Isolation and Characterization of a Trypsin/Chymotrypsin Inhibitor from the Millet *Echinocloa fruneutacea*

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A trypsin/chymotrypsin inhibitor was purified 600-fold from the millet *Echinocloa fruneutacea* by ammonium sulfate fractionation and chromatographs on DEAE-cellulose and trypsin-Affigel columns. The homogeneity of the final product was confirmed by gel chromatography on Sephadex G-100 and by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The size of the inhibitor was found to be near 14000 daltons. The inhibitor reacted with bovine trypsin in 1:2 and with bovine α-chymotrypsin in 1:1 stoichiometric ratios. Chemical modification studies showed both amino and guanido groups to be essential for antitryptic activity, while only guanido groups were found to be necessary for anti-chymotryptic activity. Linearity studies without preincubation gave sigmoidal curves of inhibition. The mode of inhibition of both enzymes was found to be noncompetitive. The K_i value for trypsin was 1.51 $\times 10^{-7}$ M and for chymotrypsin 2.26 $\times 10^{-7}$ M.

Even though proteinase inhibitors from legumes have been extensively investigated (Laskowski and Kato, 1980) the corresponding factors from the millets, which are minor grains, have received attention only recently. Inhibitors from sorghum (Harish Kumar et al., 1978), finger millet (Shivaraj and Pattabiraman, 1981), pearl millet (Chandrasekher and Pattabiraman, 1982) and setaria (Udupa and Pattabiraman, 1984) have been isolated and characterized. During a screening study on the comparative action of millet extracts on the bovine and human pancreatic proteinases (unpublished observations) we observed that the extract of the millet *Echinocloa fruneutacea* inhibited the tryptic and chymotryptic activities of both the pancreatic systems. Since this millet is consumed by human subjects, it was considered worthwhile to study the inhibitor from this grain in detail. In this communication we report the isolation and some of the properties of a trypsin/chymotrypsin inhibitor from the *Echinocloa* seeds.

MATERIALS AND METHODS

Materials. *Echinocloa fruneutacea* (var, TNAU-25) grains were obtained from Tamil Nadu Agricultural University, Coimbatore, India. Bovine trypsin (salt free, twice crystallized), bovine α-chymotrypsin (salt free, three times crystallized), and porcine elastase (twice crystallized) were obtained from Millipore Biochemical Corporation, Freehold, NJ. Porcine pepsin (three times crystallized) was from CalBiochem, San Diego, CA. Subtilisin BPN′, pronase, *A. Oryzae* protease, *S. Caespitosus* protease, peroxidase, myoglobin, cytochrome c, Dalton Mark VI reference protein kit, 2-mercaptoethanol, sodium 2,4,6-trinitrobenzene sulfonate (TNBS), benzoyl DL-arginine *p*-nitroanilide (BAPNA), *N*-acetyl-L-tyrosine ethyl ester (ATEE), porcine, and rabbit pancreatic acetone powders were the products of Sigma Chemical Company, St. Louis, MO. Ethyl acetamidate, *O*-methylisourea and 1,2-cyclohexanedione were purchased from Aldrich Chemical Company, Milwaukee, WI. Ninhydrin and 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB) were procured from Pierce Chemical Company, Rockford, IL. Affigel-10 and DEAE-cellulose were from Bio-Rad Laboratories, Richmond, CA.

Partially purified (up to the ammonium sulfate stage) bovine enterokinase was prepared according to the method of Liepnicks and Light (1979). Acetone powder preparations of bovine pancreas (obtained from a slaughter house) and human pancreas (collected during autopsy) were the sources of proenzymes. These proenzymes were activated by treating 1 g of the powder with 0.1 mL of enterokinase solution in 25 mL of 0.01 M phosphate buffer, pH 7.6, for 2 h at 37 °C. The mixture was centrifuged at 10000*g* for 20 min and the supernatants were used as sources of pancreatic proteinases.

Trypsin-Affigel Was Prepared as Follows. Bovine trypsin (350 mg) in 50 mL of 0.1 M phosphate buffer (pH 7.0) was added to 50 mL (wet vol) of Affigel-10 at 4 °C and gently stirred for 6 h. The suspension was filtered under suction and washed extensively with 1.0 M ethanolamine hydrochloride in 0.1 M phosphate buffer (pH 7.0) followed by the buffer containing 0.1 M NaCl until the washings showed no absorption at 260 nm. The immobilized enzyme was stored at 4 °C in 50 mM HCl until use.

