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Influence of naturally acid-soluble proteins from beans (*Phaseolus vulgaris* L.) on *in vitro* digestibility determination

Maria Inés Genovese & Franco M. Lajolo*

Departamento de Alimentos e Nutrição Experimental, Fac. Ciências Farmacêuticas, Universidade de São Paulo, Caixa Postal 66083, CEP 05389-970, São Paulo, SP, Brazil

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The *in vitro* digestibility of bean (*Phaseolus vulgaris*) protein fractions was studied using a pepsin–pancreatin system. Enzymatic hydrolysis was stopped by adding a strong acid and the extent of proteolysis determined by measurement of free amino groups in the soluble fraction. The *in vitro* digestibility of bean protein fractions was low when in the native state and was differently affected by denaturation. For phaseolin, the main reserve protein, heating caused a significant increase of susceptibility to hydrolysis, whereas heat had no apparent effect on digestibility of glutelins and albumins (II). For the PIL (protease inhibitor-lectin rich) fraction, which was shown to have a composition similar to total albumins, there was a decrease of digestibility, probably associated to disulfide bond formation upon heating. Results of *in vitro* digestibility were shown to be strongly dependent on the utilization of a sample blank to account for proteins naturally soluble in the acid used to interrupt hydrolysis, which would otherwise be estimated as digested protein. These proteins are characterized by a high carbohydrate content, probably responsible for their high solubility and low digestibility.

INTRODUCTION

Bean (*Phaseolus vulgaris*) proteins have a low nutritional value due to reduced digestibility and low methionine content and bioavailability (Bressani, 1993). Phaseolin (or G1 globulin) is the major seed storage protein and represent, along with albumins, near 80% of total bean proteins. Phaseolin is very poorly digested when in its native state, but effectively hydrolysed after heat treatment (Liener and Thompson, 1980; Deshpande and Nielsen, 1987*a*). The cleavage sites of native phaseolin by pepsin and trypsin have already been determined (Jivotovskaya *et al.*, 1996). In contrast, as we observed in previous papers, the *in vitro* digestibility of albumin proteins is low (26–32%) and after heating it is reduced even more, to 13–18%, independently of bean variety (Marquez and Lajolo, 1981; Genovese and Lajolo, 1996*a*,*b*). Methionine content is similar for the two fractions; although the *in vitro* bioavailability, after heat treatment, is high for phaseolin but very low for albumins, as expected from digestibility values (Genovese and Lajolo, 1996*a*). On the other hand, Coelho and Sgarbieri (1995*a*), working with albumins separated into acid- and alkali-extracted fractions, did not observe any reduction of digestibility provoked by heating, and a degree of *in vitro* hydrolysis similar to that of phaseolin was attained. The purpose of this study was to further investigate the *in vitro* digestibility of these fractions in order to determine albumin components that could be responsible for the thermal reactions leading to the decrease of digestibility, and/or try to explain the differences found in values of *in vitro* digestibility of albumins when they are obtained by different techniques.

*To whom correspondence should be addressed. Fax: 55 011 815 4410; e-mail: fmlajolo@usp.br

MATERIALS AND METHODS

Dry seeds of *Phaseolus vulgaris* L. cv. Carioca were obtained from the Instituto Agronômico de Campinas, Campinas, Brazil. After dehulling, the seeds were ground in a mill and the resultant flour passed through a 0.25 mm sieve. Pepsin (P-7012, 2690 units/mg), pancreatin (P-1750, activity equivalent to 4×U.S.P. specifications) and α-amylase (A-4268, from porcine pancreas) were purchased from Sigma Chemical Co. Dialysis tubing (Spectra/Por) with molecular weight cut-off (MWCO) of 10 000 was purchased from Spectrum

Medical Industries, Inc. All other chemicals used were of analytical grade.

Total albumins extraction

A flour sample of 20 g was extracted with distilled water (1:20 flour:water w/v) for 2 h under agitation at room temperature (25°C). After centrifugation (30 000 *g* for 30 min), the supernatant was dialysed for 48 h against distilled water followed by a 24 h dialysis against deionized water, at 4°C (Marquez and Lajolo, 1981). The precipitated globulins were separated by centrifugation and discarded, and the supernatant (albumin fraction) freeze-dried (see Fig. 1).

Phaseolin isolation

Phaseolin (G1 globulin) was isolated by the method of Hall *et al.* (1977). The flour (20 g) was extracted under acidic conditions (0.5 M NaCl in 0.025 M HCl; 20 ml/g flour); after centrifugation, the supernatant was diluted with 5 vol of distilled water (4°C) and the precipitated phaseolin separated by centrifugation (30 000 *g* for 30 min). The supernatant left was used for Albumins (II) and the PIL fraction obtention (see next and the scheme in Fig. 1). Phaseolin was ressuspended and precipitated two more times, and finally dialysed against distilled water for 24 h at 4°C and freeze-dried. Purity, shown by electrophoresis, was more than 95%.

