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NATURAL PLANT ENZYME INHIBITORS

VI. STUDIES ON TRYPSIN INHIBITORS OF *COLOCASIA ANTIQUORUM* TUBERS

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Summary

A trypsin inhibitor was purified from the tubers of *Colocasia antiquorum*. The inhibitor acted on bovine trypsin, human trypsin and weakly on bovine chymotrypsin. The inhibitor, which had a molecular weight of 40 000, contained trace amounts of carbohydrates. The purified inhibitor was stable over a pH range of 2.0—12.0 and was more thermostable than the crude preparations. Trinitrobenzene sulphonate treatment resulted in the inactivation of the inhibitor. Chymotrypsin, pepsin and pronase digested the inhibitor. Pretreatment with trypsin at neutral pH resulted in the partial loss of antitryptic activity, whereas treatment at pH 3.7 led to complete inactivation. Evidence for the formation of a trypsin-inhibitor complex at pH 7.6 is provided. During the plant growth, in the early phase (0—40 days) there was a gradual increase in protein content and in antitryptic activity. The middle phase (40—55 days) was characterized by a rapid fall and abolition of the antitryptic activity and a diminution in protein content in the tubers. The immature tubers had low antitryptic activity compared to the mature ones. Mild heat treatment caused a sharp rise in antitryptic activity in the extracts of immature tubers but not with the mature tuber preparations.

Introduction

Protease inhibitors of potato [1—4] and sweet potato [5,6] tubers have been studied extensively by several workers. In an earlier communication we

Abbreviations BAPNA, α-*N*-benzoyl-DL-arginine *p*-nitroanilide; V_e, elution volume; V_0 void volume.

reported the presence of a relatively thermostable trypsin inhibitor in the tubers of the plant *Colocasia antiquorum* [7]. In this paper, studies on trypsin inhibitor levels in different varieties of colocasia tubers and the variations in the level of the factor during different stages of plant growth are reported. Purification and properties of the major inhibitor from the tubers of the wild variety of colocasia are also presented.

Materials and Methods

Tubers: Four varieties of *Colocasia* tubers were used on these studies. Both the common cultivated variety (referred to as garden variety) and the wild variety have a dark brownish skin and are small in size (2—5 cm long). They can however be distinguished by their protein content. The wild variety has a higher protein content than the garden variety. The red variety is large and long (15—25 cm) with concentric rings on the surface. This variety is mainly cultivated in the rain-fed regions of Karnataka State, India. The medium sized variety (8—10 cm long) with a brown skin is cultivated mainly in Kerala State. It has a low protein content and the aqueous extracts are not mucilagenous unlike the other varieties. The wild variety was picked locally after the monsoon season. The other varieties were obtained through commercial sources.

Enzymes: Bovine trypsin (salt free, 2X crystallized, EC 3.4.4.4) and bovine α-chymotrypsin (salt free, 3X crystallized, EC 3.4.4.5) were purchased from Worthington Biochemical Corporation, U.S.A.; Subtilisin BPN′ (EC 3.4.4.16) from Nagase Company, Osaka, Japan; porcine pepsin (3X crystallized, EC 3.4.4.1) from Calbiochem, U.S.A.; Pronase from Sigma Chemical Company, U.S.A.; human cationic trypsin was prepared from pancreas by the method of Navak et al. [8].

Chemicals: DEAE-Cellulose was obtained from Bio-Rad Chemicals, California, U.S.A.; Sephadex G-100 and phenyl Sepharose CL-4B from Pharmacia Fine Chemicals, Sweden; α-*N*-benzoyl-DL-arginine *p*-nitroanilide and trinitrobenzene sulfonate from Sigma Chemical Company, U.S.A.; diacetyl from British Drug House, London. Other chemicals used were of analytical grade.

Active site titrations of bovine trypsin and chymotrypsin were done according to the method of Kezdy and Kaiser [9] using *p*-nitrophenyl acetate. The concentrations of these two enzymes mentioned in the text represent the active enzymes. Trypsin and chymotrypsin samples were found to be 60% and 70% active, respectively.

Caseinolytic activity of neutral proteases was performed as described earlier [7]. One unit of enzyme is defined as that amount which forms 1 mg protein equivalent of trichloroacetic acid soluble fragments under the assay conditions. One unit of inhibitor is the amount which depresses the proteolytic activity by one unit. Proteolytic activity of pepsin was measured at pH 2.0 using hemoglobin as substrate as described by Chow and Kassell [10]. Amidolytic activity of trypsin was done according to the method of Erlanger et al. [11] with BAPNA as substrate as described previously [12]. Total carbohydrate was assayed by the phenol-H_2SO_4 method [13]. Protein was determined by the method of Lowry et al. [14] using bovine serum albumin as standard.

Preparation of tuber homogenates: After removing the skin, the tubers were

homogenized with 3 vols. (w/v) 0.1 M phosphate buffer (pH 7.6). After stirring for 1 h, the homogenates were centrifuged at 12 000 × *g* for 20 min at 30°C. The supernatant solutions were used for assaying inhibitory activity against trypsin and chymotrypsin.

