

A Recombinant Precursor of the Mustard Allergen Sin a 1 Retains the Biochemical and Immunological Features of the Heterodimeric Native Protein

Oscar Palomares^a Javier Cuesta-Herranz^b Rosalía Rodríguez^a
Mayte Villalba^a

^aDepartamento de Bioquímica y Biología Molecular I, Facultad de Ciencias Químicas, Universidad Complutense y

^bServicio de Alergia, Fundación Jiménez Díaz, Madrid, Spain

Key Words

Food allergy · Mustard · 2S albumin · *Pichia pastoris* ·
Recombinant protein

Abstract

Background: Mustard has been an important cause of food allergy of increasing incidence in the last years. Sin a 1, a storage 2S albumin, is the most relevant allergen from this spice. **Methods:** *Pichia pastoris* has been used as host for the recombinant production of the precursor form of Sin a 1 (rproSin a 1). rproSin a 1 was purified to homogeneity by three chromatographic steps: gel filtration, anion exchange and reverse-phase HPLC. Molecular characterization was performed using Edman degradation, mass spectrometry, amino acid composition, and circular dichroism. Immunological properties were analyzed by immunoblotting, ELISA, and ELISA inhibition experiments. **Results:** We overexpressed rproSin a 1 as a single polypeptide with both large and small chains linked by an internal processed fragment at high yield. The purified rproSin a 1 (>95%) was obtained as a monomeric and soluble protein. rproSin a 1 showed equivalent structural and immunological properties to natural heterodimeric Sin a 1 allergen. rproSin a 1 was recognized by 75% of the patients allergic to mustard. The

inhibitory capacity of rproSin a 1 to the total allergenicity of mustard extracts varied from 13 to 83% in different patients, with a mean value of 54%. **Conclusions:** rproSin a 1 is a good candidate to replace natural allergen in diagnosis protocols of mustard allergy. *P. pastoris* has been demonstrated to be a suitable expression system for the production of allergenic derivatives of Sin a 1 that could be used for immunotherapy purposes in future.

Copyright © 2005 S. Karger AG, Basel

Introduction

Many spices have been described as inducers of allergic reactions [1]. Among them, mustard is one of the most significant in terms of widespread use and allergenic potency. For many years clinical cases of mustard allergy have been reported [2, 3], and recently its prevalence has notably increased in countries such as France, where this spice is commonly consumed, becoming the fourth most important food allergen for children [4]. The interest in mustard as a cause of food hypersensitivity reactions has increased because (1) the associated symptoms often involve anaphylactic reactions, (2) mustard is frequently consumed as a hidden food, which makes avoidance measures more difficult, and (3) mustard allergy has been

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2005 S. Karger AG, Basel
1018–2438/05/1371–0018\$22.00/0

Accessible online at:
www.karger.com/iaa

Correspondence to: Dr. Mayte Villalba
Departamento de Bioquímica y Biología Molecular
Facultad de Ciencias Químicas, Universidad Complutense
ES–28040 Madrid (Spain)
Tel. +34 91 3944155, Fax +34 91 3944159, E-Mail mayte@bbm1.ucm.es

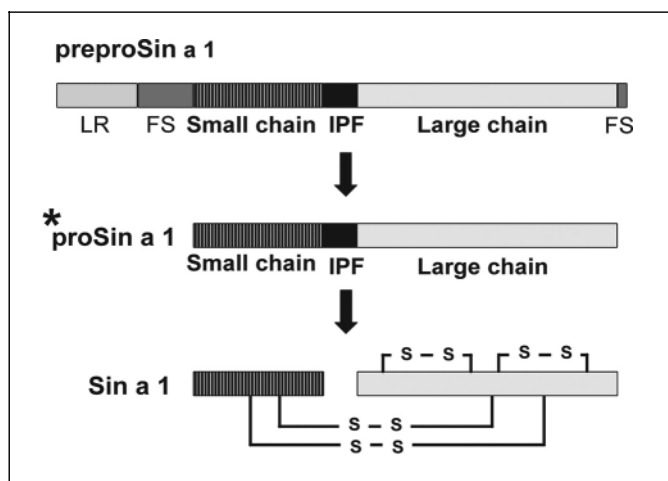


Fig. 1. Scheme of the processing of Sin a 1. LR = Leader region; FS = flanking sequence; * = precursor form produced in *P. pastoris*. Disulfide bridges are indicated in Sin a 1.

associated with hypersensitivity to other plant-derived foods [2–9].

Table mustard is made from mixtures of varied assorted crushed seeds. Yellow (*Sinapis alba* L.) and black (*Brassica nigra*) mustards are the most commonly used in Europe, while the oriental mustard (*Brassica juncea*) is the most abundant component in the manufactured mustard from the USA and Japan [10]. All these seeds belong to the Brassicaceae family. To date, only two allergens, Sin a 1 from *S. alba* [11] and Bra j 1 from *B. juncea* [12], have been reported from mustard seeds. Sin a 1 has been characterized as a polymorphic protein that belongs to the 2S albumin family, whose members are widely distributed in plant seeds. Sin a 1 is composed of two different polypeptide chains of 39 and 88 amino acids joined by two disulfide bonds [13]. This structure is the result of a complex posttranslational process in which several sequences are proteolytically cleaved from a precursor form: an N-terminal leader region, an internal processed fragment (IPF) and several flanking sequences [14] (fig. 1). The amino acid sequence of the allergen was determined by the Edman degradation [11] and its antigenic properties studied by using monoclonal antibodies [15].

