Purification and Partial Characterization of Chickpea 2S Albumin

Javier Vioque,[†] Raul Sánchez-Vioque,[†] Alfonso Clemente,[†] Justo Pedroche,[†] Juan Bautista,[‡] and Francisco Millán*[,†]

Instituto de la Grasa, Avenida Padre García Tejero 4, 41012 Sevilla, Spain, and Departamento de Bioquímica y Bromatología, Facultad de Farmacia, Universidad de Sevilla, 41012 Sevilla, Spain

A chickpea 2S albumin has been purified by solubilization in 60% methanol and ion-exchange chromatography. Under denaturing conditions it is composed of two peptides of 10 and 12 kDa. Native molecular mass determined by gel filtration chromatography is 20 kDa. Amino acid composition shows that it is rich in sulfur amino acids, mainly cysteine with 4.6% of the total. On the other hand, it has antinutritional characteristics of being allergenic for chickpea-sensitive individuals and inhibitory against porcine chymotrypsin with a lesser degree toward trypsin. The results of interest from a nutritional point of view are discussed.

Keywords: *Chickpea; 2S albumin; allergens; protease inhibitors*

INTRODUCTION

Proteins of seed legumes are usually classified in two main groups: globulins or salt soluble proteins and albumins or water soluble proteins.

Globulins represent ~70% of legume seed proteins and are composed of two major groups, characterized by their sedimentation coefficients, the 11S or legumin and the 7S or vicilin (Casey et al., 1986). Globulins function as storage proteins representing a source of energy, carbon, and reduced nitrogen for germination and seedling growth.

The albumin fraction is more heterogeneous, including most of the proteins needed for cellular metabolism. Thus, most of the albumin proteins have some physiological function, such as the enzymatic activities of lipoxygenases, glycosidases, or proteases involved in the degradation of storage proteins. Other albumins, such as protease inhibitors or lectins, are implicated in defensive mechanisms (Gueguen, 1991). Among the diverse range of proteins found in albumins two are more abundant. These have been described in pea as the PA2 albumin and the PA1 or 2S albumin (Casey et al., 1993). PA2 albumin is a homodimer that has been purified and characterized in pea (Croy et al., 1984; Schroeder, 1984). Recently it has been described as a lectin able to agglutinate papainized human erythrocytes in pea and chickpea (Vioque et al., 1998).

PA1 was initially defined as a group on the basis of their sedimentation coefficients ($S_{20,w}$) of ~2 (Youle and Huang, 1981) and hence denominated as 2S albumins. They are composed of peptides of low molecular mass, ranging between 4 and 10 kDa (Shewry et al., 1995). They are synthesized as single precursor proteins that are proteolytically cleaved with the loss of a linker peptide and short peptides from both the N and C ends (Shewry et al., 1995). The best 2S albumin studied is from *Brassica*, known as napin, which is composed of two peptides of 4 and 9 kDa linked by interchain disulfide bonds (Ericson et al., 1986). A similar structure has been described in pumpkin (Hara-Nishimura et al., 1993), cotton (Galau et al., 1992), castor bean (Sharief and Li, 1982), and lupin (Lilley and Inglis, 1986). Nevertheless, in sunflower the 2S albumin remains uncleaved (Anisimova et al., 1994), whereas in pea the peptides lack interchain disulfide bonds (Higgins et al., 1986). In general, the 2S albumin appears to be compact globular proteins, as suggested by the disulfide structure reported for the 2S albumin of lupin (Lilley and Inglis, 1986).

It seems that the major role of the 2S albumin is as storage protein. However, the high cysteine content and low molecular mass of this protein suggest a possible relationship with protease inhibitors. Few appear to have been tested for activity as inhibitors of hydrolytic enzymes and with contradictory results. For example, Gatehouse et al. (1985) reported that pea 2S albumin lacks antiprotease activity, whereas Svendsen et al. (1989) reported that 2S albumin from *Brassica napus* var. Rapifera was an inhibitor of bovine trypsin. Thus, the physiological and functional roles of these proteins in the seeds remain unclear.

Another important characteristic is that some 2S albumins are major allergens (Shewry, 1995). This influences the nutritional quality of the seed because even small amounts of the allergen can trigger an adverse reaction. Allergenic proteins have been found in a wide variety of seeds, including castor been, soybean, cottonseed, and pea (Spies, 1974). These allergens are frequently low molecular mass proteins with a high cysteine content.

