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Purification and biochemical characterization of tepary bean (*Phaseolus acutifolius*) major globulin†

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Tepary bean (*Phaseolus acutifolius* var. *lactifolius*) major storage globulin (>90% purity by electrophoresis) was composed of three glycosylated polypeptides with estimated molecular weights of 49 500, 45 890, and 44 510. The protein had an absorption maximum at 278 nm and an $A^{1\%}_{280\ nm}$ of 6·61 in 20 mM Tris-HCl pH 8·1 containing 0·5 M NaCl. The carbohydrate content of the protein was 8·23% (w/w). The hydrodynamic radius (Stokes' radius) of the native protein was 53·3 Å. Amino acid composition of the globulin indicated that S-containing amino acids were the first limiting amino acids. Acidic, basic, uncharged polar, and hydrophobic amino acids accounted for 28·75, 12·42, 21·94, and 36·91% (by weight), respectively of the total protein. The native globulin was resistant to in-vitro proteolysis. Heat denaturation (100°C, 30 min) of the globulin in aqueous 0·5 M NaCl did not improve the in-vitro proteolysis of the globulin. Heat denaturation (100°C, 30 min) of the globulin in the digestion buffer in the absence of NaCl facilitated complete in-vitro proteolysis of the globulin by chymotrypsin, trypsin, and pepsin.

INTRODUCTION

Dry beans are an important source of proteins, carbohydrates, dietary fiber, minerals, and vitamins. Among dry beans, the *Phaseolus* beans are cultivated and consumed world-wide in a variety of food preparations. The global acceptance of *Phaseolus* beans is partly due to the wide variety in these beans as well as their ability to grow in a wide range of environmental conditions. Within the *Phaseolus* beans several varieties remain underutilized primarily because of lack of detailed information on their growing characteristics as well as limited knowledge of their nutritional and functional properties. One such *Phaseolus* bean is the tepary bean (*Phaseolus acutifolius*).

Tepary bean is an indigenous legume of the arid and semiarid regions and is believed to be of southwestern United States and southern Mexico origin (Freeman, 1912; Kaplan, 1956). Native Americans consumed these beans as early as 5000 years ago; however, large scale production and utilization has not yet been achieved (Nabhan & Felger, 1978). These beans are drought- and disease-resistant (Coyne & Schuster, 1973; Thomas *et al.*, 1983) and can provide as much as 2020 kg seed/ha. The seed yield can be more than 4630 kg/ha in water-supplemented fields (Nabhan & Felger, 1978).

Typically tepary beans contain 2–5% moisture, 15–32% protein, 1% fat, 6–7% acid detergent fiber, 65–70% carbohydrates, 4–5% ash, 18–21% total dietary fiber (0·5–1% soluble fiber and 17–20% insoluble fiber); and their mineral and vitamin contents are comparable to other common beans (Nabhan & Felger, 1978; Scheerens *et al.*, 1983; Taggart *et al.*, 1983; Nabhan *et al.*, 1985; Idouraine *et al.*, 1991*a*). Tepary bean proteins have been recently investigated for protein fractionation (Idouraine *et al.*, 1993), lectin activity (DeMejia *et al.*, 1990), nutritive value (Idouraine *et al.*, 1992*a*), antinutritional factors (Idouraine *et al.*, 1992*b*), and functional properties (Idouraine *et al.*, 1991*b*). Based on literature data and our investigations, tepary bean proteins are dominated by proteins soluble in aqueous media. Among these, the globulins account for up to 50% of the total proteins. Consequently, the globulins significantly influence the functional properties (including nutritional quality) of tepary bean proteins. To date, however, the major globulin of tepary beans has not been biochemically characterized.

The purpose of this investigation was to purify and biochemically characterize major globulins in tepary beans.

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MATERIALS AND METHODS

Materials

Tepary beans (*Phaseolus acutifolius* var. *lactifolius*) were from the University of Arizona, Tucson, Arizona. Sources of electrophoresis chemicals and protein molecular weight (MW) standards were the same as reported earlier (Sathe, 1993). Proteinases TPCK-trypsin, TLCK-chymotrypsin, and pepsin were from Sigma Chemical Company, St. Louis, MO; DEAE DE 53 was from Whatman, Hillsboro, OR; Sephacryl S300 HR and gel filtration calibration standards were from Pharmacia, Inc., Piscataway, NJ. All other chemicals were from either Sigma Chemical Company, St. Louis, MO or Fisher Scientific Company, Orlando, FL.