The production of well-defined recombinant allergens in high yield as soluble and homogeneous proteins is an important achievement in allergy research as it could help to improve the in vitro and in vivo diagnosis protocols of this immunological disorder [16, 17] as well as to study in

depth their structures, immunological properties and specific biological roles [18]. The precursor form of Sin a 1 was cloned and produced as a recombinant protein in the bacteria *Escherichia coli* [19, 20]. However, this expression system failed in terms of yield and solubility probably due to the incorrect formation of disulfide bonds. *Pichia pastoris* is a methylotrophic yeast that constitutes an eukaryotic system used for the production of recombinant proteins in which posttranslational modifications such as glycosylation or properly formed disulfide bonds are needed during the biosynthesis of the molecules [21–23].

In this work, the recombinant production of the precursor form of Sin a 1 allergen in the yeast *P. pastoris* is reported. The structural and immunological properties of the recombinant allergen are also analyzed and compared to those of the natural protein. Due to the high prevalence of this allergen, possible applications of the recombinant protein as marker for mustard allergy diagnosis are also discussed.

Materials and Methods

Strains and Plasmids

E. coli strains DH5αF' and TG1 were used as hosts for plasmids dephosphorylated *Sma*I-linearized pUC18 (Pharmacia Biotech) and pPIC9 (Invitrogen), respectively. GS115 his4 *P. pastoris* strain (Invitrogen) was used as host for transformations using the plasmid pPIC9.

Sera, Antibodies and Protein Extracts

Serum samples were obtained from 28 patients who were diagnosed with mustard allergy at the Department of Allergy of the Fundación Jiménez Díaz following the criteria previously reported by the Food Allergy Committee of the Sociedad Española de Alergia [24]. All the patients were untreated hypersensitive individuals, who exhibited a positive skin prick test and RAST (classes 3–6, determined by the CAP System of Pharmacia Diagnostics) to mustard. In addition to the oral allergy syndrome, more than 50% of the patients had systemic symptoms and 20% reported anaphylaxis. A polyclonal antiserum raised against Sin a 1 was obtained as described by Palomares et al. [23] and used to analyze the antigenic properties of both natural and recombinant allergens. Yellow mustard seed extract was prepared as previously described [11].

Production of Recombinant proSin a 1

Cloning procedures and bacteria manipulation were carried out according to standard methods [25]. The cDNA encoding the precursor of Sin a 1 (proSin a 1), with the exception of its leader sequence, was amplified by PCR using the pUC18/SIN2 clone [19] as template, two nondegenerate primers, a sense primer SINPIC (5'-cgtctcgagaa-aagaGCCCCGCCCCATTTAGG-3'), and an antisense primer SA-4 (5'-ccggaattctcaGGAGGGTCCAGGCAT-3') and the DynaZyme™ DNA polymerase (Finnzymes Oy). The sense primer contains an

*Xho*I restriction site (underlined), a sequence that allows the fusion of the proSin a 1 encoding region in-frame with the sequence coding for the preprosequence of the α -mating factor present in pPIC9 and that corresponding to the amino acids at positions 2–6 of the N-terminal end of the small chain of Sin a 1 (AGPFR). The antisense primer contains an *Eco*RI restriction site (underlined), a stop codon and the sequence corresponding to the last 5 residues of the C-terminal end of the large chain of Sin a 1 (MPGPS). The purified DNA fragment was treated with T4 DNA polymerase, with T4 polynucleotide kinase and ligated into a dephosphorylated *Sma*I-digested pUC18 plasmid as described [26]. This construct was used to transform DH5 α *E. coli* cells. Several clones were sequenced to confirm the correct sequence. The DNA fragment was digested with *Xho*I/*Eco*RI restriction enzymes and subcloned into the same sites of the pPIC9 vector, and used to transform TG1 *E. coli* cells in order to obtain the construct pPIC9/proSin a 1.

pPIC9/proSin a 1 recombinant plasmid (10 μ g) was linearized with the *Bgl*III restriction enzyme and the purified larger fragment was integrated by gene replacement into GS115 cell genome using lithium acetate treatment [27]. Transformed cells were incubated on minimal dextrose plates at 30°C for 4–6 days until colonies appeared. For the production of recombinant proSin a 1 (rproSin a 1) as a secreted protein, selected (His⁺Mut^s) transformed plates were processed as described previously [22]. The culture medium of GS115-induced cells was cleared by centrifugation at 3,000 g at 4°C. The presence of rproSin a 1 in the supernatant was analyzed at different times of culture (0, 24, 72 and 96 h) by SDS/PAGE. Large-scale production of rproSin a 1 was performed under similar conditions using the colony that rendered the best yield in the small-scale experiments.

Purification of rproSin a 1

The extracellular medium obtained after centrifugation of the yeast cells was subjected to dialysis against 50 mM ammonium bicarbonate pH 8.0, using membranes in 6,000–8,000 MW cutoff dialysis tubing and lyophilized. The sample was dissolved in 150 mM ammonium bicarbonate, pH 8.0, and applied to a Sephadex G-50 column. Fractions containing rproSin a 1 were pooled and lyophilized. This sample was dissolved in 3 mM pyrophosphate-HCl buffer, pH 9.0, and the resulting material was chromatographed on an SP-Sephadex C-25 column equilibrated in the same buffer. The rproSin a 1 was eluted with a gradient from 3 to 50 mM of the pyrophosphate buffer. The fractions containing rproSin a 1 were further purified by RP-HPLC on a Nucleosil C₁₈ column with a gradient (25–45%) of acetonitrile in 0.1% trifluoroacetic acid.

