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# Analysis of the Structural and Immunological Stability of 2S Albumin, Nonspecific Lipid Transfer Protein, and Profilin Allergens from Mustard Seeds

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**ABSTRACT:** This work investigates the resistance to proteolysis and heating of the yellow mustard (*Sinapis alba* L.) allergens Sin a 1 (2S albumin), Sin a 3 (nonspecific lipid transfer protein, LTP), and Sin a 4 (profilin) to explain their potential capability to induce primary sensitization at the gastrointestinal level. Sin a 1 and Sin a 3 resisted gastric digestion showing no reduction of the IgE reactivity. Intestinal digestion of Sin a 1 and Sin a 3 produced a limited proteolysis but retained significant IgE-binding reactivity. Sin a 1 was stable after heating, and although Sin a 3 was modified, most of its structure was recovered after cooling back. These two allergens would be therefore able to sensitize by ingestion. Sin a 4 was completely digested by gastric treatment and its conformational structure markedly modified at 85 °C. Thus, this allergen can be described as a nonsensitizing mustard allergen.

**KEYWORDS:** *Sinapis alba, L., yellow mustard, mustard allergens, 2S albumin, nsLTP, profilin, protein stability, protein digestibility, heat treatment*

## INTRODUCTION

Around 5% of young children and 4% of adults suffer from some type of adverse immune responses to foods in westernized countries.[1] Nuts and seeds are among the most allergenic foods of plant origin, and the methodologies employed to detect and quantify their allergens are a current matter.[2] Mustard is one of the most significant spices in terms of allergenic potency and widespread use causing IgE-mediated food allergy.[3−5] IgE-mediated allergy to mustard has been increasingly reported in recent years. The ingestion of mustard seed flour as well as manufactured foods containing this spice has been frequently associated with the development of systemic symptoms such as generalized urticaria, angioedema, or anaphylaxis in hypersensitive patients.[6,7] Mustard is worldwide consumed in homemade meals, and some traces can be inadvertently masked in many sauces, salad dressings, or manufactured and processed products for flavoring, which makes its avoidance difficult and increases the risk of suffering unexpected allergic reactions.[7] Therefore, and according to the European Union guidelines for food labeling, the mustard content must be declared.[8] In addition, and although mustard seeds are mainly used with culinary purposes, mustard oil and seed pastry have been traditionally used in oriental medicine and now are gaining popularity as natural health cosmetic and ayurvedic products, and thus are able to trigger different adverse skin reactions such as atopic dermatitis.

Four proteins of yellow mustard (*Sinapis alba* L.) seeds have been identified as allergens so far. They have been structurally and immunologically characterized: Sin a 1 (2S albumin with 14 kDa of molecular mass and constituted by two different subunits of 10 and 4 kDa),[9] Sin a 2 (11S globulin of the cupin family, multimeric protein with subunits of 50−60 kDa molecular mass),[10] Sin a 3 (LTP, with a single chain of 92 amino acid length and 12.3 kDa apparent molecular mass in SDS−PAGE),[11] and Sin a 4 (profilin, a single chain of 14.2 kDa, 131 amino acids).[11] Sin a 1 and Sin a 2 are specific seed-storage proteins that represent the main protein components of yellow mustard seeds. Due to the great level of expression in the seeds, Sin a 1 and Sin a 2 can be purified from the natural source.[12,13] In contrast, Sin a 3 and Sin a 4 belong to widespread families of panallergens and are contained at very low amounts in yellow mustard seeds. Thus, Sin a 3 and Sin a 4 should be produced by molecular biology procedures in heterologous systems[11] in order to achieve scientific or clinical goals. The obtained recombinant allergens displayed equivalent structural and immunological properties to their natural counterparts, and are suitable to be employed for in vitro diagnosis purposes. Recent studies have determined that Sin a 1 is a diagnostic marker for sensitization to mustard, Sin a 2 is a marker to predict severity of symptoms, and Sin a 3 and Sin a 4 are allergens associated with sensitization to other plant-derived foods and pollens in mustard-allergic patients.[14]

Although thousands of dietary proteins come in contact with the immune system, only a small number of them are able to sensitize an individual through the gastrointestinal tract and trigger allergic symptoms.[15] Multiple mechanisms are involved in protecting the organism against these foreign proteins, either degrading or preventing the contact of immunogenic molecules with the immunological structures. In this way, diverse enzymes

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are expressed in saliva, stomach, or intestine and are able to break down allergenic dietary proteins into peptides without antigenic capacity. Most known allergens able to sensitize through the gastrointestinal tract belong to the prolamin and cupin families,[16] which are proteins with a high resistance to heat denaturation or degradation by peptidases.[17,18] Although there is some controversy, it has been suggested that allergenicity is linked to the stability against gastrointestinal digestion.[17,19−21] In this concern, an immunologically active form of the allergen will be able to reach the intestinal mucosa where absorption and sensitization can occur. Therefore, the digestibility trials using simulated gastric fluid and simulated intestinal fluid (SGF and SIF, respectively) have been considered as a useful tool for evaluating the potential allergenicity of a certain protein.[18,19,22,23]

The aim of this work is to analyze the resistance of the low molecular mass yellow mustard allergens Sin a 1 (2S albumin), Sin a 3 (LTP), and Sin a 4 (profilin) to proteolytic digestion, high temperature, and reductive agents, in in vitro systems to assess their ability to induce primary sensitization at the gastrointestinal level. The valuation of the IgE-binding capability of the remaining products after these treatments provides crucial information for the clinical management of patients hypersensitive to mustard derived foods.