Purification of trypsin inhibitor from the wild variety tubers: All operations were carried out at room temperature (28—30°C) unless mentioned otherwise. Tubers (45 g) were homogenized with 135 ml 0.01 M phosphate buffer (pH 7.8) and the supernatant fraction was separated as described above.

The 135 ml mucilagenous crude extract was mixed with 70 ml DEAE-cellulose equilibrated with 0.01 M phosphate buffer (pH 7.8). The mixture was stirred at low speed for 30 min and centrifuged at 1000 × *g* for 10 min. The sediment was stirred with 160 ml of the buffer and centrifuged. Both the mucilagenous supernatant fraction and the washings were devoid of inhibitory activity and were discarded. The sediment was extracted with 160 ml 0.01 M phosphate buffer (pH 7.8) containing 1 M NaCl and centrifuged. The clear supernatant fraction contained nearly 90% of the antitryptic activity.

To the DEAE-cellulose supernatant fraction, $(NH_4)_2SO_4$ added to 80% saturation. After 1 h stirring at 4°C, the mixture was centrifuged at 12 000 × *g* for 20 min. The precipitate was dissolved in 0.01 M phosphate buffer, pH 7.8 (total vol. 44 ml) and dialyzed against buffer for 16 h at 4°C. The cloudy solution was centrifuged at 12 000 × *g* for 10 min to give a clear supernatant solution.

The $(NH_4)_2SO_4$ fraction (total vol. 45 ml) was applied to a column of DEAE-cellulose (1.8 × 27.5 cm, bed vol. 65 ml) equilibrated with 0.01 M phosphate buffer (pH 7.8). The column was washed with 120 ml equilibration buffer and the washings were discarded. The column was eluted with 140 ml 0.01 M phosphate buffer (pH 7.8), 0.15 M NaCl, followed by 130 ml 0.01 M phosphate buffer, 0.2 M NaCl (flow rate 30 ml/h; 10-ml fractions). The fractions were assayed for protein and antitryptic activity. The elution profile is shown in Fig. 1. The active fractions (tube Nos. 7—10) were pooled and dialyzed against distilled water for 16 h at 4°C. This fraction is designated as DEAE-cellulose major fraction.

Electrophoresis: This was done on cellulose acetate strips in 0.04 M sodium barbitone/HCl buffer (pH 8.3) for 90 min at 200 V. The strips were stained with 0.5% acid red in 5% trichloroacetic acid. To determine antitryptic activities of the protein bands, the corresponding regions of the unstained strips were cut and extracted with 0.2 M Na_2HPO_4 containing 1 M NaCl and the extracts were used in the caseinolytic assay method.

Molecular weight determination: The molecular weight of the purified inhibitor was determined by gel chromatography on Sephadex G-100 (0.9 × 55 cm, bed vol. 35.0 ml). 5 M urea was used as eluant. Insulin, egg white trypsin inhibitor, bovine trypsin, bovine serum albumin and Dextran T-40 were used as standards.

Effect of pH on the stability of the inhibitor: The purified inhibitor (1.15 mg protein) in 0.5 ml was incubated with 0.5 ml 0.1 M buffers of different pH values (pH 2.0, HCl/KCl; 3.5, acetate; 4.0, acetate; 7.0, phosphate) or with 0.5 ml 2 M HCl (final pH, 1.0), 0.2 N NaOH (final pH, 12.7) and 2 N NaOH (final pH, 13.6) for 17 h at 30°C. Aliquots corresponding to 11.5 μg inhibitor protein

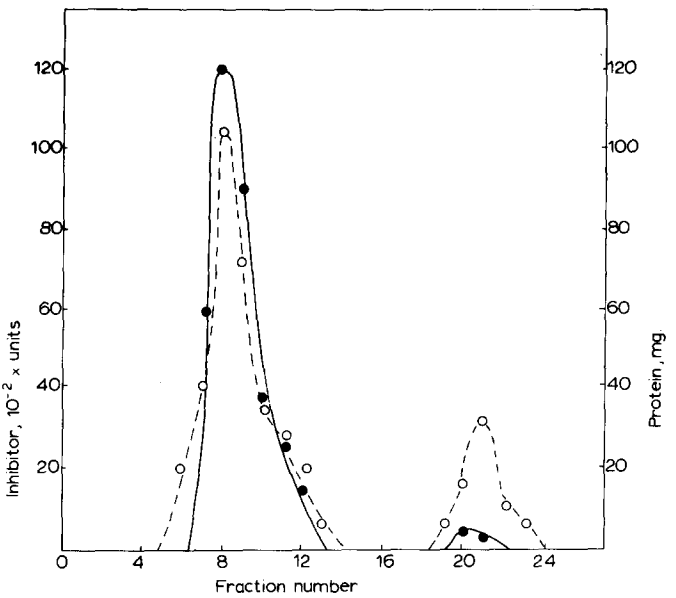

Fig. 1. Chromatography of ammonium sulphate fraction on a DEAE-cellulose column (bed dimensions: 1.8 X 27.5 cm, bed vol: 65 ml). Fraction vol: 10 ml. Flow rate: 30 ml/h. ●———●, total trypsin inhibitory activity; ○- - - - -○, total protein.

were withdrawn and assayed for antitryptic activity by the caseinolytic method.

