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An acid stable trypsin-chymotrypsin inhibitor from horse gram *(Dolichos biflorus)*

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Abstract. An inhibitor of trypsin and chymotrypsin was purified from horse gram (*Dolichos biflorus*) beans. The concentration of the inhibitor which provided total inhibition was 0.27 µg/µg tryptic enzyme and 0.46 µg/µg chymotryptic enzyme. The inhibitor was stable at 37°C between pH of 3 to 11 and at 97°C, upto pH 5.0 only. While the activities were rapidly lost in 0.1N NaO H the loss was only 5 0% in 0.1N HCl when kept for 2 h at 97°C. On heating at pH 7.8, it remained stable upto 80°C with a gradual loss in activities at 97°C and a total loss occurring by autoclaving at 15 psi for 10 min. Reduction of disulphide bonds by 2-mercapto-ethanol, pronase digestion and boiling in the presence of 1 M NaCl led to reduction in the activities. However, the inhibitor was resistant to the action of pepsin and subtilisin and to urea at 37°C.

Keywords. Trypsin-chymotrypsin inhibitor; horse gram; protease inhibitor; *Dolichos biflorus.*

Introduction

Legume seeds invariably contain inhibitors of proteases. The most widely studied of these inhibitors are the trypsin and chymotrypsin inhibitors (Liener and Kakade, 1970). These inhibitors are generally grouped under three categories—those inhibiting either trypsin or chymotrypsin alone and those which inhibit both trypsin and chymotrypsin. Recently trypsin inhibitors from mung bean (Chrispects and Baumgartner, 1978), Faba beans (Bhatty, 1977) and kintoki bean (Miyoshi *et al.,* 1978) have bean studied. Ray (1970) reported that horse gram (*Dolichos biflorus*), a commonly used pulse of India, could not support growth in rats but on autoclaving this effect was reversed. The presence of a trypsin inhibitor in the horsegram was suspected. Subbalakshmi *et al.* (1976) found that both the horse gram and moth bean exhibited trypsin inhibiting activity, and this was reduced upon heat treatment. In the light of these reports it was felt useful to purify the trypsin inhibitor from horse gram and to study its properties. In the present study this inhibitor was found to inhibit both trypsin and chymotrypsin.

Abbreviations used: BTEE, benzoyltyrosine ethyl ester; BAEE, benzoylarginine ethyl ester.

Materials and methods

Horse gram was obtained from the local market. Pancreatic α-chymotrypsin (4x crystallised) and trypsin (3x crystallised) were purchased from British Drug House Ltd., Poole, England and Sigma Chemical Co., St. Louis, Missouri, USA, respectively. All other chemicals, enzymes and proteins were from Sigma Chemical Co., St. Louis, Missouri, USA; British Drug House Ltd., Poole, England or E. Merck, Darmstadt, Germany.

Assay of trypsin and chymotrypsin inhibiting activities

(a) *With casein as the substrate:* The method of Kunitz (1948) was used. 0.5 ml of the inhibitor solution was incubated for 15 min at 37°C with an equal volume of trypsin (62.9 μg in 5mM $CaCl_2$) or chymotrypsin (50 μg) in 0.2 M borate buffer, pH 7.8. One ml of 1% casein solution in the same buffer was added and incubation continued for 20 min. Reaction was stopped by the addition of 3 ml of 5 % trichloro-acetic acid. The trichloroacetic acid-soluble product in the supernatant was measured by Folin-Ciocalteu procedure, the absorbance at 620 nm providing a measure of enzyme activity. One unit (U) of the inhibitor activity was expressed as decrease by one unit of absorbance measured at 620 nm in 20 min.