Albumins (I)

The residue left after the acidic extraction of the flour was re-extracted with sodium phosphate buffer, pH 7.5, containing 0.5 M NaCl (Fig. 1) according to Coelho and Sgarbieri (1995*a*). The extract was dialysed against deionized water for 48 h at 4°C and, after centrifugation (30 000 *g*/30 min), the supernatant (Albumins (I)) and the precipitate (globulins) were freeze-dried.

Glutelins

The residue left after the extraction with sodium phosphate buffer (pH 7.5, containing 0.5 M NaCl) was re-extracted with 0.1 M NaOH (Fig. 1). After centrifugation (30 000 *g*/30 min), the supernatant (glutelins) was dialysed against deionized water for 24 h at 4°C and freeze-dried.

PIL (protease inhibitor-lectin) fraction

A protein fraction rich in protease inhibitors and lectins (G2 globulins) was obtained according to Coelho and Sgarbieri (1995*a*). Proteins in the supernatant left after phaseolin precipitation were concentrated by ammonium sulfate addition until 100% saturation. The precipitated proteins were dialysed against distilled water for 48 h at 4°C and freeze-dried (Fig. 1).

Albumins (II)

Alternatively, proteins in the supernatant left after phaseolin precipitation were dialysed against deionized water for 48 h (4°C). The precipitated residual globulins, composed mainly of G2 globulin (Sun and Hall, 1975), were separated by centrifugation and discarded, and the supernatant (Albumins (II)) freeze-dried.

Protein content

Protein (N×6.25) content was determined in triplicate by the micro-Kjeldahl method (method no. 955.04, AOAC, 1990).

Carbohydrate content

Total carbohydrate content was determined in triplicate by the method of Dubois *et al.* (1956), using D-mannose in the standard calibration curve (0–60 μg).

Heat treatment

Aqueous solutions of proteins (10 mg/ml) were heated in a boiling water bath for 30 min, and then freeze-dried. Alternatively, samples were autoclaved at 121°C (15 psi) for 15 min.

***In vitro* digestibility**

Enzymatic hydrolysis was carried out according to the method of Mauron (1973), with slight modifications. Protein solutions (10 mg/ml KCl–HCl buffer, pH 2, in triplicate) were first digested with pepsin (enzyme to substrate ratio of 1:66.7) at 37°C for 3 h. Then, the samples were diluted with the same volume of 0.2 M sodium phosphate buffer (pH 8) and, after addition of pancreatin (1:25), digestion was allowed to proceed for 4 h at 37°C. Samples were taken at 1 h intervals and the digestion interrupted with 1.8 M $HClO_4$ (0.2 ml/ml hydrolysate). After centrifugation (10 000 *g*/10 min), the supernatants were assayed for increase in free amino groups to determine the extent of hydrolysis. Two blanks, one containing only the enzymes and the other only the proteins, were run simultaneously, in triplicate.

Extent of hydrolysis

The extent of hydrolysis was determined, based on the measurement of free amino groups released with picryl sulfonic acid (TNBS), according to Fields (1972). The absorbances at 420 nm of the controls were subtracted from those of the digested samples, and the percentage of peptide bond hydrolysis was the ratio of the number of new amino groups released (calculated using $2.2\times10^4\,M^{-1}\,cm^{-1}$ as the molar extinction coefficient for TNP-α amino groups) to the total number of peptide bonds in the protein (calculated by dividing g of

