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Effects of sodium chloride, phytate and tannin on *in vitro* proteolysis of phaseolin[1]

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Native phaseolins isolated from the Great Northern bean (*Phaseolus vulgaris* L.) and the tepary bean (*Phaseolus acutifolius* L.) were resistant to TPCK-trypsin, TLCK-chymotrypsin, and pepsin proteolysis *in vitro*. Sodium chloride (1 M) significantly decreased the initial rate of *in vitro* proteolysis of both phaseolins. However, moist heat (30 min, 100°C) denaturation of the phaseolins facilitated complete *in vitro* proteolysis by all the proteinases tested. Added phytate (phaseolin:phytate ratios 10:1, 5:1, and 1:1; w/w) decreased the initial *in vitro* proteolysis rates of the Great Northern bean phaseolin (GNP) by chymotrypsin and pepsin (but not trypsin) and that of the tepary phaseolin (TP) by pepsin (TP:phytate ratios 5:1 and 1:1, w/w). Tannin addition decreased the initial rate of *in vitro* proteolysis of both phaseolins digested with chymotrypsin (phaseolin:-tannin ratios 100:1 and 50:1, w/w) and TP digested with pepsin (TP:tannin ratio of 10:1, w/w). This inhibitory effect of added phytate and tannin was lower than the inhibitory effect of 1 M sodium chloride. Since moist heat denaturation was able to overcome the inhibitory effect of sodium chloride, the inhibitory effects of added phytate and tannin should not impede *in vitro* phaseolin proteolysis.

INTRODUCTION

On a global basis, dry beans make an important contribution to the human diet (Salunkhe, 1982; Sathe *et al.*, 1984). Among dry beans, *Phaseolus* beans are cultivated and consumed in the greatest quantity on a worldwide basis. On an average, dry beans contain 15–30% protein on a dry weight basis (Sathe *et al.*, 1984). The dry bean protein digestibility is, however, lower than that of the animal proteins (Sathe *et al.*, 1984; Deshpande & Damodaran, 1990; Deshpande, 1992). This low protein digestibility partly accounts for the underutilization of dry beans as human food.

Over the years, several factors have been suggested to be at least partly responsible for the low digestibility of the dry bean proteins. These factors include presence of proteinase inhibitors; product inhibition; presence of lectins; deficiency of sulfur amino acids (notably methionine); compact structure of the major globulin (a glycoprotein), phaseolin; steric hindrance by the carbohydrate moiety of phaseolin; protein interactions with phytates, tannins, minerals, carbohydrates (starch); and the presence of water soluble proteins that are resistant to proteolysis. Phaseolin accounts for about 50% of the total protein in mature seeds (Osborn, 1988; Sathe & Deshpande, 1991). Phaseolin digestibility must, therefore, significantly affect the overall dry bean protein digestibility. Phaseolin proteolysis therefore has been intensely investigated, especially during the last 20 years (for recent reviews, please see Nielsen, 1988; Deshpande & Damodaran, 1990; Deshpande, 1992). These investigations often cite the resistance of native phaseolin to both *in vitro* and *in vivo* proteolysis is due to one or more possible mechanism(s) mentioned earlier. They also report significantly improved proteolysis when the phaseolin is subjected to moist heat.

Literature review also indicates that there are conflicting data for phaseolin digestibility (Romero & Ryan, 1978; Antunes & Sgarbieri, 1980; Marquez & Lajolo, 1981, 1983; Deshpande *et al.*, 1983; Deshpande, 1992) depending on the experimental conditions used. Sathe *et al.* (1984) had reported earlier that when 0·5 M NaCl was present in the digestion buffer, although moist heat (30 min, 100°C) improved the *in vitro* proteolysis of the Great Northern bean proteins, it did not facilitate complete proteolysis (in 30 min). More recently, Sathe *et al.* (1994) confirmed this inhibitory effect of added NaCl on the *in vitro* digestibility of tepary bean phaseolin.

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Since beans are often cooked prior to consumption, heat-labile factors, such as the trypsin and chymotrypsin inhibitors or lectins, are of little or no practical significance to human nutrition (Nielsen, 1988; Deshpande, 1992). We were interested, therefore, in learning how the *in vitro* digestibility of phaseolin is affected by the presence of NaCl, phytate, and tannin; the heat-stable factors most commonly encountered in cooked dry beans.

