

Purification and characterization of an allergen of mustard seed

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A protein allergen has been isolated from mustard seed extracts by gel filtration. In RAST-inhibition experiments the allergen inhibited more than 60% of the binding of mustard-specific IgE from a pool of seven patients' sera. The allergenic activity was also demonstrated by histamine release from whole blood from two sensitive patients. The allergen had an isoelectric point close to 10.5 and an apparent molecular weight of 14,000. It was stable in alkaline and acid solutions and showed resistance to heating and to degradation by proteolytic enzymes.

INTRODUCTION

Spices and aromatic plants are responsible for a small percentage of food hypersensitivity in atopic patients.¹⁻⁴ The most frequently involved spices are caraway, coriander, paprika, cayenne, and mustard. They usually produce local reactions such as itching and smarting of the oral cavity; however, anaphylactic shock after ingestion of mustard and occupational asthma due to inhalation of powdered spices have also been reported.^{5,6}

Patients allergic to vegetable foods are often sensitive to pollens and to other vegetables.⁷⁻¹¹ A high correlation between hypersensitivity to spices and to tree pollens, fruits and vegetables has been found in several studies.^{2,12,13} Two hypotheses have been proposed to explain these correlations between botanically unrelated species: the presence of common antigenic determinants in these foods and the involvement of lectins with IgE-binding properties.^{7,11}

In this article, we describe the characterization of a highly allergenic protein from mustard seed.

MATERIAL AND METHODS

Sera

Sera were obtained from seven patients with clinical hypersensitivity to mustard. They suffered angioedema, urticaria, or anaphylactic shock after the ingestion of mustard. Sera from these individuals were stored at -20°C in aliquots. Sera from patients with hypersensitivity to pollens, fungi, and foods but without clinical symptoms after the ingestion of mustard were also aliquoted and frozen at -20°C .

Antigens

Mustard powder and paprika were obtained in a local food store. Commercially available caraway, coriander, celery, and black pepper seeds were ground in a domestic mill. The powders were defatted three times in acetone and then extracted in 10% wt/vol of 0.15 M borate buffer pH 8 for one hour at room temperature. After centrifugation at 2,000 g for 15 minutes the supernatants were filtered and frozen in aliquots at -20°C .

REIA

This assay was performed as previously described.¹⁴ Briefly, the coating of a microplate with monospecific anti-human IgE (Tago Lab) permits isolation of the IgE from the sera, whose specificities are revealed using a peroxidase-labeled allergen. Basically, the method has five steps: (1) coating of microtiter plates with a monospecific anti-human IgE, (2)

incubation with sera, (3) incubation with peroxidase-labeled allergen, (4) incubation with substrate; and (5) stopping the reaction and reading at 492 nm.

Labeling Procedure

Horseradish peroxidase was activated using the periodate oxidation method of Nakane¹⁵ as described previously.¹⁴ Once oxidized, 1 mg of peroxidase in 0.1 mL was mixed with 0.5 mL of the allergenic extract (1 mg/mL) and with 0.1 mL of carbonate-bicarbonate buffer (1 M, pH 9.5). After overnight incubation at 4°C , 1 mg of sodium borohydride was added and the mixture dialyzed against phosphate buffered saline (PBS) containing 25% glycerol. Then, 5 mL of 1% bovine serum albumin (BSA) in PBS-0.01% sodium merthiolate were added to stabilize the conjugate, which was aliquoted and stored frozen.

REIA Inhibition Assay

To test the cross-reactivity between different antigens, a previous determined concentration of allergen conjugate was mixed with different amounts of these antigens in 0.2 mL of the incubation buffer and added to the wells in step c following the assay as above described.

RAST Inhibition Assay

RAST inhibition was carried out using commercial paper discs (Pharmacia). Fifty microliters of a pool of the seven patients' sera were mixed with 50 μL of serial dilutions of the allergen extract and incubated

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at room temperature for one hour. Then, a paper disc coupled with mustard allergens was added to the mixtures and incubated for three hours at room temperature. Then, 50 μ L of ^{125} I-anti-IgE (Pharmacia) was added and incubated for 18 hours at room temperature, washed and the bound radioactivity determined. The inhibition was expressed as a percentage.