SDS/PAGE and Analytical Procedures

SDS/PAGE was performed using 15% polyacrylamide gels [28]. Proteins were loaded in the presence or absence of 2-mercaptoethanol (2-ME), and stained with Coomassie blue. N-terminal amino acid sequence was determined by Edman degradation using an Applied Biosystems model 477 sequencer. Mass spectrometry (MS) analysis was carried out on a Bruker Reflex II matrix-assisted laser desorption ionization time-of-flight mass spectrometer, as described [22]. Amino acid composition of the protein was determined on a Beckman 6300 amino acid analyzer after hydrolysis of 1–2 nmol of the purified protein with 5.7 N HCl at 105°C for 24 h. The concentrations of purified samples were determined from their absorbance measurements at 280 nm, using a theoretical molar extinction coefficient of 0.8.

Spectroscopic Analyses

Circular dichroism (CD) spectra were obtained on a Jasco J-715 spectropolarimeter as described [22] with minor modifications. The sample concentration was 0.2 mg/ml in 20 mM phosphate buffer, pH 7.0 and 25°C. Mean residue mass ellipticities were calculated based on 111.65 or 114.19 for Sin a 1 and rproSin a 1, respectively, as the average molecular mass/residue values obtained from the amino acid compositions, and expressed in terms of θ (degree·cm²·dmol⁻¹).

Immunological Analyses

IgE quantification was performed by ELISA in microtiter plates coated with 100 μ l/well of Sin a 1 and rproSin a 1 (2 μ g/ml) according to Tejera et al. [29]. After blocking with 3% nonfat milk, plates were incubated with individual allergic sera (diluted 1:5). Binding of human IgE was detected by mouse anti-human IgE antibodies (kindly donated by ALK-Abelló, Madrid, Spain; diluted 1:5,000), followed by horseradish peroxidase-labelled goat anti-mouse IgG (diluted 1:5,000; Pierce). The peroxidase reaction was developed using fresh enzyme substrate (0.03% H₂O₂, and 0.63 mg/ml of *o*-phenylenediamine in 0.1 M sodium citrate, pH 5.0) and measuring optical density at 492 nm. Each value is the mean of two determinations.

ELISA inhibition assays were performed as described [30]. After coating with 0.2 μ g of rproSin a 1, plates were treated with the rabbit polyclonal antiserum raised against Sin a 1 (diluted 1:300,000) previously incubated with six different amounts of Sin a 1 or rproSin a 1 (10⁻¹ to 10⁴ ng) as inhibitors. This reaction was followed by a treatment with horseradish peroxidase-labelled goat anti-rabbit IgG (diluted 1:3,000; TAGO). IgE-binding inhibition to Sin a 1- or rproSin a 1-coated wells was carried out with a pool of sera (diluted 1:5) previously incubated with the same amounts of Sin a 1 or rproSin a 1 used in the IgG-binding inhibition experiment. IgE-binding inhibition to yellow mustard seed extract (40 μ g) was performed with 21 individual sera (diluted 1:5), using 10 μ g of BSA as negative control or 10 μ g of rproSin a 1 as inhibitor. In both experiments, mouse anti-human IgE mAb and horseradish peroxidase-labelled goat anti-mouse IgG were used. The percentage of inhibition of the binding after preincubation with the different inhibitors was calculated as [(OD – OD_{inh})/OD] $\times$ 100, where OD and OD_{inh} indicate the absorbance values obtained without or with inhibitors before incubation, respectively. Each value is the mean of two determinations.

After SDS-PAGE of the purified rproSin a 1 and transferred to nitrocellulose membranes, immunodetection was achieved by using a Sin a 1-specific pAb (diluted 1:80,000) or a pool of sera (n = 5) from patients allergic to mustard (diluted 1:10) as described [31]. The signal was developed by the ECL Western blot reagent (Amersham Pharmacia Biotech) [31].

Results

Production in *P. pastoris* and Purification of rproSin a 1

The cDNA encoding the precursor form of Sin a 1 (proSin a 1) was amplified by PCR using two nondegenerate primers corresponding to the N-terminal region of the small chain and C-terminal end of the large chain of Sin a 1. The DNA fragment covered the small and large

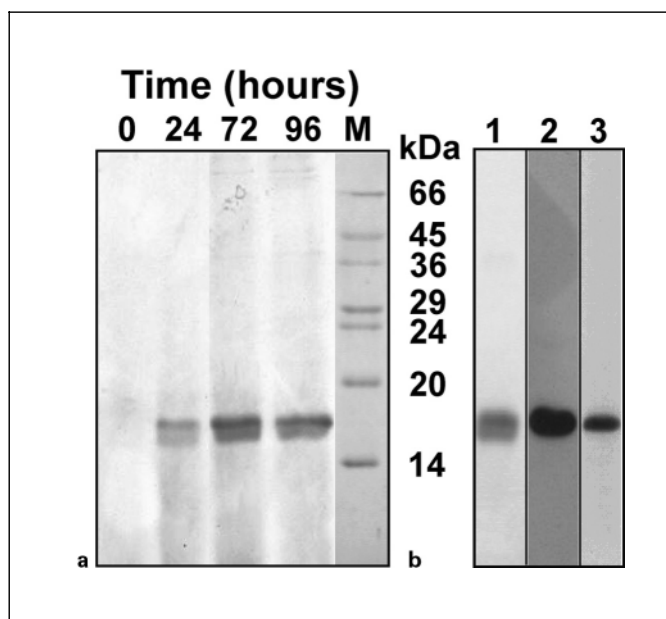


Fig. 2. SDS/PAGE analysis of rproSin a 1 production and purification. **a** Time course of the expression of rproSin a 1 in *P. pastoris* cells. Supernatants from cultures were harvested at different times (in hours) after methanol induction and stained with Coomassie blue. M = Molecular mass markers. **b** Purified rproSin a 1 was analyzed by Coomassie blue staining (lane 1), and by immunostaining with a polyclonal antiserum raised against Sin a 1 (lane 2) and with a pool of sera from patients allergic to mustard (lane 3) after transfer to membranes.

chains of Sin a 1 including the 15 amino acids that constitute the IPF of the molecule. It was cloned downstream of the AOX1 promoter and in-frame with the α -factor secretion sequence into the pPIC9 vector. The linearized construction pPIC9/proSin a 1 was used to transform GS115 yeast cells. rproSin a 1 was purified from the culture medium as described below.