MATERIALS AND METHODS

Materials

The Great Northern beans (*Phaseolus vulgaris* L.) were purchased from a local supermarket. Tepary beans (*Phaseolus acutifolius* L. var. *lactifolius*) were provided kindly by Prof. C. W. Weber of the University of Arizona, Tucson, Arizona, USA. Sources of electrophoresis chemicals and protein molecular weight (MW) standards have been reported earlier (Sathe, 1993). Proteinases TPCK-trypsin, TLCK-chymotrypsin, pepsin, and phytate (dodecasodium salt, from corn) were from Sigma Chemical Company, St Louis, MO, USA. Tannin (MW 1701; C:53·7, H: 3·1, water ~10%; Control No. 1442) was from Nutritional Biochemical Corporation, Cleveland, OH, USA. Hammersten casein was from United States Biochemicals, Cleveland, OH, USA. All other chemicals were from either Sigma Chemical Company, St Louis, MO, USA or from Fisher Scientific Company, Orlando, FL, USA.

Methods

Preparation of bean flour and phaseolin purification
The beans were ground in a hammer mill to pass through a 40 mesh screen. The flour was stored in an air-tight container at −20°C until further use. The phaseolins were prepared by the method of Hall *et al.* (1977). Details of phaseolin preparation have been described earlier (Sathe *et al.*, 1994).

Enzyme assay
Proteinase activities were determined using Hammersten casein as the substrate. Final assay conditions were: Hammersten casein 2 mg ml^{-1}, buffer 0·05 M (Tris–HCl pH 8·1 containing 0·02 M $CaCl_2$ for trypsin and chymotrypsin and HCl for pepsin), substrate-to-enzyme ratio 100:1 (w/w), incubation temperature 37°C, incubation time 30 min, and assay volume 1·0 ml. When present, NaCl concentrations were 0·01, 0·025, 0·05, 0·075, 0·1, 0·2, 0·3, 0·4, 0·5, 1·0, 1·5, or 2·0 M. Assay was terminated by adding 0·2 ml of 60% (w/v) cold (4°C) trichloroacetic acid (TCA) solution. The samples were immediately cooled in an ice bath for 30 min, centrifuged for 5 min in a Beckman Microfuge E, and the absorbance of the supernatant measured at 280 nm (A280 nm). All assays were done in triplicate in 1·5 ml plastic microcentrifuge tubes and appropriate blanks were included simultaneously. Unit enzyme activity was defined as that enzyme activity which caused a change of 0·001 in A280 nm under the assay conditions.

Phaseolin digestions
Stock phaseolin solutions were prepared in appropriate solvent at 5 mg/ml. Typically, phaseolin was dissolved in the solvent (containing 1 mM NaN_3) at 25°C with constant magnetic stirring for at least 2 h followed by centrifugation (4°C, 12 000*g*, 10 min) and filtration (Whatman filter paper No. 4) to remove aggregates. Soluble protein content of the sample was determined by the method of Lowry *et al.* (1951). The stock protein solutions were stored at 4°C until further use (typically they were used within 5 days from the preparation day).

When required, phaseolins (distilled water as the solvent) were heat denatured at 100°C (boiling water bath) in the presence or absence of the appropriate additive for 30 min.

Final digestion conditions were: phaseolin 2 mg ml^{-1}; phaseolin:enzyme ratio of 100:1 (w/w); incubation temperature 37°C; incubation time, variable; amount of additive, variable; digestion volume 0·5 ml. For A280 nm measurements, assay was terminated by adding 0·7 ml of 8·57% (w/v) cold (4°C) TCA, immediately cooling the sample in an ice-bath (4°C) for 30 min, followed by centrifugation (Beckman Microfuge E, 5 min). The A280 nm of the supernatant was measured. For the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), an equal volume of the SDS–PAGE sample buffer [containing 2% β-mercaptoethanol (β-ME)] was added to the sample and the mixture was heated for 5 min in a boiling water bath (100°C). Samples were electrophoresed on the same day the digestions were done. All A280 nm assays and the SDS–PAGE assays were done in duplicate. Appropriate blanks and controls were included in all assays.

SDS–PAGE
The SDS–PAGE was done according to the method of Fling & Gregerson (1986) as described by Sathe (1991, 1993). Gels were typically 8–25% linear acrylamide gradient and 1·5 mm thick.

