

Isolation and identification of an 11S globulin as a new major allergen in mustard seeds

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Background: Although mustard seed allergy has been largely reported during the preceding 20 years, currently only 2 allergens, Sin a 1 and Bra j 1, have been identified.

Objective: To improve the characterization of the allergenic profile of yellow mustard seeds by reporting the identification and biochemical characterization of an 11S globulin as a new major allergen.

Methods: Mustard seed proteins were separated using size exclusion and ion-exchange chromatographic columns, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and 2-dimensional polyacrylamide gel electrophoresis. Separation of different polypeptide chains was achieved by reverse-phase high-performance liquid chromatography. Mass spectrometry after tryptic digestion and Edman degradation were used to determine amino acid sequences of peptides. IgE binding assays were performed with 13 serum samples from mustard allergic patients in immunoblotting and enzyme-linked immunosorbent inhibition assays.

Results: A protein of 51 kDa was recognized as a major allergen by patients allergic to mustard and called Sin a 2. The allergen was dissociated in 2 chains of 36 and 23 kDa, which also bound IgE. N-terminal end and internal amino acid sequences allowed identification of the new allergen as a seed storage 11S globulin belonging to the Cupin super family. Purified allergen was able to inhibit the IgE binding of sera from allergic patients to mustard seeds extract in up to 55% of the responses.

Conclusions: An 11S globulin storage protein has been isolated and identified as a novel major allergen of mustard seeds.

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INTRODUCTION

Mustard allergy has been largely reported for the past 20 years.^{1,2} Among allergenic spices, mustard is one of the most significant in terms of allergenic potency and widespread use and elicits a considerable number of allergic reactions.^{3–6} In addition, mustard allergy has several features that have aroused interest in this spice as a cause of food allergy reactions: (1) its severity often involves systemic reactions such as anaphylaxis^{1–8}; (2) mustard allergy has been associated with hypersensitivity of other plant-derived foods,³ such as nuts, legumes, or Rosaceae fruits; and (3) mustard is frequently consumed as a hidden or masked food,^{1,7} which makes avoidance measures difficult. Mustard is extensively consumed as a condiment in homemade meals and frequently presents as a concealed additive in processed foods, such as fish and meat sauces, salad dressings, or flavoring powders, which increases the risk of adverse reactions.^{1,7,8} Thus, patients allergic to mustard are at high risk of experiencing life-threatening reactions.^{1–6}

Table mustard is made from varied mixtures of crushed seeds from different species according to the country of origin or the manufacturer. Yellow (*Sinapis alba*) and black (*Brassica nigra*) mustards are the most common mustards used in Europe, whereas the oriental mustard (*Brassica juncea*) flour is the most abundant in the United States and Japan. All these seeds are members of the Brassicaceae family, which includes *Brassica napus*, *Brassica rapa*, *Brassica napa*, *Brassica oleracea*, *Raphanus sativus*, and *Raphanus raphanistrum*, many of which have been reported to be allergenic.^{9,10} Currently, only 2 allergens, Sin a 1, which is from *Sinapis alba* (yellow mustard), and Bra j 1, which is from *Brassica juncea* (oriental mustard), have been reported for mustard seeds.^{11,12} These allergens are storage proteins and belong to the 2S albumin family, which constitutes abundant components in dicotyledonous seeds.

Identification and characterization of new major allergens have been key points in the advancement of food allergy for the last decades, since they provide a basis for producing well-characterized food allergen extracts, quantifying its major allergens contents, and understanding possible cross-reactivities at the molecular level. This will result in an increase of sensitivity for diagnostic purposes, for both skin testing and in vitro testing, as well as for the detection of hidden food allergens in processed food or meals. The aim of the study is to improve the characterization of the allergenic profile of yellow mustard seeds by reporting the identification and biochemical characterization of an 11S globulin as a new major allergen.