Histamine Release Assay

This was performed according to the technique described by Siraganian¹⁶ and modified by Moneo et al.¹⁷ Two hundred microliters of whole blood was mixed with 20 μ L of serial five-fold dilutions of the different allergenic extracts in 12 $\times$ 75-mm plastic tubes. After incubation for 30 minutes at 37 $^{\circ}$ C, 400 μ L of PBS-10 mM

EDTA were added and the tubes were centrifuged for five minutes at 400 g. The histamine content in the supernatant was measured using an Autoanalyzer (Technicon Instruments Corp, Tarrytown, NY, USA).

Gel Filtration Chromatography

A column (100 $\times$ 2.6 cm) was packed with Sephadex G-75 equilibrated with ammonium bicarbonate (0.15 M, pH 8). Five milliliters of crude mustard extract was applied to the column. Elution was carried out with the same buffer at room temperature with a flow rate of 25 mL/h. Fractions of 4 mL were collected and tested for allergenic activity by REIA inhibition. A_{280} was measured on a Beckman R-115 spectrophotometer.

SDS-PAGE

It was performed in 12.5% acrylamide gels (120 $\times$ 80 mm) as described by Swank and Munkres.¹⁸ The gels were stained with Co-

Table 1. Mustard-Specific IgE Determination by REIA

Serum	Normal Value	Serum Heating 56 $^{\circ}$ C, 2 hours	Antigen Inhibition*	Unrelated Antigen Inhibition*
1	2.49	0.14	0.20	1.87
2	0.50	0.08	0.10	0.58
3	2.46	0.10	0.17	2.45
4	0.82	0.11	0.13	0.87
5	8.76	0.20	0.19	9.04
6	1.69	0.16	0.10	1.57
7	0.68	0.09	0.12	0.73

10 nonrelated sera 0.16 ± 0.05
Blank 0.09 ± 0.02

* Inhibition induced by mixing the antigen (<0.5 μ g of peroxidase per well) with 20 μ g of unlabeled antigen or 20 μ g of unrelated antigen (*O. europaea*). Values are expressed as OD₄₉₂.