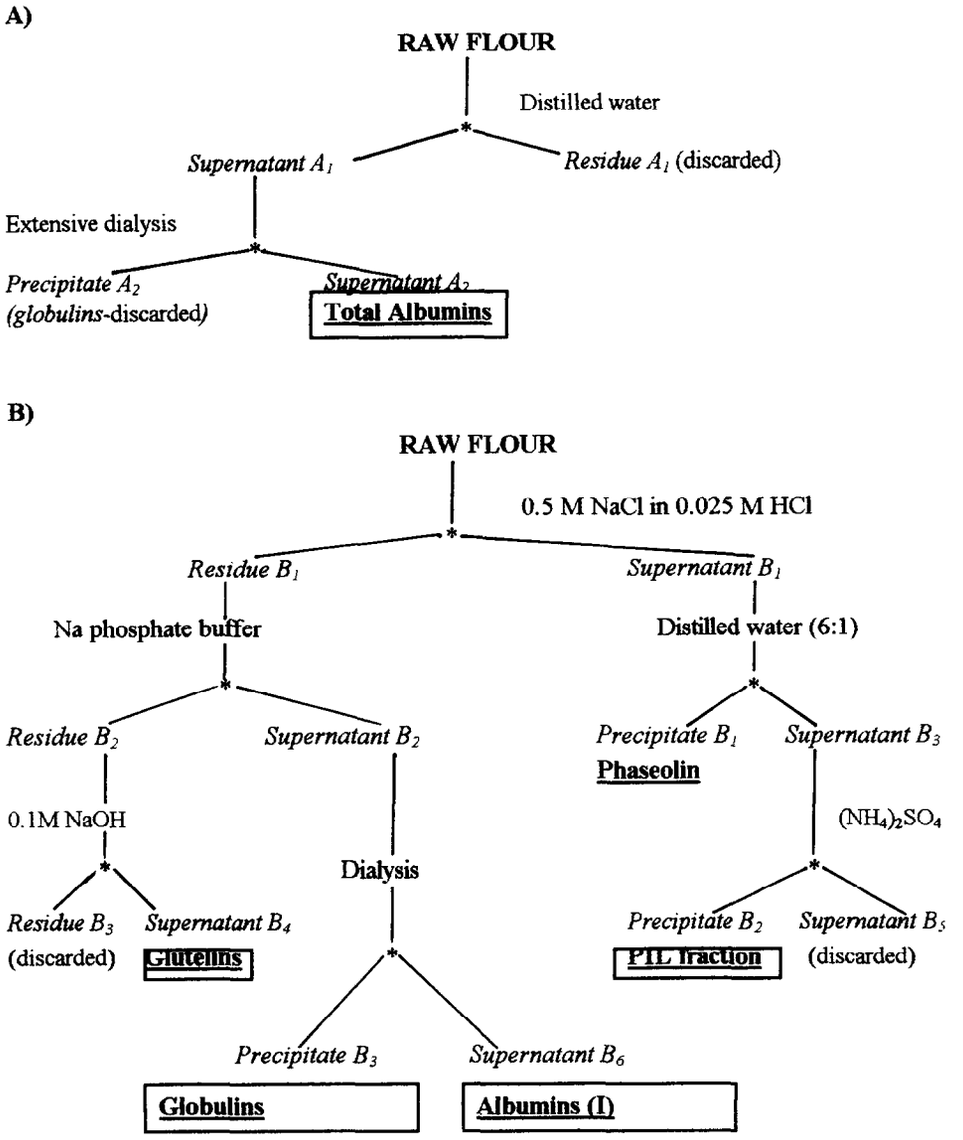

Fig. 1. Scheme for extraction and fractionation of bean proteins. (A) Total albumins extraction; (B) extraction and fractionation of Phaseolin, PIL fraction, Glutelins, Albumins (I) and Globulins. *Centrifugation 30 000 *g*/30 min. In another experiment, instead of concentrated by ammonium sulfate addition, supernatant B_3 was dialysed against deionized water for globulins precipitation and Albumins (II) obtention.

substrate by 113, the average molecular weight of amino acid residues in the protein). Analyses were made in triplicate.

Trypsin inhibitory activity

Trypsin inhibitory activity was measured according to Kakade *et al.* (1969) using BAPA (benzoyl-DL-arginine-*p*-nitroanilide) as substrate. One trypsin unit (TU) was arbitrarily defined as an increase of 0.01 absorbance units at 410 nm. Results were expressed as the number of trypsin units inhibited (TUI) per mg of protein.

Hemagglutinating activity

Hemagglutinating activity of bean protein fractions was determined by the plate microtitration method as described by Figueroa and Lajolo (1997). A 2% rabbit red blood cell suspension, previously activated by incubation with 0.1% of trypsin at 37°C for 1 h, was used. The agglutinating titer was determined as the reciprocal of the last dilution at which agglutination was observed.

α-Amylase inhibitor activity

α-Amylase inhibitor activity was determined based on the method of Bernfeld (1955) for amylase activity determination, according to Iguti and Lajolo (1991), in triplicate. Inhibitor and α-amylase were preincubated for 45 min at 37°C, after which the residual α-amylase activity was measured. One unit of α-amylase inhibitory activity (AIU) was the amount that caused total inhibition of 10 units of α-amylase activity under the assay conditions.

