Natural plant enzyme inhibitors

Characterization of an unusual α -amylase/trypsin inhibitor from ragi (*Eleusine coracana* Geartn.)

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An inhibitor I-1, capable of acting on both α -amylase and trypsin, was purified to homogeneity from ragi (finger-millet) grains. The factor was found to be stable to heat treatment at 100°C for 1 h in the presence of NaCl and also was stable over the wide pH range 1–10. Pepsin and Pronase treatment of inhibitor I-1 resulted in gradual loss of both the inhibitory activities. Formation of trypsin–inhibitor I-1 complex, amylase–inhibitor I-1 complex and trypsin–inhibitor I-1–amylase trimer complex was demonstrated by chromatography on a Bio-Gel P-200 column. This indicated that the inhibitor is ‘double-headed’ in nature. The inhibitor was retained on a trypsin–Sephadex 4B column at pH 7.0. Elution at acidic pH resulted in almost complete recovery of amylase-inhibitory and trypsin-inhibitory activities. α -Amylase was retained on a trypsin–Sephadex column to which inhibitor I-1 was bound, but not on trypsin–Sephadex alone. Modification of amino groups of the inhibitor with 2,4,6-trinitrobenzenesulphonic acid resulted in complete loss of amylase-inhibitory activity but only 40% loss in antitryptic activity. Modification of arginine residues by cyclohexane-1,2-dione led to 85% loss of antitryptic activity after 5 h, but no effect on amylase-inhibitory activity. The results show that a single bifunctional protein factor is responsible for both amylase-inhibitory and trypsin-inhibitory activities with two different reactive sites.

Protein proteinase inhibitors are widely distributed in the plant kingdom (Vogel *et al.*, 1968). Most of these factors are known to inactivate trypsin, chymotrypsin and related enzymes (Vogel *et al.*, 1968). A number of such inhibitors have been isolated and characterized from legume seeds, cereals and tubers (Birk, 1976; Sumathi & Pattabiraman, 1977, 1979). Proteinaceous α -amylase inhibitors of plant origin have received increasing attention during the last few years. α -Amylase inhibitors have been studied in detail from wheat (Buonocore *et al.*, 1977), rye (Granum, 1978) and kidney bean (Marshall & Lauda, 1975). Previously we reported the isolation and characterization of two proteinaceous factors from ragi (finger-millet) grains that inhibited human salivary, human pancreatic and pig pancreatic amylases (Shivaraj & Pattabiraman, 1980). One of the inhibitors, designated inhibitor I-1, was found to be resistant to tryptic digestion, whereas the second one, inhibitor I-2, was inactivated by trypsin. In the present paper we report a remarkable property of inhibitor I-1, namely its capacity to inhibit both α -amylase and trypsin. To

the best of our knowledge, this is the first report on the existence of such an unusual bifunctional protein.

Experimental

Materials

Pig pancreatic amylase (EC 3.2.1.1), pig pepsin (EC 3.4.4.1), Pronase (type VI proteinase), lysozyme (hen's egg-white, EC 3.2.1.17), myoglobin (horse heart), α -chymotrypsinogen A (bovine) and the sodium salt of 2,4,6-trinitrobenzenesulphonic acid were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Bovine trypsin (EC 3.4.21.4), salt-free and twice-crystallized, and bovine α -chymotrypsin (EC 3.4.21.1), salt-free and thrice-crystallized, were the products of Worthington Biochemical Corp., Freehold, NJ, U.S.A. Soluble potato starch was obtained from E. Merck, Darmstadt, W. Germany. Cyclohexane-1,2-dione was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. CNBr-activated Sepharose 4B and Sephadex G-50 were procured from Pharmacia

Fine Chemicals, Uppsala, Sweden. Bio-Gel P-60 and P-200 were obtained from Bio-Rad Chemicals, Richmond, CA, U.S.A. Bovine serum albumin fraction V was the product of Fluka, Buchs, Switzerland. All other reagents were of analytical grade.

