

Detection, isolation and complete amino acid sequence of an aeroallergenic protein from rapeseed flour

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Summary

Background Seed proteins have been found to cause hypersensitivity by ingestion or inhalation. Rapeseed flour was responsible for allergic symptoms in a patient, who develops into allergy to mustard spice.

Objective To determine the presence of allergenic proteins in rapeseed flour, and analyse the structure of the main component and its crossreactivity with the mustard allergen.

Methods SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and subsequent immunoblotting with a serum from a rapeseed allergic patient were performed to detect IgE-binding proteins. Proteolytic digestions and high performance liquid chromatography were used to obtain the peptides from the allergenic BnIII napin from rapeseed flour. Automatic Edman degradations were carried out to determine their amino acid sequences, which were compared with other sequences in nucleotide and amino acid sequence databases. Crossreactivity assays were carried out by ELISA inhibition using sera from a rapeseed allergic patient and from patients allergic to mustard.

Results The 2S albumins of rapeseed were recognized by the serum from a patient allergic to this seed. The most abundant isoform of the allergenic napins, BnIII, was used for structural and immunological analysis. The protein consists of two different chains of 9.5 and 4.5 kDa. Their complete amino acid sequences were determined. The protein exhibited structural relationships with other napin-like storage proteins from seeds. IgE and IgG crossreactivity between rapeseed and mustard allergens was also demonstrated. Considering the structural and immunological data, certain polypeptide regions are suggested to be involved in the allergenicity of these proteins.

Conclusions Rapeseed contains 2S storage proteins which may cause allergy in hypersensitive individuals. These proteins exhibit great sequence similarity with 2S albumins from different seeds. Crossreactivity between mustard and rapeseed flours can be explained by sequence homology.

Keywords: 2S albumin, amino acid sequence, *Brassica napus*, crossreactivity, napin, rapeseed, seed allergen

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Introduction

Occupational allergy is a common disease among workers in seed-processing industries [1–3]. The allergenic components

of the seeds are mainly soluble, low molecular weight proteins, as it has been found in wheat [4], cottonseed [5], soybean [6], and mustard [7]. Rapeseed (*Brassica napus* L.) is one of the most important oil seed annual crops in the world, since it represents one of the main resources for animal nutrition. There are two major types of storage proteins: 2S albumins (also called napins) and 12S globulins

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[8]. These proteins are synthesized in developing embryos during seed maturation and have a nitrogen storage function.

In this study, we report on the allergenicity of rapeseed storage napins, as well as on the complete amino acid sequence of BnIII, the most abundant allergenic isoform expressed in *Brassica napus*. Moreover, its crossreactivity with Sin a 1, the major allergen from yellow mustard seeds [7], is demonstrated.

Case report

A 48-year-old man suffered episodes of wheezing with shortness of breath after exposure to rapeseed flour. The patient had no previous history of atopy, asthma or other pulmonary diseases. There was no family history of atopy and the rest of his personal medical history was unremarkable.

The patient had been working in a feed processing plant, handling cereals, soybean, sunflower seeds, meat, fish and feather flour for 20 years. After 5 years with intermittent exposure to rapeseed flour, the patient noted sneezing and rhinorrhoea whenever he drove to the rapeseed flour store while the rapeseed flour was being discharged from the mill. The symptoms developed into wheezing and shortness of breath by the time he arrived at the store and they went away two hours after the exposure ended. Four years later, the patient suffered some episodes of oedema and pruritus in the lips, oral mucosa and the pharynx and facial urticaria immediately after ingestion of a small amount of mustard sauce. At present he is following a mustard-free diet, is avoiding inhaling rapeseed, and is not exhibiting any further symptoms.

The chest roentgenogram revealed no abnormality. Skin tests by the skin-prick method with common allergens (dust, mites, pollen, moulds and dander) and with cereals, soybean, sunflower seeds, fish, egg, mustard and rapeseed flour extracts were positive only to rapeseed flour and mustard extracts.

Materials and methods

Extract and skin tests

Proteins were extracted by incubation of rapeseed (*Brassica napus* L. seeds) flour in saline solution at a concentration of 10 mg/mL for 1 h with magnetic stirring at 20°C. The material was centrifuged at 5000 r.p.m. at 5°C for 15 min, and the supernatant filtered through Millipore membranes (0.45 µm). The filtered solution was used the same day. Other extracts were provided by Bayer.

