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Isolation and Characterization of a Major Allergen from Oriental Mustard Seeds, *Bra j* I

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Abstract. A 2 S albumin from oriental mustard (*Brassica juncea*) seeds has been isolated and characterized as an allergen. This protein, *Bra j* I, was recognized by human IgE from mustard-sensitive individuals, as proved by using two different enzyme immunoassays. *Bra j* I was found to be closely related to *Sin a* I, the major allergen from yellow mustard seeds. Many fractions with molecular weights ranging from 16,000 to 16,400 and with differences in charge were separated by ion-exchange chromatography. They exhibited small but significant amino acid composition differences for Glx, Val, Ile, Lys, and Arg contents. The heterogeneity of *Bra j* I can be explained by size and charge differences of its heavy and light chains. All of the isoallergenic forms of *Bra j* I gave a single precipitation band in double diffusion immunoassays when using a *Sin a*-I-specific rabbit polyclonal serum.

Introduction

In sensitive individuals, spice ingestion has been reported to develop urticaria, eczema, and gastrointestinal pain and, occasionally, leads to anaphylactic shocks [1, 2]. During a screening of 1,129 atopic patients, 6% had noticed symptoms from eating spices, mustard being one of the most reactive when tested in vivo [3]. Table mustard is made of flour obtained from the seeds of two species of Brassicaceae: *Sinapis alba* L. (yellow mustard) and *Brassica juncea* L. (Czern and Coss.; oriental mustard). The relative amount of each type of mustard in the commercial product may be different, depending on the manufacturer, though the yellow species is the commonest used in Europe, while the oriental mustard flour is most abundant in mustard extracts from USA or Japan. The major allergen from yellow mustard seeds, *Sin a* I, has been characterized as a basic, low-molecular-weight protein (a 2 S albumin) composed of two polypeptide chains held together by two disulfide bonds. The whole primary structures of its light and heavy chains (39 and 88 amino acids, respectively) were obtained and its relation to other seed proteins

established [4, 5]. Some of these proteins are inhibitors of α -amylase or trypsin, such as those isolated from maize or wheat. Moreover, the 2 S albumins obtained from castor bean, soybean, pea, and other seeds have also been described as allergens and their structures related to *Sin a* I. The yellow mustard allergen, which shows a high content of α -helical structure, is resistant to proteolytic and thermal treatments [6] and exhibits chemical properties of storage seed proteins such as a high glutamine content. Moreover, the immunological characterization of *Sin a* I has been recently started [7]. In this paper we describe the isolation and structural properties of *Bra j* I, a major allergen from oriental mustard seeds, and its similarities to the yellow mustard allergen are discussed.

Materials and Methods

Purification and Fractionation of Bra j I

Pure oriental mustard (*B. juncea* L; Czern and Coss.) flour was kindly provided by Prof. Albert A. Schneiter (North Dakota State University) who obtained it from Minn-Dak Milling Inc. (North Grand Forks, N.Dak., USA). Five grams of oriental mustard flour

was suspended in 150 mM sodium borate buffer, pH 8.0, at 5% (w/v). The undissolved material was removed by centrifugation at 8,700 g for 30 min at 4°C and reextracted twice. The clear supernatants were collected, lyophilized, and extracted with 10% (w/v) acetone. The precipitates obtained were dissolved in 150 mM ammonium bicarbonate buffer, pH 8.0, and applied to a Sephadex G-50 fine column (6×32 cm) equilibrated in the same buffer. Fractions containing the 2 S allergenic protein were pooled, lyophilized, and desalted on a Sephadex G-25 column eluted with 0.1 M acetic acid. The excluded protein was then lyophilized and applied to a SP-Sephadex C-25 column (2.6×25 cm) previously equilibrated in 3 mM pyrophosphate-HCl buffer (pH 9.0). The 2 S albumins were fractionated with a gradient of 3–60 mM pyrophosphate-HCl at pH 9.0. This procedure is similar to that described for *Sin a I* and rapeseed napins [4, 8]. Protein estimations were carried out according to the method of Lowry et al. [9], using bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12.5% acrylamide gels, as described by Lämmler [10]. Prior to electrophoresis, the samples were incubated at 80°C for 20 min. Molecular weights were calculated as described by Weber and Osborn [11], using the marker proteins provided in the Dalton-Mark VII-L kit from Sigma (St. Louis, Mo., USA).

