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Changes in protein fractions, trypsin inhibitor and proteolytic activity in the cotyledons of germinating chickpea

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SUMMARY. The chickpea seed germination was carried out in 6 days. During the period it was observed a little variation on total nitrogen contents, however the non protein nitrogen was double. A decrease of 19.1 and 20.6% in relation to total nitrogen was observed to the total globulin and albumin fractions, respectively. The gel filtration chromatography on Sepharose CL-6B and SDS-PAGE demonstrated alterations on the distribution patterns of the albumin and total globulin fractions between the initial and the sixth day of germination, suggesting the occurrence of protein degradation in the germination process.The assay for acid protease only appeared in the albumin fraction with casein and chickpea total globulin as substrates, whereas the former was more degradated than the latter, however the transformations detected in the protein fractions apppear indicated that others enzymes could be acting during the process. The trypsin inhibitor activity had a little drop after six day of germination indicating a possible increase on the digestibility of the proteins.
Key words: Chickpea, *Cicer arietinum* L., protein fractions, germination.

RESUMEN. Variación de las fracciones proteínicas, inhibidor de tripsina y actividad proteolítica durante la germinación de las semillas de garbanzo. La germinación de las semillas de garbanzo fue realizada durante 6 días. En ese período fue observada una pequeña variación en el contenido de nitrógeno total, sin embargo el contenido de nitrógeno no proteico duplicó. Una disminución de 19,1 y 20,6% en relación al nitrógeno total fue observada para las fracciones de globulina total y albúmina, respectivamente. La cromatografía de filtración en gel de Sepharose CL-6B y PAGE-SDS demostraron alteraciones en el perfíl de distribución de las fracciones albúmina y globulina total entre el inicio y final de la germinación, sugiriendo que ocurrió degradación de proteina durante el proceso de germinación. La actividad de la enzima proteasa ácida solamente fue detectada en la fracción albúmina utilizándose caseína y globulina total como substrato de la reacción, siendo la primera mas degradada que la última, entretanto las transformaciones detectadas en las fracciones proteicas parecen indicar que otras enzimas podrían estar actuando durante ese proceso. La actividad del inhibidor de tripsina presentó pequeña alteración despues de 6 días de germinación indicando posiblemente un aumento en la digestibilidad de las proteínas.
Palabras clave: Garbanzo, *Cicer arietinum* L., fraccion protéica, germinación.

INTRODUCTION

Legumes constitute an important protein source in the human diet for large segments of the world's population, but this protein is of poor value unless subjected to heat treatments (1-4). The digestibility of legume proteins is relatively low due to the presence of antiphysiological factors and structural characterisitics of the storage proteins (1,2,5). Although the benefical effect of heat treatment has been attributed to protease inhibitor destruction, others evidences suggest that the compact structure of storage proteins could represent a resistance to mammalian digestive enzymes (3, 6-8), a factor contributing to the poor nutritive values of some legumes seeds. The albumin protein are minor and the globulin protein are major proteins from legume seeds. Unlike the latter, which are storage proteins, the former are mostly enzymic or non-storage proteins, however the albumins were degraded during germination like the globulins (9). The protein characteristics and its mobilization during the seed germination could be a fact well explored for some species (10-16) indicating a slow hydrolysis whose mechanism of degradation control are still uncertain. Some species behaviour alteration in the principals polimeric constituents, as carbohidrates and proteins, during germination with direct consequences for nutritional value (4, 14-19). The proteins are degraded due to increased activities of endoproteases during germination of the seed (11,13). The aim of this work was to study the variations caused by germination seeds of *Cicer arietinum* L., var. IAC-Marrocos, in the nitrogen constituents, protein fractions, trypsin inhibitor activity and the proteolytic activities.