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Table 1. Clinical Characteristics of Patients With Mustard Allergy

Patient No./sex/age, y	Mustard allergy			Pollen allergy	
	Symptoms	Skin test wheal diameter, mm	Specific IgE*	Pollen allergy	Respiratory symptoms
1/F/45	OAS	10 × 8	0.267	Yes	R
2/M/24	Anaphylaxis	22 × 20	0.934	Yes	R, A
3/F/23	OAS	14 × 9	0.200	Yes	R
4/M/41	OAS, GIS	18 × 11	3.500	Yes	R, A
5/F/16	Anaphylaxis	11 × 6	0.228	Yes	R
6/F/35	OAS, U	7 × 5	0.258	Yes	R
7/M/25	OAS	9 × 9	1.196	Yes	R
8/F/53	OAS, U	11 × 11	0.799	Yes	R, A
9/M/24	OAS	21 × 19	0.267	Yes	R
10/F/46	Anaphylaxis	18 × 15	3.500	No	
11/M/34	OAS	31 × 11	3.500	Yes	R, A
12/F/28	OAS, AE, GIS	11 × 8	0.238	Yes	R, A
13/F/37	OAS, AE	12 × 6	0.157	Yes	R, A

Abbreviations: A, asthma; AE, angioedema; GIS, gastrointestinal symptoms; OAS, oral allergy syndrome; R, rhinitis; U, urticaria.

* Determined by enzyme-linked immunosorbent assay as absorbance at 492 nm.

METHODS

Patient Serum Samples

Serum samples were obtained from patients with mustard allergy (Table 1) in the Allergy Department of Fundación Jiménez Díaz (Madrid, Spain) and stored in aliquots at -70°C . Serum samples from individuals allergic to olive pollen were used as controls.

Yellow Mustard Seed Extract and Sin a 1 Purification

Mustard powder obtained from seeds of *Sinapis alba* L was suspended in 0.15M sodium borate buffer, pH 8, at a 10% (wt/vol) ratio and gently stirred for 1 hour and centrifuged at 4°C . The pellet was extracted twice with borate buffer, and the supernatants were lyophilized. The resulting material was then extracted 3 times with 10% (wt/vol) acetone. The pellets were air desiccated and dissolved in 0.15M ammonium bicarbonate, pH 8. After centrifugation at 8,700g, at 4°C , supernatants were lyophilized and stored at -20°C . This starting material was used to purify Sin a 1, which was performed as previously described.¹¹

The protein extract was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in 15% (wt/vol) polyacrylamide gels under nonreducing conditions. Proteins were visualized by Coomassie blue staining, and the molecular mass of protein bands was estimated by comparison with those of standard protein markers (MW-SDS-70L) from Sigma-Aldrich (St Louis, MO). The protein concentration was determined using the method of Lowry et al,¹³ with bovine serum albumin (BSA) as the standard.

IgE Immunoblotting

Protein samples were separated by 15% SDS-PAGE under nonreducing conditions and blotted onto nitrocellulose membranes. After blocking with 3% (wt/vol) nonfat milk and 0.1% (vol/vol) Tween 20 in phosphate-buffered saline, pH 7.4, the membranes were incubated with patients' sera (di-

luted 1:10) at room temperature for 2 hours. Bound IgE antibodies were detected by incubating with mouse anti-human IgE antibodies (diluted 1:5,000) and then with a horseradish peroxidase–labeled goat anti-mouse IgG anti-serum (diluted 1:5,000; Pierce, Rockford, IL). The peroxidase reaction was developed using the enhanced chemiluminescence Western blotting reagent (Amersham Biosciences, Barcelona, Spain).

Two-dimensional Analysis

Protein samples were separated according to their isoelectric point (pI) by using the protean isoelectrofocusing (IEF) system (BioRad, Hercules, CA) following the manufacturer's protocol, which uses dithiothreitol as the reducing agent. Standard proteins from Bio-Rad (IEF standards; pI range, 3.6–9.3) were used to calibrate the strips. After IEF, the proteins were separated by SDS-PAGE in 15% polyacrylamide gels. Proteins were detected by Coomassie blue staining or transferred to membranes and immunostained with a pool of sera from patients allergic to mustard seeds, as described herein.