Skin tests were performed by conventional skin-prick test on the volar surface of the forearm with inhalants (dust, mites, pollen, moulds and dander) and with cereals, soybean, sunflower seeds, fish, egg, rapeseed flour and mustard

extracts. Glycerol saline solution and histamine hydrochloride (10 mg/mL) were used as negative and positive controls, respectively. Readings were taken at 15 min; the greatest diameter and perpendicular of the resulting weal were measured. The mean of both diameters was used as the size of the test. Tests greater than 3 mm were assessed as positive [9].

Antigens

Napin BnIII was isolated from rapeseed flour as described by Monsalve and Rodríguez [10]. Separation of the small and large chains constituting the native protein was carried out after reduction of the disulphide bonds with 2-mercaptoethanol and carboxyamidomethylation with [¹⁴C]-iodoacetamide as previously described [7]. The radioalkylated chains were chromatographed on a Nucleosil C-18 reverse phase column, using a high pressure liquid chromatography (RP-HPLC) system, with a 20–40% linear gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid. The eluent was continuously monitored at both 214 and 280 nm.

Mustard allergen was isolated as described by Menéndez-Arias *et al.* [11].

Protein characterization

Amino acid analyses of protein and peptides were performed on a Beckman 6300 Amino Acid Analyzer after acid hydrolysis under the conditions previously described [7]. Tryptophan was determined spectrophotometrically according to the method of Bencze and Schmid [12]. Protein concentrations were calculated by amino acid analysis.

Electrophoresis and immunoblotting

Protein samples (5 µg) were analysed by SDS-PAGE according to Laemmli [13] in polyacrylamide gels. Proteins were visualized by Coomassie Brilliant Blue staining or electrophoretically transferred onto nitrocellulose membranes (Bio-Rad) for immunostaining as described [14].

For the IgE binding analysis, the membranes were incubated for 2 h at room temperature in blocking buffer (10 mM sodium phosphate, pH 7.2, containing 140 mM NaCl, 2.7 mM KCl, and 0.1% v/v Tween 20 and 3% w/v skim milk). The strips were then incubated 2 h at room temperature with the serum from a patient allergic to rapeseed flour (diluted 1:5), or with a pool of sera from patients allergic to mustard flour (diluted 1:10) prepared as described [15]. The nitrocellulose strips were incubated 1 h with a mouse anti-human IgE monoclonal antibody (kindly provided by Dr Lombardero, Abello S.A.) diluted 1:5000 in blocking buffer, and then incubated 1 h with a goat anti-mouse IgG antibody labelled with horseradish peroxidase (Pierce

Chemical Co.) diluted 1:5000 in blocking buffer. The peroxidase reaction was developed with the ECL Western blotting reagent (Amersham Corp.) according to the manufacturer's recommendations.

ELISA titration and inhibition assays

ELISA IgE titration and inhibition assays were performed as previously described [15] with minor modifications. Microtitre plates (high-binding, flat-bottomed, 96-well; Costar) were coated with 100 μ L per well of antigen (0.1 μ g/mL) in phosphate buffered saline (PBS), and saturated with blocking buffer. Afterwards, 100 μ L aliquots of the different serum dilutions, or the inhibitor-serum co-incubations, were added and incubated at 37°C for 2 h. These co-incubations were carried out with the serum from the rapeseed-sensitive patient (diluted 1:5), or with the pool of sera from mustard-sensitive individuals (diluted 1:10), or with rabbit antisera raised against Sin a 1 (diluted 1:100 000). Binding of the human IgE was detected by mouse anti-human IgE monoclonal antibody 1:5000 diluted in blocking buffer, followed by horseradish peroxidase-labelled goat anti-mouse IgG diluted 1:5000 in blocking buffer. Binding of polyclonal IgG was detected by horseradish peroxidase-labelled goat anti-rabbit IgG (Bio-Rad) diluted 1:3000 in blocking buffer. The peroxidase reaction was performed as described [15].