Effect of temperature on the stability of the inhibitor: Both the purified inhibitor (11.5 μg protein) and the crude homogenate (51.0 μg protein) in 0.1 ml were kept in a water bath at 95°C for different times, cooled and assayed for residual antitryptic activity by the caseinolytic method. To determine the effect of cooking, the whole tubers (after removing the skin) were suspended in 10 vols. (w/v) H_2O and maintained at 95°C for 20 min. The mixture was cooled, ground in a mortar, centrifuged at 1000 X *g* for 15 min and the supernatant fraction was assayed for antitryptic activity.

Pre-treatment of the inhibitor with proteases: The purified inhibitor (11.5 μg protein) was treated with 10 μg pronase or 7.0 μg bovine chymotrypsin and 10 μmol phosphate buffer (pH 7.6) in 0.15 ml for different times. Pronase was inactivated by heat treament at 75–80°C for 10 min and chymotrypsin by heat treatment at 95°C for 5 min. Residual antitryptic activity in the reaction mixture was determined by the caseinolytic method. Pre-treatment with 10 μg porcine pepsin was done at pH 2.0. The pH was raised to 7.5 by addition of 0.05 ml 0.02 M NaOH and the residual inhibitory activity was determined. The inhibitor (11.5 μg protein) was also pre-treated with 6 μg trypsin or 7 μg chymotrypsin at pH 3.7, in presence of 10 μmol acetate buffer in 0.15 ml for different times, neutralized and the residual antitryptic activity was determined after inactivating the enzymes by heat treatment at 60°C for 30 min. Suitable controls with the purified inhibitor without the proteases were performed simultaneously.

Preincubation of the inhibitor with trypsin at pH 7.6: The inhibitor (5.8 μg–11.5 μg protein) was incubated with 6.0 μg bovine trypsin in presence of 20 μmol phosphate buffer (pH 7.6) in 1 ml for different times at 37°C. The enzyme reaction was initiated by the addition of 1 ml buffered casein (20 mg) and the assay was performed as usual. Control experiments without inhibitor were done to correct for loss in tryptic activity.

Preparation of 'modified inhibitor' after trypsin treatment at pH 3.7: The inhibitor (345 μg protein) was treated with 180 μg bovine trypsin in presence of 300 μmol acetate buffer (pH 3.7) in 4.6 ml for 2 h. The solution was dialyzed against phosphate buffer (pH 7.8) for 4 h at 4°C. The dialyzed solution was applied to a column of DEAE-cellulose (0.8 × 12.0 cm, bed volume 5.3 ml) equilibrated with 0.01 M phosphate buffer (pH 7.8). The column was eluted first with 15 ml equilibration buffer and then with 15 ml buffer containing 0.2 M NaCl (2.5-ml fractions; flow rate, 15 ml/h). The fractions were assayed for tryptic activity, protein and antitrypsin activity. Active trypsin was eluted with the equilibration buffer. A protein fraction that had neither enzyme nor inhibitor activity was eluted with the buffer containing NaCl. Active inhibitor was also processed similarly without trypsin and fractionated. It was eluted in the same region as the inactive protein but with full antitryptic activity.

Modification of amino groups of the inhibitor: This was done as described by Haynes et al. [15]. The inhibitor solution (6 ml, 6.90 mg protein) was subjected to heat treatment at 95°C for 5 min and cooled. TNBS solution (60 mg, 6 ml) was added followed by 12 ml 0.1 M phosphate buffer (pH 7.6). The system was incubated at 30°C. At definite intervals of time, 2 ml aliquots were withdrawn and dialyzed against distilled water for 16 h at 4°C. The dialyzed samples were used for determining residual inhibitory activity by the caseinolytic method and also for determining the amino groups. Inhibitor solution without prior heat treatment was also used in another set of study. Prior heat treatment of the inhibitor enhanced the rate of inactivation during TNBS treatment.

Estimation of amino groups of the inhibitor: This was done as described by Fields [16]. Isoleucine was used as standard.

Modification of arginyl residues of the inhibitor: This was performed according to the method of Yankeelov [17]. Both the native and the heat treated (at 95°C for 5 min) inhibitor solutions (0.8 ml, 460 μg protein) were mixed with 0.5 ml diacetyl solution (2.5 mg) and 0.5 ml 0.05 M borate buffer (pH 7.5), 1.0 M NaCl. After 48 h reaction at 30°C the solution was dialyzed against distilled water for 17 h at 4°C. The dialyzed samples were assayed for antitryptic activity by the caseinolytic method.

Sprouting studies: Mature tubers of the garden variety were allowed to sprout under soil, under controlled conditions. Tubers at different stages of plant growth were collected and total antitryptic activity and protein were estimated in the homogenates prepared as described earlier. The leaves, roots, petioles and stems were also screened for antitryptic activity at different stages of growth.

