Phytochemistry, Vol. 22, No. 11, pp. 2349–2357, 1983.
Printed in Great Britain.

0031–9422/83 $3.00+0.00
Pergamon Press Ltd.

ISOLATION AND CHARACTERIZATION OF A TRYPSIN INHIBITOR FROM FINGER MILLET

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(*Revised received* 14 *April* 1983)

Key Word Index—*Eleusine coracana*; Gramineae; finger millet trypsin inhibitor; isolation of trypsin inhibitor; characterization of trypsin inhibitor; α-amylase inhibitor; trypsin–proteinase inhibitor interactions; α-amylase–trypsin inhibitor interaction; double-headed inhibitor.

Abstract—A trypsin inhibitor was isolated from finger millet (*Eleusine coracana*) by ammonium sulphate fractionation, chromatography on CM-Sephadex and Sephadex G-50. The purified preparation appeared to be homogeneous by both chromatographic and electrophoretic analyses. The finger millet trypsin inhibitor (FMTI) had a MW of 12000 and amino acid analysis showed that it contained eight half-cystines and was rich in basic amino acids and glutamic acid. Glutamic acid was the *N*-terminal amino acid of the inhibitor. FMTI formed a 1 : 1 complex with bovine trypsin and the K_i (dissociation constant) of the complex was 1.2×10^{-8} M. FMTI possesses the unique property of inhibiting α-amylases. The FMTI–trypsin complex exhibited α-amylase inhibitory activity suggesting the 'double-headed' nature of the inhibitor. The interaction of trypsin and FMTI was accompanied by spectral changes in the 290–306 nm region of the spectrum. 1-Chloro-3-tosylamido-7-amino-L-2-heptanone–trypsin and trypsinogen did not interfere with the activity of FMTI towards catalytically active enzyme. The chemical modification of lysine and arginine residues indicated that $-NH_2$ groups are essential for the activity of FMTI towards trypsin.

INTRODUCTION

Proteins which inhibit the activity of proteolytic enzymes are widely distributed both in animal and plant tissues. The distribution, properties and biological significance of these inhibitors have been reviewed [1–7]. Research on plant proteinase inhibitors for the most part has been concerned with leguminous plants because of the importance of legumes as rich sources of proteins for animal and human consumption. The inhibitors are also widely distributed among cereal grains such as wheat, rye, oats, barley, rice, corn [1, 2] and sorghum [8]. The mode of action of the proteinase inhibitors with the proteinases is now well established [5]. A few aspects of the interaction, including the splitting of a specific peptide bond on the surface of the molecule (the reaction site) by the proteinases, have been well documented [9, 10]. Two protein α-amylase inhibitors have been isolated and characterized from finger millet and one of them has been shown to inactivate trypsin [11, 12].

In view of the importance of finger millet as a food grain and the paucity of information on the proteinase inhibitors of this millet, an investigation was initiated to isolate and characterize the proteinase inhibitors. This paper describes the isolation, properties and mode of action of a tryspin inhibitor from finger millet which also possesses α-amylase inhibitory activity.

RESULTS

Purification of FMTI

FMTI† was isolated in an apparently homogeneous form by the following procedure. All operations were carried out at 25–27° unless otherwise stated and all centrifugations were done in a Zanetski (Model K-70) refrigerated centrifuge using a 25 cm rotor at 0°.

Acetone-defatted finger millet flour (50 g) was stirred with 500 ml of 50 mM sodium phosphate buffer (pH 7.6) for 4 hr at 5°. The extract was centrifuged at 2500 rpm for 30 min. The pH of the supernatant was adjusted to 5.0 with 50% acetic acid and the suspension was heated to 70° with stirring. After 10 min at 70° the suspension was rapidly chilled in an ice-bath and centrifuged at 2500 rpm for 15 min to remove the precipitate.

The pH of the supernatant from the previous step was adjusted to 7.0 with dilute ammonium hydroxide. Solid ammonium sulphate was added to 60% saturation with constant stirring at 0°. After stirring for 1 hr at 0°, the suspension was centrifuged at 3000 rpm for 15 min. The precipitate was dissolved in 100 mM sodium acetate buffer (pH 5.0) and dialysed. A small amount of precipitate formed during dialysis was removed by centrifugation.

CM-Sephadex C-25 was suspended in 100 mM sodium acetate buffer (pH 5.0) and packed into a column (2 × 15 cm). The ammonium sulphate fraction (80 mg pro-

†Abbreviations: FMTI, finger millet trypsin inhibitor; BAPNA, *N*-benzoyl-D,L-arginine-*p*-nitroanilide HCl; ATEE, *N*-acetyl-L-tryosine ethyl ester; APNE, *N*-acetyl-D,L-phenylalanyl-β-naphthyl ester; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); TNBS, trinitrobenzene sulphonic acid; CHD, 1,2-cyclohexanedione; TLCK, 1-chloro-3-tosylamido-7-amino-L-2-heptanone; TIA, trypsin inhibitory activity; AIA, α-amylase inhibitory activity; CIA, α-chymotrypsin inhibitory activity; TIU, trypsin inhibitory units; AIU, α-amylase inhibitory units.

tein) was applied to the column and eluted with 100 ml of the starting buffer. Thereafter, a stepwise elution with increasing concentrations of sodium chloride in the starting buffer was performed as detailed in the legend to Fig. 1. Fractions containing trypsin inhibitory activity (TIA) (48–56) were pooled, dialysed against distilled water and lyophilized. The electrophoretic pattern of this preparation is shown in Fig. 3.

