows the chromatographic profile obtained from reversed-phase HPLC, on the C_8 column. We observed two main peaks with different elution times (20.084 min, fraction 1, and 21.929 min, fraction 2) and several smaller fractions.

3.4.6. Amino acid sequencing

The N-terminal sequence of fraction 20.084 was: Ala–Arg–Asp–Leu–Pro–Arg–Arg–Cys–Asn–Met–Arg–Pro–Gln–Gln–Cys–Ala–Phe, which corresponds to an internal fragment of the large chain of the 2S albumin of castor bean (Acc.-No. P01089).

Fraction 21.929 was blocked and had to be digested with trypsin before sequencing was possible.

Fig. 4 shows the chromatographic profile obtained from reversed-phase HPLC, on the C_{18} column, performed to separate the tryptic peptides. We observed two main peaks with different elution times (39.883 min, fraction 3; and 40.737 min, fraction 4) and several smaller fractions. Fraction 39.883 was blocked.

The N-terminal sequence of fraction 40.737 was:

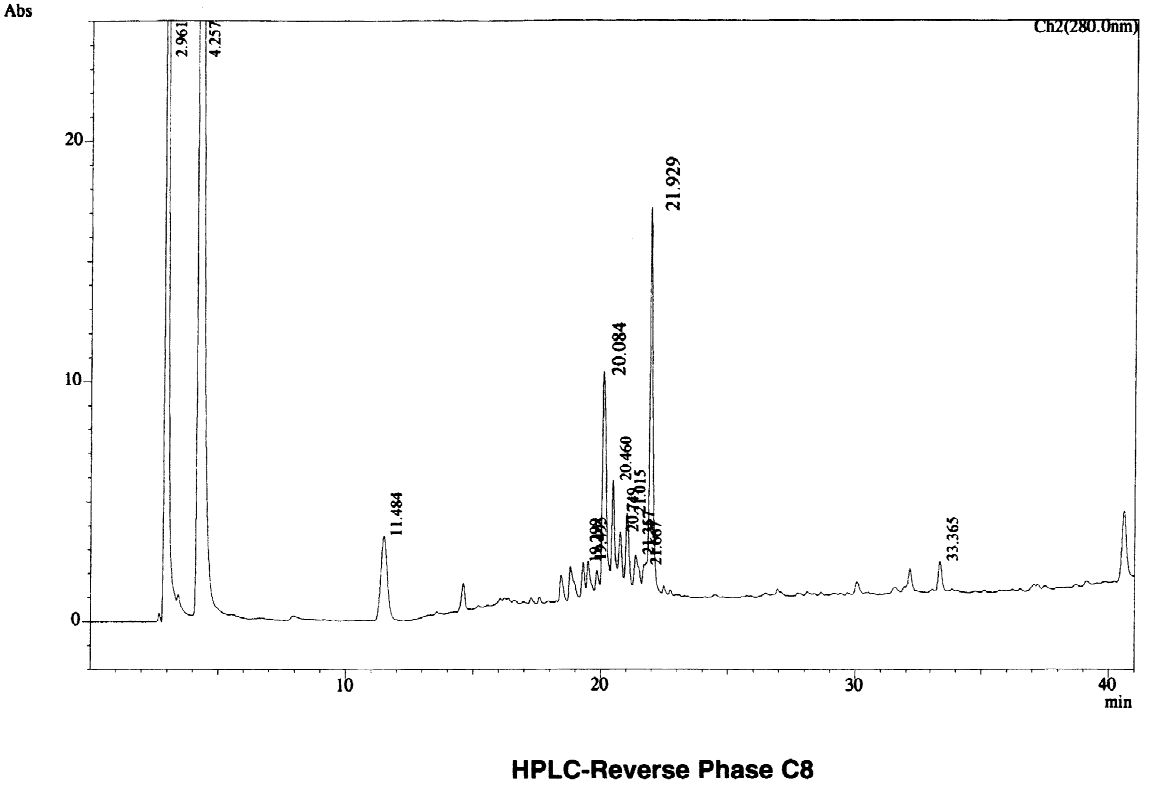

Fig. 3. Chromatographic profile of reversed-phase HPLC on a C_8 column of the purified 2S albumin.