MATERIALS AND METHODS

Material

Chickpea (*Cicer arietinum* L.), cv IAC-Marrocos, were purchased from Instituto Agronômico de Campinas, Campinas, São Paulo, Brazil. Chickpea seeds were washed with water, soaked in distilled water and the seeds immersed for one minute in a solution containing 0.001% of Benlate (methyl-1-butyl carbamoyl-2- benzimidazol carbamate). After this time the solution was drained and the seeds involved in an appropriate paper (germitest paper) previously treated with Benlat solution. Germination was carried out in a chamber with excess of humidity at the temperature of 16-18°C in darkness. Only distilled water was sprayed daily during germination period. Cotyledons of 0, 2, 4 and 6 day of germinated seeds were taken up for protein studies. At the differents germination periods (0, 2, 4, 6 days) seeds were taken and the sprouting separated. The seeds were manually dehuled, the air dried cotyledons and seed coats were ground, separately, to pass through a 60 mesh sieve and the cotyledon flours deffated in n-hexane (1:8), filtered and dried at room temperature. Deffated flours obtained were utilized for total, protein, non-protein nitrogen determination and for isolation of the different protein fractions.

Methods

Nitrogen determination

The total nitrogen and protein nitrogen (TCA-precipitable) were estimated by the micro Kjeldhal method (20). The non-protein N was extracted from the flour with TCA (trichloroacetic acid) 10%, homogenized in a magnetic stirrer for 1 h at room temperature and centrifuged (15000 g/40 min). The residue was reextracted (2 X), the supernatants were combined and the N determined as cited above.

Protein fractionation

Albumins and total globulins were successively extracted from deffated chickpea flour at each day germinated (1:20 flour to solvent ratio) with deionized water and 0.5 M NaCl solution as described by Sathe and Salunkhe (21). The albumins and total globulins were ressuspended in distilled water and lyophilized. The lyophilized protein fractions from the ungerminated and germinated seeds were determined (triplicate) by its nitrogen contents.

Gel chromatography

Aliquots (40-60 mg) of the albumins and total globulins from ungerminated and germinated seeds were solubilized in 5 mM potassium phosphate buffer, pH 7.5 with 0.5 M NaCl and applied, separately, to a column packed with Sepharose CL-6B resin (2.5x100 cm), equilibrated with the same buffer. Fractions of 5.5 ml were collected with a FRAC-100 fraction collector and the protein was monitored. The Vo (void volume) of the column was determined by the elution of Blue dextran 2000.

Protein determination.

Protein concentration of the various solutions were determined by the method of Lowry *et al.* (22), using bovine serum albumin as a standard. On extraction procedures were utilized the nitrogen contents multiplied by the factor 6.25. Absorbance at 280 nm was also used to monitor protein in the column eluates.

Polyacrylamide gel electrophoresis.

The SDS-PAGE were performed by the method of Laemmli (23) with monomer concentration of 12.5%, bromophenol blue was used as a front marker and the proteins visualized on gels by Comassie Blue. The MW markers employed were: cytochrome C (12,4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhidrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (67 kDa). Relative mobility was calculate relative to the migration of the bromophenol marker dye.

Protease activity

The presence of proteases in the protein extracts and the isolated fractions, germinated and ungerminated were verified by the measure of the hydrolysis grade utilizing casein at 1% as substrate. The proteolitic activity was determined utilizing as enzyme source the salt soluble extracts (NaCl 0.5 M soluble proteins) and albumin fractions for ungerminated and germinated seeds. Aliquots in triplicate were prepared for: solution of enzyme:casein, casein whitout enzyme and only enzyme; followed by incubation of the sealed tubes with parafilm in a water bath at 37°C in a buffer (potassium phosphate-citrate, pH 5.5) mixture. The reactions were initiated by the enzyme addition (NaCl soluble extract and albumin) and interrupted at different times of incubation (0, 30, 60, 90, 120 e 240 min). The tubes were removed to the bath, diluted with cold distilled water (10x) and utilized for amino nitrogen determination. The extent of hydrolysis was determined by the increase of free amino groups using 2,4,6-trinitrobenzenesulphonic acid (TNBS) according to the method of Fields (24) as modified by Spadaro *et al.* (25). The percent of peptide bond hydrolysis was calculated from the changes in the ratio of new amino groups in the digestion to the total number of peptide bonds in the mixture. The molar extinction coefficient for TNP-α-amino groups of 16500 M^{-1} cm^{-1} and an average weight of 113 g/mol for aminoacid residues in protein were used for calculation. All hydrolysis assays were performed in triplicate on 1:10 and 1:5 enzyme:substrate ratio with casein and isolated total globulin as substrates.