Assay methods

Amylase activity was measured as described by Bernfeld (1955), with the following modification. A portion of amylase (0.1 ml; 0.2 μ g of protein of pig pancreatic amylase) was added to a solution containing 30 μ mol of sodium phosphate buffer, pH 6.9, and 10.5 μ mol of NaCl in a volume of 1.5 ml. After 20 min incubation at 37°C, 0.5 ml of 1.0% starch solution was added. The enzyme reaction was arrested after 5 min incubation by the addition of 1 ml of dinitrosalicylate reagent (Bernfeld, 1955). The solution was kept in a boiling-water bath for 10 min, cooled and diluted to 11.0 ml with water. Amylolytic activity was calculated as maltose equivalents liberated. One unit of enzyme is defined as the amount of enzyme that liberated 1 μ mol of maltose equivalent under the assay conditions.

To measure the amylase-inhibitory activity, suitable amounts of the purified ragi inhibitors were preincubated with the enzyme solution in a total volume of 1.5 ml containing 30 μ mol of phosphate buffer, pH 6.9, and 10.5 μ mol of NaCl for 20 min at 37°C. Controls without inhibitors were run simultaneously. The enzyme reaction was initiated by the addition of starch solution, and the systems were processed as described above. The decrease in enzyme activity was a measure of the inhibitory activity. One unit of inhibitor is defined as the amount that decreased amylase activity by 1 unit.

Trypsin and chymotrypsin activities were assayed by the caseinolytic method as described before (Sumathi & Pattabiraman, 1975). The assay system contained 20.0 mg of casein, 120 μ mol of phosphate buffer, pH 7.6, and trypsin or chymotrypsin (6–10 μ g of active enzyme) in a total volume of 2.0 ml. After 10 min incubation at 37°C, the reaction was stopped by the addition of 3.0 ml of 5% (w/v) trichloroacetic acid. After 1 h standing, the precipitate was sedimented by centrifugation at 2500 g for 10 min. Then 1 ml samples of the supernatant were analysed by the method of Lowry *et al.* (1951). One unit of proteolytic activity is defined as the amount of enzyme that released 1 mg of trichloroacetic acid-soluble peptide fragments under the assay conditions. For the purpose of calculation bovine serum albumin was used as standard in this method. Proteinase inhibitory activities of the purified ragi inhibitors were determined by including suitable amounts of inhibitor solutions in the assay system. Decrease in caseinolytic activity in the test system compared with the control was a measure of the

inhibitory activity. One unit of inhibitor is defined as the amount that suppressed proteolytic activity by 1 unit.

Protein content was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Purification of inhibitors

The details of purification of amylase inhibitors I-1 and I-2 from ragi grains were as described previously (Shivaraj & Pattabiraman, 1980). Ground ragi grains were homogenized with 3 vol. (w/v) of 0.15 M-NaCl and filtered. To the filtrate, solid $(\text{NH}_4)_2\text{SO}_4$ was added to 55% saturation. The precipitate formed was collected by centrifugation and dissolved in 2 mM-sodium acetate buffer, pH 5.0, containing 75 mM-NaCl and dialysed overnight against the same buffer. The dialysis residue was centrifuged, and the clear supernatant was subjected to chromatography on a CM-cellulose column equilibrated with 2 mM-acetate buffer, pH 5.0, containing 75 mM-NaCl. The two inhibitors that were exchanged on the column were eluted separately with 20 mM-acetate buffer, pH 5.0, containing 0.15 M-NaCl and the same buffer containing 0.3 M-NaCl. The two fractions were further purified by gel chromatography on Sephadex G-50 and rechromatography on CM-cellulose. Inhibitor I-1 accounted for 86.5% of the recovered activity and inhibitor I-2 for 13.5% of the activity.

Molecular-weight determination

The molecular weights of purified inhibitors I-1 and I-2 were determined by electrophoresis on polyacrylamide gel (10% acrylamide) in the presence of sodium dodecyl sulphate as described by Weber *et al.* (1972). Lysozyme (egg-white, mol.wt. 14300), myoglobin (horse heart, mol.wt. 17200), α -chymotrypsinogen A (bovine, mol.wt. 25700) and pepsin (pig, mol.wt. 35000) were used as standard proteins.