Sulfhydryl and disulfide contents

Sulfhydryl groups were determined by a modification of the method of Beveridge *et al.* (1974). Proteins were resuspended in 0.086 M Tris–0.09 M glycine–0.006 M EDTA (pH 8.0) buffer containing 8 M urea. To 3 ml of these solutions, 30 μl of DTNB (4 mg/ml Tris–glycine buffer, pH 8.0) were added and the absorbance at 412 nm measured 10 min later. Determination of disulfide bonds was carried out according to Li-Chan (1983): 0.01 g of proteins were solubilized in 10 ml of Tris–glycine–EDTA (pH 8) buffer containing 10 M urea and 2% β-mercaptoethanol. After 1 h incubation at room temperature, 10 ml of 24% trichloroacetic acid (TCA) were added and the samples centrifuged. The pellets were twice resuspended in 10 ml of 12% TCA and finally dissolved in Tris–glycine–EDTA (pH 8) buffer with 8 M urea. To 3 ml of this solution 50 μl DTNB were added and $A_{412\,nm}$ was read after 10 min. Half the value obtained after subtracting the sulfhydryl (SH) content from the total SH was defined as a measure of the disulfide (SS) content. Sample and reagent blanks were included for each determination and the analyses were made in triplicate.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Slab SDS–PAGE was carried out according to the procedure of Laemmli (1970) at gel concentrations of 12% (%C of 2.6) and constant current. Gel slabs were fixed and stained with 0.2% Coomassie Blue R in methanol–acetic acid–water (10:7:83). Sample volumes were of 10 μl (lanes 1, 2, 4, 5, from Figs 2 and 3), 20 μl (lanes 3 and 6 from Figs 2 and 3), and 3 μl for MW markers. The mixture of molecular weight markers (Dalton Mark VII-L, Sigma Chem. Co.) consisted of α-lactalbumin (14.3 kDa), soybean trypsin inhibitor (20.1 kDa), trypsinogen (24.0 kDa), carbonic anhydrase (29.0 kDa), glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa), egg albumin (45.0 kDa), and bovine albumin (66.0 kDa). The electrophoresis equipment used was the Mini-Protean II cell from Bio-Rad.

RESULTS AND DISCUSSION

Total yield, protein and carbohydrate content of bean protein fractions

As can be seen in Table 1, Phaseolin (the main storage bean globulin) and Total albumins together represented nearly 63% of total bean proteins. Protein fractions obtained in the scheme of extraction and fractionation proposed by Coelho and Sgarbieri (1995*a*) [Phaseolin, Albumins (I), Globulins, PIL fraction and glutelins] together represented nearly 98% of total bean proteins. However, we were not able to separate Albumins (I) from Globulins by dialysis against deionized water, as they did. As a consequence, the amount of Globulins obtained was too low to allow further characterization. Actually, as shown by SDS–PAGE (Fig. 2), Globulins corresponded to phaseolin, which was not completely extracted under acidic conditions. The protein content of these fractions varied from 51.5 (Glutelins) to 86.4% (Phaseolin), and total carbohydrate content from 5.3% (Phaseolin) to 20.2% (Albumins (II)). Dialysis of the PIL fraction against deionized water, for globulin precipitation, seemed to concentrate carbohydrates in the soluble fraction (Albumins (II)). Deshpande and Nielsen (1987*a*,*b*) also found a high carbohydrate content associated to dry bean albumins (17–30%). The only report found in the literature of a bean albumin component highly glycosylated is of a heterodimeric α-amylase inhibitor composed of an α-subunit and a β-subunit, each one containing, respectively, 28% and 7% by weight of carbohydrate (Yamaguchi, 1991, 1993).

Trypsin inhibitory activity

The presence of protease inhibitory activity in the albumin fraction has been related to its low digestibility (Deshpande and Nielsen, 1987*c*). As expected, phaseolin showed no trypsin inhibitory activity and albumins a great amount of it (Table 2). Also, the protease inhibitory activity seemed to be concentrated in acid extracted fractions, both the PIL fraction and the Albumins (II), which showed a similar inhibitory activity. However, it should be more concentrated in Albumins (II), as this fraction corresponds to the PIL fraction after elimination of contaminants globulins (mostly G2) by dialysis against deionized water. The most probable explanation is that there was also a loss of some trypsin inhibitors during dialysis, as they have low molecular weights—8.5–9.2 kDa (Wu and Whitaker, 1990)—and a dialysis tubing with molecular weight cut off of 10 kDa was used. Albumins (I), which were shown to be contaminated with phaseolin, had nearly half the amount of trypsin inhibitory activity of the acid-extracted fractions.

