Characterization of a new oriental-mustard (*Brassica juncea*) allergen, *Bra j* IE: detection of an allergenic epitope

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Bra j IE, a major allergen from oriental-mustard (*Brassica juncea*) seeds, has been isolated and characterized. Its primary structure has been elucidated. This protein is composed of two chains (37 and 92 amino acids) linked by disulphide bridges. The amino acid sequence obtained is closely related to that previously determined for *Sin a* I, an allergen isolated from yellow mustard (*Sinapis alba*). A common epitope has been detected in the large chain of both *Bra j* IE and *Sin a* I by means of electroblotting

and immunodetection with 2B3, which is a monoclonal antibody raised against the yellow-mustard allergen. A histidine residue of the large chain of both mustard allergens has been found to be essential for the recognition by 2B3 antibody. A synthetic multiantigenic peptide containing this His was recognized by 2B3 as well as by sera of mustard-hypersensitive individuals. Therefore this antigenic determinant must be involved in the allergenicity of these proteins.

INTRODUCTION

Atopic allergy is a clinical disorder which can induce severe symptoms in human beings and shows an increasing incidence during the last few years. More than 10 % of the world population suffers atopic Type I allergy. Pollens, hymenopteran venoms, house-dust mites, animal danders and foods are the most relevant sources of allergens. Many spices have been described as leading to allergic diseases after their ingestion [1]. Among them, mustard seeds were one of the most reactive when tested *in vivo* [2]. Table mustard is made of flour obtained by grinding the seeds of two species of Brassicaceae: *Sinapis alba* L. (yellow mustard) and *Brassica juncea* (L.) Czern and Coss (oriental mustard). The commercial products may contain different amounts of these species, although *S. alba* is the most commonly used in Europe and *B. juncea* in U.S.A. and Japan. *Sin a* I (M_r 14100) is the major allergen of *S. alba* seeds and belongs to the 2 S albumin class of storage proteins of these seeds. This protein has been isolated and its whole sequence determined [3,4]. It is composed of two polypeptide chains (39 and 88 amino acids) held together by two disulphide bridges. An epitope mapping of this allergen has recently been reported, and it was found that one out of ten mouse monoclonal antibodies raised against the yellow-mustard allergen seemed to recognize a continuous epitope on the protein [5]. This antigenic determinant involves the only tyrosine residue of the molecule, which is located on the large chain in a region which has been proposed to be an immunodominant portion of the protein [5].

Allergenic activity has been reported in the 2 S albumin fraction of *B. juncea* seeds [6]. Moreover, five isoallergenic fractions from these 2 S albumins of *B. juncea* have been partially resolved and characterized [6]. The existence of sequence similarity among the 2 S albumins [7,8], as well as the importance of the knowledge of antigenic determinants on allergens, led us to study the oriental-

mustard 2 S fraction. Here we describe the amino acid sequence elucidation of a new allergen (*Bra j* IE) obtained from oriental-mustard seeds. Moreover, the existence of an epitope common to mustard allergens, which seems to be involved in their allergenicity, is proposed from these studies.

EXPERIMENTAL

Purification of *Bra j* I

Pure mustard flours were kindly provided by Professor Albert A. Schreier (North Dakota State University, Fargo, ND, U.S.A.), and *Brassica napus* (rape) seeds by the Instituto Nacional de Investigaciones Agronómicas, Madrid, Spain. Yellow-mustard allergen (*Sin a* I) and napin BnIII were purified from *Sinapis alba* L. and *B. napus* seeds respectively as previously described [3,9]. Oriental-mustard allergen (*Bra j* I) was obtained from *Brassica juncea* (L.) Czern and Coss. flour as described by González de la Peña et al. [6]. Five isoallergenic fractions were obtained during this procedure, *Bra j* IE being the most abundant isoallergenic fraction [6].

Amino acid analysis

Proteins and peptides (1–5 nmol) were hydrolysed under conditions previously described [4]. Hydrolysates were analysed on a Beckman system 6300 amino acid analyser with an analog interface module of System Gold. Half-cystine was determined as carboxymethylcysteine after reduction and alkylation of the protein [3]. Tryptophan was determined spectroscopically [10].