Complex-formation

Complex-formation between inhibitor I-1 and trypsin or amylase was demonstrated by gel chromatography on a Bio-Gel P-200 column (0.9 cm $\times$ 53.5 cm, bed volume 34.0 ml) equilibrated with 20 mM-phosphate buffer, pH 7.0, containing 0.15 M-NaCl. In each experiment the components were applied to the column in a total volume of 0.3 ml containing 6 μ mol of phosphate buffer, pH 7.0, and 45 μ mol of NaCl. The column was eluted with equilibration buffer at a flow rate of 3 ml/h, and 1 ml fractions were collected. To obtain the trypsin-inhibitor I-1 complex, 400 μ g of protein of bovine trypsin was incubated with 400 μ g of protein of inhibitor I-1 at room temperature (28°C)

for 30 min, and the mixture was applied to the column. The fractions were assayed for protein content and α -amylase-inhibitory activity. Similarly, the mixture of pig pancreatic amylase (550 μ g of protein) and inhibitor I-1 (400 μ g of protein) was applied to the column after incubation for 30 min in order to obtain the amylase-inhibitor I-1 complex. Formation of trimer complex between trypsin, inhibitor I-1 and amylase was shown by passing a mixture of 400 μ g of protein of trypsin, 400 μ g of protein of inhibitor I-1 and 550 μ g of protein of pig pancreatic amylase through the column after incubation for 30 min at pH 7.0. Protein content was determined in each fraction. In separate experiments inhibitor I-1 (400 μ g of protein), bovine trypsin (400 μ g of protein) and pig pancreatic amylase (550 μ g of protein) were passed through the column to determine their elution patterns.

Affinity chromatography

Affinity chromatography of inhibitor I-1 was performed at 4°C on a trypsin-Sepharose 4B column. Trypsin (25 mg) was treated with CNBr-activated Sepharose 4B (3.5 ml wet volume) in the presence of 0.1 M-sodium borate buffer, pH 8.3, containing 0.5 M-NaCl. The trypsin-Sepharose 4B was packed into a column (0.9 cm $\times$ 4.7 cm, bed volume 3.0 ml) and equilibrated with 20 mM-phosphate buffer, pH 7.0, containing 50 mM-NaCl. Then 0.5 mg of protein of inhibitor I-1 in a total volume of 0.4 ml containing 8 μ mol of phosphate buffer, pH 7.0, and 20 μ mol of NaCl was applied to the column. The column was washed with 15.6 ml of the equilibration buffer. The inhibitor bound to the column was then eluted with 50 mM-HCl containing 50 mM-NaCl. Fractions of volume 2 ml were collected during washing and elution at a flow rate of 4 ml/h. Protein and inhibitory activities were determined in the fractions.

Formation of trimer complex between trypsin, inhibitor I-1 and α -amylase was also demonstrated on a trypsin-Sepharose column. First, 0.5 mg of protein of inhibitor I-1 was bound to the trypsin-Sepharose column (bed volume 3.0 ml) as indicated above. The column was washed extensively with 20 mM-phosphate buffer, pH 7.0, containing 0.15 M-NaCl. Then 250 μ g of protein of pig pancreatic amylase in a volume of 0.25 ml containing 5 μ mol of phosphate buffer, pH 7.0, and 37.5 μ mol of NaCl was applied to the column. The column was again washed with 20 mM-phosphate buffer, pH 7.0, containing 0.15 M-NaCl. The washings (20 ml) were assayed for protein content and α -amylase activity. In a separate control experiment 250 μ g of protein of pig pancreatic amylase alone was applied to a trypsin-Sepharose column, and protein as well as α -amylase activity were determined in the washings.

Effect of temperature

For studying the effect of heat treatment on the amylase-inhibitory and trypsin-inhibitory activities of inhibitor I-1, 3.2 μ g of protein of the inhibitor in the presence of 1.5 μ mol of NaCl in a volume of 20 μ l was kept in a boiling-water bath at 100°C for 1 h. The contents were cooled and assayed for residual inhibitory activities.

Effect of pH

Effect of pH on the inhibitory activities of inhibitor I-1 was measured as follows. A sample (8 μ g of protein) of inhibitor I-1 was preincubated with 0.1 M buffers of different pH values (sodium citrate, pH 3.0; acetate, pH 5.0; phosphate, pH 6.9 and 8.0; glycine/NaOH, pH 9.0 and 10.0), 0.2 M-HCl, pH 0.9, and 0.2 M-NaOH, pH 12.6, for 16 h at 4°C in a total volume of 0.1 ml. After addition of 0.5 ml of 0.2 M-phosphate buffer, pH 7.6, suitable samples were withdrawn and assayed for residual inhibitory activities.

