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Purification and characterization of trypsin inhibitor from seeds of faba bean (*Vicia faba* L.)

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Abstract

A trypsin inhibitor from seeds of faba bean (*Vicia faba* L.) was purified to near homogeneity as judged by native-PAGE with about 11 % recovery using ammonium sulphate fractionation, ion-exchange chromatography on DEAE-cellulose and gel filtration through Sephadex G-100. The inhibitor had a molecular weight of 18 kD as determined by SDS-PAGE and Sephadex G-100. The inhibitor inhibited trypsin and chymotrypsin to the extent of 48 and 12 %, respectively. The inhibtion was of non-competitive type with dissociation constant for the enzyme inhibitor complex in the region of 0.07 $mg \cdot ml^{-1}$. The inhibtor was stable between pH 4 and 5. It completely lost its activity when heated at 125 °C for 1 h or at 100 °C for 2 h. The inhibtor also lost its activity on exposure to 2-mercaptoethanol. Based on these properties, it could be concluded that *Vicia faba* trypsin inhibitor belongs to Bowman-Birk type of inhibitors, as it has molecular weight lower than generally observed for Kunitz type inhibitors.

List of abbreviations: **PAGE**, polyacrylamide gel electrophoresis; **DEAE**, diethyl amino ethyl; **SDS**, sodium dodecyl sulphate; **kD**, kilodalton; **CM**, carboxy methyl.

Introduction

Protease inhibitors are widespread in plants, animals and microorganisms. These have the ability to inactivate the proteolytic action of certain enzymes in humans (Prabhu *et al.* 1984) and cause pancreatic carcinogenesis (Gumbmann *et al.* 1986). Recently, these were implicated in defence mechanisms against microbial and insect attack and genes for trypsin inhibitor from cowpea were already cloned in a number of agricultural crops (Hilder *et al.* 1987, Ryan 1990, Jouanin *et al.* 1998). They have also been proposed to function as storage proteins particularly in legume seeds (Liener and Kakade 1980), from where they were purified and characterized quite extensively (Pustzai *et al.* 1991, Godbole *et al.* 1994, Ferrason *et al.* 1997). The study of these inhibitors attracted the attention of nutritionists as they are rich in S-containing amino acids. Thus for evolving an appropriate nutritional regime for diets containing legume seeds as a major source of proteins, it is necessary to have detailed information on the protease inhibitors present in these crops. Most of the information present in the literature is from the conventional food legumes, but not much is known about the protease inhibitors of unconventional food legumes like faba bean (*Vicia faba*). *Vicia faba* containing about 26 % protein has

recently gained importance due to its high productivity even under marginal conditions of cultivation. Keeping in view the importance of this legume as a food crop, an investigation was initiated to isolate, purify and characterize trypsin inhibitor from the seeds of this crop.

Material and methods

Seeds of *Vicia faba*, var. VH 82-1 were procured from the department of Plant Breeding of CCS Haryana Agricultural University, Hisar. All the biochemicals were purchased either from Sigma Chemical Co. (St. Louis, MO, USA) or from SRL or E. Merck (India).

Extraction and assay of trypsin inhibitor

Defatted seed flour was extracted with 0.1 M phosphate buffer, pH 7.5 (1:20, w/v) for 2 h at room temperature in a shaking water bath. The homogenate/slurry obtained was centrifuged at 10,000 x g for 30 min and the supernatant collected. Trypsin inhibitor activity in the clear supernatant was determined by the method of Kakade *et al.* (1969). The assay mixture contained trypsin solution made to 2.0 ml with phosphate buffer (pH 7.5) and 2.0 ml of 2 % casein solution. The reaction mixture was incubated for 20 min at 37 °C and trypsin activity was determined by caseinolytic assay. Trypsin activity was assayed in presence (x) and absence (y) of the flour extract and the trypsin units inhibited (TUI) were calculated as the difference of the two (y-x). One inhibitor activity unit was defined as decrease in 0.01 absorbance unit of trichloroacetic acid soluble casein hydrolysis products (liberated by trypsin action) at 280 nm in 20 min at 37 °C in the given assay volume (10 ml).