## MATERIALS AND METHODS

**Human Sera, Allergens, and Specific Antibodies.** The patients included in this study were allergic to mustard *(Sinapis alba* L.*)* seeds. Sera from 15 patients allergic to mustard were used in the study of protein digestibility: 5 sera contained specific IgE to Sin a 1, 5 other sera with specific IgE to Sin a 3, and 5 other sera with specific IgE to Sin a 4. With these sera, 3 different mixtures were prepared, each mixture combining equal volumes of sera from the 5 patients with IgEs to the same allergen. Each serum pool was used for the stability analysis of such allergen. Moreover, 15 additional sera were used in the thermal treatment experiments, 5 sera containing, as above, IgE specific to each allergen. Written informed consent was obtained from all patients and control subjects, and the study was approved by the Ethics Committee of the Hospital Fundación Jiménez Díaz (Madrid).

Mustard Sin a 1 was purified from yellow mustard seeds by assessed procedures for 2S albumins,[12,24] and their specific antibodies were obtained from rabbit as described.[24] Briefly, polyclonal serum (IgG-pAb) was prepared by immunizing a New Zealand white rabbit by weekly injection of Sin a 1 in complete Freund's adjuvant. Sin a 3 and Sin a 4 were produced as recombinant allergens and purified as previously described.[11] Specific polyclonal antisera against these two mustard allergens were produced in mice BALBc as described:[25] specific mouse IgG-pAbs to Sin a 3 or Sin a 4 were prepared by weekly intraperitoneal injection of BALBc mice with 4 $\mu$g of the corresponding allergen preincubated for 1 h with $Al(OH)_3$ adjuvant in PBS. After 21 days of treatment sera were obtained by centrifugation of the mouse blood.

**Electrophoresis and Immunoblotting.** SDS−PAGE was performed in 17% polyacrylamide gels, alternatively with or without 0.5% $\beta$-mercaptoethanol. Proteins were visualized by Coomassie Blue staining (CBS) or transferred to nitrocellulose membranes. The protein concentration was determined using the method of bicinchoninic acid.

Immunodetection of proteins in membranes was achieved as described[12] by using a pool of sera from patients allergic to mustard (diluted 1:5) or polyclonal antisera to Sin a 1 (diluted 1:5000), Sin a 3, or Sin a 4 (diluted 1:2500). The binding of human IgE was detected with mouse anti-human IgE antibodies (diluted 1:5000) kindly provided by ALK-Abelló (Madrid, Spain), followed by horseradish peroxidase-labeled goat anti-mouse IgG (1:2500 diluted; Pierce, Rockford, Illinois). Reaction to pAb against Sin a 1 was detected by horseradish peroxidase-labeled goat anti-rabbit IgG (diluted 1:3000; BioRad, Richmond, CA) or in the case of pAbs against Sin a 3 and Sin a 4 with horseradish peroxidase-labeled goat anti-mouse IgG (diluted 1:2500; Pierce). 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.

**Preparation of Phospholipid Vesicles.** Ten milligrams of dried phosphatidylcholine (PC) (Avanti Polar Lipids, Alabama, USA) was rehydrated with 1 mL of SGF without enzyme (30 mM NaCl, 48 mM HCl, pH 1.2) at 37 °C.[26] The solution was stirred every 10 min at 37 °C during 1 h, and then sonicated for 10 min. The vesicles were stored at 4 °C and used within 48 h.

**Simulated Gastric Digestion.** Digestions were performed both in the presence and in the absence of PC, as described,[26] with minor modifications. Purified mustard allergens (40 $\mu$g) were dissolved in SGF without enzyme (0.3 mg/mL of allergen concentration) and mixed with PC vesicle solution for a final lipid concentration of 6.7 mM. The mixture was maintained with shaking at 37 °C, and then porcine pepsin (Sigma, St. Louis, MO, USA; activity: 4720 U/mg) was added at a ratio of enzyme:substrate 1:20 w/w to a final volume of 200 $\mu$L of SGF. The digestion was performed at 37 °C with moderate shaking. Aliquots of 15 $\mu$L were taken at 0, 10, and 30 s and 1, 5, 10, 20, 30, 60, and 120 min for SDS−PAGE analysis. The treatment was stopped by increasing the pH with 5 $\mu$L of $Na_2CO_3$ 0.2 M, adding 10 $\mu$L of 3× loading buffer, and keeping samples in liquid nitrogen until use. For the experiment in the absence of PC, the PC solution was replaced by the same volume of the SGF without enzyme. Control experiments, without enzyme, or with BSA instead of allergen, were also performed. The mixtures were frozen and stored at −20 °C until SDS−PAGE analysis.