Purification of IgE-Reactive Protein and Separation of Subunits

Lyophilized seed extract was dissolved in 0.15M ammonium bicarbonate buffer, pH 8.0, and applied onto a Sephadex G-50 column. Fractions that contained protein bands of approximately 50 kDa were pooled and resolved by ion-exchange chromatography on a SP-Sephadex C-25 column equilibrated in 10mM phosphate buffer, pH 7.0. Proteins were eluted with a gradient from 10 to 100mM phosphate buffer, pH 7.0. Chemical reduction of the protein was performed with 5% (vol/vol) 2-mercaptoethanol and alkylation with iodoacetamide. The carboxyamidomethylated chains were separated on a reverse-phase high-performance liquid chromatographic (RP-HPLC) nucleosil C-18 column with a

gradient (20%–60%) of acetonitrile in 0.1% trifluoroacetic acid. The N-terminal amino acid sequence was determined by Edman degradation using a sequencer (model 477A, Applied Biosystems, Foster City, CA).

Protein Digestion and Mass Spectrometry Analyses

Bands of interest were manually excised from gels, alkylated, and digested with trypsin according to Sechi and Chait.¹⁴ Bands were shrunk with 100% acetonitrile and dried. Then the samples were reduced with 10mM dithiothreitol and alkylated with iodoacetamide. Finally, samples were digested with sequencing-grade trypsin (Roche Molecular Biochemicals, Indianapolis, IN) in 25mM ammonium bicarbonate (pH 8.5). Matrix-assisted laser-desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) analyses were performed in a Voyager-DESTR instrument (PerSeptive Biosystems, Framingham, MA). All mass spectra were calibrated externally using a standard peptide mixture (Sigma-Aldrich). The MS/MS sequencing analyses were performed using the MALDI-tandem-TOF MS spectrometer 4700 Proteomics Analyzer (Applied Biosystems).

Enzyme-Linked Immunosorbent Assay Inhibition

Experiments

Enzyme-linked immunosorbent assay (ELISA) plates were coated with 50 µg/mL of mustard seed extract and incubated with patients' sera (1:10 diluted) previously adsorbed with 5 µg of purified antigens, 50 µg of mustard seed extract, or 5 µg of BSA for control purposes. Binding of human IgE was detected with mouse anti-human IgE antibodies (1:5,000 diluted) and afterward with horseradish peroxidase-labeled goat anti-mouse IgG antiserum (1:5,000 diluted). Peroxidase reaction was developed using fresh enzyme substrate and measuring the absorbance (OD) at 492 nm. Each OD value was calculated as the mean of 2 determinations (SD was <5%). The percentage of inhibition was calculated as $(OD_{BSA} - OD_{inh})/OD_{BSA} \times 100$, where OD_{BSA} and OD_{inh} indicate the OD values after preincubation with BSA and the inhibitors, respectively.

RESULTS

Detection of a New Major Allergen in Yellow Mustard Seed Extract

The allergenic profile of yellow mustard seeds was obtained by separation in SDS-PAGE under nonreducing conditions, blotting onto nitrocellulose membranes, and immunostaining using 13 serum samples from patients allergic to mustard (Fig 1). The Coomassie blue staining of the whole extract is also displayed in Figure 1. The IgE-binding pattern is composed of allergens with the following molecular masses: (1) 2 main components at 14 and 51 kDa, (2) several slight bands between 25 and 46 kDa, and (3) a high-molecular-weight protein cluster of 85 to 110 kDa. The protein of 14 kDa could correspond to the major allergen from yellow mustard seeds previously described, Sin a 1.¹¹ Interestingly, 12 of the 13