(b) *With synthetic substrate:* The method described by Rhodes *et al.* (1960) was followed. A solution of trypsin (62.9 μg in 0.3 ml) or chymotrypsin (50 μg) in 0.004 M acetic acid and 0.02 M $CaCl_2$ was mixed with 0.7 ml of inhibitor solution in 0.006 M Tris-HCl buffer, pH 8.8, followed by 1 ml of 0.006 M Tris-HCl buffer, pH 8.2. After incubation at 37°C for 15 min, 2 ml of substrate-indicator mixture [0.02 M benzoylarginine ethyl ester (BAEE) for trypsin and 0.008 M benzoyltyrosine ethyl ester (BTEE) for chymotrypsin in suitable buffer containing indicator 0.2% *m*-nitrophenol] was added and change in absorbance at 450 nm was measured. The results are expressed as per cent residual enzyme activity.

Estimation of protein

Protein in the fractions from column chromatography was determined according to the method of Lowry *et al.* (1955). Total nitrogen in the lyophilised material was determined by the micro Kjeldahl method of A.O.A.C. (1955), total protein being obtained by using a factor of 6.25.

Polyacrylamide disc-gel electrophoresis

It was done according to the method described by Othmer (1971) using 10% separating gel at pH 7.5 and a constant current of 4 mA per tube for 2 h at room temperature. Two identical gels were run and one was stained with Amido black 10B for protein. The corresponding protein bands from the unstained gel were cut out and protein was extracted with 0.2 M borate buffer pH 7.8. Inhibitory activities were tested in the extracted protein solution.

Activities of trypsin or chymotrypsin complex with inhibitor

The inhibitor (25 μg) in 0.6 ml of 0.006 M Tris HCl buffer pH 8.6 were mixed with 0.3 ml of trypsin (88.1 μg) or chymotrypsin (55.6 μg) solution in 0.004 M acetic acid containing 0.02 M $CaCl_2$ and incubated for 10 min at 37°C to form respective

complexes. To the chymotrypsin-inhibitor or trypsin-inhibitor complex, 0.3 ml of trypsin solution (87.36 µg) or chymotrypsin solution (50 µg) was added, respectively. It was incubated for 10 min at 37°C before addition of the respective substrate-indicator solution and the absorbance at 450 nm was measured. Enzym activities were also measured with and without the inhibitors as a control.

Stability studies

For these studies solutions of the inhibitor were prepared in buffers of different pH values, in 0.l N NaOH, in 0.1 N HCl, in 1 M NaCl and in 6 M urea and these solutions were incubated at temperatures of 37°C and 97°C for different time periods. The concentrations of the inhibitor used in these studies were 71 µg/ml for antitrypsin activity and 40 µg/ml for antichymotrypsin activity. After cooling, aliquots were removed at various time intervals and pH was adjusted to 7.8 where ever needed by addition of 0.2 M borate buffer of suitable pH. The activities were measured against trypsin and chymotrypsin using casein as the substrate. The buffer systems used in this study were 0.05 M citrate buffer pH 3.0 and 5.0, 0.05 M borate buffer pH 7.0 and 9.0 and 0.05 M glycine-NaOH buffer pH 11.0.

Digestion with protease enzymes

Solutions of the inhibitor in 0.2 M borate buffer, pH 7.8 were incubated with proteases subtilisin BPN and pronase (150 µg enzyme/mg inhibitor) at 37°C for 48 h. For pepsin digestion, the inhibitor and enzyme solutions were made in 0.06 N HCl (100 µg pepsin/mg inhibitor) and incubated at 37°C for 48 h. Aliquots were removed and placed in boiling water bath for 2 min to inactivate the enzymes and adjusted to pH 7.8 after cooling before assaying for inhibitor activity. A control with heat inactivated enzyme was also run.

Reduction and reoxidation

Reduction of disulphide bond was done as described by Simlot *et al.* (1966) by dialysing the inhibitor solution against 0.1 M 2-mercaptoethanol for 10 h followed by dialysing against 0.05 M borate buffer pH 7.0 in cold. Reoxidation of reduced disulphide bond was done by exposing the solution to air at pH 8.2 for 10 and 20 h at room temperature.