Preincubation of inhibitor I-1 with trypsin at pH 3.7

Inhibitor I-1 (8.0 μ g of protein) was preincubated with 26 μ g of trypsin at 25°C in a volume of 0.1 ml containing 2.0 μ mol of acetate buffer, pH 3.7, and 10 μ mol of NaCl for different time intervals. The pH of the system was adjusted to 7.6 by adding 0.4 ml of 0.2 M-phosphate buffer, pH 7.6. The contents were made up to 1.0 ml with water and assayed for residual trypsin activity. Unlike the procedure mentioned above, the reaction was started by adding 1.0 ml of substrate (casein) and incubated for 10 min. Controls were run simultaneously without addition of the inhibitors. Similarly the preincubation mixtures were assayed for amylase-inhibitory activity after neutralization of the contents by adding 0.4 ml of 0.2 M-phosphate buffer, pH 6.9.

Results

The isolation of two proteinaceous α -amylase inhibitors, I-1 and I-2, in homogeneous forms from ragi has already been described (Shivaraj & Pat-tabiraman, 1980). Both the inhibitors were basic proteins, and the apparent molecular weights based on gel chromatography on Sephadex G-50 and Bio-Gel P-60 were about 9000 and 8000 respectively for inhibitors I-1 and I-2. However, the inhibitors were non-diffusible on dialysis. This led to a re-investigation of the molecular weights of the inhibitors by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The electrophoretic patterns of inhibitors I-1 and I-2 are shown in Fig. 1. The calculated molecular weights based on gel electrophoresis of inhibitors I-1 and I-2 were 14 300 and 16 500 respectively, which are at wide variance

Fig. 1. Polyacrylamide-gel electrophoresis of inhibitors I-1 and I-2 in the presence of sodium dodecyl sulphate

The electrophoresis was performed on polyacrylamide gel (10% acrylamide) in the presence of 0.1% sodium dodecyl sulphate in both gel and electrode buffer as described by Weber *et al.* (1972). The samples (inhibitors I-1 and I-2) were treated with 1% sodium dodecyl sulphate and 1% β -mercaptoethanol for 2 h at 37°C at pH 7.2, and subjected to electrophoresis for 4 h with a current of 6 mA/tube. The protein bands were stained with Coomassie Brilliant Blue R-250 (0.1% in methanol/acetic acid/water, 5:1:5, by vol.).

with the values obtained by gel-permeation chromatography. The discrepancy is probably due to the fact that the two inhibitors, being basic proteins, are retarded on Sephadex G-50 and Bio-Gel P-60 columns, exhibiting anomalous elution behaviour. Similar observations have been reported for other proteins (Starkey & Barrett, 1976).

When inhibitor I-2 (16 μ g of protein) was incubated with bovine trypsin (10 μ g) for 2 h at pH 7.6 and at 37°C, the amylase-inhibitory activity was almost completely lost. On the other hand, inhibitor I-1 was fully resistant to tryptic attack under identical conditions. This prompted us to study the effects of inhibitors I-1 and I-2 on tryptic activity. Although inhibitor I-2 had no effect on the caseinolytic activity of bovine trypsin even when used in a molar ratio of 4:1, inhibitor I-1 was found

Fig. 2. Polyacrylamide-gel electrophoresis of inhibitor I-1 (a) At pH 5.0 with 40 mM-acetate buffer (continuous buffer system). The electrophoresis was run for 3 h with a current of 3 mA/tube. (b) At pH 8.6 with 25 mM-Tris/Veronal buffer (continuous buffer system) for 45 min with a current of 3 mA/tube. The protein bands were stained with 0.1% Amido Schwartz in 7% (v/v) acetic acid.

to be a powerful inhibitor of trypsin. This suggested that inhibitor I-1 is an unusual bifunctional inhibitor capable of acting on α -amylase as well as on trypsin. The following studies corroborated this conclusion.

In order to prove that inhibitor I-1 is a single protein responsible for both trypsin-inhibitory and amylase-inhibitory activities, the homogeneity of the preparation was established by several criteria. Inhibitor I-1 moved as a single band towards the cathode at both pH 5.0 and pH 8.6 when electrophoresis was performed on polyacrylamide gel (Fig. 2) and cellulose acetate strips (Fig. 3). Inhibitor I-1 was also found to be homogeneous on the basis of gel-filtration studies on Sephadex G-50 in the presence and in the absence of 6 M-urea (Fig. 4). Similar elution behaviour was also obtained during gel filtration on Bio-Gel P-60.