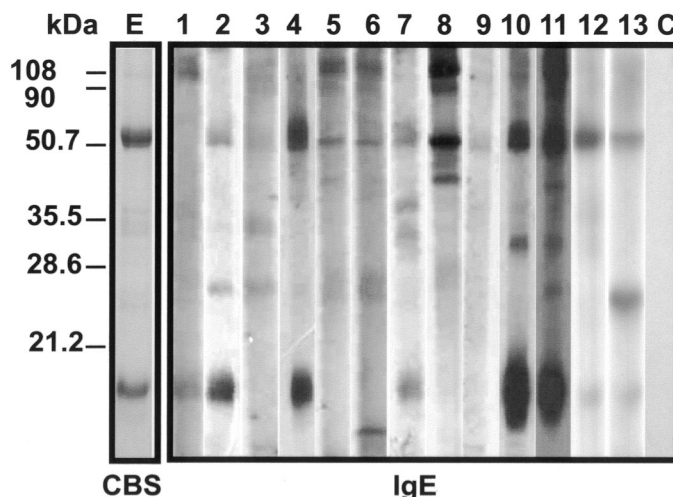


Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis for yellow mustard seed extracted proteins. Left, Coomassie blue staining (CBS) for total protein content. Right, IgE reactivity profile of sera from patients allergic to mustard (lanes 1 to 13) to blotted protein extract; the reactivity of the serum from a patient allergic to olive pollen is included (lane C). Molecular mass markers are indicated on the left margin.

tested patients gave a positive response to an allergenic band of 51 kDa.

Two-dimensional Analysis and IgE Immunoblotting

A fraction enriched in high-molecular-weight proteins of mustard extract was obtained from the size-exclusion chromatography on a Sephadex G-50 column. The SDS-PAGE analysis of this fraction under nonreducing conditions is shown in Figure 2A. This sample was used for 2-dimensional separation, which combines IEF and PAGE-SDS under reducing conditions. Figure 2B shows the Coomassie blue staining of the resolved components. Although a single protein band of 51 kDa was detected in the monodimensional SDS-PAGE (Fig 2A), protein bands with molecular masses higher than 36 kDa were not detected in the 2-dimensional gel. This result suggested that the protein of 51 kDa would consist of different subunits linked by disulfide bonds, which were separated under reducing conditions. Figure 2C shows the immunostaining of the 2-dimensional transferred gel with a pool of sera allergic to mustard (patients 2, 4, 10, and 11 from Fig 1). A main basic component appeared at 36 kDa, and it is accompanied by another less IgE-reactive spot at 23 kDa. In addition, 2 acidic (pI 3.5–4.6) IgE-reactive proteins were stained in the range of 50 to 66 kDa, which were not detected by Coomassie blue staining.

IgE-Reactive Protein of 51 kDa

To remove the acidic IgE-reactive proteins, the sample obtained after the Sephadex G-50 was applied on an SP-Sephadex C-25 ion-exchange column. Figure 3A shows the SDS-PAGE and Coomassie blue staining of the retained protein in the absence and presence of 2-mercaptoethanol. In the first

