

Short Communication

Is the Cross-Reactivity of Sin a 1, 2S Albumin from Mustard Seeds, Exclusively Restricted to *Brassicaceae* Members?

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Abstract

Food allergy is an important health problem that is gradually growing worldwide. The most prevalent food allergens are those of vegetal origin, affecting approximately 2-4% of the European adult population and 8% of childhood.

2S albumins have been described as relevant food allergens and their availability as purified molecules could constitute important clinical diagnostic advantage for food allergies. Despite the relatively low sequence similarity between members of this plant protein family, especially from distant species, studies focused on a potential role for these allergens in cross-reactivity and unexpected reactions have been approached.

In this manuscript, different extracts from *Brassicaceae* family, tree nuts and other seeds have been isolated. Sin a 1, the 2S albumin from mustard seeds (*Sinapis alba*) and 2S albumin from pine nuts were purified and identified by mass-spectrometry. These proteins display typical features as their homologues from the 2S albumin family retaining the ability to bind IgE. Immunoblotting assays with a pool of Sin a 1-allergic patients' sera revealed the allergenic capacity of members from the *Brassicaceae* family across the recognition of Sin a 1 and the IgE binding ability to pine nuts and sesame even though their different phylogenetic family.

In conclusion, although cross-reactivity related to Sin a 1 is mainly assigned to *Brassicaceae*, other seeds, such as pine nut have to be kept in mind in order to unexpected reactions. These characterized allergens could be used as clinical tools elaborating a more accurate diagnosis and therefore a more effective allergy treatment.

ABBREVIATIONS

BCA: Bicinchoninic Acid Method; βME: 2- Mercaptoethanol; OAS: Oral Allergy Syndrome; RP-HPLC: Reverse Phase High Performance Liquid Chromatography; SMP: Skim Milk Powder; SPT: Skin Prick Test

INTRODUCTION

Type I hypersensitivity is a common immunological disorder, that affects about 5% of young children and 8% of adult population in western countries [1,2]. Therefore, the identification and characterization of new antigens involved in IgE-mediated reactions is essential for understanding the

mechanisms of allergy.

In comparison with aeroallergens, food allergens are usually stable proteins, which are capable of crossing the gut barrier and triggering all the reactions. Among plant foods, tree nuts and seeds constitute important allergenic sources. Allergic reactions to these foods begin at an early age and can persist throughout the life of the individual, with severe symptoms associated such as anaphylaxis [3]. Therefore, the study of these allergenic sources is crucial to develop therapies that improve the quality of life of allergic patients.

2S albumins are one of the major groups of seed storage proteins and belong to the prolamin family with low prevalence

among population but triggering severe and unexpected symptoms. These proteins are synthesized *in vivo* as a polypeptide chain precursor. They undergo proteolytic processing of three fragments rendering the mature protein: an N-terminal leader region, an internal processed fragment and an extra amino acid residue in the C-terminal region [4]. They have sizes between 10 and 14 kDa and generally consist of two polypeptide chains of around 3-5 kDa and 8-10 kDa, stabilized by a pattern of conserved inter/intra chain disulfide bridges scaffold [5]. These proteins are encoded by a multigene family leading to numerous is forms, in such a way that natural 2S albumin patterns show a high level of polymorphism. Furthermore, they exhibit a high stability to thermal and enzymatic treatments, which enable them to sensitize the environment of the gastrointestinal tract.

These proteins are relevant and constitute often major allergens of several tree nuts and seeds, involved in severe symptoms such as anaphylaxis [6]. A great amount of 2S albumins have been described as allergens that lead to allergic reactions after their ingestion.

Their structural properties have a great influence in the immunological activity. Sirvent et al. [7], showed that secondary structure of Sin a 1, the 2S albumin from mustard seeds, did not significantly change during heating treatment or by the addition of pepsin or trypsin proteases, without no appreciable variations on the ability to bind IgEs from sera of allergic patients after treatment.