**Simulated Intestinal Digestion.** Digestions of purified Sin a 1, Sin a 3, and Sin a 4 were performed in SIF (4 mM sodium taurocholate, 4 mM sodium glycodeoxycholate, 26.1 mM bis-Tris buffer, 30 mM NaCl, pH 6.5, 35 $\mu$g/mL trypsin, and 1.76 $\mu$g/mL $\alpha$-chymotrypsin).[26] Briefly, 40 $\mu$g of purified mustard allergens were dissolved in the SIF (0.2 mg/mL). The solution was tempered at 37 °C with shaking before adding the mixture of peptidases constituted by trypsin and $\alpha$-chymotrypsin (Worthington Biochemical Co., Freehold, NJ; 200 U/mg and 50 U/mg, respectively) at a trypsin:chymotrypsin:substrate ratio of 34.5 U:0.44 U:1 mg and final volume of 200 $\mu$L. The digestion was performed at 37 °C with moderate shaking. Aliquots of 15 $\mu$L were taken at 0, 10, and 30 s and 1, 5, 10, 20, 30, 60, and 120 min for further analysis. The digestion was stopped by adding phenylmethylsulfonyl fluoride at a final concentration of 2 mM. BSA was also treated as positive control. The mixtures were frozen and stored at −20 °C until use in SDS−PAGE. The proteolytic digestion was analyzed by CBS, while the IgE-binding capacity of proteolytic fragments was established by Western blot with sera from patients allergic to mustard as above-described.

**Circular Dichroism (CD) Analysis and Heat Treatment.** The CD spectra were obtained using a JASCO J-715 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) fitted with a 150 W xenon lamp and connected to a Westlab RTE-111 thermostabilizer bath, at 25 °C, as described.[27] Far-UV spectra (190−250 nm wavelength) were registered using optical-path cell of 0.1 cm. The protein concentration was 0.2 mg/mL in 20 mM sodium phosphate buffer, pH 7.2. Mean residue mass ellipticities were calculated based on 119, 103, and 108 as the average molecular mass/residue for Sin a 1, Sin a 3, and Sin a 4 respectively, obtained from the amino acid composition (by acid hydrolysis for Sin a 1, and from the values deduced of the nucleotide sequences for Sin a 3 and Sin a 4), and expressed in terms of $\theta$ (degree × $cm^2$ × $dmol^{-1}$). Final spectra were corrected by subtracting the corresponding baseline spectrum obtained for the buffer alone under identical conditions.

Thermal unfolding was monitored by recording the ellipticity at 220 nm while heating (25−85 °C) or cooling (85−25 °C) at 1 °C/min with a computer-controlled circulation water bath. Sin a 1, Sin a 3, and Sin a 4 IgE-binding capability were analyzed by Western blot with sera from mustard allergic patients after heating at 95 °C during 30 min in