Methods. Assay of Proteinases. Caseinolytic assays of neutral proteinases were performed as described earlier (Sumathi and Pattabiraman, 1975). In routine experiments, 10 μg of bovine trypsin and 9.0 μg of bovine α-chymotrypsin were used to provide 1.4 units of activity. One unit is defined as the amount that coverts 1 mg of casein to trichloroacetic acid soluble fragments under the assay conditions (pH 7.6, 37 °C, 10 min). In other experiments 12.5 μg of porcine elastase, 40 μg of pronase, 13 μg of subtilisin BPN′, 400 μg of *A. oryzae*, 70 μg of *S. caespitosus* protease, 150 μg of human pancreatic extract, 75 μg of bovine pancreatic extract, 68 μg of porcine pancreatin, and 190 μg of rabbit pancreatin were used to get comparable caseinolytic units.

Amidolytic activity was measured according to the method of Erlanger et al. (1961) with BAPNA as substrate. In routine investigations, 10 μg of bovine trypsin, 160 μg of bovine pancreatin, 270 μg of porcine pancreatin, 190 μg of rabbit pancreatin, and 370 μg of human pancreatin were used to get an absorbance of 0.6 (λ 410) under the assay conditions (pH 7.6, 37 °C, 30 min). Esterolytic activity with ATEE as substrate was measured according to the method of Prabhu and Pattabiraman (1977). Bovine α-chymotrypsin (0.9 μg), 12 μg of activated human pancreatin, 6 μg of bovine pancreatin, 28 μg of porcine pancreatin, and 150 μg of rabbit pancreatin were used to get an OD value of 0.6 under the assay conditions (pH 7.6, 37 °C, 10 min). Enterokinase was assayed according to Bhat et al. (1981).

To determine the inhibitory activity, suitable aliquots of the inhibitor solution were included in the assay systems and the magnitude of inhibition compared to a control

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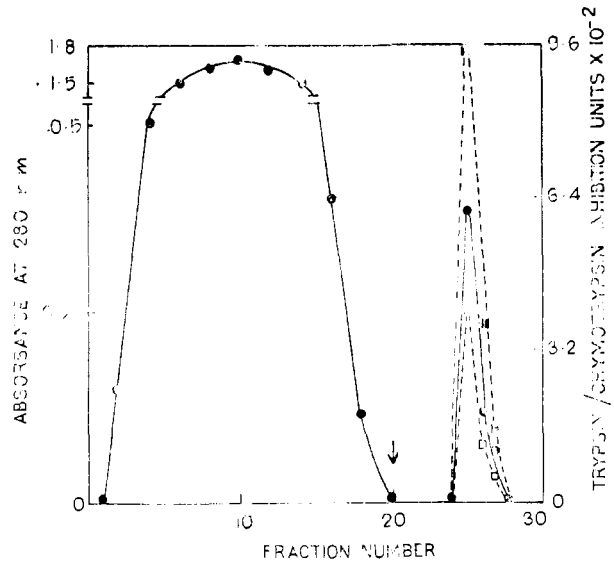

Figure 1. Chromatography of the DEAE-cellulose fraction on trypsin-Affigel. (O–O) Protein. (■–■) Trypsin inhibitory activity. (□–□) Chymotrypsin inhibitory activity. The arrow indicates the start of elution with 0.05 M HCl.

system was assessed. For routine purpose one unit of inhibitory activity is defined as the amount that depressed the caseinolytic activity by one unit.

Other Assay Methods. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. UV absorption spectrum of the purified inhibitor was measured in a Beckman DB recording spectrophotometer in 50 mM HCl.

Isolation of the Inhibitor. Finely ground *Echinocloa* grain (200 g) was homogenized with 1000 mL of 0.02 M phosphate buffer, pH 7.0, containing 0.15 M NaCl and stirred for 3 h at 4 °C. The homogenate was centrifuged at 10 000*g* for 20 min at 4 °C. The supernatant (crude extract, 755 mL) was subjected to heat treatment at 85 °C for 10 min and centrifuged at 10 000*g* for 20 min at 4 °C. The supernatant (heat treated fraction, 715 mL) was subject to 80% saturation with ammonium sulfate (400 g). The precipitate collected by centrifugation at 10 000*g* for 20 min at 4 °C was dissolved in 0.02 M phosphate buffer, pH 7.6, and dialyzed for 6 h at 4 °C against 2000 mL of the buffer. The cloudy solution was centrifuged.