Even though 2S albumins have similar three-dimensional (3D) folding, cross-reactivity seems to be rarely frequent in this protein family. This lack of cross-reactivity has been attributed the low similarity of the regions corresponding to IgE-binding sites, called hyper variable regions, located in the large chain of these proteins. However, certain cases of cross-reactivity have been reported; an example is the almond or Brazil nut allergy and peanut allergy which are caused by their homologous 2S albumins [8], due to the conservation of a continuous epitope.

Mustard seed, as many other spices, is frequently consumed and sometimes appears as a hidden allergen in foods, resulting in unexpected allergic reactions [9]. For many years clinical cases of mustard allergy have been reported [10,11] and its prevalence has notably increased in developed countries, becoming one of the most important food allergen for children [12]. On the other hand, allergy to pine nuts, belonging to gymnosperm group, has been documented in the scientific literature since 1958, being described to occur after consumption of pine nuts as part of salads, meatballs, cakes, candies or cookies [13], where small amounts of pine nut can induce dangerous reactions in sensitized patients [14]. No cross-reactivity has been reported in previous studies between gymnosperms and angiosperms such mustard seeds.

Because of that, the aim of this study was to use the natural 2S albumin from mustard seeds to determine its IgE-binding capacity and its potential cross-reactivity with other 2S albumins, in order to show that these proteins may be used as clinical tools in Component-Resolved Diagnosis (CRD). These results could allow us to elaborate a more accurate diagnosis and therefore a more effective treatment of food allergies.

MATERIALS AND METHODS

Patients sera

5 Mustard allergic patients were recruited by Allergy Services of Fundación Jiménez Díaz of Madrid. Inclusion criteria were a well-defined clinical history of mustard allergy and elevated levels of specific IgE against Sin a 1. As negative controls, we included a group of individuals with tolerance to mustard and without food allergic episodes. The study was approved by the Ethic Committee of the Hospital and written informed consent was obtained from all subjects.

Specific IgE determination in the studied population

Specific IgE to mustard seeds and natural proteins were determined by ELISA with serum samples.

Protein extracts and purification of 2S albumins from mustard seeds and pine nuts

Seeds and nuts used for this study were crushed under liquid nitrogen to obtain their flour. Proteins were extracted by homogenization in sodium borate buffer (0.15 M, pH 8.0) containing PMSF (1 mM) and shaking during one hour at 4 °C; for tree-nut extracts, we used ammonium bicarbonate (0.2 M) instead of sodium borate. The slurry was centrifuged at 12000 x g for 30 min at 4°C and filtered and delipidated in acetone. The slurry was centrifuged again at 5500 x g for 15 min at 4 °C. This process was repeated two more times and finally, organic phase was discarded and the sediment resuspended in ammonium bicarbonate buffer (20 mM, pH 8.0), stored at -20 °C. Extract concentrations were determined by Lowry method [15].

Pine nut 2S albumin was isolated by a size exclusion chromatography using a Sephadex G-50 Medium column equilibrated 0.2 M ammonium bicarbonate at a flow-rate of 0.6 ml/min. After analyzing by SDS-PAGE, and the low molecular-mass protein fractions were pooled together for a second step in a reverse phase- High Performance Liquid Chromatography (RP-HPLC), using a C-18 reverse phase column, with an acetonitrile gradient from 0% to 60% at a flow-rate of 0.5 ml/min. Fractions again were analyzed again by SDS-PAGE.

For isolation of mustard seed 2S albumin, Sin a 1, a second size exclusion chromatography with Sephadex G-50 Fine was loaded with fractions resulted from the first step, using 0.15 M ammonium bicarbonate at a flow rate of 1 ml/min. Finally, an ion exchange chromatography in a SP-Sephadex C25 column, using a sodium pyrophosphate gradient from 3 to 50 mM at a flow rate of 1.3 ml/min was performed. Isolated proteins were stored at -20 °C.

Analytical procedures

SDS-PAGE was performed in 17% polyacrylamide gels in the presence and absence of β ME. Proteins were visualized by means of Coomassie blue staining or alternatively transferred to nitrocellulose membranes (Amersham, Hybond, Germany). Protein concentration was determined using the bicinchoninic acid method (BCA) (Pierce Chemical, Rockford, Ill) [16].

