

nsLTP and profilin are allergens in mustard seeds: cloning, sequencing and recombinant production of Sin a 3 and Sin a 4

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Summary

Background Patients allergic to mustard are frequently sensitized to peach.

Objective To identify and analyse new yellow mustard allergens that could be involved in IgE cross-reactivity.

Methods Sera from mustard-allergic patients with symptoms to peach were studied. Mass spectrometry analyses provided sequences of IgE-reactive proteins. cDNAs encoding Sin a 3 and Sin a 4 were amplified by polymerase chain reaction, cloned and sequenced. The recombinant allergens were obtained in *Pichia pastoris* and *Escherichia coli*, respectively, and used for ELISA, immunoblotting and inhibition experiments. Sequence alignment was used to identify common IgE epitopes.

Results Sin a 3- and Sin a 4-specific cDNAs encode for mature proteins of 92 and 131 amino acids that belong to nsLTP and profilin protein families, respectively. Sin a 3 and Sin a 4 showed 54% and 80% identity with allergenic nsLTP from peach and profilin from melon, respectively. Both recombinant allergens were IgE-reactive in ELISA and immunoblotting. Peach pulp and peel, and melon extracts nearly abolished the IgE binding to recombinant Sin a 3 or recombinant Sin a 4 in immunoblotting.

Conclusion Sin a 3 (nsLTP) and Sin a 4 (profilin) were identified as new mustard allergens and showed IgE cross-reactivity with fruits such as peach or melon, respectively. The knowledge of these two allergens will contribute towards better understand with cross-reactivity between mustard and other plant food allergens, and their availability will provide physicians with useful tools for molecular diagnosis.

Keywords cross-reactivity, mustard allergy, nsLTP, profilin, Sin a 3, Sin a 4

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Introduction

Mustard is one of the most significant spices in terms of allergenic potency and widespread use. Mustard allergy has been frequently associated with severe symptoms, such as urticaria, angioedema, or anaphylaxis. IgE-mediated allergy to mustard has been increasingly reported in recent years [1, 2]. Mustard or derivate products are added for flavoring manufactured and processed foods, and frequently appear as hidden condiments on sauces and salad dressings, which increase the risk of unexpected reactions. According to the European Union guidelines for food labelling, the mustard content must be declared [3]. Mustard oil is also used in cosmetic and traditional medicine products. Yellow mustard (*Sinapis*

alba L.) is the variety most commonly used in Europe, whereas oriental mustard (*Brassica juncea*) is used in United States and Asia. Rapeseed, cabbage and broccoli are also allergenic members of the Brassicaceae family [4–6].

Two families of allergenic proteins have been identified in mustard seeds: 2S albumins (Sin a 1 from yellow mustard and Bra j 1 from oriental mustard) [7, 8] and 11S globulins (Sin a 2) [9, 10]. 2S albumins (14 kDa) are highly stable to proteolytic and heat treatments. 11S globulins (51 kDa) are salt-soluble proteins composed of cupin domains. Both allergens are specific seed-storage proteins.

More than 50% of patients allergic to mustard present hypersensitivity to mugwort pollen and several vegetable foods [11], mainly Rosaceae fruits, but also nuts and legumes, the former being among the most frequent

causes of food allergy in the adult population of the Mediterranean area [12, 13]. Cross-reactivity between mustard and peach extracts cannot be explained by Sin a 1 or Sin a 2 because they belong to protein families specifically expressed in seeds. However, IgE antibodies to Rosaceae nsLTPs reacted to broccoli [6], and an allergenic nsLTP, Bra o 3, has been identified in cabbage leaves showing significant cross-reactivity with mustard, mugwort pollen and peach [14]. These findings strongly suggest the existence of non-tissue-specific allergens in mustard seeds responsible for IgE cross-reactivity between mustard and fruits or vegetable foods.

Sera of mustard-allergic patients with symptoms to peach were used to identify two new allergens from yellow mustard seed, belonging to the nsLTP and profilin families. Their amino-acid sequences, recombinant production and immunological features are reported.

Materials and methods

Subjects

Serum samples were obtained from 15 mustard-allergic patients with peach allergy. All patients had clear IgE-mediated symptoms with mustard and positive skin prick tests (SPT) to a commercial mustard extract (ALK-Abelló, Madrid, Spain) or specific IgE to a mustard extract [9] determined in ELISA. In addition, all patients had symptoms with fresh peach and positive SPT to a commercial peach extract (ALK-Abelló) as well as prick by prick with fresh peach peel [13]. Open oral challenge tests to fresh peach were performed as reported previously [13].