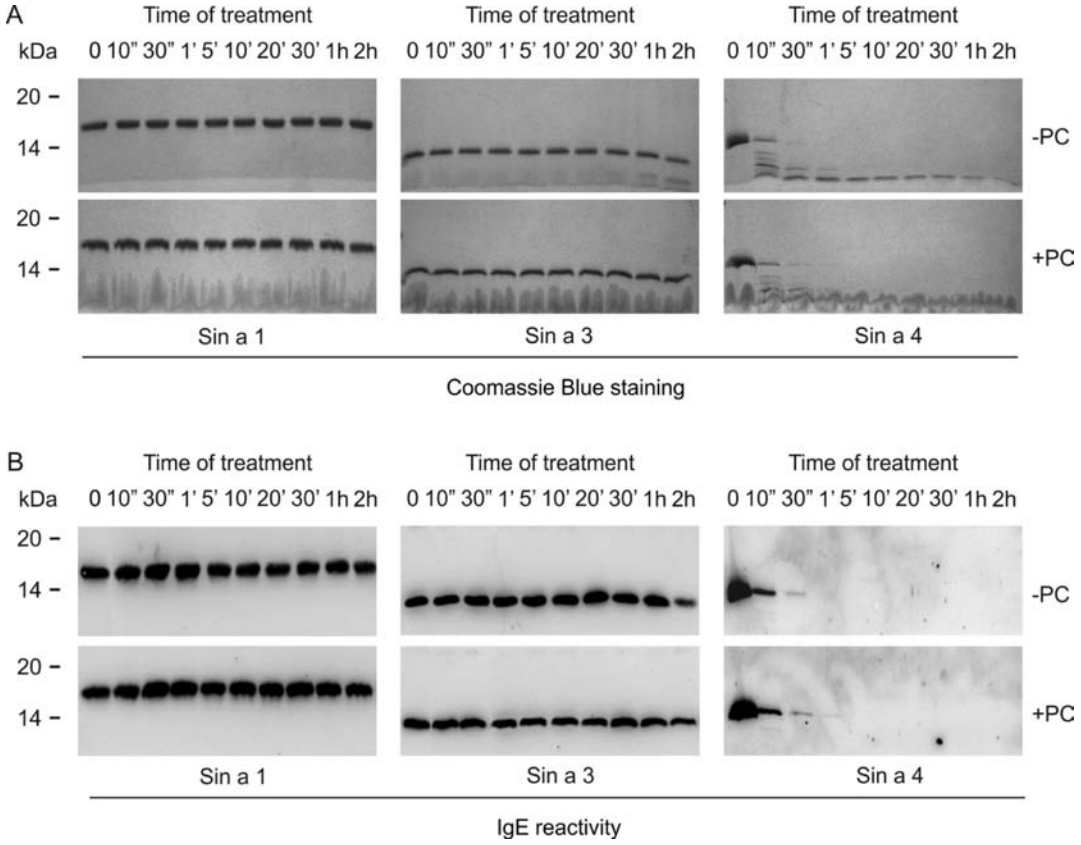


**Figure 1.** Simulated gastric digestion of mustard allergens. (A) Coomassie blue staining (CBS) of the digestion products in the absence (−PC) or presence (+PC) of phosphatidylcholine after different times of treatment. (B) Western blot of the digestion products with a pool of sera from patients sensitized to Sin a 1, to Sin a 3, or to Sin a 4 in the absence (−PC) or presence (+PC) of phosphatidylcholine. Molecular mass markers are included in kDa.

the presence or absence of 2-mercaptoethanol and maintained at 25 °C during 30 min.

**Three-Dimensional Modeling.** The three-dimensional structures of Sin a 1, Sin a 3, and Sin a 4 (accession numbers P15322, EF626938, and EF626939, respectively) were modeled by using the services of the Swiss-Model Protein Modeling Server[28] and the structures of the 2S albumin from rapeseed BnIb (PDB accession number 1SM7), the LTP from peach Pru p 3 (PDB 2B5S), and the profilin from latex Hev b 8 (PDB 1G5U) as templates, respectively. Graphical processing of the three-dimensional structures was accomplished with PDB viewer PyMOL program. The theoretical cleavage sites (TCS) for peptidases were predicted using the program Peptide Cutter of the ExPASy Server.[29]

## RESULTS

**Gastric Digestion.** Gastric digestion was carried out using a preparation of pepsin in SGF. Aliquots were collected at different times, and the enzyme was inactivated by increasing the pH. SDS−PAGE and CBS were used to separate and visualize the digestion products (Figure 1A). No fragments were detected for Sin a 1 after 2 h of incubation. Gastric digestion of Sin a 3 produced fragments that were visible after 1 h of treatment. Sin a 4 was totally digested after 1 min of treatment. No differences were detected in the proteolytic degradation pattern for the three allergens either in the presence or in the absence of PC vesicles (Figure 1A). BSA, used as positive control for digestive activity, was also rapidly processed (data not shown).

IgE reactivity was analyzed by Western blot of the digestion products using the different pools of sera sensitized to each allergen. Sin a 1 and Sin a 3 were proved to be very resistant to gastric digestion, showing no reduction of the IgE reactivity after 2 h of treatment (Figure 1B). By contrast, the IgE-binding capability of Sin a 4 was completely abolished within less than 1 min (Figure 1B).

**Intestinal Digestion.** Considering that several factors such as the use of antacids or the presence of protective molecules within the food matrix can affect the digestibility of certain proteins in the stomach allowing allergens to reach the intestine in an immunologically active form, we also performed separate SIF treatments. Intestinal digestions of Sin a 1 and Sin a 3 produced a limited degree of proteolysis detected by CBS (Figure 2A). Fragments of slightly lower molecular mass than intact Sin a 1 were visible by CBS after 5 min of treatment, whereas the Sin a 3 digestion products were visible within the first seconds of the treatment. Digestion of Sin a 4 resulted in the appearance of several small proteolytic fragments during the first seconds of the assay (Figure 2A), indicating that this allergen is more susceptible to degradation than Sin a 1 and Sin a 3. In addition, Sin a 4 was much less susceptible to degradation in SIF than SGF.

The IgE reactivity of the intestinal digestion mixture was tested by immunoblotting with sera from patients allergic to mustard (Figure 2B). Sin a 1 showed a reactive band of around 1.5 kDa lower molecular mass than the intact protein after 30 min of intestinal digestion. Although the IgE reactivity of both bands did not disappear after 2 h of treatment, a decrease of 23% was observed in the signal. In the same way, intestinal digestion of Sin a 3 resulted in the partial proteolysis of the


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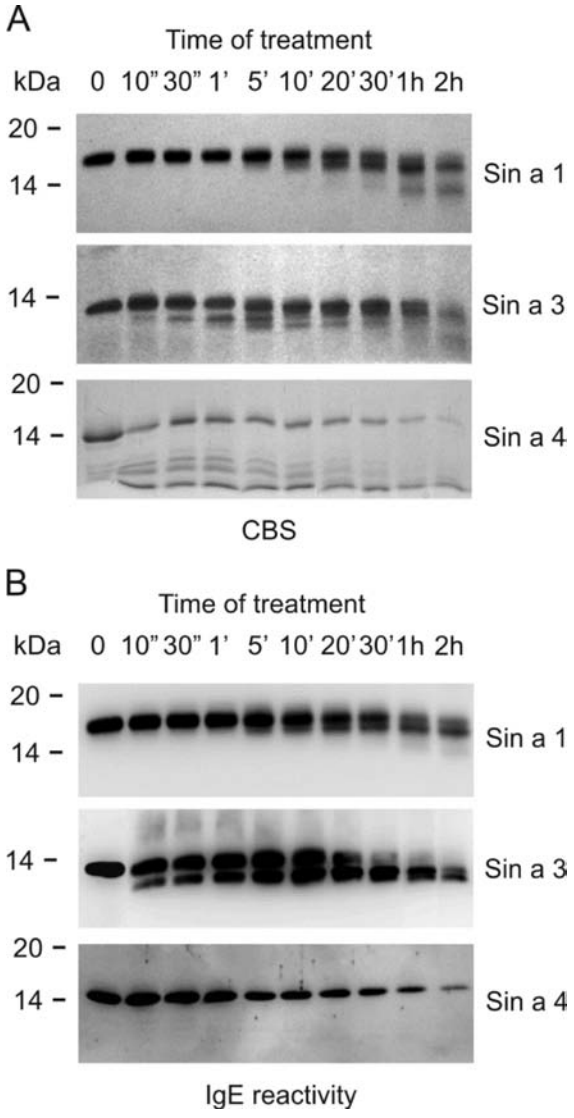