Proteolytic treatments and amino acid sequence for *Bra j* IE

Separation of the large and small chains of *Bra j* IE was performed by Sephadex G-50 chromatography after reduction

Abbreviations used: DMF, *N,N*-dimethylformamide; Fmoc: 9-fluorenylmethyl derivative; mAb: monoclonal antibody; MAP, multiple antigenic peptide; tBoc: *t*-butoxycarbonyl derivative; TFA, trifluoroacetic acid.

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of the disulphide bonds and radioalkylation with iodo- $[^{14}\text{C}]$ acetamide ($1\ \mu\text{Ci}/5\ \mu\text{l}$; Amersham International) [3]. Tryptic digestions of small (10–15 nmol) and large (20–30 nmol) chains were performed as previously described [11]. No insoluble material was detected after these treatments. Cleavage of the N-terminal 5-oxoproline of the alkylated large chain (20 nmol) was performed by using pyroglutamate aminopeptidase (EC 3.4.19.3; Boehringer) as described in [12]. Proteolytic digestions were loaded on a Nucleosil C-18 reverse-phase h.p.l.c. column with an acetonitrile gradient in 0.1% TFA, designed for each sample. The fractionated peptides were hydrolysed and their amino acid compositions determined.

N-terminal Edman degradations of the alkylated chains and isolated peptides (0.5–1.0 nmol) were performed on an Applied Biosystems model 477A gas-phase protein sequencer by standard methods. Alignments were obtained by using the NEWAT-85 program kindly provided by Dr. Russell F. Doolittle, and based on the algorithm of Needleman and Wunsch [13,14].

Human sera, specific-antisera and enzyme immunoassay

Sera from 11 allergic individuals exhibiting a positive radio-allergosorbent test (values 3–4) were used. The *Sin a* I-specific monoclonal antibody (mAb) 2B3 was obtained in BALB/c mice as previously described [5]. Rabbit polyclonal sera against *Sin a* I were prepared after immunizing two New Zealand White rabbits over a 6-week period by weekly injection of the protein (100 μg) in Freund's adjuvant. Sera were collected after bleeding. Two other rabbits were immunized with a peptide chemically synthesized (see below) by using a multipuncture method [15] to obtain the corresponding antisera.

For e.i.s.a.s, microtitre plates were coated with antigen (1 μg /well). The binding of the hybridoma supernatants and the reaction with the horseradish peroxidase-labelled goat anti-mouse IgG (H + L) (Pierce, Oud-Beijerland, The Netherlands) were performed under conditions previously described [5]. The peroxidase reaction was also developed as in [5].

Acidic pH electrophoresis, blotting and immunodetection

Electrophoresis was essentially performed as described in [16], on a Bio-Rad Mini Protean II system and containing 15% (w/v) acrylamide/0.1% (w/v) bisacrylamide/2.5 M urea/1 M acetic acid. Samples were applied in 0.9 M acetic acid/15% (w/v) sucrose. Protein staining was performed with 0.5% Amido Black in 7% (v/v) acetic acid, and the non-stained gels to be transferred were soaked in 0.7% acetic acid for 5 min.

Electrophoretic transfer of proteins and peptides was carried out as described in [17], on a LKB Novablot 2117 Electrophoretic Transfer Unit (LKB, Bromma, Sweden). Sheets of ProBlott (Applied Biosystems) were equilibrated in 0.7% acetic acid for 5 min. The membrane was cathodically oriented to the gel and the current intensity was kept at 40 mA for 60 min.

