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Isolation and *in vitro* hydrolysis of lentil protein fractions by trypsin

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SUMMARY. The albumin and globulin fractions from lentil seeds were isolated and characterised by gel filtration. The latter was shown to be homogeneous and the former heterogeneous on PAGE. the aminoacid analysis revealed high values of amidic amino acids for both fractions with great differences in the sulphur-containing amino acids. Native albumin, globulin and salt-soluble proteins were markedly resistant to trypsin hydrolysis compared to casein. The SDS-PAGE of native salt-soluble proteins indicated that the globulin fragments (20 to 30 kD) were slowly digested in the presence of albumin. The heating increased the hydrolysis of the proteins in the order: salt-soluble, albumin and globulin. The facilitated hydrolysis of the heated salt-soluble fraction seemed to be due to protein-protein interactions induced by heat.

RESUMEN. Aislamiento e hidrólisis «*in vitro*» de las fracciones proteínicas de la lenteja por la tripsina. Las fracciones proteínicas albumina y globulina de lentejas fueron aisladas y caracterizadas por cromatografía en gel de filtración. Las globulinas mostraron ser homogéneas y las albúminas heterogéneas en electroforesis en gel de poliacrilamida. El análisis de aminoácido reveló alto contenido de amino ácidos amídicos para las dos fracciones con diferencias pronunciadas en los sulfurados. La albúmina, globulina y las proteínas solubles en la sal, en la forma nativa, se mostraron resistentes a hidrólisis con tripsina, cuando fueron comparadas con la caseína. La PAGE-SDS de las proteínas solubles en sal, en la forma nativa, indicaron que fragmentos de la globulina (20 a 30 kD) eran menos digeridas en presencia de albuminas. El calentamiento acarreó un aumento de la hidrólisis en las proteínas del orden: solubles en la sal, albumina y globulina. La hidrólisis facilitada de las proteínas solubles en la sal pareció suceder debido a las posibles interacciones proteína-proteína en el calentamiento.

INTRODUCTION

Legume seeds are an important source of proteins for human consumption in developing countries. The qualitative and quantitative composition of the protein is one of the basic factors in the selection of plants for nutritive value, and since globulins are the major proteins in legumes there have been many studies about their characterization in many species (1). Different factors appear to contribute to the poor nutritive value of legume proteins such as the presence of antinutrients, amino acid composition and digestibility (2,3,4). Although there is considerable information about legume proteins in the literature, most of these studies have been concentrated on a limited number of species such as beans and soybean.

Lentil seeds are a valuable source of protein, their protein content being 19.5 to 35.5 %; the studies on lentil seeds are mostly related to overall composition, amino acid composition and nutritional quality of total protein, while little information appears to be available regarding to isolation, fractionation, electrophoretic analysis, subunit composition as well as enzymatic susceptibility of the lentil protein fractions. The observed low digestibility of the legume proteins results from different factors (2,4,5,6), whereas the enzymatic susceptibility of the protein fractions appears to be important, since protein associations, a common fact in legume protein systems, also affect proteolysis. A considerable variation has been reported for lentil protein digestibility in the literature (3); however, contrary to other species, nothing is known about the role of lentil protein fractions.

The purpose of this investigation was to isolate and characterize by gel filtration and electrophoresis the lentil protein fractions albumin and globulin and to determine their in vitro hydrolysis by trypsin.

MATERIAL AND METHODS

Material: Lentil seeds (*Lens culinaris* Medik) of a commercial Chilean type were soaked in distilled water at 4 °C for 6h. and dehulled manually, and the cotyledons were dried in an oven at 40 °C with forced air. The dried material was ground to a meal until it passed through a 100 mesh sieve and defatted in n-hexane at room temperature.

Bovine serum albumin, egg white albumin, soy trypsin inhibitor, carbonic anhydrase, cytochrome C, 2,4,6-trinitrobenzenesulphonic acid (TNBS), acrylamide, bis-acrylamide, bovine trypsin, 2-mercaptoethanol, and TEMED were purchased from Sigma Chemical Co. St. Louis, MO. All other chemicals were reagent grade.