**Figure 2.** Simulated intestinal digestion of mustard allergens. (A) Coomassie Blue staining (CBS) of the digestion products in the absence of phosphatidylcholine after different times of treatment. (B) Western blot of the digestion products with a pool of sera from patients sensitized to Sin a 1, to Sin a 3, or to Sin a 4.

intact protein and the appearance of low molecular mass fragments with IgE reactivity (62% of the initial value) (Figure 2B). Sin a 1 and Sin a 3 maintained a significant IgE binding capability after 2 h of digestion. On the other hand, intestinal treatment of Sin a 4 did dramatically reduce its IgE reactivity. However, and by contrast with gastric digestion, IgE reactivity to Sin a 4 was slightly visible (20% residual activity) after 1 h of proteolysis (Figure 2B).

**Potential Peptidase Cleavage Sites.** The TCS for pepsin were located in the three-dimensional structure of Sin a 1, Sin a 3, and Sin a 4. Although they were mostly distributed in the inner parts of these proteins (Figure 3A), Sin a 4 displayed a higher number of TCS located in the surface of the molecule than in Sin a 1 or Sin a 3.

Analyzing the location of the TCS for trypsin and chymotrypsin in the three-dimensional structure of mustard allergens, a certain correlation between these sites and the level of degradation of these proteins was observed (Figure 3B). The number of TCS in Sin a 1 for trypsin and chymotrypsin ($n$ = 14 + 8, respectively) was larger than for pepsin ($n$ = 17). Their distribution throughout the three-dimensional structure of the allergen and the exposure of these sites to the solvent could explain the sensitivity of Sin a 1 to these pancreatic enzymes. All the TCS for trypsin are located on the surface of Sin a 3, whereas only 55% of the sites for chymotrypsin are located on the surface of Sin a 3 (Figure 3B). Sin a 4 presents the TCS for intestinal enzymes both inside and outside of the three-dimensional structure.

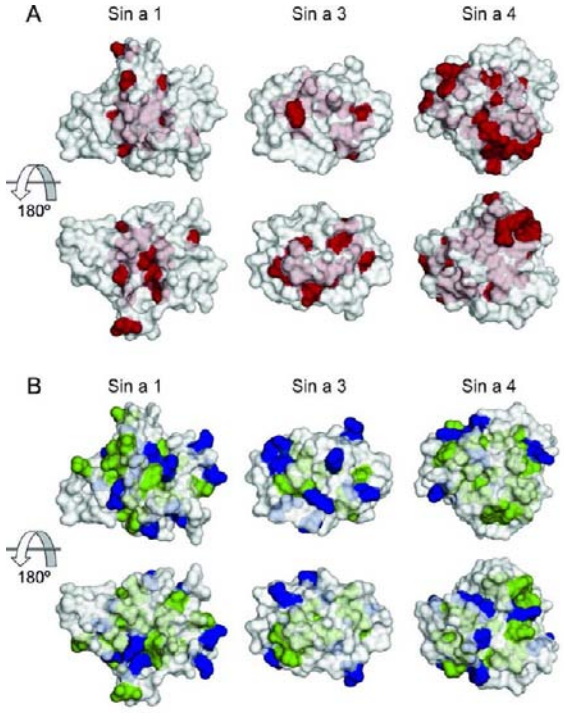


**Figure 3.** Theoretical cleavage sites for (A) gastric enzyme at pH < 2 and (B) intestinal enzymes, in the three-dimensional modeling of mustard allergens Sin a 1, Sin a 3, and Sin a 4, which have been colored in red for pepsin, blue for trypsin, and green for chymotrypsin. Sensitive peptide bonds located inside the protein structures are shown in pale color. Modeling was performed on the basis of the experimentally determined structures of rapeseed napin, LTP from peach, and profilin from latex.