Protein sequencing

The alkylated small chain (30–50 nmol) was alternatively digested with trypsin and chymotrypsin, and treated with cyanogen bromide (CNBr). Alkylated large chain (50–100 nmol) was digested with chymotrypsin and clostridiopeptidase B, and CNBr treated. All of these treatments were carried out as previously described [7]. Separation of the peptides was performed by RP-HPLC on a μ Bondapak C-18 column. The flow rate was 1.5 mL/min. Different acetonitrile gradients, in 0.1% (v/v) trifluoroacetic acid, were employed for the elution of the digested materials. Detection at 214 nm wavelength was continuously monitored. Radioactive peptides were detected by counting 25 μ L aliquots from each collected fraction.

NH₂-terminal Edman degradations of the whole alkylated chains and of the obtained peptides were performed on an Applied Biosystems model 477 A sequencer [16]. The resulting phenylthiohydantoin-amino acid derivatives were identified by using a model 120 A on-line phenylthiohydantoin-analyser and the standard Applied Biosystems program.

The alignment of homologous protein sequences was obtained according to Needleman and Wunsch algorithm, by using the PAPA programs of Doolittle and Feng [17].

Results

IgE binding to rapeseed extract and napins

The protein components present in the saline extract of rapeseed flour were analysed for their binding to the serum of a patient with allergic symptoms to rapeseed (Fig. 1). After blot immunodetection, only three protein components, with apparent molecular mass about 14, 16 and 30 kDa, were recognized in the extract by the IgE antibodies.

The napins isolated from rapeseed can be separated in five discrete fractions by a gel filtration Sephadex G-50 column followed by ion exchange chromatography [10]. The IgE immunoblotting performed on the fractions eluted from gel filtration indicates that only napins possess significant IgE binding activity (data not shown). After ion-exchange fractionation of napins, four peaks containing BnI, BnII, BnIII, and BnIV were analysed by Coomassie Blue staining and immunoblotting (Fig. 1A). Figure 1 also shows the strong tendency of napins to form dimer and trimer structures (bands of 30 kDa and 45 kDa, respectively). All these proteins are recognized by the IgE antibodies of the serum from the individual allergic to rapeseed (Fig. 1B), although with different response intensities, BnI being the least reactive. Interestingly, the protein aggregates showed strong IgE binding capability as was determined by densitometric analysis of the Coomassie staining and immunoblotting patterns of monomers and aggregates.

Sequencing of BnIII

BnIII is the most abundant napin in rapeseed [10], representing more than 30% of all the 2S storage proteins isolated from these seeds. This napin is an homogeneous protein, since the RP-HPLC fractionation of its reduced and carboxyamidomethylated polypeptide chains shows only one main component for both small (SC) and large chain (LC). The remaining rapeseed napins exhibit a certain degree of microheterogeneity [18]. These factors were taken into account when selecting this napin for structural and immunological analysis.

The amino acid compositions of BnIII and its polypeptide chains are given in Table 1. They show the characteristic composition of a storage protein, with a high content of Gln, Pro and Cys.

In order to determine the complete primary structure of BnIII, its polypeptide chains were hydrolysed by different enzymatic and chemical treatments, and the resulting peptides fractionated on RP-HPLC. SC was digested alternatively with trypsin and chymotrypsin, and the LC was treated with clostridiopeptidase B and chymotrypsin (Fig. 2). Some of the peptides obtained on the HPLC column were rechromatographed by using a different gradient of acetonitrile concentration. Amino acid compositions of the

purified peptides were determined and most of them were sequenced by means of Edman degradation (Fig. 3). The NH₂-terminal sequence of each reduced and alkylated chains were determined. With the exception of position 21 of the SC, in which Lys and Arg were found, a single residue was obtained at each cycle of sequence. Both polypeptide chains were also cleaved using CNBr in order to confirm the COOH-terminal sequences. The strategy for sequencing and the obtained primary structure are shown in Fig. 3. Both polypeptide chains exhibited microheterogeneities in their sequences: besides the Lys/Arg 21 of SC, Gly and Ser were found at the 3rd position of LC, as well as Lys and Arg in position 68. Microheterogeneities and secondary cleavages resulted in the appearance of a number of peptides higher than expected (e.g. peptides C-2' and C-2'' in SC, and peptides C-3 and C-3' in LC).