TABLE I

INHIBITORY ACTIVITIES IN VARIETIES OF *COLOCASIA* TUBERS

Antitryptic and antichymotryptic activities were measured by the caseinolytic method as described under Materials and Methods.

Variety	No. of samples	Antitryptic activity			Antichymotryptic activity		
		Units/g wet wt		Units/mg protein	Units/g wet wt		Units/mg protein
		Mean	Range	Mean	Mean	Range	Mean
Wild	5	1176	1090—1260	22.7	41.4	36.0—48.0	0.8
Garden	8	314	255—368	30.0	12.8	10.8—15.3	1.2
Red	4	663	630—690	23.5	20.1	15.6—27.0	0.7
Medium	5	Nil	—	Nil	Nil	—	Nil

Results

The levels of antitryptic and antichymotryptic activities in the different varieties of colocasia tubers are shown in Table I. The medium sized variety did not show any inhibitory activity. The wild variety showed the highest antitryptic activity when the values were expressed as units/g wet wt. However, the specific activity of antitrypsin(s) in the garden variety, wild variety and the red variety are comparable. All these three varieties of tubers displayed low antichymotryptic activity. The ratio of antitryptic activity to antichymotryptic activity was found to be around 25—40 in the different varieties of tubers.

The details of purification of the major trypsin inhibitor from the wild variety tubers are summarized in Table II. The major inhibitor was purified 4.8-fold with a recovery of 60% of activity. Further attempts to purify the inhibitor by rechromatography on DEAE-cellulose at pH 8.5 and 7.0, chromatography on Sephadex G-100 or by chromatography on phenyl Sepharose CL-4B were not successfull.

During chromatography of the ammonium sulphate fraction on DEAE-cellulose, a minor trypsin inhibitor which was more tightly bound to the column was identified (Fig. 1). This fraction which accounted for only 2.5% of the recovered activity was not processed further.

The molecular weight of the major inhibitor was found to be around 40 000 as determined by gel filtration on Sephadex G-100 (Fig. 2). The inhibitor had nearly the same elution volume when 5 M urea, 0.01 M phosphate buffer (pH

TABLE II

PURIFICATION OF A TRYPSIN INHIBITOR FROM *COLOCASIA* TUBERS

Fractions	Trypsin inhibitor (units)	Total protein (mg)	Specific activity	Yield (%)
Crude extract	50 960	2 015	25.2	100
DEAE-cellulose supernatant	43 890	1 031	42.6	86.1
Ammonium sulphate fraction	32 400	630	51.4	63.6
DEAE-cellulose major fraction	30 400	254	120.0	59.7

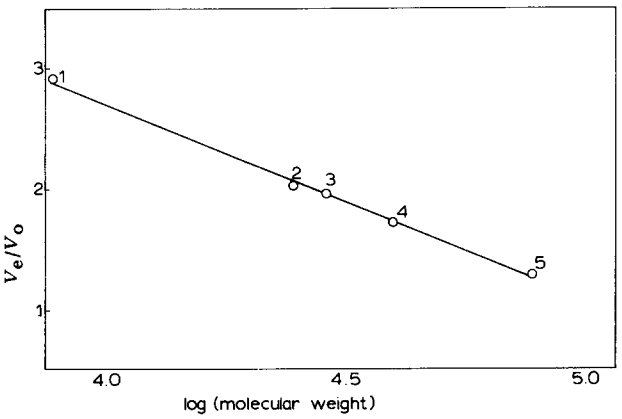

Fig. 2. Molecular weight determination of the purified inhibitor on Sephadex G-100 column (bed dimensions: 0.9 X 55.0 cm, bed vol: 35.0 ml), Flow rate: 10 ml/h. 1, insulin; 2, hen egg white trypsin inhibitor; 3, bovine trypsin; 4, Dextran (T-40) and purified inhibitor; 5, bovine albumin.

7.8) containing 0.2 M NaCl or water was used as eluant. The inhibitor was found to contain trace amounts of carbohydrates based on the phenol-H_2SO_4 reaction. When the carbohydrates were expressed as galactose, 1 mol of the inhibitor was found to contain 3.09 monosaccharide units.

When the purified inhibitor was subjected to cellulose acetate electrophoresis three protein bands could be separated (Fig. 3). All the three bands showed antitryptic activity. However, the specific activities of the three regions could not be determined due to low levels of proteins extracted from the cellulose acetate strips.

The purified inhibitor was 25 times and 2.5 times more active on bovine trypsin compared to bovine chymotrypsin and human trypsin respectively. Bovine trypsin (5.0 μg), bovine chymotrypsin (5.0 μg) and human trypsin (37 μg protein) showed comparable caseinolytic activity. The amounts of the inhibitor required to cause 45% inhibition were 11.5 μg for bovine trypsin, 276 μg for bovine chymotrypsin and 28.8 μg for human trypsin.

