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NATURAL PLANT ENZYME INHIBITORS

V. A TRYPSIN/CHYMOTRYPSIN INHIBITOR FROM *ALOCASIA MACRORHIZA* TUBER

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Summary

A trypsin/chymotrypsin inhibitor was isolated from the tubers of *Alocasia macrorhiza* by extraction at pH 7.6, heat teratment at 80°C, ammonium sulphate precipitation and successive column chromatography on CM-cellulose, DEAE-Sephadex A-50 and Sephadex G-100. The inhibitor was pure by cellulose acetate electrophoresis. The molecular weight was approximately 32 000 as determined by gel filtration on Sephadex G-100. The inhibitor acted on bovine trypsin, human trypsin and bovine chymotrypsin. It had no action on human chymotrypsin, subtilisin BPN', pronase, *Aspergillus oryzae* protease, human and porcine pepsins. The bindings sites for bovine trypsin and chymotrypsin are not mutually exclusive. The inhibitor was stable over a pH range of 1—10. The purified inhibitor was far more thermostable than the crude inhibitor. The purified inhibitor lost only 33% of activity on heat treatment at 95°C for 2 h. Trinitrobenzene sulphonate treatment resulted in the loss of antichymotryptic activity faster than the antitrypsin activity of the inhibitor.

Introduction

Protease inhibitors of plant tubers have received increasing attention since the isolation of a crystalline chymotrypsin inhibitor from potato by Balls and Ryan [1]. The iso-inhibitors of potato acting on trypsin, chymotrypsin and other proteases have been studied by several groups of workers [2—4]. Three different inhibitors for trypsin were isolated from sweet potato by Sugiura et al. [5]. In an earlier communication we reported the presence of a relatively thermostable trypsin inhibitor in the tubers of *Colocasia antiquorum* a common edible tuber of India [6]. In this paper we report the presence of a trypsin chymotrypsin inhibitor in the tubers of *Alocasia macrorhiza* another edible

tuber grown in the rain-fed regions of Western India. The properties of the purified inhibitor are presented.

Materials and Methods

Fresh and mature *Alocasia macrorhiza* tubers used in these studies were collected locally.

Bovine trypsin (salt free, twice crystallized, Worthington Biochemical Corporation, U.S.A., EC 3.4.21.4), bovine α-chymotrypsin (salt free, thrice crystallized, Worthington, EC 3.4.21.1), and subtilisin BPN′ (Nagase company, Osaka, Japan, EC 3.4.21.14) were the generous gifts of Dr. William B. Lawson, New York State, Department of Health, Albany, N.Y., U.S.A. Porcine pepsin (thrice crystallized, EC 3.4.23.1) was purchased from Calbiochem, U.S.A. Pronase (Type VI protease) and *Aspergillus oryzae* protease were procured from Sigma Chemical Company, U.S.A. A 0—80% ammonium sulphate precipitate of human gastric juice dissolved in 0.01 M HCl was used as the source of human pepsin. Human cationic trypsin and chymotrypsin were prepared from pancreas according to the method of Narenda Nayak et al. [7].

DEAE-Sephadex A-50 and Sephadex G-100 were purchased from Pharmacia Fine Chemicals, Sweden. CM-Cellulose was obtained from Bio-Rad Chemicals, California, U.S.A. α-*N*-Benzoyl DL-arginine *p*-nitroanilide, ethyl *N*-acetyl L-tyrosinate and trinitrobenzene sulfonate were procured from Sigma Chemical Company, U.S.A. Diacetyl was the product of British Drug House, London. Other chemicals used were of analytical grade.

The caseinolytic activity of neutral proteases was measured as described earlier [6]. The assay system consisted of 20 mg of casein, 120 μmol of phosphate buffer pH 7.6 and suitable quantities of the inhibitor in a volume of 1.9 ml. The reaction was initiated by the addition of 0.1 ml of enzyme solution. After 10 min incubation at 37° C, the reaction was stopped by the addition of 3 ml of 5% trichlotoacetic acid solution. After standing for 1 h the solution was centrifuged at 2000 × *g* for 10 min. 1 ml of the supernatant was analysed by the method of Lowry et al. [8]. One unit of enzyme is defined as the amount of enzyme that will cause an increase of one mg of Lowry sensitive materials in the trichlotoacetic acid soluble fraction under the assay conditions. For calculation purposes, the values were based on a reference curve using bovine serum albumin as standard in the Lowry procedure.