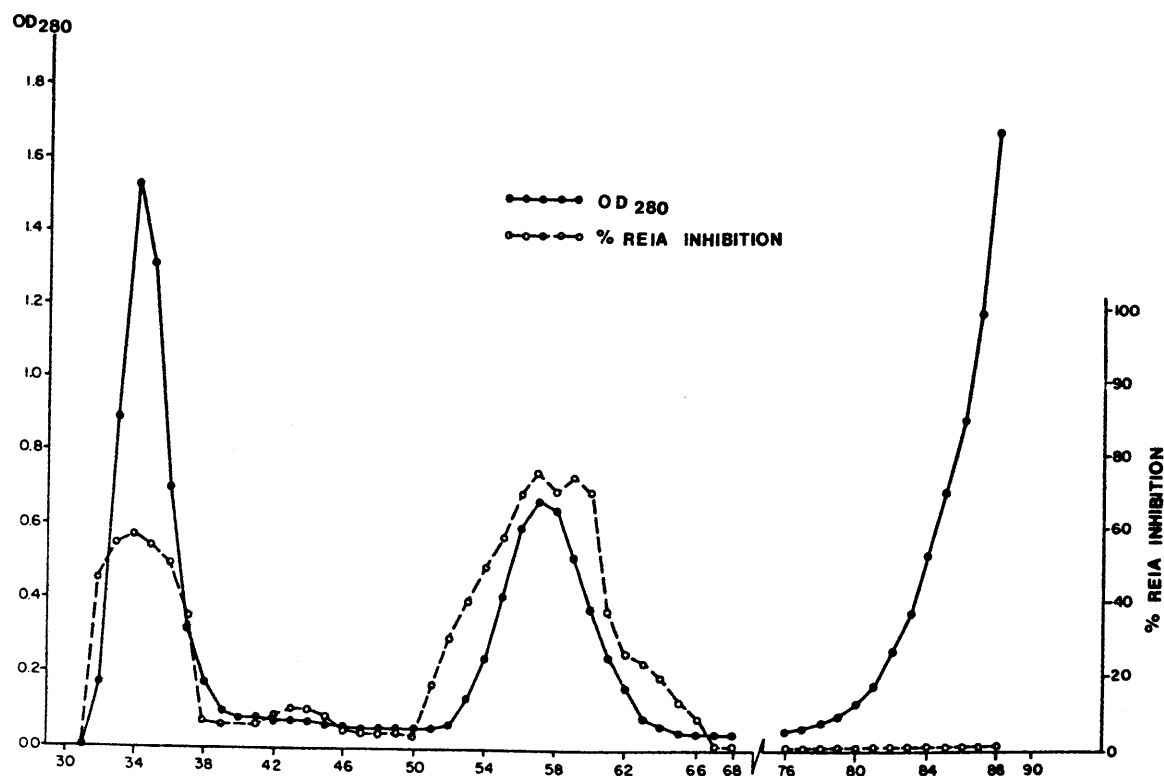


Figure 1. Gel filtration of mustard extract on a Sephadex G-75 column. Fractions were analyzed for proteins by ultraviolet light adsorption at 280 nm and allergenic activity by REIA inhibition assay.

massie Blue G-250. Molecular weights were determined using the MW-SDS-17 Kit (Sigma).

Isoelectric Focusing

This was carried out in 5% acrylamide gel containing 4.8% ampholine (LKB) ranging from pH 7.8 to pH 10. The gels were stained with Coomassie Blue G-250. Papain (pI 8.7) and cytochrome C (pI 10.5) were used as pI markers.

Allergen Stability Experiments

They were carried out to determine the resistance of the antigen to different treatments that it could undergo in cooking or while passing through the gastrointestinal tract.

A solution of purified allergen (1 mg/mL) in PBS was incubated for 60 minutes at 37 °C, 56 °C, and 100 °C. The same concentration of the allergen was incubated in glycine-HCl (0.1 M, pH 2.0) and in glycine-NaOH (0.1 M, pH 11.0) for 60 minutes at room temperature. The solution was then dialyzed against PBS.

An identical aliquot was reduced with 2-mercaptoethanol (0.1 M) for 30 minutes, and alkylated with iodoacetamide (0.5 M) for 30 minutes at room temperature. Then it was dialyzed against PBS.

Samples of 1-mg purified allergen were treated with proteolytic enzymes: trypsin, chymotrypsin, and pepsin, which are present in the gastrointestinal tract. Papain, and less selective proteases such as subtilisin, pronase, and protease V from *Streptomyces griseus* (Sigma) were also used. These enzymes were incubated with the allergen, in a 1:100 wt/wt ratio in the optimal buffer for each enzyme as previously described for both,¹⁹ for one hour at 37 °C and 18 hours at room temperature.

The presence of carbohydrates in the determinants recognized by the IgE was tested by periodate treatment, performed at room temperature in the dark with 30 mM NaIO₄ in acetate buffer (0.1 M, pH 5). After 30 minutes, the sample was

dialyzed and sodium borohydride was added to reach a final concentration of 50 mM. Last, the sample was dialyzed against PBS.

All samples were tested for allergenic activity by REIA inhibition.

RESULTS

Mustard-Specific IgE Determination by REIA

The characteristics of sensitivity, specificity, low cost, and easy performance convinced us to use the REIA assay, which we also used to detect specific IgE to other allergens.^{14,20-22} Table 1 shows the OD₄₉₂ values obtained with seven sera from patients with clinical hypersensitivity to mustard as compared with ten nonrelated atopic sera. A decrease higher than 90% was obtained by heating the sera at 56 °C for two hours or by the addition of unlabeled mustard extract. On the contrary, an *Olea europaea* extract

did not induce inhibition even at the highest concentration tested.

Isolation of Mustard Allergens

Figure 1 shows the Sephadex G-75 profile of the crude mustard extract. Three different fractions were obtained. The first, named Sa-1, corresponding to high molecular weight components, only partially inhibited the mustard REIA. The allergenic activity was mainly present in the second fraction (Sa-2), as determined by REIA and RAST inhibition. More than 70% of the binding activity of the IgE antibody in the pooled sera to the mustard disc was inhibited with 10 µg of this fraction, indicating that IgE to Sa-2 accounted for a large proportion of the IgE against mustard (Fig 2).