When inhibitor I-1 was passed through a column of trypsin-Sepharose 4B, both the inhibitory activities were bound to the immobilized enzyme. The binding was not due to weak interaction, since elution with 0.5 M-NaCl did not dissociate the amylase-inhibitory or trypsin-inhibitory activities. The inhibitor protein was eluted with 50 mM-HCl containing 50 mM-NaCl. Complete recovery of trypsin-inhibitory activity and 92% recovery of amylase-inhibitory activity were obtained in a single

Fig. 3. Cellulose acetate electrophoresis of inhibitor I-1. The cellulose acetate electrophoresis was performed both at (a) pH 5.0 (0.1 M-acetate buffer) and (b) pH 8.6 (40 mM-sodium barbitone buffer) on Celagram (Shandon) strips. The time of run was 1 h at 200 V. The strips were stained with 0.5% Acid Red in 5% (v/v) acetic acid.

fraction (elution volume 20 ml) during affinity chromatography. The eluted inhibitor I-1 resembled the native inhibitor with respect to chromatographic behaviour on Sephadex G-50 and electrophoretic pattern on polyacrylamide gel. Moreover the fraction could be again immobilized on a trypsin-Sepharose column at neutral pH. There was no interaction or binding of inhibitor I-1 to Sepharose alone, since the inhibitor was not retained on a chymotrypsin-Sepharose column.

α -Amylase was not retained on a trypsin-Sepharose column and was eluted in the washings (20 mM-phosphate buffer, pH 7.0, containing 0.15 M-NaCl). However, when amylase was passed through the column on which inhibitor I-1 was immobilized, no amylase activity or protein was eluted in the washings. These findings show that amylase is bound to immobilized inhibitor I-1. This demonstrates that amylase-inhibitory activity and trypsin-inhibitory activity are associated with one and the same protein.

When inhibitor I-1 solution was subjected to heat treatment in the presence of 75 mM-NaCl at 100°C for 1 h, there was no loss of antitryptic activity as well as amylase-inhibitory activity. Dialysis of the inhibitor against water resulted in complete precipitation, indicating that salt (NaCl) is essential for the solubility of the inhibitor.

Fig. 4. Gel filtration of inhibitor I-1 on a Sephadex G-50 column (0.9 cm $\times$ 60 cm, bed volume 38 ml)

O, Inhibitor I-1 (0.9 mg of protein) in 0.4 ml containing 8 μ mol of phosphate buffer, pH 7.6, and 60 μ mol of NaCl was applied to the column previously equilibrated with 20 mM-phosphate buffer, pH 7.6, containing 0.3 M-NaCl. ●, Inhibitor I-1 (0.9 mg of protein) was incubated with 6 M-urea in a volume of 0.4 ml containing 8 μ mol of phosphate buffer, pH 7.6, and 60 μ mol of NaCl for 2 h at 37°C. The sample was then applied to the column previously equilibrated with 20 mM-phosphate buffer, pH 7.6, containing 0.15 M-NaCl and 6 M-urea. In both cases the inhibitor was eluted with equilibration buffers at a flow rate of 7 ml/h, and 1 ml fractions were collected. The fractions were assayed for protein content.

When the inhibitor was exposed to different pH values varying from 1 to 10 for 16 h at 4°C there was no loss of either inhibitory activity. However, at pH 12.6 about 30% of the amylase-inhibitory activity was lost, though the antitryptic activity was not diminished.

The effects of pepsin and Pronase treatment on the inhibitory activities of inhibitor I-1 are shown in Figs. 5 and 6 respectively. There was a gradual loss of the activities with increasing time of treatment with the proteinases. However, pepsin destroyed preferentially the amylase-inhibitory activity of inhibitor I-1, whereas the antitryptic activity was preferentially removed by Pronase.