Sera from a non-atopic subject (C1) and from an olive pollen-allergic patient only sensitized to Ole e 1 (C2) were used as controls. The study was approved by the Fundación Jiménez Díaz Ethic Committee, and written informed consent was obtained from all subjects.

Extracts, proteins and cloning materials

Yellow mustard seed extract for cloning and *in vitro* experiments was obtained as described [9]. Peach and melon for *in vitro* assays were obtained from local food stores. Ground peel and pulp of peach were defatted with acetone and ethanol:ether, and extracted in ammonium bicarbonate 150 mM, 10 mM ascorbic acid and 1 mM phenylmethylsulphonyl fluoride. Protein extract from melon was prepared as described [15]. Sin a 1 and Sin a 2 were prepared from the mustard seed extract as described previously [7, 10].

The TOPO TA-cloning kit (Invitrogen, Groningen, The Netherlands) was used for cloning the PCR products [10]. *Escherichia coli* strain DH5 α F' (Invitrogen) was used as a host to obtain the pET11b/Sina4 and pPICZ α A/Sina3 constructs. *Pichia pastoris* KM71 (Invitrogen) and *E. coli* BL21-CodonPlus (DE3) (Stratagene, La Jolla, CA, USA)

strains were used for expression of rSin a 3 and rSin a 4, respectively.

Analytical procedures

Sodium dodecyl sulphate (SDS-PAGE) was performed in 17% polyacrylamide gels under non-reducing conditions or, alternatively, with 2-mercaptoethanol. Proteins were visualized by Coomassie Blue or transferred to nitrocellulose membranes. Digestion, mass spectrometry (MS) and MS/MS analyses of the excised bands were carried out as described [9]. Bands of interest were manually excised from gels and digested with sequencing-grade trypsin, after reducing with dithiothreitol and alkylation with iodoacetamide. Matrix-assisted laser-desorption ionization (MALDI) time-of-flight (TOF) MS analyses were performed in a Voyager-DESTR instrument (PerSeptive Biosystems, Framingham, MA, USA). The MS/MS sequencing analyses were performed using the MALDI-tandem-TOF MS spectrometer 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA). N-terminal Edman degradation of proteins was performed using a 477A sequencer (Applied Biosystems).

Enzyme-linked immunosorbent assay, Western blot and immunoglobulin E immunoblot inhibition

Specific IgE levels were tested by ELISA in microtitre plates coated with 0.5 μ g/well of purified protein, as described [16]. Plates were incubated with individual allergic sera (1 : 5 diluted). Bound IgE antibodies were detected with mouse anti-human IgE antibodies (1 : 5000 diluted), followed by horseradish peroxidase-coupled goat anti-mouse IgG (diluted 1 : 2500; Pierce Chemical Co, Rockford, IL, USA). Peroxidase reaction was developed and optical density (OD) was measured at 492 nm [17]. Each value was the mean of two determinations after blank subtraction. Values <0.1 were considered negative.

After SDS-PAGE under non-reducing conditions or, alternatively, in the presence of 2-mercaptoethanol, membranes containing blotted extracts or purified proteins were incubated with patient's sera (1 : 5 diluted for proteins and 1 : 10 for extracts) at 4 °C overnight. IgE binding was detected as described above. Peroxidase reaction was developed using ECL Western blotting reagent (Amersham Biosciences, Barcelona, Spain) [10]. In immunoblotting inhibitions, the pool of sera (1 : 10 diluted) reactive to rSin a 3 was preadsorbed with 250 μ g of peach peel or pulp extracts, or 2 μ g of purified rSin a 3, before the incubation of rSin a 3 (0.2 μ g) blotted to membranes. IgE-binding inhibition of sera positive to rSin a 4 was performed as for rSin a 3 but using a melon extract (250 μ g) or rSin a 4 (2 μ g) as inhibitors. Phosphate-buffered saline or a mixture of bovine serum albumin, lysozyme and ovalbumin (3 μ g per protein) were used as controls. Volummograms of the reactive bands from two experiments were analysed by

double scanning densitometry using UVIBand V97 (UVItec, St. John's Innovation Center, Cambridge, UK).