Protein (20 mg) from fractions 48–56 was applied to a Sephadex G-50 column (1 × 80 cm) and chromatographed using 100 mM sodium acetate buffer (pH 5.0) as the effluent. The elution profile of this gel-permeation chromatography is shown in Fig. 2. The active fractions (21–27) were pooled, dialysed and lyophilized.

Recoveries and relative purification at each step for a typical purification from 100 g of defatted flour are shown in Table 1. By this procedure, *ca* 20 mg of inhibitor protein was obtained from 100 g of flour. The α-amylase inhibitory activity (AIA) copurified with the TIA and the ratio of trypsin inhibitory units (TIU) to α-amylase inhibitory units (AIU) remained constant at various stages of purification. The final preparation showed a single protein band in polyacrylamide gel electrophoresis (PAGE) and this band was shown to possess TIA by a specific staining method (Fig. 3).

Physical and chemical properties of FMTI

MW. The MW of the FMTI was determined by gel filtration on Sephadex G-75, SDS-PAGE and ultracentrifugation. FMTI eluted as a single symmetrical protein and activity peak when chromatographed on a calibrated Sephadex G-75 column. A MW of 12 000 ± 100 was calculated from the plot of V_e/V_o vs log MW for the calibrating proteins and FMTI. In the ultracentrifuge FMTI sedimented as a single symmetrical peak and its $S_{20,w}$ value was calculated to be 1.4 S. The MW from the sedimentation analysis was 12 100. The inhibitor showed a single band on 1% SDS-PAGE. From the SDS-PAGE technique, the MW of the inhibitor was estimated to be 11 900 ± 100. From these electrophoretic and chromatographic techniques, FMTI appears to be homogeneous.

UV. The inhibitor had a typical protein spectrum with an absorption maximum at 280 nm, a minimum at 255 nm and a shoulder at 290 nm. $E^{1\%}_{1\,cm}$ (280 nm) was calculated to be 7.75.

Amino acid composition. The amino acid composition of FMTI is shown in Table 2. The inhibitor was composed of 113 residues with the formula weight of 12 300 ± 100. FMTI contained eight half-cystine residues per molecule. It is noteworthy that FMTI contained two tryptophan residues per molecule. FMTI was rich in basic acids, glutamic acid, glycine, valine, alanine and leucine, which together accounted for more than 70% of the total amino acid content. The partial specific volume was calculated to be 0.715 based on the amino acid composition. No free sulphydryl groups were found in the inhibitor. The absence of sulphydryl groups indicates that all the half-cystine residues presumably participate in disulphide linkages. FMTI is not a glycoprotein since the protein band by PAGE did not take up the PAS-stain [15].

Amino terminal analysis. The *N*-terminal group of FMTI, as determined by dansylation of the protein, was glutamic acid.

Stability of the inhibitor. The effects of heat (25–121°), pH (2–12) and 8 M urea on the trypsin and α-amylase

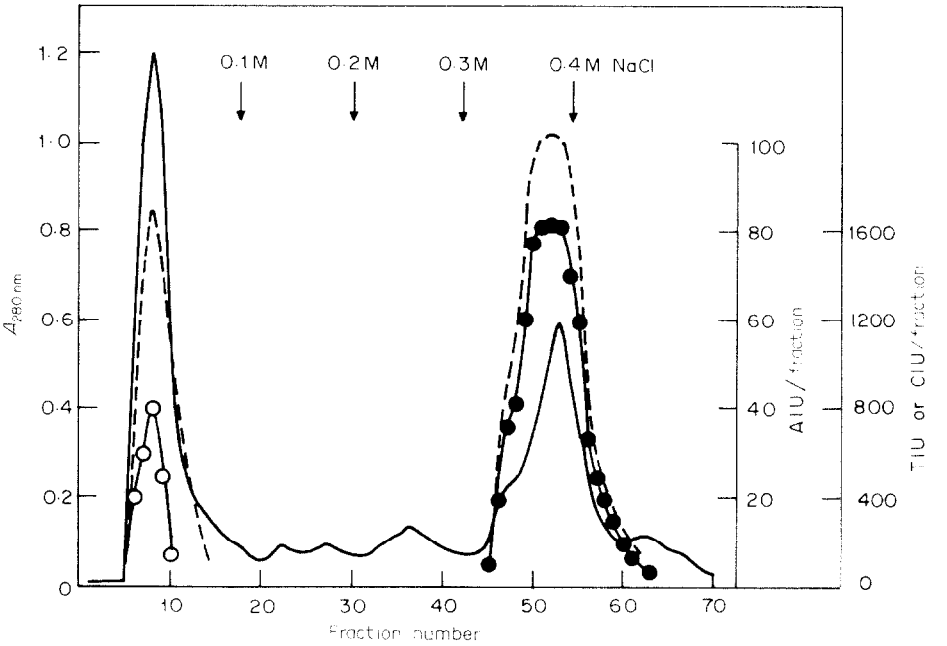

Fig. 1. CM-Sephadex C-25 column chromatography of 60% ammonium sulphate fraction. Protein (80 mg) was applied to the column (2 × 15 cm) in 100 mM sodium acetate buffer, pH 5.0, and the adsorbed proteins were eluted stepwise with 0.1, 0.2, 0.3 and 0.4 M NaCl in the starting buffer. Fractions, each 4.5 ml, were collected at a flow rate of 30 ml/hr. Fractions from 48–56 were pooled, dialysed and lyophilized. ——, Absorbance at 280 nm; ●——●, TIA; ○——○, CIA; – – –, AIA.