Hemagglutinating and α-amylase inhibitory activity of bean protein fractions

Hemagglutinating activity was the highest for the PIL fraction, confirming that this fraction concentrates both trypsin inhibitors and lectins (Coelho and Sgarbieri, 1995*a*). Total albumins also had a high hemagglutinating activity, contrarily to Albumins (I) and (II) (Table 2). Lectins (or G2 globulin) are in reality globulins requiring very low salt concentrations for solubility and partially soluble in distilled water (Sun and Hall, 1975). This explains why they are co-extracted with Total albumins. Also, during dialysis of the PIL fraction against deionized water there was probably an almost total precipitation of lectins, responsible for the low hemagglutinating activity of Albumins (II). α-Amylase

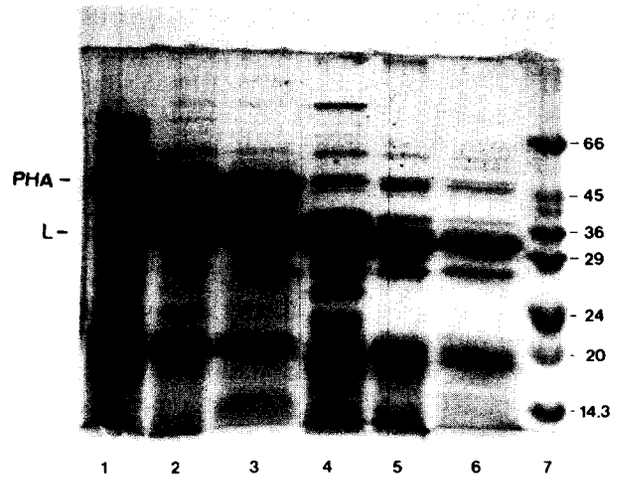

Fig. 2. SDS–PAGE (12%) profile of native Albumins (I) and the PIL fraction sequentially digested with pepsin–pancreatin. Lane 1, Native Albumins (I) (38.2 μg); Lane 2, After 3 h pepsin hydrolysis (32.9 μg); Lane 3, After 4 h pancreatin hydrolysis (29.8 μg); Lane 4, Native PIL fraction (41.3 μg); Lane 5, After 3 h pepsin hydrolysis (35.5 μg); Lane 6, After 4 h pancreatin hydrolysis (32.2 μg); Lane 7, MW markers. PHA. phaseolin; L.

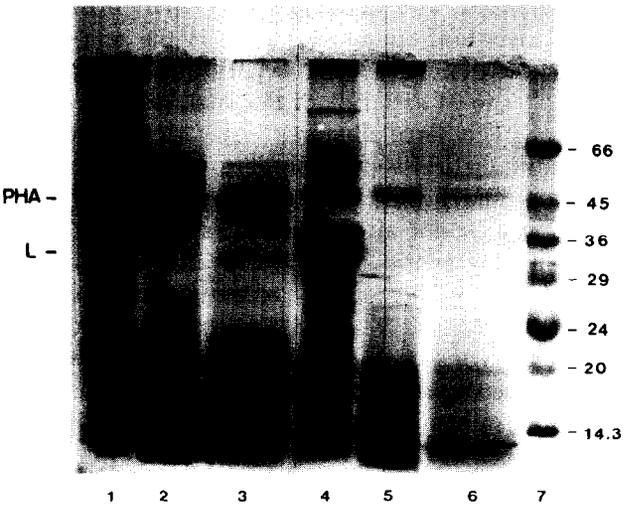

Fig. 3. SDS–PAGE (12%) profile of denatured Albumins (I) and the PIL fraction sequentially digested with pepsin–pancreatin. Lane 1, Heated (99°C/30 min) Albumins (I) (38.2 μg); Lane 2, After 3 h pepsin hydrolysis (32.9 μg); Lane 3, After 4 h pancreatin hydrolysis (29.8 μg); Lane 4, Heated (99°C/30 min) PIL fraction (41.3 μg); Lane 5, After 3 h pepsin hydrolysis (35.5 μg); Lane 6, After 4 h pancreatin hydrolysis (32.2 μg); Lane 7, MW markers. PHA. phaseolin; L. lectins.

inhibitors, on the other hand, concentrated in this fraction (Table 2) and, as expected, were also found in high amounts in the PIL fraction. Albumins (I) showed a low content of α-amylase inhibitors (Table 2). The low hemagglutinating and α-amylase inhibitory activity of Albumins (I) was expected due to their high contamination with phaseolin.