The clear supernatant (ammonium sulfate fraction, 57 mL) was allowed to flow through (30 mL h^{-1}) a column of DEAE-cellulose (2.5 × 16.3 cm^2, bed vol, 80 mL) equilibrated with 0.02 M phosphate buffer, pH 7.6. The column was washed with 10 bed volumes of the buffer. The inhibitor bound was eluted with a linear salt gradient formed between 200 mL of the buffer and 200 mL of buffer containing 0.5 M NaCl. Fractions (20 mL) were collected. Fraction numbers 8–11 containing the activity were pooled.

This DEAE fraction was passed through a column of trypsin-Affigel (0.9 × 35 cm^2, bed volume 20 mL) equilibrated with 0.02 M phosphate buffer pH 7.6 containing 0.5 M NaCl at a flow rate of 10 mL h^{-1}. The column was washed with 200 mL of the equilibration buffer. The inhibitor was eluted with 0.05 M HCl and 10-mL fractions were collected. The active fractions (tube no. 25–27, Figure 1) were pooled, dialyzed against 2000 mL of deionized water, and concentrated by lyophilization to 3.0 mL. This solution (trypsin-Affigel fraction) was used for further studies.

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was performed by using 0.04 M Tris-glycine buffer pH 8.3 at a current of 3 mA/tube for 1 h. Electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol was done for 10 h at 7 mA/tube at pH 7.2 with 10% gel. Protein bands were stained with Coomassie Brilliant Blue R-250. For molecular weight determination, Dalton Mark VI containing lysozyme, β-lactalbumin, trypsinogen, pepsin, ovalbumin, and bovine serum albumin was used as standard.

Gel Chromatography. The inhibitor (950 μg protein) was subjected to gel chromatography on Sephadex G-100 (0.9 × 58 cm^2, bed vol 37 mL, equilibration buffer and elution buffer 0.02 M phosphate, pH 7.6, containing 0.1 M NaCl, flow rate 5 mL h^{-1}, and fraction volume 1.0 mL). The fractions were analyzed for protein, antitryptic, and antichymotryptic activities. For molecular weight determination, lysozyme, cytochrome c, myoglobin, and α-chymotrypsin were used. For studies on complex formation, 145 μg of protein of the inhibitor was mixed with 580 μg of bovine trypsin in the presence of 40 μmol of phosphate buffer, pH 7.6, at 4 °C and subjected to chromatography. Fractions were analyzed for tryptic activity, antitryptic activity, and protein. Similarly a mixture of inhibitor (145 μg protein) with α-chymotrypsin (260 μg of protein) was subjected to gel chromatography and the fractions were analyzed. The mixtures at the concentrations of inhibitor and enzymes used did not have either residual inhibitory activity or caseinolytic activity. The enzyme solutions for these studies were dialyzed overnight against 10^{-3} M HCl at 4 °C with one change of the outer solution. The protein concentrations were measured in the dialyzed solutions.

Effect of pH and Temperature on the Stability of the Inhibitor. The inhibitor was exposed to different pHs and temperatures for varying periods of time (details are described elsewhere, Udupa and Pattabiraman, 1984) and the residual antitryptic and antichymotryptic activities were measured.

Effect of Pepsin and Pronase. The inhibitor (15 μg of protein) was incubated with 75 μg of pepsin at 37 °C in the presence of 50 mM HCl–KCl buffer, pH 2.0, in a volume of 2.5 mL. Aliquots were withdrawn at definite intervals of time into tubes containing 0.5 mL of 0.2 M phosphate buffer, pH 7.6. Residual inhibitory activities of the solutions were measured. The inhibitor (15 μg protein) was also treated with 150 μg of pronase in the presence of 50 mM phosphate buffer, pH 7.6, at 37 °C in a volume of 2.5 mL. Aliquots at definite intervals of time were subjected to heat treatment at 85 °C for 15 min to inactive pronase and assayed for residual inhibitory activities.