Proteolytic activity of pepsin was measured at pH 2 using hemoglobin as substrate as described by Chow and Kassell [9].

The esterolytic activity of bovine trypsin was determined according to the method of Erlanger et al. [10]. The assay system consisted of 5 μmol of α-*N*-benzoyl-DL-arginine-*p*-nitroanilide, 20 μmol of phosphate buffer 7.6 and the inhibitor in a volume of 1.9 ml. The reaction was initiated by the addition of 0.1 ml of trypsin solution. After 15 min incubation at 37° C, the reaction was stopped by the addition of 3 ml of 30% acetic acid and *p*-nitroaniline liberated was estimated. The esterolytic activity of chymotrypsin was determined by the method of Prabhu and Pattabiraman [11]. The assay system consisted of 5 μmol of ethyl-*N*-acetyl · L-tyrosinate, 100 μmol of phosphate buffer, pH 7.6, suitable amounts of chymotrypsin and the inhibitor, in a volume of 2 ml. After

15 min incubation at 37°C, the reaction mixture was extracted with 5 ml of ethyl acetate. Acetyltyrosine in the aqueous phase was estimated by the method of Lowry et al. [8].

Total carbohydrates was assayed by the phenol-sulphuric acid method [12]. Protein was determined by the method of Lowry and others [8] using serum albumin as standard.

Polyacrylamide gel electrophoresis was performed by the method of Davis [13] using 0.04 M Tris/glycine buffer pH 8.3 with a current of 4 mA/tube. Gels were stained with 0.1% amido Schwartz in 7% acetic acid. Electrophoresis on cellulose acetate strips was performed at pH 8.3 in 0.04 M barbitone/hydrochloric acid buffer for 1 h at 200 V. The strips were stained with 0.5% acid red in 5% trichloroacetic acid.

Modification of free amino groups was done with trinitrobenzene sulphonic acid. The purified inhibitor (25 μg) was treated with 200 μg of the reagent in presence of 10 μmol of phosphate buffer, pH 7.6, in a total volume of 0.25 ml at room temperature for different time intervals. The mixture was dialyzed against water for 3 h at 4°C and the residual inhibitor activity was determined.

Modification of arginyl residues in the inhibitor was done with diacetyl [15]. The inhibitor (100 μg protein) was treated with 500 μg of diacetyl in presence of 20 μmol of borate buffer, pH 7.5, in a total volume of 1 ml for different time intervals at room temperature. The mixture was dialyzed at 4°C overnight and the residual inhibitory activity was assayed.

Purification of the inhibitor

Alocasia tuber (100 g) was homogenized with 300 ml of 0.1 M phosphate buffer pH 7.6. After stirring for 1 h the mixture was centrifuged at 12 000 × g for 20 min. The cloudy supernatant (crude extract, volume 285 ml) was subjected to heat treatment at 80°C for 10 min and left at 4°C overnight. To the clear supernatant obtained after centrifugation at 12 000 × g for 20 min (heat-treated fraction, volume 275 ml), 147 g of ammonium sulphate was added to a saturation of 80%. After 1 h stirring at 4°C, the mixture was centrifuged. The precipitate was dissolved in water (Total volume 54 ml) and dialyzed against one liter of 0.002 M citrate buffer, pH 6.2, for 16 h. The cloudy solution was centrifuged at 12 000 × g for 20 min to give a clear supernatant (ammonium sulphate fraction, volume 58 ml).

The above solution was applied to a column of CM-cellulose (1.8 × 22.5 cm, bed volume 65 ml) equilibrated with 0.002 M citrate buffer, pH 6.2. The column was washed with 75 ml of the equilibration buffer and the washings containing 158 mg of inactive protein was discarded. Elution was performed with 0.002 M citrate buffer pH 6.2 containing 0.2 M NaCl (flow rate 35 ml/h). 10-ml fractions were collected and assayed for protein and inhibitor activity. The active fractions (Tube Nos. 5 to 10, Fig. 1a) were pooled and saturated with ammonium sulphate (53.3 g) to 80%. The precipitate collected by centrifugation at 12 000 × g for 20 min, was dissolved in 0.05 M phosphate buffer pH 7.8 containing 0.1 M NaCl and dialyzed against one litre of the same buffer for 16 h. The dialyzed solution was centrifuged to give a clear supernatant (CM-cellulose fraction, volume 7 ml).