For immunodetection, the membranes were equilibrated in PBS (10 mM, pH 7.2)/0.5% Tween 20 and maintained in 2% (w/v) BSA in PBS for 2 h at room temperature. After two washes with PBS/0.05% Tween 20, a solution of the *Sin a* I-specific mAb 2B3 or human sera or rabbit peptide-specific antisera, depending on the experiment, was added. The 2B3 solution was prepared by diluting the corresponding hybridoma supernatant at 1:75, in PBS/0.1% BSA, and the membranes were incubated for 3 h. Rabbit sera were used at 1:200 dilution and incubation was also for 3 h. Human sera were 1:5 diluted in the same solution containing 0.2% Tween 20, and the incubation was

performed for 16–24 h. Afterwards, membranes were washed with PBS/0.05% Tween 20. Then anti-mouse IgG (H + L), anti-human IgE (Nordic Immunology, Tilburg, The Netherlands) or anti-rabbit IgG (H + L) (Tago inc., Burlingame, CA, U.S.A.), all of them from goat and horseradish peroxidase-labelled, and diluted with PBS/0.1% BSA (1:2000, 1:1000 and 1:1000 respectively) were added in each case and incubated for 3 h. The peroxidase reaction was developed after three washing treatments with PBS/0.05% Tween 20. For such purpose, the membrane was soaked in 0.3 mg/ml solution of 3,3'-diaminobenzidine in PBS, containing 0.005% H_2O_2 . Reaction was eventually stopped by substituting this solution with glass-distilled water.

Peptide synthesis

The synthesis of an octameric multiple antigenic peptide (MAP) was carried out manually by a stepwise solid-phase procedure [18] with the modifications described in [19,20]. The synthesis was performed according to the 9-fluorenylmethyl derivative (Fmoc) strategy. A solid support composed of a polyamide resin and a branched core of seven lysines ($\text{Lys}_4\text{-Lys}_2\text{-Lys}_1$) was prepared. Pepsin gel resin (Milligen) was maintained overnight in ethylenediamine. The resin was then washed with dimethylformamide (DMF) and with 10% (w/v) *NN*-di-isopropylethylamine in DMF. Afterwards, the coupling was achieved by adding the *p*-hydroxyphenoxyacetic active ester, which was prepared by mixing 1-hydroxybenzotriazole in DMF and di-isopropyl carbodi-imide with a *p*-hydroxyphenoxyacetic acid solution in dichloromethane at 25 °C. The ester was diluted with DMF and added to the resin. The mixture was incubated under nitrogen bubbling for 45 min. One β -Ala residue was attached to the resin by adding a mixture of *t*Boc- β -Ala-OH and Fmoc- β -Ala-OH. A symmetric anhydride was prepared with a 5 M excess of these amino acid derivatives, dissolved in dichloromethane and cooled. Upon the addition of di-isopropyl carbodi-imide the sample was incubated at 25 °C for 10–15 min. After mixing with the resin, dimethylaminopyridine in dichloromethane and *N*-methylmorpholine were also added and incubated for 60 min under nitrogen bubbling. Fmoc-Cys(acetamidomethyl)-OH was added to the β -Ala-polyamide resin, after deprotection of NH_2 groups by washing twice with 20% (v/v) piperidine in DMF. The amino acid derivative is coupled with a 3 M excess of an active ester. Recoupling was achieved with a 1.5 molar excess of the symmetric anhydride. Afterwards, the lysine-core was prepared on the Cys(acetamidomethyl)- β -Ala-OH-resin with three levels of Fmoc-Lys(Fmoc). This was obtained by the same deprotection/coupling/recoupling procedure described in the preceding paragraph. Finally, eight (QGPHVISRIYQTAT) sequences were synthesized, attaching each amino acid derivative to the lysine core by the same protocol previously described. An aliquot (10 nmol) of the peptide-resin complex was removed at this step to be sequenced on the Applied Biosystems model 477A sequencer described above. Afterwards, the N-terminal ends of the synthetic peptide were capped by acetylation with acetic anhydride in DMF. The protected peptide-resin was treated with 90% (v/v) TFA/5% (v/v) anisole/5% (v/v) ethane-1,2-dithiol in order to cleave the MAP from the resin support. The crude peptide was then washed with cold diethyl ether and extracted with 10% (v/v) acetic acid. The peptide was gel-filtered through a Sephadex G-10 column equilibrated in 1% (v/v) acetic acid. The freeze-dried MAP gave a satisfactory amino acid analysis and eluted as a pure component in reverse-phase h.p.l.c. Moreover, the unprotected peptide rendered the expected sequence by the automatic Edman degradation and by using the appropriate amino acid phenylthiohydantoin-standards.