Chickpea (*Cicer arietinum* L.) is an important source of vegetable proteins in many countries, being extensively grown in India, Mexico, and the Mediterranean region. It is a rich source of starch and protein, accounting for 50 and 25% (w/w), respectively. As in other legumes, globulins represent the main protein fraction, and albumins constitute about one-third of the former. Among albumins, the 2S or low molecular mass albumins constitute one of the main components. In spite of the interest in chickpea and 2S albumins from a

* Corresponding author (telephone 34-54611550; fax 34-54616790; e-mail frmillan@cica.es).

[†] Instituto de la Grasa.

[‡] Universidad de Sevilla.

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nutritional point of view, no study on the characteristics of this albumin fraction in chickpea is available. To clarify the function and nutritional characteristics of 2S albumin, we have purified and partially characterized chickpea 2S albumin.

MATERIALS AND METHODS

Material. Pea seeds *(Pisum sativum* L.), commercial variety Jumbo, were obtained locally; seeds of chickpea (*Cicer arietinum* L.) kabuli type cultivar Athenas were a gift from Koipesol Semillas S.A. (Sevilla, Spain). The Sephadex G- 100 column and Mono Q HR 5/5 column standard proteins were from Amersham Pharmacia (Uppsala, Sweden). Acrylamide, *N*,*N*′-methylenebisacrylamide, and Coomassie Brilliant Blue G-250 were from Serva (Heidelberg, Germany). Diethylethoxymethylenemalonate was supplied by Fluka (Buchs, Switzerland). Nitrocellulose membranes, Tween 20, sodium dodecyl sulfate, casein, trichloroacetic acid, D,L-α-aminobutyric acid, porcine trypsin, and chymotrypsin were purchased from Sigma Chemical Co. (St. Louis, MO). Nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl-phosphate, and alkaline phosphatase conjugate mouse antihuman IgE were from Boehringer Mannheim GmbH (Mannheim, Germany). All other chemicals were of analytical grade.

Purification of Chickpea 2S Albumin. To produce flour, seeds were ground with a domestic blender. Fractionation of chickpea proteins was performed according to the method of Singh et al. (1988). For this, flour was extracted three times with 0.1 M borate buffer, pH 8.3 (1:10, w:v), during 1 h. Extracts were centrifuged at 8000*g* and the supernatants pooled and dialyzed against 25 mM sodium citrate buffer, pH 4.6 (1:10, v:v), for 48 h at 4 °C. Dialyzed extracts were centrifuged as above. The resulting supernatants and pellets were the albumin and globulin fractions, respectively. Albumins were concentrated by lyophilization. An extract enriched in 2S albumin was prepared by treatment of albumins with methanol at 60% (v:v, aqueous methanol) at which concentration 2S albumin remains soluble. Proteins soluble in 60% methanol were concentrated by lyophilization, and chickpea 2S albumin was finally purified by ion-exchange chromatography in a FPLC Mono Q HR *5/5* column, which has been previously equilibrated with 0.05 M Tris-HCl, pH 8.0, buffer. After the sample had been loaded, the column was washed with 20 mL of the above-mentioned buffer and the proteins were eluted with a 30 mL linear gradient of 0.0−0.5 M NaCl with a flow rate of 0.5 mL/min. The column effluent was monitored at 280 nm, and 1 mL fractions were collected. Column fractions containing 2S albumin were pooled and concentrated by lyophilization. This preparation of 2S albumin was used for all further studies.

M_r Determination. For native molecular weight determination, purified chickpea 2S albumin was loaded on a Sephadex G-100 gel filtration column (100 × 2 cm) at a flow rate of 0.5 mL/min using 20 mM phosphate buffer/0.5 M NaCl, pH 7. The column effluent was monitored at 280 nm. The column was calibrated with the following standard proteins: blue dextran, lipoxygenase (108 kDa), bovine serum albumin (67 kDa), ovoalbumin (43 kDa), ribonuclease (13.7 kDa), and cytocrome *c* (12.2 kDa).

Protein Measurements. Protein concentrations were determined according to the method of Bradford (1976). Bovine serum albumin was used as standard.