Statistics
Appropriate data were analyzed using linear regression and Fisher's LSD (protected test, $P = 0.05$) as described by Ott (1977).

RESULTS AND DISCUSSION

Phaseolin homegeneity

Phaseolins were prepared from the Great Northern and tepary beans because they represent, respectively, a

colorless and a colored variety. They were selected also because they represent some of the variety in the *Phaseolus* genus. The phaseolin in both these beans is, however, of the same electrophoretic type ('S' type in the classification suggested by Brown *et al.*, 1981). The major reason for choosing the 'S' type phaseolin was that it represents the dominant type variant (69%) of the reported 107 bean cultivars (Brown *et al.*, 1982). The phaseolins that we prepared were >90% pure based on the SDS–PAGE analysis (Fig. 1) and were homogenous by the Sephacryl S300 HR column chromatography (data not shown).

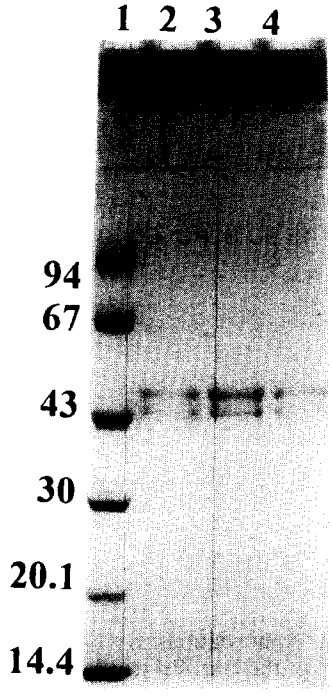

Fig. 1. The SDS–PAGE for the Great Northern bean phaseolin (GNP) and tepary bean phaseolin (TP). Lane 1, molecular weight (MW) markers (MWs indicated in the left-hand margin in kDa). Lane 2, TP (5 μg); Lane 3, TP (5 μg) + GNP (5 μg); Lane 4, GNP (5 μg).

Phaseolin digestibility

As expected, the native phaseolins were resistant to proteolysis by the trypsin, chymotrypsin, and pepsin (Table 1, Figs 2–4). Heat denaturation significantly improved the rate of proteolysis regardless of the phaseolin type, enzyme type, or the incubation period. Among the three proteinases used in present investigations, pepsin was the most effective in hydrolyzing the phaseolins. This observation is consistent with literature (Deshpande & Nielsen, 1987; Nielsen *et al.*, 1988). Initial proteolysis rate of the GNP was significantly higher than that of the TP for the corresponding enzyme. This was somewhat unexpected, since both phaseolins were electrophoretically and column chromatographically very similar. A closer look at the published amino acid composition of the TP and other phaseolins (Sathe *et al.*, 1994) does, however, indicate some differences in the amino acid compositions of these two phaseolins. For example, the total sum for the basic amino acid residues (lysine + arginine + histidine) in the TP is 12·42 g/100 g compared to 12·6 and 13·2 g/100 g reported by Doyle *et al.* (1986) and Derbyshire *et al.* (1976), respectively. The corresponding figures for aromatic amino acids are, respectively, 10·34 compared to 10·9 and 9·2 g/100 g. The leucine and methionine content of the TP are 8·72 and 0·98 g/100 g, respectively. The corresponding figures for these amino acids reported by Derbyshire *et al.* (1976) and Doyle *et al.* (1986) are respectively, 9·10 and 10·02 and 0·70 and 1·20 g/100 g. These compositional differences, as well as subtle differences in the tertiary and quaternary structures, may be at least partly responsible for the differences in susceptibility to proteolysis. In the case of pepsin, the higher initial proteolysis rate of GNP must be due to better accessibility of the susceptible bonds compared to those in TP. This difference in accessibility of susceptible bonds must, in turn, be due to small but important differences in the tertiary and quaternary structures of the two phaseolins.