This solution was applied to a column of DEAE-Sephadex A-50 (1 × 48 cm,

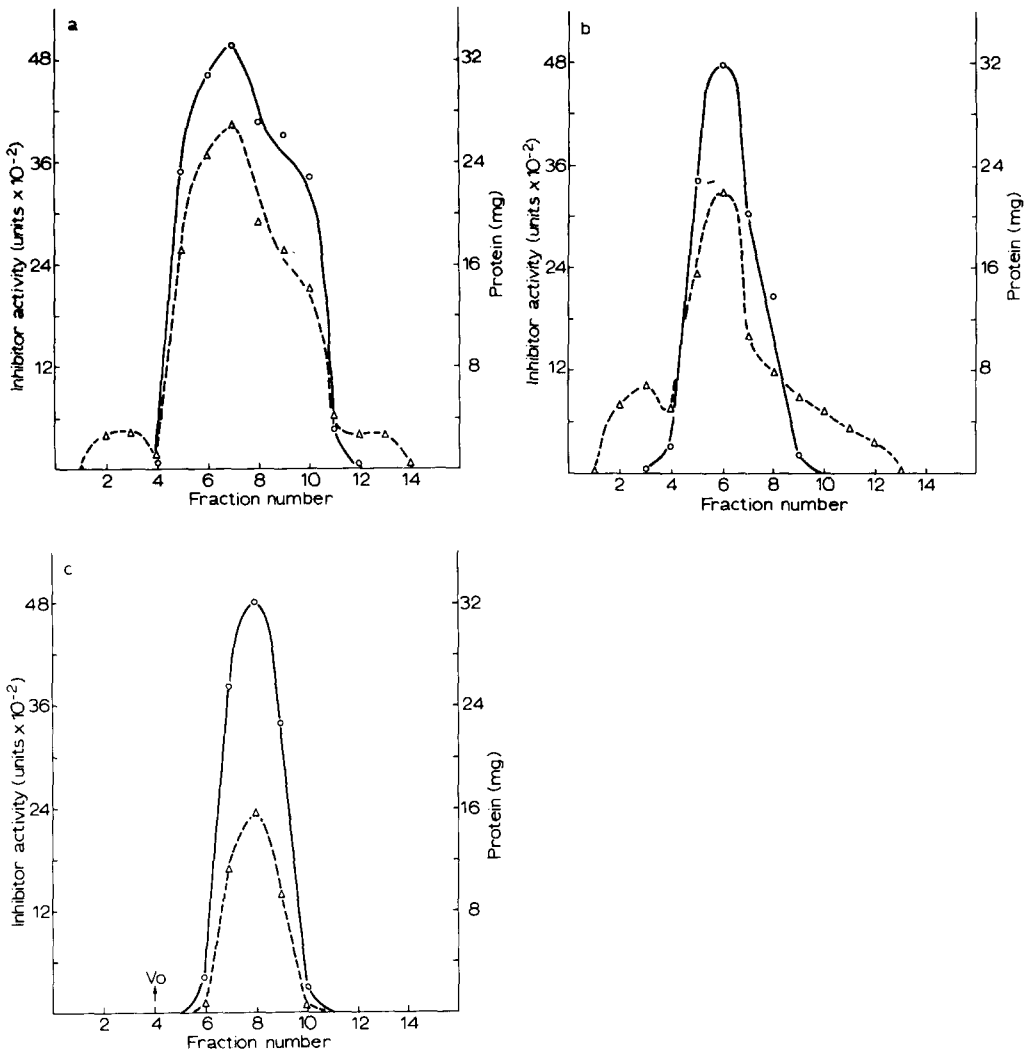

Fig. 1. (a) Purification of ammonium sulphate fraction on CM-cellulose, ○———○, total trypsin inhibitor activity in the fractions; △———△, total protein in the fractions. (b) Purification of CM-cellulose fraction on DEAE-Sephadex A-50. ○———○, total trypsin inhibitor activity in the fractions; △———△, total protein in the fractions. (c) Purification of DEAE-Sephadex fraction on Sephadex G-100. ○———○, total trypsin inhibitor activity in the fractions; △———△, total protein in the fractions.