Methods

1. Protein fractions: The albumin and total globulin fractions were obtained by sequential extraction as described by Sathe and Salunkhe (7) with deionized water and NaCl. The meal solvent ratio was 1:10, the NaCl concentration 0.55 M, and centrifugation was carried out at 10000 rpm/40 min. After separation, the water-soluble albumin and salt-soluble globulin fractions were lyophilized. The salt-soluble proteins used in the hydrolysis experiments were extracted

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with 0.05 M KPi buffer, pH 7.8, containing 0.5 M NaCl. Nitrogen content was determined by the microkjeldhal method (8). The 6.25 conversion factor was used for conversion of nitrogen to protein.

2. In vitro hydrolysis: Casein (Hammarsten), albumin, globulin and salt-soluble lentil proteins were solubilized in 0.05 M KPi buffer, pH 7.8 (1-2 mg). Aliquots containing 0.5-1.0 mg of the proteins were incubated individually in a small test tube and trypsin was added to give a 1:10 (w/w) enzyme protein ratio in a total volume of 0.7 ml. The tubes were sealed with parafilm and incubated at 37 °C for up to 60 min. At various incubation time tubes were removed from the bath, the reaction was stopped by adding distilled deionized water up to a volume of 7.0 ml and the tubes were stored in an ice bath. Protein solutions were also heated at 121 °C/15 min. and hydrolyzed as cited above for native proteins. The extent of hydrolysis was determined by the increase of free amino groups using TNBS according to Fields (9) as modified by Romero and Ryan (10). To aliquots of the diluted digests (unheated and heated) we added 0.5 ml of 0,1 M $Na_2B_4O_7$, pH 9.5 to make a final volume of 1.0 ml and 20 µl of a freshly prepared solution of TNBS (1:1 M in water) and the mixture was incubated for 30 min. at room temperature. The reaction was stopped by the addition of 2.0 ml of 0.1 M NaH_2PO_4 containing 1.5 mM Na_2SO_3 and the absorbance measured at 420 nm against a buffer solution containing all the reagents. Appropriate blanks containing enzyme and substrate only were prepared simultaneously. The absorbances for the no-enzyme and enzyme-only control were subtracted from the values of the appropriate samples. The percent of peptide bond hydrolysis was the ratio of the number of new amino groups in the digestion mixtures to the number of peptide bonds in the proteins. This number of new amino groups was calculated using 22000 as the molar extinction coefficient for TNP-amino groups. The total number of peptide bonds was obtained by dividing grams of proteins in the digests by 113, the average MW of the amino acids. Means of triplicate determinations are reported. Samples were digested separately and submited to SDS-PAGE using 12.5 % acrylamide according to Laemmli (11). In these cases the reaction was stopped by adding 0.7 ml sample buffer (0.025 TRIS-glycine, 1.0 % SDS, 20 % glycerol, 2.0 % 2-mercaptoethanol and 0.01 % bromophenol blue), immediately heating the tubes at 99 °CV for 3 min and frozen until the gels were run.

3. Trypsin inhibitor activity: Benzoyl-DL-arginine-p-nitroanilide (BAPA) was used as a substrate for determination of the trypsin inhibitor activity according to Kakade et al (12).

4. Extraction-determination of tannins: The tannic substances were extracted-reextracted (2X) from an integral and decorticated meal using methanol/ 1 % HCl for 1 h in a bottle protected from light. After centrifugation (5000 rpm/ 30 min) the tannins in the supernatant were determined by the vanillin-HCl method as modified by Deshpande and Cheryan (13) using (-) catechin as a standard.

5. Gel chromatography: The salt-soluble extract and the isolated albumin and globulin fractions were solubilized in 50 mM KPi-buffer, pH 7.8 with 0.5 M NaCl and applied separately to a column packed with Sephadex G-200 resin (2.5 x 100 cm), equilibrated with the same buffer. Fractions of 3.5 ml were collected with a FRAC-100 fraction collector and the protein was monitored. Apparent MWs were estimated by the method of Whitaker (14) and the MW standards used were: ferritin (480 kD), lactate dehydrogenase (130 kD), egg white albumin (43 kD), soy trypsin inhibitor (21.5 kD) and cytochrome C (12.4 kD).