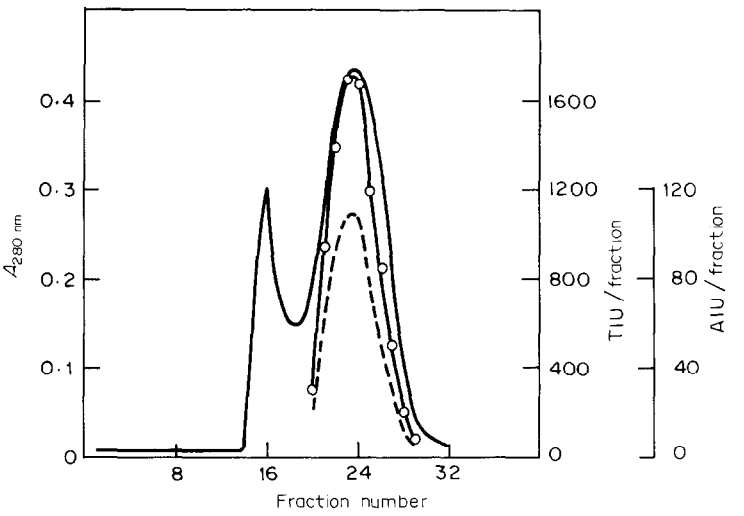

Fig. 2. Sephadex G-50 gel filtration of the CM-Sephadex C-25 preparation. Protein (20 mg) was applied to the column (1 × 80 cm) equilibrated with 100 mM sodium acetate buffer, pH 5.0, and eluted with the same buffer. Fractions (2 ml) were collected at a flow rate of 12 ml/hr. ——, Absorbance at 280 nm: ○——○, TIA; - - - -, AIA.

Table 1. Summary of the purification of FMTI*

Purification steps	Total protein (mg)	Total activity (units)		Specific activity (units/mg protein)		Ratio TIU / AIU	Yield† (%)
		TIU‡	AIU‡	TIA	AIA		
Crude extract	986	54400	4820	55	4.8	11.28	100
Heat-treated extract	483	50400	4510	104	9.3	11.19	93
60% $(NH_4)_2SO_4$ fraction (dialysed)	178	41700	3720	233	20.8	11.21	77
CM-Sephadex preparation	50	36700	2260	734	45.2	16.23	68
Sephadex G-50 preparation (final preparation)	20	25300	1540	1265	77.0	16.42	47

*Starting material was 100 g of defatted flour.
†Yield was calculated on the basis of TIU.
‡TIU and AIU are defined according to refs. [13, 14].

inhibitory activities of FMTI were tested. The inhibitor was found to be stable up to 80° for 10 min (Table 3). About 43 and 77% residual TIA and AIA, respectively, were observed on heating the inhibitor at 97° for 10 min. However, there was complete loss of both activities on autoclaving (121°). FMTI was quite stable in the presence of urea, and contact with 8 M urea for 24 hr did not affect either TIA or AIA. FMTI was stable over a wide pH range from 2 to 12.

Specificity of FMTI. Specificity of the inhibition of different classes of proteinases and α-amylases by FMTI was tested against the enzymes from various sources (Table 4). FMTI inhibited only bovine trypsin and showed very weak or no activity against other proteolytic enzymes tested.

The inhibition of pancreatic α-amylase by FMTI was linear up to 50% inhibition, and 100% inhibition was not achieved even after using a 10-fold excess of inhibitor concentration. The activity of FMTI against pancreatic α-amylase (AIU/mg inhibitor = 78) was four times higher than that against salivary α-amylase. It did not inhibit the activity of *Bacillus subtilis* α-amylase.

The inhibitory activity of FMTI against bovine trypsin with BAPNA as substrate is shown in Fig. 4. The inhibition was linear up to 60% inhibition. From the amount of inhibitor required for 50% inhibition, it was seen that 1 mol of FMTI inhibited 1 mol of trypsin. From these data, K_i (apparent) was calculated using the formula

$$K_i = \frac{[e]\,[I-(E-e)]}{[E-e]},$$

where [E] is the concentration of active plus inactive enzyme (total enzyme), [e] is the concentration of active enzyme and [I] is the concentration of free plus bound inhibitor (total inhibitor) [16]. The K_i of the FMTI–trypsin complex was calculated to be 1.2×10^{-8} M.

Complex formation between bovine trypsin and FMTI. Isolation of the FMTI–trypsin complex by gel-permeation chromatography on Sephadex G-75 column calibrated with marker proteins and free FMTI is shown in Fig. 5. Apart from the protein peak corresponding to

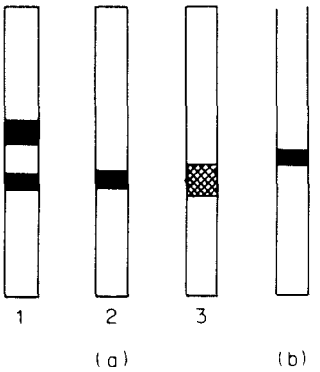

Fig. 3. Electrophoretic analysis of FMTI. (a) PAGE was carried out at pH 4.3 in 7.5% gels. Direction of migration is from top (anode) to bottom (cathode). 1, CM-Sephadex preparation; 2, purified FMTI; 3, visualization of the trypsin inhibitory band on the gel. For the visualization the gels after electrophoresis were first incubated with trypsin solution (0.1 mg/ml) and then with the substrate (APNE)–dye (Diazoblue-B) mixture [41]. The inhibitory activity appeared as a clear zone against a pink background. (b) SDS-PAGE of purified FMTI in a 10% gel. The gel was stained with Coomassie brilliant blue G. Direction of migration is from top (cathode) to bottom (anode).

