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Enzymatic Hydrolysis of Sweet Lupin, Chickpea, and Lentil 11S Globulins Decreases their Antigenic Activity

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We investigated the effects of treatments with the enzymes pepsin and trypsin on the in vitro immunological reactivity of the major globulins found in the seeds of sweet lupin, chickpea, and lentil. Polyclonal major globulin-specific antiserum was obtained by immunization of rabbits with a solution of the 11S globulin of each legume. The globulins were hydrolyzed with pepsin and trypsin for 1, 5, 15, and 30 min. The native globulins and their hydrolysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting to identify the polypeptide bands with antigenic activity, and the hypoantigenicity of the hydrolysates was analyzed by enzyme-linked immunosorbent assay. Our results show that enzymatic treatment of the major storage protein (11S globulin) of sweet lupin, chickpea, and lentil with pepsin or trypsin lead to the formation of large amounts of short peptides and free amino acids that do not allow antibody binding, resulting in a weakened immunoreactivity.

KEYWORDS: Sweet lupin; chickpea; lentil; enzymatic hydrolysis; antigenic activity.

INTRODUCTION

Legumes make a significant contribution to human and animal nutrition because they represent a rich source of proteins and carbohydrates (*1*). Protein contents of the different varieties of legume seeds range from 17 to 40%, and the nutritional value of these proteins is highly variable, being generally lower than that of control diets based on casein (*2*). This fact seems to be multifactorial and related to the low content of sulfur-containing amino acids (*3*), the compact proteolysis-resistant structure of native seed proteins, (*4*) and the presence of antinutritional factors, including protease inhibitors, tannins, and lectins. These factors negatively affect protein digestibility, as they decrease protein hydrolysis, disturb the absorption of peptides and amino acids, and consequently increase the loss of nitrogen (N) of both endogenous and exogenous origins (*5*).

Storage globulins make up the bulk of the protein in legume seeds and they comprise two main proteins, differentiated by their sedimentation coefficients, the 11S or legumin-like and the 7S or vicilin-like proteins (*1*). These proteins constitute about 70−80% of the total protein in the legume seeds; however, some of the globulins are poorly digestible and associated with intolerance and allergy in humans and animals (*6*). Various immunoassay studies of proteins in a lot of legume species have indicated that the 7S or vicilin-type proteins are more immunogenic than the 11S or legumin type (*7*−*9*).

The use of protein hydrolysates in the food industry has expanded considerably in the past few years. Thus, plant protein hydrolysates are widely used to obtain value-added products from dietary proteins because of the improvement in nutritional and functional characteristics, delayed deterioration, and removal of antinutritional, toxic, or inhibitory ingredients (*10*). In addition, protein hydrolysates show technological advantages such as improved solubility, heat stability, and relatively high resistance to precipitation by many agents such as pH or metal ions (*11*).

Antibodies can be used to detect structural changes that occur in a protein molecule during the various processes to which it is subjected. Most commercial hypoallergenic hydrolysates are assayed for antigenicity or allergenicity by enzyme-linked immunosorbent assay (ELISA) because of its sensitivity and relative simplicity (*12*). Thus, the effect of the degree of hydrolysis on antigenicity has been studied in milk proteins (*13*).

Several investigators have evaluated the immunogenicity or allergenicity of proteins that are enzymatically hydrolyzed for potential use in formulas to feed people with food allergies (*14*, *15*). Storage proteins from common bean (*Phaseolus vulgaris*), soybean (*Glycine max*), and pea (*Pisum sativum*) have been studied extensively (*1*, *4*, *16*, *17*). However, research on the major globulins in sweet lupin (*Lupinus albus* L.), chickpea (*Cicer arietinum* L.), and lentil (*Lens esculenta* M.) is very

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incomplete, which means that a conclusive account of the biochemical and immunological characteristics of the various protein fractions of these legumes is still lacking. A detailed understanding of storage protein structure and diversity is an important prerequisite for attempts to improve quality because it indicates the extent to which the structure of the proteins can be manipulated without affecting their biological properties (*1*).