Cloning of *Sin a 3* and *Sin a 4*

Total RNA from yellow mustard seeds [17] was extracted as described previously and used as a template to synthesize total cDNA using the SMART RACE cDNA amplification kit (BD Biosciences-Clontech, Madrid, Spain).

The coding region of the *Sin a 3* cDNA was amplified by PCR with *Taq Advantage2* DNA polymerase (BD Biosciences-Clontech) using the mustard cDNA as a template and two degenerate primers, a sense 5'GGNTGYTGACNGG NGTNAC3' and an antisense 5'RTARGGDATRTTRACDCCRC ANG3', which were designed from sequences deduced from peptides obtained from MS analyses (GCCTGVT and ACGVNIPY). An internal fragment of 170 base pairs was obtained and its nucleotide sequence allowed the synthesis of two non-degenerate oligonucleotides 5'GCTCGTACAACC CCGGACCGTCAG3' and 5'CTGACGGTCCGGGGTTGTACGA GC3' (corresponding to the amino acid sequence ARTTP DRQ), which were used together with a non-specific primer included in the kit to amplify the 5' and 3' ends of the *Sin a 3*-encoding region, respectively. The full-length nucleotide sequence codifying for *Sin a 3*, without the signal peptide, was amplified using in a single round of PCR a sense 5'cgtctcgagaaaagaGCTCTGAGCTGTGGAACC3' and an antisense 5'ctgcgggcgcgtcaTCTAACGCTGTTGCAG TT3' primers, corresponding to the amino acid sequences ALSCGT and NCNSVR. The sense primer contains (in lower case letter), besides an *XhoI* restriction site (underlined), a sequence that allows fusion of the protein-encoding region in-frame with the preprosequence of the α -mating factor from *Saccharomyces cerevisiae* in the pPICZ α A plasmid. The antisense primer contains (in lower case letter) a *NotI* restriction site (underlined) and a stop codon. The signal peptide sequence processing site was deduced from the N-terminal sequence determination by Edman degradation of the protein isolated from seeds.

The cDNA encoding *Sin a 4* was amplified by PCR as described above using the total cDNA from mustard seeds as a template and two primers designed from the sequences of profilins from olive and chenopod: a sense primer 5'atacatATGTCNTGGCARACNTAYGT3' (amino-acid sequence MSWQTYV) with an *NdeI* restriction site (underlined) and an antisense primer 5'gcgaattcttaGAGNC CYTGTYCHATNAG3' (amino-acid sequence LIEQGL) with an *EcoRI* restriction site (underlined) and the stop codon.

The PCR products obtained were cloned into the pCR2.1 plasmid and the constructs (pCR2.1/*Sina3* and pCR2.1/*Sina4*) used for sequencing.

Homology searches were performed using the BLAST program [18] and multiple amino acid sequence alignments using the CLUSTAL W program [19].

Expression of *Sin a 3* in *Pichia pastoris* and *Sin a 4* in *Escherichia coli*

The pCR2.1/*Sina3* construct was digested with *XhoI/NotI* restriction enzymes and the fragment obtained was sub-cloned into the same sites of the pPICZ α A plasmid. The pPICZ α A/*Sina3* construct was linearized with the *BstXI* restriction enzyme, and the purified fragment was used to electroporate KM71 cells. To produce r*Sin a 3* selected transformed colonies (His⁺ Mut^s phenotype), growing under YPDS/zeocin, were cultured for 48 h at 30 °C in a buffered minimum glycerol medium, and afterwards cells were collected by centrifugation and resuspended in one-fifth of the original volume of buffered methanol minimum medium for induction of the AOX1 promoter. After 4 days, the KM71-induced cells were sedimented by centrifugation at 5000 rpm and 4 °C and the supernatant containing the recombinant protein was dialysed and used as the starting material. The presence of r*Sin a 3* in the extracellular culture medium was analysed by SDS-PAGE.

