
Rafael I. MONSALVE,* Manuel A. GONZALEZ DE LA PEÑA,* Luis MENENDEZ-ARIAS,*§ Carlos LOPEZ-OTIN,† Mayte VILLALBA* and Rosalia RODRIGUEZ‡

*Departamento de Bioquímica y Biología Molecular, Facultad de Química, Universidad Complutense, 28040 Madrid, and †Departamento de Biología Funcional, Facultad de Medicina, Universidad de Oviedo, 33006 Oviedo, Spain

*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 1*, 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 1* 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 1* (Mr 14,100) 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 1***

Pure mustard flours were kindly provided by Professor Albert A. Schneiter (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 1*) and napin BnIII were purified from *Sinapis alba* L. and *B. napus* seeds respectively as previously described [3,9]. Oriental-mustard allergen (*Bra j 1*) 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 1* 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 spectrophotically [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
of the disulphide bonds and radioalkylation with iodo-
[14C]acetamide (1 μCi/5 μl; 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 pyrroglutamate 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 immunodetection
Sera from 11 allergic individuals exhibiting a positive radio-
allergosorbent test (values 3–4) were used. The Sin a 1-specific
monoclonal antibody (mAb) 2B3 was obtained in BALB/c mice
as previously described [5]. Rabbit polyclonal sera against Sin a
1 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.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)
succrose. 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 Electro-
phoretic 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 1-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 1:100 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 washed
in 0.3 mg/ml solution of 3,3'-diaminobenzidine in PBS,
containing 0.005 % H2O2. 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 (Lys5-Lys5-Lys5) was
prepared. Pepsin gel resin (Milligen) was maintained overnight
in ethylenediamine. The resin was then washed with dimethyl-
formamide (DMF) and with 10 % (w/v) N,N-di-isopropyl
amine in DMF. Afterwards, the coupling was achieved by
adding the p-hydroxyphenyloxycetic active ester, which was
prepared by mixing 1-hydroxybenzotriazole in DMF and
diisopropyl carbodi-imide with a p-hydroxyphenyoxycetic 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 tBoc-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 NH2
groups by washing twice with 20 % (v/v) piperidine in DMF.
The amino acid derivative was 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 (QGPHVISRITYQTAT)
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/3 % (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
primary 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 carboxymidomethylation 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](image1.png)

**Figure 1** Curves for the binding of the Sin a 1-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 1; and ●, napin BnIII) and binding was measured by adding horseradish peroxidase-labelled goat anti-mouse IgG (G+H).

![Graph](image2.png)

**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.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Small chain</th>
<th>Large chain</th>
<th>Native protein</th>
<th>Native protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys</td>
<td>2</td>
<td>6</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Asx</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Thr</td>
<td>–</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Ser</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Gln</td>
<td>8</td>
<td>23–24</td>
<td>32</td>
<td>28</td>
</tr>
<tr>
<td>Pro</td>
<td>3</td>
<td>12</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Gly</td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>8.5</td>
</tr>
<tr>
<td>Ala</td>
<td>4</td>
<td>3</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Val</td>
<td>–</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Met</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Ile</td>
<td>0–1</td>
<td>5</td>
<td>5–6</td>
<td>4</td>
</tr>
<tr>
<td>Leu</td>
<td>2</td>
<td>7</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Tyr</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Phe</td>
<td>2–3</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>His</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Lys</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Arg</td>
<td>4</td>
<td>5</td>
<td>9</td>
<td>5.5</td>
</tr>
<tr>
<td>Trp</td>
<td>1</td>
<td>–</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>37–38</td>
<td>90–91</td>
<td>128–129</td>
<td>127</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Small chain</th>
<th>Large chain</th>
<th>Native protein</th>
<th>Native protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys</td>
<td>2</td>
<td>6</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Asx</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Thr</td>
<td>–</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Ser</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Gln</td>
<td>8</td>
<td>23–24</td>
<td>32</td>
<td>28</td>
</tr>
<tr>
<td>Pro</td>
<td>3</td>
<td>12</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Gly</td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>8.5</td>
</tr>
<tr>
<td>Ala</td>
<td>4</td>
<td>3</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Val</td>
<td>–</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Met</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Ile</td>
<td>0–1</td>
<td>5</td>
<td>5–6</td>
<td>4</td>
</tr>
<tr>
<td>Leu</td>
<td>2</td>
<td>7</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Tyr</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Phe</td>
<td>2–3</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>His</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Lys</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Arg</td>
<td>4</td>
<td>5</td>
<td>9</td>
<td>5.5</td>
</tr>
<tr>
<td>Trp</td>
<td>1</td>
<td>–</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>37–38</td>
<td>90–91</td>
<td>128–129</td>
<td>127</td>
</tr>
</tbody>
</table>

**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. a titration with 2B3, a monoclonal antibody raised against the yellow-mustard allergen Sin a 1. It has been proposed that 2B3 recognizes a continuous epitope in the large chain of Sin a 1, since it binds to
the unfolded polypeptide [5]. This antibody recognizes the five 
Braj 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 1. 
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 Braj I and Sin a 1 [7,8].

The five Braj 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, Braj IA, Braj IB 
and Braj IC display great heterogeneity. However, Braj ID 
and Braj 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, Braj 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 carboxamidomethylated small and large chains of Braj 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 1 and Braj 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.i.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 albums.