Both fractions, Sa-1 and Sa-2, were able to induce histamine release from the whole blood of two mustard-sensitive patients. Sa-2, however, triggered histamine release

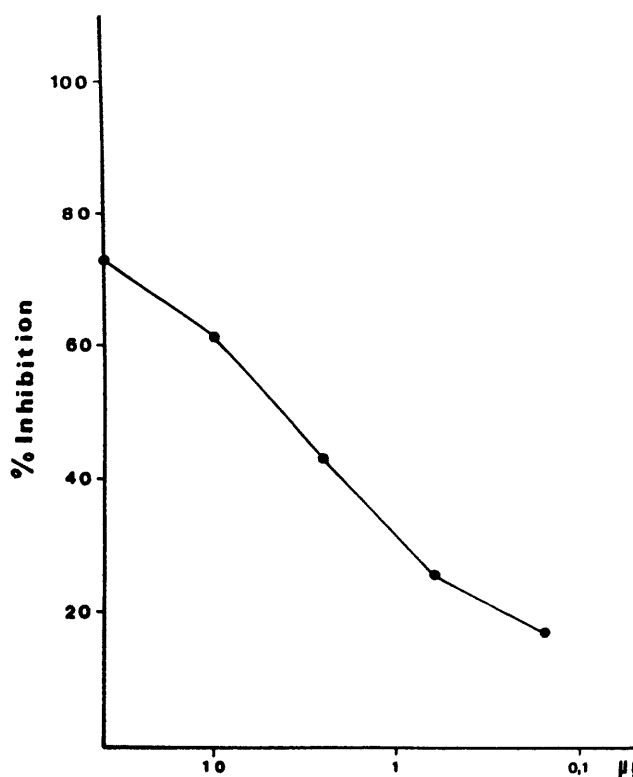


Figure 2. RAST inhibition by Sa-2 antigen. Fifty microliters of pooled sera from seven mustard-sensitized patients was mixed with different amounts of Sa-2 antigen and incubated on mustard discs.

at lower concentrations than Sa-1 in these patients (Fig 3).

The third fraction showed a high A_{280} but did not induce inhibition in the REIA assay. This fraction had a bright yellow color and a very low protein content as measured by Bradford assay.²³

Purity and Molecular Weight Determination

The purity of antigen Sa-2 was assessed by SDS-PAGE and IEF (Fig 4). By SDS-PAGE, antigen Sa-2 gave a single band, with an apparent molecular weight of 14,000 under nonreducing conditions. In the

presence of 0.1 M 2-mercaptoethanol, two bands were observed with apparent molecular weights of 10,500 and 6,300.

By IEF only a faint basic band, with a pI close to the cytochrome C (pI = 10.5) was detected.

Stability of Allergic Activity

The stability of the allergic activity of Sa-2 was measured by REIA inhibition (Table 2). The allergen was stable at temperatures below 56 °C. Heating to 100 °C for 60 minutes resulted in a loss of 50% of the activity. Treatment in acid (pH =

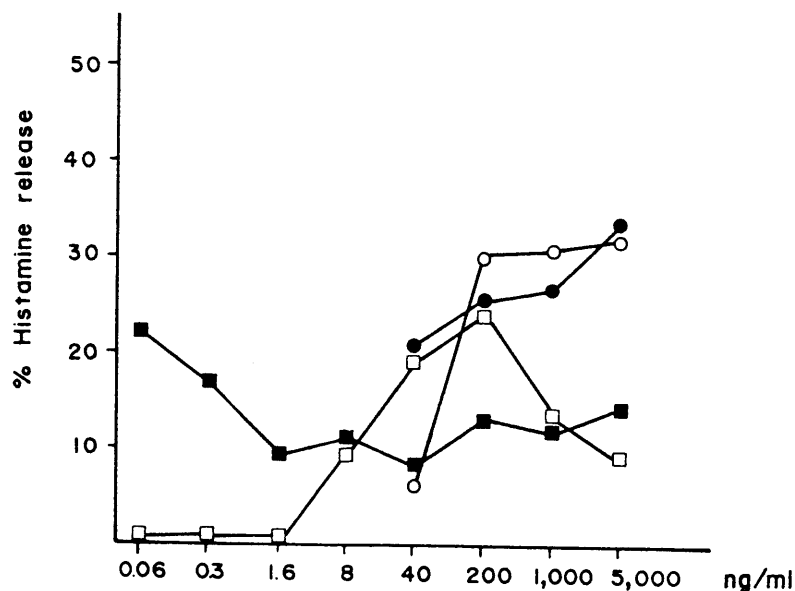


Figure 3. Histamine release from whole blood. Whole blood from two patients (●, ■) was challenged with varying doses of Sa-1 (open symbols) or Sa-2 antigen (black symbols).