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to the method of Laemmli (1970). The gel system, containing 0.2% (w:v) SDS, consisted of a 15% polyacrylamide resolving gel (pH 8.8) and a 3% stacking gel (pH 6.8). The lengths of the resolving and stacking gels were 10 and 2 cm, respectively, with a gel thickness of 0.75 mm. Electrophoresis was performed at a constant current of 25 mA. Protein bands were stained by immersion of the gels in a 0.05% (w:v) Coomassie Brilliant Blue G-250 solution, in 45% methanol, and in 9% acetic acid solution. Molecular masses were determined using the low molecular weight standard kit from Amersham Pharmacia.

Amino Acids Analysis. Samples (10 mg) were hydrolyzed with 4 mL of 6 N HCl. The solutions were sealed in hydrolysis tubes under nitrogen and incubated in an oven at 110 °C for 24 h. Amino acids were determined in the acid hydrolysis after derivatization with diethylethoxymethylenemalonate by reverse phase high-performance liquid chromatography (HPLC), according to the method of Alaiz et al. (1992), with D,L-α-aminobutyric acid as internal standard. Losses of amino acids, sensitive to acid hydrolysis, especially methionine and cysteine, were considered for accurate quantification. The HPLC system consisted of a model 600E multisystem with a 484 UV−vis detector (Waters, Milford, MA). Separations were attained with a 300 × 3.9 mm i.d. reverse phase column (NovaPak C_{18}, 4 μm, Waters) using a binary gradient system. The solvents used were (A) 25 mM sodium acetate containing 0.02% sodium azide (pH 6.0) and (B) acetonitrile. The solvent was delivered to the column at a flow rate of 0.9 mL/min as follows: time 0.0−3.0 min, linear gradient from A:B (91:9) to A:B (86:14); 3.0−13.0 min, elution with A:B (86:14); 13.0−30.0 min, linear gradient from A:B (86:14) to A:B (69:31); 30.0−35.0 min, elution with A:B (69:31). The column was maintained at 18 °C with a temperature controller.

Immunochemical Assay. Dot blotting was developed as described by Weiss et al. (1997) with modifications. Pea and chickpea 2S albumins samples were blotted on 0.45 μm nitrocellulose membranes. Membranes were dried, and the remaining active sites were blocked by incubation with 5% defatted milk in TBS (20 mM Tris-HC1/0.05 M NaCl, pH 7.5) for 2 h. The nitrocellulose membranes were then washed three times with TTBS (0.05% Tween 20 in TBS) for 15 min and incubated with the serum of chickpea-sensitive individuals (1:1000, v/v dilution) overnight at 4 °C. The nitrocellulose blot was washed three times with TTBS for 15 min each, and alkaline phosphatase conjugate mouse antihuman IgE (1:1000 v/v in TTBS) was added and incubated at room temperature for 2 h. The nitrocellulose membrane was washed two times with TTBS and once with 1 M borate buffer, pH 8.3, for 15 min each. The blot was developed with 50 μL of nitro blue tetrazolium and 37.5 μL of 5-bromo-4-chloro-3-indolyl-phosphate in 10 mL of 1 M borate buffer at room temperature for 10 min. The reaction was stopped in distilled water for 15 min and the membrane air-dried. Protein extracts of chickpea (10 μg) and BSA (3 μg) were used as positive and negative controls, respectively. Membranes were loaded with 1 μg of chickpea and pea 2S albumins samples.

Protease Inhibitor Activity. Porcine trypsin and chymotrypsin inhibitory activities of chickpea total albumins and 2S albumin were determined by assaying trypsin and chymotrypsin activities in the presence and absence of albumins and 2S albumin, using casein as the substrate for the proteases as described by Batra et al. (1986) with modifications. The assay mixture contains 1 mL of phosphate buffer (0.1 M, pH 7.6), 0.5 mL protease solution (1 mg/mL 1 mN HCl), 0.5 mL 1 mN HCl, 2 mL of casein solution (2% in phosphate buffer, 0.1 M, pH 7.6), and 1 mL of inhibitory solution in phosphate buffer (0.1 M, pH 7.6). Before addition of the substrate, the mixture was incubated for 30 min to allow binding of the inhibitors to the proteases. Casein was added and the reaction incubated for 20 min at 37 °C. A blank set was prepared by adding trichloroacetic acid before the addition of the protease solution. A control set was also prepared in which inhibitory solution was deleted from the assay mixture. The reaction was stopped by addition of 6 mL of 5% trichloroacetic acid. The samples were filtered through Whatman No. 1 filter paper, and the released tyrosine was determined in an aliquot of the filtrate according to the method of Lowry et al. (1951). The inhibitory activity was determined by subtracting the amount of tyrosine released in the experimental set from that in the control set. Percentage of inhibition was calculated by comparing the reduction in enzyme activity on the addition of inhibitory solution with that given by the same enzyme concentration in the absence of any inhibitor.