Amino acid sequence of Braj 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 
Braj 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 
trypptic peptides was performed by considering the structural 
similarity between Sin a 1 and Braj 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 Braj IE small chain (Ile/Phe and 
Arg/Lys, at positions 6 and 20 respectively) would explain the 
minor heterogeneity displayed on both h.p.l.c. (Figure 2) and 
acidic electrophoresis (Figure 3). The large chain of Braj IE 
contains four more residues than the corresponding chain of 
Sin a 1: 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 1, Braj 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, electrophoresis and immunodetection of: (1) Sin a 1; (2) napin BnIII; (3) Braj 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 d) Immunoblot of replicates of the gel (a) and line 
(d). 4 respectively immunostained with the Sin a 1-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 Braj 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 spectrophotometrically. 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

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Peptide</th>
<th>T1</th>
<th>T2</th>
<th>T2'</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T5'</th>
<th>T6</th>
<th>T6*</th>
<th>T7</th>
<th>T7'</th>
<th>Complete chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys</td>
<td>0.6 (1)</td>
<td>0.9 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asx</td>
<td>1.0 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>1.0 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>0.8 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glx</td>
<td>2.4 (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>4.2 (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>0.9 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>0.9 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>0.9 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Met</td>
<td>0.7 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>0.9 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>0.9 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>0.9 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>0.9 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>0.9 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>0.9 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp</td>
<td>+ (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amount (nmol)</td>
<td>10.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sequence position</td>
<td>1–5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(b) Large chain

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Peptide</th>
<th>T1</th>
<th>T1'</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
<th>T7</th>
<th>T8</th>
<th>T9</th>
<th>T10</th>
<th>Complete chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys</td>
<td>3.5 (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asx</td>
<td>1.4 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>1.0 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>0.8 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glx</td>
<td>2.4 (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>2.4 (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>2.9 (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>1.0 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>1.1 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Met</td>
<td>0.8 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>1.0 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>1.0 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>1.0 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>0.9 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>0.9 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>0.9 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amount (nmol)</td>
<td>6.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sequence position</td>
<td>1–32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Characterization of a new mustard allergen

Table 2 presents the 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 spectrophotometrically. 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

- **Amino acid Composition**:
  - Cys: 0.6 (1) 0.9 (1)
  - Asx: 1.0 (1)
  - Thr: 1.0 (1)
  - Ser: 0.8 (1)
  - Glx: 2.4 (2)
  - Pro: 4.2 (4)
  - Gly: 0.9 (1)
  - Ala: 0.9 (1)
  - Val: 0.9 (1)
  - Met: 0.7 (1)
  - Leu: 0.9 (1)
  - Tyr: 0.9 (1)
  - Phe: 0.9 (1)
  - His: 0.9 (1)
  - Lys: 0.9 (1)
  - Arg: 0.9 (1)
  - Trp: + (1)
  - **Amount (nmol)**: 10.0
  - **Sequence position**: 1–5

(b) Large chain

- **Amino acid Composition**:
  - Cys: 3.5 (4)
  - Asx: 1.4 (1)
  - Thr: 1.0 (1)
  - Ser: 0.8 (1)
  - Glx: 2.4 (2)
  - Pro: 2.4 (2)
  - Gly: 2.9 (3)
  - Ala: 1.0 (1)
  - Val: 1.1 (1)
  - Met: 0.8 (1)
  - Leu: 1.0 (1)
  - Tyr: 1.0 (1)
  - Phe: 1.0 (1)
  - His: 0.9 (1)
  - Lys: 0.9 (1)
  - Arg: 0.9 (1)
  - **Amount (nmol)**: 6.0
  - **Sequence position**: 1–32

**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 1. The sequence data obtained for the non-acetylated peptide match perfectly with the expected data. 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.
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.

**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 1*, 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 1 (4.8 µg) and MAP (3 µg). 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 1 on the right.

chain of the major allergen from yellow-mustard (Sinapis alba) seeds, Sin a 1 [5]. Such epitope involves the only Tyr residue of this allergen, and it is recognized by the Sin a 1-specific mAb 2B3 [5]. This mAb also reacts with the crude 2S fraction from oriental mustard, but does not recognize the 2S albumin fractions isolated from other seeds of related crucifers, like rapeseed, turnip (Brassica napus), cabbage (Brassica oleracea) or radish (Raphanus sativus) (results not shown). Therefore it could be expected that Sin a 1 and Bra j 1, 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 1 allergen shows a significant microheterogeneity on ion-exchange chromatography, the five isoallergenic fractions (Bra j 1A–Bra j 1E) obtained from it bind mAb 2B3. Therefore all of them would bear the epitope recognized by that Sin a 1-specific mAb. Structural studies require the analysis of well-defined molecular species. Thus an efficient method was employed to determine the heterogeneity in the 2S albumins from seeds [22,23]. This procedure involves the reduction of the native protein and reverse-phase hplc. of the resulting products. By using this method we observed that the Bra j 1E isoallergenic fraction displays very a low degree of heterogeneity (Figure 2). Moreover, Bra j 1E is the main component of the 2S fraction from B. juncea. Consequently, this allergenic fraction was selected among the B. juncea 2S 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 1 and Bra j 1E, 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 1E were determined. The alignment of this sequence with that of Sin a 1 reveals an 89% identity. Most of the differences between Bra j 1 and Sin a 1 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) 2S 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 1, Bra j 1E and BnIII in this region (Figure 6) shows that positions 60–71 (numbering according to that for Bra j 1E) are identical in the three proteins. Moreover, Bra j 1E 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 1 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 1E 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.

This research was supported by grant PB89/087 from the Dirección General de Investigación Científica y Técnica (Spain). We thank Dr. M. L. Gonzalez and Dr. M. I. Esteban (Unidad de Alergía, Hospital General de Segovia) for providing the mustard-
sensitive human sera and Dr. C. Lahoz for olive-pollen-sensitive patients sera. We also acknowledge Dr. J. G. Gavilanes for his helpful discussions.

REFERENCES


Received 24 July 1992/9 February 1993; accepted 16 February 1993