Methods

Preparation of flour

Tepary beans were ground in a hammer mill to pass through a 40 mesh screen. The flour was stored in an air-tight container at 4°C until further use.

Purification of the major globulin

Tepary bean major globulin was prepared by the method of Hall *et al.* (1977). Typically, 100 g of flour was extracted with 0·5 M NaCl containing 0·025 M HCl (flour-to-solvent ratio 1 : 10 (w/v)) at room temperature (25°C) for 2 h with constant magnetic stirring. The slurry was centrifuged (15 000 × *g*, 4°C, 20 min) and the supernatant was filtered through a Whatman filter paper to remove any floating debris. To the filtrate was then added five volumes of cold (4°C) distilled deionized water to precipitate the globulin. The precipitate was collected after centrifuging (15 000 × *g*, 4°C, 20 min) the diluted filtrate. The precipitate was dissolved in a minimum amount of 0·5 M NaCl and further subjected to cold precipitation and centrifugation as above three more times. The final precipitate was dissolved in 0·5 M NaCl and dialyzed against distilled deionized water at 4°C for 24 h (6 changes, 5 litres each) and lyophilized. The lyophilized globulin was stored at −20°C in an air-tight container until further use.

Analytical methods

Soluble protein content was determined by the method of Lowry *et al.* (1951).

Total carbohydrate content (expressed as glucose equivalents) of protein sample was determined by the phenol–sulfuric acid method (Dubois *et al.*, 1956). Glycoprotein staining was done using periodic acid-Schiffs stain (Dubray & Bezard, 1982).

Stokes' radius was measured using a 1·6 cm × 93·5 cm Sephacryl S300 HR column calibrated with standard proteins of known Stokes' radii according to the method of Siegel and Monty (1966).

The ultraviolet absorption spectrum for the globulin was recorded using a Perkin Elmer λ_3 UV/VIS spectrophotometer. Protein was dissolved in 20 mM Tris-HCl pH 8·1 buffer containing 0·5 M NaCl.

Trypsin inhibitory activity and the phytate content of the globulin were determined as reported earlier (Idouraine *et al.*, 1992*b*).

Amino acid composition was determined using a dedicated Applied Biosystems Model 410A Amino Acid Analyzer with automatic hydrolysis (vapor phase, 160°C for 1 h 40 min using 6 N HCl) and precolumn phenylthiocarbamyl-amino acid (PTC-AA) analysis. Tryptophan content was determined by the calorimetric method of Opieńska-Blauth *et al.* (1963) as described by Yensen and Weber (1987).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the method of Fling and Gregerson (1986) as described by Sathe (1991).