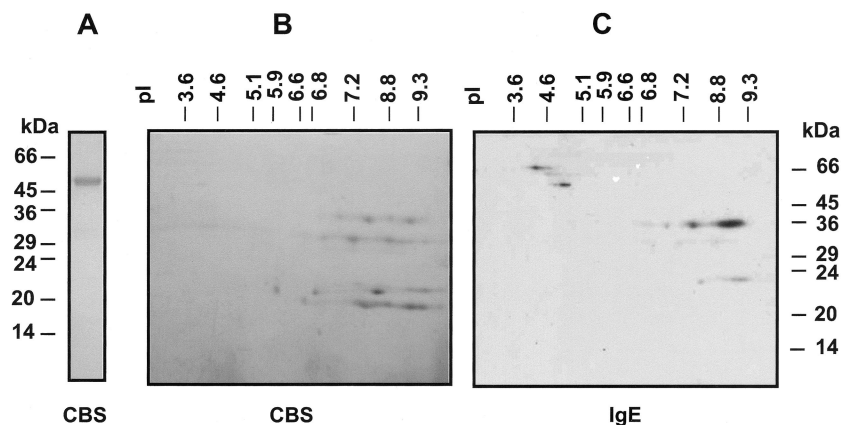


Figure 2. Proteomic analysis of fractions enriched in the new allergen. A, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining (CBS) of the sample eluted from Sephadex G-50 column. B, Two-dimensional separation by isoelectrofocusing and SDS-PAGE and CBS. C, Immunostaining of the sample in B with a pool of sera from representative patients allergic to mustard (IgE). Molecular mass and isoelectric point (pI) markers are indicated.

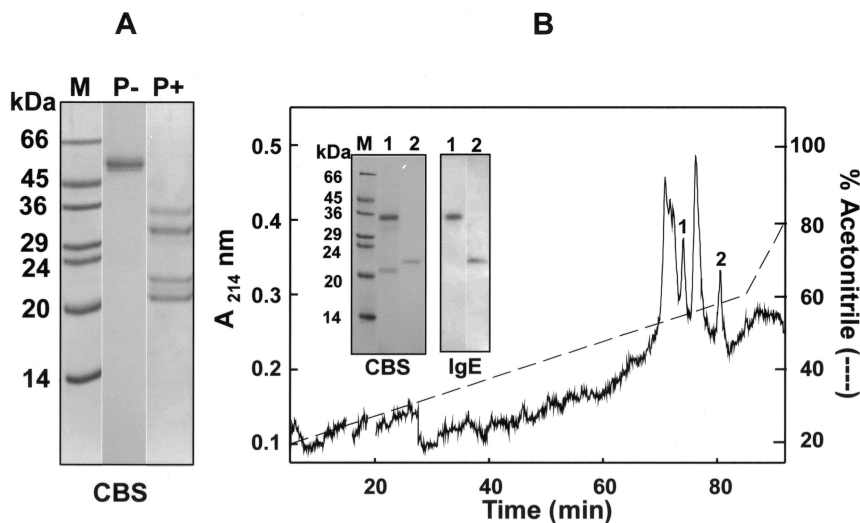


Figure 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis and purification of the chains from Sin a 2. A, SDS-PAGE and Coomassie blue staining (CBS) of purified Sin a 2 treated (lane P+) or untreated (lane P-) with 2-mercaptoethanol. B, Reverse-phase high-performance liquid chromatographic elution profile of the reduced and alkylated protein bands; inset, SDS-PAGE and CBS or immunostaining (IgE) with a pool of sera from mustard allergic patients of the proteins contained in the numbered peaks. A_{214} indicates absorbance at 214 nm; M, molecular mass markers.

case, a protein band of 51 kDa was observed; however, in the presence of the reducing agent, 4 protein bands of 36, 34, 23, and 20 kDa were detected. These protein bands were separated by means of a RP-HPLC column after reduction and alkylation of the sample (Fig 3B). The proteins obtained in peaks 1 (36 kDa) and 2 (23 kDa) became allergenic (Fig 3B, inset). Edman degradation of the N-terminal end of the 36-kDa protein band gave the sequence RQSLGVPPQ (Table 2), which was identical to residues 24 through 32 of 11S legumin-type globulin from *B napus* (GeneBank Database accession No. CAA44042). No sequence was obtained from the

Table 2. Amino Acid Sequences of Peptides From Sin a 2

Sequence and position in 11S globulin from <i>Brassica napus</i>	Method	Molecular weight, Da	Chain, kDa
¹ RQSLGVPPQ ⁹	ED		36
²⁷⁸ GPFQVVRPPLR ²⁹⁸	MS/MS	1,265	36
²⁷⁶ VKGPFQVVRPPLR ²⁹⁸	MS/MS	1,492	36
³⁵¹ TSVNSYTLPIQYIR ³⁶⁵	MS/MS	1,867	20
⁴⁶³ ALPLEVITNAFQISLEEAR ³⁸¹	MS/MS	2,130	20

Abbreviations: ED, Edman degradation; MS/MS, mass spectrometry/mass spectrometry.