Purification and sequence analysis of peptides from a 2 S albumin isolated from *B. napus*

The small and large chains of napin BnIII (100 nmol), a 2 S albumin obtained from *B. napus* [9], were separated after reduction and carboxyamidomethylation as described above for the chains of *B. juncea*. Alkylated large chain (40 nmol) was digested with trypsin and the peptides were isolated by reverse-phase h.p.l.c. on the Nucleosil column described above for peptide purification. Acidic electrophoresis, amino acid analysis, electrotransfer and immunostaining, sequence and other analyses were performed as described for *Bra j* IE.

Figure 1 Curves for the binding of the *Sin a* I-specific mAb 2B3 to the solid-phase-fixed isoallergenic fractions of *B. juncea*

Polystyrene wells were coated with the antigens (■, *Bra j* IA; △, *Bra j* IB; ○, *Bra j* IC; ▲, *Bra j* ID; ●, *Bra j* IE; □, *Sin a* I; and ●, napin BnIII) and binding was measured by adding horseradish peroxidase-labelled goat anti-mouse IgG (G + H).

Table 1 Amino acid compositions of *Bra j* IE and of its small and large chains

Data were obtained from standard acid hydrolyses and the corresponding amino acid analysis. Cysteine was determined as its carboxymethylated derivative and tryptophan was determined spectroscopically. The composition of *Sin a* I is also included for comparison.

Amino acid	Composition (residues/molecule or chain)			<i>Sin a I</i>
	<i>Bra j</i> IE			
	Small chain	Large chain	Native protein	
Cys	2	6	8	8
Asx	—	2	2	2
Thr	—	4	4	4
Ser	2	4	6	7
Glx	8	23–24	32	28
Pro	3	12	16	16
Gly	3	5	8	8.5
Ala	4	3	7	7
Val	—	4	4	7
Met	1	1	2	2
Ile	0–1	5	5–6	4
Leu	2	7	9	9
Tyr	—	1	1	1
Phe	2–3	1	3	3
His	2	3	5	5
Lys	2	4	6	9
Arg	4	5	9	5.5
Trp	1	—	1	1
Total	37–38	90–91	128–129	127

RESULTS

Binding analysis of *Bra j* I isoallergens to mAb 2B3

Five 2 S albumin isoallergenic fractions can be isolated from oriental-mustard (*B. juncea*) seeds [6]. These protein fractions, called *Bra j* IA, *Bra j* IB, *Bra j* IC, *Bra j* ID and *Bra j* IE according to [21], have now been analysed by e.l.i.s.a titration with 2B3, a monoclonal antibody raised against the yellow-mustard allergen *Sin a* I. It has been proposed that 2B3 recognizes a continuous epitope in the large chain of *Sin a* I, since it binds to

Figure 2 Analysis of the heterogeneity of the isoallergenic fractions of *Bra j* I

H.p.l.c. elution profiles of the reduced isoallergenic fractions isolated from *B. juncea*: (a) *Bra j* IA; (b) *Bra j* IB; (c) *Bra j* IC; (d) *Bra j* ID; and (e) *Bra j* IE. Each fraction (0.5 mg) was incubated in the presence of 5% (v/v) 2-mercaptoethanol at 80 °C for 20 min before the injection on to the column. The small (S) and large (L) chains from each isoallergenic fraction were eluted from the Nucleosil C-18 reverse-phase column with an acetonitrile gradient (20–40%) in 0.1% TFA. The flow rate was 1 ml/min, and the eluate was continuously monitored at 214 nm.

the unfolded polypeptide [5]. This antibody recognizes the five *Bra j* I isoallergens (Figure 1). A 50% maximum binding was obtained for the five isoallergens at 1:200 culture supernatant dilution, which is a similar value to that required for *Sin a* I. Therefore, the epitope recognized by 2B3 would be borne by all the oriental-mustard isoallergens. On the other hand, according to the results shown in Figure 1, this epitope would not be present in napin BnIII, although this protein is a 2 S albumin isolated from rapeseed (*B. napus*) and shows sequence similarity to *Bra j* I and *Sin a* I [7,8].