Results

Extraction of the inhibitor from horse-gram

200 g. of ground horse gram (*Dolichos biflorus*) was defatted with carbon tetrachloride until the pigment was removed. The cake was dried at 40°C for 24 h to remove the solvent completely. The defatted meal (100 g) was extracted with 300 ml of 0.2 M acetate buffer, pH 4.2, in cold for 8 h and then centrifuged for 30 min at 900 *g*. Supernatant was collected and the residue reextracted twice with 125 ml each of the buffer in cold. All the three supernatants were pooled and dialysed against distilled water for 36 h in cold with changes of distilled water after every 6 h Antitrypsin, antichymotrypsin activities and protein were determined in the lyophilised material. The specific antitrypsin and antichymotrypsin activities were found to be 8.1 U/mg and 1.8 U/mg protein, respectively.

Preparation of trichloroacetic acid soluble fraction

To the dialysed extract from the previous step, 50% trichloroacetic acid solution was gradually added in cold with continuous stirring till the final concentration of trichloroacetic acid solution was 2.5%. The mixture was kept overnight in cold followed by centrirugation for 20 min at 900 *g.* The supernatant was decanted off and the precipitate was washed twice with 40 ml each of cold 2.5% trichloroacetic acid solution. Supernatants were pooled and dialysed against distilled water for 40 h in cold with change of water after every 6 h. The dialysed extract was lyophilised and designated as trichloroacetic acid-soluble fraction. The specific antitrypsin and antichymotrypsin activities of this fraction were 25 U/mg and 4.8 U/mg protein, respectively.

DEAE-cellulose chromatography of trichloroacetic acid-soluble fraction

Lysophilised trichloroacetic acid-soluble fraction (30 µg) dissolved in 5 ml of 0.01 M Tris-HCl buffer, pH 8.4, was applied to a column (20r1.7 cm) of DEAE-cellulose equilibrated with 0.01 M Tris-HCl buffer, pH 8.4, stepwise elution with the same buffer containing 0, 0. 1, 0.15 and 0.2 M sodium chloride was carried out at room temperature. Fifty five fractions of 5 ml each were collected and protein and antitrypsin activity in each fraction was estimated. The result is shown in figure 1A.

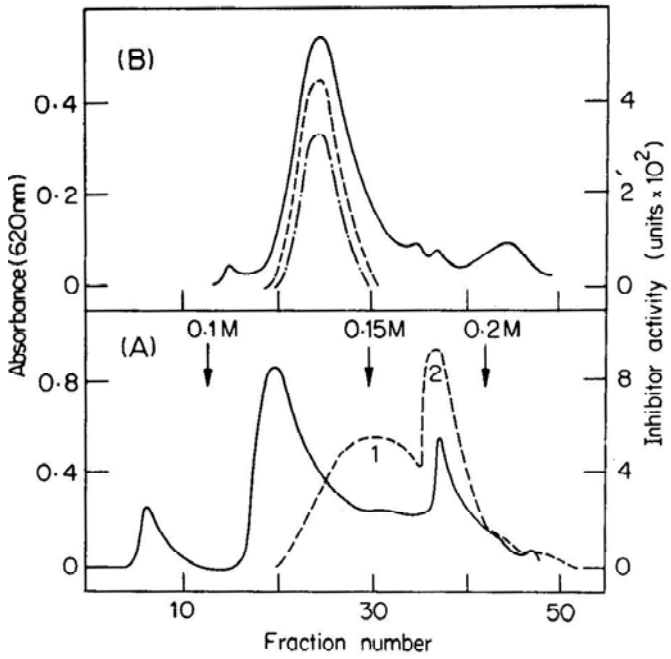

Figure 1. Purification of the inhibitor of trypsin and chymotrypsin.

A) DEAE-cellulose chromatography. Stepwise elution with increasing concentrations of NaCl (0, 0.1 M, 0.15 M and 0.2 M) in buffer 0.01 M Tris-HCl, pH 8.4. Arrows indicate the place of change in eluent. Fraction volume 5 ml/tube.

B) G-50 Sephadex column chromatography of peak-1 from (A) Eluent was double distilled water. Fraction vol. 4 ml/tube.

Protein —— ; Trypsirinhibitor -----; Chymotrypsin inhibitor(– . –).