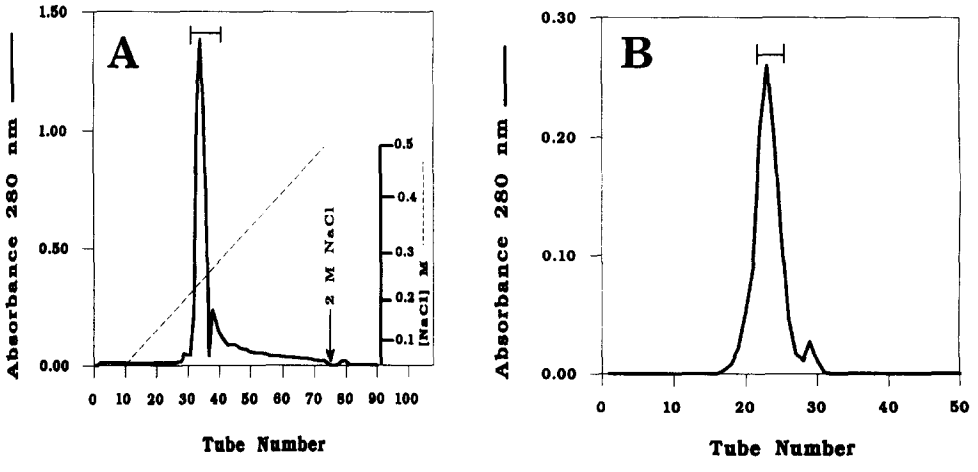

Fig. 1. Elution profiles of tepary globulin off: (A) DEAE DE 53 (2·6 cm × 21·2 cm) column. Equilibrium buffer was 20 mM Tris-HCl pH 8·1 and elution buffer was equilibrium buffer containing 0–0·5 M NaCl linear gradient (500 ml each) followed by 2 M NaCl. Fractions were collected every 15 min and the column flow rate was 61·5 ml/h. Horizontal bar (tubes 32–45) indicates fractions pooled (containing the globulin). Globulin (approximately 200 mg) in 22 ml of equilibrium buffer was loaded onto the column. (B) Sephacryl S300 HR (1·6 cm × 93·5 cm) column. Equilibrium and elution buffer was 20 mM Tris-HCl pH 8·1 containing 0·1 M NaCl and 1 mM NaN_3. Fractions were collected every 15 min. The column flow rate was 19·3 ml/h. Horizontal bar (tubes 21–26) indicates fractions pooled (containing the globulin). Ten mg of globulin in 1 ml equilibrium buffer was loaded onto the column.

Protein in-vitro digestibility was assessed using TPCK-trypsin, TLCK-chymotrypsin, and pepsin. Substrate protein was dissolved in either 0·5 M NaCl (5·0 mg/ml) or directly in the digestion buffer. For heat denaturation, globulin was heated for 30 min (boiling water bath) in 0·5 M NaCl alone or in the digestion buffer. For the globulin dissolved in 0·5 M NaCl, the final digestion conditions were as follows: globulin concentration of 2 mg/ml, 0·2 M NaCl, globulin-to-enzyme ratio 100 : 1 or 10 : 1 (w/w), 0·1 M buffer (Tris-HCl pH 8·1 for trypsin and chymotrypsin and HCl for pepsin), 37°C, and appropriate digestion time. For the globulin dissolved in buffer, the final digestion conditions were as described by Deshpande and Nielsen (1987*a*): globulin concentration of 2 mg/ml, buffer concentration of 0·05 M (Tris-HCl pH 8·1 for trypsin and chymotrypsin and HCl for pepsin), 0·02 M $CaCl_2$ (for trypsin and chymotrypsin digestions only), 37°C, and 30 min. For these digestions, the globulin was heat-denatured in the appropriate buffer and the enzymes were dissolved in the appropriate digestion buffer. For the first set (globulin dissolved in 0·5 M NaCl) of digestions, the enzymes were dissolved in 0·001 M HCl. Digestion was initiated by adding the enzyme. At the end of the digestion period, an equal volume of SDS-PAGE sample buffer (0·05 M Tris-HCl, pH 6·8, 1% SDS, 0·01% bromophenol blue, 30% glycerol, and 2% β-mercaptoethanol (β-ME) was added to the digestion mixture and the samples immediately heated for 5 min in a boiling water bath to stop enzyme action. Appropriate protein and enzyme controls were run simultaneously.

All experiments were done at least in duplicate and averages were reported.