It has been shown previously that treatment of inhibitor I-1 with starch for 10 min resulted in complete abolition of amylase-inhibitory activity

Fig. 5. Changes in amylase-inhibitory and trypsin-inhibitory activities of inhibitor I-1 on treatment with pepsin

Inhibitor I-1 (15 μ g of protein) was treated with 5 μ g of pepsin in the presence of 20 μ mol of HCl/KCl buffer, pH 2.0, in a total volume of 0.2 ml. The contents were incubated for different time intervals at 37°C. Pepsin action was arrested by the addition of 0.2 ml of 0.2 M-phosphate buffer, pH 7.6. Residual amylase-inhibitory and trypsin-inhibitory activities were measured in the samples. O, Amylase-inhibitory activity; ●, trypsin-inhibitory activity.

Fig. 6. Changes in amylase-inhibitory and trypsin-inhibitory activities of inhibitor I-1 on treatment with Pronase

Inhibitor I-1 (15 μ g of protein) was incubated with 5 μ g of protein of Pronase in a volume of 0.2 ml containing 20 μ mol of phosphate buffer, pH 7.6, at 37°C. At different time intervals the Pronase was inactivated by heat treatment at 75°C for 10 min. The contents were assayed for residual inhibitory activities. O, Amylase-inhibitory activity; ●, trypsin-inhibitory activity.

Table 1. Modification of inhibitor I-1 with 2,4,6-trinitrobenzenesulphonic acid and cyclohexane-1,2-dione and changes in amylase-inhibitory and trypsin-inhibitory activities

Amino groups were modified by treatment with 2,4,6-trinitrobenzenesulphonic acid at pH 7.6 (Sumathi & Pattabiraman, 1977). A sample (520 μ g of protein) of inhibitor I-1 was treated with 3.3 mg of 2,4,6-trinitrobenzenesulphonic acid in the presence of 400 μ mol of phosphate buffer, pH 7.6, in a volume of 4.0 ml at 28°C. Samples were withdrawn at intervals of 1, 2, 3, 4 and 5 h and each was dialysed against 2 litres of 50 mM-NaCl for 5 h at 4°C. The dialysed samples were assayed for residual amylase-inhibitory and trypsin-inhibitory activities. Arginine residues of inhibitor I-1 were modified by treatment with cyclohexane-1,2-dione (Toi *et al.*, 1967). Inhibitor I-1 (520 μ g of protein) was treated with 1.7 mg of cyclohexane-1,2-dione in the presence of 200 μ mol of sodium borate buffer, pH 9.0, at 28°C in a total volume of 4.0 ml. At intervals samples were withdrawn, dialysed as described above and tested for residual inhibitory activities.

Assay components	Time (h)	Residual inhibitory activity (%)	
		Antiamylase	Antitrypsin
Inhibitor I-1	Control	100	100
Inhibitor I-1 + cyclohexane-1,2-dione	1	100	64
	2	100	49
	3	100	28
	4	100	19
	5	100	16
Inhibitor I-1 + 2,4,6-trinitrobenzenesulphonic acid	1	6	78
	2	3	68
	3	0	66
	4	0	60
	5	0	60

(Shivaraj & Pattabiraman, 1980). To study the effect of such a treatment on the antitryptic activity, 4.0 μ g of protein of inhibitor I-1 was mixed with 0.3 ml of starch solution (1%) at pH 6.9 and at 7.6. After 10 min, the contents were assayed for trypsin-inhibitory activity. There was no detectable loss of proteinase-inhibitor activity after preincubation with starch, indicating that the binding site for trypsin on inhibitor I-1 is mutually exclusive of the binding site for amylase. This was also proved by preincubation of inhibitor I-1 with amylase. A sample (4.0 μ g of protein) of inhibitor I-1 was incubated with 10 μ g of protein of pig pancreatic amylase in 0.3 ml in the presence of 6 μ mol of phosphate buffer, pH 6.9, and 15 μ mol of NaCl at 37°C for 20 min. When this mixture was directly assayed for antitryptic activity, 100% of the proteinase-inhibitory activity was retained compared with the control without amylase.