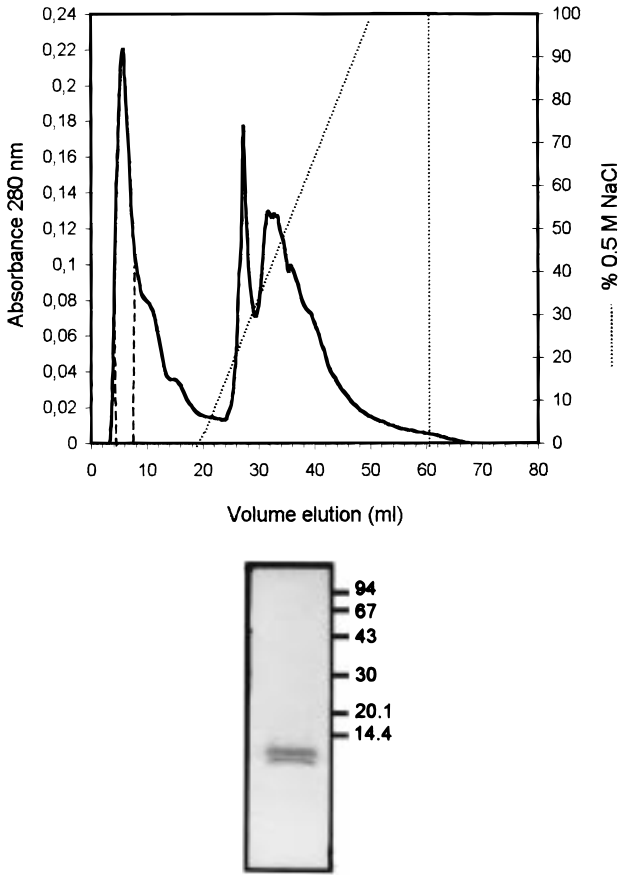

Figure 1. (A, top) Ion exchange elution profile of chickpea albumins on a Mono Q HR 5/5 column. Fractions of pure chickpea 2S albumin are between dotted lines. (B, bottom) SDS−PAGE analysis of purified chickpea 2S albumin. Molecular weight markers are indicated on the right.

RESULTS AND DISCUSSION

Chickpea 2S Albumin Purification. Albumins were extracted according to the method of Singh et al. (1988). This procedure was more effective to purify chickpea albumins than the classical water extraction method described by Osborne (1924). Using the Osborne method, the purified albumins were contaminated with globulins (data not shown). This is probably because a fraction of chickpea globulins is soluble in water and extracted together with albumins. Chickpea 2S albumin was further purified by solution of a total chickpea albumin extract in methanol at 60%. At this concentration 2S albumin was soluble while most of the other chickpea albumins were insoluble. The protein was further purified by fractionation of proteins soluble in methanol at 60% by ion-exchange chromatography on a Mono Q HR 5/5 column (Figure 1A). In the conditions assayed, chickpea 2S albumin was not retained to the column eluting as a single peak in the flow-through. SDS−PAGE of this peak showed that the protein was pure (Figure 1B). Chickpea 2S albumin under denaturing conditions is formed by two peptides of 10 and 12 kDa. These values are higher than those reported for pea (Gatehouse et al., 1985), lupin (Menéndez-Arias et al., 1988) or *Brassica* low molecular mass albumins (Ericson et al., 1986).

Under nondenaturing conditions purified chickpea 2S albumin was eluted from a Sephadex G-100 gel filtration column as a single symmetrical peak, corresponding to a molecular mass of ~20 kDa. This value together with the fact that chickpea 2S albumin peptides always eluted together when chromatographed on ion-exchange column, using different buffering conditions, suggests that in chickpea, as observed in lupin (Lilley and Inglis, 1986) and to the contrary of pea (Higgins et al., 1986), the protein subunits are most likely linked by interchain disulfide bridges.