For expression of *Sin a 4*, construct pCR2.1/*Sina4* was digested with *NdeI/EcoRI* restriction enzymes, and sub-cloned into the plasmid pET11b. Production as a non-fusion recombinant protein was achieved after transforming *E. coli* BL21 (DE3)-competent cells with the pET11b/r*Sina4* construct as described [10]. Briefly, overnight cultures were 10-fold diluted with an LB medium containing 0.1 mg/mL ampicillin, and grown up to $A_{600} = 1.0$. After induction with isopropyl- β -D-thiogalactopyranoside to a final concentration of 0.4 mM, cultures were maintained for 4 h at 37 °C and centrifuged. The cellular pellet resuspended in 20 mM ammonium bicarbonate, 1 mM phenylmethylsulphonyl fluoride, was lysated by three cycles of freezing and thawing at 42 °C. The soluble fraction obtained after centrifugation was used as the starting material for purification of the recombinant protein.

r*Sin a 3* and r*Sin a 4* were purified by two consecutive chromatographic steps in fine and superfine Sephadex G-50 (Amersham Biosciences), preceding a reverse-phase high-performance liquid chromatography in a μ -bondapak C₁₈ column eluted with an acetonitrile gradient in 0.1% trifluoroacetic acid.

Results

Patients

Fifteen mustard-allergic patients with symptoms to peach were included in this study (Table 1). Nine patients were female and six were male. The mean age was 30.5 ± 10.0 years, ranging from 18 to 54. The majority of them suffered from systemic symptoms when eating mustard (13/15) as well as OAS (Table 1). All patients had OAS on eating fresh peach and 5/15 patients (patients # 2, 4, 6, 12 and 15) also suffered from systemic symptoms. Mustard SPT ($n = 13$) were $67.0 \pm 53.1 \text{ mm}^2$ (mean $\pm$ SD) and peach SPT ($n = 15$) were $71.2 \pm 57.7 \text{ mm}^2$. Most of them also

Table 1. Clinical and immunological features of the patients included in this study

Mustard Allergy						
Patient no.	Age/sex	Symptoms with mustard	Yellow mustard seeds		Other food allergies	Pollen allergy
			SPT*	ELISA [†] OD ₄₉₂		
1	30/F	OAS, CU	21	Neg	r, n, p, m	Yes
2	26/F	OAS	122	0.200	r, k, n, p, m	Yes
3	30/M	OAS, U, AE, TT, A, RC, D	131	1.327	r, n, p	Yes
4	29/M	OAS, U, RC, D	54	2.809	r, l, m	Yes
5	31/F	OAS, U, AE	ND	0.238	r, k, n, p, m	Yes
6	20/F	OAS, U, AE, RC, D	18	0.228	r, n, p, l, m	Yes
7	31/M	OAS	ND	0.267	r, n, p, m	Yes
8	28/F	TT, RC	42	0.101	r, k, n	Yes
9	29/M	OAS, U, AE, A	22	Neg	r, k, m	Yes
10	54/F	OAS, U, TT, A, RC, D	43	0.847	r, k, m	Yes
11	30/M	OAS, AE, A, RC	196	0.856	r, k, n, p, m	Yes
12	18/M	OAS, U, AE, RC	72	0.267	r, n, p, l, m	No
13	31/F	OAS, AE, CU, TT, A	76	0.441	r, k, n, p, l, m	Yes
14	51/F	OAS, AE, TT	25	Neg	r, k, m	Yes
15	19/F	U, AE, TT, RC, D	49	0.113	r, k, n, p, l	No
C1	20/M	No	ND	Neg	No	No
C2	25/F	No	ND	Neg	No	Yes

*Skin prick test: weal area in mm².

[†]Specific IgE determined in ELISA as OD at 492 nm.

M/F, male/female; OAS, oral allergy syndrome; AE, angioedema; U, urticaria; CU, contact urticaria; TT, throat tightness; A, asthma; RC, rhinoconjunctivitis; D, digestive symptoms; r, rosacea (excluding almond); k, kiwi; m, melon; l, legumes (excluding peanut); p, peanut; n, nuts (including almond); ND, not done. Neg, negative (<0.100).

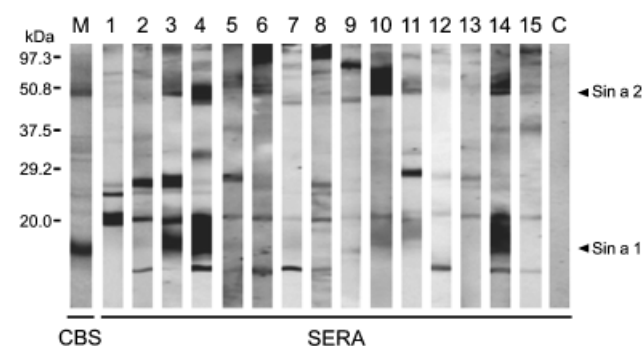


Fig. 1. IgE reactivity of yellow mustard seed extract. SDS-PAGE analysis, Coomassie Blue staining (lane CBS) and immunoblotting with sera from patients (lanes 1–15) included in the study. Lane C, serum from a non-atopic subject. Sin a 1 and Sin a 2 positions are indicated by arrowheads.

presented symptoms with other plant-derived foods, such as nuts (11/15), legumes (5/15), peanut (10/15), melon (12/15) or kiwi (9/15). Patients' IgE response to yellow mustard seed extract was analysed by immunoblotting (Fig. 1). Major protein bands at 14 and 50 kDa were identified as Sin a 1 and Sin a 2, respectively, using these purified allergens as inhibitors.