Subunit Structure

The molecular weights of the polypeptide components of the isolated 2 S albumins were determined after sample reduction in 5% (v/v) β -mercaptoethanol (β -ME) by SDS-PAGE (19.3% acrylamide) in the presence of 6 M urea, as described [12]. Alternatively, the native proteins were reduced with dithiothreitol and alkylated with ¹⁴C-iodoacetamide. The carboxyamidomethylated chains were separated by Sephadex G-50 as previously described [4].

Charge microheterogeneity was studied by urea-polyacrylamide electrophoresis under acidic conditions. This was essentially performed as described by Panyim and Chalkley [13] with 15% acrylamide, 0.1% bisacrylamide, and 2.5 M urea in 1 M acetic acid. Preelectrophoresis in 0.9 M acetic acid was performed for 3 h at 15 mA. Samples were applied in 0.9 M acetic acid containing 15% (w/v) sucrose, and if disulfide bond reduction was required, β -ME was added to a 5% (v/v) final concentration. Prior to application the samples were incubated at 80°C for 20 min. Sperm whale cytochrome c and hen egg white lysozyme were used as basic marker proteins. Protein staining was done with 0.5% (w/v) amido black in 7% acetic acid. Excess stain was removed by successive washes with the acetic acid solution.

Amino Acid Analysis

Proteins were hydrolyzed at 105°C for different lengths of time (24, 48, 72, 96 and 120 h) with 5.7 N HCl containing 0.1% (w/v) phenol in evacuated and sealed tubes. Hydrolysates were analyzed on a Durrum D-500 amino acid analyzer. For each hydrolysis time, at least three separated determinations were performed. Half-cystine was determined as cysteic acid after oxidation of the protein with performic acid at -10°C [14]. Tryptophan was spectroscopically determined [15, 16].

Human Sera

Serum samples were obtained from 10 individuals who had not previously been hyposensitized and were selected from a larger population which had the highest radioallergosorbent test and histamine release values to crude mustard extracts and also to the pure allergen *Sin a I* [6, 7]. A pool of human sera from healthy individuals without *Sin a I*-specific IgE was used as control.

Sin a I-Specific Monoclonal Antibodies and Polyclonal Sera

The *Sin a I*-specific monoclonal antibodies used were obtained from BALB/c mice by following the previously described procedure [7]. Rabbit polyclonal sera against *Sin a I* were prepared after immunizing white New Zealand rabbits over a 6-week period by weekly injections of 100 μ g of the protein in Freund's adjuvant. Serum was collected after bleeding of the rabbits.

Enzyme Immunoassays

Reverse Enzyme Immunoassay (REIA)

This assay was essentially performed as previously described [6]. Microtiter plates were coated with 100 μ l/well of a monospecific antihuman IgE solution at 10 μ g/ml in PBS (10 mM sodium phosphate buffer containing 0.9% NaCl, pH 7.4). After 18 h of incubation at 4°C, the plates were washed with 0.5% (v/v) Tween 20 in PBS and emptied. 200 μ l of a 2% (w/v) bovine serum albumin (BSA) solution in PBS was added to each well and incubated at 37°C during 2 h. After washing the plates with 0.5% Tween 20 in PBS, these were emptied, and 100 μ l per well of 0.5 dilution of human serum in calf fetal serum was added and left overnight at 4°C. Then the plates were washed and emptied, and 200 μ l of a solution containing 100 ng of the horseradish peroxidase (HRPO) labeled allergen was added to each coated well. The labeled conjugate was prepared according to the method of Farr and Nakane [17], as described by Domínguez et al. [6]. After 3 h of incubation at room temperature, the plates were washed again and emptied, and the peroxidase reaction was performed as previously described [7].

Enzyme-Linked Immunosorbent Assay (ELISA)

Human IgG Binding. Microtiter plates were coated with 1 μ g/well of the allergenic protein and left at 4°C overnight. After washing the plates with 0.5% Tween 20 in PBS, they were emptied and incubated with a BSA solution as described above. Once the albumin is removed from the corresponding wells, the plates were washed and emptied. Then, 100 μ l of a 0.5 dilution of human sera in calf fetal serum was added to each well. After this step, the plates were kept at 37°C during 2 h. Then, they were washed and emptied and incubated again for another 1 h at 37°C with 100 μ l/well of a 1:1,000 dilution of HRPO-labeled goat antihuman IgE (Tago, Burlingame, Calif., USA) in PBS/25% calf serum/0.5% Tween 20. Afterwards, extensive washing was carried out and the peroxidase reaction developed as described [16].