Table 1. Amino Acid Composition (Grams per 100 g of Protein) of Chickpea Flour, Globulins, Albumins, and Purified 2S Albumin[a]

amino acid	flour	globulins	albumins	2S albumin
Asp + Asn	11.8 ± 1.0	12.9 ± 0.3	12.1 ± 0.9	9.3 ± 1.6
Glu + Gln	16.9 ± 0.9	18.8 ± 0.1	15.8 ± 1.0	17.7 ± 2.1
Ser	5.8 ± 0.1	5.7 ± 0.1	5.8 ± 0.2	6.2 ± 0.1
His	2.8 ± 0.1	2.8 ± 0.0	3.5 ± 0.1	3.4 ± 0.2
Gly	4.1 ± 0.1	3.9 ± 0.0	5.0 ± 0.2	5.8 ± 0.5
Tre	4.0 ± 0.1	3.1 ± 0.1	6.5 ± 0.2	6.7 ± 0.1
Arg	10.6 ± 0.1	11.5 ± 0.2	8. 1 ± 0.4	8.5 ± 0.3
Ala	4.6 ± 0.0	4.5 ± 0.1	4.7 ± 0.1	4.0 ± 0.5
Tyr	3.4 ± 0.1	2.5 ± 0.1	4.3 ± 0.1	2.9 ± 0.1
Val	4.9 ± 0.1	5.3 ± 0.1	4.0 ± 0.1	3.9 ± 0.1
Met	1.8 ± 0.0	1.3 ± 0.3	2.9 ± 0.0	2.4 ± 0.1
Cys	1.4 ± 0.2	1.3 ± 0.1	3.9 ± 0.0	4.6 ± 0.3
Ile	5.2 ± 0.1	5.2 ± 0.2	3.9 ± 0.1	3.9 ± 0.2
Leu	8.6 ± 0.1	8.3 ± 0.1	6.1 ± 0.2	6.7 ± 0.1
Phe	6.8 ± 0.1	6.5 ± 0.1	4.0 ± 0.1	2.6 ± 0.1
Lys	7.3 ± 0.1	6.4 ± 0.2	9.4 ± 0.2	11.4 ± 0.4

[a] Each value represents the mean ± SD of three determinations.

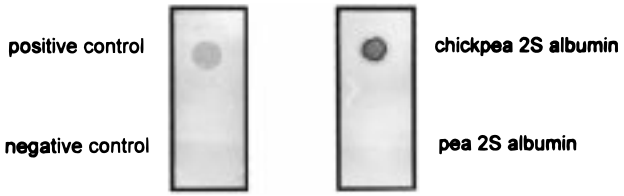

Figure 2. Dot blots of chickpea and pea 2S albumins against sera of a person sensitive to chickpeas. Chickpea total proteins were used as positive control. BSA was used as negative control.

Amino Acid Composition of Chickpea 2S Albumin. Low molecular mass or 2S albumins have been described as unusually rich in sulfur-containing amino acids (Shewry, 1995). Thus, the search for high-sulfur amino acid proteins for the improvement of sulfur amino acids deficient feeds has made this albumin fraction a target for further study (De Lumen et al., 1997).

Chickpea 2S albumin has a high content of sulfur amino acids in relation to total chickpea seeds proteins and globulins (Table 1). In this sense, cysteine contents of chickpea 2S albumin are 3 times higher than in the flour or globulins. Also, methionine percentages are higher in chickpea 2S albumin than in the flour and globulins. Cysteine contents in chickpea 2S albumin are slightly lower than those reported for pea (Gatehouse et al., 1985) or lupin (Menéndez-Arias et al., 1988) 2S albumin, although methionine percentages are higher in chickpea 2S albumin than in the homologous protein of lupin or pea. Thus, the total amount of sulfur amino acids in 2S albumin is similar for the three crops. In this sense, chickpea 2S albumin also represents a reserve of sulfur amino acids contributing to the nutritional quality of chickpea storage proteins.