The purified inhibitor affected both the caseinolytic and amidolytic activities of bovine trypsin. The effects of varying concentrations of the inhibitor on these activities are presented in Fig. 4. At equimolar concentration of the inhibitor the inhibition observed by the caseinolytic method was slightly higher. The magnitude of inhibition was linear over a wide range of inhibitor levels (upto

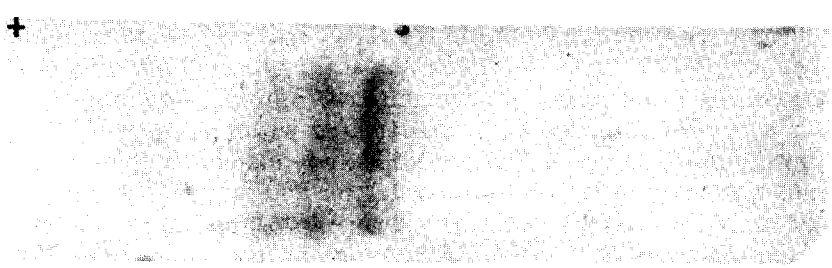

Fig. 3. Cellulose acetate electrophoresis of the purified inhibitor. Details are given under Materials and Methods.

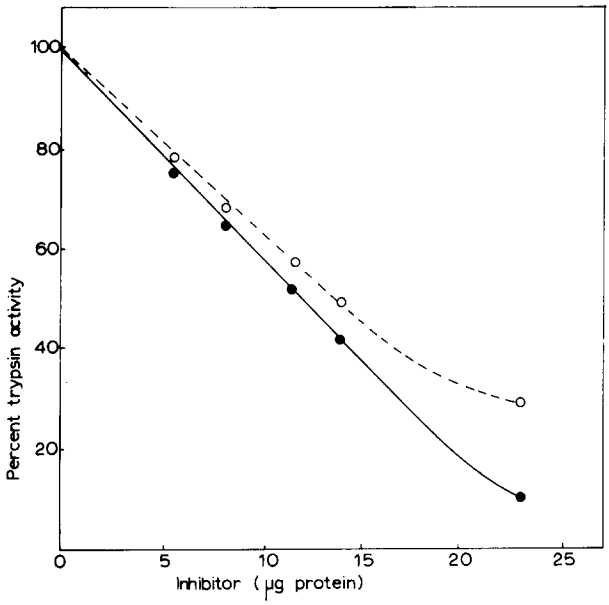

Fig. 4. Effect of varying concentration of the purified inhibitor on the caseinolytic and amidolytic activities of bovine trypsin, 6 µg of trypsin was used. ●———●, caseinolytic inhibition; ○- - - - -○, amidolytic inhibition.

90%) by the caseinolytic method. By the amidolytic method, linearity was observed upto 50% inhibition. Both the activities of trypsin were found to be non-competively inhibited by the inhibitor. The calculated K_i value from the inhibition data by the caseinolytic method for the inhibitor was $1.46 \cdot 10^{-7}$ M.

The inhibitor was found to be completely stable on exposure to wide range of pH 1.9—12.7. At highly acidic and highly alkaline conditions beyond this range, the biological activity was destroyed rapidly. The inhibitory activity of the crude extract was completely destroyed on exposure to 95°C for 10 min. On the other hand, the purified inhibitor lost only 50% of its activity under the same condition. When the tuber was cooked for 20 min in water at 95°—98°C the inhibitory activity was completely lost.

The effect of treatment of the purified inhibitor with porcine pepsin, pronase and bovine chymotrypsin on the antitryptic activity was investigated. Treatment with chymotrypsin for 10 min at pH 7.6 destroyed the activity completely, whereas complete destruction with pepsin or pronase treatment took 45 min and 120 min respectively. When the inhibitor was pre-incubated with trypsin for 10 min at pH 7.6 at 37°C, 30% of the antitryptic activity was lost. However, increasing the pre-incubation time upto 40 min did not cause any further loss in activity. When the inhibitor was treated with trypsin at 37°C at pH 3.7 for 60 min, the antitrypsin activity was lost completely. Similar treatment with chymotrypsin at pH 3.7 did not cause any loss in antitryptic activity. These data suggest that treatment at pH 3.7 with trypsin resulted in a proteolytic cleavage of the inhibitor that caused complete loss in activity. The inactive trypsin modified inhibitor was separated from the active enzyme by DEAE-cellulose chromatography. The modified inhibitor was eluted nearly in

the same region during chromatography on DEAE-cellulose as the native inhibitor (Fig. 5). When the modified inhibitor was incubated at pH 7.6 with trypsin upto 120 min, no recovery of biological activity was noticed.

Complex formation between trypsin and purified inhibitor at pH 7.6 was studied by gel chromatography on Sephadex G-100. The results are presented in Fig. 6. Analysis of the enzyme-inhibitor mixture revealed the presence of a protein fraction that was eluted earlier than the native inhibitor. This fraction was without any inhibitory activity and tryptic activity. Besides this major protein fraction, a second protein peak was eluted with a V_e/V_0 value of 3.53. This fraction that could have arisen by a partial proteolytic cleavage, had no inhibitory activity and was found to be dialyzable. The excess tryptic activity in the mixture was eluted in fractions 9 and 10 (elution volume 20—25 ml) and is not shown in the figure. The elution profiles of the native inhibitor and trypsin, determined in separate experiments are also shown in Fig. 6.