RESULTS AND DISCUSSION

Molecular properties

Typically, we obtained 3 g of globulin from 100 g of the bean flour. The globulin was >90% pure by SDS-PAGE. The globulin was homogeneous because it eluted as a single major peak off the anion exchange DEAE DE 53 (Fig. 1(A)) and gel filtration (Fig. 1(B)) columns. It is a trimeric protein composed of at least 3 polypeptides with estimated MWs (mean ± s.e.m.) 49 550 ± 980; 45 890 ± 310, and 44 510 ± 1290. Based on the banding pattern of these polypeptides on SDS-PAGE, the major globulin in tepary beans appears to be the 'S' type in the phaseolin classification suggested by Brown *et al.* (1981). The MW range of the subunit polypeptides is consistent with the reported MW range of 43 000–54 000 for phaseolins (Osborn, 1988). The typical ultraviolet absorption spectrum of the globulin (Fig. 2) indicated maximal absorption at 278 nm. The $A^{1\%}_{280\ nm}$ in 20 mM Tris-HCl pH 8·1 containing 0·5 M NaCl was 6·61 ± 0·04 and was similar to the reported value of 7·0 for common bean phaseolin (Deshpande & Damodaran, 1989*a*). The carbohydrate content (%) of the globulin, 8·23 ± 1·05 (n = 4), is comparable to the reported carbohydrate content ranges of 5·55–13·76% for *P. vulgaris* (Deshpande & Nielsen, 1987*b*), 1·2–5·5% for *P. vulgaris* (Wright, 1987), and 11·7% for carioca bean globulin (Marquez & Lajolo, 1981). Glycoprotein staining of the globulin after the SDS-PAGE showed that all of the polypeptides were glycosylated (data not shown), which is consistent with the literature (Osborn, 1988). The globulin had a trypsin inhibitory activity of 7·88 ± 0·12 units/mg protein which was similar to the trypsin inhibitory activity of the 0·5 M NaCl soluble protein fraction of tepary flour (9·54 units/mg protein) reported earlier (Idouraine *et al.*, 1992*b*). Unlike the small red-bean (*Phaseolus vulgaris*) phaseolin, (Suzuki *et al.*, 1983) we could not detect any phytic acid in our globulin preparations. The Stokes' radius of the tepary globulin was 53·3 ± 1·9 Å (n = 6) which was similar to the Stokes' radii for several phaseolins (Deshpande, S. S. & Sathe, S. K. 1987, unpublished). From the plot of K_{av} *versus* $\log_{10}$MW (gel filtration data) we determined the MW of tepary globulin to be 231 031 ± 23 898 (n = 6), which is similar to the value of 240 000 for phaseolin determined using the Sepharose CL-6B gel filtration method of Blagrove *et al.* (1984). From the polypeptide composition and the MWs of subunit polypeptides one would expect the MW of the native tepary globulin to be 139 950. The discrepancy in MW determinations by gel filtration and SDS-PAGE is quite large but consistent with the results of Blagrove and coworkers. Our results of MW determination by gel filtration are internally consistent because the protein with a Stokes' radius closest to tepary globulin is catalase (52·2 Å), whose MW is 232 000. We do not exclude the possible non-specific weak interactions of the protein with the gel matrix which may influence the MW value determined using gel filtration. However, we had included 0·1 M NaCl in the gel filtration buffer (20 mM Tris-HCl pH 8·1 containing 1 mM NaN_3) to minimize such interactions. These data are, however, consistent with the assertion of Siegel and Monty (1966) that 'The behavior of each of a series of proteins during chromatography on columns of Sephadex G-

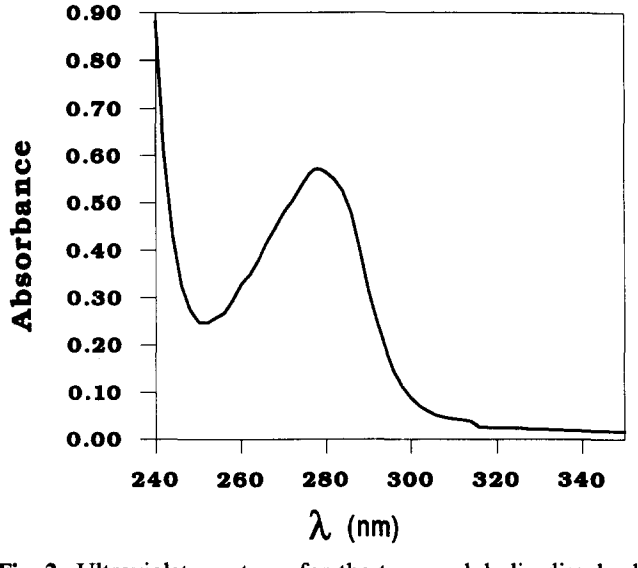

Fig. 2. Ultraviolet spectrum for the tepary globulin dissolved in 20 mM Tris-HCl pH 8·1 containing 0·5 M NaCl. Protein concentration was 0·854 mg/ml.