**Spectroscopic Analyses and Heat Treatment.** The CD spectra of Sin a 1, Sin a 3, and Sin a 4 as well as the composition of the secondary structure elements obtained by application of the convex-constrain-analysis method are shown in Figure 4A. The stability of Sin a 1 was confirmed as the $\alpha$-helix content did not significantly change during heating at 85 °C. In contrast, the secondary structure of Sin a 3 was notably affected by temperature at 85 °C, as demonstrated by the significant loss of $\alpha$-helix (from 55% to 28%). In addition, the initial values were not completely recovered upon cooling back at 25 °C (Figure 4A). When analyzing the thermal denaturation curve of Sin a 3 (Figure 4B), an abrupt change in the secondary structure is observed at approximately 37 °C. This would be the result of partial denaturation of the structure and preservation of a stable core of $\alpha$-helix which continued diminishing until 85 °C. A fraction of the $\alpha$-helix content was partially recovered upon cooling back the sample. The secondary structure of Sin a 4, which consists of equal parts of $\alpha$-helix and $\beta$-sheet, was markedly modified by heat treatment at 85 °C but not reconstituted upon cooling back the protein to 25 °C.

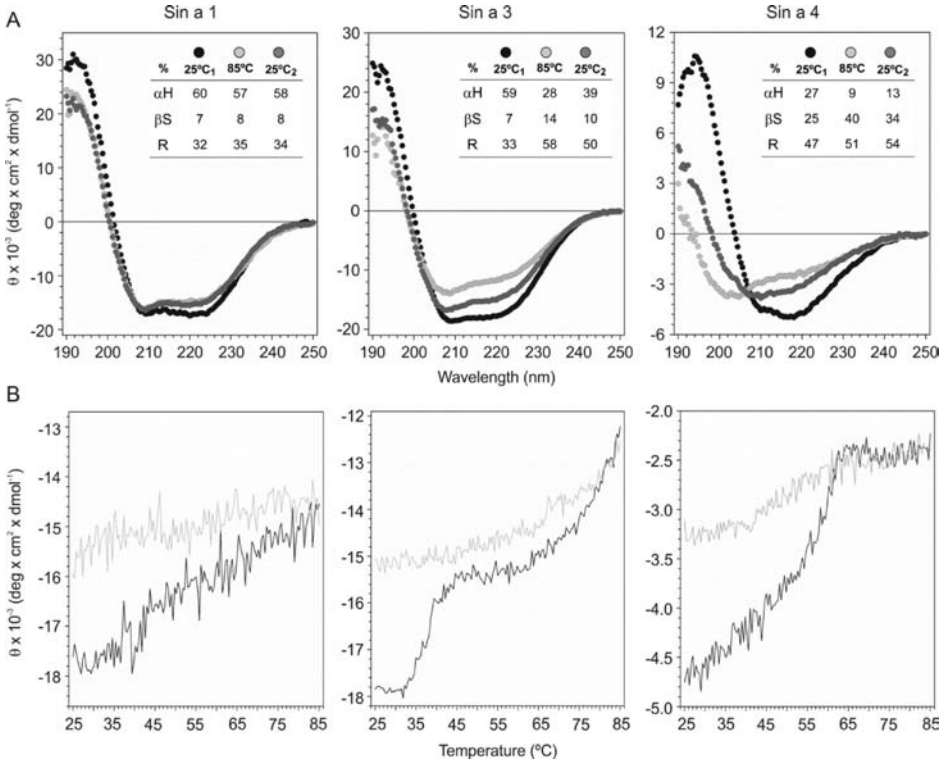


**Figure 4.** CD spectra of mustard allergens in the far-UV at different temperatures (A), heating from 25 °C ($C_1$) to 85 °C and then cooling back to 25 °C ($C_2$). Secondary structure contributions ($\alpha$H, $\alpha$-helix; $\beta$S, $\beta$-sheet; R, random coil) are shown inside the graphs. Thermal unfolding (B) was monitored by measuring the change in ellipticity at 220 nm during heating from 25 to 85 °C (black) and during cooling from 85 to 25 °C (gray).

The three allergens were tested for their IgE- and IgG-binding capability after heat (95 °C during 30 min) and reductive (with $\beta$-mercaptoethanol) treatments, in comparison to the nontreated samples by Western blot (Figure 5). After heating, Sin a 1 and Sin a 3 showed no appreciable variations—less than 10%—on the ability to bind IgEs from sera of allergic patients and specific IgG from the polyclonal antisera (Figure 5A,B). However, reducing treatment decreased drastically their IgE and IgG reactivity; moreover, as a consequence of the rupture of the disulfide bridges, the two polypeptide chains of Sin a 1 were separated, and the binding of the individual chains to specific IgEs was nearly abolished (Figure 5A). The IgE reactivity of reduced Sin a 3 was almost eliminated for 8 out of 10 sera, whereas it was increased for patients 4 and 7 (about 22% and 84%, respectively). Concerning Sin a 4, and apart from the frequently reported appearance of two bands on profilins in the absence of reducing agent,[30] the heat treatment has little impact on the specific IgE binding capacity of this allergen as a high response (>85%) for all the sera was preserved (Figure 5C). In contrast with Sin a 1 and Sin a 3, the reducing treatment of Sin a 4 produced minor variations on its IgE and IgG reactivity.

## DISCUSSION

Digestion stability cannot be used as the single criterion for defining a protein as a food allergen since several studies have demonstrated that the correlation between resistance to proteolytic treatments and allergenic activity of proteins contained in foods is not conclusive in all the cases.[17,19,20] However, the trials simulating the gastrointestinal digestion can be useful tools for evaluating the potential allergenicity of certain proteins since the resistance to digestion in the gastrointestinal tract is one of the factors that may contribute to the allergenicity of diet proteins.[18,21,31] Considering these aspects, in this study we wanted to compare the mustard allergens Sin a 1, Sin a 3, and Sin a 4, which show similar low molecular weight.