Purification

For purification of the inhibitor, the crude extract was prepared by homogenizing 100 g of defatted flour with 1L of 0.1 M phosphate buffer (pH 7.5). After stirring for 2 h in shaking water bath at room temperature, the supernatant was centrifuged at 10,000 x g for 30 min. To the supernatant, $(NH_4)_2SO_4$ was added to 85 % saturation and the protein was allowed to precipitate overnight at 4 °C. The precipitated protein was collected by centrifugation at 10,000 x g for 15 min and the pellet dissolved and dialysed against phosphate buffer. The dialysed preparation was then fractionated by DEAE-cellulose column (26 x 2.6 cm; flow rate 35 $ml \cdot h^{-1}$) chromatography using linear salt gradient of 0.1-0.5 M NaCl in phosphate buffer (0.1 M; pH 7.5). Hundred and fifty fractions of 5 ml each were monitored for protein (A_{280}) and trypsin inhibitor activity. The fractions (8-32) showing trypsin inhibitor activity were pooled, concentrated by osmosis against solid sucrose and chromatographed on Sephadex G-100 column (70 x 1.6 cm; flow rate 12 $ml \cdot h^{-1}$) that were pre-equilibrated with the same buffer. Ninety fractions of 2 ml each were collected and monitored for protein (A_{280}) and trypsin inhibitor activity. The active fractions were pooled and used for further studies.

The purity of the trypsin inhibitor in different fractions was checked by native polyacrylamide gel electrophoresis (native- PAGE) using 10 % acrylamide gel and anionic system of Davis (1964) on a vertical slab gel electrophoresis (Atto, Japan).

Determination of molecular weight and sub-unit composition

The polypeptide composition was determined by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) using a linear gradient of 5-20 % acrylamide (Laemmli 1970). Molecular weight of the purified inhibitor was determined by gel filtration through Sephadex G-100 column and SDS-PAGE. Sephadex G-100 column was calibrated with standard marker proteins viz. bovine serum albumin (66 kD), carbonic anhydrase (29 kD) and cytochrome C (12.4 kD), while molecular weight markers used for SDS-PAGE were bovine serum albumin (66 kD), egg albumin (45 kD), β-lactoglobulin (18.4 kD) and lysozyme (14.3 kD).

Protein estimation

Protein content in various fractions was quantitatively measured according to the method of Lowry *et al.* (1951).

Results and discussion

Preliminary experiments were conducted to find out the relationship between the inhibitor activity and the amount of inhibitor extract and also the time of incubation. The trypsin inhibitory activity (TIA) was linear up to 70 % inhibition and up to 25 min of incubation. For further studies, the amount of inhibitor which gave 40-50 % inhibition (0.2 ml) was always incubated at 37 °C for 20 min.

To investigate the effect of extraction medium, the trypsin inhibitor was extracted from the defatted seed flour in three different extraction media *viz.*, distilled water, 0.2 % NaCl and 0.1 M phosphate buffer (pH 7.5). The inhibition profile of trypsin as a function of increasing level of extract was linear upto 0.3 ml with all extracts. However, percent inhibition was more (70 %) when the seed flour was extracted with phosphate buffer (0.1 M, pH 7.5). The deviations at high levels of inhibitor concentration were attributed to the partial dissociation of the inhibitor complex (Pichare and Kachole 1996).

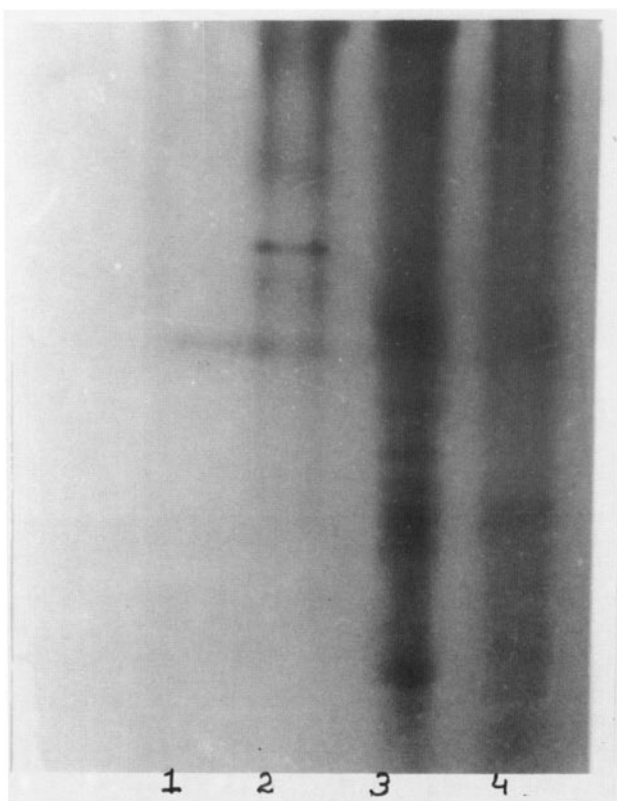

Fig. 1. Native PAGE pattern of purification fractions of *Vicia faba* trypsin inhibitor. Lane 1, Sephadex G-100 fraction; lane 2, DEAE-cellulose fraction; lane 3, $(NH_4)_2SO_4$ fraction; lane 4, crude extract.