The inhibitor concentration required to completely inactivate 1 mol of trypsin was found to be 1.9 mol, based on values in the linear range of inhibition in the caseinolytic method.

Treatment of the native inhibitor with diacetyl upto 48 h did not result in the loss of antitryptic activity. Treatment with TNBS for 48 h resulted in the loss of 48% of the inhibitory activity. When the inhibitor was subjected to heat treatment at 95°C for 5 min and cooled prior to TNBS treatment, the activity was lost completely in 48 h. However, heat treatment did not cause any loss of activity on incubation with diacetyl for 48 h. The results on the percent loss of activity and the number of amino groups modified, as a function of time during

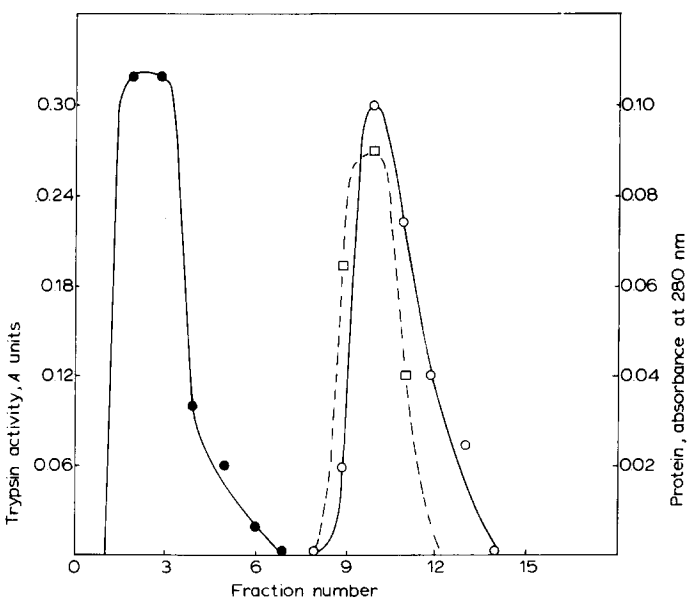

Fig. 5. Preparation of trypsin modified inhibitor. Details are given under Materials and Methods. ●———●, caseinolytic activity of trypsin, *A* units. ○———○, absorbance at 280 nm of modified inhibitor. □- - - - -□, absorbance at 280 nm of native purified inhibitor (300 μg protein).

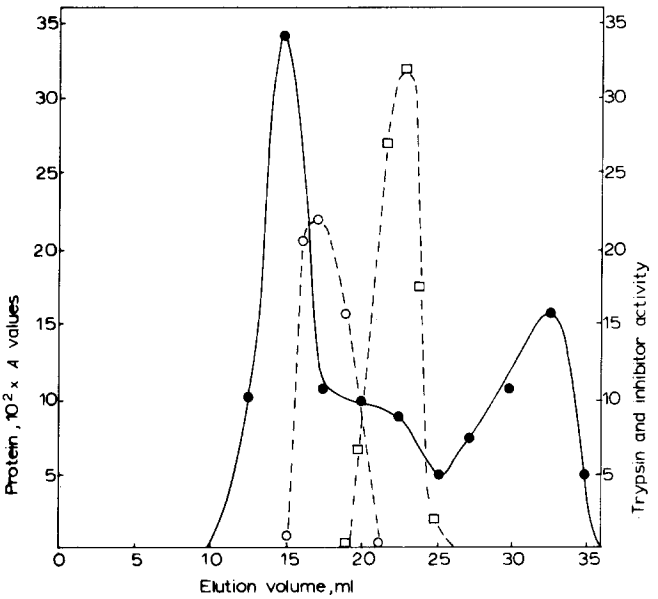

Fig. 6. Studies on complex formation between trypsin and purified inhibitor at pH 7.6 on Sephadex G-100. The purified inhibitor (1.70 mg protein) was incubated with 0.90 mg of trypsin in presence of 50 μmol of phosphate buffer (pH 7.8) in a vol of 1.0 ml at 30°C for 2 h. The solution was applied on a Sephadex G-100 column (bed dimensions: 0.9 X 52 cm, bed vol: 33 ml) equilibrated with 0.01 M phosphate buffer (pH 7.8) containing 0.2 M NaCl. Elution was performed with the same buffer at a flow rate of 10 ml/h. Fraction volume was 2.5 ml. The purified inhibitor (230 μg protein) and trypsin (180 μg) were also chromatographed in separate experiments. ●———●, enzyme-inhibitor mixture, protein 10^2 X A values. ○- - - - -○, inhibitor activity, scale 10^2 X A values; □- - - - -□, enzyme activity, scale 50 X A values.