bed volume 35 ml) equilibrated with 0.05 M phosphate buffer, pH 7.8, containing 0.1 M NaCl. The column was washed with 35 ml of the equilibration buffer and the washings containing 35 mg of inactive protein was discarded. Elution was performed with 0.05 M phosphate buffer, pH 7.8, containing 0.2 M NaCl (flow rate 25 ml/h). 10-ml fractions were collected. Active fractions (Tube Nos. 5 to 8, Fig. 1b) were pooled and saturated with ammonium sulphate (21.3 g) to 80%. The precipitate was collected by centrifugation, dissolved in 0.1 M phosphate buffer pH 7.6 containing 0.2 M NaCl, dialyzed against one litre of the same buffer for 16 h and centrifuged at 12 000 × *g* for

20 min. A clear solution (DEAE-Sephadex fraction, volume 3 ml) was obtained.

The above solution was subjected to gel filtration on a column of Sephadex G-100 (1.4 × 78 cm, bed volume 120 ml) equilibrated with 0.1 M phosphate buffer pH 7.6, containing 0.2 M NaCl. Elution was performed with the same buffer at a flow rate of 20 ml/h and 10 ml fractions were collected. The active fractions (Tube Nos. 7 to 9, Fig. 1c) were pooled and dialyzed against water for 16 h at 4°C. The solution was freeze-dried and dissolved in water (Sephadex G-100 fraction, volume 2 ml).

Results

The protease inhibitor of *Alocasia macrorhiza* tuber was purified 13.6 fold with 31% recovery of the activity. The different steps leading to the purified inhibitor are summarized in Table I. The units of inhibitor activity were arrived at, using the caseinolytic assay method. We have determined the effect of preincubation, by treating trypsin with the inhibitor in phosphate buffer medium, pH 7.6, for time intervals varying from 0 to 60 min before the assay. It was observed that preincubation did not alter the magnitude of trypsin inhibition by the purified inhibitor.

On cellulose acetate strip electrophoresis (Fig. 2) at pH 8.3, the inhibitor gave a single protein band moving towards the anode. On polyacrylamide gel electrophoresis, apart from a major band a faint band moving faster towards anode could be detected (Fig. 3). The molecular weight of the inhibitor as determined by gel filtration on Sephadex G-100 was around 32 000 (Fig. 4). A 5 M urea solution was used as eluant in this experiment. The elution pattern was similar when water was used. The inhibitor (1 mg) did not show a positive phenol/sulphuric acid reaction, indicating the absence of carbohydrates.

The purified inhibitor displayed activity against bovine trypsin and chymotrypsin in the same ratio of 1.8 as the crude extract. Further, the ratio remained roughly same during different stages of purification suggesting that the same inhibitor is responsible for inhibitory activities against the two enzymes. The action of the inhibitor on the caseinolytic activities of trypsin and chymotrypsin individually and on the mixture of these enzymes was studied and the data are presented in Table II. It was found that the magnitude of inhibition of the mixture did not exceed that of trypsin alone. This suggests

TABLE I

PURIFICATION OF TRYPSIN CHYMOTRYPSIN INHIBITOR FROM ALOCASIA TUBER

Fraction	Total protein (mg)	Trypsin inhibitor activity (units)	Specific activity	Yield (%)
Crude extract	1596.0	37620	23.6	100.0
Heat treated fraction	811.0	44540	54.9	118.0
Ammonium sulphate fraction	342.0	26680	78.0	70.9
CM-cellulose fraction	119.8	24560	205.0	65.3
DEAE-Sephadex fraction	56.5	13360	236.0	35.5
Sephadex G-100 fraction	36.5	11680	320.0	31.1