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.* (5). All determinations were performed in triplicate.

RESULTS AND DISCUSSION

When the seeds were sprayed with 0.01% of sodium azide, small colonies of molds could be discerned around the hilum area on the seed coat after 5 days of germination. The use of Benlat allowed the seeds to germinate until 8 days, however microbial colonies appeared after 9 to 12 days of germination. Germination of Chickpea seeds were uniform under experimental conditions during 6 days. The results of Table 1 indicated 5.61% variation in the total nitrogen during germination. The non-protein nitrogen, 10% TCA non precipitable, increases about 120.96% after 6 days of germination and in relation to total nitrogen, however this represented a decrease of only 2.02% from the protein nitrogen.

TABLE 1

Changes in chickpea (vc Marrocos) nitrogen fractions *during germination*

Time (days)	Nitrogen (mg/g flour)[a] Total	Non protein	Protein[b]
0	36.88 ± 0.39	2.29 ± 0.15	34.59
2	37.52 ± 0.88	3.81 ± 0.16	33.71
4	37.92 ± 0.87	4.40 ± 0.17	33.52
6	38.95 ± 0.73	5.06 ± 0.16	33.89

[a] N expressed as mg/g decorticated flour, [b]expressed as the difference between total N_2 and non-protein N_2. Mean values ± standard deviations of three replicates.

In relation to the results in this paper, a progressive decrease in protein nitrogen was observed during 72 h germination of *Cow pea*, *Chick pea* and *Green gram* (26). Khaleque *et al* (27) and Khalil & Mansour (4) observed an increase in the protein content after 4 and 3 days germination to chickpea and faba beans, respectively; contrary to observed by Ganesh Kumar & Venkataraman (28). The total nitrogen remained constant in 96 h germination of *Cicer arietinum* and *Phaseolus aureus* seeds with a decrease in protein nitrogen (29).

The Table 2 shows that globulins and albumin were 19.13 and 20.77% degraded after 6 days of germination, however the salt-soluble proteins remained whitout variation. These values for albumin and globulin turn into relatively small values when we only consider the protein nitrogen. The dialysable nitrogen was increased in 66.86% until 6 days of the experiment and this values quantitatively represented 28.26% of the sum of albumin and globulin fractions at the initial time and 58.59% in the 6 days. Considering a litlle error inerent to each analysis the differences corresponded to the sum of albumin and globulin fractions degraded (27.41%). This observation indicates that these protein fractions were reduced to fragments lower than 10 kDa, as confirmed by the chromatographic and electrophoretic studies.

TABLE 2

Composition of chickpea protein fractions during germination.

Protein fraction	Nitrogen (%) 0 days	6 days	Total Protein* (N x 6.25). 0 days*	6 days*
Flour	3.69	3.90	23.05	24.34
Salt-soluble	2.86	3.00	77.55	77.16
Albumins	0.54	0.45	14.59	11.56
Globulins	1.69	1.44	45.85	37.08
Dialysable[a]	0.63	1.11	17.08	28.50
Insoluble[b]	0.83	0.89	22.50	22.85

*(0) (6) days germinated. [a]Difference between salt-solubles proteins and the sum of albumin and globulin.Equivalent to difference between total N and salt-soluble N.

The results presented here suggest that the degradation of albumin during chickpea germination could represent a storage function as the globulins, a fact observed with others species (9,13). Results in the literature and those presented here show a degradation of seed storage proteins during germination with a strong variation interspecies and intervarieties (11-15,18,19,26).