Table 1. Amino acid composition of tepary globulin: Comparison with phaseolin[a]

Amino acid	Tepary globulin	Phaseolin		
		1[b]	2[c]	3[d]
Asx	13·81 ± 0·63	12·4	13·2	12·1*
Thr	2·74 ± 0·09	3·4	7·1	3·3
Ser	7·92 ± 0·11	6·7	8·3	9·3
Glx	14·94 ± 0·13	15·1	16·0	14·1**
Gly	7·90 ± 0·10	2·7	4·5	5·5
Ala	5·61 ± 0·20	3·0	4·4	5·9
Cys/2	0·04 ± 0·00	0·3	0·2	0·0
Val	5·68 ± 0·50	5·2	5·8	6·4
Met	0·98 ± 0·06	0·7	0·5	1·2
Ile	4·61 ± 0·31	5·6	5·2	5·9
Leu	8·72 ± 0·43	9·1	9·1	10·9
Tyr	3·36 ± 0·10	3·5	3·6	3·1
Phe	5·49 ± 0·18	6·6	6·6	5·9
Lys	5·25 ± 0·37	5·6	5·8	5·7
His	1·94 ± 0·08	2·6	1·5	2·4
Arg	5·23 ± 0·18	5·0	5·2	4·5
Pro	4·33 ± 0·16	2·9	3·4	3·6
Trp	1·49 ± 0·01	0·8	***	0·2

[a] Data are expressed as g amino acid/100 g protein. Data for tepary globulin are mean ± s.e.m. for three determinations.
[b] From Derbyshire *et al.* (1976).
[c] From Johnson *et al.* (1982).
[d] From Doyle *et al.* (1986).
* Asn (7·1) + Asp (5·0).
** Gln (5·5) + Glu (8·6).
*** Not reported.

200 may be correlated with the Stokes' radius of the protein, but does not correlate with molecular weight.'

The amino acid composition of tepary globulin (Table 1) was comparable to that of phaseolin. The acidic, basic, uncharged polar, and the hydrophobic residues were 28·75, 12·42, 21·94, and 36·91%, respectively by weight. Compared to the Food and Agriculture Organization (FAO) of the United Nations Committee on Protein Requirements recommendations, S-containing amino acids and threonine were the first and second limiting amino acids, respectively. Threonine content of the globulin (2·72 g/100 g) was very close to the FAO recommendation of 2·8 g/100 g protein. Tepary globulin, with the exception of S-containing amino acids, is therefore a good source of food protein. Since legumes are often consumed with cereals rich in S-containing amino acids, the deficiency of sulfur amino acids should not be considered as a serious limitation (Deshpande, 1992).

In-Vitro digestibility

When the tepary globulin was digested (proteinase-to-globulin ratio of 1 : 100 (w/w), 30 min digestion time) in a buffer system containing 0·2 M NaCl (final concentration) with trypsin, chymotrypsin, and pepsin, it was evident that the native protein was resistant to proteolysis (Fig. 3). This resistance of the tepary globulin to proteolysis is consistent with the findings of several investigators reporting the resistance of bean globulins to proteolysis (Romero & Ryan, 1978; Marquez & Lajolo, 1981; Scheerens *et al.* 1983; Bradbear & Boulter, 1984; Deshpande & Nielsen, 1987*a*; Nielsen, 1988; Santoro *et al.* 1988; Deshpande & Damodaran, 1989*b*; Idouraine *et al.* 1992*a*). The native globulin was degraded by trypsin to two major polypeptides with estimated MWs of 21 880 and 22 390, which remained resistant to further proteolysis, and a few minor polypeptides in the MW range 4 680–20 510. Chymotrypsin did not cause major hydrolysis of the native globulin under the same digestion conditions. Pepsin, on the other hand, pro-teolyzed the native globulin to five major polypeptides with the estimated MWs of 38 910, 33 880, 28 840, 22 390, and 12 020. Increasing the ratio of the proteinase to the native globulin to 1 : 10 (w/w) did not alter the proteolysis pattern by these proteinases (data not shown). The tepary globulin heat denaturation did not facilitate improved proteolysis by trypsin, chymotrypsin, or the pepsin. This was unexpected since the majority of phaseolins are completely proteolyzed within 30 min by these proteinases (Deshpande & Nielsen, 1987*a*; Nielsen *et al.*, 1988). We also note that unlike the resistance of native phaseolins to pepsin digestion (Deshpande & Nielsen, 1987*a*) the native tepary globulin was hydrolyzed by pepsin under the experimental conditions used in this investigation. The SDS-PAGE patterns for the tepary globulin, native or heat-denatured, digestions by a particular proteinase were similar, suggesting that the tepary globulin was quite resistant to the proteinases even after heat-denaturation of the globulin. The time course of tepary globulin digestion with trypsin (Fig. 4)