The primary structure of BnIII has been compared with those deduced from the nucleotide sequence data of 2S albumins from rapeseed [19–22], with the amino acid sequence of the major allergen from yellow mustard seeds [7], and with a low molecular weight napin, BnI [23] (Fig. 4). The alignment shows the great similarity between BnIII and the other 2S albumins.

Crossreactivity between BnIII and the major mustard allergen

Indirect ELISA titration of purified proteins showed that the major mustard allergen, Sin a 1, is recognized by the IgE antibodies present in the serum of the rapeseed allergic patient at a rate similar to that of BnIII (Fig. 5a).

In order to detect allergenic determinants shared by BnIII and Sin a 1, ELISA inhibition experiments were performed with the serum from the patient allergic to rapeseed flour. BnIII and Sin a 1 showed differences in their capability to inhibit the binding of IgE from the allergic serum to each other. Sin a 1 inhibited only a 60% of the IgE recognition of solid-phase fixed BnIII by these antibodies (Fig. 5b), whereas BnIII is able to completely inhibit the binding to Sin a 1 (Fig. 5c).

The presence of common epitopes in BnIII and Sin a 1 was also corroborated by using anti-Sin a 1 IgG and IgE antibodies. The results showed that binding to solid-phase fixed Sin a 1 of both rabbit anti-Sin a 1 IgG (Fig. 6a) and IgE from mustard allergic human sera was 80–90% inhibited by BnIII (Fig. 6b). The inhibition capability of BnIII was slightly greater than that of Sin a 1.

Discussion

Hypersensitivity to seed flour has been documented among workers of seed-processing manufacturers [1–3]. However, oilseed rape allergy has been reported as an apparent

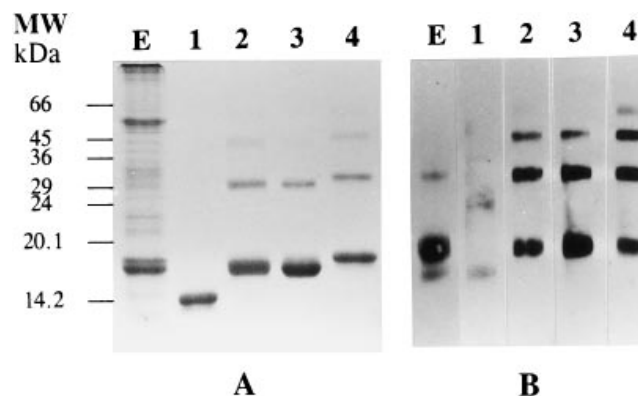


Fig. 1. SDS-PAGE of rapeseed extract (lane E) and purified rapeseed napins BnI (lane 1), BnII (lane 2), BnIII (lane 3) and BnIV (lane 4). (A) Coomassie Brilliant Blue staining; (B) autoradiography of the same samples after immunoblotting with serum from a rapeseed-sensitive patient. (MW) molecular weight markers.

consequence of the inhalation of pollen particles [24–26], since respiratory damage and hay fever are suffered by individuals exposed during the flowering period [25,27]. Allergy to other members of the *Brassicaceae* family, such

Table 1. Amino acid composition of BnIII and its small (SC) and large (LC) chains. Numbers in parenthesis indicate sequence data

	SC	LC	BnIII*
Cys**	1.8 (2)	5.6 (6)	8
Asx	– (0)	2.1 (2)	2
Thr	– (0)	3.4 (4)	4
Ser	2.9 (3)	3.5 (4)	7
Glx	8.2 (8)	22.4 (23)	31
Pro	2.3 (2)	11.3 (11)	13
Gly	3.2 (3)	5.4 (95)	8
Ala	3.9 (4)	3.0 (3)	7
Val	– (0)	6.3 (7)	7
Met	0.7 (1)	0.7 (1)	2
Ile	0.8 (1)	2.4 (3)	4
Leu	1.5 (2)	6.7 (7)	9
Tyr	– (0)	0.9 (1)	1
Phe	2.0 (2)	1.0 (1)	3
His	1.9 (2)	2.1 (2)	4
Lys	3.4 (3)	3.4 (5)	8
Arg	2.6 (3)	3.0 (3)	6
Trp**	(1)	(0)	1
Total	(37)	(88)	125

* Data obtained from sequencing.