The aim of this study was to investigate the in vitro antigenic response of hydrolysates of 11S globulins from lupin, chickpea, and lentil produced by the action of pepsin and trypsin.

MATERIALS AND METHODS

Samples. Seeds of sweet lupin (*Lupinus albus* L.), chickpea (*Cicer arietinum* L.), and lentil (*Lens esculenta* M.), were purchased from the Campinas Agronomic Institute (IAC), Campinas, Brazil. Seeds of each legume were washed in running water and soaked for 8 h at 4 °C. Seed coats were then removed by hand, and the grains were dried in an oven at 40 °C. Dried grains were ground into flour (60 mesh), and this was defatted with hexane (1:8 w/v flour to solvent ratio) by rocking at room temperature for 4 h. The defatted flour obtained was dried at room temperature and used for protein extraction.

Reagents and Chemicals. Sephadex G-25, Sepharose CL-6B, pepsin, trypsin, molecular weight standards for chromatography column and electrophoresis, Tris-HCl, Coomassie Brilliant Blue R-250, diaminobenzidine, Tween 20, and orthophenylene diamine were purchased from Sigma Co. (St. Louis, MO). Complete and incomplete Freund's adjuvants were provided by Difco Laboratories (Detroit, MI). The polyvinylidene difluoride (PVDF) membrane and molecular weight standards for immunoblotting (Biotinylated SDS-PAGE Standard, Broad Range) were obtained from Bio-Rad Laboratories (Hercules, CA). ELISA microtiter plates were provided by Corning Costar Corp. (Cambridge, MA). Peroxidase-labeled goat antirabbit IgG was from Southern Biotechnology (Birmingham, AL). All other chemicals were of reagent grade.

Extraction of Major Storage Globulins from Legumes. *Sweet Lupin.* Major globulin (11S) was successively extracted from defatted sweet lupin flour as described by Melo et al. (*18*). A suspension of defatted flour in deionized water (1:10 w/v) was centrifuged at 15000*g* for 30 min. The supernatant was removed and the residue adjusted to pH 8.0 with Tris/HCl buffer solution containing 100 g/L NaCl, 0.05 g/100 g NaN_3, and 0.1 mmol/L EDTA. The suspension was centrifuged at 30000*g* for 30 min. The residue was discarded and $(NH_4)_2SO_4$ was added to the supernatant to 80% saturation. The resulting protein suspension was centrifuged at 30000*g* for 30 min. The supernatant was discarded and the residue, containing the total globulins, was suspended in potassium phosphate buffer (0.15 mol/L K_2HPO_4 pH 7.0). This protein suspension was applied to a Sephadex G-25 column and the eluate obtained was transferred to a Sepharose CL-6B-column (100 cm × 2.5 cm). The fractions containing 11S protein were pooled and used for the study. The protein isolated was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE (*19*).

Chickpea. The major storage protein was obtained by the method described by Kumar and Venkataraman (*20*). Aliquots of defatted chickpea flour were suspended in 100 g/L NaCl solution (1:10 w/v) and stirred gently for 2 h at 4 °C. The suspension was centrifuged at 5000*g* for 30 min. The supernatant was diluted with distilled water (1:10 v/v supernatant to water ratio) and centrifuged at 5000*g* for 30 min. The precipitate was resuspended in 100 g/L NaCl solution. The solution obtained was diluted with distilled water (1:20 v/v solution to water ratio) and centrifuged (5000*g* for 30 min). The precipitated pellet represented the major chickpea globulin. Aliquots of this protein fraction were resuspended in 0.5 mol/L NaCl and applied to a Sepharose CL-6B column (100 cm × 2.5 cm), previously equilibrated with potassium phosphate buffer (5 mmol/L K_2HPO_4 pH 7.5 containing 0.5 mol/L NaCl). Fractions of 4.2 mL were collected. The elution profile was followed by absorbance at 280 nm. The presence of the major globulin (10.3S) in the fractions was confirmed by SDS-PAGE (*19*). The protein content in all procedures was measured by the Lowry method (*21*).