Antitrypsin activity was present in two peaks. Fractions corresponding to these were pooled, dialysed against distilled water in cold and then lyophilised. Pooled fractions 24-34 was designated as peak-1 and from 36-40 as peak-2. The specific inhibitory activities against trypsin and chymotrypsin were 68.7 U/mg protein and 13.1 U/mg protein in peak-1 and 43 U/mg protein and 8.5 U/mg protein in peak-2, respectively. Peak-1 with higher specific activity was used for further purification of the inhibitor.

Gel filtration on Sephadex G-50

Lyophilised peak-1 was chromatographed at room temperature on a column of Sephadex G-50 (81 × 1.7 cm) and eluted with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M KCl. Fifty fractions of 4 ml each were collected and this was analysedforprotein, antitrypsin and antichymotrypsin activities. When the pooled fractions were dialysed against distilled water in cold prior to lyophilisation, 70% of the total inhibitory activity was found to have been lost. As an alternative, the gel filtration chromatography was then carried out using double distilled water in place of the buffer. The elution pattern with respect to the two inhibitory activities did not change with distilled water and is shown in figure 1B. Fractions corresponding to the peak of antitrypsin and antichymotrypsin activities were pooled and lyophilised. The specific activities of this material were antitrypsin 138.8 U/mg protein and antichymotrypsin 29.7 U/mg protein. This material was used for the study of the properties of the inhibitor. Table 1 records the stepwise purification of the inhibitor. The final purification achieved during the whole

Table 1. Stepwise purification of inhibitor from horse gram.

Steps	Antitrypsin activity				Antichymotrypsin activity			
	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)
1. Crude extract	42,770	8.1	100	—	9396	1.8	100	—
2. TCA soluble fraction	8,254	25.0	19.3	3	1578	4.8	16.8	3
3. DEAE-Celluiose chromatography								
Peak-1	4,439	68.7	10.4	8.5	847	13.1	9.0	7
Peak-2	1,318	43.0	3.1	5	259	8.5	2.8	5
4. G-50 sephadex chromatoraphy of peak-1	2,891	138.8	6.8	17	619	29.7	6.6	16.5

process was 17 fold for both the inhibitor activities. It may be noted that during each step of purification, the antitrypsin and antichymotrypsin activities remained together suggesting that the inhibitor could be a single component having dual activities.

Polyacrylamide disc gel electrophoresis of the purified inhibitor

Two closely positioned protein bands were observed and both were active against both trypsin and chymotrypsin. The ratio of antitrypsin and antichymotrypsin activities was almost the same (5.2 and 5.4) in both these bands indicating that the inhibitors could be isoinhibitors.

Molecular weight of the inhibitor

Tentative molecular weight of the inhibitor was found to be 13,500 daltons from its elution pattern on a G-50 Sephadex column (not shown) determined according to the method of Andrews (1964). The reference proteins used were alpha chymotrypsiongen (25,000), myoglobin (17,800), ribonuclease (13,700) and cytochrome C (12,400). Elution was carried out with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M KCl.

Inhibition of trypsin and chymotrypsin

Inhibition of the esterolytic activities of the trypsin and chymotrypsin using BAEE and BTEE as the synthetic substrates, respectively, was studied with increasing concentrations of the horse gram inhibitor (figure 2). Even at high concentraions