The five *Bra j* I isoallergenic fractions were chemically reduced and the resulting chains separated by h.p.l.c. in order to analyse their compositions. As shown in Figure 2, *Bra j* IA, *Bra j* IB and *Bra j* IC display great heterogeneity. However, *Bra j* ID and *Bra j* IE have a main component for the large chain and only two well-defined components, obtained in a similar yield, for the small chain. Among these two isoallergenic fractions, *Bra j* IE was selected for further structural and immunological analyses, since it is the most abundant 2 S albumin in the mature seeds of oriental mustard [6]. The amino acid compositions of the reduced and carboxyamidomethylated small and large chains of *Bra j* IE are given in Table 1.

Immunodetection with 2B3 of the reduced chains of the mustard allergens

PAGE under acidic conditions and in the presence of 2.5 M urea allows one to separate the constituent polypeptide chains of 2 S albumins after treatment with 2-mercaptoethanol (Figure 3a). The immunoblots obtained for reduced *Sin a* I and *Bra j* IE by staining with 2B3 are shown in Figure 3(b). Reduced napin BnIII was also considered for comparison, since it was not detected by mAb 2B3 by e.l.i.s.a. Immunostained bands appeared at the position of the large chains of both mustard proteins, whereas napin BnIII chains were not recognized by the mAb. This result indicates that the antigenic determinant recognized by 2B3 seems to be conserved in both mustard allergens, but not in other closely related 2 S albumins.

Amino acid sequence of *Bra j* IE

Figure 4 and Table 2 show the h.p.l.c. profiles and the amino acid compositions of the peptides obtained from the two chains of *Bra j* IE. The amino acid sequence of the tryptic peptides and the N-terminal analyses of the whole chains elucidate the primary structure of this allergen (Figure 5). The final alignment of the tryptic peptides was performed by considering the structural similarity between *Sin a* I and *Bra j* IE on the basis of their amino acid compositions, c.d. and fluorescence spectra [3,4,6], proteolytic h.p.l.c. fingerprints and peptide compositions. The heterogeneities found in the *Bra j* IE small chain (Ile/Phe and Arg/Lys, at positions 6 and 20 respectively) would explain the microheterogeneity displayed on both h.p.l.c. (Figure 2) and acidic electrophoresis (Figure 3). The large chain of *Bra j* IE contains four more residues than the corresponding chain of *Sin a* I: besides a Gln residue at the N-terminal end of the molecule, three extra amino acids are located in an internal region of this chain (Figure 5).

Amino acid sequence of peptides from napin BnIII

In order to analyse the differences between the three molecules, *Sin a* I, *Bra j* IE and BnIII, around the site recognized by 2B3, the amino acid sequences of several BnIII peptides were de-

Figure 3 Immunodetection with antibody 2B3 of the yellow- and oriental-mustard allergens and the synthetic peptide

Acid PAGE, electrotransfer and immunodetection of: (1) *Sin a* I; (2) napin BnIII; (3) *Bra j* IE; and (4) MAP. (a and d) Amido Black staining of the reduced samples in 5% (v/v) 2-mercaptoethanol at 80 °C for 20 min. (b and c) Immunoblot of replicas of the gel (a) and line (d,4) respectively immunostained with the *Sin a* I-specific mAb 2B3. Abbreviations: H, hen's-egg-white lysozyme; P, cytochrome c; and M, a mixture of both lysozyme and cytochrome marker proteins as negative control of the binding.

Figure 4 Elution profiles of the reversed-phase h.p.l.c. on a Nucleosil C-18 column of the tryptic hydrolysates of the small (SC) and large (LC) chains from *Bra j* IE allergen

The peptides were eluted with an acetonitrile gradient in 0.1% (v/v) TFA. The flow rate was 1 ml/min. Fractions (1 ml) were collected. Hatched peaks correspond to radioactive material (cysteine-containing peptides).

Table 2 Amino acid compositions of the tryptic peptides from the carboxyamidomethylated small and large chains of *Bra* / IE

Cysteine was determined as its carboxymethylated derivative, and tryptophan was determined spectroscopically. The number of residues determined by sequence analysis is shown in parentheses. The composition of the complete chains deduced from the protein sequence is also shown; * indicates the total number of amino acids of the corresponding chain.