Competition Assays. Inhibition assays were performed either with *Sin a I* or *B. juncea* 2 S fraction. 100 μ l of different dilutions of inhibitor were coincubated with the appropriate amount of

monoclonal antibodies to give 50% maximum binding on ELISA [6]. The incubations were done for 2 h at 37°C, and, after completion, 0.1-ml aliquots were removed and transferred to the previously *Sin a*-I-coated and BSA-treated wells of the microtiter plate. After 2 h of incubation, the procedure continued as described above, but using HRPO-labeled goat antimouse IgG (Nordic Laboratories, Tilburg, The Netherlands).

Immunodiffusion Assays

These assays were done in plastic Petri dishes with a diameter of 54 mm. 5 ml of a 1% (w/v) agarose solution in PBS, containing 0.5% (w/v) sodium azide, was poured onto the lower dish to make a 3–4 mm thick gel. Samples were applied to wells previously made in the agarose gel, and 20 µl of the rabbit polyclonal serum was applied to the center well, while 20 µl of the corresponding allergens at 1 mg/ml was applied into the external wells. After 12–24 h, precipitation bands appeared.

Results

The 2 S albumin fraction from oriental mustard (*B. juncea*) seeds was isolated after saline extraction, delipidation, and chromatography on a Sephadex G-50 column; 5 g of pure seed flour rendered about 650 mg of defatted saline extract which gave three fractions after gel filtration chromatography. The profile obtained (fig. 1) was similar to that reported for the yellow mustard and other Brassica extracts [4, 8]. The two first fractions correspond to the major reserve proteins in those seeds, known as 12 S globulins and 2 S albumins. The 2 S fraction from oriental mustard represented around 20% of the initial defatted material and showed to be apparently homogeneous in molecular weight by SDS-PAGE. Its allergenicity was demonstrated by the ELISA and REIA methods with the sera of mustard-sensitive patients. Comparative analysis of the purified fraction from oriental mustard seeds and *Sin a* I (the major allergen from the yellow species) revealed that specific IgE for both 2 S albumins was present in the sera of the sensitive individuals (table 1). This suggested that both allergens would bear common epitopes which would be recognized by the IgE from the sera used. Moreover, the 2 S fraction obtained from *B. juncea* seeds was also recognized by rabbit polyclonal sera raised against *Sin a* I (data not shown) and by six mouse *Sin a*-I-specific monoclonal antibodies: 4A11, 1G5, 3F3, 2B3, 7A7, and 2B9 [7]. As can be seen from figure 2, the 2 S fraction obtained from *B. juncea* exhibited a binding capacity similar to *Sin a* I itself in competition assays.

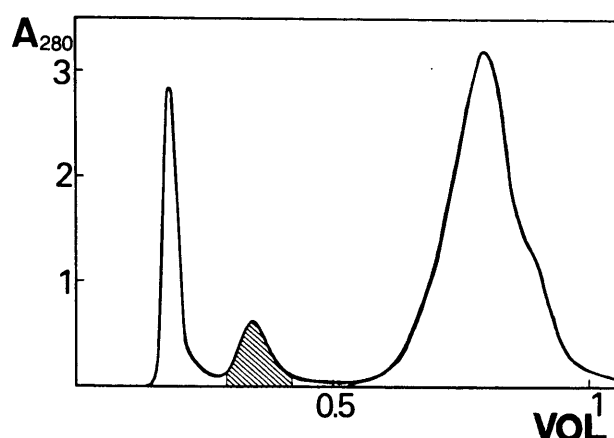


Fig. 1. Elution profile analysis of the delipidized saline extracts from oriental mustard seeds performed using a Sephadex G-50 column (6 × 32 cm) equilibrated in 150 mM ammonium bicarbonate, pH 8.0. The elution volume is expressed in liters. The allergenic 2 S fraction is represented as a dashed peak.