Chromatographic studies

On fractionation on Sepharose CL-6B gel column the albumin fraction of ungerminated chickpea flour (Figure 1) gave 4 fractions with the first eluted near the void volume (Vo) of the column and the others with Ve/Vo values of 2.20, 2.39 and 2.90, respectively. The elution behaviour and the distribution of the fractions from albumin were modified during germination. The fraction IV showed a spread distribution (Ve/Vo), and this may indicates that have been improved from fractions of greater molecular weight (II and III) due to degradation. The Figure 2 shows the SDS-PAGE of salt-soluble proteins and albumin. The albumin fraction from ungerminated seeds shows about 19 bands with distinct colour intensities and presenting a tick and diffuse band with a molecular weight between 12 to 20 kDa. After 6 days all bands were reduced except a majoritary one between 20-30 kDa and a diffuse band below to 20 kDa that increased its intensity. All transformations appeared to have processed after 6 days germination, indicating that the little fragments below to 12 kDa and no detected on PAGE could be resulted of these degradations.

FIGURE 1
Gel filtration chromatography on Sepharose CL-6B of albumin protein from chickpea. 47.5 mg of protein were applied to thc column and fractions of 5.5 ml were collected. A to D are: 0, 2, 4 and 6 days germination, respectively

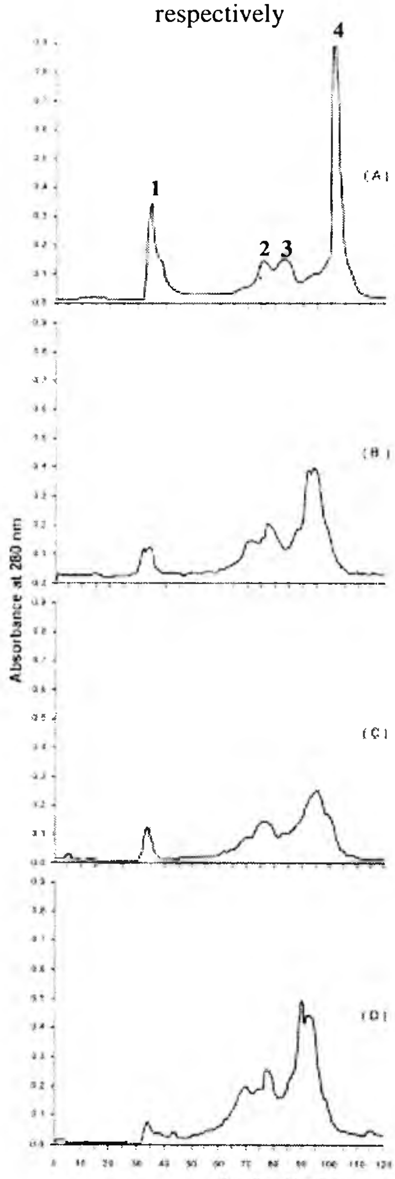
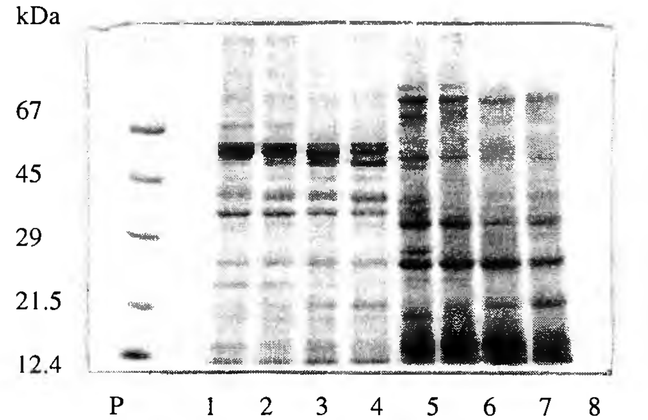