Chemical Modification Studies. Arginine residues are modified with ninhydrin (Chaplin, 1976) and with CHD (Abe et al., 1978). Amino groups were modified with TNBS (Haynes et al., 1967), ethyl acetamidate (Hunter and Ludwig, 1972), and *O*-methylisourea (Kimmel, 1967). Sulfhydryl groups were modified with DTNB (Ellman, 1959). The details of the methods were described earlier (Udupa and Pattabiraman, 1984).

RESULTS

The protease inhibitor from *Echinocloa* grains was purified about 600-fold with 51% recovery of the activity (Table I). The ratio of antitryptic to antichymotryptic activity remained fairly constant during different stages of purification indicating that a single factor is responsible for both the activities. The inhibitor had a λ_{max} at 276 nm and λ_{min} at 254 nm with an absorption ratio of 1.36. The inhibitor was found to be homogeneous by PAGE and SDS-PAGE (Figure 2). During gel chromatography on Sephadex G-100 the inhibitor was eluted as a single peak

Table I. Purification of the Protease Inhibitor from *Echinocloa* Grains

steps	total protein, mg	antitryptic activity: inhibitor units	antitryptic activity: specific activity	antitryptic activity: percent recovery	antitryptic/ antichymotryptic activity
crude extract	2720	2900	1.07	100	2.28
heat treated raction	2230	2750	1.23	94.6	2.27
ammonium sulfate fraction	520	2330	4.47	80.2	2.30
DEAE-cellulose fraction	1.54	1610	105	55.6	2.38
Trypsin-Affigel fraction	2.35	1470	625	50.7	2.35

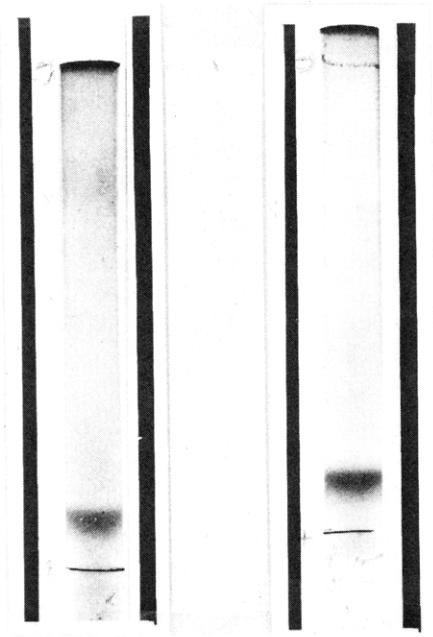

Figure 2. (a) SDS-PAGE and (B) PAGE of the purified inhibitor.

with M_r of 13 500. This value agreed well with 13 800 obtained by SDS-PAGE.

Heat treatment at 100 °C for 1 h at pH 7.0 resulted in the loss of 35% of the antitryptic activity and 50% of the antichymotryptic activity of the pure inhibitor. Pressure cooking (10^5 Pa, 15 min) resulted in the loss of 40% of the antitryptic activity and almost complete loss of the antichymotryptic activity. In contrast, the inhibitory activity in the crude extracts of echinocloa grains was less stable to heat. Heat treatment at 100 °C for 1 h or autoclaving for 15 min caused complete abolition of both antitryptic and antichymotryptic activities of the crude extract. These data and the data on the stability of the purified inhibitor on exposure at 90 and 100 °C for different intervals of time (data not shown) indicated that the antichymotryptic activity is more heat labile. The studies on the effect of pH on the stability of the purified inhibitor also led to the same conclusion. Both the antitryptic and antichymotryptic activities of the inhibitor were fully stable on exposure to solutions with a wide range of pH (1–9) for 24 h at 4 °C. Beyond pH 10 the inhibitor gradually lost its activity, the loss of antichymotryptic activity being faster (pH 10.0, 30 h loss 17%, 72 h loss 37%; pH 12.0, 30 h loss 61%, 72 h loss 69%) than the loss of antitryptic activity (pH 10.0, 30 h and 72 h loss nil; pH 12.0, 30 h and 72 h loss 50% and 55%). The inhibitor was highly resistant to proteolysis with pepsin and pronase. Treatment with pepsin up to 3 h did not result in any loss of either antitryptic or antichymotryptic activity. With pronase there was a loss of 40% of the antitryptic activity after 1 h of treatment. thereafter there was no further loss up to 6 h. In regard to antichymotryptic activity, there was a gradual loss culminating in 60% loss of activity after 6 h of treatment.