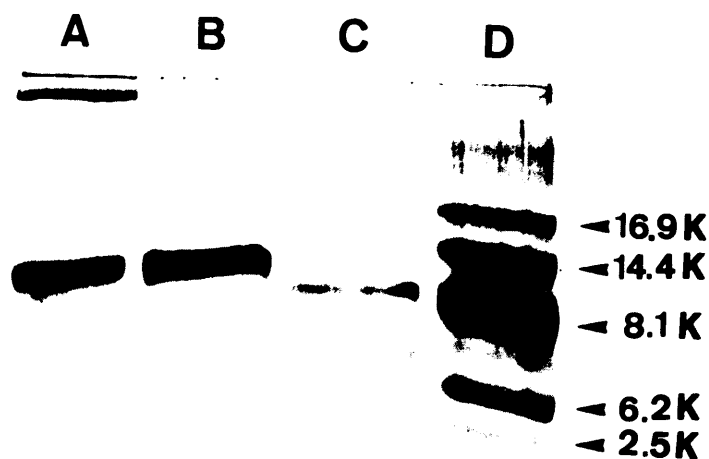


Figure 4a. Evaluation of the molecular weight of Sa-2 antigen by SDS-PAGE electrophoresis (12.5%). Proteins were stained with Coomassie brilliant blue G-250. (A) Crude mustard extract (30 μ g) under nonreducing conditions, (B) Sa-2 antigen (20 μ g) under nonreducing conditions, (C) Sa-2 antigen (20 μ g) with 0.1 M 2-mercaptoethanol, and (D) molecular weight markers.

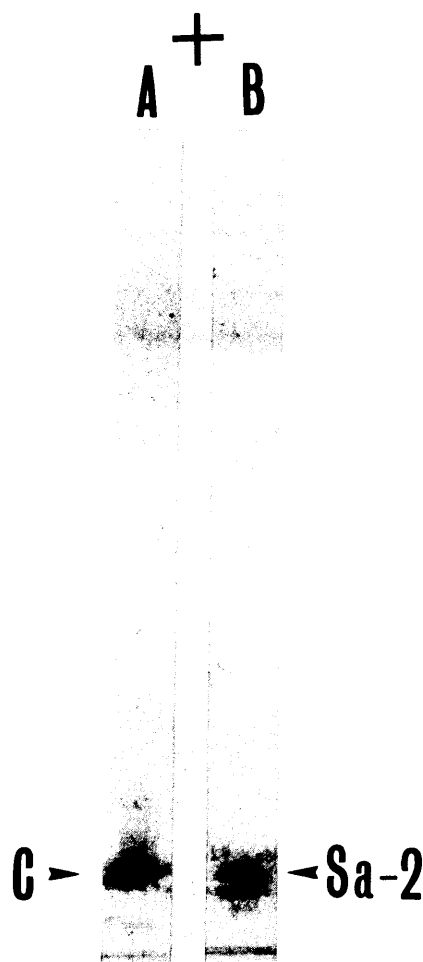


Figure 4b. IEF, pH range from 7.8 to 10. The anode is at the top. (A) Cytochrome C (pI = 10.5) and (B) Sa-2 antigen, 15 μ g.