(a) Small chain

		Composition (residues/peptide)												
Amino acid	Peptide ...	T1	T2	T2'	T3	T4	T5	T5'	T6	T6'	T6''	T7	T7'	Complete chain
Cys					0.6 (1)	0.9 (1)			1.0 (1)	0.9 (1)				2
Asx														—
Thr														—
Ser												1.6 (2)	1.8 (2)	2
Glx							4.2 (4)	3.9 (4)	2.2 (2)	2.4 (2)		2.0 (2)	1.0 (1)	8
Pro		1.2 (1)	1.1 (1)	0.8 (1)								1.0 (1)	1.1 (1)	3
Gly		1.1 (1)										2.4 (2)	1.8 (2)	3
Ala		1.0 (1)					1.0 (1)	1.2 (1)	1.0 (1)	1.0 (1)		1.0 (1)		4
Val														—
Met												0.7 (1)		1
Ile				1.0 (1)										0.4
Leu							0.9 (1)	0.7 (1)	1.0 (1)		1.1 (1)			2
Tyr														—
Phe		1.1 (1)	1.0 (1)				0.8 (1)	1.1 (1)						2.6
His							1.0 (1)	0.9 (1)	1.0 (1)		1.0 (1)			2
Lys						1.0 (1)		0.6 (1)	0.7 (1)		0.7 (1)			2.5
Arg		1.1 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.2 (1)	0.9 (1)							4.5
Trp									+(1)	+(1)				1
Amount (nmol)...		10.0	6.0	2.2	13.4	2.2	6.4	2.6	7.5	3.0	1.5	3.1	3.0	
Sequence position ...		1–5	6–8	6–8	9–10	9–11	12–20	12–20	21–28	21–25	26–28	29–37	32–37	37*

(b) Large chain

Composition (residues/peptide)													
Amino acid	Peptide ...	T1	T1'	T2	T3	T4	T5	T6	T7	T8	T9	T10	Complete chain
Cys		3.5 (4)	2.4 (3)						0.5 (1)	0.6 (1)			6
Asx		1.2 (1)	1.4 (1)						1.0 (1)				2
Thr		1.0 (1)	1.2 (1)					1.6 (2)				1.0 (1)	4
Ser				0.8 (1)			0.8 (1)			0.9 (1)		0.9 (1)	4
Glx		9.4 (10)	5.4 (4)			2.4 (2)	11.8 (11)	1.4 (1)			0.9 (1)		25
Pro		6.4 (6)	3.2 (2)					1.3 (1)	1.0 (1)	0.7 (1)		1.5 (2)	11
Gly		1.1 (1)		1.0 (1)			2.9 (3)					1.1 (1)	6
Ala				1.0 (1)	1.0 (1)			1.0 (1)					3
Val		1.2 (1)	1.4 (1)		1.1 (1)				1.1 (1)	1.2 (1)			4
Met												0.8 (1)	1
Ile						1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)			5
Leu		5.0 (5)	3.5 (3)				1.0 (1)	1.0 (1)					7
Tyr								0.9 (1)					1
Phe										1.1 (1)			1
His		1.1 (1)	1.1 (1)				0.9 (1)	1.0 (1)					3
Lys		0.9 (1)	1.0 (1)	1.1 (1)	0.9 (1)						1.0 (1)		4
Arg		1.0 (1)				1.0 (1)	0.9 (1)	1.0 (1)	1.0 (1)				5
Amount (nmol) ...		6.0	2.7	11.5	12.3	9.1	11.6	18.3	16.5	9.1	12.7	8.5	
Sequence position ...		1–32	16–32	33–36	37–39	40–43	44–62	63–72	73–78	79–84	85–86	87–92	92*

terminated. Reduction, alkylation and tryptic digestion of its large chain, as well as peptide separation, were performed. Two tryptic peptides were sequenced. The amino acid sequences of these peptides were aligned with those of the same region of the mustard allergens (Figure 6).