Table 1. Results of the IgE-binding enzyme immunoassays for the two allergens, *Sin a* I and *Bra j* I

Human sera	REIA		ELISA	
	<i>Sin a</i> I	<i>Bra j</i> I	<i>Sin a</i> I	<i>Bra j</i> I
<i>High responders</i>				
1	0.971	0.747	0.826	1.588
2	0.726	0.319	0.388	0.572
3	0.226	0.278	0.566	0.614
4	0.433	0.378	0.428	0.544
<i>Low responders</i>				
5	0.160	0.233	–	–
6	0.026	0.193	–	–
7	0.359	0.385	–	–
8	0.107	0.248	–	–
9	0.161	0.146	–	–
10	0.162	0.237	–	–

The optical density at 492 nm was measured, and controls were subtracted to get the corresponding value. The absorbances obtained in controls using sera from individuals without mustard-specific IgE were around 0.25 and 0.10 in REIA and ELISA experiments, respectively. Significant positive reactions were those over 0.12 in REIA experiments and over 0.04 in ELISA assays. No specific IgEs are detected in low responders by using the ELISA method (–), whereas this method renders higher absorbances in high responders. It has been observed that solid-phase attached allergens can be saturated by no specific IgE when low amounts of the specific IgE are present in the corresponding sera [25] which would explain such an apparent discrepancy.

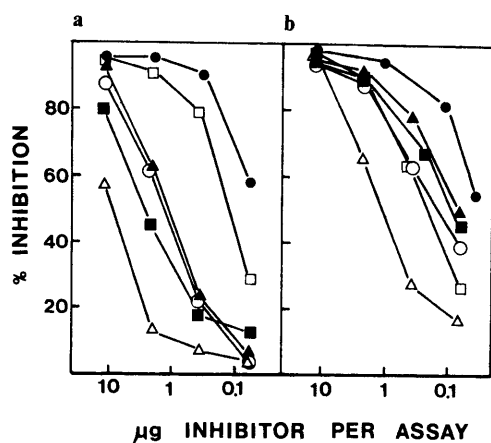


Fig. 2. Monoclonal antibody binding inhibition by *Bra j I* (a) in comparison with *Sin a I* (b). Polystyrene plates were coated with *Sin a I*. The inhibitor was added after coincubation with each monoclonal antibody: ● = 7A7; □ = 2B9; ▲ = 4A11; ○ = 3F3; ■ = 1G5; △ = 2B3. Binding was determined after addition of the HRPO-labeled goat antimouse IgG (H+L).

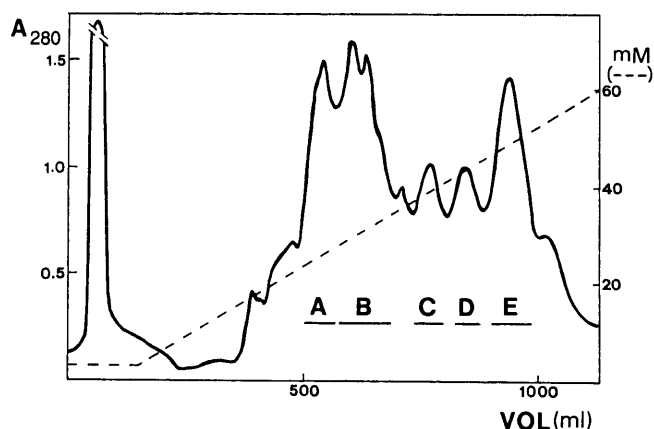


Fig. 3. Elution profile analysis of the 2 S fraction from *B. juncea* seed performed using an ion-exchange SP-Sephadex C-25 column (2.6 × 25 cm) equilibrated in 3 mM pyrophosphate-HCl buffer, pH 9.0. The 2 S fraction was eluted by using a 3- to 60-mM pyrophosphate gradient (---). The horizontal bars represent the five fractions selected for further studies.

Thus, the 2 S albumin from oriental mustard might be considered an allergen homologous to *Sin a I*, and its assigned systematic name is *Bra j I* according to Marsh et al. [18].