The family of 2S albumins has been described as a family of proteins very stable against proteolytic degradation and heat denaturation. These seed-specific proteins have a compact three-dimensional structure with four $\alpha$-helices and four disulfide bridges[32] which help their stability. In vitro digestion assays of members of this allergenic family showed that the resistance to gastrointestinal degradation is a property shared by many of them such as BnIb,[18] Ses i 11,[33] and Ber e 1,[34] and it could be related with their common folding. Therefore, it has been considered that the conformation of protein allergens is an important factor for their ability for resisting enzyme degradation. We have probed that Sin a 1 is very stable both structurally and immunologically during gastric digestion. However, this allergen suffers a certain degree of damage by intestinal proteolysis although retaining significant capability to bind specific IgEs from patients allergic to mustard. Sin a 1 is a basic protein (p$I$ > 10) and displays a high number of peptide bonds theoretically sensitive to trypsin and chymotrypsin. The analysis of the peptides released from the allergen by the intestinal digestion allowed us to determine the presence of small fragments: one set of peptides with 5 and 6 amino acids (peptides PAGPF and PAGPFR) located at the N-terminal end of the light chain[9] and other set of peptides containing 6–8 amino acids (KKTMPGPS, KTMPGPS, and TMPGPS) from the C-terminus of the large chain[9] (data not shown). These segments of 2S albumins have random structures and are completely exposed to the solvents,[18,32] being thus very

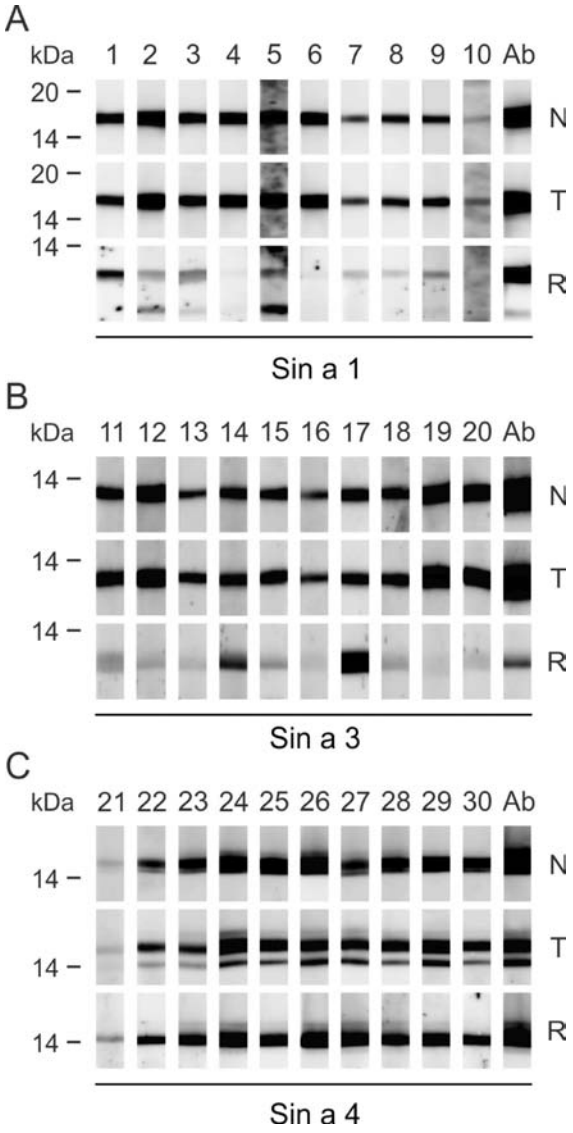


**Figure 5.** Analysis of the IgE binding capability of Sin a 1, Sin a 3, and Sin a 4 at 25 °C (N), after heating at 95 °C during 30 min (T), and after heating at 95 °C during 30 min in the presence of $\beta$-mercaptoethanol reducing agent (R). Thirty individual sera (1−30) from patients allergic to mustard were used: sera 1−10 were reactive to Sin a 1; sera 11−20 were reactive to Sin a 3; and sera 21−30 were reactive to Sin a 4. Specific antisera for each allergen (lanes Ab) were also used. Molecular mass marker positions are indicated in kDa.

accessible to pancreatic proteases. These results also explain the appearance of intestinal digestion products of Sin a 1 with slightly lower molecular mass than the intact protein.

Sin a 1 was shown to be able to strongly interact with phospholipid vesicles promoting lipid mixing between vesicles and leakage of vesicles' aqueous contents.[35] This interaction was attributed to the neutralization of the charge because of the cationic character of the protein since acidic phospholipids were required.[35] It was also suggested that the interaction would account for an increased permeability of the intestinal barrier for Sin a 1—the extracellular leaflet of the intestinal brush border membranes has larger amounts of acid phospholipids when compared to other plasma membranes—and thus the allergen would have a shortened transit in the gut.[35] However, the presence of PC vesicles in the digestion mixtures of our experiments did not seem to have any effect on the obtained products. The most plausible explanation would be related to the experimental design: for instance, we used PC derivates instead of phosphatidylglicerol, and different lipid/protein ratios (following the recent methods[26]), and different pH according to the protease activity to be assayed.