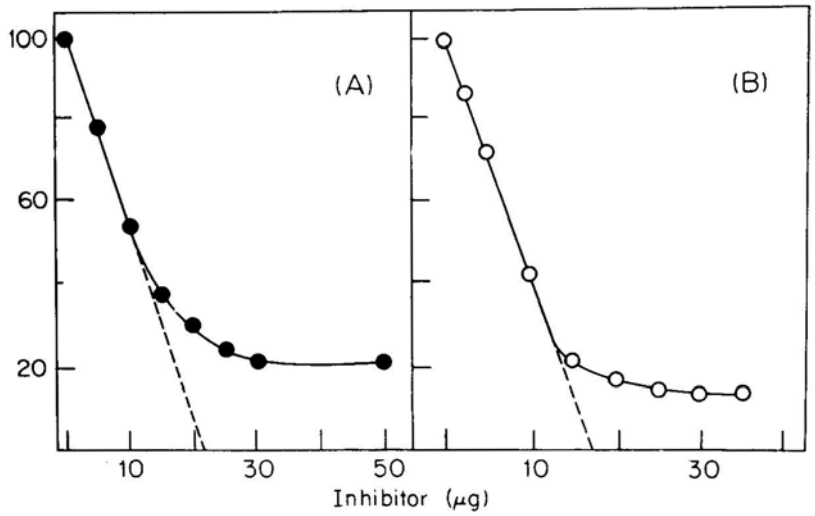

Figure 2. Inhibitor pattern against trypsin and chymotrypsin (for details see text).
A) Chymotrypsin inhibition. Amount of chymotrypsin = 5 0 µg
B) Trypsin inhibition. Amount of trypsin=62.9 µg
Broken line denotes the extrapolation of the linear part of the curve for total inhibition.

of the inhibitor, complete inhibition of the enzymes did not occur. 22% of the original alpha-chymotryptic and 13 % of the original tryptic activities remained unaffected after treatment with the inhibitor. An equilibrium between the enzyme and the inhibitor appears to have been reached at the high concentration. Extrapolation of linear part of the curves gave values of the amount of inhibitor required to completely inhibit the enzymes. 17.25 µg of inhibitor was required to

inhibit 62.9 µg of trypsin and 22.5 µg of inhibitor was needed to inhibit 50µg of chymotrypsin. On a molar basis inhibitor to trypsin and inhibitor to chymotrypsin ratios came out to be 0.49 and 0.82, respectively.

Activities of inhibitor-enzyme complexes

Preformed inhibitor-chymotrypsin or inhibitor-trypsin complexes when tested for inhibitor activities against trypsin or chymotrypsin, respectively, showed that the inhibitor-chymotrypsin complex inhibited trypsin to the same extent as inhibitor alone whereas inhibitor-trypsin complex inhibited chymotrypsin activity to 60% of that brought about by inhibitor alone (figure 3).

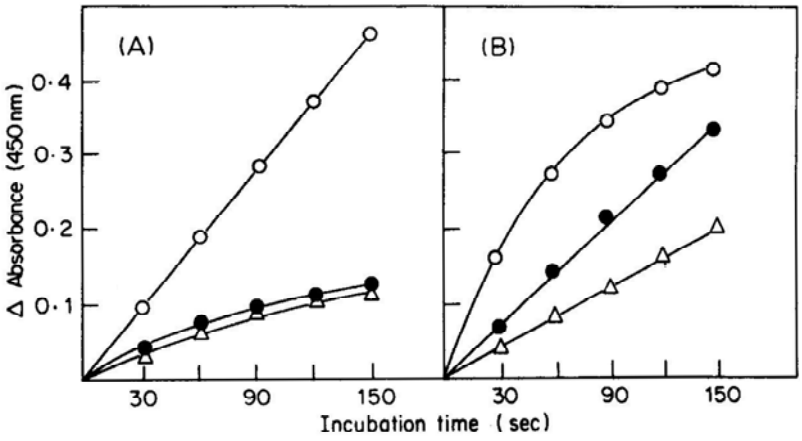

Figure 3. Inhibitor activities of enzyme—inhibitor complexes. Inhibitor was complexed with the enzymes by preincubation with trypsin or chymotrypsin for 10 min in equivalent amounts and the inhibitor activity of the complex was measured (for details see text).