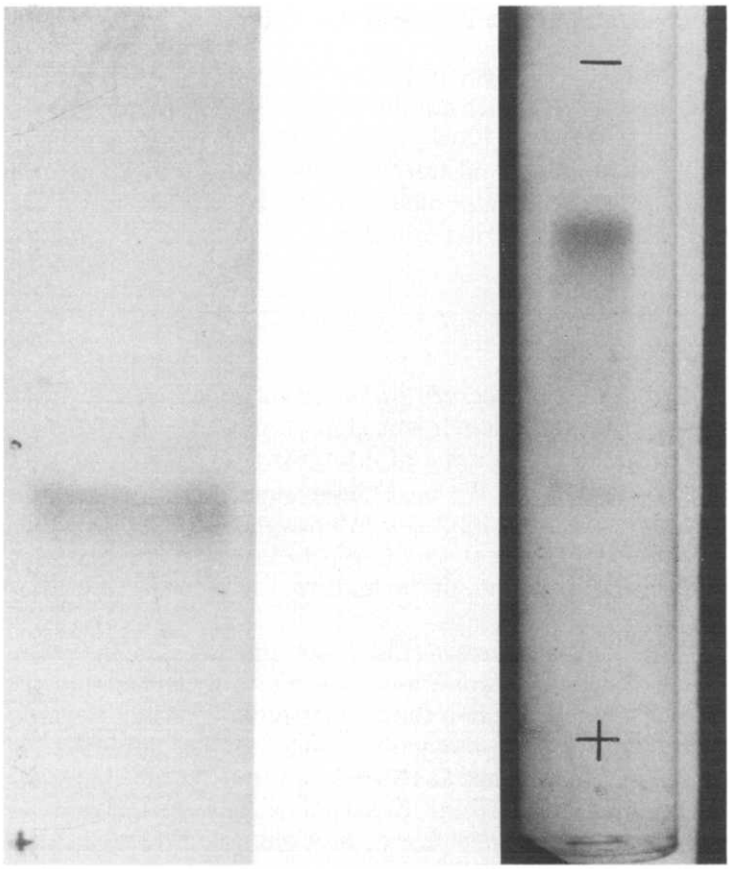

Fig. 2. Cellulose acetate electrophoresis of the purified inhibitor. Details are given in Materials and Methods.

Fig. 3. Polyacrylamide gel electrophoresis of the purified inhibitor. Other details are given in Materials and Methods.

TABLE II

INHIBITION OF TRYPSIN, CHYMOTRYPSIN AND THE MIXTURE OF ENZYMES

The assay system is described in Materials and Methods. The additions were bovine trypsin (6 μg protein), bovine chymotrypsin (5.6 μg protein) and the inhibitor (3.6 μg protein).

Additions	Caseinolytic activity (units)	Inhibition units
Trypsin	1.70	—
Trypsin + inhibitor	0.63	1.07
Chymotrypsin	1.80	—
Chymotrypsin + inhibitor	1.23	0.57
Trypsin + chymotrypsin	3.47	—
Trypsin + chymotrypsin + inhibitor	2.42	1.05

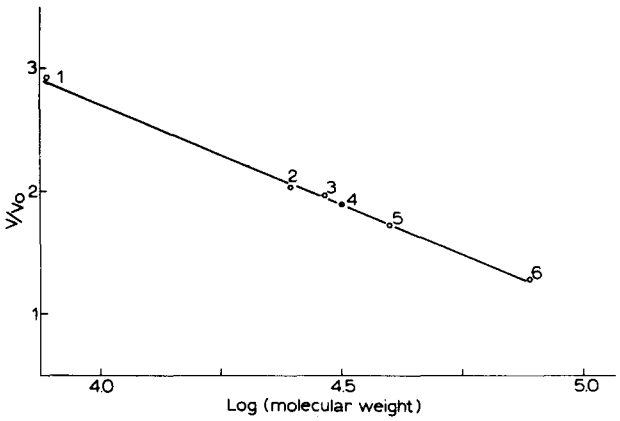

Fig. 4. Molecular weight determination of the purified inhibitor on Sephadex G-100. Protein samples were applied on a column of Sephadex G-100 (1 × 48 cm, bed volume 35 ml) and eluted with 5 M urea at a flow rate of 20 ml/h. 1, insulin; 2, hen egg-white inhibitor; 3, bovine trypsin; 4, purified inhibitor; 5, Dextran (T-40); 6, bovine albumin.

that the binding sites for trypsin and chymotrypsin of the inhibitor are not mutually exclusive.