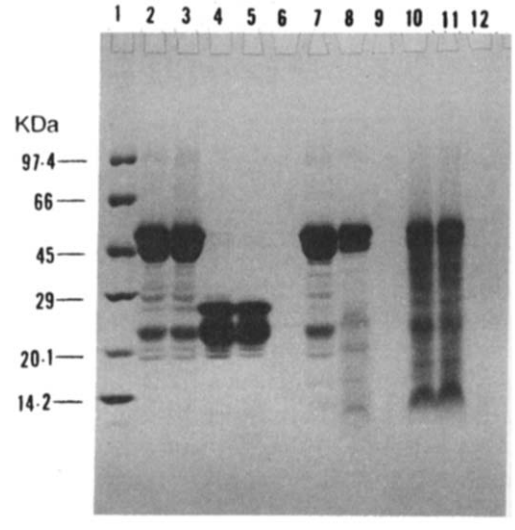

Fig. 3. SDS-PAGE for tepary globulin digested with different proteinases. For digestion details, refer to materials and methods section. This is an 8–25% linear acrylamide gradient gel (1·5 mm thick). Lane 1, low molecular weight markers (MWs indicated in the margins as kDa). Lanes 2 and 3, native and heat-denatured globulin controls respectively. Lanes 4, 7, and 10, native globulin digested with TPCK-trypsin, TLCK-chymotrypsin, and pepsin, respectively. Lanes 5, 8, and 11, heat-denatured globulin digested with TPCK-trypsin, TLCK-chymotrypsin, and pepsin, respectively. Lanes 6, 9, and 12 TPCK-trypsin, TLCK-chymotrypsin, and pepsin enzyme controls, respectively. Tepary globulin load in each lane was 100 μg and that for the enzyme controls was 1·0 μg. Proteinase to globulin ratio was 1 : 100 (w/w) and the digestion time was 30 min.

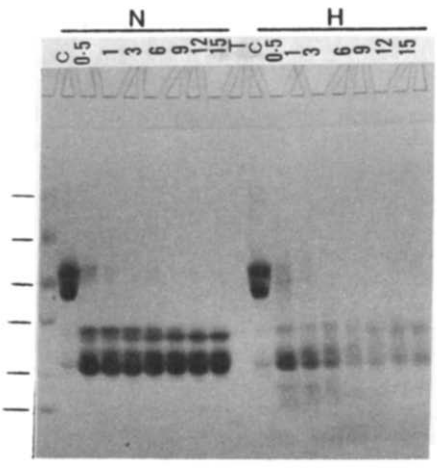

Fig. 4. SDS-PAGE for TPCK-trypsin digestion of the native (N) and heat denatured (H) tepary globulin. Protein to enzyme ratio was 100 : 1 (w/w). This is an 8–25% linear acrylamide gradient gel (1·5 mm thick). Digestion time (min) is indicated on top of each lane. C = globulin control, T = TPCK-trypsin control. The MWs of standard proteins (leftmost lane) are the same as in Fig. 3. The protein load was 30 μg and that for the enzyme was 0·3 μg.