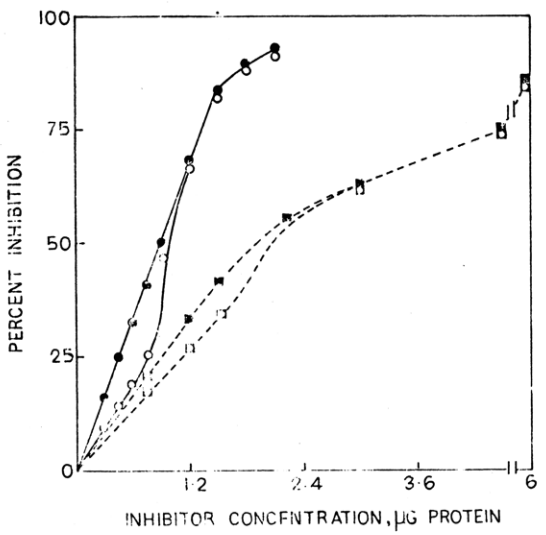

Figure 3. Effect of inhibitor concentration on trypsin activity (●–●) with preincubation for 10 min and (○–○) without preincubation and on chymotrypsin activity (■–■) with preincubation and (□–□) without preincubation.

The inhibitor had no action on the proteolytic activity of subtilisin BPN′, *A. oryzae* protease, *S. caespitosus* protease, pronase, or porcine elastase when assayed by the caseinolytic method. Inhibitor had no effect on bovine enterokinase. The data on the effect of preincubation for 10 min of the inhibitor with trypsin or chymotrypsin are compared with the inhibitory profiles without preincubation in Figure 3. While with preincubation the inhibition was linear with respect to inhibitor concentration over a wide range, without preincubation the patterns obtained were sigmoidal. The phenomenon was observed irrespective of whether casein or synthetic substrates were used. The mode of inhibition of trypsin as well as chymotrypsin was found to be noncompetitive. The calculated K_i values for the two enzymes were 1.51×10^{-7} M and 2.26×10^{-7} M, respectively. On the basis of preincubation data the stoichiometric ratio of interaction between the inhibitor and trypsin and between the inhibitor and α-chymotrypsin were found to be 1:2.1 and 1:1, respectively.

The data on complex formation between the inhibitor and target enzymes are shown in Figure 4. With trypsin, the inhibitor formed a complex with an M_r of 36 000 (Figure 4 part A). Since the stoichiometric ratio indicated the possible formation of a trimer complex between one inhibitor molecule and two trypsin molecules (hypothetical M_r 64 000) we used twice the amount of trypsin compared to inhibitor in terms of molar concentration. However, no evidence for a trimer complex formation was obtained. Apart from the protein peak corresponding to the complex (M_r 36 000), a peak corresponding to trypsin (with enzyme activity, not shown in the figure) and a minor peak corresponding to inhibitor (but without inhibitory activity) were discerned. In the case of chymotrypsin also (Figure 4 part B) a protein peak corresponding to M_r of 36 000 was observed, indicating the formation of a 1:1 chymotrypsin