The time course of the production of rproSin a 1 was followed by SDS/PAGE analysis of the extracellular medium of the yeast culture (fig. 2a). A major band of around 17 kDa appeared at 24 h of induction. After small-scale expression, the best producing colony was used for large-scale purification. The rproSin a 1 was purified from the extracellular medium by three sequential chromatographic steps: gel filtration on a Sephadex G-50, ion exchange on a SP-Sephadex C-25, and RP-HPLC on a nucleosil C₁₈ column. The final yield of the purified protein was 15 mg/l of cell culture.

rproSin a 1 was analyzed by Coomassie blue staining after SDS/PAGE (fig. 2b, lane 1) and by immunoblotting

with a polyclonal antiserum raised against the Sin a 1 allergen as well as with a pool of sera from patients allergic to mustard with a positive response to Sin a 1 (fig. 2b, lanes 2 and 3). As shown in figure 2b, these IgG and IgE antibodies recognized rproSin a 1.

Molecular Characterization of rproSin a 1

The allergen Sin a 1 showed an apparent molecular mass of 16.5 kDa in SDS/PAGE under nonreducing conditions (fig. 3a, lane 1). As this allergen is composed of two polypeptide chains of 9.5 and 5 kDa linked by disulfide bonds [13], both chains were dissociated after treatment with 2-ME (fig. 3a, lane 2). However, rproSin a 1 consists of a single polypeptide chain, and under nonreducing conditions (fig. 3a, lane 3) as well as after reductive treatment (fig. 3a, lane 4) a single band was detected. The higher mobility exhibited by rproSin a 1 after 2-ME treatment could be attributed to the unfolding of the molecule as a consequence of the disulfide bond disruption.

Edman degradation of the N-terminal end of rproSin a 1 gave the sequence RIPK indicating that the molecule was processed in the yeast four amino acids downstream of the expected proteolytic site and lacked the first four amino acids (AGPF) (fig. 3b).

MS analysis of the recombinant product was carried out and the spectrum showed a peak of 16,069 Da (fig. 3c). The amino acid composition of rproSin a 1 determined after acidic hydrolysis fitted well to that calculated from the sequence deduced from the clone lacking the four amino acids from the N-terminal end (data not shown). Both results supported the conservation of the rest of the primary structure of the protein.

CD in the far UV informs about the secondary structure of polypeptide chains, thus giving a measurement of the folding of the molecule. Figure 2d shows the CD spectra in the far-UV range (195–245 nm) obtained for Sin a 1 and rproSin a 1. No differences were detected either in the shape or in the ellipticity values for both molecules.

Antigenic and Allergenic Properties of rproSin a 1

To analyze the antigenic equivalence between rproSin a 1 and Sin a 1, ELISA inhibition experiments were performed using a Sin a 1-specific polyclonal antiserum. Figure 4 shows that the binding of IgG antibodies to rproSin a 1 was totally abolished when Sin a 1 or rproSin a 1 were used as inhibitors. The extent of inhibition with both molecules was equivalent with minor differences, indicating that rproSin a 1 conserved the antigenic determinants of Sin a 1.