A) Trypsin activity B) Chymotrypsin activity

Enzyme activity in the absence of inhibitor (O); Inhibition of enzyme by inhibitor (Δ); Activities of the enzyme-inhibitor complexes (●)

Effect of pH

At 37°C, there was no loss of the inhibitor activities in the pH range 3 to 11 and also in 0.01 N HCl up to 48 h of testing (data not shown). In 0.1 N NaOH the inhibitory activity towards both enzymes decreased with time (table 2). At boiling water temperature, the inhibitor was stable at pH 3 and 5 whereas in 0.1 N HCl, a reduction in inhibitory activity was observed (figure 4). At higher pH values the inhibitory activity showed progressive decrease with time. The loss in activity was rapid at pH 11 and in 0.1 N NaOH, 86% and 100% of the activity being lost in 10 min, respectively.

Table 2. Effect of alkali, urea and NaCl upon the activities of the inhibitor at 37°C

Period of incubation (h)	Inhibition (%)					
	Antitrypsin activity			Antichymotrypsin activity		
	0.1 M NaOH	6 M Urea	1 M NaCl	0.1 M NaOH	6 M Urea	1 M NaCl
0	100	100	100	100	100	100
2	95.5	100	100	91.2	97.8	101.1
6	82.5	100.6	98.7	70.8	93.5	100.6
12	65.0	100.6	98.7	41.6	91.3	101.7
24	39.5	100	98.7	10.9	87.0	101.1
36	25.5	99.4	98.1	0	83.7	101.7
48	20.4	99.4	97.5	0	82.6	100

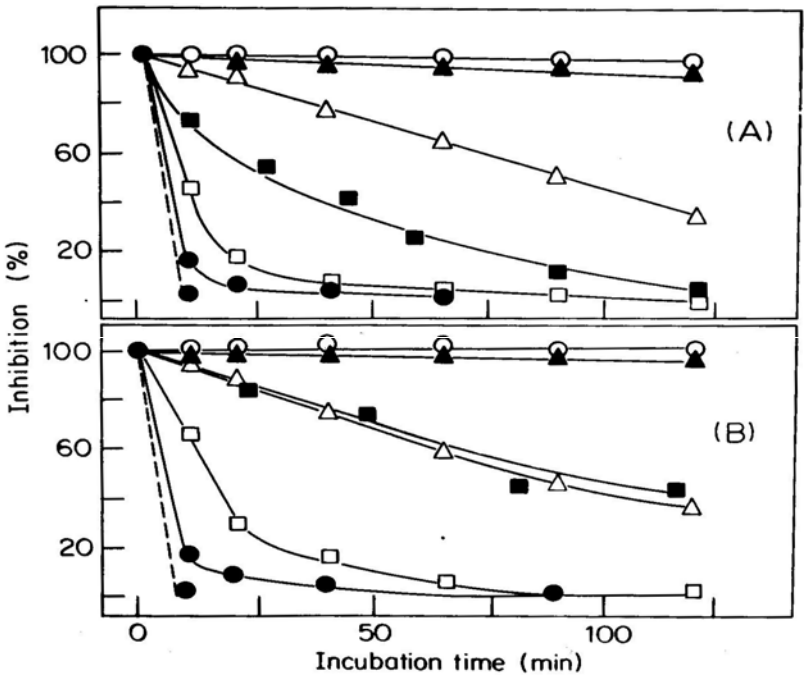

Figure 4. Effect of pH, acid and alkalin on the stability of the inhibitor. Inhibitor was incubated in buffer of different pH and in 0.1 N HCl and 0.1 N NaOH in boiling water and assayed after cooling and bringing the pH to 7.8 (for details see text).

A) Antichymotrypsin activity. B) Antitrypsin activity.
pH 3.0(▲); pH 5.0 (O); pH 7.0(△); pH 9.0(□); pH 11.0 (●); 0.1 N HCl (■) 0.1 N NaOH (--●--)

Effect of temperature

At 40°, 60° and 80°C there was no significant loss of the activities, but at 97°C, there was a gradual decrease in both the inhibitory activities. Autoclaving of the inhibitor solution at 15 psi in 10 min completely destroyed the two activities (figure 5).