Immunodetection of a synthetic multiantigenic peptide by 2B3 and human sera

A MAP has been chemically synthesized in order to study the

antigenic determinant specific of the mustard allergens. This peptide (13.8 kDa) was designed considering the sequence around the only Tyr of the large chains (Figure 6). This Tyr is involved in the recognition by 2B3 [5]. The segment selected comprises the 14-amino-acids sequence QGPHVISRIYQTAT, which corresponds to the positions 55–68 of *Sin a* I. The sequence data obtained for the non-acetylated peptide match perfectly with the expected molecule. As shown in Figure 3, this synthetic peptide was recognized by 2B3, demonstrating that the amino acid sequence studied contains the same antigenic determinant present

Figure 5 Amino acid sequences obtained for the small (a) and large (b) chains of *Bra j* IE (B) in comparison with those of *Sin a* I (S)

Tryptic peptides are prefixed by a 'T'. Right-pointing arrows correspond to residues identified by automatic Edman degradation. Continuous thick arrows indicate the residues identified from the whole polypeptide chains. The Gln N-terminal residue of the large chain was previously removed by L-pyroglutamate aminopeptidase treatment (see the Experimental section). 'x' denotes gaps on these positions for *Sin a* I. The sequence data reported will appear in the SWISS-PROT database under the accession no. P80207.

Figure 6 Amino acid sequences of *Bra j* IE, *Sin a* I and napin BnIII around the site recognized by 2B3

Numbering is given according to the primary structure of *Bra j* IE. Large arrows indicate sequenced peptides isolated from BnIII. The segment covered by a thick line constitutes the sequence of the chemically synthesized peptide (MAP).

in the large chains of the mustard allergens. These facts are corroborated by the results shown in Figure 7: the MAP-specific sera obtained after immunization of rabbits bind to the reduced large chains of the mustard allergens. It is also noteworthy that the MAP synthesized was a very good immunogen, since a high titre was obtained in rabbits after the second immunization.

More interestingly, this synthetic peptide was recognized by the IgE of mustard-allergic patients (Figure 8). In fact, most of the mustard-sensitive human sera tested (7 among eleven) bound the MAP. Therefore this synthetic peptide would contain one of the polypeptidic structures responsible for the allergenicity of the mustard allergens. On the other hand, all the mustard-sensitive human sera recognized the large chains of these proteins (Figure 8). This is a specific reaction, since none of the samples tested was recognized either by the sera of olive (*Olea europaea*)-pollen-allergic individuals or by that of non-allergics used as negative controls.

Figure 7 Immunoblotting with MAP-specific rabbit antiserum staining

Immunodetection with MAP-specific polyclonal serum after acidic PAGE and electrotransfer to ProBlott membranes, of reduced *Sin a* I (S) and MAP (M). The samples (2 µg) were reduced prior to electrophoresis by heating at 80 °C for 20 min in the presence of 2-mercaptoethanol. The electrophoretic patterns of the samples analysed are shown in (a) and the corresponding immunoblottings in (b).

DISCUSSION

The allergenicity of the 2 S albumin fraction from *B. juncea* seeds has been previously reported [6]. Thus this allergen was termed *Bra j* I, as recommended [21]. On the other hand, the existence has been suggested of a continuous epitope on the large

Figure 8 Immunoblotting with sera from allergic patients

Immunodetection with IgE of mustard-sensitive individuals, after acidic PAGE and electrotransfer to ProBlott membranes, of reduced *Sin a* I (4.8 μ g) and MAP (3 μ g). The sera from the 11 patients available are identified by their initials. Immunodetection of the same samples was carried out with olive-pollen-hypersensitive human sera (RM) as negative control. For each serum the strip corresponding to MAP is located on the left and that of *Sin a* I on the right.

chain of the major allergen from yellow-mustard (*Sinapis alba*) seeds, *Sin a* I [5]. Such epitope involves the only Tyr residue of this allergen, and it is recognized by the *Sin a* I-specific mAb 2B3 [5]. This mAb also reacts with the crude 2 S fraction from oriental mustard, but does not recognize the 2 S albumin fractions isolated from other seeds of related crucifers, like rapeseed, turnip (*Brassica nap*a), cabbage (*Brassica oleracea*) or radish (*Raphanus sativus*) (results not shown). Therefore it could be expected that *Sin a* I and *Bra j* I, yellow- and oriental-mustard allergens respectively, would bear the same, or a very similar, epitope conserved in their structures. This antigenic determinant would be specific of mustard allergens.