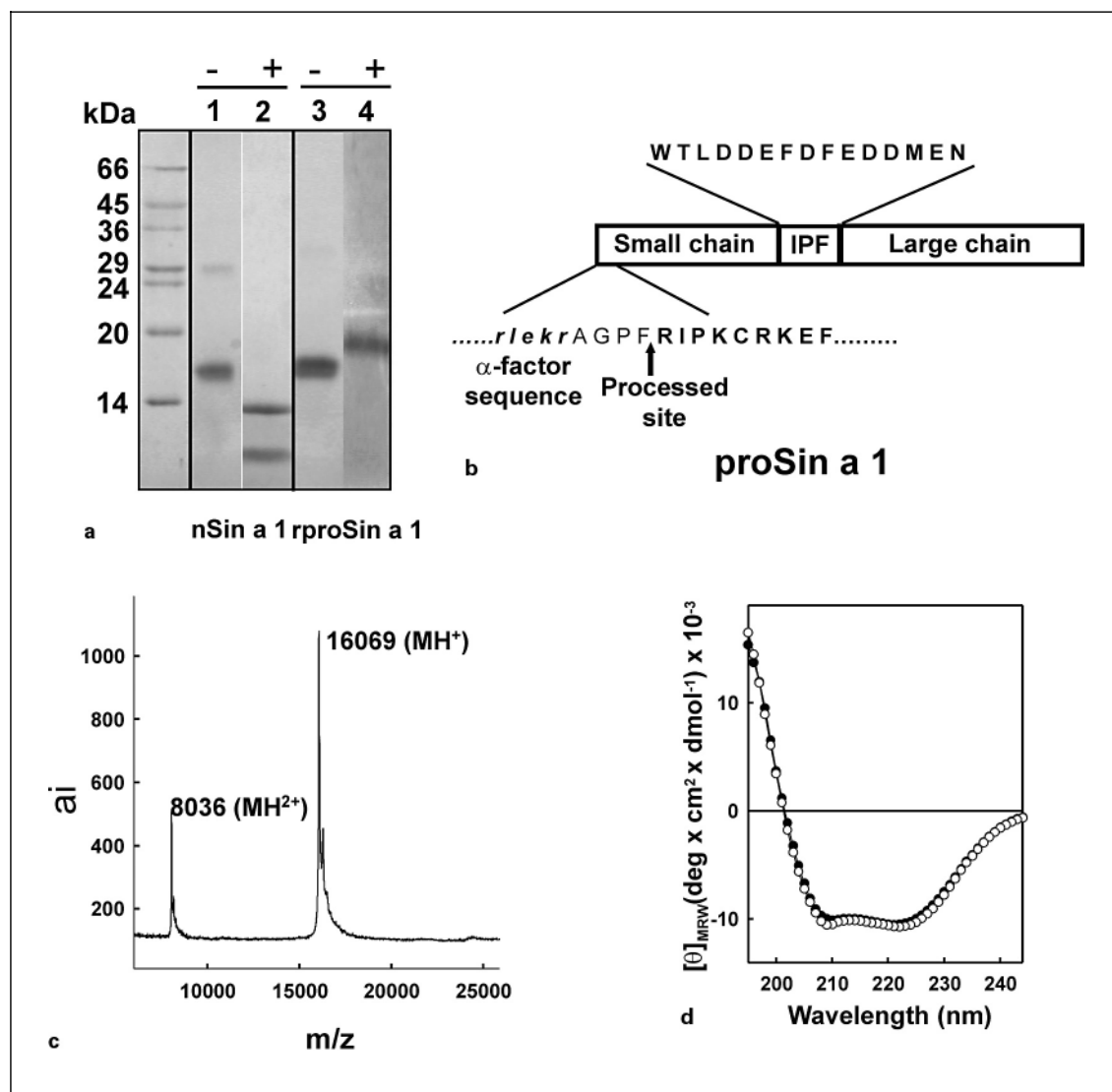
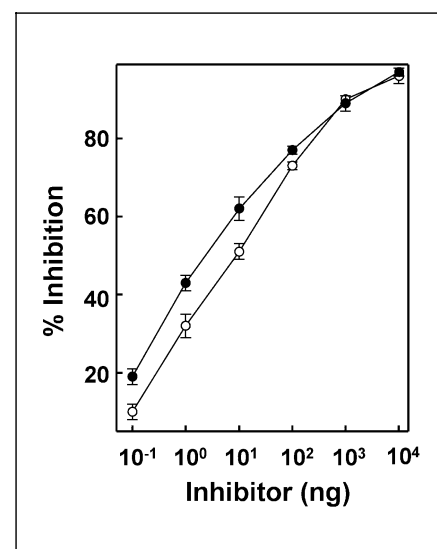


Fig. 3. Molecular properties of rproSin a 1. **a** Analysis of Sin a 1 (lanes 1 and 2) and rproSin a 1 (lanes 3 and 4); purified proteins in the absence (lanes -) and the presence (lanes +) of 2-ME were subjected to SDS/PAGE and stained with Coomassie blue. **b** Scheme of rproSin a 1. The amino acid sequence of the IPF joining the small and large chains is displayed. The site where rproSin a 1 is processed is indicated with an arrow. In lower case italics the amino acids belonging to the *Saccharomyces cerevisiae* α-mating factor peptide are shown. **c** MS analysis of purified rproSin a 1; the mass/charge ratio (m/z) is given as arbitrary intensity (ai). **d** CD spectra in the far-UV range (195–245 nm) of Sin a 1 (●) and rproSin a 1 (○) at pH 7.0 and 25 °C. $[\theta]_{MRW}$ = Mean residue weight ellipticity.

Fig. 4. Analysis of the IgG-binding capability of rproSin a 1. ELISA inhibition assays of the binding of a rabbit polyclonal antiserum raised against Sin a 1 to rproSin a 1-coated wells when Sin a 1 (●) and rproSin a 1 (○) were used as inhibitors.