Lentil. Major lentil globulin (11S) was isolated from defatted lentil flour as described by Neves and Lourenço (*22*). Defatted lentil flour was suspended in a solution containing 0.5 mol/L NaCl and 0.25 mol/L ascorbic acid (1:20 w/v flour:solution ratio), adjusted to pH 3.5. The suspension was stirred for 1 h at 4 °C and filtered through Whatman No. 2 filter paper. The solution obtained was centrifuged at 60000*g* for 30 min, and deionized water was added to the supernatant (1:5 v/v supernatant to water ratio). The diluted supernatant was centrifuged at 60000*g* for 30 min. The pellet, which represented the major lentil globulin (11S), was redissolved and loaded on a Sepharose CL-6B column (100 cm × 2.5 cm) and eluted in a single peak that was pooled. The protein isolated was checked by SDS-PAGE (*19*).

In Vitro Protein Hydrolysis. *Pepsinolysis.* Major globulins were dissolved in 1 mmol/L KCl buffer adjusted to pH 1.5 with HCl. Aliquots in triplicate (0.5−1.0 mg/mL) were incubated for 1−30 min with pepsin (1:66 w/w enzyme to protein ratio) at 37 °C in wrapped test tubes.

Trypsinolysis. Aliquots in triplicate (0.5−1.0 mg/mL) of major globulins were dissolved in 20 mmol/L phosphate buffer, pH 7.8, and incubated for 1−30 min with trypsin at 1:10 w/w enzyme to protein ratio at 37 °C in wrapped test tubes.

Samples from both enzymatic digestions (taken after 1, 5, 15, and 30 min hydrolysis) were diluted with Tris-SDS-mercaptoethanol buffer and heated at 99 °C for 3 min and used for the immunoassays.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Native globulins and hydrolysates were subjected to SDS-PAGE (12 g/100 g acrylamide resolving gel) under reducing conditions, as described by Laemmli (*19*).

Production of Polyclonal Antibodies. Immunogens (1 mg of each native major globulin), dissolved in 1 mL of sterile saline (0.9 g/L NaCl) and emulsified in 1 mL of Freund's complete adjuvant, were administered (1 mL in total) to different rabbits as multiple-site subcutaneous injections on the back. Three booster injections followed at 14-day intervals, consisting of 0.5 mg immunizing antigen in 0.5 mL of incomplete Freund's adjuvant. Rabbits were bled from the marginal ear vein 30 days after the booster injections. After centrifugation of the blood, the serum was removed and stored at −20 °C until required. Polyclonal antisera were thus produced to the major globulins of sweet-lupin, chickpea, and lentil. These experiments were approved by the Research Ethic Committee of the School of Pharmaceutical Sciences, São Paulo State University (UNESP).

Immunoblot Analysis. Proteins separated by SDS-PAGE were electrophoretically transferred to a PVDF membrane for 75 min at 0.8 mA/cm^2, using a semidry transblot apparatus (Amersham Pharmacia Biotech). After transfer, the gel was stained with Coomassie Brilliant Blue R-250 and destained to ensure the proteins were completely transferred to the membrane. The membrane was saturated with 3 g/100 g nonfat milk powder in Tris-buffered saline (TBS, pH 7.3) for 2 h and incubated in a 1:2000 dilution of antimajor globulin serum in phosphate-buffered saline containing 0.05 g/100 g Tween 20 (PBST) containing 1 g/100 g nonfat milk powder overnight at 4 °C with gentle rocking. After three washings with PBST, the membranes were incubated for 90 min with goat antirabbit IgG labeled with peroxidase (dilution 1:8000) at room temperature. Finally, binding was revealed with diaminobenzidine as substrate in 50 mmol/L Tris/HCl buffer (pH 7.4) containing 0.03 g/100 g H_2O_2 for 15 min at room temperature. After this step, the membrane was washed three times for 5 min each with distilled water.