Table 2. Amino acid composition of FMTI

Amino acid	No. of residues/ mol	Nearest integer
Lys	4.86	5
His	3.00	3
Arg	9.34	9
Asp	5.08	5
Thr	5.34	5
Ser	2.97	3
Glu	14.24	14
Pro	7.12	7
Gly	11.60	12
Ala	11.63	12
Cys*	8.01	8
Val	9.79	10
Met	1.78	2
Ile	4.22	4
Leu	7.31	7
Tyr	1.98	2
Phe	2.67	3
Trp†	2.17	2
Total	112.54	113

*Half-cystine was estimated as cysteic acid after performic acid oxidation.
†Determined spectrophotometrically.

FMTI, another protein peak was eluted from the column soon after the void volume at an elution position corresponding to a MW of *ca* 35 000, which agreed with the FMTI–trypsin complex (35 600). The protein fraction corresponding to the FMTI–trypsin complex was devoid of both trypsin activity and TIA, but it exhibited AIA to the same extent as that of the uncomplexed FMTI. Apparently all the trypsin had complexed with the inhibitor and was eluted out at the first peak position, corresponding to a 1 : 1 complex of trypsin and FMTI.

Table 3. Effect of heat on FMTI

Temp. (°)	TIA remaining* (%)	AIA remaining* (%)
25	100	100
40	100	97
50	91	100
60	90	100
70	91	101
80	87	93
97†	43	77
121‡	0	0

*Inhibitory activity at 25° was taken as 100%.
†Boiling water.
‡Autoclaving at 1.04 kg/cm^2 pressure.

Table 4. Activity of FMTI on proteinases

Enzyme	μg of enzyme inhibited/μg of inhibitor* Proteolytic activity	Esterolytic activity
Bovine trypsin	2.00	2.04†
Porcine trypsin	0.21	0.21†
Pronase	0.16	0.10†
Subtilisin-BPN′	—	0.08‡
Subtilisin-A	—	0.18‡
Elastase	0.09§	—
Bovine α-chymotrypsin	0.0	0.0
Papain	0.0	—
Chymopapain	0.0	—
Bromelin	0.0	—
Pepsin	0.0	—

*Calculated at 50% inhibition of enzyme activity.
†Using BAPNA as substrate.
‡Using ATEE as substrate.
§Using elastin–Congo red as substrate.

Difference spectra. The interaction of bovine trypsin and FMTI, both at a concentration of 8×10^{-5} M, resulted in a difference spectrum in the region 290–306 nm (Fig. 6). The difference spectrum, showed a maximum at 297 nm.

Mode of inhibition. Trypsin activity in the presence (10 and 20 μg) and absence of FMTI was measured at different substrate concentrations. The double-reciprocal plot of the kinetic data is shown in Fig. 7. It can be seen that FMTI inhibited trypsin by a competitive mechanism involving competition of the substrate and inhibitor for the same site on the enzyme.

Interaction of FMTI with inactive trypsin. To ascertain whether the zymogen or the inactive derivatives of the enzyme could compete with the active enzyme for the

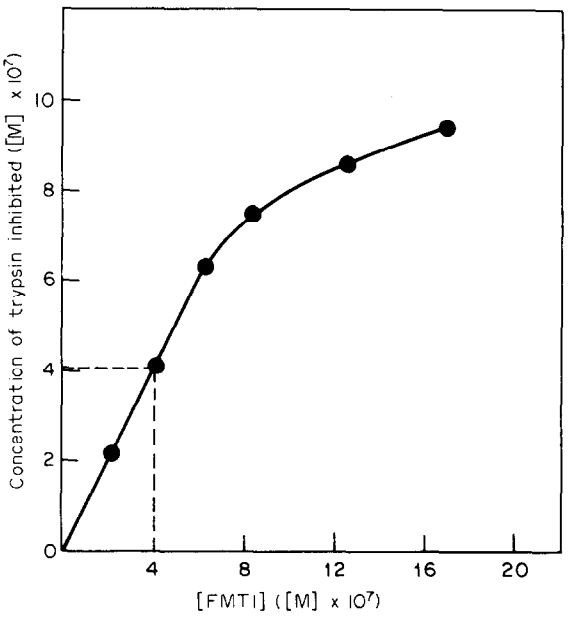

Fig. 4. Inhibition of bovine trypsin by FMTI using BAPNA as substrate. Bovine trypsin (10.5×10^{-7} M) was titrated with various amounts of the inhibitor. The apparent K_i was calculated at 4×10^{-7} M FMTI.

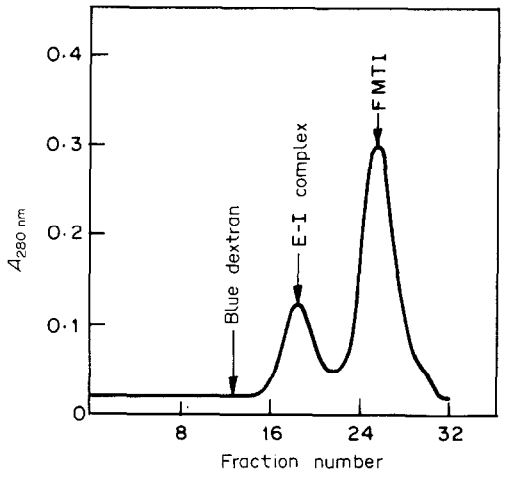

Fig. 5. Gel filtration on Sephadex G-75 (superfine) of a mixture of bovine trypsin and FMTI. FMTI (2 mg) and 1 mg bovine trypsin in 2 ml of 100 mM sodium phosphate buffer, pH 7.6, was applied to the column (1 x 80 cm) and eluted with the same buffer. Fractions, each 2 ml, were collected at a flow rate of 15 ml/hr. Protein was monitored at 280 nm (——).

inhibitor, inhibition of trypsin by FMTI in the presence of TLCK–trypsin or trypsinogen was determined. It was observed that the activity of trypsin was not altered in the presence of trypsinogen or TLCK–trypsin. Similarly, trypsinogen or TLCK–trypsin did not have any effect on the inhibitory activity of FMTI towards trypsin, even when they were present at 5- to 10-fold weight excess.