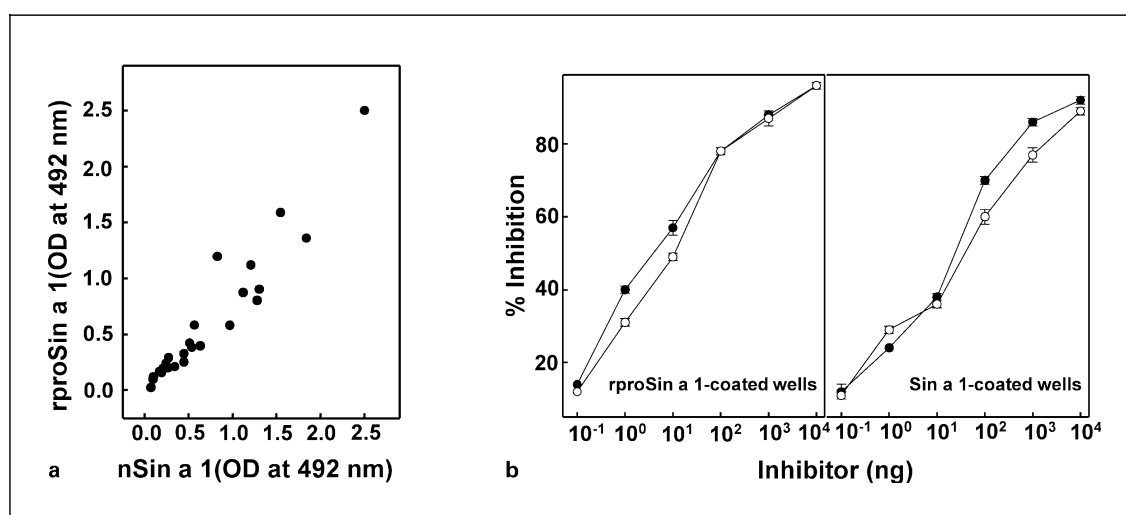


Fig. 5. Analysis of the IgE-binding capability of rproSin a 1. **a** Estimation of the levels of specific IgE by ELISA. OD values at 492 nm of the IgE binding to Sin a 1 versus those obtained for rproSin a 1 using 28 sera from patients allergic to mustard. **b** ELISA inhibition analyses of the IgE binding from a pool of sera from patients allergic to mustard to rproSin a 1- or Sin a 1-coated wells using Sin a 1 (●) or rproSin a 1 (○) as inhibitors.

To compare the IgE-binding capability of Sin a 1 and rproSin a 1, both molecules were tested in ELISA with 28 individual sera from patients allergic to mustard. Seventy-five percent (21 out of 28) of the tested sera were positive to Sin a 1. All these sera were also reactive to rproSin a 1, with a linear correlation (0.95) for the responses to both proteins (fig. 5a).

The IgE-binding capability equivalence between Sin a 1 and rproSin a 1 was analyzed in ELISA inhibition experiments using a pool of sera from patients allergic to mustard. The IgE binding to each molecule was determined after preadsorbing the pool of sera with different amounts of Sin a 1 or rproSin a 1 as inhibitors. Both proteins showed equivalent IgE inhibition curves (fig. 5b). These results indicated that Sin a 1 and rproSin a 1 shared IgE epitopes.

Contribution of Sin a 1 to the Allergenicity of Yellow Mustard Seeds

To determine the contribution of Sin a 1 to the overall allergenicity of yellow mustard seed extract, ELISA inhibition experiments were carried out using the 21 individual sera from patients allergic to mustard with a positive response to Sin a 1. Yellow mustard seed extract-coated wells were treated with individual sera preincubated alternatively with BSA or rproSin a 1. Preincubation with rproSin a 1 led to a considerable reduction of the IgE bind-

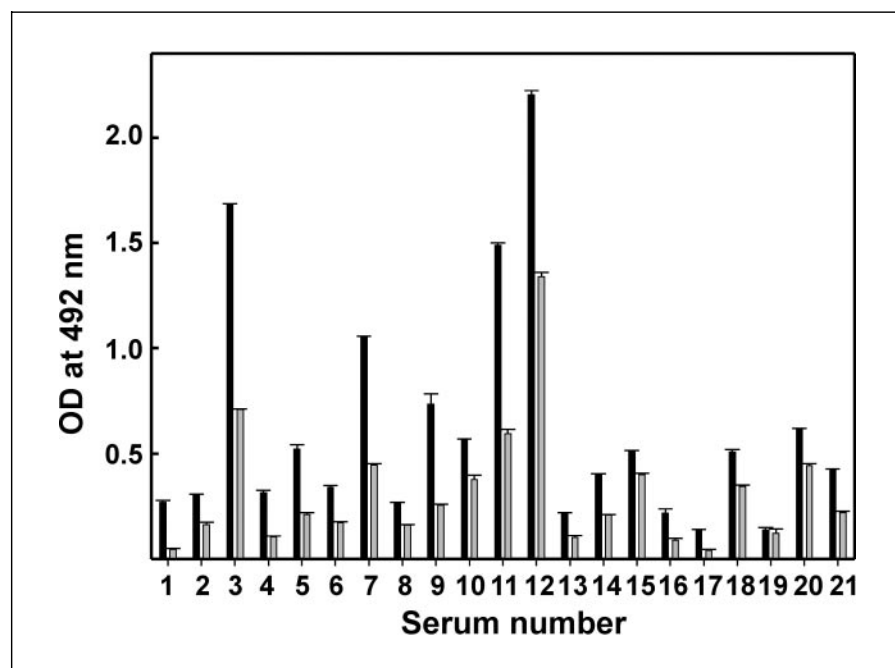
ing to ELISA plate-bound yellow mustard seed extract for most of the sera (fig. 6). The percentage inhibition ranged between 13% (serum 19) and 83% (serum 1) with an inhibition average of 54%. Interestingly, 76% of the tested sera (16 out of 21) presented a percentage of inhibition higher than 50%.

Discussion

Mustard has been reported for many years as an allergenic food. Sin a 1, a 2S albumin storage protein from yellow mustard seeds, has been largely recognized as the major allergen from this spice. Our group isolated the allergen from its natural source [13]. It was sequenced [11], and immunologically characterized [15].