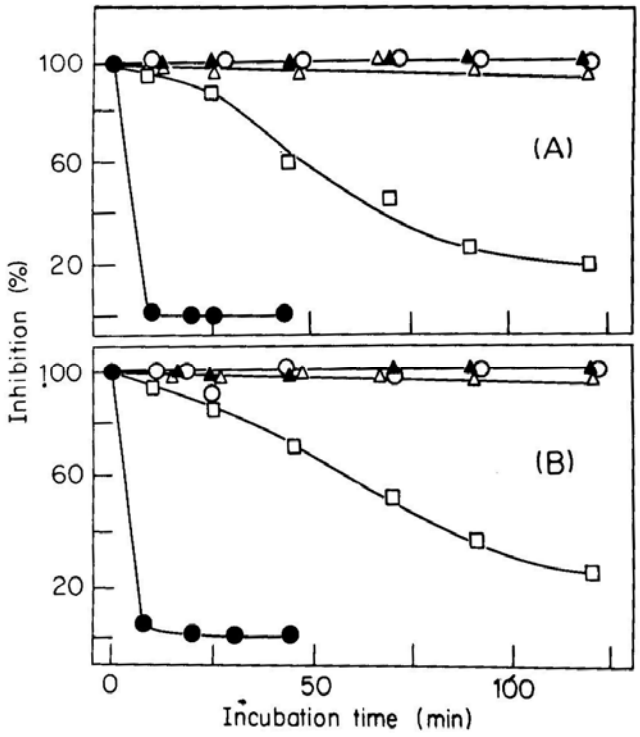

Figure 5. Effect of heating on the stability of the inhibitor (for details see text). A) Antitrypsin. B)Antichymotrypsin
40°C(▲); 60°C (O); 80°C(Δ); 97°C (boiling water) (□); Autoclave at 15 psi (●).

Effect of urea and sodium chloride

At 37°C the loss in inhibitory activity in the presence of urea and NaCl was insignificant upto 48 h testing except 18% loss in antichymotrypsin activity in 6 M urea (table 2). In 6 M urea at 97°C (table 3) there was rapid initial inactivation — 52% loss in antitrypsin activity in the first 25 min and 10% in next 95 min; during the same period the loss in antichymotryptic activity was 30% and 26% respectively. At this temperature the controls themselves showed considerable loss in activity by 95-120 min (table 3) .

Digestion with proteolytic enzymes

Digestion with subtilisin had no effect upon antichymotryptic activity, but 20 % loss ocurred in trypsin inhibitor activity (figure 6). Similarly pepsin digestion had

Table 3. Effect of urea and NaCl upon the activities of inhibitor at 97°C.

Period of incubation (min)	Inhibition (%)					
	Antitrypsin activity			Antichymotrypsin activty		
	Control	6 M Urea	1 M NaCl	Control	6 M Urea	1 M NaCl
0	100	100	100	100	100	100
10	96	56.2	95.5	92	80	89.1
25	93	47.9	85.8	87	70	70.7
45	80	43.2	75.4	75	64	43.5
70	63.5	41.1	53.7	59.5	58	19.6
95	47	38.4	35.8	43	50	8.2
120	36	37.0	26.9	33	44	2.7