Competition studies with FMTI. Inhibitory activities of

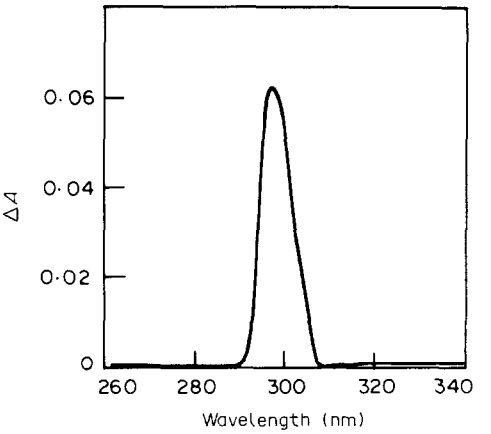

Fig. 6. Difference spectrum between FMTI, trypsin and the FMTI–trypsin complex at individual protein concentrations of 8 $\times 10^{-5}$ M in 100 mM sodium phosphate buffer, pH 7.6.

FMTI on trypsin in the presence of varying concentrations of α-amylase and on α-amylase in the presence of varing concentrations of trypsin were studied to ascertain whether the reactive sites on the inhibitor for the two enzymes were common or different. It was found that varying the concentrations of α-amylase had no effect on the TIA of FMTI and vice versa (Table 5).

Chemical modification of FMTI. (a) Modification of the amino groups. The free amino groups on FMTI were modified using TNBS. Loss of inhibitory activity with modification of the $-NH_2$ groups was observed (Fig. 8). Modification of as few as 30% of the amino groups resulted in nearly 50% inactivation and modification of 75% of the amino groups resulted in an almost complete inactivation of the TIA. Although the modification of the amino groups also had a deleterious effect on the AIA, the effect was less marked compared to that of the TIA. This indicates that the amino group(s) of FMTI were essential for the inhibitory action against trypsin.

(*b*) Modification of arginyl residues. The effects of modification of arginyl residues with 1,2-cyclohexanedione (CHD) on the TIA and AIA of FMTI were investigated. The modified inhibitor showed only a 20% loss of TIA. This indicates that the arginyl residues in the inhibitor did not have any significant role in the inhibition of trypsin by FMTI. On the other hand, modification of the arginyl residues has a more profound effect on the AIA, resulting in nearly 90% loss of activity.

DISCUSSION

A trypsin inhibitor was obtained from finger millet in an apparently pure state. The CIA and TIA present in the ammonium sulphate fraction were separated by CM-Sephadex-ion-exchange chromatography (Fig. 1). This indicates the presence of more than one proteinase inhibitor in finger millet and substantiates our earlier findings [13]. The AIA was separated into two peaks. The major portion of the AIA of the seed extract was associated with the trypsin inhibitor and copurified with it during the purification steps. The ratio of TIU to AIU was constant in the column eluates from CM-Sephadex as well as Sephadex G-50 (Table 1). This suggests that the two

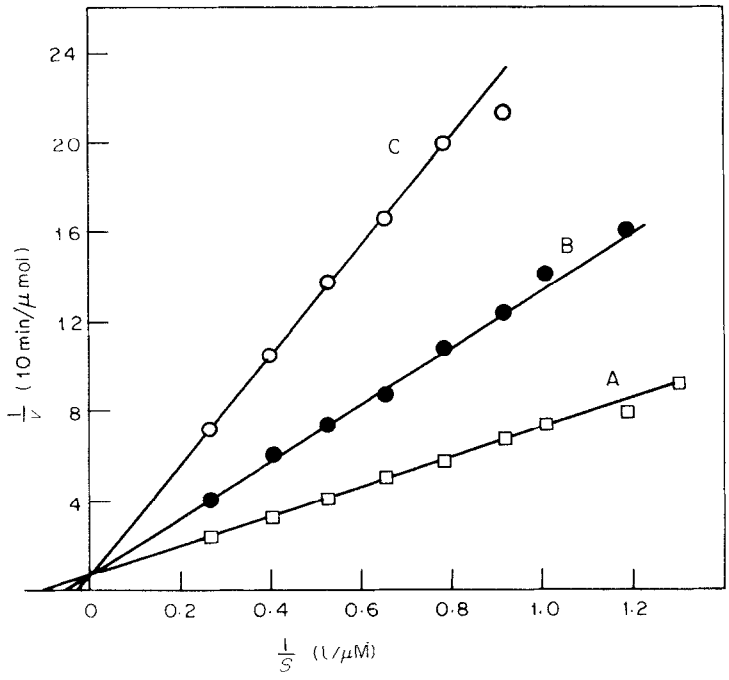

Fig. 7. Lineweaver–Burk plot of the inhibition of esterolytic activity of trypsin by FMTI. Experimental conditions are described in the text. BAPNA solution (77–383 μM) and 50 μg trypsin were added to the reaction system containing 10 or 20 μg FMTI. □——□, Without inhibitor; ●——●, with 10 μg FMTI; O——O, with 20 μg FMTI.