ELISA Development. An ELISA format was developed to quantify the reduction of antigenic activity of the protein hydrolysates relative to the protein isolates. The antigens (major globulins of sweet lupin, chickpea, and lentil, in the native form and treated with pepsin and trypsin) were diluted to 1.25 μg/mL in 60 mmol/L sodium carbonate−bicarbonate buffer (pH 9.6). These solutions were added to the microtiter plate (100 μL in each well) and incubated overnight at 4 °C. After washing three times in PBST for 5 min, rabbit antiserum specific for each globulin was diluted in PBST containing 1 g/100 g bovine serum albumin (BSA) and added to the rows (100 μL per well) in serial dilutions ranging from 1:250 to 1:512,000 (v/v) and left to stand for 1 h at 37 °C. Rows were then washed three times, for 5 min each time, with PBST and filled with a solution of goat antirabbit IgG labeled with peroxidase, diluted 1:4000 (v/v) in PBST containing 1 g/100 g BSA. After 1 h of incubation at 37 °C followed by three washes for 5 min with PBST, 100 μL of 1 mg/mL orthophenylene diamine in 0.1

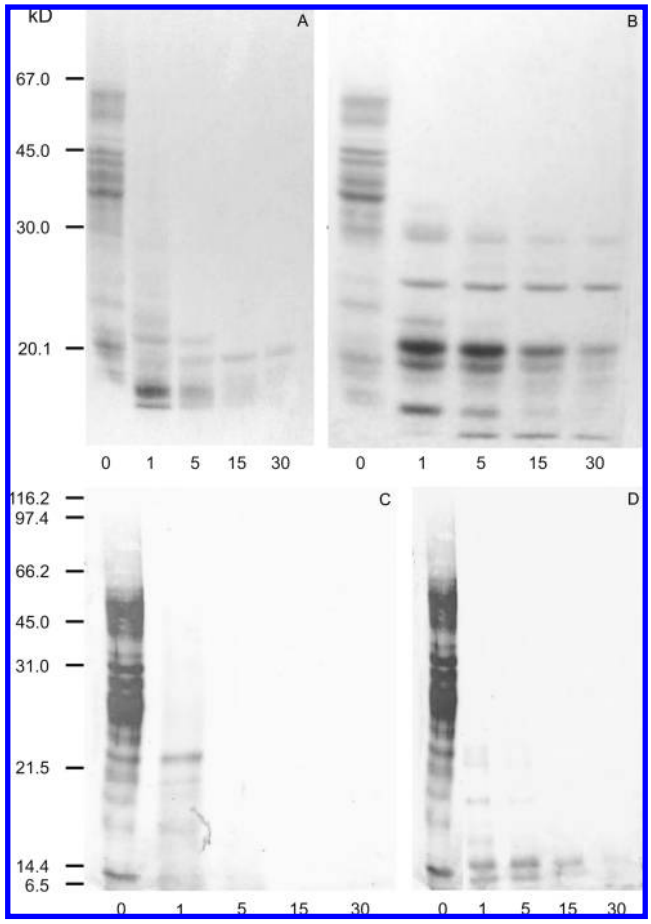

Figure 1. Sweet lupin globulin: SDS-PAGE pattern of native globulin and globulin exposed to pepsin (**A**) and trypsin (**B**) for varying periods of time (0, 1, 5, 15, and 30 min). IgG-specific immunoblot of native globulin, pepsin hydrolysates (**C**), and trypsin hydrolysates (**D**) with the sweet lupin-specific rabbit antiserum. Molecular weight markers are indicated left in kD.