Table 1. Effect of NaCl on the initial proteolysis rate of phaseolins[a]

	Treatment			
	UH		H	
Enzyme	No Salt	1·0 M NaCl	No Salt	1·0 M NaCl
Great Northern Bean				
Trypsin	390 (7·38)[b]	200 (2·86)	725 (10·55)	410 (4·88)
Chymotrypsin	300 (2·83)	250 (1·52)	1080 (16·87)	500 (5·82)
Pepsin	1370 (19·92)	695 (6·38)	2305 (33·82)	990 (8·95)
Tepary bean				
Trypsin	295 (4·28)	150 (2·19)	1000 (14·27)	500 (4·63)
Chymotrypsin	255 (2·81)	150 (1·63)	900 (11·97)	440 (2·89)
Pepsin	835 (9·45)	500 (3·82)	1740 (19·68)	1120 (11·08)
LSD ($P=0·05$)[c]		13·32		

[a]Initial proteolysis rate calculated based on up to 5 min proteolysis; UH = native phaseolin, H = heat-denatured phaseolin. Rate of proteolysis = ΔA280 nm (5 min)/mg enzyme, min (ΔA280 nm of 0·001 was defined arbitrarily as = 1·0).

[b]Figures in parentheses for proteolysis rates are slope of the curve (A280 nm versus time of proteolysis plot) ×1000. The LSD ($P=0·05$) values for the Great Northern bean and tepary bean phaseolin digestions (for all the enzymes) for these were respectively, 0·156 and 0·226.

[c]Differences between two means exceeding this LSD value are significant.

The other possibility is the presence of non-protein material bound to the phaseolins may also influence the proteolysis.

Adding NaCl (1 M) to the digestion mixture clearly caused a significant decrease in the initial proteolysis rate of both phaseolins. This effect was seen in both the native and heat denatured phaseolins for all enzyme digestions (Table 1). Electrophoretic analyses (Figs 2–4) support these observations. It was equally clear from Figs 2–4 that this initial decrease in proteolysis rate may not be of nutritional concern since both heat denatured phaseolins were completely digested (data for GNP electrophoresis not shown). The initial decrease in phaseolin proteolysis rate may be due to: (1) enzyme inhibition by the NaCl; (2) improved phaseolin stability due to increased hydrophobic interactions responsible for protein stabilization; or (3) a combination of (1) and (2). Since heat-denatured phaseolins could be proteolyzed completely (after overcoming the initial resistance) by all the proteinases tested, contribution by the hydrophobic interactions towards phaseolin stability towards proteinase attack must be minimal and important only in the beginning (1 min or less) of the digestion. This is because it is known that once the initial cuts are made,

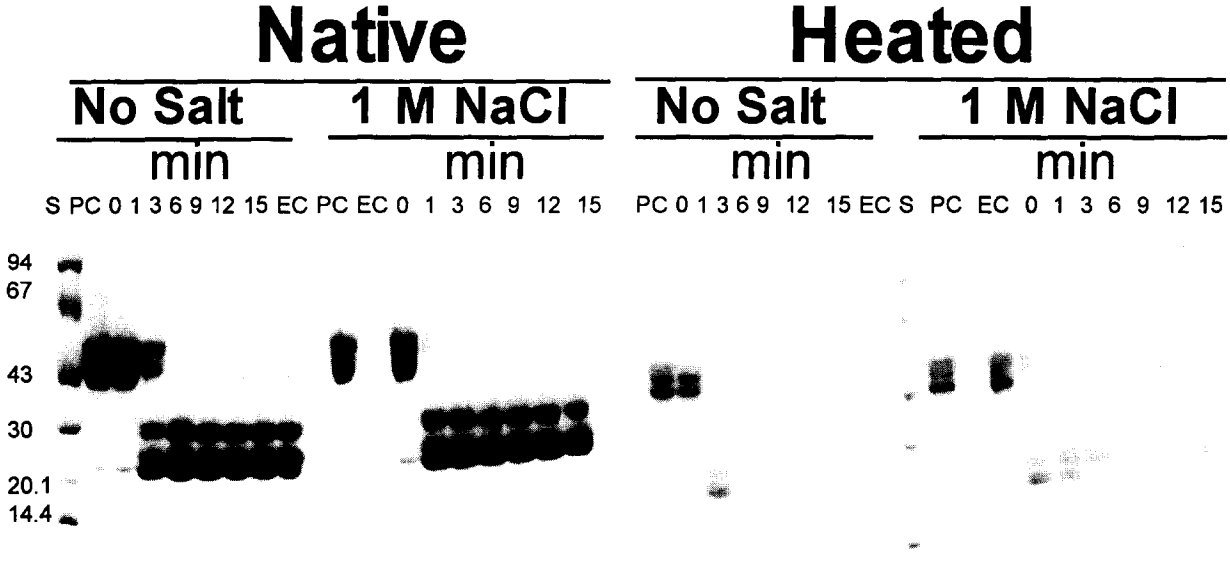

Fig. 2. Effects of heat denaturation and NaCl on TP proteolysis by TPCK-trypsin. Abbreviations: S = MW markers, PC = phaseolin control, EC = TPCK-trypsin control. Digestion time (min) indicated at the top of the lane. Protein load was 30 μg each and the enzyme control load was 0·33 μg each.