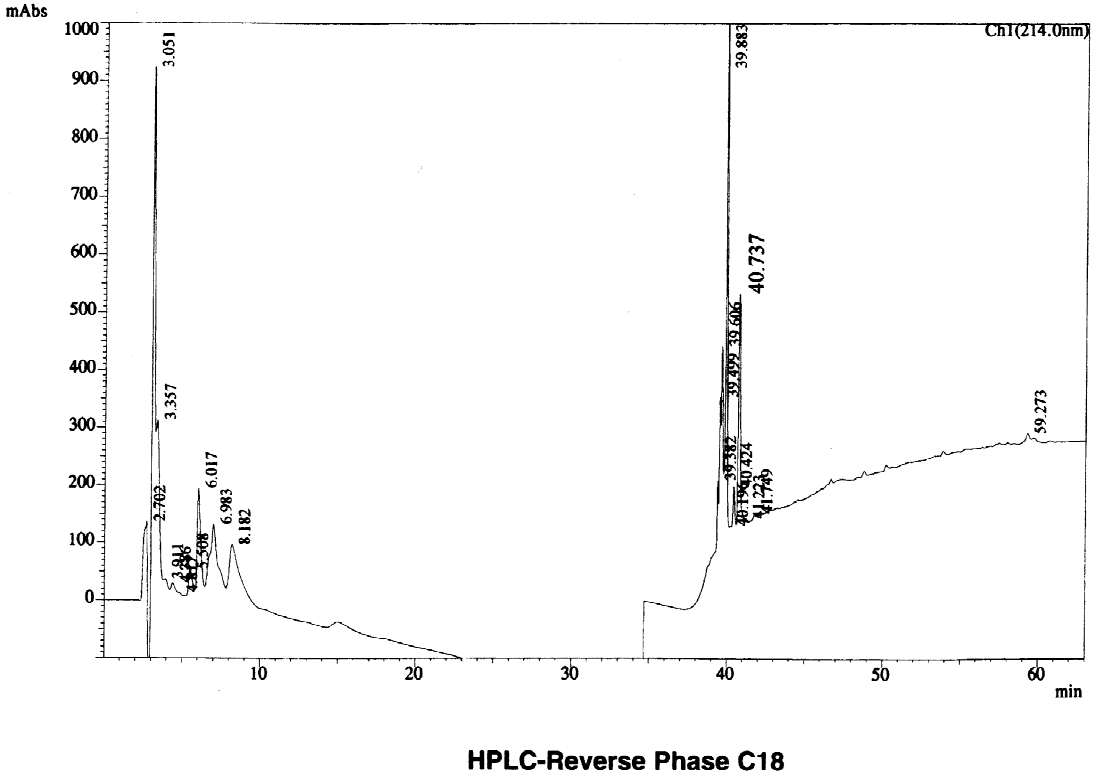

Fig. 4. Chromatographic profile of reversed-phase HPLC on a C_{18} column of the tryptic peptides obtained from the 21.929 peak of the C_8 column.

Cys–Asn–Met–Arg–Pro–Gln–Gln, which corresponds to an internal fragment of the large chain of the 2S albumin of castor bean and is also included in the sequence of fraction 1. It can thus be deduced that HPLC C_8 fraction 21.929 corresponds to the large chain, with the NH_2-terminal blocked due to the formation of pyroglutammic acid.

In conclusion, we identified in this sample an heterodimeric 2S albumin comprising a small chain, identified from its NH_2-terminal sequence, and a large chain, identified from an internal sequences, that had its NH_2-terminal blocked. This molecule, from research in the Swiss Prot data bank, was found to be homologous to the 2S albumins of Brazil nuts (similarity 87%, identity 47%, Acc.-No. P04403), castor beans (similarity 65%, identity 41%, Acc.-No. P01089) and sunflowers (similarity 93%, identity 43%, Acc.-No. P15461) as shown in Table 2; it is the most important major allergen of sesame seed responsible for allergic reactions to food.