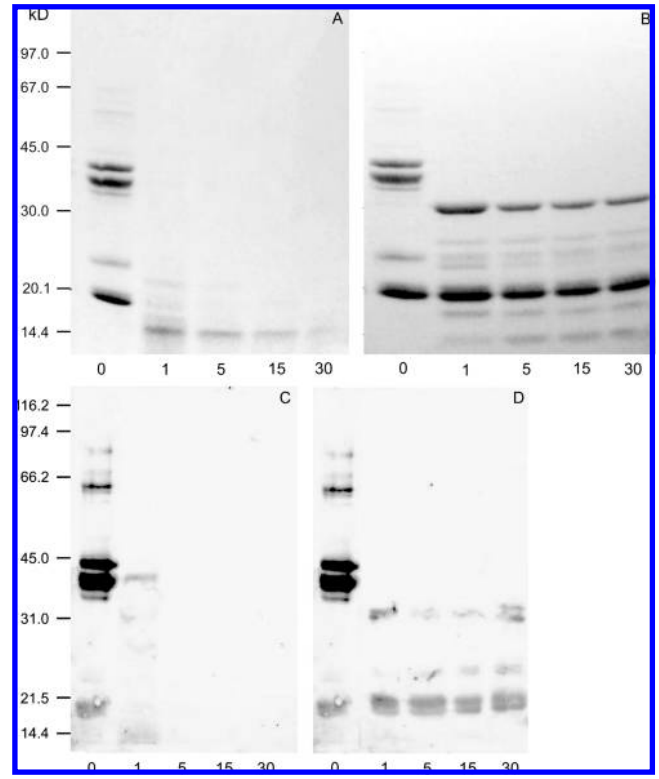

Figure 2. Chickpea globulin: SDS-PAGE pattern of native globulin and globulin exposed to pepsin (**A**) and trypsin (**B**) for varying periods of time (0, 1, 5, 15, and 30 min). IgG-specific immunoblot of native globulin, pepsin hydrolysates (**C**), and trypsin hydrolysates (**D**) with the chickpea-specific rabbit antiserum. Molecular weight markers are indicated left in kD.

M citrate phosphate buffer, pH 5.0, containing 0.03 g/100 g hydrogen peroxide, was added to each well. After 15 min, 100 μL of 3 mol/L HCl was added to stop the reaction. The intensity of the resulting color developed at room temperature was read in a microplate spectrophotometer model 550 (Bio Rad) at 492 nm. To ensure the validity of the results, every assay included a control blank and negative (nonimmunized rabbit serum) and positive (immunized rabbit serum) controls, which were processed similarly to samples. All samples were run in triplicate. The results were expressed as percentage inhibition, calculated with the formula:

$$(1 - A_H/A_N) \times 100$$

where A_H is the absorbance value obtained by hydrolyzed samples and A_N is the absorbance value of native globulins.

RESULTS AND DISCUSSION

Electrophoretic and Antigenic Profiles of Major Globulin Proteins and Their Hydrolysates. *Sweet Lupin.* Comparing the profiles of the hydrolysates to those of the native proteins shows that enzymatic digestion led to the disappearance of a number of protein bands. Hydrolysis by pepsin after 5 min led to the disappearance of all proteins with molar masses from 20.1 to 97 kDa (**Figure 1A**), while the action of trypsin resulted in only partial digestion (a gradual decrease in intensity of protein bands between 30 and 97 kDa after 1 min) (**Figure 1B**). The majority of the proteins were hydrolyzed to components of lower molar mass, which enriched the hydrolysates in small peptides with molar masses between 20 and 30 kDa and lower than 20 kDa. As the hydrolysis proceeded, the intensity of the bands decreased.

The reaction of the sweet lupin-specific antiserum with the hydrolysates revealed bands of low molecular weight, as expected. After 5 min of treatment with pepsin, the antigenicity of the protein had been annulled (**Figure 1C**). Thus, at the start of hydrolysis, a rapid loss of sweet lupin antigenic activity was observed. The immunoblotting pattern of the trypsin hydrolysates revealed a reduced number of bands (**Figure 1D**). Thus, the trypsin promotes a gradual hydrolysis of the lupin globulin and the protein bands of low molar mass become weaker as the hydrolysis continues.