One of the major goals in allergy research is to obtain well-defined recombinant allergens that could be used to better define its structural and immunological properties as well as to be included in the diagnosis and specific immunotherapy protocols. 2S albumins possess two structural properties that make their recombinant production difficult: first, they are Cys-enriched proteins with a high concentration of disulfide bonds, and second, they present a complex molecular design with two different subunits joined by this type of Cys-Cys bridges. Previous attempts at expressing a properly folded rSin a 1 in *E. coli*

Fig. 6. Contribution of rproSin a 1-specific IgE epitopes to the overall allergenicity of yellow mustard seeds measured in ELISA. OD at 492 nm of the IgE binding of 21 sera from allergic patients to mustard extract-coated wells after preincubation with 10 µg of BSA (black bars) or 10 µg of rproSin a 1 (grey bars).



always failed [19, 20]. The genetic organization of their genes makes feasible a biotechnology strategy using the precursor forms for the recombinant production of these heterodimeric proteins. The combination of this molecular biology design and the use of *P. pastoris* have made it possible to obtain a variant of the mustard allergen with IgG- and IgE-binding capabilities similar to those of the wild-type allergen. We have produced the precursor form of Sin a 1, both chains linked by the IPF peptide, as a recombinant protein in the eukaryotic methylotrophic yeast *P. pastoris*. This strategy is supported by different facts: the IPF is required for the correct folding of 2S albumins [32, 33]. Supporting these results, the attempts for reconstituting the heterodimeric napins with the isolated chains were unsuccessful [34]. On the other hand, the precursor form of BnIb, a 2S albumin from rapeseed (*Brassica napus*), was produced in *P. pastoris* and showed structural and immunological properties identical to its mature counterpart isolated from rapeseed [23]. Finally, the yield of the recombinant products in *P. pastoris* is usually high, and the maturation mechanisms involve disulfide bond formation and N-glycosylation processes. Moreover, the recombinant proteins can be obtained as secreted products in the extracellular medium making the purification protocol easier since this medium is poor in endogenous proteins.

rproSin a 1 has been obtained as a soluble form and purified to homogeneity. The rproSin a 1 is produced as a

single polypeptide chain, as expected for the expression design, without suffering any processing of their internal sequences in the yeast, since both chains (small and large) remained connected by the IPF. Previous works have reported the presence of charged residues in the protein sequence as a potential target for a secondary processing of the recombinant proteins produced in *P. pastoris* [35]; however, such cleavage processes were not detected in rproSin a 1. Despite of the differences between rproSin a 1 and Sin a 1 in their primary structures, both proteins showed identical conformation as their CD spectra in the far-UV range showed.

Recombinant allergens should be validated comparing their immunological properties with those of their natural forms. Therefore, IgG- and IgE-binding properties of rproSin a 1 were compared to those of Sin a 1 using ELISA inhibition experiments, and they indicated that most of the epitopes of Sin a 1 were conserved in rproSin a 1. The very small differences should be due to the polymorphic character of Sin a 1. In addition, both forms of the mustard allergen were equally recognized by all the tested sera from patients allergic to mustard with a positive response to Sin a 1. These results pointed out that rproSin a 1 showed equivalent allergenic properties to Sin a 1, thus supporting the fact that the IPF is needed in the folding of the molecule and its presence has little effect on the immunological features of the allergen.

The availability of rproSin a 1 has provided a powerful tool for studying the allergenic relevance of Sin a 1 within the mustard-allergic population and the particular contribution to the overall allergenicity of this protein for each patient. rproSin a 1 was able to block from 13 to 83% of the IgE-binding response of the whole yellow mustard seed extract. The high structural and immunological stability of Sin a 1 and its resistance to digestion [36] give it the potential capability to act as a main food allergen sensitizer, and therefore the use of rproSin a 1 for the in vitro diagnosis could help to identify patients who are primarily sensitized to mustard. This later aspect together with the fact that most of the patients allergic to mustard present with specific IgE antibodies directed to Sin a 1 (prevalence of 75%) makes it possible to consider

rproSin a 1 as a good candidate to be included in diagnosis protocols of type I allergy to mustard.

Finally, this work also provides the strategy to design and produce other allergenic recombinant variants of rproSin a 1 that could be used for specific immunotherapy protocols in future.

Acknowledgments

This work was supported by grants SAF2002-02711 from the Ministerio de Ciencia y Tecnología (Spain) and Red Temática de Investigación Cooperativa de Alergia a Alimentos (G03/094, Fondo de Investigación Sanitaria, Ministerio de Sanidad y Consumo, Spain).