Food cooking or processing usually implies heat treatments such as boiling, baking, or frying. Accordingly, the resistance to denaturation during food processing is another factor supporting potential allergenicity of food proteins. Sin a 1 showed the characteristic resistance of the 2S albumin family[18,36] to denaturation during heat treatments, since its global folding is conserved during heating, and keeps the integrity of both IgE and IgG epitopes. Apart from the high concentration of Sin a 1 in the mustard seeds (about 19% of total protein)[12] and its capability of interaction with phospholipid bilayers,[35] the conformational stability would be an additional reason to increase the amount of intact allergen that reaches the immunological structures with capacity of sensitization via gastrointestinal tract.

LTP-sensitized patients experience a higher percentage of severe reactions than those sensitized to other food allergens such as profilins.[37,38] These symptoms are attributed to the resistance of the LTPs to in vivo proteolytic degradation within the gastrointestinal tract.[20,31] Sin a 3 is reasonably stable to gastric and intestinal digestions in vitro and keeps its IgE-binding capability. Although Sin a 3 suffered a certain degree of intestinal degradation, the combination of tryptic and chymotryptic activities would have led to big peptide fragments linked by disulfide bonds that resulted in the presence of two IgE-reactive bands in SDS−PAGE. On the other hand, the secondary structure of Sin a 3 is affected by temperature, as it is partially unfolded by losing a certain content of $\alpha$-helix conformation, which is partially refolded when cooling again. This behavior is similar to that reported for sunflower LTP and can be due to a local unfolding.[36] LTPs from barley and apple were found remarkably stable to high temperatures.[39,40] In contrast, LTP from peach was thermolabile when heating at 95 °C and pH 7, whereas it appeared more stable at the same temperature and pH 3.[41] Despite its conformational change, Sin a 3 maintained the IgE and IgG reactivity after the strong thermal treatment at 95 °C during 30 min. Interestingly, both Sin a 1 (2S albumin) and Sin a 3 (LTP) belong to the prolamin superfamily and share a structural folding with four $\alpha$-helices core stabilized by covalent disulfide bonds. This common folding has being related to the high structural stability shown by this protein superfamily and, in the case of mustard allergens Sin a 1 and Sin a 3, seems to be essential for the IgE binding. In addition, although reducing treatment with $\beta$-mercaptoethanol abolished most of the IgE reactivity of these proteins, some epitopes seem to be recognized by IgEs of any mustard allergic patients and a few specific IgGs from the polyclonal population.

The profilin Sin a 4 did not resist gastric digestion since it disappeared within the first minute of treatment. The multiple TCS all along the protein structure, as well as the lack of disulfide bonds that could have held together the produced fragments, could explain its high susceptibility to pepsin treatment. The gradual cleavage of Sin a 4 would involve the disappearance of the intact protein and the accessibility of an increasing number of sensitive sites, which led this protein to lose the IgE reactivity. However, we cannot completely rule out that the presence of the matrix food might mask some peptidase cleavage sites thus increasing the resistance to

digestion of this allergen. The secondary structure of Sin a 4 was unfolded by heat treatment in an irreversible way, to which the absence of disulfide bonds could also have contributed. Despite the unfolding of the profilin, the heat treatment or the reducing agent do not reduce its IgE reactivity. These results reveal the low contribution of secondary structure to the formation of IgE epitopes. Sin a 4 showed the characteristic behavior of food allergens frequently associated with symptoms restricted in general to the oral cavity,[42] although systemic reactions have also been reported.[43] The clinical relevance of this type of allergen is a subject of discussion,[44] but recent studies have shown their importance for patients allergic to melon, banana, watermelon, tomato, pineapple, and orange.[45]

In summary, we have shown that two clinically relevant allergens[14] from yellow mustard seeds, Sin a 1 and Sin a 3, are structurally stable enough to high temperatures and proteolysis digestions retaining most of their IgE and IgG binding capacity. These types of processing performances are usually involved in the preparation and consuming of mustard derived foods, but they seem not to significantly affect the immunogenic capabilities of these proteins. Therefore, these results, together with the immunological and clinical features previously reported for these molecules,[9,11,14] indicate that Sin a 1 and Sin a 3 might well act as genuine food allergens as they would be able to reach the gut immune system and trigger systemic reactions.[17,21] Finally, taking into account (i) the low prevalence of Sin a 4 sensitization in mustard allergic patients,[14] (ii) the low level of expression of mustard profilin in yellow mustard seeds,[12] and (iii) the extreme susceptibility of this protein to gastric digestion here shown, Sin a 4 may not act as a genuine sensitizing mustard allergen at the gastrointestinal level but rather as a cross-reactive agent involved in pollen and/or other food allergies.[25]

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### Notes

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## ABBREVIATIONS USED

BSA, bovine serum albumin; CBS, Coomasie Blue staining; CD, circular dichroism; nsLTP, nonspecific lipid transfer protein; PC, phosphatidylcholine; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; TCS, theoretical cleavage sites

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