TNBS reaction are presented in Table III. A total of 18 amino groups were modified per molecule of the inhibitor by TNBS in 48 h with complete loss of activity. The inhibitor was found to contain a total of 32 amino groups per molecule. A sharp fall in activity was noticed once in the earlier phase of the reaction (5—6 h) and again during the later phase of reaction (36—48 h) as shown in Table III.

During the screening studies with *Colocasia* tubers it was observed that the

TABLE III

EFFECT OF TNBS ON THE TRYPSIN INHIBITOR

The experimental details are described under Materials and Methods

Time of treatment (h)	No. of amino groups modified per molecule of inhibitor	Percent loss of antitryptic activity
0	0	0
5	1.9	00
6	3.4	60
8	4.5	60
12	14.5	76
24	16.0	76
36	16.6	76
48	17.7	100

TABLE IV

ANTITRYPTIC ACTIVITY IN IMMATURE TUBERS

Antitryptic activity was measured by the caseinolytic procedure. The details are given under Materials and Methods

Sample No.	Protein (mg/g wet wt.)	Inhibitor (units/ml extract)	Inhibitor after heat treatment (units/ml extract)
1	3.45	10.8	51.6
2	11.70	55.2	110.4
3	12.60	43.2	88.8

TABLE V

VARIATION OF TRYPSIN INHIBITOR AND PROTEIN DURING PLANT GROWTH

Antitryptic activity was measured by the caseinolytic procedure. Protein was determined by Lowry's method. Details are given under Mateirals and Methods.

No. of days of growth	Protein (mg/g wet wt.)	Inhibitor (units/g wet wt.)
0	25.5	396
17	26.6	468
32	36.4	756
37	39.0	792
45	24.5	360
57	12.2	0

immature tubers of all the three varieties (red, wild and garden varieties) contained low antitryptic activity (10—40%) compared to the corresponding mature tubers. It was also observed that the protein content of the immature tubers were about 40—50% of the mature ones. When the homogenates of the immature tubers were subjected to heat treatment at 80°C for 10 min there was a 2—5 fold increase in antitryptic activity. No such increase was noticed with the mature tuber homogenates. Typical data on three immature tubers of the garden variety are presented in Table IV. The levels of antitryptic activity in the garden variety tubers during different stages of sprouting and plant growth were investigated and the results are presented in Table V. It was observed that there was a gradual increase in the trypsin inhibitor levels and protein content upto 37 days during sprouting. Thereafter, there was a fall in activity and by 55 days there was complete disappearance of antitryptic activity. At this stage the protein content also decreased markedly. It was around this stage nodules started developing which ultimately became new tubers. The leaves, stem and petioles and roots of the colocasia plant were screened for antitryptic activity at different stages of plant growth. No detectable antitryptic activity was observed in any of these tissues.

Discussion

Tubers of three different varieties of the plant *Colocasia antiquorum* are shown to contain high levels of antitryptic activity. Trypsin inhibitor concentrations in these tubers are higher than the reported values in potato and sweet

potato [7] the two most extensively investigated tubers for protease inhibitors.

The major trypsin inhibitor has been purified from the wild variety tubers of colocasia and was found to be homogenous by gel chromatography and ion-exchange chromatography studies. The inhibitor may not have a role in determining the nutritional status of the tubers, since pepsin and chymotrypsin digested the inhibitor rapidly. The inhibitor also was inactivated completely during cooking.

The purified inhibitor was found to be more thermostable than the activity in the crude preparations. A similar observation had been made with the trypsin-chymotrypsin inhibitor from the tubers of *Alocasia macrorhiza* [12]. It is probable that factors responsible for labilizing the inhibitors in crude preparations of these tubers were removed during the purification process.

The inhibitor lost its antitryptic activity rapidly on treatment with chymotrypsin at pH 7.6 but there was no loss on treatment at pH 3.7. On the other hand, preincubation with trypsin at pH 7.6 for 10 min at 37°C resulted only in a partial loss of antitryptic activity. This could be due to a restricted proteolytic cleavage of the inhibitor resulting in a modified, but less active form which is bound to trypsin far more tightly than with chymotrypsin. Lack of stoichiometric interaction between trypsin and the inhibitor could be partly explained by this reasoning.

When the inhibitor was treated with trypsin at pH 3.7, there was complete loss of antitryptic activity. The difference in the action of trypsin at pH 3.7 and 7.6 could be due to the following reasons. It is known that trypsin inhibitors form stable complexes with the enzyme at neutral conditions but not acidic condition [18]. It is also known that trypsin causes site-specific, limited cleavage of the inhibitors which is discussed below. Presumably after a partial cleavage at pH 3.7, the modified inhibitor undergoes a conformational change to become inactive, whereas at pH 7.6 the modified inhibitor may be still held tightly by the enzyme preventing a conformational change that can lead to complete inactivation. The observation that an enzyme inhibitor complex in the case of the colocasia inhibitor could be isolated after interaction at pH 7.6 but not at pH 3.6 supports this thesis.