Among the several proteases tested, bovine trypsin was found to be most powerfully inhibited. The inhibitor was less active against bovine chymotrypsin and human trypsin (Fig. 5). In the linear range of inhibition when expressed in terms of protein content these three enzymes were inactivated by the purified inhibitor in the ratio 2.34 : 1.28 : 1.0. With bovine trypsin the inhibition was linear upto 85% as a function of inhibitor concentration. However, with human trypsin and bovine chymotrypsin linearity was not observed beyond 50% inhibition.

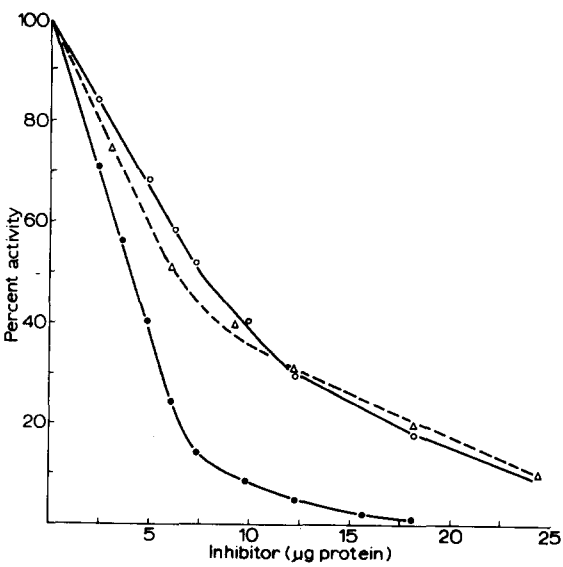

Fig. 5. Action of the purified inhibitor on the proteolytic activity of bovine trypsin (9 μg protein) ●——●, human trypsin (16 μg protein) ○——○, and bovine α-chymotrypsin (8 μg protein) △——△.

TABLE III

EFFECT ON THE CASEINOLYTIC AND ESTEROLYTIC ACTIVITIES OF TRYPSIN AND CHYMOTRYPSIN

The assay systems are described in the text. Bovine trypsin (9 μg protein) and bovine chymotrypsin (8 μg protein) were used.

Inhibitor (μg protein)	Trypsin		Chymotrypsin	
	% caseinolytic inhibition	% esterolytic inhibition	% caseinolytic inhibition	% esterolytic inhibition
2.4	24.3	21.6	—	—
3.6	38.6	35.0	21.9	18.0
4.8	53.0	43.3	—	—
7.2	—	—	40.3	33.0

No inhibition of proteolytic activity of human chymotrypsin (20 μg protein) subtilisin BPN' (25 μg), pronase (20 μg), *A. oryzae* protease (50 μg), porcine pepsin (15 μg) and human pepsin (25 μg) was observed when 25 μg (protein) of the inhibitor was used. At half this concentration, bovine trypsin (9 μg protein) was inhibited nearly completely.

While the inhibitor had no effect on human pepsin when included in the assay medium, pre-incubation of pepsin with the inhibitor for 15 min at pH 6.9 caused partial inactivation of pepsin. There was 27% loss of peptic activity when 10 μg of the inhibitor was used and 67% loss was observed with 50 μg of the inhibitor. The amount of pepsin used in these studies was 25 μg.

The magnitude of inhibition of the caseinolytic activity of bovine trypsin was slightly higher than the inhibition of the esterolytic activity. A similar observation was made with bovine chymotrypsin. The results are shown in Table III. The inhibition of the proteolytic as well as the esterolytic activities was found to be non-competitive for both the enzymes. The double reciprocal