In vitro digestibility of bean proteins

In vitro digestibility of phaseolin, glutelins and albumins, both native and after heat treatment, was determined using the successive enzymatic system pepsin–pancreatin (Table 3). Phaseolin was extensively hydrolyzed after heat treatment (88% of hydrolysis). However, when native, its digestibility was smaller than that of native Total albumins and glutelins, as previously observed (Marquez and Lajolo, 1981; Genovese and Lajolo, 1996*a*). The increased susceptibility to hydrolysis of phaseolin after heating, both *in vitro* and *in vivo*, has been reported by several authors (Romero and Ryan, 1978; Liener and Thompson, 1980; Bradbear and Boulter, 1984; Deshpande and Nielsen, 1987*a*; Ahn *et al.*, 1991). Total albumins, as previously reported (Marquez and Lajolo, 1981; Genovese and Lajolo, 1996*a*), had a drop of pepsin–pancreatin digestibility after denaturation, more severe when heating was performed at 121°C (Table 3). Contrary to what was reported by Coelho and Sgarbieri (1995*a*), this phenomenom was also observed for the PIL fraction. Albumins (II), obtained after dialysis of the PIL fraction, showed the smallest pepsin–pancreatin digestibility of the proteins studied, either native or denatured. This fact could be related to their high carbohydrate content (Table 1), which may impair protease action by steric hindrance. Heating had no significant effect on Albumins (II) digestibility, although any decrease would be difficult to detect due to the already very low degree of hydrolysis of the native proteins.

Table 1. Total yield, protein and carbohydrate content of bean protein fractions

	Yield (% of total bean protein)	Protein content (%)	Carbohydrate content (%)
Phaseolin[a]	35.4 ± 1.5	86.4 ± 0.6	5.3 ± 0.1
Albumins (I)[a]	12.5 ± 0.8	76.4 ± 1.1	10.4 ± 0.1
Globulins[a]	0.4 ± 0.0	—	—
PIL fraction[a]	27.1 ± 1.4	82.6 ± 0.8	11.5 ± 0.1
Glutelins[a]	22.9 ± 1.0	51.5 ± 0.8	15.6 ± 0.2
Total albumins	27.8 ± 1.1	65.2 ± 1.5	13.3 ± 0.1
Albumins (II)[b]	17.8 ± 1.2	55.7 ± 0.2	20.2 ± 0.2

[a]Obtained concomitantly in the same scheme of extraction, as described in Materials and Methods.
[b]Obtained after dialysis of the PIL fraction, against deionized water, for residual globulin precipitation. All determinations were made in triplicate.

Table 2. Hemagglutinating activity, trypsin and α-amylase inhibitory activity of bean protein fractions

	TUI/mg of protein[a]	HT/μg protein[b]	AIU/mg protein[c]
Phaseolin	traces	n.d.	n.d.
Total albumins	278.9 ± 4.3	1005.2	422.9 ± 16.6
Albumins (II)	420.6 ± 22.7	147.1	802.5 ± 6.7
Albumins (I)	218.3 ± 6.9	214.5	168.3 ± 7.3
PIL fraction	446.5 ± 27.5	1983.5	602.1 ± 17.4

[a]Trypsin units inhibited per mg of protein
[b]Hemagglutinating titer per μg of protein.
[c]α-Amylase inhibitor units per mg of protein.
n.d. not detected.

Table 3. Pepsin-pancreatin digestibility of native and denatured (99°C/30 min or 121°C/15 min) bean proteins

	% Hydrolysis[a]	
	After 3 h pepsin digestion	After pepsin digestion, followed by 4 h pancreatin digestion
Phaseolin		
Native	9.8 ± 0.4	22.7 ± 0.7
Heated 99°C/30 min	25.5 ± 0.9	88.2 ± 1.3
Total albumins		
Native	19.1 ± 0.4	28.8 ± 0.9
Heated 99°C/30 min	10.8 ± 0.7	19.4 ± 0.4
121°C/15 min	9.2 ± 0.5	15.3 ± 0.8
PIL fraction		
Native	20.1 ± 0.6	28.2 ± 0.1
Heated 99°C/30 min	14.8 ± 0.9	25.0 ± 1.5
121°C/15 min	10.7 ± 1.4	15.7 ± 1.0
Albumins (II)		
Native	10.7 ± 0.5	14.1 ± 0.7
Heated 99°C/30 min	9.8 ± 0.5	13.4 ± 0.4
121°C/15 min	8.3 ± 1.9	11.1 ± 1.0
Albumins (I)		
Native	19.9 ± 0.8	25.6 ± 1.1
Heated 99°C/30 min	14.5 ± 2.1	26.1 ± 2.1
121°C/15 min	17.2 ± 1.6	36.5 ± 2.7
Glutelins		
Native	14.9 ± 2.4	42.1 ± 3.6
Heated 99°C/30 min	13.1 ± 1.5	43.5 ± 2.4
121°C/15 min	13.8 ± 1.0	39.6 ± 0.6

[a]Results were the mean of three determinations.

Albumins (I) digestibility apparently was not affected by heating at 99°C for 30 min, but increased moderately after heating at 121°C for 15 min. In reality, as this fraction was not pure, two different effects of heating were probably occurring: the increase of contaminant phaseolin hydrolysis opposite to the decrease of albumins hydrolysis. Along this, residual heat-stable trypsin inhibitory activity must also be responsible for the final low digestibility (Genovese and Lajolo, 1996*b*). Glutelins showed the highest digestibility of the proteins studied when native, and, similarly to Albumins (II), heating had no apparent effect upon it.