Table 2. Stability of the Allergenic Activity of Mustard Allergen Sa-2

Procedure	Allergenic Activity*
37 °C, 60 min	100%
56 °C, 60 min	100%
100 °C, 60 min	46%
pH 2.0, 60 min	100%
pH 11.0, 60 min	100%
8 M Urea	100%
6 M Guanidine-HCl	100%
Reduction in 0.1 M 2-ME and alkylation in 0.5 M iodoacetamide	0%
Trypsin digestion	70%
Chymotrypsin digestion	100%
Pepsin digestion	55%
Papain digestion	90%
Periodate oxidation (30 mM)	100%

* Determined by REIA inhibition assay. Calculated by the following formula:

$$\frac{\mu\text{g Sa-2 untreated that induce 50\% inhibition}}{\mu\text{g Sa-2 treated that induce 50\% inhibition}} \times 100$$

2.0), alkali (pH = 11.0) and denaturing solutions (urea 8M, guanidine-HCl 6 M) did not produce any decrease of the allergenic activity. A complete loss of allergenic potency was observed after reduction with 0.1 M 2-mercaptoethanol and alkylation with 0.5 M iodoacetamide, indicating that the integrity of disulfide bonds are essential for this allergen.

When the allergen was treated with proteases such as trypsin, chymotrypsin, pepsin, or papain, only slight losses of allergenic activity were obtained. This high resistance could be due to poor accessibility of these enzymes to their specific residues on the native allergen since treatment with 4 M urea rendered it susceptible to degradation by these enzymes (data not shown). Less selective proteolytic enzymes such as pronase, subtilisin, or protease V from *Streptomyces griseus* produced a total loss of the allergenic activity, providing evidence of the protein nature of the allergenic determinants. The oxidation with sodium periodate did not alter the allergenic reactivity, which suggests that carbohydrate residues are not relevant for the allergenicity.

Cross-reactivity of Mustard Allergens with Other Spices

The cross-reactivity of mustard allergens with those present in caraway, celery, coriander, paprika, and

Table 3. Cross-reactivity of Mustard Allergens with Other Spices

	Patient Serum				Sera Pool*
	1	2	3	4	
Caraway	1	0	0	0	ND
Coriander	8	4	1	2	ND
Pepper	0	4	11	1	ND
Celery	48	0	27	46	ND
Paprika	ND	ND	ND	ND	0%

All values correspond to the % of inhibition obtained mixing 5 µg of protein of these antigens with 0.25 µg of labeled mustard allergen.

* Pool of sera from the seven patients sensitive to mustard.

ND = not determined.

pepper was tested by REIA inhibition. The results are summarized in Table 3. Significant inhibition was observed only with celery showing values close to 50% in two of the four sera tested from patients sensitive to mustard.

DISCUSSION

Mustard is a very common spice obtained from the seeds of *Sinapis alba*, a member of the Cruciferae family. Other foods belonging to this family are cabbage, cauliflower, broccoli, horseradish, Brussel sprouts, etc.

As has been shown for other Cruciferae seed extracts,²⁴ gel filtration on Sephadex G-75 is a particularly useful method for the isolation of proteins. By this approach, a large amount of highly purified antigen Sa-2, as determined by SDS-PAGE, was obtained. This antigen is composed of two subunits linked by disulfide bonds and has a strongly cathodic pI, close to 10.5. All these features are characteristic of a family of seed storage proteins called napins, present in rapeseed (*Brassica napus*), one of the members of the Cruciferae.²⁵

By REIA assays this antigen reacted with each of the seven patients' sera and displayed a high IgE binding activity, reacting with more than 70% of specific IgE present in these sera as determined by RAST inhibition. Although the number of sera tested was low, these data suggested that Sa-2 is one of the most important mustard allergens.

High heat, acid, and alkali treatment stability, together with high

resistance to proteolytic degradation by trypsin, chymotrypsin, and pepsin indicated that this allergen could remain potentially active after the degradation steps of the physiologic food processing in the gastrointestinal tract.

The presence of common IgE binding structures in botanically unrelated spices such as coriander, ginger, or paprika has been suggested.²⁶ We were unable, however to find such cross-reactivity between mustard and other spices. Only two of the patients' sera showed a 50% inhibition in the mustard REIA with a high concentration of celery. Monoclonal antibodies to Sa-2 produced so far (manuscript in preparation) detected the allergen in an extract of celery and Cruciferae seeds but not in leaf tissues obtained from cabbage, Brussel sprouts, and cauliflower. This fact suggests that this allergen is a seed reserve protein, unlike the cabbage leaf allergen described by Blaiss et al.²⁷ This may explain the clinical tolerance of the seven patients to these foods.

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