Identification of allergenic nsLTP in yellow mustard seeds

A fraction enriched in low-molecular mass proteins (≤ 18 kDa) was obtained from a mustard extract after

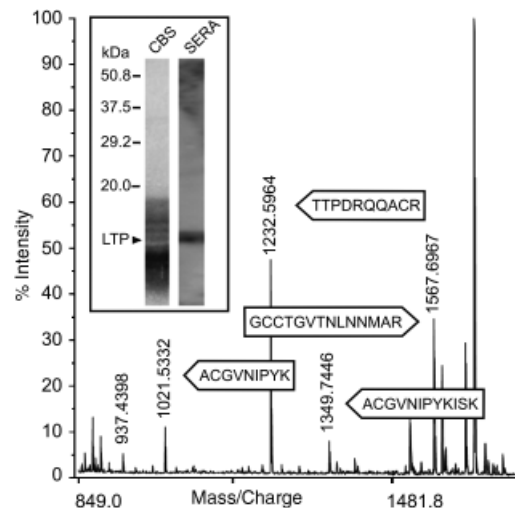


Fig. 2. Detection and identification of nsLTP in mustard extract. Mass spectrometry analysis of the IgE-reactive band excised from the membrane after SDS-PAGE (inset) of the low-molecular-mass protein fraction.

two size-exclusion chromatographies. Coomassie Blue staining after SDS-PAGE and the IgE response of this fraction with serum #12 is shown in Fig. 2 (inset). The IgE-reactive band was digested with trypsin. Peptides were analysed by MALDI-TOF-MS (Fig. 2) and compared with those from databases. The protein was identified as

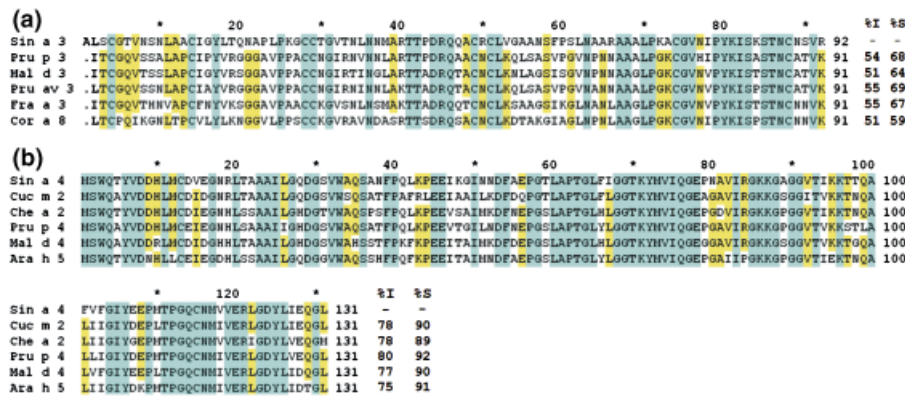


Fig. 3. Amino acid sequence of Sin a 3 and Sin a 4, and alignment with nsLTP or profilin allergens. (a) Yellow mustard (Sin a 3), peach (Pru p 3), apple (Mal d 3), apricot (Pru av 3), strawberry (Fra a 3) and hazelnut (Cor a 8) (Accession numbers EF626938, CAB98876, CAB96874, Q9M5X8, AAY83346, AAK28533, respectively). (b) Yellow mustard (Sin a 4), melon (Cuc m 2), chenopod (Che a 2), peach (Pru p 4), apple (Mal d 4) peanut (Ara h 5) (Accession numbers EF626939, Q7X9Q0, Q84V37, Q8GT39, Q9XF40 the Q9SQI9, respectively). Residues conserved in all proteins are in blue, and those conserved in 5 sequences are in yellow. %I and %S, percentages of identity and similarity, respectively.

an nsLTP and was named Sin a 3 according to the official nomenclature.