Chickpea. The pepsin exerted a gradual action on the chickpea globulin, and after 30 min, the bands had practically disappeared (**Figure 2A**). The protein bands from 45 to 97 kDa were degraded after 1 min, and some components of low molar mass appeared below 20 kDa. After 5 and 15 min, the formation of a faint polypeptide band near 14.4 kDa was observed, and after 30 min, the hydrolysate of the globulin showed no protein fragments of molecular mass above 14.4 kDa (**Figure 2A**). Hydrolysis by trypsin altered the electrophoretic pattern after 1 min; however, a lot of minor and two stronger bands between 14.4 and 30 kDa are present in the hydrolysates, and apparently these are peptides resistant to trypsin (**Figure 2B**).

The pepsin hydrolysate obtained soon after 1 min of treatment reacted with the chickpea-specific antiserum, and weakly reactive antigenic bands of molar masses below 31 kDa can be seen (**Figure 2C**). On the other hand, the antiserum reacted with the resistant protein bands (below 31 kDa) of the trypsin hydrolysate at all time points (**Figure 2D**).

Lentil. The pepsin treatment destroyed bands between 45 and 67 kDa after 1 min and provoked the appearance of bands of

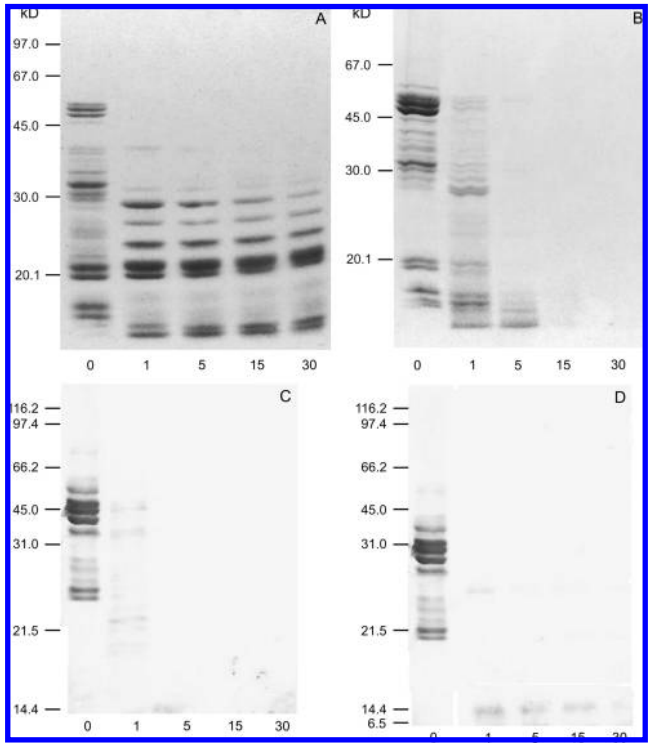

Figure 3. Lentil globulin: SDS-PAGE pattern of native globulin and globulin exposed to pepsin (**A**) and trypsin (**B**) for varying periods of time (0, 1, 5, 15, and 30 min). IgG-specific immunoblot of native globulin, pepsin hydrolysates (**C**), and trypsin hydrolysates (**D**) with the lentil-specific rabbit antiserum. Molecular weight markers are indicated left in kD.

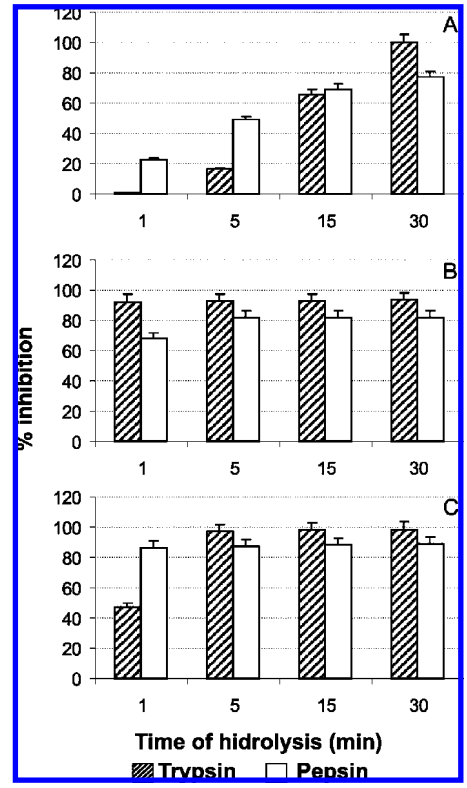

Figure 4. Inhibition of antigenic activity of sweet lupin (**A**), chickpea (**B**), and lentil (**C**) hydrolysates with respect to native globulins. The hydrolysates were obtained by individual treatment with trypsin and pepsin, as described in Materials and Methods. All samples were run in triplicate.