Cation-exchange chromatography on SP-Sephadex C-25 revealed the heterogeneity of *Bra j I* (fig. 3). The excluded peak of the profile contains no protein, since its absorbance maximum appears at 260 nm, and

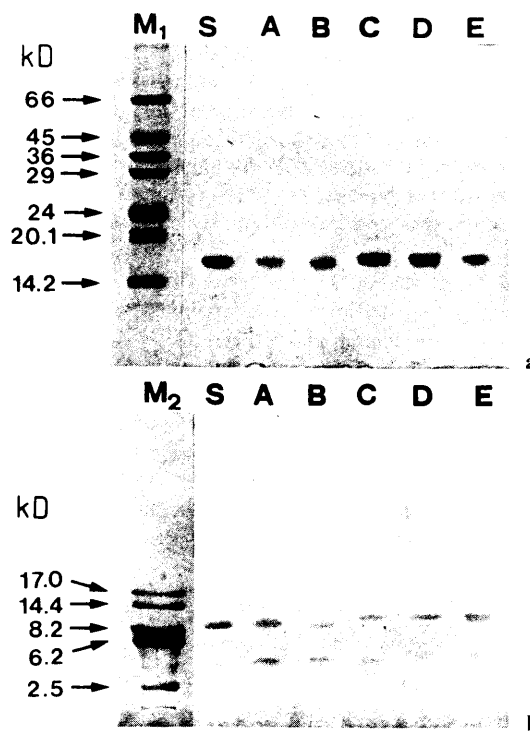


Fig. 4. Electrophoretic patterns of *Bra j I* isoallergenic fractions. a SDS-PAGE (12.5% acrylamide) of native proteins. b SDS-PAGE (19.3% acrylamide), in the presence of 6 M urea, of the polypeptide components obtained after reduction of the native proteins by heating at 80°C for 20 min in the presence of 5% β-ME. M₁ and M₂ stand for standard kits for molecular weight determination obtained from Sigma (MW-SDS-70L and MW-SDS-17, respectively). S, A, B, C, D, and E represent *Sin a I* and fractions A, B, C, D, and E from *Bra j I*, respectively.

no protein bands are observed after SDS-PAGE. The remaining material elutes with a gradient of 3–60 mM pyrophosphate buffer and shows a high degree of heterogeneity. From this material, five fractions (A–E) were selected for further studies on the basis of their relative absorbance and chromatographic resolution.

The five selected fractions displayed a molecular weight similar to *Sin a I* using SDS-PAGE, but exhibited slight differences in their electrophoretic mobility under nonreducing conditions (fig. 4a). Thus, fractions C, D, and E had smaller mobility than fractions A and B. Accordingly, the molecular weight of the components of *Bra j I* would range from 16,000 to 16,400. These five fractions were also analyzed by urea-PAGE under reducing conditions (fig. 4b). According to this analysis, the observed molecular

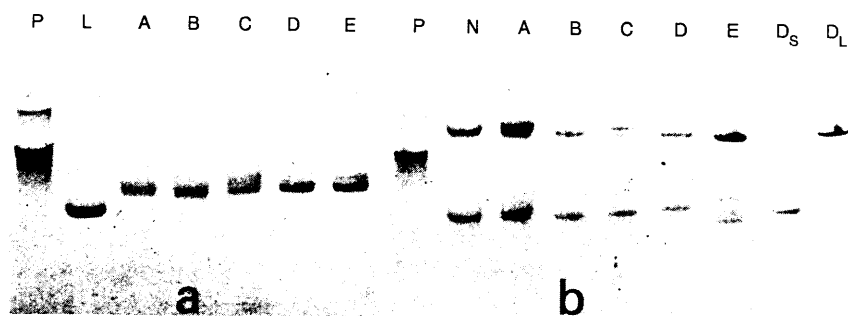


Fig. 5. Electrophoretic patterns of *Bra j* I components under acidic conditions. A, B, C, D, and E represent the fractions selected from the SP-Sephadex chromatographic profile. **a** Native components. **b** Polypeptide chains obtained after reductive treatment with β -ME during 20 min at 80°C (A–E) and after separation of the small (D_s) and large (D_L) carboxyamidomethylated chains of fraction D. P, L, and N stand for standard proteins, cytochrome c, lysozyme, and rapeseed 2 S albumin, respectively.

weight differences should be related to the corresponding differences in the heavy chains. In fact, the heavy chain in C, D, and E fractions had a relative molecular weight of 10,000 versus 9,000 in fractions A and B, while the small chains exhibited an identical molecular weight for all fractions (4,300).