Identification of 2S albumins by Mass-Spectrometry

Proteins were identified by matrix-assisted laser desorption/ionization (MALDI)-Time of flight (TOF) Mass-Spectrometry. For this purpose, SDS-PAGE was performed in sterile conditions, staining the gel with colloidal Coomassie Blue and conserving it in ultrapure Milli-Q® water.

Samples were analyzed in a mass spectrometer 4800 Proteomics Analyzer (AB SCIEX). This spectrometer has a MALDI ionization source and two TOF analyzers in tandem, allowing knowing the molecular masses of proteins and their fingerprint patterns.

According to the 2S albumin from pine nuts, 90% of sequence from rendered peptides matched to the Pin p 1 allergen.

IgE immunoblotting analysis

Immuno detection of proteins in nitrocellulose membranes was achieved as described Menéndez-Arias et al (1987), by using a pool of sera above reported (1:5). The binding of human IgE was detected with a mouse anti-human IgE monoclonal antibody (diluted 1:5000) kindly provided by ALK-Abelló (Madrid, Spain), followed by HRP-labeled rabbit anti-mouse IgG (1:2500 diluted; Pierce, Rockford, Illinois). The signal was developed by the ECL-Western Blotting reagent, and detected in a luminescent imager analyzer LAS3000. Quantitation of the signal was performed in triplicate using the computer program Multigauge V3.0.

For the inhibition immunoblotting, sera were diluted with 6% skim milk powder (SMP) in PBS-T, and were pre-incubated with tree nut or seed extracts, using BSA as negative control, at room temperature for 2 h. The inhibition mixtures or whole sera (diluted 1:5 3% SMP in PBS-T) were added to the membrane and incubated at room temperature for 2 h.

Amino acid sequence alignment

Due to the results obtained at IgE-binding assays, we focused on the analysis of the amino acid sequence similarities between 2S albumins from pine nut, sesame seed and *Brassicaceae* members. Sequences alignments were performed with the informatics

program GeneDoc and selected sequences were: *Pinus pinea* 2S albumin (Pin p 1), *Sinapis alba* 2S albumin (Sin a 1) and *Brassica napus* 2S albumin (Bra n 1).

RESULTS AND DISCUSSION

Clinical features of patients

Mustard seeds allergic patients (N=5) included in this study had the clinical features summarized in Table (1). The average age of the patients was 35.2 ± 6.2 years showing a predominance of male individuals (60%). Clinical symptoms of all patients can be described as OAS and systemic reactions. The most frequent of these systemic reactions was general urticaria in 80% of the patients' sample, while each individual presented other symptoms such as asthma, angioedema, contact urticarial, digestive symptoms, throat tightness and rhino conjunctivitis. In addition, they showed positive SPT with other mustard seed allergen, Sin a 2, but negative ELISA to Sin a 3 and Sin a 4.

Only one patient was allergic exclusively to mustard seed, meanwhile four patients (80%) were also allergic to olive pollen (*Olea europaea*) and other vegetables. Among these individuals, three of them showed allergy to foods including kiwi, nuts, melon or *Rosaceae* family and the other one presented symptoms only with mustard and pollen.

2S albumins isolation and identification

Protein extracts from *Brassicaceae* species (mustard seed, rapeseed, cabbage seed and radish seed), grass pea seed, sesame seed, lentil and tree nuts (sunflower seed, hazelnut, cashew nut, peanut and pine nut) were obtained from their respective natural sources; then, the isolation of low molecular-mass proteins using a combination of three chromatographic steps: size exclusion, ion exchange and RP-HPLC were performed. The eluted proteins were quantified and visualized by SDS-PAGE. Proteins with a molecular mass around 12 kDa were identified by their fingerprint using MALDI-TOF-TOF mass-spectrometry techniques. The results obtained by mass-spectrometry were analyzed by BLASTp program, confirming they belonged to the 2S albumins super family. The 2S albumin from pine nuts

Table 1: Clinical characteristics of patients with mustard allergy.