** Cys was determined as carboxymethylcysteine; Trp was determined spectroscopically.

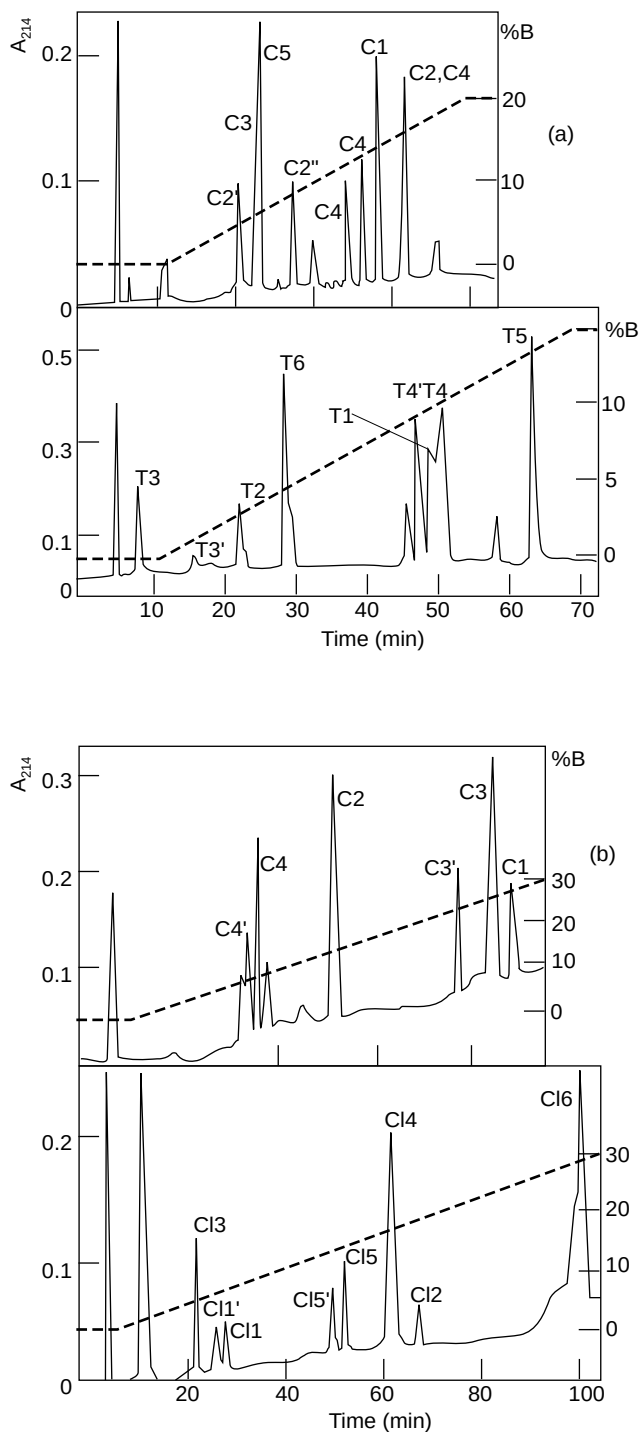


Fig. 2. Elution profiles of the RP-HPLC on a μ Bondapak C-18 column of: (a) chymotryptic (C peptides, above) and tryptic (T peptides, below) hydrolysates of the small chain of BnIII; (b) chymotryptic (C peptides, above) and clostridiopeptidase B (Cl peptides, below) hydrolysates of the large chain of BnIII. The nomenclature of the peptides corresponds to their position in the sequence. The peptides were eluted with an acetonitrile gradient (%B; —) in 0.1% trifluoroacetic acid.

as cabbage or mustard, has only been reported as induced by their seeds, by ingestion or by contact [28–30].