6. PAGE/SDS-PAGE: Polyacrylamide gel electrophoresis of the proteins was performed on 7.5 % gel using the method of Davis (15). SDS-PAGE was carried out according to Laemmli (11); monomer concentration was 12.5 % bromophenol blue was used a as a front marker and the protein visualized on gels by Comassie Blue. The MW markers employed were: bovine serum albumin (67 kD), egg white albumin (43 kD), carbonic anhydrase (29 kD), soy trypsin inhibitor (21.5 kD) and cytochrome C (12..4 kD).

7. Protein determination: Protein was determined by the method of Lowry et al (16) using bovine serum albumin as a protein standard. Absorbance at 280nm was used to monitor protein in column eluates.

8. Amino acid analysis: Amino acid analysis was performed using an amino acid analyzer after hydrolysis of the samples at 110 °C for 24h in 6 N HCl. Half cystine residues were determined after performic acid oxidation, and tryptophan after alkaline hydrolysis.

RESULTS AND DISCUSSION

The protein content of the lentil meal was 27.8%, a value similar to that large number of cultivated lentil genotypes grown in other countries (3). The fractionation procedure solubilized 75.7% of the meal nitrogen as a salt soluble compound, the globulin and albumin fractions corresponded to 42% and 11% of the meal nitrogen and the remaining 22.7% diffused out during the dialysis process. These values were higher than that reported for common Chilean type lentil and lower than that for Laird lentil (17). Variation in these fractions has been observed within and among legume species and appears to be influenced by the procedures adopted in each case. Table 1 shows the amino acid composition of albumin and the major globulin from lentils; both fractions had a high values of amidic amino acids, with albumin presenting a higher content of Lys, Thr, Gly, and Ala than globulin; however the greatest differences were observed in the sulphur amino acids. The higher values of Met in albumin were practically identical to data from another report (18). Sulphur amino acids were the limiting in globulin, and Trp was in albumin. The uniformity observed in various lentils species has been sugested by Bhatty (17) to be related to a relative homogeneity of the genus Lens. Nutritionally, albumin appears to be the best fraction, although more information is needed.

On gel chromatography in Sephadex G-200 (Figure 1) the lentil globulins were resolved into 2 fractions with Ve/Vo of 1.25 and 3.23 respectively. The major fraction (I) with a calculated MW of 340 kD was found to be fairly homogeneous by PAGE analysis, revealing only one band stained. The second peak containing 30X less protein than the former, calculated by the method of Lowry et al (16), corresponded to a MW of less than to 12.4 kD; the quantitative contribution of this fraction to total globulin seemed to be nonsignificant. The albumins showed the presence of 3 peaks based on absorbance at 280 nm. The first of them eluted with the void volume of the column and probably consists of aggregated protein material since it did not appear on Sepharose CL-4B chromatography (data not shown), and failed to enter the gel on PAGE. The others two, with a Ve/Vo of 1.75 and 2.47, corresponded to a calculated MW of 160 kD and 20 kD, respectively (Figure 2). The PAGE of these

fractions was not homogeneous, with a large number of electrophoretic components and appearing to indicate considerable overlapping between fractions.

TABLE 1
Amino acid composition of lentil protein fractions (g/100 g of protein)

Amino Acid	Protein Fraction	
	Albumin	Major Globulin
Asp	11,77	13,78
Thr	5,50	2,36
Ser	4,88	5,67
Glu	18,30	21,26
Pro	3,90	3,68
Gly	5,17	3,08
Ala	6,45	3,20
1/2 Cys	1,26	nd
Val	5,39	4,98
Met	2,27	0,16
Ile	3,97	2,68
Leu	6,31	9,85
Tyr	4,40	3,41
Phe	4,15	7,19
His	3,10	2,17
Lys	9,63	7,69
Arg	8,73	10,29
Trp	1,12	0,35

nd= not detected

FIGURE 1
Gel filtration chromatography of total globulins from lentil on Sephadex G-200. Apparent MWs of the fractions I and II estimated by the method off Whitaker (14).