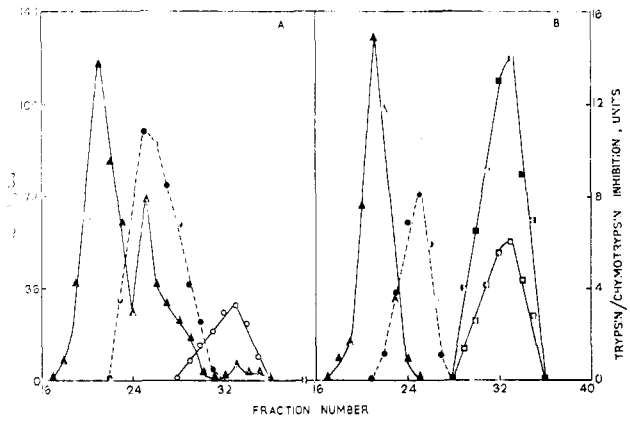

Figure 4. Studies on complex formation. (A) Inhibitor and Trypsin. A mixture of 145 μg of inhibitor and 580 μg of trypsin was subjected to gel chromatography on Sephadex G-100 as described under materials and methods and the protein (▲–▲) elution profile was determined. Native trypsin protein (●–●) profile. Native inhibitor protein (○–○) profile. (B) Inhibitor and chymotrypsin. A mixture of 145 μg of inhibitor and 260 μg of α-chymotrypsin was subjected to gel chromatography on Sephadex G-100 as described under materials and method and the protein elution profile (▲–▲) was determined. Native α-chymotrypsin protein (●–●) profile. Native inhibitor antitryptic activity (■–■) and antichymotryptic activity (□–□). The elution profile suggests that the same protein is responsible for both the inhibitory activities.

Table II. Action of *Echinocloa* Inhibitor on Different Pancreatic Extracts

enzyme system	inhibitor concentration (μg of protein) to cause 50% inhibition		
	caseinolytic activity	amidolytic (BAPNA) activity	esterolytic (ATEE) activity
bovine pancreatin	1.50	0.83	0.063
human pancreatin	1.88	1.20	0.130
rabbit pancreatin	1.40	0.83	1.07
bovine trypsin	0.85	0.75	
bovine chymotrypsin	2.00		0.21

inhibitor complex. Unlike the trypsin-inhibitor complex which had no antichymotryptic activity, this complex had weak antitryptic activity (not shown in the figure). The capability of chymotrypsin-inhibitor complex to inhibit trypsin was further confirmed by the following experiment. The inhibitor (60 μg of protein) was mixed with 100 μg of α-chymotrypsin in the presence of 400 μmol of phosphate buffer, pH 7.6. After 20 min, aliquots were withdrawn and assayed for antitryptic activity by using BAPNA as substrate. The antitryptic activity of chymotrypsin-treated inhibitor was about 60% of the native inhibitor.

The effect of the inhibitor on the caseinolytic, amidolytic (BAPNA), and esterolytic (ATEE) activities of four pancreatic preparations is shown in Table II. Even though the magnitude of caseinolytic and amidolytic inhibitions are roughly of the same order for the different pancreatic preparations, the inhibitor had relatively poor action on the chymotryptic activities of rabbit pancreas and porcine pancreas.

The data on chemical modification of the inhibitor and the concomitant changes in inhibitory activities are summarized in Table III. It was observed that the antichymotryptic activity was lost rapidly compared to antitryptic activity on modification of the guanido groups as

Table III. Effect of Chemical Modification on the Action of the Inhibitor

modifier	time, h	percent residual activity against	
		trypsin	chymotrypsin
ninhydrin	1	58	63
	3	56	36
	24	54	10
ninhydrin[a]	1	47	28
	2	40	0
CHD	6	92	57
	24	67	21
TNBS	3	67	100
	24	42	100
TNBS[a]	3	58	100
	24	25	100
ethyl acetamide	1	60	100
	3	40	100
O-methylisourea	3	60	100
	6	32	100
TNBS + CHD	3	54	
	24	0	
TNBS[a] + CHD	3	21	
	6	0	
DTNB	48	100	100

[a] Chemical modification after subjecting the inhibitor solution to heat treatment at 100 °C for 3 min and cooling.

evidenced by the action of ninhydrin and CHD on the native inhibitor. When the inhibitor was subjected to heat treatment (100 °C, 3 min) prior to the addition of the modifiers the loss of inhibitory activity was more. Treatment with TNBS, ethyl acetamidate, and *O*-methylisourea caused loss of antitryptic activity alone. The heat treated inhibitor on treatment with TNBS lost more of the antitryptic activity, but the antichymotryptic activity was not affected. Treatment with DTNB had no effect on the inhibitor.

While the purified inhibitor in solution was stable with respect to antitryptic and antichymotryptic activities when stored at room temperature (28–30 °C) for 10–15 days, the crude extract of the *Echinocloa* seeds behaved differently when stored under asceptic conditions. While the antitryptic antivity was stable, there was a sharp fall in the antichymotryptic activity. After 24 h, there was a loss of 50% of the activity and by the 5th day there was complete abolition of antichymotryptic activity.