Allergy to inhaled rapeseed flour is caused by the 2S storage napins. These polypeptide components of the seed have been fractionated, and the presence of napin-binding IgE antibodies in the serum of a patient allergic to rapeseed has been demonstrated (Fig. 1). Considering the two types of napins found in rapeseed -15 kDa and 12 kDa variants-, the former showed greater capability to bind IgE from the allergic patient. The major allergens from yellow and oriental mustard seeds, which induce allergy by ingestion, have also been found in their 2S albumin fractions [7,31]. Therefore, inhalant rapeseed flour allergy and food mustard allergy are caused by their homologous 2S albumins, BnII, BnIII, BnIV and Sin a 1. This fact contrasts with the respiratory and food allergies to soybean (*Glycine max*) seeds, which have been described as induced by different type of seed proteins. The hydrophobic proteins of soybean (HPS, 8 kDa) have been shown to be responsible for the inhalant symptoms, asthma and rhinitis after exposure to soybean dust and flour [32], whereas 2S albumins (21 kDa trypsin inhibitor), 7S albumins (β -conglycinin) and 11S fraction (glycinin) were identified as those involved in food symptoms [33]. On the other hand, Baker's Asthma, an occupational allergy of high prevalence in the baking industry, is caused by the inhalation of cereal flours, in which the 12–15 kDa albumins (α -amylase inhibitor proteins) are the allergens responsible for this hypersensitivity [4,34].

Although the nucleotide sequences of several genes encoding napins are known, scarce data exist about their whole mature structures. Moreover, the exact processing position of the polypeptide precursor of these proteins is goal of controversy. Therefore, in order to study the rapeseed allergy at the molecular level, the amino acid sequence of BnIII, the most abundant natural napin expressed in the seeds, was determined by Edman degradation. The protein exhibits the general properties of a 2S albumin, but its sequence does not match exactly with any other known napin, confirming the high polymorphism of these proteins [7,19,20]. The great sequence similarity exhibited by these isoforms explains the positive reactions of the ion-exchange fractionated napins (BnII, BnIII and BnIV) against the serum from the patient allergic to rapeseed flour. Recently, it has been shown that *Brassica napus* and *B. rapa* pollen extracts contain proteins recognized by the IgE from sera of patients allergic to rape [35]. These proteins, which have been considered as allergens and named Bra n I, Bra n II, Bra r I and Bra r II, have been cloned and sequenced and consist of a single polypeptide chain of about 83 amino acids [35]. Their primary structures do not show similarity with that of BnIII. Moreover, the IgE from the serum of the patient allergic to rapeseed flour did not bind to proteins

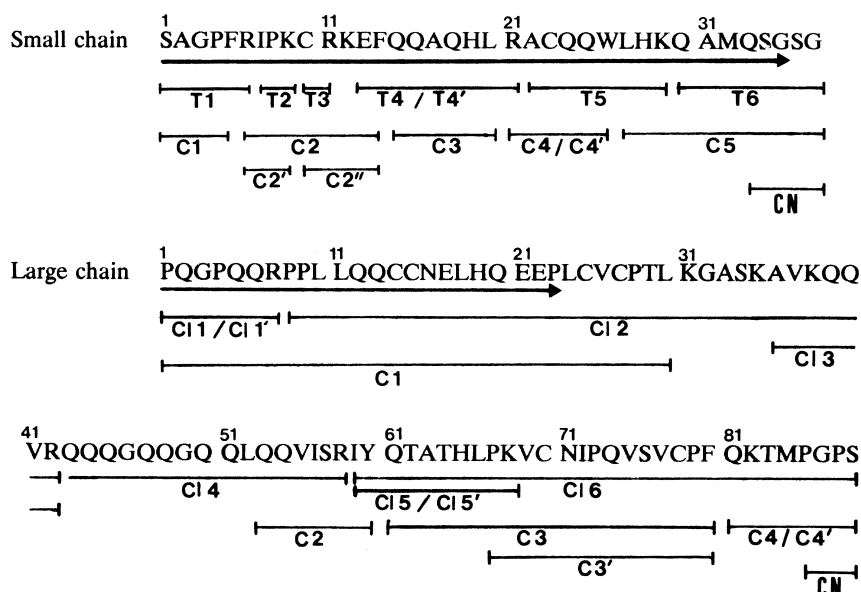


Fig. 3. Primary structure of the small and large chains of BnIII. Sequenced peptides obtained by using trypsin (T), chymotrypsin (C), clostridiopeptidase B (Cl) and cyanogen bromide (CN), are shown included in bars. The arrows indicate the automatic NH_2 -terminal Edman degradations of the whole chains. Nomenclature of the peptides as in Fig. 2. The accession number in the SWISS-PROT database is P80208.