23-kDa protein band, which indicates that the first amino acid is blocked.

Internal sequences of the 2 IgE-reactive polypeptides of 36 and 23 kDa were determined by digestion with trypsin of the bands excised from the gel. The peptides were analyzed by MALDI-TOF-MS. Thereafter, MS/MS analysis of the 2 major peptides selected from each MS profile was performed. All the obtained sequences (Table 2) were aligned with segments of the large or the small subunit of the 11S globulin from *B napus*. These results confirm that the new allergen detected in yellow mustard seeds is an 11S legumin-type globulin and was called *Sin a 2*.

Immunologic Characterization

The IgE response to *Sin a 2* was analyzed for 13 serum samples from mustard allergic patients in immunoblotting (Fig 4). Nine (69%) of 13 serum samples presented specific IgE antibodies to the allergen. To quantify the contribution of the 11S globulin to the allergenicity of the whole mustard extract, ELISA inhibition experiments were performed with 4 individual serum samples from the patients allergic to mustard seeds (samples 2, 4, 10, and 11 from Figs 1 and 4). Either *Sin a 1* or *Sin a 2* or an equimolar mixture of both purified allergens and the whole extract were used as inhibitors. The IgE binding to mustard seed extract was notably reduced after preincubation of the 4 serum samples with purified *Sin a 1* (66.5% average inhibition) (Table 3). When the purified 11S globulin was used as the inhibitor, values ranging from 19% to 55% (average, 36.5%) were reached, and when the sera were preincubated with the mixture of both allergens, the IgE binding to the whole extract was nearly abolished, since the inhibition was higher than 90% for all patients, with an average of 94.5%.

DISCUSSION

The results of this study provide, for the first time to our knowledge, identification and physicochemical characterization of a new major mustard allergen (*Sin a 2*), an 11S globulin storage protein. This study was mainly based on 2 key points: (1) the knowledge that 11S globulins are involved as major allergens in mustard allergy and (2) the characterization of mustard allergenic components for the improvement of food extracts standardization.

Mustard flour has been known to be allergenic for many decades^{1,2}; however, little is known about the allergen profile

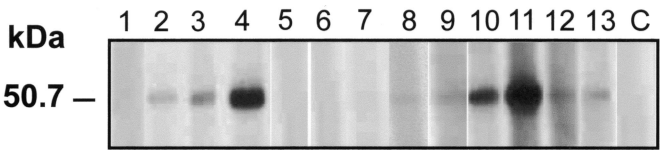


Figure 4. Analysis of the IgE binding of purified *Sin a 2*. Immunodetection of nitrocellulose-blotted *Sin a 2* with 13 individual serum samples (lanes 1 to 13) from patients allergic to mustard; the serum of a patient allergic to olive pollen was used as the negative control (lane C); molecular mass of the protein is indicated.

Table 3. Inhibition of IgE Binding to Yellow Mustard Seed Extract When Patients' Serum Samples Are Preincubated With the Indicated Inhibitors

Patient No.	Inhibition, mean $\pm$ SD, %			
	<i>Sin a 1</i> (2S albumin)	<i>Sin a 2</i> (11S globulin)	<i>Sin a 1</i> and <i>Sin a 2</i>	Mustard extract
2	74 $\pm$ 1	49 $\pm$ 1	93 $\pm$ 1	97 $\pm$ 1
4	55 $\pm$ 2	55 $\pm$ 2	97 $\pm$ 1	99 $\pm$ 1
10	81 $\pm$ 1	19 $\pm$ 1	98 $\pm$ 1	99 $\pm$ 1
11	56 $\pm$ 1	23 $\pm$ 1	90 $\pm$ 1	99 $\pm$ 1