DISCUSSION

Trypsin is known to cause a selective cleavage of a peptide bond at the reactive site of the proteinase inhibitors (Laskowski and Kato, 1980) or proteolysis at secondary sites (Schneider et al., 1974). We have used immobilized trypsin during purification of the *Echinocloa* inhibitor. The possibility of limited cleavage of the inhibitor during this step cannot be ruled out. However, the inhibitor did not lose its activity during this step and the studies on homogeneity did not indicate any evidence for extensive modification.

The protease inhibitor from *Echinocloa* resembles the counterparts from the millets setaria (Udupa and Pattabiraman, 1984), pearl millet (Chandrasekher and Pattabiraman, 1982), fingler millet (Shivaraj and Pattabiraman, 1981) and sorghum (Harish Kumar et al., 1978) in molecular size, heat stability, and stability under acidic conditions. However, it shows some interestingly different properties. Preincubation with the target enzymes was found to be essential to elicit maximal inhibitory response only when the inhibitor concentration was low. This resulted in biphasic curves when preincubation was not performed. Similar patterns have been observed with the

inhibitor from germinating barley seeds (Boisen et al., 1982) and for the thiol protease inhibitor of rat epidermis (Takeda et al., 1983).

The sites of interaction with trypsin and chymotrypsin even though are not mutually exclusive in the inhibitor, they appear to be partially distinct. Chemical modification studies reveal that amino groups are not essential for the action of the inhibitor on chymotrypsin whereas they are necessary for tryptic action, whereas guanido groups were essential for both the activities. Thus the chymotrypsin-inhibitor complex was found to inhibit trypsin, although to a reduced extent, whereas the reverse was not found to be true. Even though based on stoichiometric studies the inhibitor was expected to form a complex with trypsin in a 1:2 molar ratio, gel chromatographic studies did not provide evidence for such a stable complex. It is probable that at the second site of binding with trypsin the interaction is weak and could not be identified during the long period employed for gel chromatographic studies.

The chymotrypsin site of the inhibitor appears to be more exposed and easily susceptible to alterations. Thus heat treatment, exposure to high alkaline condition, treatment with pronase, and guanido groups modification resulted in more rapid loss of antichymotryptic activity. This is further supported by the observation that in the crude extracts of *Echinocloa* seeds there is rapid loss of antichymotryptic activity whereas the antitryptic activity was fully stable on storage. It has been reported earlier that the *Echinocloa* extracts did not possess antichymotryptic activity (Chandrasekher et al., 1982). The present observation on the disappearance of antichymotryptic activity explains this difference.

The relative ridigity of the trypsin site is supported by the observations on chemical modification. Heat treatment of the inhibitor prior to addition of amino group modifiers enhanced the rate of disappearance of the antitryptic activity. A similar observation has been made with alocasia trypsin/chymotrypsin inhibitor (Sumathi and Pattabiraman, 1977).

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Registry No. Trypsin inhibitor, 9035-81-8; proteinase inhibitor, 37205-61-1; chymotrypsin, 9004-07-3; trypsin, 9002-07-7.

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Isolation of an Amylase Inhibitor from *Setaria italica* Grains by Affinity Chromatography on Blue-Sepharose and Its Characterization

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An α-amylase inhibitor from *Setaria italica* grains was purified 150-fold by chromatography on Blue-Sepharose after neutralization of the acid extract and ammonium sulfate fractionation. The inhibitor was found to be homogenous by polyacrylamide gel electrophoresis and gel chromatography on BioGel P-30. The molecular weight was found to be 24 K. SDS-PAGE showed that it is made up of two dissimilar polypeptides. Affinity chromatography on immobilized porcine pancreatic amylase and analysis showed that both the polypeptides are essential for the action of the inhibitor. The setaria inhibitor acted on human salivary amylase, human pancreatic amylase, and porcine pancreatic amylase but had no action on *B. subtilis* and *A. oryzae* amylases. It was labile to heat and to extreme acidic and alkaline conditions. Pronase, pepsin, trypsin, and α-chymotrypsin inactivated the inhibitor. Amino groups and guanido groups were found to be essential for its action.

Several of the proteinaceous α-amylase inhibitors from the plant kingdom have been purified by conventional methods (Buonocore et al., 1977; Warchalewski, 1983) or by affinity chromatography with immobilized α-amylase (Marshall and Lauda, 1975; Chandrasekher and Pattabiraman, 1983). During attempts to isolate the amylase inhibitor which was reported to be present in the millet

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