lower molar mass (near 30 kDa and lower than 20.1 kDa). After 5 min, bands of molar mass below 20.1 kDa were formed, while after 15 min, protein bands were no longer observed (**Figure 3A**). The treatment with trypsin provoked the destruction of the larger molar mass bands (between 30 and 67 kDa) and the appearance of bands of lower mass (between 20.1 and 30 kDa and lower than 20.1 kDa) after 1 min (**Figure 3B**). The immunoblotting of the pepsin hydrolysate with the lentil-specific antiserum showed weakly immunogenic bands only after 1 min of treatment (**Figure 3C**). The antiserum reacted only with bands lower than 14.4 kDa of the trypsin hydrolysate, which resulted from the enzymatic action. As the treatment proceeded, those bands lost their immunogenic capacity gradually (**Figure 3D**).

Hypoantigenicity of Hydrolysates. Loss of antigenicity was defined as a decrease in the capacity of antigenic determinants to interact with immunologically specific antibodies. The hypoantigenicity of the hydrolysates was analyzed by ELISA.

Sweet Lupin. The antigenic activity of the hydrolysates from sweet lupin major globulin was progressively reduced by the exposure of native protein to enzymatic hydrolysis. The pepsin gradually destroyed the epitopes on the sweet lupin major globulin, and after 30 min, 77.12% inhibition of the antigenicity can be observed in the pepsin hydrolysate (**Figure 4A**). Differently from pepsin, the effect of trypsin can be observed to start from 5 min of action, and at 30 min, the antigenic epitopes present in the hydrolysate were completely destroyed (**Figure 4A**).

Chickpea. Pepsin led to a 68.31% inhibition of the antigenicity after 1 min. However, after 5 min, the action of this enzyme reached its maximum, with 81.8% inhibition, which remained the same until 30 min (**Figure 4B**). Thus, although at 30 min we did not observe any immunoreactive bands by immunoblotting, ELISA showed that the hydrolysate still produced a faint reaction with the specific serum. The residual antigenicity was probably due to the presence of monovalent peptide fragments. Trypsin exerted a total action on the protein by 1 min. Thus, trypsin hydrolysis led to a 93.6% decrease in recognition of the antigen−antibody reaction and caused the destruction of almost all antigenic epitopes of chickpea major globulin (**Figure 4B**). The immunoblotting (**Figure 2D**) shows that at all the times bands were seen from 14.4 to 31 kDa and this result implies that the epitopes with antigenic activity detected by ELISA represent less than 10% of the peptides present in the hydrolysate.

Clemente et al. (*12*) found that the antigenic activity of the hydrolysates from chickpea protein isolate obtained with flavourzyme or alcalase was significantly reduced by enzymatic hydrolysis, but the most effective reduction was observed when an extended hydrolysis was performed by the sequential action of both enzymes. Hindi-Tamelikecht et al. (*23*), using water-soluble concentrate of chickpea proteins, found that enzymatic hydrolysis by bromelain and chymotrypsin resulted in reductions of 58 and 45% of the antigenic character of the proteins, respectively, in an ELISA inhibition system with chickpea protein antiserum raised in rabbits. Salgado et al. (*8*) carried out an immunological investigation of legume proteins and showed that proteins of the 7S family were more immunogenic than the 11S proteins in the small intestinal digesta of weaned piglets.

In the present study, under the conditions employed for hydrolysis by pepsin and trypsin, it was shown that pepsin hydrolysis led to approximately 80% and trypsin to 100% loss of the antigenic character of the major globulin (10.3 S) from chickpea.