FIGURE 2
SDS-PAGE patterns of salt-soluble protein (1-4) and albumin (5-8) from chickpea during germination at 0, 2, 4 and 6 days respectively. P – standard proteins as described in methods



The elution pattern of total globulins on Sepharose CL-6B column chromatography (Figure 3) shows four fractions with the first one eluting next to the void volume (Vo) and the others with Ve/Vo of 1.81, 2.25 and 2.90, respectively. The first fraction shown very turbid but it wasn't protein material as confirmed by PAGE and protein determination by Lowry method (22). In the sixth day this fraction had an abrupt increase as compared to the absorbance values for ungerminated one. The fraction III disappeared after two days germination while the fraction IV was duplicated in the same period, this could indicate that the latter has been enriched by the former degradation. The elution volume of the fraction II (Ve/Vo = 1.81) appeared indicates a very little molecular weight modification, confirmed on SDS-PAGE, while the fraction IV was increased quantitatively, probably resulting of the enrichment by fragments from greater fractions.

The Figure 4 shows the SDS-PAGE from salt-soluble and total globulins. Accord to Table 2 the globulins corresponded to 59.12% and 48.06% of the salt soluble proteins in the initial and 6 days of seed germination. The PAGE indicates that the peptides between 45 to 66 kDa have little alteration on its relative mobility behaviour, however the bands intensities were reduced while new peptides arose in the 20-30 kDa interval and below to 20 kDa. Ahmed *et al.* (10) observed a reduction in the total protein bands number on PAGE of chickpea germinated by 7 days. G. Kumar & Venkataraman (28) results revealed a maximum degradation of the chickpea proteins in the 6° day of germination, the authors verified alterations on the elution behaviour of the proteins on gel filtration indicating degradation as a result of germination, the same was confirmed on SDS-PAGE with a decrease in the larger bands and the appearance of smaller ones.

FIGURE 3
Gel filtration chromatography on Sepharose CL-6B of total globulin protein from chickpea. 44.5 mg of protein were applied to the column and fractions of 5.5 ml were collected. A to D are: 0, 2, 4 and 6 days germination, respectively

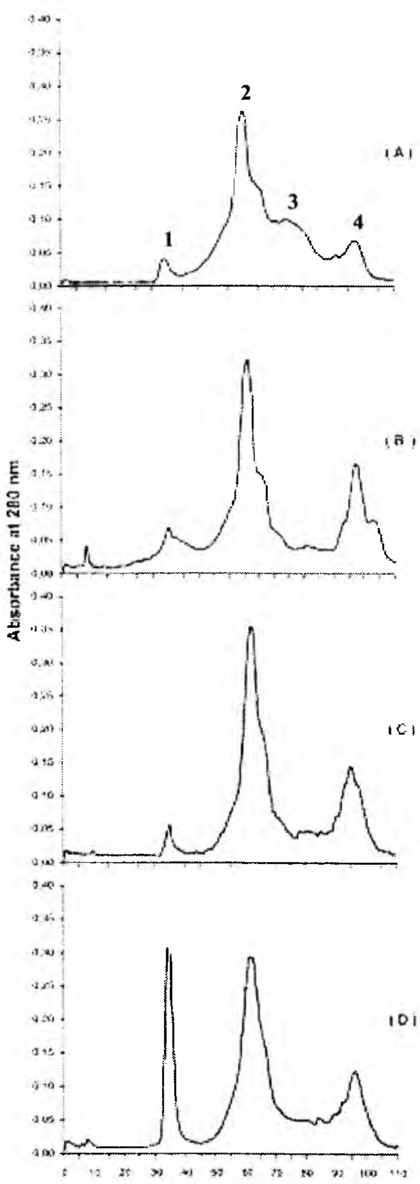
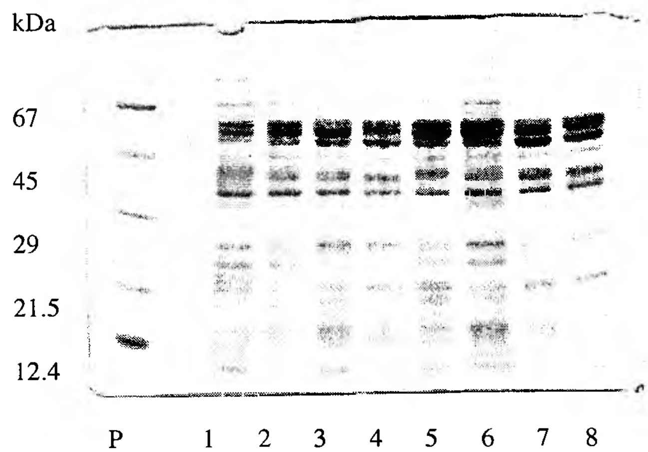