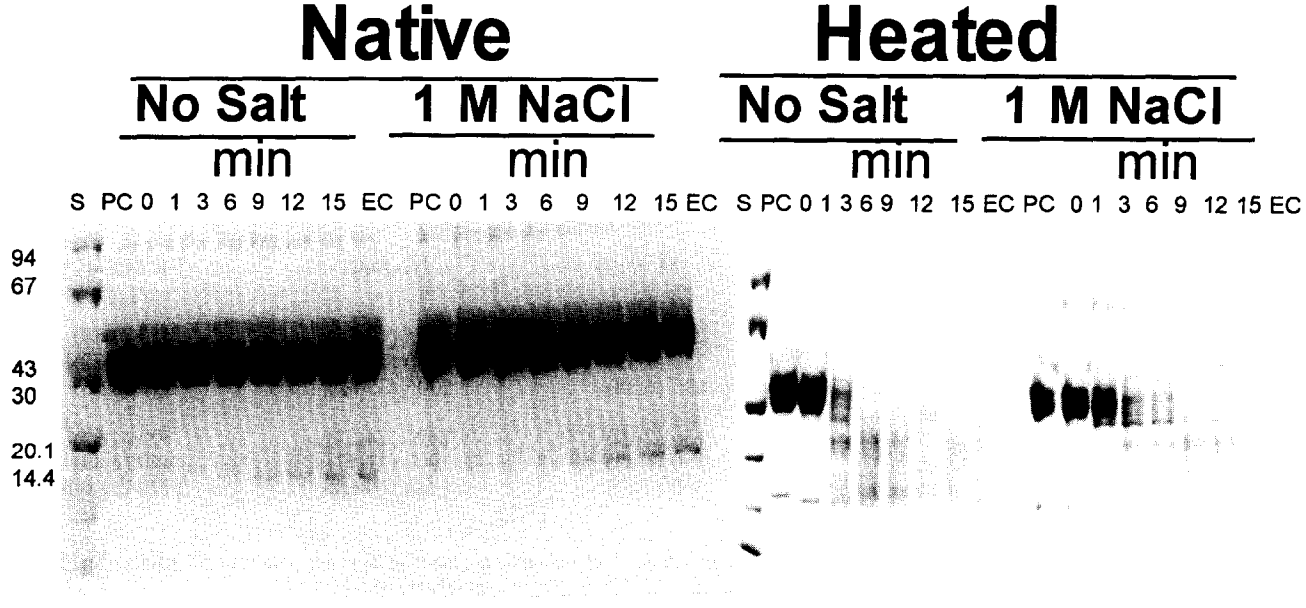

Fig. 3. Effects of heat denaturation and NaCl on TP proteolysis by the TLCK-chymotrypsin. Abbreviations: PC = phaseolin control, EC = TLCK-chymotrypsin control. Digestion time (min) indicated at the top of the lane. Protein load was 30 μg each and the enzyme control load was 0·33 μg each.