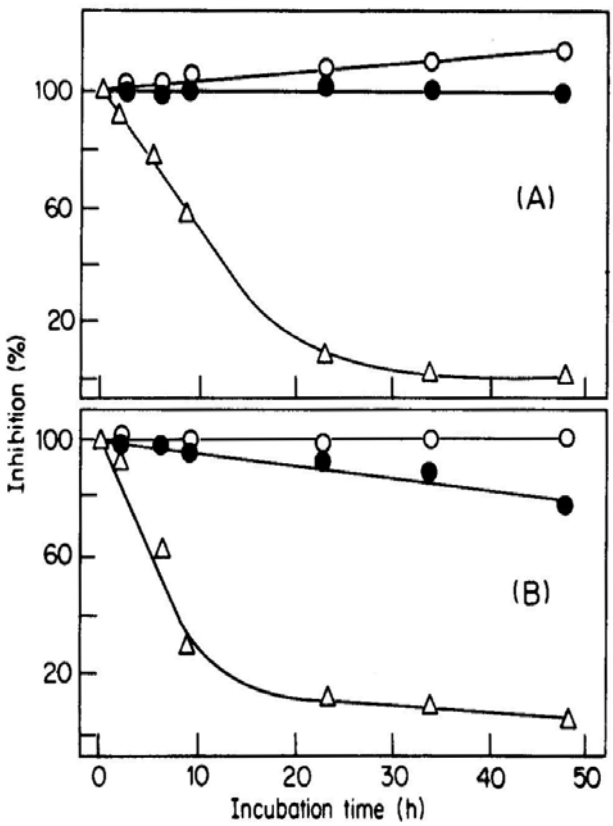

Figure 6. Effect of action of proteases on the activities of the inhibitor (for details see text)
A) Inhibition against chymotrypsin. B) Inhibition against trypsin.
Pepsin digestion (O); Subtilisin digestion (●); Pronase digestion (Δ).

marginal to no effect upon chymotryptic and tryptic inhibitory activities respectively. Pronase did inactivate the inhibitor in about 36 h, the relative rate of loss of antitrypsin activity was greater than that of antichymotrypsin activity.

Effect of reduction and reoxidation

Upon reduction 69% to 90% of the inhibitory activities were lost (table 4). There was, however, no appreciable reactivation upon reoxidation even after 20 h exposure to air.

Table 4. Effect of reduction and reoxidation of disulphide bonds of inhibitor on its activity.

Activity	Inhibition (%)			
	Control	Reduced	Reoxidised	
			10 h	20 h
Antitrypsin	100	30.9	36.3	38.1
Antichymotrypsin	100	9.5	10.1	10.1

Inhibitor was reduced by dialysing its solution (0.5 mg/ml) in 0.02 M phosphate buffer, pH 7.4 against 0.1 M 2-mercaptoethanol solution in the same buffer for 10 h and then against .05 M borate buffer, pH 7.0 in cold to remove the reagent. Reduced material was reoxidised by exposure to air at pH 8.2 for 10 and 20 h. A control was also run through these steps.

Discussion

An inhibitor purified from horse gram (*D. biflorus*) has been found to inhibit the activity of both chymotrypsin and trypsin. The presence of the two activities in the same molecule was implied by the fact that they could not be separated during the various steps of purification and the two close bands observed on polyacrylamide disc gel electrophoresis contained both the activities in almost the same ratio. The inhibitor activities of the enzyme-inhibitor complexes further supported this.

Trypsin and chymotrypsin inhibitors of plant origin are known for their stability under harsh treatments (Kassel, 1970). The inhibitor from horse gram was resistant to acidic pH (pH 3 and 5) even at high temperature upto 2 h. Inactivation could be obtained only using a combination of strong acid (0.1N HCl) and high temperature (97°C). At alkaline pH the rate of loss of inhibitor activities depended upon the pH of the solution, temperature and period of incubation Strong alkali at 37°C and 97°C and autoclaving at 15 psi resulted in rapid destruction of both the activities. Besides being stable to acidic conditions, this inhibitor was also stable to temperatures of upto 80°C at pH 7.8. Even the rate of inactivation at 97°C was rather slow.

Like in the case of inhibitors from soyabean, (Birk, 1961) lima bean, (Jones *et al.,* 1963) kidney bean (Pusztai, 1968) and kintoki bean (Miyoshi *et al.,* 1978) the present inhibitor was also not affected by pepsin action. But pronase, an enzyme of bacterial origin and of broad specificity, could destroy the activities of the inhibitor. It was fairly resistant to the denaturing action of 6 M urea. On the otherhand 2-mercaptoethanol, a disulphide reducing agent, could inactive the inhibitor;

reoxidation however, did not result in recovery of activity. In general, the antichymotrypsin activity was more susceptible to the various treatments compared to antitrypsin activity. The behaviour of horse gram inhibitor was similar to those already reported from various beans in regard to their stability and interaction with trypsin and chymotrypsin. It, however, differed from others in being more resistant to various treatments and in having higher specific activity against trypsin.

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