The trypsin inhibitor was purified to near homogeneity as judged by native-PAGE (Fig. 1) with 83 fold purification and about 11 % recovery using $(NH_4)_2SO_4$ fractionation, ion exchange chromatography on DEAE-cellulose and gel filtration through Sephadex G-100 (Table 1). Different recoveries and purification factors for trypsin inhibitors were reported in several different crops (Godbole *et al.* 1994a, Ferrason *et al.* 1997). The specific activity increased from 11 in crude extract to 894.0 in Sephadex G-100 fraction. The inhibitor during ion-exchange chromatography became eluted in the unbound fraction. Asao *et al.* (1991) also observed the exclusion of one of the trypsin inhibitor with elution buffer prior to application of NaCl gradient on CM-Sephadex.

SDS-PAGE under reducing conditions revealed a single protein band, proving that the inhibitor from faba bean seeds is a monomer with molecular weight of 18 kD. Similar value for molecular weight was obtained when determined by gel filtration. The results obtained in the present investigation are in close agreement with those reported by Bergeron and Nielsen (1993) for Great Northern beans.

The inhibition of trypsin was measured at different substrate concentrations in the presence and absence of two fixed concentrations of 0.5 and 1.0 mg of trypsin inhibitor protein (Fig. 2). The Michaelis-Menten constant (Km) for trypsin as determined by Lineweaver-Burk plot (Fig. 3) was found to be 1.02 $mg \cdot ml^{-1}$. The faba

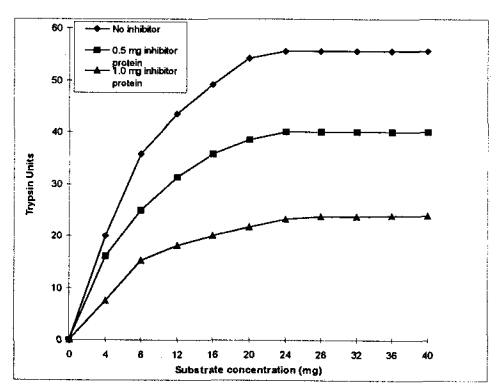

Fig. 2. Trypsin activity at different substrate concentrations in the absence and presence of 0.5 and 1.0 mg inhibitor protein.

Table 1. Data on trypsin inhibitor purification from *Vicia faba* flour

Step	Total protein (mg)	Total TIA	Specific activity	Recovery (%)	Fold purification
Crude extract	11786.0	126800.0	10.75	100	1.00
$(NH_4)_2SO_4$ ppt	7915.0	105810.0	13.36	83	1.24
DEAE cellulose	264.0	39375.0	149.14	31	13.87
Sephadex G-100	15.0	13416.0	894.40	11	83.20

bean inhibitor inhibited trypsin non-competitively. The dissociation constant, Ki for enzyme-inhibitor complex was 0.07 $mg \cdot ml^{-1}$. Such type of non-competitive inhibition of trypsin and chymotrypsin by the inhibitor of Jackfruit seeds was reported by Annapurna *et al.* (1991).

The inhibitor of *Vicia faba* inhibited both trypsin and chymotrypsin while pepsin was inhibited only slightly (Fig. 4). The percent inhibition of trypsin, chymotrypsin and pepsin observed by the inhibitor concentration used in the present studies (0.2 ml extract) was 50, 15 and <10 %, respectively. The inhibition of trypsin and chymotrypsin to the extent of 58 and 28 %, respectively has been reported in *Cajanus cajan* seeds (Godbole *et al.* 1994a).

The pH stability of enzyme inhibitor at different pH ranging from 3.0 to 12.0 was studied by preincubating the enzyme inhibitor in different buffers for 24 h and then assaying the inhibitor activity (Table 2). The trypsin inhibitor was stable at pH 4.0 and pH 5.0 and started losing activity at pH values higher than 5.0. The inhibitor was completely unstable at extreme pH values. This may be due to the conformational changes in the structure of trypsin inhibitor. Annapurna *et al.* (1991) reported the trypsin inhibitor from jack fruit seeds to be stable over a still wider range of pH from 3.0 to 12.0. On the other hand, the trypsin inhibitor solution from pigeonpea seeds retained its full activity between pH 7.0 and 10.0, but when exposed to acidic pH between 3.0 to 5.0, 20 % of the activity was lost (Godbole *et al.* 1994a).