SMALL CHAIN		1	10	20	30	37
BnIII		SAGPFRIPKCRKEFQQAQHLRACQQLWHLKQAMQSGSG				
Sin a 1	P					PS
BnI		Q PQ	QR	E	IRQ	L--AGSPF
gNa	P					P·G·SG
pN2						G--G
pN1				K		G--G
napA						G--G
BngNAP1	P			K		--G

LARGE CHAIN		1	10	20	30	40	45
BnIII		PQGPQQRPPLLQCCCNELHQEEPLCVCPTLKGASKAVKQQVRRQQ					
Sin a 1							L
BnI		QS	G·W·RE	Y	DQV	Q·A·S·RV	----
gNa	Q		PP			R	
pN2						I	
pN1	Q		H·Y	Q		R	R·I
napA						I	
BngNAP1							

		46	50	60	70	80	88
BnIII		GQQ	QQLQQVISRIYQTATHLPKVCNIPQVSVCPFKQKTMGPSP				
Sin a 1	E		GPH			K	
BnI		-----	HGPFQST	I·KN	N	MK·IGT	IAI
gNa		·MQ	·M	·V	·R	·R	·I
pN2		-----	GK	MV			Y
pN1	E	-----	GK	MVN			Y
napA		-----	GK	MV			Y
BngNAP1							Y

Fig. 4. Alignment of the sequences of the small and large chains of napin BnIII, Sin a 1 allergen [7], napin BnI [23], and the deduced amino acid sequences from several napin genes: gNa [20], pN2 and pN1 [19], napA [21], and BngNAP1 [22]. (·) show the identity among the sequences (referred to BnIII); (—) stand for gaps created for the best alignment.

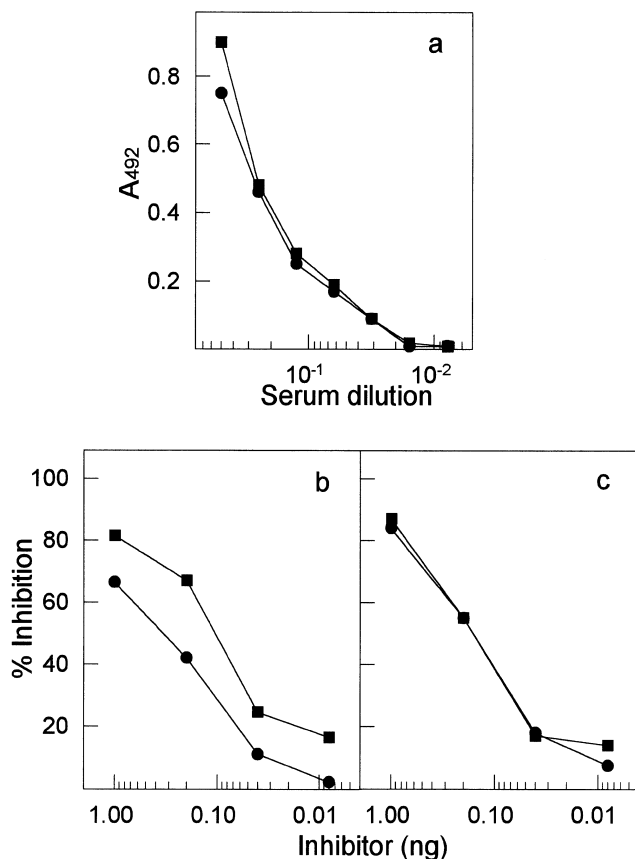


Fig. 5. Indirect ELISA (a) for IgE titration of a rapeseed flour-sensitive patient's serum to solid-phase fixed BnIII (■) or Sin a 1 (●). ELISA inhibitions of human rapeseed-sensitive IgE-binding to solid-phase fixed BnIII (b) and Sin a 1 (c), using BnIII (■) and Sin a 1 (●) as inhibitors.

from saline extract of rape pollen (data not shown). Therefore, rape pollen allergens are different proteins than the rapeseed flour allergen BnIII.