Molecular cloning of mustard nsLTP (Sin a 3) and profilin (Sin a 4)

Total cDNA from yellow mustard seeds was used as a template for PCR amplification of the cDNAs encoding Sin a 3 and Sin a 4 (the official name for mustard profilin). The sequence obtained for Sin a 3 (accession number EF626938 in the GenBank Database) encodes a mature nsLTP of 92 amino-acid length (Fig. 3a), with a deduced molecular mass of 9465.9 Da and pI 9.41. The cDNA sequence obtained for Sin a 4 (EF626939) encodes a profilin of 131 amino acids (Fig. 3b) with a deduced molecular mass of 14167.1 Da and pI 4.75. Alignments of Sin a 3 and Sin a 4 with homologous counterparts as well as their identity and similarity percentages are shown in Fig. 3.

Recombinant expression, purification and immunoglobulin E binding to rSin a 3 and rSin a 4

Recombinant Sin a 3 was obtained as an extracellular secretion protein, and no degradation or aggregation products were detected (Fig. 4a). The protein was purified by gel filtration chromatographies and RP-HPLC, and showed a 12.3 kDa apparent molecular mass in SDS-PAGE. Edman degradation of the N-terminal of rSin a 3 yielded the sequence ALSXG, and MS yielded 9467.3 Da.

Sin a 4 was expressed as a non-fusion intracellular recombinant protein (Fig. 4a) and was purified from the soluble fraction by gel filtration and RP-HPLC. The protein migrates as two close bands at 13–14 kDa in SDS-PAGE, and as a single band (13 kDa) after reductive treatment (Fig. 4a). Edman degradation yielded SWQTY

and MS yielded 14039.1 Da, indicating the correct bacterial processing of the Met located at the N-terminal of the expression product, and thus a mature rSin a 4 of 130 amino-acids length.

The IgE-binding capacity of purified rSin a 3 and rSin a 4 was assayed by ELISA and immunoblotting after SDS-PAGE using all the patients' sera (Fig. 4b). Both proteins displayed IgE-binding activity. Nine sera were clearly positive to rSin a 3 and four sera were positive to rSin a 4. Several patients did not react to any of these proteins, and thus they should be reactive to other proteins of the mustard extract as can be seen in Fig. 1.

nsLTP and profilin immunoglobulin E-binding sites are highly conserved in Sin a 3 and Sin a 4

The potential cross-reactivity of Sin a 3 and Sin a 4 with other allergenic nsLTPs and profilins was examined by comparison of the corresponding amino acid sequences of the IgE epitopes reported for Pru p 3 from peach [20] and Cuc m 2 from melon [21], respectively (Figs 5a and b). Peach nsLTP epitopes E1, E2 and E3 displayed, respectively, identity values of 33%, 73% and 70% (47%, 93% and 70% similarity) with the corresponding peptides on Sin a 3, whereas 89%, 54%, 87% and 93% were identity values (89%, 64%, 96% and 93% similarity) for E1, E2, E3 and E4 of Cuc m 2 and Sin a 4.

Experimental data on the cross-reactivity were obtained by immunoblotting after SDS-PAGE of rSin a 3 and rSin a 4. The inhibition of the IgE reactivity to rSin a 3 of a pool of positive sera (sera 2, 7, 12 and 15) was assayed with peach peel and pulp extracts as inhibitors (Fig. 5c), obtaining 89% and 87% of inhibition, respectively, while 95% was obtained with rSin a 3. The IgE reactivity to rSin a 4 of a pool of sera positive to this profilin (sera 4, 5, 9 and 14) was