Although the *Bra j* I allergen shows a significant microheterogeneity on ion-exchange chromatography, the five isoallergenic fractions (*Bra j* IA–*Bra j* IE) obtained from it bind mAb 2B3. Therefore all of them would bear the epitope recognized by that *Sin a* I-specific mAb. Structural studies require the analysis of well-defined molecular species. Thus an efficient method was employed to determine the heterogeneity in the 2 S albumins from seeds [22,23]. This procedure involves the reduction of the native protein and reverse-phase h.p.l.c. of the resulting products. By using this method we observed that the *Bra j* IE isoallergenic fraction displays a very low degree of heterogeneity (Figure 2). Moreover, *Bra j* IE is the main component of the 2 S fraction from *B. juncea*. Consequently, this allergenic fraction was selected among the *B. juncea* 2 S albumins for further analyses.

Acidic PAGE in the presence of urea, blotting and immunostaining reveal the specific binding of mAb 2B3 to the reduced large chains of both *Sin a* I and *Bra j* IE, whereas no binding was detected for the reduced light chains of these allergens, nor for reduced chains of rapeseed napin BnIII. Therefore the epitope of mustard allergens recognized by 2B3 must be located in the large chain of these molecules.

The complete sequences of the two polypeptide chains of the oriental-mustard allergen *Bra j* IE were determined. The alignment of this sequence with that of *Sin a* I reveals an 89% identity. Most of the differences between *Bra j* I and *Sin a* I are located on a Gln-rich segment (Figure 5). This region, near the tyrosine residue involved in the recognition by mAb 2B3 [5], has been described as an hypervariable region in napin-like proteins, as deduced from the nucleotide sequences of the *B. napus*, *Arabidopsis thaliana* (thale cress) and *Raphanus sativus* (radish)

2 S albumin-encoding genes [8,24–26]. Other differences are related to the presence of extra residues at both terminal ends of the chains (Figure 5), which could result from the different specificity of maturation proteinases during the post-translational processing and would depend on the protein sequence recognized, as was pointed out for napins [23].

The amino acid sequence of the napin BnIII large chain around the Tyr residue has been also elucidated. The alignment of *Sin a* I, *Bra j* IE and BnIII in this region (Figure 6) shows that positions 60–71 (numbering according to that for *Bra j* IE) are identical in the three proteins. Moreover, *Bra j* IE and napin BnIII show total identity between positions 47 and 57. Thus His-58 is the only amino acid appearing in both mustard allergens but not in napin BnIII in this polypeptide segment. According to these results and the immunological data, this residue, His-58, would appear to have a crucial role in defining the antigenic properties of the specific epitope of mustard allergens. It is well known that peptides with sequence variations at the level of a single amino acid residue can greatly affect the immunological response [27,28], and the absence of His in that position of the large chain of BnIII would justify its lack of binding by 2B3. On the other hand, Val-55 of *Sin a* I large chain does not seem to play a significant role in the recognition by mAb 2B3, since it is replaced by Glu in *Bra j* IE without a remarkable effect on the binding.

The binding of mAb 2B3 to a synthetic multiple antigenic peptide (MAP), which contains the region considered, around His-58 and Tyr-64, as well as the binding of MAP-specific rabbit sera to the large chains of mustard allergens, would confirm the existence of a common epitope on such region of these proteins, at least as a 'primary' interaction site [29]. Moreover, this antigenic determinant seems to be involved also in the allergic response of mustard-sensitive individuals or at least in a significant number of them, although the existence of other allergenic epitopes cannot be ruled out. This finding could be of importance for future analyses, since this epitope, contained in a limited polypeptide segment, could be used or modified for studying the mechanisms that originate allergic responses in human beings.

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