Napin BnIII is homologous to other napin-type proteins, such as Sin a 1 and Bra j 1E, the yellow and oriental mustard 2S albumins, which have been described as allergens [7, 31]. Their alignment shows that the most important differences are found at the NH₂- and COOH-terminal ends of the mature chains. A region of the large chain (positions 38–59 in Fig. 4) has been demonstrated to be immunodominant in Sin a 1, and contains a continuous epitope (positions 50–62) described for this mustard allergen [31]. This region of the protein could be involved in the allergenicity of BnIII, since its length and hydrophilicity are very similar in Sin a 1. Moreover, the differences of sequence that have been observed in these positions in the case of the 12 kDa napins (BnI, Fig. 4) may explain their strongly decreased capability to bind IgE (Fig. 1). Nevertheless, the immunological relevance of other regions of napins cannot be

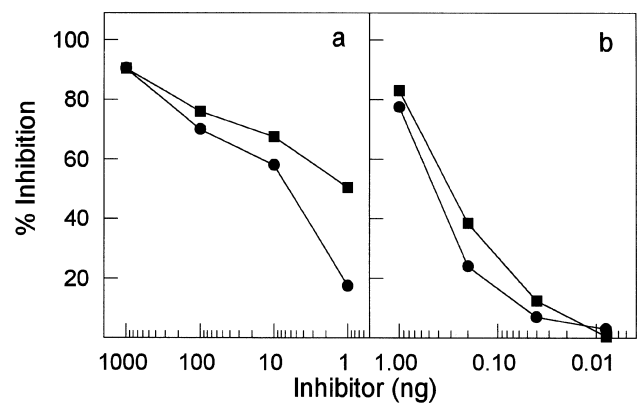


Fig. 6. (a) ELISA inhibition of Sin a 1-specific rabbit IgG-binding to solid-phase fixed Sin a 1, using BnIII (■) and Sin a 1 (●) as inhibitors. (b) ELISA inhibition of human mustard-sensitive IgE-binding to solid-phase fixed Sin a 1, using BnIII (■) and Sin a 1 (●) as inhibitors.

excluded since conformational epitopes have also been detected [36].

The amino acid sequences of rapeseed napin BnIII and Sin a 1 allergen are similar enough to explain their IgE and IgG cross-reactivities. Both allergens are recognized by IgE of sera of mustard-sensitive individuals as well as by those present in the serum of the rapeseed-hypersensitive patient. ELISA inhibition experiments clearly showed that they share common allergenic epitopes. The observed differences could be due to the degree and nature of the microheterogeneity of each allergen, and to the degree of antigenicity of each isoform. It has been shown that the IgE-binding capability of an homogeneous isoform may be lower than that of the whole polymorphic allergen [37]; moreover, single isoforms can escape recognition by individual allergic sera [38]. Future structural and immunological studies on homogeneous and well-defined isoforms of homologous allergenic proteins will help to better understand their implications in the hypersensitivity reaction.

It is worth noting the marked lack of bibliographical sources about rapeseed flour allergy in countries where rapeseed crops are widely extended. To our knowledge, no allergy to rapeseed flour has been reported to the date, but only to inhaled rape pollen [24–26]. This allergy could have been masked because of the benignity of its symptoms, for being concurrent in the workplace with other allergens such as soybean and cereals, or due to its expected cross-reactivity with other *Brassicaceae* seed flours.

As it has been shown in this study, sensitization to oilseed rape flour can develop into allergy to other seeds closely related botanically and employed for many uses, such as condiments, meals or feed; thus, hypersensitization

to rapeseed flour or related seeds may occur in patients allergic to mustard spice. As rapeseed is being employed in farmer animal feeds, allergy to its components should be considered when studying patients who exhibit allergic reactions to meat products from these animals. The relevance of this assessment is increased if the high physical and chemical stabilities of the napins against thermal and proteolytic treatments are taken into account [37,39], since these allergens would be affected neither by food cooking and processing nor by gut digestion.

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