		Mustard allergy										
		SPT*			ELISA † OD ₄₉₂							
Patient no.	Sex/Age (y)	Symptoms	Mus-tard	Sin a 1	Sin a 2	Mustard	Sin a 1	Sin a 2	Sin a 3	Sin a 4	Other food allergies	Pollen allergy
1	F/51	OAS, U, AE, D	221	170	69	3.336	3.186	0.98	Neg	Neg	No	Yes
2	F/30	OAS, CU, TT, D	119	97	46	3.477	3.532	2.138	Neg	Neg	k, n	Yes
3	M/30	OAS, U, A	163	544	156	2.876	2.345	2.404	Neg	Neg	No	No
4	M/36	OAS, U	137	353	139	3.5	3.5	2.138	Neg	Neg	r, m	Yes
5	M/30	OAS, U, AE, TT, A, RC	171	135	83	0.934	0.92	0.166	Neg	Neg	n	Yes

Abbreviations: A: Asthma; AE: Angiodema; CU: Contact Urticaria; D: Digestive Symptoms; F: Female; k: kiwi; m: Melon; M: male; n: Nuts (including almond); Neg: Negative (< 0.100 for ELISA and wheal area < 7 mm² for SPT); OAS: Oral Allergy Syndrome; r: Rosaceae Family (peach, apple, pear, apricot, plum, cherries, and strawberries and excluding almond); RC: Rhino Conjunctivitis; TT: Throat Tightness; U: Generalized Urticaria

*Skin prick test: wheal area in mm²

†Specific IgE determined in ELISA as OD at 492 nm

was identified by *de novo* sequencing of two peptides, which correlated with *Pinus strobus* 2S albumin (100% identity), and *Pseudotsuga menziesii* 2S albumin (64% identity). As reported Cabanillas et al. [17], the pine nuts 2S albumin presents only one chain since only one band appears in the presence of β ME. However, Sin a 1 purified from mustard seed present two chains (9 and 4 kDa) under reduced conditions (Figure 1A).

Detection of IgE-reactive proteins in the protein extracts

The IgE-binding capacity of purified proteins using sera allergic to Sin a 1 was assayed by immunoblotting. In Figure (1B), specific IgE recognition to *Brassicaceae* members besides mustard and other seeds and nuts extracts is shown.

Previous studies revealed that Sin a 1 and rapeseed albumin, Bra n 1, were recognized either by mustard seed or rapeseed allergic patients' sera, suggesting the existence of common antigenic regions between these two *Brassicaceae* members due to the great identity of their amino acid sequences[18]. However, it is interesting that the sera here used, who are allergic to Sin a 1, recognize low molecular mass proteins from pine nuts and sesame seed, and not to other nuts.

Sin a 1 cross-reactivity with other vegetables extracts

To confirm the possibility of cross-reactivity between 2S albumins from different plant families, inhibition immunoblotting experiments were carried out using the same pool of sera (Figure 1C). IgE binding to Sin a 1 was nearly completely inhibited when rapeseed, cabbage seed, radish seed, sesame seed and pine nuts (95, 96, 94, 95 and 95%, respectively, measured by densitometry) were used as inhibitors, and is significantly inhibited by grass pea (56%).

Moreover, cross-reactivity between purified Sin a 1 and pine nut 2S albumin was tested, showing IgE recognition of both purified proteins (Figure 1D).

Cross-reactivity in 2S albumins usually occurs between vegetables from the same family, because they have a higher sequence similarity and they can share common linear epitopes. In fact, the amino acid identity degree of members from the same protein family is very high. This fact occurs with Sin a 1 and other 2S albumins from *Brassicaceae* family with values of 81 % with Bra n 1 (Table 2). In the case of Sin a 1 and 2S albumin described from pine nuts, Pin p 1, there is an %I and %S of 13 and 26, respectively. However, as the sequence alignment between these two allergens shows in Figure (2), there are conserved regions along the large chain, which probably could correlate with IgE