Table 2. pH stability of *Vicia faba* trypsin inhibitor

pH	TUI	% control
Control	15.0	100
2	12.0	80
3	11.0	73
4	17.0	113
5	15.0	100
6	12.0	80
7	14.0	93
8	13.0	87
9	12.0	80
10	14.0	93

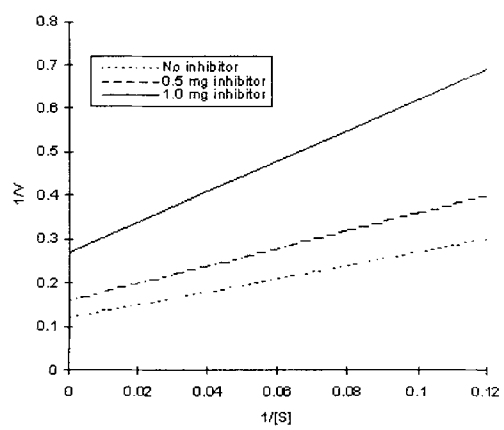

Fig. 3. Lineweaver-Burk plot of trypsin activity in the absence and presence of 0.5 and 1.0 mg inhibitor protein.

Heating of the trypsin inhibitor protein at 125 °C for 1 h resulted in complete loss of its activity (Fig. 5). The inhibitor also lost its activity when heated at 100 °C for 2 h. Heat stability of legume inhibitors may be attributed to the compact structure stabilized by a number of disulphide linkages. Therefore, pressure cooking is the suggested method for removing *Vicia faba* trypsin inhibitors which in turn can improve the nutritional quality of this legume.

Trypsin-inhibitor complex showed about 25 % residual trypsin activity. However, incubation of trypsin-inhibitor complex with 0.2 M and 0.4 M of 2-ME resulted in 46 and 85 % restoration of trypsin

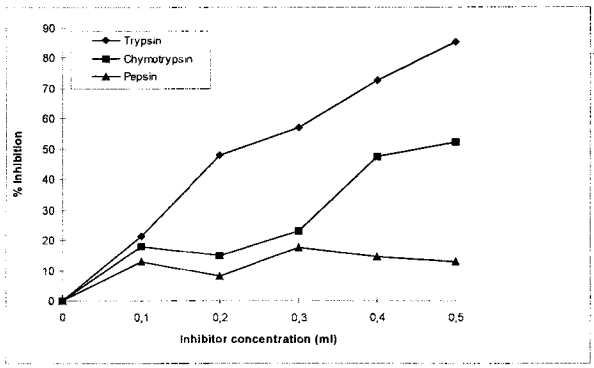

Fig. 4. Effect of inhibitor concentrations on the activity of some proteases.

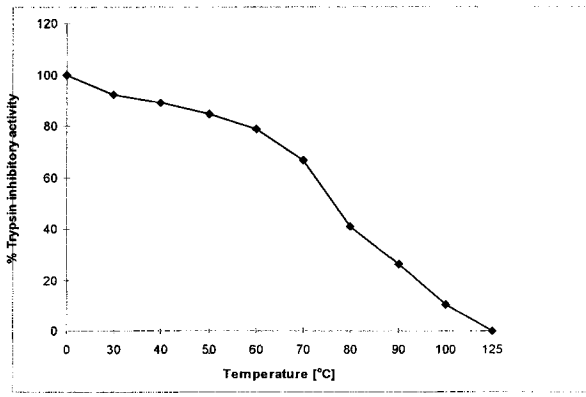

Fig. 5. Thermostability of inhibitor at different temperatures.

activity, respectively, indicating that disulphide linkages of trypsin inhibtors are responsible for TIA which became cleaved on exposure to 2-ME and disrupted the three dimensional conformation of the inhibitor resulting into immediate dissociation of enzyme-inhibitor complex. Such type of restoration was also reported by other workers (Godbole *et al.* 1994b).

From the results obtained in the present study, it appears that the trypsin inhibitor of *Vicia faba* is of Bowman-Birk type because it inhibited both trypsin and chymotrypsin independently. The Bowman- Birk inhibitors (BBIs) have in general low molecular weight (8,000-10,000) as compared to Kunitz type (20,000). In our studies, the average molecular weight of 18 kD seems to be slightly higher than that of Bowman-Birk type inhibitors. It may be due to monomer-dimer equilibrium of the inhibitor protein (Whitley and Bowman 1975) or due to oxidation of cysteine residues as has been reported by Ferrasson *et al.* (1997). High molecular weights of Bowman-Birk inhibitors were also reported in other legumes as well (Norimura *et al.* 1990, Godbole *et al.* 1994a). Noroika *et al.* (1988) demonstrated proteinase inhibitors in legumes to change from Kunitz type to Bowman-Birk type as they evolved.

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