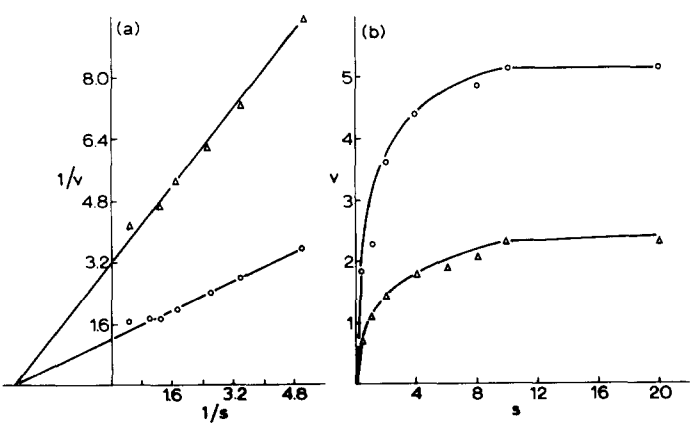

Fig. 6. Non-competitive inhibition of the caseinolytic activity of bovine trypsin (a) and bovine chymotrypsin (b). ○———○, velocity in the absence of inhibitor; △———△, velocity in presence of inhibitor. 4.8 μg (protein) of the inhibitor and 9.6 μg (protein) of the inhibitor were used respectively. The amount of enzymes were 9 μg trypsin and 8 μg chymotrypsin.

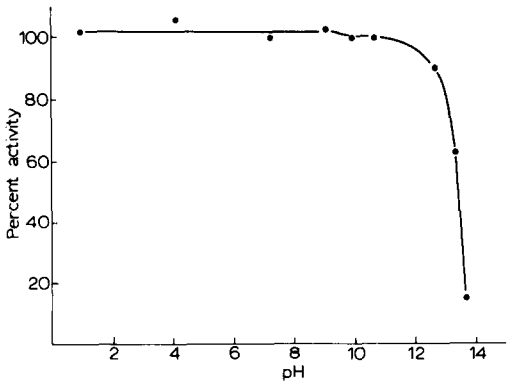

Fig. 7. Effect of pH on the stability of the purified inhibitor (caseinolytic inhibition of bovine trypsin). Inhibitor (4.8 μg) and trypsin (9 μg) were used. The inhibitor solution was adjusted to the different pH indicated with 1 N HCl or 1 N NaOH and left at 4°C for 16 h. The residual inhibitory activity was assayed with suitable aliquots.

plot for the caseinolytic activity of trypsin is shown in Fig. 6a. The normal plot for the proteolytic activity of chymotrypsin is shown in Fig. 6b.

The effect of hydrogen ion concentration on the stability of the inhibitor was studied and the results are presented in Fig. 7. The inhibitor was stable over a wide range of pH from 1 to 10. A sharp fall in activity was observed on exposure to highly alkaline conditions.

The data on the studies of the effect of heat treatment of the purified and crude inhibitors are summarized in Fig. 8. The inhibitory activity of the crude

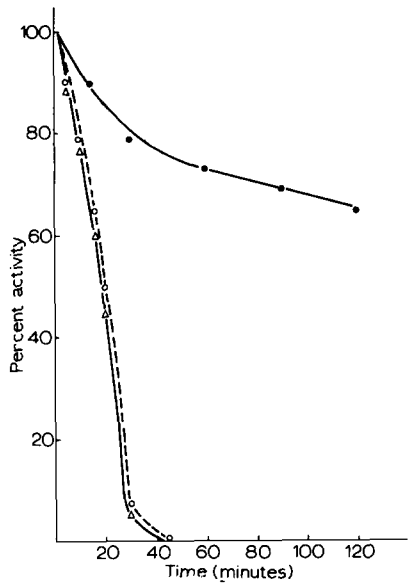

Fig. 8. Effect of heat treatment at 95°C. ●———●, purified inhibitor (4.8 μg protein); ○———○, crude alocasia extract (56 μg protein); △———△, mixture of crude (28 μg protein) and purified inhibitors (2.4 μg protein). The antitryptic activity was measured by the caseinolytic method using 9 μg protein of the bovine enzyme.

TABLE IV

EFFECT OF TRINITROBENZENE SULPHONATE TREATMENT

The caseinolytic assay conditions are described in the text.

Conditions	Trypsin inhibitory activity (%)	Chymotrypsin inhibitory activity (%)
Control	100	100
+ trinitrobenzene sulphonic acid, 4 h	100	94
+ trinitrobenzene sulphonic acid, 16 h	65	0
+ trinitrobenzene sulphonic acid, 40 h	48	0
Heated control	100	100
+ trinitrobenzene sulphonic acid, 4 h	100	47
+ trinitrobenzene sulphonic acid, 16 h	38	0
+ trinitrobenzene sulphonic acid, 48 h	0	0

extract was found to be completely destroyed on heat treatment at 95°C for 30 min. On the other hand, the purified inhibitor was highly thermostable. Nearly two thirds of the inhibitory activity was retained even after 2 h heat treatment. When a mixture of the purified inhibitor and the crude extract was subjected to heat treatment, the total inhibitory activity was destroyed in 30 min. The heat stability profiles did not change when dialyzed preparations of crude extract or purified inhibitor solutions were used.