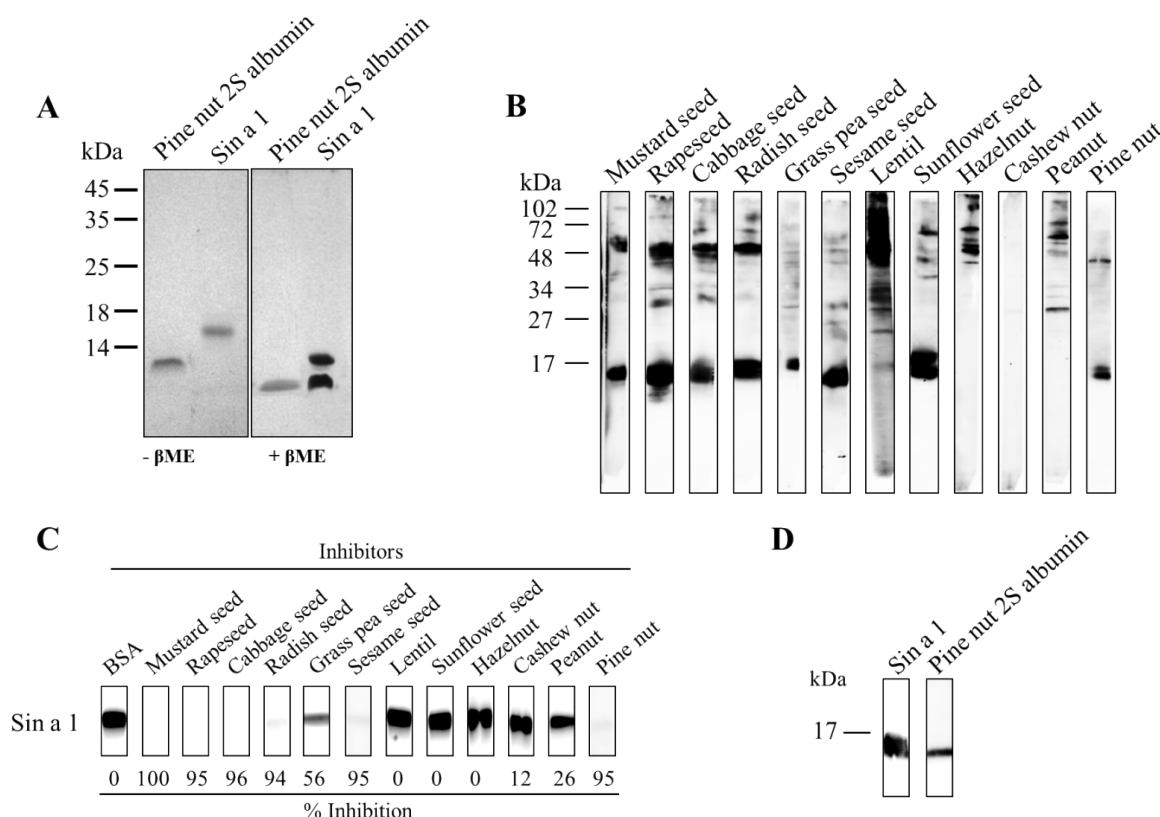


Figure 1 (A) SDS-PAGE of purified 2S albumins from pine nut and mustard seed (Sin a 1), under reducing (+ β ME) and non-reducing conditions (- β ME), with Coomassie Blue Staining. (B) Immunoblotting of several seeds and tree nuts extracts tested with a pool of mustard seed-allergic patients' sera. (C) Inhibition of the IgE binding to Sin a 1 by several inhibitors using a pool of sera from mustard-allergic patients sensitized to Sin a 1. (D) IgE recognition to Sin a 1 and pine nut 2S albumin by mustard seeds allergic patients' sera.

Table 2: Amino acid sequences percentages of identity (%I) and similarity (%S) of 2S albumins from *Brassicaceae* family Sin a 1 and Bra n 1, and from pine nut (Pin p 1).