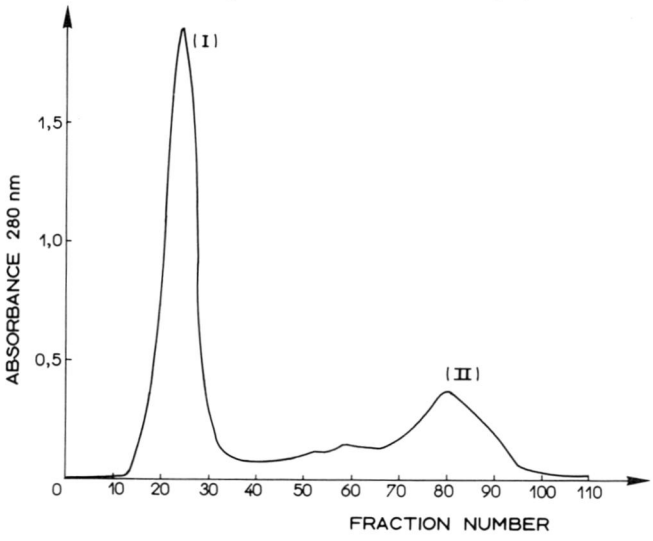
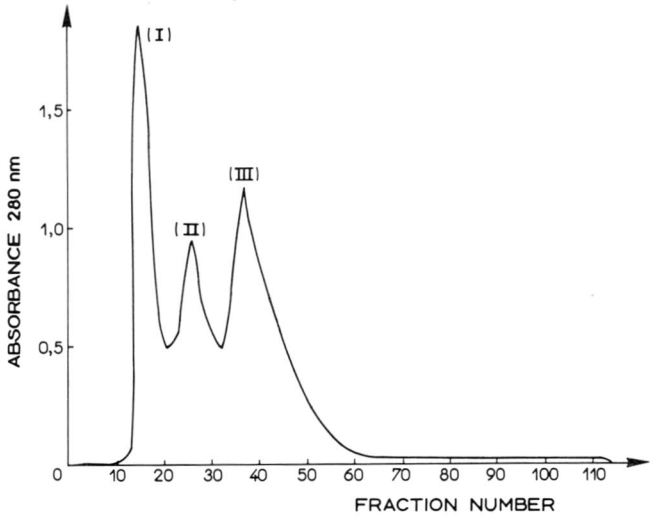


FIGURE 2
Gel filtration chromatography of albumin proteins from lentil on Sephadex G-200 Apparent MWs of the fractions I, II and III estimated by the method of Whitaker (14).



The observed homogeneity of the globulin fraction appears to be a characteristic of stored legume proteins SDS-PAGE of the total albumin and globulin produced 23 and 12 components, respectively (Figure 3A-1 and 3B-1).

FIGURE 3A
SDS-PAGE patterns of native albuumins subjected to trypsin hydrolysis (1) protein bands of native albumin under dissociation conditions with 2- mercaptoethanol (control); (2 to 9) protein bands of native albumin after trypsin hydrolysis for: 15s, 30 s, 1, 5, 15, 30, 60 and 120 min. respectively