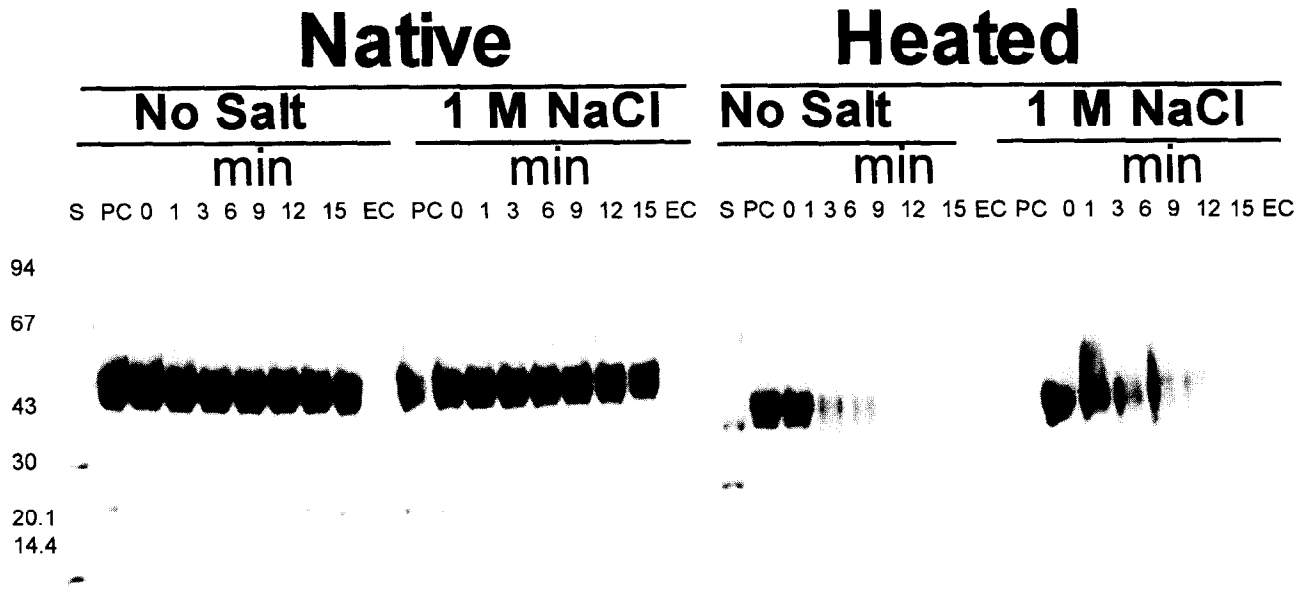

Fig. 4. Effects of heat denaturation and NaCl on TP proteolysis by pepsin. Abbreviations: S = MW markers, PC = phaseolin control, EC = pepsin enzyme control. Digestion time (min) indicated at the top of the lane. Protein load was 30 μg each and the enzyme control load was 0·33 μg each.

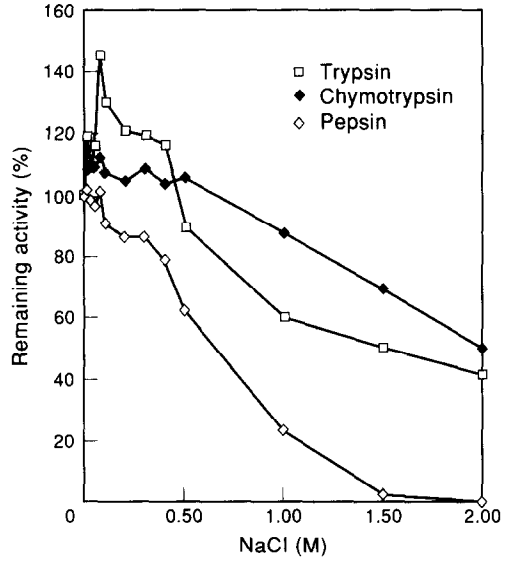

Fig. 5. Effect of NaCl on the caseinolytic activity of the TPCK-trypsin, TLCK-chymotrypsin and pepsin.

phaseolin is rapidly proteolyzed by several common proteinases (Deshpande & Nielsen, 1987; Nielsen, 1988). Consequently, enzyme inhibition was considered a major probable cause for this decrease in initial proteolysis rate. When these enzymes were assayed in the presence of different concentrations of NaCl (0–2 M) a significant decrease in the enzyme activity was seen (Fig. 5), especially at $\geq$1 M NaCl. At 1 M NaCl concentration, the % remaining activity for the trypsin, chymotrypsin, and pepsin was respectively, 60·6, 87·9, and 23·6. The complete proteolysis of heat-denatured phaseolins (Figs 2–4), however, suggests that the partial enzyme inhibition by NaCl may not be of nutritional concern. It should be mentioned here that 1 M NaCl concentration (5·85 g/100 g) is also very unlikely to be encountered in cooked beans under home cooking conditions (for comparative purposes, typical commercial fat-free refried beans contain 480 mg Na/6 g proteins which would be equivalent to 0·984 g/100 g product). At low NaCl concentrations, there was a significant increase in the enzyme activities (up to 0·4, 0·5 and 0·075 M for trypsin, chymotrypsin, and pepsin, respectively) which may suggest that small amount of NaCl may indeed be beneficial with respect to improving the initial proteolysis rate of phaseolin.