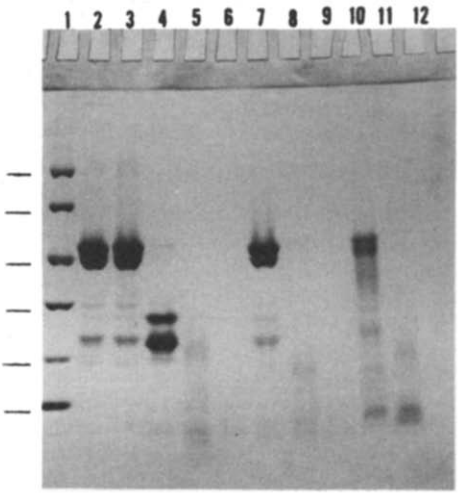

Fig. 5. SDS-PAGE for tepary globulin digestion according to Deshpande and Nielsen (1987*b*). This is an 8–25% linear acrylamide gradient gel (1·5 mm thick). Lane 1, low molecular weight markers (same as in Fig. 3). Lanes 2 and 3, tepary globulin native and heat denatured controls respectively, (in 0·05 M Tris-HCl pH 8·1 buffer containing 0·02 M $CaCl_2$). Lanes 4, 7, and 10, native globulin digested with TPCK-trypsin. TLCK-chymotrypsin, and pepsin, respectively. Lanes 5, 8, and 11, heat denatured globulin digested with TPCK-trypsin, TLCK-chymotrypsin, and pepsin, respectively. Lanes 6, 9, and 12, TPCK-trypsin, TLCK-chymotrypsin, and pepsin enzyme controls, respectively. Tepary globulin load in each lane was 50 μg and that for the enzyme controls was 0·5 μg. Proteinase to globulin ratio was 1 : 100 (w/w) and the digestion time was 30 min.

indicated that the native or heat-denatured globulin was degraded to two sets of polypeptides (30 550, 28 180 and 22 390, 21 880) within the first 0·5 min followed by rapid generation of several smaller MW fragments within 3 min (MWs 4680 to 20510). The major polypeptides generated by trypsin digestion of native globulin remained resistant to further proteolysis. When the globulin was heat-denatured, the polypeptides initially generated although proteolyzed to some extent, were not completely degraded.

In view of the published literature, the proteinase digestion results seemed contradictory. We were therefore interested to find whether these contradictory results were due to digestion conditions used in the experiments or some true differences between tepary globulin and other phaseolins. We therefore evaluated the in-vitro proteolysis of the tepary globulin (native as well as heat-denatured) under the experimental conditions described by Deshpande and Nielsen (1987*a*). As can be seen (Fig. 5), under the digestion conditions described by Deshpande and Nielsen, heat-denaturation of tepary globulin did facilitate complete proteolysis of the globulin within 30 min by all of the proteinases tested, while the native globulin was resistant to these proteinases. These results are consistent with those of Deshpande and Nielsen (1987*a*) for several phaseolins. Improvement in proteolysis due to heat-denaturation is most likely due to quaternary and tertiary structural changes in the globulin, since it is known that the major globulin in *Phaseolus* beans has very little α-helix content (Blagrove *et al.*, 1984; Deshpande & Damodaran, 1989*b*, 1990). The phaseolin denaturation (protein unfolding) at pH 8·0 has been recently shown to start at 50°C (Dyer *et al.* 1992).

The differences observed in susceptibility of tepary globulin to proteinases, especially after heat-denaturation, under two different digestion conditions are quite significant. In the first system the globulin was heat-denatured in 0·5 M NaCl alone, without any buffer, while in the second set heat denaturation was in the presence of a buffer (0·05 M Tris-HCl pH 8·1 containing 0·02 M $CaCl_2$ for trypsin and chymotrypsin and 0·05 M HCl for pepsin). Since NaCl can indirectly promote hydrophobic interactions, it may offer some protection against protein denaturation. Calcium chloride is known to destabilise protein structure and therefore may facilitate protein denaturation and thereby improving susceptibility to proteinases trypsin and chymotrypsin. Calcium chloride is also known to protect trypsin and chymotrypsin against autolysis, which may help these proteinases to remain active for longer periods, thus improving proteolysis of the tepary globulin. In the case of pepsin digestions the primary differences between the two systems is the presence of NaCl (0·2 M in the final digestion mixture) in one set and heat denaturation of the globulin in the presence of 0·05 M HCl in the second set. It is possible that heating the globulin in 0·05 M HCl may cause some protein hydrolysis, prior to pepsin action, which may enhance the ability of pepsin to digest the globulin. It is therefore important to precisely define the in-vitro digestion conditions used in such experiments.

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