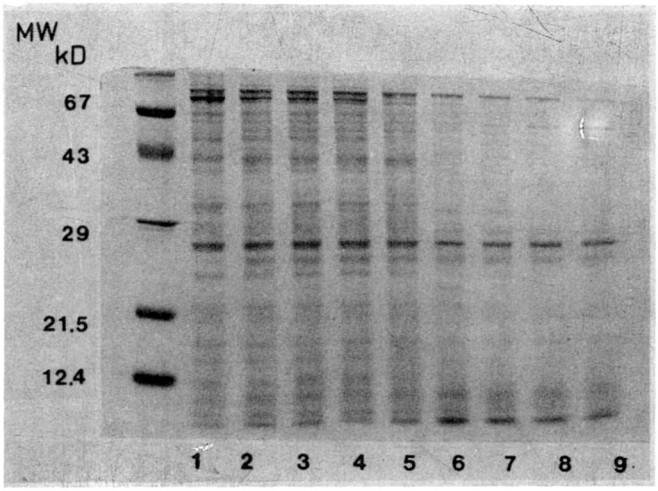


FIGURE 3B
SDS-PAGE patterns of native total globulin subjected to trypsin hydrolysis (1) protein bands of native globulin under dissociation conditions with 2-mercaptoethanol (control); (2 to 9) protein bands of native globulin after trypsin hydrolysis for 15 s, 30 s, 1, 5, 15, 30, 60, and 120 min respectively.

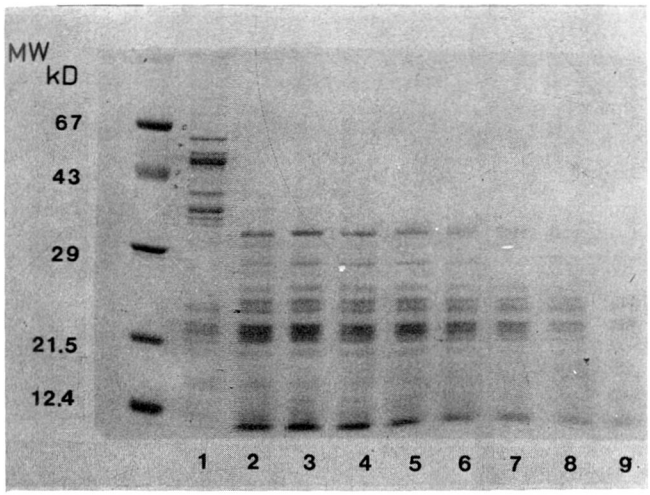


FIGURE 3C
SDS-PAGE patterns of native salt-soluble proteins subjected to trypsin hydrolysis (1) protein bands of native salt-soluble proteins under dissociation conditions with 2-mercaptoethanol (control), (2 to 9) protein bands of native sal-soluble proteins after trysin hydrolysis during 15 s, 30 s, 1, 5, 15, 30, 60 and 120 min, respectively.

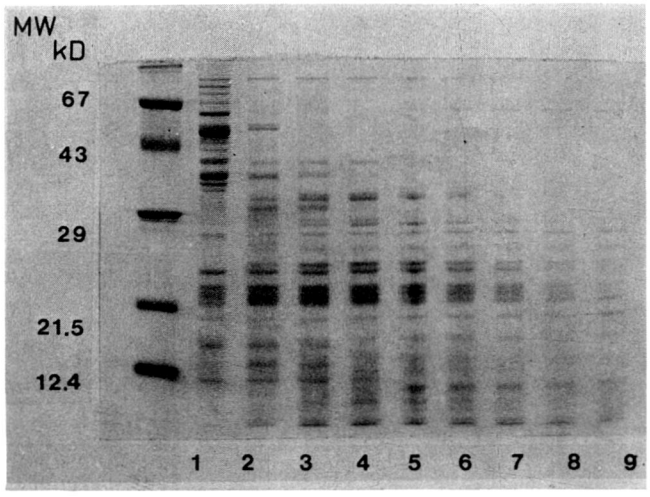


Table 2 shows the results of the in vitro hydrolysis of the lentil proteins by trypsin All of the native fractions were more resistant than casein. The hydrolysis of a salt-soluble extract containing a mixture of native albumin and globulin fractions presentd a value of 53 % in relation to casein (100 %). The isolated native fractions showed that albumiin was markedly resistant compared to globulin, with 38 % and 63 % of casein hydrolysis, respectively. The effect of heat treatment (121 °C/15 min) of the isolated fractions on hydrolysis was more marked for albumin than for globulin, however, in each case the heating was not sufficient to supplant the casein. Hydrolysis of the heated salt-sluble extract was higher than observed for the separate fractions and similar to casein.