Phytate-protein (Reddy & Salunkhe, 1981; Reddy *et al.*, 1989; Idouraine *et al.*, 1992) and tannin–protein interactions (Reddy *et al.*, 1985; Deshpande & Sathe, 1991) and their possible nutritional implications are of concern. This concern arises due to the fact that both phytate and tannin are heat stable and therefore are not destroyed during cooking and that these interactions often involve minerals possibly adversely affecting the mineral bioavailability. Therefore, we investigated the effects of added phytate and tannin on the initial *in vitro* rate of proteolysis. In these experiments, the protein was heat denatured in the presence of the additive prior to digestion. During heat denaturation, no buffers were used to simulate the normal water cooking of beans. The levels of phytate and tannin were selected to represent their naturally occurring wide range of concentrations in intact beans (taking into account the amount of phaseolin that would be present in the intact bean). Adding phytate or tannin did cause a decrease in the initial proteolysis rate in certain instances (Table 2) when compared to the proteolysis rate of the corresponding control (e.g. the rate was 16·87 for GNP proteolysis compared to 8·73 when tannin was added at phaseolin:tannin ratio of 50:1). When one compares

Table 2. Effect of NaCl, phytate, and tannin on the initial rate of phaseolin proteolysis[a]

Phaseolin:additive (w/w)	Great Northern bean phaseolin			Tepary bean phaseolin		
	T[b]	C[c]	P[d]	T[b]	C[c]	P[d]
No additive	10·55	16·87	33·82	14·27	11·97	19·68
NaCl (0·034:1)	4·88	5·82	9·95	4·63	2·89	11·08
Phytate						
10:1	17·55	12·01	18·48	20·93	14·10	24·18
5:1	15·93	12·37	15·68	18·01	13·52	11·19
1:1	12·08	11·68	8·21	16·21	12·20	8·59
Tannin						
100:1	13·32	11·85	39·35	8·70	10·32	24·33
50:1	19·98	8·73	67·96	13·73	11·60	23·81
10:1	26·29	29·05	70·65	59·78	67·06	11·86
LSD ($P=0·05$)[e]	0·677	0·785	1·487	0·797	0·436	0·779

[a]Rate of proteolysis = slope of the curve (A280 nm versus proteolysis time (up to 5 min)plot] ×1000. Only heat-denatured phaseolins were used for these experiments.
[b]T = TPCK-trypsin.
[c]C = TLCK-chymotrypsin.
[d]P = pepsin.
[e]Differences between two means (within the same column) exceeding this value are significant.

these proteolysis rates to those for corresponding samples containing 1 M NaCl (Table 2 and Figs 2–4), it is apparent that this initial decrease in proteolysis rate should not be of nutritional consequence. Vaintraub & Bulmaga (1991) have reported that phytate inhibits the action of pepsin on the proteins. In this sense, our data are consistent with regard to the decrease in the initial proteolysis rates for pepsin (in the presence of added phytate). However, when one compares them with corresponding rates in the presence of NaCl as well as results from Fig. 4, it is apparent that this initial decrease should not adversely affect the phaseolin proteolysis, which is consistent with earlier observations reported by Reddy *et al.* (1988). That the phytate was unable to adversely affect proteolysis by trypsin and chymotrypsin is also consistent with published literature (Deshpande & Damodaran, 1989; Vaintraub & Bulmaga, 1991). For a given enzyme, compared to 1 M NaCl, adding tannin helped to improve the initial proteolysis rate, which suggests that tannin addition should not adversely affect phaseolin proteolysis *in vitro*. This lack of inhibitory effect of tannin on proteolysis is similar to the findings of Neucere *et al.* (1978). These investigators reported that when arachin (the major storage protein in peanuts) was exposed to 0·1 M catechol or pyrogallol, it was more susceptible to pepsin hydrolysis *in vitro*. More recently, Mole & Waterman (1987) have observed that added tannic acid did not inhibit trypsin activity when bovine serum albumin was present in the incubation mixture.

Based on the results of this investigation and some of the published literature, it is apparent that the heat-denatured phaseolin is a highly digestible protein (at least *in vitro*). The common digestive proteinases trypsin, chymotrypsin, and pepsin should be able to overcome any initial resistance caused by NaCl, phytate, or the tannin addition, and thus accomplish complete proteolysis of phaseolin *in vitro*.

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