Table 5. Effect of varying amounts of trypsin on AIA and α-amylase on TIA of FMTI

Trypsin concn (μg)	AIU*	α-Amylase concn (μg)	TIU†
0	1.00	0	35
25	0.99	5	34
50	0.97	10	33
75	0.95	20	35
100	0.90	30	33
—	—	50	32

*Amount of α-amylase in the assay mixture was 5 μg.
†Amount of trypsin in the assay mixture was 50 μg.

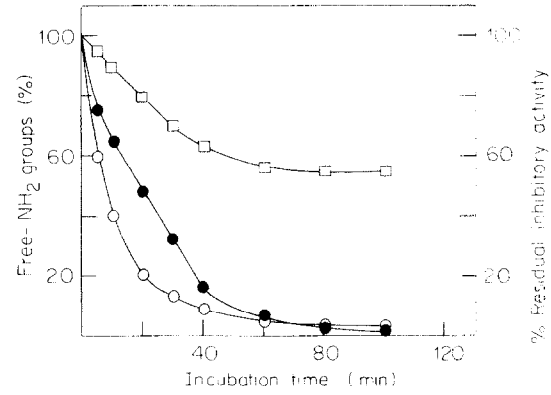

Fig. 8. Time course of modification of free amino groups with TNBS and loss of inhibitory activity in the inhibitor (FMTI). ●——●, % free amino groups; O——O, % residual TIA; □——□, % residual AIA.

inhibitory activities most probably reside in the same protein. Both activities were affected to the same extent by heat, pH and protein denaturing agents like urea, which further supports the dual action of FMTI.

The amino acid composition of FMTI is characterized by high contents of basic amino acids and glutamic acid. It is similar to the rice bran trypsin inhibitor in its amino acid composition [17]. FMTI contains eight half-cystine residues and two tryptophan residues per molecule. It is noteworthy that inhibitors isolated from rice bran [17], lettuce seed [18] and sorghum [8] contain tryptophan residues, whereas the trypsin inhibitors isolated from legumes lack tryptophan but are rich in cystine [19, 20].

Studies on the inhibition spectrum of FMTI show that the inhibition of bovine trypsin by FMTI is stoichiometric. In comparison, the inhibitory effect of FMTI on porcine trypsin is relatively weak indicating the species-specific action of the inhibitor. Similar types of observation have been made on other inhibitors also [21, 22]. FMTI showed only weak inhibition on other serine proteinases such as pronase, subtilisin-A, subtilisin-BPN′ and elastase. Bovine chymotrypsin was not inhibited by FMTI. The inhibitor had no effect on other proteinases such as papain, chymopapain, bromelin and pepsin. These observations indicate that FMTI is a potent inhibitor of trypsin. In this respect, FMTI resembles inhibitors isolated from other cereals, such as Opaque-2

corn [23], rice bran [17], sweet potato [24] and wheat [25].

An interesting feature of the specificity spectrum of FMTI is that it is endowed with a capacity to inhibit α-amylases also. It inhibited the activity of pancreatic α-amylase and salivary α-amylase, but had no effect on *Bacillus subtilis* α-amylase. The inhibitory action of FMTI against α-amylases resembles the action of α-amylase inhibitors isolated from wheat and rye [26] since the latter inhibit pancreatic α-amylase to a greater extent.

The formation of a stable FMTI–trypsin complex was demonstrated by Sephadex gel filtration studies. The isolated FMTI–trypsin complex still had AIA, indicating the presence of two different and considerably remote active sites—one for trypsin binding and the other for α-amylase binding.

The dissociation constant of the FMTI–trypsin complex was 1.2×10^{-8} M, which is in the range of dissociation constants calculated for other proteinase inhibitors [2]. The low K_i indicates a high affinity between the inhibitor and trypsin.

Spectral changes associated with the interaction of trypsin with the inhibitor resulted in the appearance of a single symmetrical peak in the difference spectrum in the region of 290–306 nm with a maximum at 297 nm. This suggests a conformational change in the FMTI, or enzyme, or both during complex formation and perhaps reflects an environmental change for tryptophan residues in the E–I complex.

The inhibition of trypsin by FMTI followed the normal competitive mechanism when enzyme activity was assayed against a synthetic substrate (BAPNA). Most of the naturally occurring proteinase inhibitors inhibit the respective enzymes competitively [1]. However, there are a few instances where the inhibition of proteolytic enzyme by proteinase inhibitors is by a non-competitive mechanism [24, 27].

Chemical modification of enzymes specifically at residues involved in the catalytic activity would appear to be one of the most powerful tools for the study of the mechanism of action of the enzymes and their protein inhibitors [10]. The competition experiments either with the zymogen (trypsinogen) or with TLCK–trypsin showed that a catalytically active enzyme is necessary for the interaction of FMTI with trypsin. These results agree with the earlier findings [28, 29] that a catalytically active enzyme is obligatory for the inhibitor–enzyme interactions, resulting in the cleavage of a specific reactive site peptide bond, as demonstrated in the case of soybean trypsin inhibitor (Kunitz) [30]. However, in the present study, it has not been demonstrated whether or not a specific peptide bond is split in the FMTI–enzyme complex.

The competition experiments with trypsin and α-amylase indicated that trypsin does not interfere with AIA, and α-amylase does not interfere with the TIA of FMTI. This suggests that two independent reactive sites are responsible for the AIA and TIA, and FMTI is a 'double-headed' protein in this respect. The fact that the isolated FMTI–trypsin complex still possessed AIA further substantiates the conclusion that the binding sites for trypsin and α-amylase on FMTI are different.