The results on complex-formation between inhibitor I-1 and the enzymes are depicted in Fig. 7. When trypsin/inhibitor I-1 mixture was subjected to gel chromatography on Bio-Gel P-200, a peak with maximal protein values at an elution volume of 24 ml was seen. In addition, there was a shoulder region (27–31 ml). Trypsin itself when subjected to gel chromatography was eluted with a peak at 27 ml, and the inhibitor alone was eluted with a peak at 30 ml. The trypsin-inhibitor complex (tube no. 24) displayed amylase-inhibitory activity. The amylase/inhibitor mixture was eluted with a protein peak at 22 ml. A second peak (30 ml) was also noticed during this experiment. This might correspond to non-reacted amylase inhibitor. Amylase alone when subjected to gel chromatography was eluted with an elution volume of 25 ml. Trypsin/amylase/inhibitor mixture was also subjected to gel chromatography, and two protein peaks were observed with maximal protein values at 18 ml and 23 ml. The first peak should represent the trimer complex, and the second peak may represent the mixture of amylase-inhibitor and trypsin-inhibitor complexes. The molecular weights of the inhibitor-enzyme complexes could not be ascertained by gel chromatography owing to the anomalous behaviour of the inhibitor during gel chromatography.

The findings from chemical modification also supported the conclusion that binding sites for trypsin and amylase on inhibitor I-1 are mutually exclusive. The effects of treatment of inhibitor I-1 with cyclohexane-1,2-dione and 2,4,6-trinitrobenzenesulphonic acid on the inhibitory activities are shown in Table 1. Modification of the arginine residues with cyclohexane-1,2-dione by treatment for up to 5 h did not result in any loss of amylase-inhibitory activity, whereas 85% of the antitryptic activity was lost under this condition. Only 7% of the trypsin-inhibitory activity was retained after 20 h treatment with cyclohexane-

Fig. 7. Complex-formation between inhibitor I-1, trypsin and amylase on a Bio-Gel P-200 column (0.9 cm $\times$ 53.5 cm, bed volume 34.0 ml)

The column was equilibrated with 20 mM-phosphate buffer, pH 7.0, containing 0.15 M-NaCl. (a) $\circ$, Pig pancreatic amylase (550 μ g of protein); $\bullet$, inhibitor I-1 (400 μ g of protein). (b) $\bullet$, Bovine trypsin (400 μ g of protein); Δ , trypsin-inhibitor I-1 complex (400 μ g of protein of each of trypsin and inhibitor I-1). (c) $\circ$, Amylase-inhibitor I-1 complex (550 μ g of protein of amylase and 400 μ g of protein of inhibitor I-1); Δ , trypsin-inhibitor I-1-amylase trimer complex (400 μ g of protein of each of trypsin and inhibitor I-1 and 550 μ g of protein of amylase). To obtain the complexes the components were mixed and incubated for 30 min at 28°C at pH 7.0 and applied to the column. Then 1 ml fractions were collected at a flow rate of 3 ml/h.

1,2-dione. Conversely, modification of amino groups by 2,4,6-trinitrobenzenesulphonic acid resulted in almost complete loss of amylase-inhibitory activity

in 1 h, whereas only 40% of the antitryptic activity was destroyed after 5 h treatment.

When inhibitor I-1 was preincubated with trypsin at pH 3.7 for 30 min, there was about 50% loss of antitryptic activity, which remained constant up to 1 h. This loss of activity could be due to limited proteolysis of inhibitor at lower pH (Smirnoff *et al.*, 1976; Sumathi & Pattabiraman, 1979). However, this treatment had no effect on amylase-inhibitory activity.

The inhibitory pattern against trypsin was found to be linear with respect to inhibitor concentration up to 75%. In the linear range of inhibition 1.0 mol of inhibitor I-1 was found to interact stoichiometrically with 1.0 mol of bovine trypsin. Inhibitor I-1 did not exhibit any inhibitory activity against bovine chymotrypsin even when excess (40 µg of protein) of the inhibitor was used. With pig pancreatic amylase, the inhibition by inhibitor I-1 was linear only up to 40%, as reported previously (Shivaraj & Pattabiraman, 1980).

Discussion

Proteinase inhibitors capable of acting on trypsin and chymotrypsin and possessing distinct binding sites for the two enzymes have been reported (Birk *et al.*, 1967; Smirnoff *et al.*, 1976). However, the present studies establish for the first time the existence of an unusual 'double-headed' enzyme inhibitor that inhibits two unrelated enzymes, namely α -amylase and trypsin. The inhibitor was found to be a basic protein with mutually exclusive binding sites for the two enzymes. The results of chemical modification suggest that arginine residues are essential for the trypsin-inhibitory activity, whereas they are not involved in the inactivation of amylase by the inhibitor. On the other hand, amino

groups on the inhibitor were found to be necessary for binding and inactivation of α -amylases.

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