of mustard seeds, and only 2S albumins have been identified as allergenic proteins.^{11,12} Storage proteins from plants were classified as globulins (11S and 7S), albumins (2S), and prolamins, the latter 2 belonging to the prolamin superfamily.¹⁵ The identification of an 11S globulin as a major allergen of mustard seeds indicates that the 2 families of storage proteins (2S albumins and 11S globulins) of these seeds are involved in allergy. In addition, 2S albumins and 11S globulins exhibit some common features: stability, presence in high amount in the seeds, function as storage materials, and frequently antifungal activity. However, these proteins display different solubility character and amino acid compositions, because the 2S albumins are water-soluble and frequently sulfur-enriched proteins.

The 11S globulins, also frequently named legumins and classified into the Cupin superfamily, are composed of 2 polypeptide chains of different molecular masses and amino acid sequences (heterodimeric form composed of a 30- to 40-kDa chain plus a 20- to 25-kDa chain), which are linked together by one disulfide bridge.¹⁶ These subunits are frequently codified by multigene families and came from a single polypeptide precursor that is cleaved during the biosynthesis of the protein in the seeds. The N-terminal portion of the immature precursor corresponds to the large subunit, and the C-terminal segment constitutes the small chain.¹⁶ In the present study, we identified the heterodimeric form (51 kDa) of the 11S globulin of mustard seeds as an IgE-reactive protein in nonreducing conditions. In addition, the 2 subunits (36 and 23 kDa) were dissociated by the reduction treatment of the 51-kDa protein and separated by both 2-dimensional techniques and RP-HPLC. These results agree with the presence of disulfide bonds that connect the 2 subunits. The immunologic staining of the sample separated by the 2-dimensional electrophoretic system with serum samples from mustard allergic patients indicated that both subunits were able to bind the IgE from the sera.

Ara h 3, G2 glycinin, and Cor a 9, which belong to this family of proteins, have been described as major allergens in seeds from peanut, soybean, and hazelnut, respectively.¹⁷⁻¹⁹ These allergens were also resolved in 2 components: the N-terminal large chain and the C-terminal small chain, and most of their IgE-binding sites were found to be located at the

large subunit.^{18–20} Whether the intensity of the IgE-stained spots of the 2 subunits of Sin a 2 (Fig 2) is related to the presence of more IgE epitopes in the large chain than in the small one remains to be investigated. Other 11S globulins have been implicated in food allergy, such as those from Brazil nut (Ber e 2), cashew nut (Ana o 2), sesame, coconut, walnut, and almond,^{21–25} suggesting an intrinsic allergenicity to this family of proteins.

Results of this study indicate the contribution of Sin a 2 to the potency of whole mustard extracts, since the allergen was able to inhibit the IgE binding of the sera to the whole extract in a range of 19% to 55% (Table 3). In addition, the mixture of Sin a 1 and Sin a 2 allergens blocked at least 90% of whole mustard specific IgE.

Most patients had oral allergy syndrome; however, systemic symptoms and anaphylaxis were reported in up to 53% and 23% of the patients, respectively. A higher frequency of systemic reactions to vegetable-derived foods has been described among non-pollen allergic patients in the central area of Spain.^{26–27} Curiously, most patients evaluated in this study were pollen allergic patients. We believe that mustard and pollen allergy are concomitant problems in atopic patients, since Sin a 1 and Sin a 2 are protein components specifically expressed in seeds and could not be related to pollen-inducing allergies.

In summary, we describe a new allergenic protein (Sin a 2) in mustard flour, identified as an 11S globulin, which is involved in mustard allergy as a major allergen. In addition to the improvement of characterization of mustard allergen extracts, these findings open a path for future investigations to evaluate the relationships with other 11S globulins reported as allergens in other plant-derived foods at the molecular, immunologic, and clinical levels.

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