4. Discussion

This study demonstrated that the major allergen of sesame seed, responsible for food allergic reactions, is an M_r 9000 2S albumin. All the patients selected for severe anaphylaxis after consumption of foods containing sesame reacted to this allergen, which may thus be considered the clinically most relevant. It should be noted that some previous research reports patients with sesame induced allergic reactions but negative RAST, suggesting the possibility of no-IgE mediated reactions. It might however be that the time elapsed between the last sesame assumption and IgE testing could have influenced the level of specific IgE, which may also become undetectable, as has been suggested by Kägi and Wüthrich [4] who described one of these cases. Otherwise a false negativity could be attributed to excessively violent handling of the sesame extract, capable of destroying its IgE binding activity. In our

Table 2
Amino acid sequences of the 2S albumins of sesame (large chain), Brazil nut, castor bean and sunflower

	1																17
Sesame	**A**	**R**	**D**	**L**	**P**	**R**	**R**	**C**	**N**	**M**	**R**	**P**	**Q**	**Q**	**C**	**A**	**F**
	123														137		
Brazil nut	**A**	E	N	I	**P**	s	R	**C**	N	L	s	**P**	M	R	**C**	–	–
(P04403)	242																258
Castor bean	**A**	a	N	**L**	**P**	s	m	**C**	G	V	s	**P**	t	E	**C**	r	F
(P01089)	277														290		
Sunflower	–	Q	N	**L**	**P**	N	Q	**C**	D	L	E	v	**Q**	**Q**	**C**	–	–
(P15461)																	

Referring to the sesame sequence, identical amino acids are written in bold underlined capital letters, similar amino acids are in capital letters and different amino acids are in small letters.

study almost all the patients had very high levels of sesame specific IgE antibodies and positive SPTs, both with fresh and commercial extracts, demonstrating this is a classical IgE-mediated food-induced reaction. By performing an immunoblotting experiment using an extract prepared following the method suggested by Vocks et al. [8] we identified the M_r 9000 allergen, which was then purified by gel filtration. This method was used since the molecular mass of the allergen and the seed nature of the source suggested it might be a 2S albumin: the majority of the seed allergens described to date, are 2S albumins. The most famous of them are the major allergens of the two types of mustard, Sin a 1 from yellow mustard *Sinapis alba* [13] and Bra j 1 from oriental mustard *Brassica juncea* [14], of Brazil nut Ber e 1 [15], of *Ricinus communis* Ric c 1 [16]. In several cases, gel filtration afforded very good purification of these proteins [13,15], which account for to 13% of the proteic content of proteins extracted with saline buffer [17] from the whole seed. This was achieved for Sin a 1 and Ber e 1, while for Ric c 1 a previous ion-exchange step was required. Bra j 1 obtained by gel filtration revealed several isoallergenic forms, which were separated by ion-exchange chromatography; to date these are the only case of different 2S molecules with the same IgE reactivity [14]. The analysis of the sequence regarding the determined residues of the large chain demonstrated it was a 2S albumin with about 40% homology to allergens of sunflower seeds, Brazil nut and castor bean [16]. The later two are known to be important allergens, as the major allergen of Brazil nut, Ber e 1, is related to food-induced anaphylactic reactions, while the major allergen of castor bean, Ric c 1, plays an important role in professionally exposed workers. 2S albumins have a typical structure, as they are heterodimeric proteins consisting of large and small subunits synthesized as a single precursor polypeptide. All the 2S albumins are compact globular proteins with conserved cysteine residues, which are responsible for the disulfur interchain bonds between the small and the large polypeptides forming the whole protein [18]. These 2S albumins are storage proteins diffused among different species and with high variability of amino acid sequences, which are contained in high amounts in plant seeds and serve to provide a store of amino acids for use during germination and seedling growth [18]. Because of their storage function, the 2S albumins contain sufficient sulfur in the form of methionine and nitrogen to support protein synthesis for the developing germling; this characteristic has been exploited to increase the methionine content of forage grasses [18] by genetic engineering. The other important feature of seed storage proteins is their antifungal activity, probably connected with protection of seeds before and during germination, which has been reported for proteins purified from the Brassicaceae family [19] and from radish seeds [20], whose growth inhibition activity is mainly directed against plant pathogenic fungi. On the basis of their aminoacidic sequences, the 2S proteins were identified as belonging to the very important and widespread family of cereal trypsin/α-amylase inhibitors. Proteins belonging to this family are the most important allergens of wheat and other cereals such as rice [21], especially giving the so-called ‘‘baker’s asthma’’ and also food allergic reactions [22].

The 2S albumins do not present a high level of

cross-reactivity, as in general their sequences are not very similar, but more extensive studies should be addressed to this topic, as few data are available at present. The 2S albumin of sesame seeds has never been described before either as a component or as an allergen; thus this study is the first demonstration that it is present in sesame seeds, where it is the most important allergen provoking food-induced anaphylactic reactions.

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