References

- van Toorenbergen AW, Dieges PH: Demonstration of spice-specific IgE in patients with suspected food allergies. *J Allergy Clin Immunol* 1987;79:108–113.
- Panconesi E, Sertoli A, Fabbri P, Giorgini S, Spallanzani P: Anaphylactic shock from mustard after ingestion of pizza. *Contact Dermatitis* 1980;6:294–295.
- Niinimäki A, Hannuksela M: Immediate skin test reactions to spices. *Allergy* 1981;36:487–493.
- Rance F, Kanny G, Dutau G, Moneret-Vautrin DA: Food hypersensitivity in children: Clinical aspects and distribution of allergens. *Pediatr Allergy Immunol* 1999;10:33–38.
- Caballero T, San-Martin MS, Padial MA, Contreras J, Cabanas R, Barranco P, López-Serrano MC: Clinical characteristics of patients with mustard hypersensitivity. *Ann Allergy Asthma Immunol* 2002;89:166–171.
- Monreal P, Botey J, Pena M, Marin A, Eseverri JL: Mustard allergy. Two anaphylactic reactions to ingestion of mustard sauce. *Ann Allergy* 1992;69:317–320.
- Rance F, Dutau G: Labial food challenge in children with food allergy. *Pediatr Allergy Immunol* 1997;8:41–44.
- Kanny G, Fremont S, Talhouarne G, Nicolas JP, Moneret-Vautrin DA: Anaphylaxis to mustard as a masked allergen in 'chicken dips'. *Ann Allergy Asthma Immunol* 1995;75:340–342.
- Rance F, Dutau G, Abbai M: Mustard allergy in children. *Allergy* 2000;55:496–500.
- Kay AB: Food allergens; in Kay AB (ed): *Allergy and Allergic Diseases*. Oxford, Blackwell, 1997, p 974.
- Menéndez-Arias L, Moneo I, Domínguez J, Rodríguez R: Primary structure of the major allergen of yellow mustard (*Sinapis alba* L.) seed, Sin a I. *Eur J Biochem* 1988;177:159–166.
- Monsalve RI, González de la Peña MA, Menéndez-Arias L, López-Otín C, Villalba M, Rodríguez R: Characterization of a new oriental-mustard (*Brassica juncea*) allergen, Bra j IE: Detection of an allergenic epitope. *Biochem J* 1993;293:625–632.
- Menéndez-Arias L, Monsalve RI, Gavilanes JG, Rodríguez R: Molecular and spectroscopic characterization of a low molecular mass seed storage protein from yellow mustard (*Sinapis alba*). *Int J Biochem* 1987;10:899–907.
- Crouch ML, Tenbarger KM, Simon AE, Ferl R: cDNA clones from *Brassica napus* seed storage proteins: Evidence from nucleotide sequence analysis that both subunits of napin are cleaved from a precursor polypeptide. *J Mol Appl Genet* 1983;2:273–283.
- Menéndez-Arias L, Domínguez J, Moneo I, Rodríguez R: Epitope mapping of the major allergen from yellow mustard seeds, Sin a I. *Mol Immunol* 1990;27:143–150.
- Valenta R, Lidholm J, Niederberger V, Hayek B, Kraft D, Gröndlund H: The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CDR and CRIT). *Clin Exp Allergy* 1999;29:896–904.
- Heiss S, Mahler V, Steiner R, Spitzauer S, Schweiger C, Kraft D, Valenta R: Component-resolved diagnosis (CRD) of type I allergy with recombinant grass and tree pollen allergens by skin testing. *J Invest Dermatol* 1999;113:830–837.
- Valenta R, Kraft D: From allergen structure to new forms of allergen-specific immunotherapy. *Curr Opin Immunol* 2002;14:718–727.
- González de la Peña MA, Villalba M, García-López JR, Rodríguez R: Cloning and expression of the major allergen from yellow mustard seeds, Sin a I. *Biochem Biophys Res Commun* 1993;190:648–653.
- González de la Peña MA, Monsalve RI, Batanero E, Villalba M, Rodríguez R: Expression in *Escherichia coli* of Sin a 1, the major allergen from yellow mustard. *Eur J Biochem* 1996;237:827–832.
- White CE, Kempf NM, Komives EA: Expression of highly disulphide-bonded proteins in *Pichia pastoris*. *Structure* 1994;2:1003–1005.
- Huecas S, Villalba M, Gonzalez E, Martínez-Ruiz A, Rodríguez R: Production and detailed characterization of biologically active olive pollen allergen Ole e 1 secreted by the yeast *Pichia pastoris*. *Eur J Biochem* 1999;261:539–546.
- Palomares O, Monsalve RI, Rodríguez R, Villalba M: Recombinant pronapin precursor produced in *Pichia pastoris* displays structural and immunologic equivalent properties to its mature product isolated from rapeseed. *Eur J Biochem* 2002;269:2538–2545.
- Comité de Reacciones Adversas a alimentos: Metodología diagnóstica en la alergia a alimentos. *Rev Esp Alergol Inmunol Clín* 1999;14:50–62.
- Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual*, ed 2. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 1990.
- Villalba M, Batanero E, Monsalve RI, González de la Peña MA, Lahoz C, Rodríguez R: Cloning and expression of Ole e 1, the major allergen from olive tree pollen. *J Biol Chem* 1994;269:15217–15222.
- Gietz RD, Schiestl RH: Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. *Yeast* 1991;7:253–263.
- Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.

- 29 Tejera ML, Villalba M, Batanero E, Rodríguez R: Identification, isolation, and characterization of Ole e 7, a new allergen of olive tree pollen. *J Allergy Clin Immunol* 1999;104:797–802.
- 30 Barderas R, Villalba M, Lombardero M, Rodríguez R: Identification and characterization of Che a 1 allergen from *Chenopodium album* pollen. *Int Arch Allergy Immunol* 2002;127:47–54.
- 31 Batanero E, Villalba M, Ledesma A, Puente XS, Rodríguez R: Ole e 3, an olive-tree allergen, belongs to a widespread family of pollen proteins. *Eur J Biochem* 1996;241:772–778.
- 32 Muren E, Ek B, Rask L: Processing of the 2S storage protein pronapin in *Brassica napus* and in transformed tobacco. *Eur J Biochem* 1995;227:316–321.
- 33 D'Hondt K, Van Damme J, Van Den Bossche C, Leejeerajumneam S, De Rycke R, Derksen J, Vandekerckhove J, Krebbers E: Studies of the role of the propeptides of the *Arabidopsis thaliana* 2S albumin. *Plant Physiol* 1993;102:425–433.
- 34 Monsalve RI, Villalba M, López-Otín C, Rodríguez R: Structural analysis of the small chain of the 2S albumin, napin nIII, from rapeseed. Chemical and spectroscopic evidence of an intramolecular bond formation. *Biochim Biophys Acta* 1991;1078:265–272.
- 35 Alcocer MJC, Murtagh GJ, Bailey K, Dumoulin M, Sarabia A, Parker MJ, Archer DB: The disulphide mapping, folding and characterization of recombinant Ber e 1, an allergenic protein, and SFA8, two sulphur-rich 2S plant albumins. *J Mol Biol* 2002;324:165–175.
- 36 Astwood JD, Leach JN, Fuchs RL: Stability of food allergens to digestion in vitro. *Nat Biotechnol* 1996;14:1269–1273.