Naturally acid-soluble proteins

As the beans used were from the same variety, the differences between our results and those found by Coelho and Sgarbieri (1995*a*), mainly in relation to *in vitro* digestibility of the PIL fraction, seemed to be related to differences in the methodology used. In fact, although the authors included enzyme blanks to account for enzyme autolysis, they did not run sample (protein) blanks, to account for naturally acid-soluble proteins, which, at least in beans, seem to represent an important fraction. As previously reported, 65% of native albumins remained soluble in 0.3 M of perchloric acid (Genovese and Lajolo, 1996*b*), the same final concentration used here to interrupt proteolysis and precipitate undigested proteins. In the case of a final concentration of 5% trichloroacetic acid, as used by Coelho and Sgarbieri (1995*a*) to stop proteolysis, 54% of total albumins' N did not precipitate (Table 4), confirming the need of a sample blank to account for naturally acid soluble proteins. After heating, the amount of these proteins decreased to 17% of total albumins' N. Carbohydrates concentrated in the acid-soluble fraction (81% of the total), and suffered a less acentuated decrease (to 66% of the total) after heating (Table 4). This could indicate that carbohydrates were not covalently linked to proteins. However, albumin precipitation with ammonium sulfate until 100% saturation left a supernatant with only 14% of the total carbohydrate content of this fraction. Most probably, the high carbohydrate content is associated with the high solubility in acid of these proteins, although trypsin inhibitors, which are not glycoproteins, also concentrate in the acid-soluble fraction (Genovese and Lajolo, 1996*b*). Trypsin inhibitors are of concern because, besides their physiological effects, they are very poorly digested, making their high half-cystine content unavailable, both *in vitro* and *in vivo* (Kakade, 1974; Bradbear and Boulter, 1984). After pepsin–pancreatin digestion, the amount of N in the acid-soluble fraction increased to 73 and 45% of the total, and carbohydrates to 94 and 88% of the total, respectively, for native and denatured albumins (Table 4). If we consider that naturally acid-soluble proteins are mainly trypsin inhibitors and highly glycosylated proteins, probably very slowly or not at all digested, and that all the N solubilized by pepsin–pancreatin hydrolysis is absorbable *in vivo*, the digestibility of native and denatured albumins will be of 19 and 28%, respectively. However, when the increase of α-amino groups is taken as a measure of digestibility, a lower value is found for denatured albumins compared to native albumins

Table 4. Carbohydrate and protein (N×6.25) content of 5% TCA soluble albumins, before and after pepsin-pancreatin digestion

5% TCA soluble fraction[a]	Native albumins	Denatured albumins (99°C/30 min)
Protein content (% of total N)[b]		
Before digestion	53.6	17.3
After digestion	72.9	45.3
Carbohydrate content (% of total)[c]		
Before digestion	80.7	66.1
After digestion	94.0	88.2

[a]Aqueous solutions of native and heated albumins were separated in acid-soluble and insoluble fractions by 30% TCA addition (0.2 ml/ml albumin solution) and centrifugation (10 000 *g*/20 min). Protein and carbohydrate contents of the acid-soluble fractions were expressed as % of the total found in albumins. Results were the mean of two determinations, and the difference between values was less than 5% of the mean.
[b]Determined by the method of Kjeldahl (AOAC, 1990).
[c]Determined according to Dubois *et al.* (1956).

(Table 3). As the increase of acid-soluble N is also accompanied by an increase of carbohydrates, we cannot be sure that these glycopeptides would be really available *in vivo*, and consequently the increase of α-amino groups seems to be a more adequate measure of digestibility.

For phaseolin and glutelins, sample blanks represented less than 5% of the amino groups found in the hydrolysates. However, for Albumins (II) and the PIL fraction they contributed significantly to the values found. As an example, 46.7 ± 2.1, 46.9 ± 1.4 and 50.4 ± 1.8% of the free amino groups found in the hydrolysates from the PIL fraction, respectively, native, heated at 99°C for 30 min, and heated at 121°C for 15 min, were from naturally acid-soluble proteins, and consequently not from peptides solubilized by digestion or from the action of proteases upon the already acid-soluble proteins. This means that, when a sample blank is not included, these naturally acid-soluble proteins are considered as already digested proteins, explaining the higher values of *in vitro* digestibility found by Coelho and Sgarbieri (1995*b*) and other authors (Sathe *et al.*, 1981).