Chemical modification studies of the functional groups with selective reagents implicate the involvement of the fast reacting lysine residue in FMTI for the TIA. The modification of arginyl residues of FMTI with CHD did not have any significant effect on the TIA. However, the slight loss observed may be due to a side reaction of the reagent with lysine residues. This type of side reaction has been observed in the cases of turkey ovomucoid and lima bean inhibitor [31]. The above modification resulted in a 90% loss of AIA indicating the probable involvement of arginyl residues at the active site. This may also be due to induced conformational changes consequent to arginine modification. The chemical modification studies also support the finding that two independent reactive sites are responsible for the dual inhibitory activity exhibited by FMTI. Since lysine modification resulted in the inactivation of TIA, FMTI probably belongs to the class of 'lysine-type' inhibitors. To our knowledge, FMTI is the first proteinase inhibitor from cereal sources to possess a Lys-X reactive site. Most of the other cereal trypsin inhibitors have been identified as 'arginine-type' inhibitors [32].

Shivaraj and Pattabiraman [12] have isolated an α-amylase/trypsin inhibitor from finger millet by affinity chromatography on trypsin-Sepharose. This inhibitor appears to be different from FMTI in physico-chemical characteristics described in the present work. The MW of FMTI was determined to be 12000 ± 100 by several criteria, whereas the MW of the α-amylase/trypsin inhibitor has been reported to be 9000 and 14300 by gel filtration and SDS-PAGE, respectively [12]. The extent of thermal destruction of both TIA and AIA is also different for FMTI and the α-amylase/trypsin inhibitor. The α-amylase/trypsin inhibitor has approximately the same mobility both in cationic (pH 5.0) and anionic (pH 8.6) systems. On the other hand, FMTI exhibited virtually no mobility at pH 8.6. However, there is no report on the amino acid composition and the *N*-terminal analysis of α-amylase/trypsin inhibitor. Therefore, it is difficult to judge whether these two proteins are the same or different. In addition, there appear to be notable differences between FMTI and the α-amylase/trypsin inhibitor in their interaction with enzymes. For example, it was observed in the present investigation that modification of lysine residues in FMTI resulted in the complete loss of TIA without notable loss of AIA, whereas this modification resulted in complete inactivation of AIA of the α-amylase/trypsin inhibitor with only 40% loss in TIA [12]. Modification of the arginine residues of FMTI resulted in inactivation of AIA with a marginal loss in TIA, but this modification had no effect on the AIA of the α-amylase/trypsin inhibitor [12]. It remains to be seen whether these differences are due to intrinsic differences in the FMTI and α-amylase/trypsin inhibitor protein or due to differences in the source material.

EXPERIMENTAL

Finger millet (HPB 23-6 variety) was obtained from the germplasm collection, Main Research Station, University of Agricultural Sciences, Bangalore, India.

All biochemicals and enzymes used in this investigation were purchased from Sigma Chemical Co., except pancreatic α-amylase (pancreatin), which was obtained from Fluka. Diluted saliva (1:1000) was used as the source of salivary α-amylase. All other chemicals used were of analytical grade.

Preparation of the defatted flour. The seeds were ground to a fine powder in a Waring blender. The flour was defatted by stirring with 10 vols. of cold Me_2CO for 4 hr at 5°, filtered under suction and dried.

Assay methods. The inhibition spectrum of finger millet inhibitor was established by assay of proteolytic or esterolytic activities of the enzymes on appropriate substrates. In principle, a fixed amount of the enzyme was incubated with various amounts of the inhibitor and the residual enzyme activity was assayed.

The activities of trypsin and pronase or their inhibition were routinely assayed by the method of ref. [33], using either BAPNA or casein as substrate. The inhibitory activity towards α-chymotrypsin was determined using casein [34] or ATEE [35] as substrate. The casein digestion method [36] was used to determine the activity or inhibition of papain, chymopapain and bromelin. Elastolytic activity of elastase was determined as in ref. [37] using elastin–Congo red as substrate. The activity of pepsin was assayed by the method of ref. [38]. The esterolytic activities of subtilisin-A and subtilisin-BPN′ were assayed using ATEE as substrate in a manner similar to the α-chymotrypsin assay.

α-Amylase (salivary, pancreatic or bacterial) activity or its inhibition was measured by the method of ref. [14].

Protein was estimated by the method of ref. [39] using bovine serum albumin as standard.

PAGE was performed according to ref. [15]. The gels were stained for protein with 1% amido black in 7% HOAc and for glycoproteins by the PAS-staining technique according to ref. [40]. The PAS-staining technique was standardized using ovomucoid and ovalbumin. For visualization of the inhibitor in acrylamide gels, the gels after electrophoresis were first incubated with trypsin soln (0.1 mg/ml) and then with the substrate (*N*-acetyl-D,L-phenylalanyl-β-naphthyl ester)–dye (Diazoblue-B) mixture [41]. The TIA appeared as a clear zone against a pink background.

MW by SDS-PAGE was carried out essentially according to ref. [42]. For estimation of MW, the samples and the MW marker proteins were incubated at 50° for 1 hr in 0.1 ml Pi buffer, with 10 μl 20% SDS and 10 μl β-mercaptoethanol prior to SDS-PAGE. The method of ref. [43] was employed to determine the MW of the inhibitor by gel filtration on a Sephadex G-75 column (1 × 80 cm) equilibrated with 100 mM NaPi buffer, pH 7.0, containing 100 mM NaCl. The MW marker proteins used were bovine serum albumin (66 000), ovalbumin (45 000), chymotrypsinogen (25 400), cytochrome *c* (12 400) and insulin-B chain (3400).