%I %S	Sin a 1	Bra n 1	Pin p 1
Sin a 1		81	13
Bra n 1	84		13
Pin p 1	26	29	

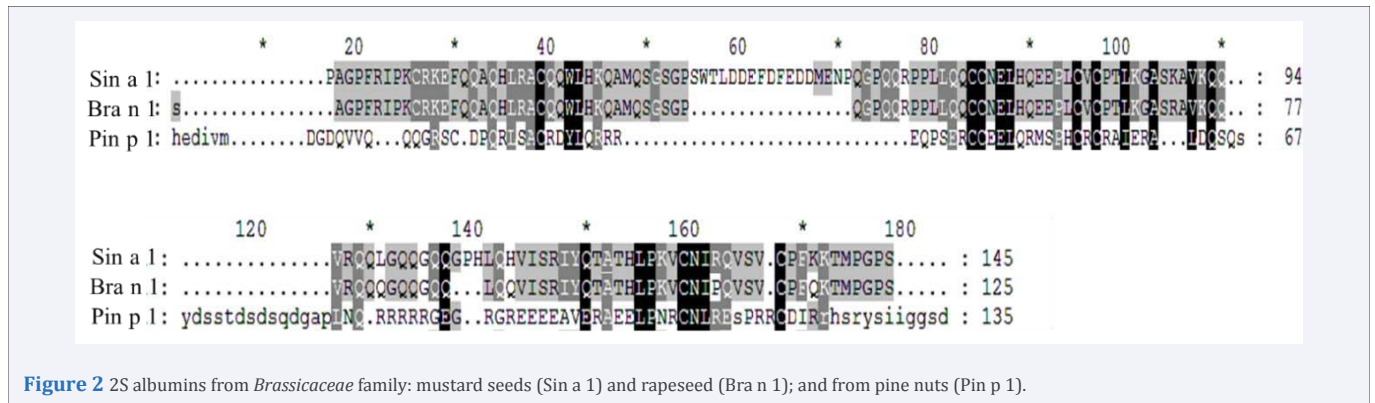


Figure 2 2S albumins from *Brassicaceae* family: mustard seeds (Sin a 1) and rapeseed (Bra n 1); and from pine nuts (Pin p 1).

binding regions, different but really close from the epitope described in Sin a 1 [19] and which may explain the results observed at the immunoassays. Cross-reactivity processes in which these proteins were involved have been attributed to more conserved lineal regions between 2S albumins phylogenetically related, as it happens with Sin a 1 and other *Brassicaceae* members [9].

In other study, it has been shown that IgE binding to proteins from *P. pinea* seeds were completely inhibited by proteins from *P. cembra* seeds, but hazelnut and peanut did not revealed inhibitory ability [20]. Besides, cross-reactivity between sunflower seeds and mustard inducing anaphylaxis has been studied [21]. In this case cross-reactivity also happens between proteins from non-related families, mustard seed, sesame seed and pine nuts, which has not been reported before and rendered interesting results for a better diagnosis.

CONCLUSION

Cross-reactivity in 2S albumins family linked to mustard allergy has been related to members of the same vegetable *Brassicaceae* family. This is due to the similarity between their amino acid sequences, so they share conserved regions that can be recognized by specific IgE. However, cross-reactivity between 2S albumins from non-related vegetable species seems to be less probable due to the big sequence differences. This can be explained phylogenetically: angiosperms, as *Brassicaceae*, and gymnosperms, as *Pinaceae*, have evolved separately; therefore, correlation between their 2S albumins was not expected. Moreover, 2S albumins belong to a multigene family, which lead them to numerous isoforms with different amino acid sequences. This makes cross-reactivity between mustard seeds and pine nuts much unexpected. In this study we have showed that pine nut and sesame seeds extracts inhibit specific IgE-binding to Sin a 1; furthermore, mustard seeds-allergic patients' sera recognized the pine nut 2S albumin either both in the extract and the purified proteins available for this study.

This kind of studies are very relevant in order to determine the allergenic cross-reactions among members of seed storage protein family, those expected and unexpected by the phylogenetical origin, because these allergens have been reported to cause severe symptoms when consumption of their biological source. For this reason, completing the allergenic 2S albumins panel is required to avoid unexpected allergic reactions that could be strongly severe as anaphylaxis and could lead to fatal consequences.

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