Nevertheless, also charge differences should exist among the components of *Bra j* I according to the chromatographic profiles obtained with the SP-Sephadex column. A useful method to analyze charge heterogeneity in basic proteins is electrophoresis at acidic pH. Under these conditions, the protein components of the oriental mustard allergen show an intermediate mobility between cytochrome c and hen's egg white lysozyme considered as standards. Figure 5a shows the electrophoretic behavior of the native proteins of the selected fractions from *Bra j* I. As it can be seen, the molecular mass/charge ratio is not identical for all the fractions, although similar. Moreover, at least fractions C and E show microheterogeneity. In the presence of β -ME or by reduction and alkylation, the polypeptide components can be separated (fig. 5b). The identity of the individual carboxyamidomethylated chains was confirmed by SDS-PAGE. Both small and large chains from the oriental mustard allergen display microheterogeneity. Thus, as an example, the large chain of fraction C shows two bands

Table 2. Amino acid composition of the fractions of *Bra j* I (A–E) given in comparison with that of *Sin a* I, the major allergen from yellow mustard seeds [5]

	<i>Sin a</i> I	<i>Bra j</i> I				
		A	B	C	D	E
Cys ^a	8	8	8	8	8	8
Asx	2	2	2	2	2	2
Thr	4	4	4	4	4	4
Ser	7	6	6	6	6	6
Glx	28	29	28	31	33	32
Pro	16	16	16–17	16–17	17	17
Gly	8.5	8	8	8–9	8–9	8–9
Ala	7	7	7	7	7	7
Val	7	7–8	6–7	5–6	5	4
Met	2	2	2–3	2–3	1–2	1–2
Ile	4	4–5	4–5	5–6	5–6	5–6
Leu	9	9–10	9	9	9	9–10
Tyr	1	1	1	1	1	1
Phe	3	3	3	3	3	3
His	5	5	4–5	4–5	5	5
Lys	9	9	8	7	6	6
Arg	5.5	6	6	8	9	10
Trp ^b	1	1	1	1	1	1
Total	127	127–130	123–128	127–133	130–133	129–133

^a Determined as cysteic acid.

^b Determined spectrophotometrically.

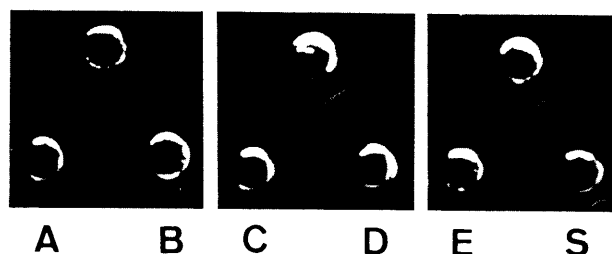


Fig. 6. Double immunodiffusion test in agarose gels. 25 μ l of rabbit polyclonal serum obtained against the yellow mustard allergen was applied to the upper wells and 25 μ l of 1 mg/ml of *Sin a* I (S) and each of the *Bra j* I fractions (A-E) to the lower wells.

and the small chain of fraction E three bands. Moreover, the electrophoretic mobilities of the light chains of fractions B, C, and D were not identical.

The charge heterogeneity of *Bra j* I can also be confirmed by considering the amino acid compositions of the selected fractions (table 2). Small but significant differences were found in the contents of basic amino acids (Lys and Arg) as well as of Glx, Val, and Ile residues. The values for the other residues are very similar among all fractions, even when compared with composition of *Sin a* I.

Despite these differences, the five fractions studied gave a single band in a double diffusion immunoassay when tested against a *Sin a*-I-specific rabbit polyclonal serum (fig. 6). All the above results were interpreted in terms of the presence of isoallergenic forms in *Bra j* I.

Discussion

Spices are a very common food additive used in human diets. Some of them have been described as responsible for hypersensitivity in atopic individuals [3]. Moreover, mustard has been reported as one of the most reactive spices, since anaphylactic shocks have been described after its ingestion [2].