Ozawa and Laskowski observed that a single Arg-Ile bond in Kunitz soya bean trypsin inhibitor is cleaved by trypsin at pH 3.7, with the resultant formation of a modified but active inhibitor [19]. Similar observations on the formation of modified but active inhibitors have been made with lima bean inhibitors [20] and Bowman-Birk soya bean inhibitor [21]. On the other hand, chick pea trypsin-chymotrypsin inhibitor [22] was found to lose 85% of its antitryptic activity on treatment with trypsin and all the antichymotryptic activity on treatment with chymotrypsin at pH 3.7. In this respect the colocasia inhibitor resembles somewhat the chick pea inhibitor. The modified Bowman-Birk and lima bean inhibitors were found to be converted to the native form on treatment with trypsin at neutral pH [23,24]. In the case of the colocasia inhibitor no antitryptic activity was recovered by such a treatment.

TNBS treatment resulted in complete loss of activity implicating the amino groups, presumably those of lysine. A short heat treatment prior to addition of TNBS enhanced the rate of inactivation of the inhibitor, indicating that the amino groups are not easily accessible for chemical modification in the native

inhibitor. Time course study indicates that at least one fast reacting amino group and a slow reacting amino group are essential for the maximal activity of this inhibitor.

During the first phase of the plant growth extending upto about 40 days, there was a gradual increase in protein content and inhibitory activity of the tubers. The middle phase (40—55 days) was characterized by a fall in protein content and complete disappearance of antitrypsin, suggesting that the stored trypsin inhibitor serves as a reserve material for metabolic activities in this phase. Immature tubers that appeared much later (80—100 days) had relatively low protein content and antitryptic activity. This could represent the onset of a phase characterized by active synthesis of trypsin inhibitor. A curious observation was the sharp increase in antitryptic activity in immature tuber extracts on heat treatment at 80°C, whereas no such increase was observed with the mature tubers. It is tempting to suggest that the newly formed trypsin inhibitor is in combination with thermolabile factors resulting in lowered activity and that the free inhibitor is released during the process of maturation. Further studies are needed to explain this phenomenon.

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References

1 Balls, A.K. and Ryan, C.A. (1963) J. Biol. Chem. 238, 2976—2982
2 Belitz, H.D., Kaiser, K.P. and Santarius, K. (1971) Biochem. Biophys. Res. Commun. 42, 420—427
3 Hochstrasser, K., Werle, E., Siegelmann, R. and Schwarz, S. (1969) Z. Physiol. Chem. 350, 867—902
4 Melville, J.C. and Ryan, C.A. (1972) J. Biol. Chem. 247, 3445—3453
5 Sugiura, M., Ogiso, T., Takeuti, K., Tamura, S. and Ito, A. (1973) Biochim. Biophys. Acta 328, 407—417
6 Ogiso, T., Tamura, S., Kato, Y. and Sugiura, M. (1974) J. Biochem. 76, 147—156
7 Sumathi, S. and Pattabiraman, T.N. (1975) Ind. J. Biochem. Biophys. 12, 383—385
8 Nayak, N., Rajaram, S.V. and Pattabiraman, T.N. (1976) Ind. J. Biochem. Biophys. 13, 335—338
9 Kezdy, F.J. and Kaiser, E.T. (1970) in Methods in Enzymology (Perlmann, G.E. and Lorand, I., eds.), Vol. 19, pp. 1—27, Academic Press, New York
10 Chow, R.B. and Kassell, B. (1968) J. Biol. Chem. 243, 1718—1724
11 Erlanger, B.F., Kokowsky, N. and Cohen, W. (1961) Arch. Biochem. Biophys. 95, 271—278
12 Sumathi, S. and Pattabiraman, T.N. (1977) Biochim. Biophys. Acta 485, 167—178
13 Du Bois, M., Killes, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Analyt. Chem. 28, 350—356
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
15 Haynes, R., Osuga, D.T. and Feeney, R.F. (1967) Biochemistry 6, 541—547
16 Fields, R. (1972) in Methods in Enzymology (Hirs, C.H.W. and Timasheff, S.N., eds.), Vol. 25, pp. 464—468, Academic Press, New York
17 Yankeelov, J.A., Jr. (1972) in Methods in Enzymology (Hirs, C.H.W. and Timasheff, S.N., eds.), Vol. 25, pp. 566—578, Academic Press, New York
18 Finkenstadt, W.R. and Laskowski, M., Jr. (1964) J. Biol. Chem. 240, 962—963
19 Ozawa, K. and Laskowski, M., Jr. (1966) J. Biol. Chem. 241, 3955—3961
20 Krahn, J. and Stevens, F.C. (1972) Biochemistry 11, 1804—1808
21 Seidl, D.S. and Liener, I.E. (1971) Biochim. Biophys. Acta 251, 83—93
22 Smirnoff, P., Khalef, S., Birk, Y. and Applebaum, S. (1976) Biochem. J. 157, 745—751
23 Frattali, V. and Steiner, R.F. (1969) Biochem. Biophys. Res. Commun. 34, 480—487
24 Krahn, J. and Stevens, F.C. (1973) Biochemistry 12, 1330—1335