Analytical ultracentrifugation was carried out in a Spinco Model E Analytical Ultracentrifuge equipped with Schlieren optics. The experiment was run at 59 787 rpm at 20° in 50 mM NaPi buffer (pH 7.0). The $S_{20,w}$ was calculated from measurements made directly from the Schlieren pattern.

Amino acid analysis was carried out with a Hitachi KLA-3B autoanalyser. The dry protein was hydrolysed at 110° in 6 M HCl in an evacuated and sealed tube for 24 hr. The tryptophan content in intact protein was determined spectrophotometrically from the alkaline spectra in 0.1 M NaOH according to the method of ref. [44]. Cystine was determined as cysteic aid after performic acid oxidation [45]. The free thiol groups in the inhibitor were estimated by reaction with DTNB [46, 47].

Amino-terminal analysis was carried out using the dansylation technique in the presence of 1% SDS [48]. Dansyl amino acid present in the hydrolysate of dansyl protein was identified by TLC on polyamide plates [49].

UV absorption spectra of the inhibitor (0.1% soln in 100 mM NaPi buffer, pH 7.0) was recorded in a UV–visible recording spectrophotometer (Carl Zeiss) using quartz cuvettes with an optical path length of 10 mm.

Isolation of the trypsin–FMTI complex. The enzyme–inhibitor (E–I) complex was isolated by gel filtration on Sephadex G-75. A mixture of inhibitor (2 mg) and trypsin (1 mg) in 2 ml 100 mM NaPi buffer, pH 7.6, was allowed to stand at room temp. for 15 min. This soln was then chromatographed on a Sephadex G-75 column (1 × 80 cm) equilibrated with 100 mM Pi buffer, pH 7.6, and the column was then developed with the same buffer soln. The $A_{280\,nm}$ of the fractions was measured. TIA and AIA in the fractions were determined.

UV difference spectra during the complex formation was recorded using a Carl Zeiss recording spectrophotometer. Two pairs of matched 10 mm cells of 2 ml capacity were placed in the reference and sample compartments in a 'tandem' fashion. The reference cuvettes contained solns of the inhibitor and of the enzyme separately on 100 mM NaPi buffer, pH 7.6. The two cells of the sample compartment contained a 1 : 1 mixture of trypsin and inhibitor solns at the same concns as in the reference cells.

Estimation of the apparent dissociation constant (K_i) of the FMTI–trypsin complex was performed according to ref. [50]. A fixed concn of enzyme (10.5×10^{-7} M) was titrated with increasing amounts of inhibitor. The equilibrium constant was obtained from the plot of enzyme concn against inhibitor concn. Molar concns were calculated assuming a MW of 12 000 for FMTI and 23 400 for bovine trypsin.

Kinetic measurements. The nature of inhibition of trypsin by FMTI was studied by incubating trypsin with varying concns of substrate (BAPNA) in the presence and absence of the inhibitor. The substrate (77–383 μM) was incubated with 50 μg trypsin in a total vol. of 9 ml for 10 min at 37°. After the incubation period, the reaction was stopped by adding 1 ml 30% HOAc. $A_{410\,nm}$ of the soln was read against a blank incubation sample, but without enzyme. The assay was repeated in the presence of 10 and 20 μg of the inhibitor in the reaction mixture.

Competition experiments. The interaction of catalytically inactive enzyme (TLCK–trypsin) and the zymogen with FMTI was measured by competitive enzymatic assays performed essentially according to ref. [51]. The preparation of TLCK–trypsin was done according to ref. [52]. In a typical experiment, 40–200 μg TLCK–trypsin or trypsinogen was incubated for 10 min at 37° with 10 μg inhibitor in 1.5 ml 67 mM NaPi buffer, pH 7.0. Then 50 μg trypsin (active) in 0.5 ml 1 mM HCl was added to this mixture and incubated further for 10 min. The residual trypsin activity was determined. In each case the control experiments were carried out without FMTI and the values were subtracted from the experimental value. FMTI was also titrated with α-amylase in the presence and absence of saturating amounts of trypsin, and with trypsin in the presence and absence of saturating amounts of α-amylase.

Chemical modification. (a) Modification of the amino groups with TNBS was carried out by the method of ref. [53]. To 1 ml protein soln (1 mg/ml), 1 ml 5% $NaHCO_3$, pH 8.5, and 1 ml 0.1% TNBS in H_2O were added. The mixture was incubated for different intervals of time at 40°. The reaction was stopped by adding 1 ml 10% SDS soln and 0.5 ml 1 M HCl, and $A_{344\,nm}$ of the soln read against a blank treated as above but containing 1 ml H_2O instead of protein soln. TIA and AIA were determined in the modified inhibitor.

(b) Modification of the arginyl residues in the inhibitor with CHD was carried out according to ref. [31]. To the inhibitor (5 mg) dissolved in 4.5 ml 1 mM triethanolamine buffer, pH 8.0, 0.5 ml CHD soln (10 mg/ml in H_2O) was added. In the control, 0.5 ml H_2O instead of CHD soln was added to the inhibitor soln. The reaction was allowed to proceed at room temp. in the dark for 12 hr. The mixture was then dialysed against H_2O for 48 hr in the cold and lyophilized. The lyophilized material was assayed for TIA and AIA.

Acknowledgements—We thank Mr. K. N. Mallanna, Plant Scientist, University of Agricultural Sciences, Bangalore, for the

supply of millet samples. N.H.M. is grateful to the Bangalore University for a Research Assistantship.

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