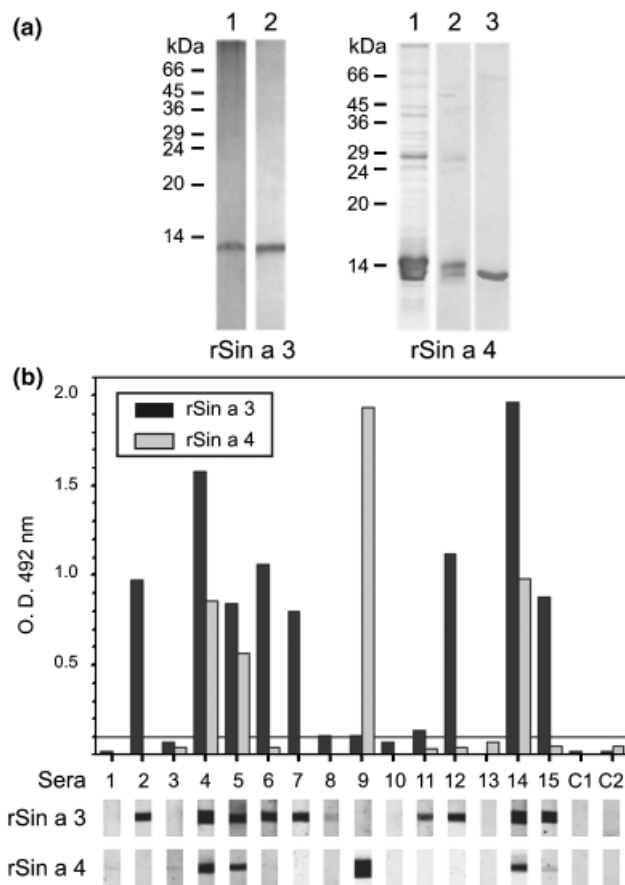


Fig. 4. Purification of rSin a 3 and rSin a 4, and IgE reactivity. (a) SDS-PAGE and Coomassie Blue staining of extracellular culture medium for rSin a 3, and soluble fraction of culture medium for rSin a 4 (lanes 1); purified allergens under non-reducing conditions (lanes 2) and rSin a 4 after β -mercaptoethanol treatment (lane 3). (b) IgE-reactivity analyses of purified rSin a 3 and rSin a 4; ELISA (upper panel), and immunoblotting (lower panel) with the sera of the 15 patients included in the study; immunoblot for rSin a 4 was performed under reducing conditions. OD, optical density. C1 and C2 are negative controls.

inhibited at 93% with melon extract and at 99% with rSin a 4 (Fig. 5c). Standard deviations were <0.9 for LTPs assays and 0.7 for profilins.

Discussion

This study was designed to identify new allergens in mustard seeds. Patients were recruited among those with mustard and peach allergy. The IgE-binding pattern of these patients against mustard seed extract revealed the presence of reactive protein bands in addition to those of 2S albumins (Sin a 1) and 11S globulins (Sin a 2). Rosaceae allergy frequently found in mustard-allergic patients [2, 11] as well as the cross-reactivity reported between peach and mustard extracts [6, 14] could not be explained by Sin a 1 and Sin a 2 sensitization as they are not appreciably expressed in organs other than seeds. Therefore, we investigated whether there are new allergens that could be involved in cross-reactivity between mustard and peach extract. Several drawbacks had to be overcome to identify Sin a 3 and Sin a 4 as new allergens: (a) the high frequency of Sin a 1 sensitivity among mustard-allergic patients together with its very high content in the seed, (b) the similar molecular weight of the three allergens Sin a 1, Sin a 3 and Sin a 4 (ranging from 12 to 14 kDa), (c) the close pI (>9) of Sin a 1 and Sin a 3 and (d) the very low amount of nsLTP and profilin in the mustard seed extract. The use of partially purified protein extracts, MS tools and database banks allowed us to obtain sequence data of the two potential allergens. Their encoding cDNAs were cloned, sequenced and recombinantly expressed in heterologous systems: *P. pastoris* for Sin a 3 because nsLTPs need disulphide bonds' formation, and *E. coli* for Sin a 4 as profilins do not need post-translational modifications. The recombinant proteins, Sin a 3 and/or Sin a 4, were recognized by the IgE of sera from 11 out of 15 patients