Lentil. Pepsin caused a destruction of all antigenic epitopes in as little as 1 min of action (**Figure 4C**). The faint bands

shown in the immunoblotting at 1 min did not react with the antiserum in ELISA. Trypsin led to 47.56% inhibition of the antigenicity after 1 min. However, after 5 min, the action of this enzyme reached a maximum, with approximately 98% inhibition, which was maintained until 30 min (**Figure 4C**).

A number of studies on the effect of enzymatic hydrolysis on the antigenicity and allergenicity of dietary proteins have been carried out on proteins of cow milk (*13*), soybean (*15*, *24*), chickpea (*12*, *23*), peanut, (*25*) and soybean, pea, fava bean, chickpea, and lupin (*8*). For all 11S globulins in our study, the enzymatic hydrolysis by pepsin (0.016 w/w) was very fast in the initial stages and led to a progressive and total disappearance of protein bands in the final stages. After 30 min, a small number of protein bands was seen near and below 14.4 kDa and practically without antigenic activity. This fact suggests that these proteins all tend to react similarly to peptic hydrolysis.

van Boxtel et al. (*26*) reported that after being exposed for 40 min to pepsin (0.001 w/w), legumins from peanut and soybean were destroyed, leaving only peptides of molar mass below 20 kDa. Our study showed that the legumins from lupin, chickpea, and lentil did not maintain their antigenic activity after hydrolysis by pepsin for 30 min and that 20% (lupin, chickpea) and 10% (lentil) residual antigenic activity detected by ELISA represented the reactivity of small peptides present in the hydrolysates of the globulins. The data indicate that the dominant allergen in the 11S globulin of lupin consists of a basic subunit of approximately 20 kDa and that it loses its antigenic activity as the hydrolysis with pepsin and trypsin proceeds. The same can be observed for globulins of chickpea and lentil, which may result from a sequence homology, especially near the sites of action of the enzymes, as observed by Magni et al. (*27*) for the 11S fraction of lupin in its cross-reactivity with the same fraction of other legumes. Lee et al. (*24*) also observed some reactivity of this basic subunit and that the hydrolysis of the 11S fraction of soybean with pepsin and pepsin−pancreatin resulted in fragments of less than 10 kDa that did not seem to be immunoreactive in the ELISA assays, suggesting that such behavior was likely to be due largely to conformational epitopes rather than sequence epitopes. However, Marcone (*28*), using a variety of immunological techniques, found substantial cross-reactivity between the IgG expressed against 11S globulin from amaranth and legumins of other plants. Sequence homologies were found to occur almost exclusively in the basic type subunit of these seed globulins, as recently also observed by Magni et al. (*27*) in lupin. On the other hand, L'Hocine et al. (*29*) have shown that the immunological response of 11S globulin from soybean is strongly modulated by its conformational structure, which in turn is affected by environmental conditions such as pH, ionic strength, and temperature. In the case of legumins from peanut and soybean, van Boxtel et al. (*26*) have observed that heating before digestion slightly increases the stability of the proteins to the action of pepsin, but this did not affect the IgE binding to the remaining peptides.

Our results show that enzymatic treatments of sweet lupin, chickpea and lentil 11S globulins with pepsin or trypsin led to the formation of large amounts of short peptides and free amino acids that did not allow antibody binding, resulting in a loss of immunoreactivity. However, it is important to remember that we focused this study only on these two enzymes, and additional research is needed to elucidate the complex effects of enzymatic hydrolysis in general on the allergenic activity of the 11S proteins. An investigation of the effects of heating the 11S and 7S globulins from lupin, chickpea, and lentil followed by digestion with pepsin, trypsin, and chymotrypsin, on their immunoreactivity are in progress in this laboratory.

ABBREVIATIONS USED

BSA, bovine serum albumin; EDTA, ethylenediamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; PBST, phosphate-buffered saline containing 0.05 g/100 g Tween 20; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

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