The inhibitor was found to lose its activity on modification with trinitrobenzenesulphonic acid as shown in Table IV. The loss of antichymotryptic activity was faster than the loss of antitryptic activity. After 16 h treatment while all the antichymotryptic activity was lost, only 35% of the antitryptic activity was abolished. Heat treatment of the inhibitor solution for 10 min at 95°C prior to trinitrobenzene sulphonic acid treatment, accelerated the rate of loss of inhibitory activity. The results are shown in Table IV. These data suggest free amino groups are essential for the interaction of the inhibitor with both trypsin and chymotrypsin. Diacetyl a specific modifier of arginine residues did not alter the inhibitory capacity of the pure inhibitor both against trypsin and chymotrypsin. Prior heat treatment of the inhibitor before reaction with diacetyl also did not result in any loss of activity.

When the crude extracts were subjected to heat treatment at 80°C for 10 min, there was an increase in the inhibitory activity to the extent of 20% (Table I). In order to find out whether this increase is due to the destruction of an endogenous protease, the crude extracts were screened for caseinolytic activities at pH 2, pH 4, pH 6 and pH 8.0 both in the presence and absence of 10^{-2} M cysteine and $5 \cdot 10^{-3}$ M NADPH. Proteolytic activity could not be demonstrated under any of these conditions after 60 min incubation at 37°C.

Discussion

The studies reported here establish the presence of a thermo-stable trypsin chymotrypsin inhibitor in the tubers of *Alocasia macrorhiza*. The antitryptic activity in this tuber per mg of extractable protein (23.6 units) is comparable to the reported levels [6] in potato (18.7) and *Colocasia* tuber (30.0). The

Alocasia tuber appear to differ from potato and sweet potato in that it contains a single protease inhibitor.

The purified inhibitor that acted on both bovine trypsin and chymotrypsin does not appear to have mutually exclusive binding sites for these two enzymes unlike in the case of the double headed inhibitors of black eyed pea [16], lathyrus seed [17], Bowman-Birk soya been inhibitor [18] and chick pea [19]. In this respect it resembles the trypsin chymotrypsin inhibitor of Indian red wood seed [20].

The *Alocasia* inhibitor is far more heat stable than the potato [6] and sweet potato [5] inhibitors. The purified inhibitor is extremely thermostable like the white bean trypsin inhibitor [21]. The differences in the heat stability of the crude and purified preparations are interesting. It was found that mixing the purified inhibitor with the dialyzed crude extract rendered it more thermolabile. It is tempting to suggest that the crude preparations contain a non-dialyzable component that complexes with the inhibitor and makes it more susceptible to heat. Gennis and Cantor [16] have reported the presence of a protease in black-eyed pea which was found in combination with the protease inhibitor. It seems unlikely that a similar complex exists in *Alocasia* tuber, since our attempts to demonstrate the presence of protease activity in alocasia preparations have not been successful. Further studies are needed to establish the nature of the labilizing factor in crude preparations of this tuber.

Protein inhibitors of trypsin are generally classified into 'arginine' and 'lysine' inhibitors [14] depending on the abolition of activity after site-specific chemical modifications. The purified alocasia inhibitor did not lose its activity on treatment with diacetyl suggesting that it is not an 'arginine inhibitor'. The activity was found to be abolished on treatment with trinitrobenzene sulphonic acid. Prior heat treatment of the inhibitor enhanced the rate of loss of activity on trinitrobenzene sulphonic acid treatment, indicating that the reacting free amino groups are not easily available in the native state. An interesting observation is the faster rate of loss of antichymotryptic activity on treatment with trinitrobenzene sulphonic acid. This emphasizes the importance of exercising caution in interpreting the data on chemical modification of protease inhibitors. Since trypsin [22] and other enzymes [23] have very extensive binding sites for macromolecular substrates and inhibitors, mere loss of activity on treatment with specific reagents cannot be taken as evidence for implicating single lysyl or arginyl residues as essential for interaction between trypsin and trypsin inhibitors.

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