The allergenic proteins of mustards are 2 S albumins stored in the protein bodies of the seeds. The most useful chromatographic procedure used to isolate this type of albumins from other Brassicaceae seeds involves a gel filtration chromatography in Sephadex G-50 [8, 19]. Under the same conditions, the profile obtained for oriental mustard does not differ from those reported for yellow mustard, rapeseed,

turnip, or cabbage. The allergenic behavior of the 2 S albumin from oriental mustard was very similar to that of *Sin a* I when tested by ELISA and REIA. These results suggested that both proteins are very closely related allergens and that they bear common epitopes. This is confirmed by polyclonal rabbit sera and monoclonal antibodies, both obtained against *Sin a* I, since they cross-react with the 2 S albumin from oriental mustard. Moreover, the amino acid compositions of both proteins showed great similarity. These results allowed us to describe *Bra j* I as the homologous allergen in *B. juncea* to that of *S. alba* (yellow mustard), *Sin a* I. Therefore, *Bra j* I would be a major allergen in oriental mustard seeds.

However, both allergens show a different degree of heterogeneity, since a further ion-exchange chromatographic step resolves several components from *Bra j* I, in contrast to what was reported for *Sin a* I [4]. In fact, the 2 S albumin from yellow mustard gives only a discrete peak eluting from the SP-Sephadex, while under the same conditions many allergenic fractions can be separated from oriental mustard. However, heterogeneity was observed when the primary structure of *Sin a* I was determined [5]. It should be noted that the diversity of components of *Bra j* I would be a consequence of the fact that *B. juncea* is an amphidiploid hybrid originated from *Brassica nigra* and *Brassica rapa* (*Brassica campestris*) [20]. The fractions obtained from *Bra j* I result from size heterogeneity in the heavy chains and charge heterogeneity in both light and heavy chains.

The molecular weight of *Bra j* I herein reported, ranging from 16,000 to 16,400, differs from that previously obtained for the 2 S albumin from *B. juncea* by Venkatesh and Rao [21], whose estimation by gel filtration chromatography was 22,900 daltons. This last value is also significantly higher than our reported value for *Sin a* I (14,600 daltons) determined by using the same method [4]. The use of hen's egg white lysozyme as a marker protein to calibrate their gel filtration column [21] is probably the reason for this disagreement. Lysozyme is not an appropriate marker, as this protein interacts with the chromatographic support [22], eluting later than it would be expected assuming its actual size.

The amino acid compositions of *Sin a* I and the five selected *Bra j* I fractions were found to be very similar, though differences were detected in some amino acids. Remarkable are the decrease of Val and Lys and the increase of Ile, Arg, and Glx contents when

we consider the composition of the fractions which elute first in the ion-exchange chromatography and those eluting later. It seems as if Val and Lys had been substituted by Ile and Arg, respectively, in the protein sequences from the fractions A–E. Fractions A and B exhibit very similar properties, but the Lys and Val contents in fraction A were always higher than in fraction B. On the other hand, the different pK value for the side chains of Lys and Arg and their respective contents along the five fractions selected would be enough to explain the separation on ion-exchange chromatography. Referring to the Glx content, it is important to mention that *Sin a I* as well as napins (the 2 S albumins from rapeseed) have a glutamine-rich region variable in length in the primary structure of their heavy chains. These segments, located around positions 38–54 of the sequence, have been described as the most variable region of the 2 S albumins of Brassicaceae [5, 23, 24]. The Glx content of the fractions isolated from *Bra j I* ranges from 28 to 33 residues per molecule. Thus, the different length of the heavy chains of *Bra j I* could be a consequence of the different Glx content in this region. This proposal would agree with the different molecular weight obtained for these chains. On the other hand, the partial deamidation of glutamine in these storage proteins should also be mentioned as a cause of differences in charge between the components of *Bra j I*.

Despite the differences in chromatographic behavior, electrophoretic mobility, and amino acid composition, the allergic response to *Sin a I* and *Bra j I* fractions was nearly identical in a series of sera from mustard-sensitive individuals. In addition, cross-reactivity and inhibition capacity between the yellow mustard allergen and the five *Bra j I* fractions were also observed by immunodiffusion tests using a rabbit polyclonal serum obtained against *Sin a I* and monoclonal antibodies specific for this protein.

The structural data of *Bra j I* presented above allow to expect a very similar primary structure for the allergen obtained from both mustard species, yellow and oriental. Moreover, it seems that the antigenic structure of *Bra j I* would be closely related to that of *Sin a I*, since it is recognized by all the *Sin a I*-specific antibodies tested. The knowledge of two very related allergens, one of which exhibits heterogeneity, opens the possibility for the study of the relative importance of certain residues in terms of antigenicity and allergenicity of these molecules, even in comparison with other seed proteins described as allergens.

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