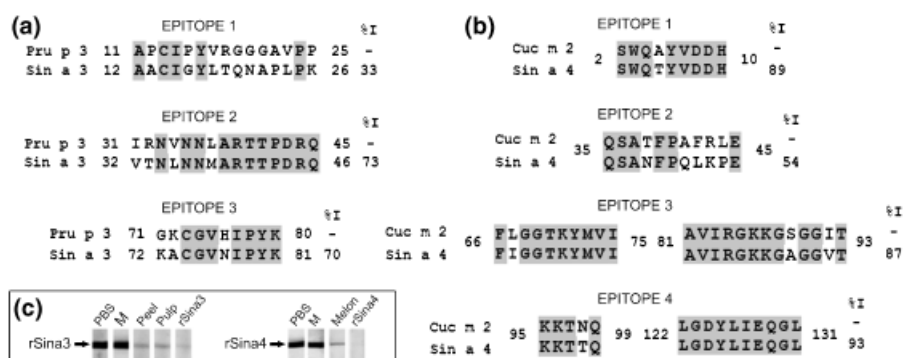


Fig. 5. IgE-epitope analysis of Sin a 3 and Sin a 4 in comparison with Pru p 3 and Cuc m 2, respectively. Comparison of the IgE epitope sequences of: (a) nsLTP allergens from peach and mustard, and (b) profilins from melon and mustard; conserved amino acids are shaded; I% and %S, percentages of identity and similarity, respectively. (c) Immunoblotting inhibition of the IgE response to rSin a 3 with peach pulp and peel extracts and to rSin a 4 with melon extract; inhibition with PBS and a mixture (M) of proteins (BSA, ovalbumin and lysozyme) were used as negative controls.

used in the study. The remaining patients recognized protein bands corresponding to major allergens such as Sin a 1 and Sin a 2 (sera #3, #10) or not yet explored allergens as in the case of sera #1 and #13.

Sin a 3 shows 65% identity (13 out of 20 residues) with the N-terminal amino acid sequence reported for Bra o 3 (nsLTP from cabbage, *B. oleraceae* var. *capitata*) [14], and moderate identity ranging from 51% to 55% with Pru p 3, Mal d 3, Pru av 3, Fra a 3 or Cor a 8. The IgE reactivity to Sin a 3 of our mustard-allergic patients was nearly abolished by both peach pulp and peel extracts. This result would agree with the findings of Asero et al. [6] and Palacín et al. [14], who demonstrated the IgE cross-reactivity between Rosaceae fruits and nsLTPs from broccoli or cabbage leaves both belonging to the Brassicaceae family. Palacín et al. [14] also observed the reactivity of anti-Pru p 3 rabbit polyclonal antibodies to mustard extract. LTPs are markedly expressed in Brassicaceae leaves; thus mustard seed represents a new tissue concerning the appearance of LTPs in this family. Interestingly, Sin a 3 displays around 70% identities to Pru p 3 on the E2 and E3 epitope regions [20], but only 33% on epitope E1, suggesting the possibility that most of the IgE reactivity would be directed to epitopes E2 and E3 and similar residues of E1. nsLTPs are widespread allergenic proteins with a diverse degree of cross-reactivity depending on the sequence similarity [22–27].

Profilins are panallergens from many biological sources with highly conserved structures [27, 28]. Sin a 4 shows around 80% of identity with other allergenic food profilins such as Cuc m 2 from melon and Pru p 4 from peach. This would be consistent with the degree of inhibition observed with melon extract to Sin a 4-reactive IgEs. Several reports suggest the existence of cross-reactive and species-specific IgE epitopes in profilins [29]. The presence of cross-reactive IgE epitopes has been shown to elevate the risk of multiple sensitization [30], while differences in IgE directed to variable epitopes may influence the clinical manifestation of profilin sensitization [29].

In this work, we have focused our study on linear epitopes of Sin a 3 and Sin a 4, in order to compare with those of well-known LTPs and profilins obtained by the SPOT membrane technique [20, 21]. However, the presence of conformational epitopes has been reported recently for both families of allergens, supporting the importance of the three-dimensional structure of the protein to the IgE binding [29, 31]. In addition, structural bioinformatic analysis of the conserved external main chains has also been useful to the assessment of potential IgE cross-reactivity [32]. Our findings broaden the mustard allergen profile and provide evidence for cross-reactivity between mustard and other vegetable food extracts for the two new allergens. Future studies, such as performing oral challenges with purified allergens or

involving different populations of allergic patients such as monosensitized individuals, would help to elucidate the actual role of each allergen in the mustard allergy clinical reaction. In addition, the availability of purified recombinant mustard allergens would allow the evaluation of patient sensitization to particular nsLTP and profilin epitopes from mustard and other sources. The identification of these proteins will probably facilitate further understanding of allergy to other botanically related foods, such as broccoli, cabbage, cauliflower and radish.

In summary, Sin a 3 (nsLTP) and Sin a 4 (profilin) have been identified as new mustard allergens, showing a high IgE cross-reactivity with homologues from peach and melon. These findings provide a better understanding of plant-derived food cross-reactivity, provide additional diagnostic tools and, perhaps, make possible new therapeutic approaches.

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