FIGURE 4
SDS-PAGE patterns of salt-soluble protein (1-4) and total globulin (5-8) from chickpea during germination at 0, 2, 4 and 6 days respectively. P – standard proteins as described in methods



The Table 3 shows the variation on trypsin inhibitor activity during seed germination. The results indicated a little change on inhibitor activity with a maximum in the fourth and a decrease until the sixth day of germination. This behaviour appeared to be followed by the increase on acid protease activity measured with casein and total globulin as substrates. No acid protease activity occured during 6 days germination utilizing casein as substrate and chickpea salt-soluble extract as a source of enzyme in an enzyme:substrate ratio of 1:10 (data not shown), however there was an enzymatic system in the albumin fraction that hydrolyses casein and total globulin molecules into aminoacids and peptides.

TABLE 3
Trypsin inhibitor activity in chickpea flours during germination

Germination (days)	UIT/mg flour	UIT/mg protein[a]	UIT reduction (%)
0	17.35	92.62	-
2	14.05	77.82	16.0
4	11.94	69.80	24.6
6	12.14	71.49	22.8

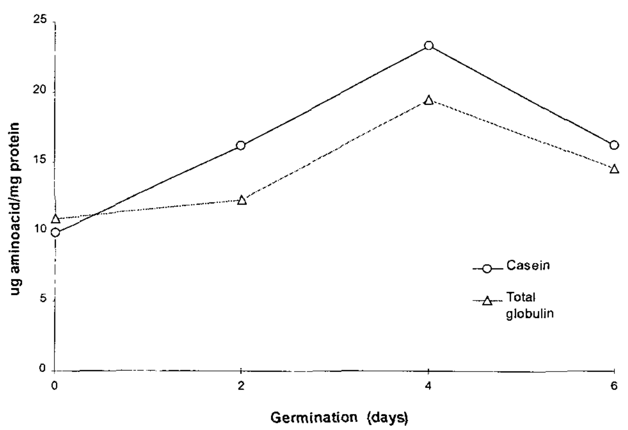
[a]UIT/mg protein- trypsin inhibitor units as defined by Kakade *et al.*(5).

The Figure 5 show the acid protease activity from albumins during germination with the substrates in a 1:5

enzyme:substrate ratio. The proteolytic activity increase with germination until 4° day for both substrates and decrease to the 6°, however the enzymatic activity was higher to casein than total globulin. Considering the albumin to globulin ratio on chickpea seeds (3:5) the proteolytic activity could be higher than obtained in vitro. The reserve behaviour of the globulins and their degradation during germination togheter with the increase in the proteolytic activity is consistent with various reports (10,11,13,17). Protein degradation in the protein bodies of lentil associated to an increase in a caseinolytic system were observed after 7 days of seed germination (11).

FIGURE 5

Proteolytic activity during chickpea germination. Enzyme source: albumin fraction from chickpea. Substrates: casein and chickpea total globulin. The assays were performed in triplicate as described in methods



The decrease in the total and storage proteins were associated with two different sets of enzymes during lentil seed germination (13). Nielsen & Liener (17) also observed a different protease activity during Phaseolus vulgaris seed germination when associated with trypsin like activity or azocasein activity.

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