Allergenic Characterization of Chickpea 2S Albumin. Dot blotting using sera from 10 chickpea-sensitive individuals gave positive reaction against chickpea 2S albumin (Figure 2). This protein has been described also as allergenic in yellow mustard seeds

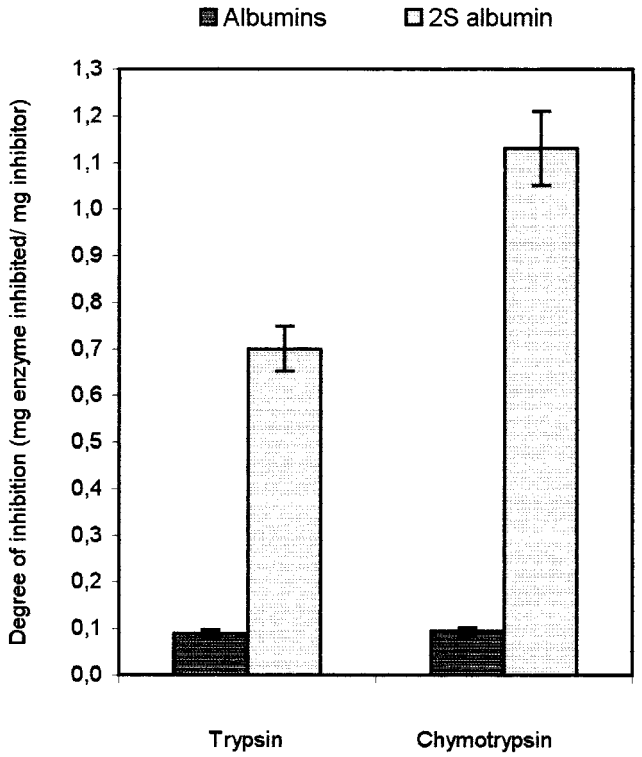

Figure 3. Inhibition of porcine trypsin and chymotrypsin by total chickpea albumins and 2S albumin.

(Menéndez-Arias et al., 1988), castor bean (Machado and Silva, 1992), and Brazil nut (Bartolome et al., 1997). It has been tested for the response of these individuals to pea 2S albumin, but in this case no positive cross-reaction was observed. This is in contrast to the results obtained with chickpea and pea PA2 albumins, both generating a positive reaction in chickpea-sensitive individuals (Vioque et al., 1998). This indicates that a close taxonomical relationship is not enough to generate the same allergenic reaction against homologous proteins from different species. The differences in the allergenic response to pea and chickpea 2S albumins may be related to the presence of disulfide bonds in the chickpea 2S polypeptide.

Protease Inhibitory Activity of Chickpea 2S Albumin. The presence of trypsin and chymotrypsin inhibitors in chickpea seeds has been reported. Different studies on the influence of protease inhibitors in the nutritional quality of chickpea seeds have been developed (Singh and Jambunathan, 1981; Shamanthaka and Murray, 1987; Bausal et al., 1988; Saini et al., 1992). They have been purified having a molecular mass between 8 and 10 kDa (Belew et al., 1975; Belew and Eaker, 1976; Smirnoff et al., 1976). Thus, they are low molecular mass proteins with inhibitory activity toward trypsin and chymotrypsin, suggesting that they correspond to the Bowman−Birk inhibitor family which exhibits separate independent sites for trypsin and chymotripsin inhibition. This type of proteinase inhibitor is widely distributed in seeds of the Leguminosae and is characterized by a relatively low molecular mass and a high cysteine content (Richardson, 1991).

We have assayed chickpea albumins and 2S albumin for inhibitory activity on porcine trypsin and chymotrypsin (Figure 3). The results show that the albumin extract and the peptides that compose chickpea 2S albumin possess inhibitory activity. As previously reported in chickpea (Saini et al., 1992), in 2S albumin this activity is higher against chymotrypsin than trypsin, although the reasons for this different inhibitory rate are unknown. In total albumins, this difference is not appreciable, probably because of the presence of other protease inhibitors with higher activity against trypsin that are not included in chickpea 2S albumins.

In conclusion, chickpea 2S albumins may be of interest from a nutritional point of view because of the high-sulfur amino acid contents. However, they are also nutritionally undesirable because of allerginicity and inhibitory activity on digestive proteases. In the yellow mustard 2S albumin, similar antinutritional characteristics have been reported; it is a major allergen and resistant to hydrolysis (González de la Peña et al., 1996). It should be noted that characteristics common to protease inhibitors and allergens are their low molecular mass and high sulfur amino acid contents (Richardson, 1991; Spies, 1974). Also, a high heat stability and resistance to acids have been reported, and the presence of disulfide linkages appears to protect them against enzymatic proteolysis (Richardson, 1991; Metcalfe, 1985; Taylor, 1992). Thus, the resistance to chemical and biochemical degradation will provide the necessary stability in the digestive tract to generate the corresponding antinutritional answer.

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