TABLE 2
Effect of heating on the trypsin hydrolysis of the lentil protein fractions

Protein	% Total peptide bond hydrolysis[a] unheated	heated[b]
Casein (control)	12,62 ± 0,14	12,53 ± 0,04
Albumin	4,80 ± 0,08	10,25 ± 0,18
Globulin	7,88 ± 0,06	9,61 ± 0,12
Salt-soluble extract	6,69 ± 0,04	12,26 ± 0,04

a Proteins were digested during 60 min
b Heated at 121 °C/15 min

SDS-PAGE of the proteins during hydrolysis showed that the higher MW fragments of albumin were progressively hydrolyzed after 60 min, while the lower MW ones persisted (Figure 3-A). The native globuline showed a fast decrease in the components near 60 kD and 40 kD during the hydrolysis and these events were followed by an increased intensity of the bands near 30 kD and between 20 and 30 kD but some of these fragments were still present after 60 min (Figure 3-B). The native salt-soluble proteins were digested more slowly than globulin, the breakdown products in the MW range of 20-30 kD were less hydrolyzed in the salt-extract even after 2hr. (Figure 3-C). The results appear to indicate that the presence of albumin in the salt-extract (3.5:10; globulin to albumin) showed globulin hydrolysis since others factors such as trypsin inhibitors were present at lower concentrations (Table 3) and the meal was decorticated and consequently tannin free (Table 4). This observation was confirmed by the results of tryptic hydrolysis of a native protein isolate with a low content of albumins (data not shown). The electrophoretic pattern of the heated proteins showed that some polypeptides failed to enter the gel, indicating that some sort of aggregation took place during heating. However, the salt-soluble proteins appeared to be completely hydrolyzed by trypsin after one hour, presenting only fragments with a faint color (data not shown). The better digestiblity of the heated salt-soluble extract suggests that possible protein-protein and protein-other component interactions facilitated hydrolysis.

TABLE 3
Effect of heating (121 °C/15 min) on the trypsin inhibitor activity in lentil protein fractions

Fraction	Specific Inhibition (UIT/ mg protein)[a] unheated	heated[b]
Salt-soluble extract	15,06	8,65
Globulin	30,50	0,0
Albumin	80,00	42,30

a UIT/mg protein - trypsin inhibitor units as defined by Kakade et al (13)
b Heated at 121, a °C/15 min

TABLE 4
Effect of heating (121 °C/15 min) on the tannin content of lentil meals

LENTIL MEAL Fraction	TANNIN CONCENTRATION (mg/ EC/100 g)	% tannin reduction
INTEGRAL:		
unheated	383,90 ± 2,10	—
heated	318,60 ± 0,80	17
DECORTICATED:		
unheated	36,20 ± 0,28	—
heated	0,0	100

EC catechin equivalents

Several reports in the literature have also demostrated an increase in the hydrolysis of the globulin fraction from various leguminous after heating. However, the same has not always been observed for the albumin fraction. Deshpande and Nielsen (4) observed more susceptiblity to trypsin hydrolysis for heated salt-soluble proteins compared to water-soluble proteins in 11 dry beans varieties with an E.S. ratio fo 1:10. Sathe et al (19) reported that native albumin was more resistant than the globulin fractions of *Phaseolus vulgaris*, however, after heating both of them increased their susceptibility with a higher percent increase for albumin, as also observed for the lentil fractions studied here. Marques and Lajolo (20) observed higher tryptic hydrolysis for the native albumin compared to globulin in *Phaseolus vulgaris*, with heating decreasing the former and increasing the latter. The in vitro digestibility of the lentil proteins reported in the literature using different methodologies is in the range of 60 to 70 % (21), nevertheless little is known about the hydrolysis of the protein fractions and its contribution to the considerable range of values observed. The only results reported by Bhatty (3) are contrary to ours, in which the native lentil albumin was hydrolyzed faster than globulin in the presence of trypsin. However, this author used different conditions of E:S ratio, time reaction and peptide bond hydrolysis. These conflicting data in the literature may be related to the characteristics of each assay as suggested by Nielsen (6). Furthermore, dry bean proteins have been studied more extensively, while little is known about other leguminous species. In this respect the results presentd here give additional insights into the study of in vitro hydrolysis of lentil protein fractions.

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