Table 5. Sulfhydryl (SH) and disulfide (SS) contents of native and heated protein fractions

	SH (μmol/g protein)	SS (μmol/g protein)
PIL fraction		
Native	6.6 ± 0.1	22.5 ± 0.1
Heated 99°C/30 min	1.9 ± 0.0	24.9 ± 0.1
121°C/15 min	0.6 ± 0.1	25.5 ± 0.1
Albumins (II)		
Native	6.6 ± 0.1	24.8 ± 0.3
Heated 121°C/15 min	0.2 ± 0.0	28.0 ± 0.3
Glutelins		
Native	n.d.	n.d.

n.d. not detected. Determinations were made in triplicate.

Sulfhydryl and disulfide contents

As previously reported (Genovese and Lajolo, 1996*a*), heat treatment of total albumins caused the formation of new disulfide bonds. This was related to their lower digestibility, as a more compact structure would impair protease access to labile peptide bonds. To determine whether this could also be associated to the decrease of digestibility observed for the PIL fraction, sulfhydryl and disulfide contents were determined before and after heating. Results are shown in Table 5. As can be seen, the PIL fraction and Albumins (II) showed a high content of disulfide groups, probably associated with the presence of trypsin inhibitors. In glutelins, contrarily, neither sulfhydryl nor disulfide were detected. Coelho and Sgarbieri (1995*b*), on the other hand, were not able to detect any half-cystine in the PIL fraction, although it concentrated trypsin inhibitors, which have 12–14 mol of half-cystine per mol of protein (Wu and Whitaker, 1990). An increase of disulfide bonds was shown to be caused by heating (Table 5), both for the PIL fraction and the Albumins (II). For the PIL fraction this seems to corroborate the role of disulfide bonds on the higher hydrolysis resistance. The decrease in sorghum protein digestibility observed after cooking has also been attributed to disulfide bond formation (Hamaker *et al.*, 1987; Oria *et al.*, 1995). In the case of Albumins (II), as a very high content of carbohydrates is present (Table 1) and the digestibility of this fraction when native is already extremely low (Table 3), this effect was not so evident.

SDS–PAGE profiles

SDS–PAGE profiles of Albumins (I) and of the PIL fraction are shown in Fig. 2. As can be observed, Albumins (I) are highly contaminated with phaseolin (band of approximately 50 kDa), as previously mentioned. The PIL fraction showed a profile very similar to the previously published for total albumins (Genovese and Lajolo, 1996*a*). SDS–PAGE of native (Fig. 2) and heated (Fig. 3) PIL fraction and Albumins (I) subjected to pepsin–pancreatin digestion showed that the profile of hydrolysis products changed upon heating. In native samples, both Albumins (I) and the PIL fraction, enzymatic hydrolysis caused minor changes in SDS–PAGE profiles, due to the compact structure of proteins resistant to the action of proteases. Only the partial or total disappearence of minor bands was observed. On the other hand, in heated samples, the hydrolysis products showed a significant alteration, with a predominance of lower molecular weight peptides (< 20 kDa) when compared to native samples. In Albumins (I) there was a significant decrease of the band corresponding to contaminating phaseolin after the action of pepsin. In the PIL fraction, peptides in the 32–34 kDa region (probably lectins), resistant to hydrolysis in the native fraction, were completely digested after heating. Although from these profiles it could be afforded that after denaturation there is an increase of digestibility, heating was shown to promote aggregation, observed by the presence of proteins that were not able to enter the stacking gel when samples for electrophoresis were prepared in the absence of β-mercaptoethanol (not shown). This fact had already been observed for total albumins (Genovese and Lajolo, 1996*a*) and confirms the role of disulfide bonds on hydrolysis resistance. SDS–PAGE profiles also shown that after the action of pepsin, little changes are caused by the action of pancreatin, either in native or in heated samples. This was shown to occur due to heat stable trypsin inhibitors present in the albumins (Deshpande and Nielsen, 1987*c*; Genovese and Lajolo, 1996*b*), as pancreatin is mainly composed of trypsin and chymotrypsin. This fact is of concern as it may not represent the real condition of bean consumption, when it seems to occur a complete inactivation of trypsin inhibitors (Rayas-Duarte *et al.*, 1992; Weder and Link, 1993).

CONCLUSIONS

The results from the present work confirmed the role of carbohydrates and disulfide bonds on the low susceptibility to proteolysis of bean proteins. Also, residual trypsin inhibitory activity interferes with pepsin–pancreatin digestibility of heat denatured protein fractions and must be taken into account when evaluating the results. Determination of *in vitro* digestibility of bean proteins must include, along with a blank for the enzymatic preparation used, a blank for the proteins studied, as naturally acid-soluble proteins represent an important fraction in beans and otherwise would be considered as already digested proteins. As they concentrate trypsin inhibitors and highly glycosylated proteins, naturally acid-soluble proteins are supposed to be only slowly or not at all digested.

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