Isolation and characterization of a specific enterokinase inhibitor from kidney bean (*Phaseolus vulgaris*)

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A specific enterokinase inhibitor from kidney bean (*Phaseolus vulgaris*) was purified to homogeneity. It showed a single protein band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in the presence of mercaptoethanol, and the M_r was 31 000. Aspartic acid was identified as the *N*-terminus of the inhibitor. The M_r by gel chromatography on Sephadex G-200 was found to be 60 000, indicating the dimeric nature of the inhibitor. The inhibitor was found to be a glycoprotein. The monosaccharide moieties were glucose, mannose, glucuronic acid and glucosamine in the proportions 3.15%, 5.0%, 0.85% and 1.3% respectively. The inhibitor was most active on pig enterokinase, followed by bovine and human enterokinases. Maximal inhibitory activity was elicited by preincubation of the inhibitor with the enzyme for 15 min. Digestion with pepsin resulted in loss of inhibitory activity. The inhibitor was stable to exposure to a wide range of pH values (2–10), and exposure to pH above 10 resulted in loss of inhibitory activity. Modification of arginine residues by cyclohexane-1,2-dione and ninhydrin led to complete loss of enterokinase-inhibitory activity.

Enterokinase-inhibitory activity from plant sources was reported only recently (Lau *et al.*, 1980; Bhat *et al.*, 1981). Lau *et al.* (1980) purified two bovine enterokinase inhibitors from peanuts (*Arachis hypogaea*) which also inhibited trypsin and chymotrypsin. The inhibitors studied by these workers may be the non-specific proteinase inhibitors previously studied by others (Tur-Sinai *et al.*, 1972; Hochstrasser *et al.*, 1969). Bhat *et al.* (1981) in a survey for enterokinase inhibitors from plant sources showed that twelve tubers and nine pulses contained enterokinase-inhibitory activities. They also showed the presence of specific enterokinase inhibitors in kidney bean (*Phaseolus vulgaris*) and field bean (*Dolichos lablab*) which were devoid of trypsin-inhibitory activity. In the present paper we report the isolation and characterization of a specific enterokinase inhibitor from kidney bean (*Phaseolus vulgaris*).

Experimental

Materials

Pig enterokinase, Pronase (type VI proteinase), myoglobin (horse heart), lysozyme (hen's-egg white),

Dalton Mark VI (a mixture of standard proteins containing bovine serum albumin, ovalbumin, pepsin, trypsinogen, β -lactoglobulin and lysozyme), dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride and the sodium salt of 2,4,6-trinitrobenzenesulphonic acid were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Bovine trypsin (salt-free and twice crystallized) and bovine α -chymotrypsin (thrice crystallized) were products of Worthington Biochemical Corp., Freehold, NJ, U.S.A. Pig pepsin (thrice crystallized) was a product of Calbiochem, San Diego, CA, U.S.A. Ninhydrin and 5,5'-dithiobis-(2-nitrobenzoic acid) were purchased from Pierce Chemicals, Rockford, IL, U.S.A. Cyclohexane-1,2-dione was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. All other reagents were analytical-grade commercial chemicals.

Bovine trypsinogen was prepared as described by Wilimowska-Pelc & Mejbaum-Katzenellenbogen (1978). Bovine enterokinase was partially purified from duodenum as described by Liepnicks & Light (1979). The procedure was followed up to the DEAE-cellulose chromatographic stage and the active enzyme fractions were passed through a Sephadex G-200 column, and the fractions that contained enterokinase activity were used for the studies. Human enterokinase and dog enterokinase preparations were made by using similar procedures.

Abbreviation used: SDS, sodium dodecyl sulphate.

Enterokinase assay

Trypsinogen was activated to trypsin by preincubation with enterokinase at pH 5.0, and the trypsin formed was assayed by the method of Erlanger *et al.* (1961). The preincubation system contained 40 μ mol of Tris/acetate buffer, pH 5.0, 1 μ mol of CaCl_2 , 100 μ g of bovine trypsinogen and 20 munits of enterokinase in a total volume of 1.0 ml. The reaction was initiated by adding trypsinogen and the reaction mixture was incubated at 37°C for 10 min. To this assay mixture 2.0 ml of 2 mM- α -N-benzoyl-DL-arginine *p*-nitroanilide in 50 mM-Tris/HCl buffer, pH 8.0, was added and incubated for 15 min. The reaction was stopped by adding 1.0 ml of 30% (v/v) acetic acid. The colour was measured at 410 nm. One unit of enterokinase is defined as the amount that produced one amidolytic unit of trypsin under the specified assay conditions. One amidolytic unit of trypsin is the amount that liberated 1 μ mol of *p*-nitroaniline from the nitroanilide in 1 min under the assay conditions. The esterolytic activity of enterokinase was measured with α -N-benzoyl-L-arginine ethyl ester as substrate (Bhat *et al.*, 1981).

To measure the enterokinase-inhibitory activity, suitable samples of inhibitor were preincubated with enterokinase for 15 min at 37°C at pH 5.0, followed by the trypsinogen-activation step, as described above. One unit of inhibitor is defined as the amount that suppressed the enterokinase activity by one unit.

Trypsin assay

Amidolytic activity of crystalline bovine trypsin was measured as follows: 1 μ mol of CaCl_2 , 40 μ mol of Tris/acetate buffer, pH 5.0, 100 μ mol of Tris/HCl buffer, pH 8.0, 4.0 μ mol of benzoylarginine *p*-nitroanilide and 20 μ g of trypsin in a final volume of 3.0 ml were incubated at 37°C for 15 min. The reaction was started by the addition of substrate and stopped by the addition of 1.0 ml of 30% (v/v) acetic acid, and the A_{410} was measured. To measure trypsin-inhibitory activity, suitable samples of inhibitor were preincubated with the trypsin for 15 min in the assay system. One amidolytic unit of trypsin is the amount that liberated 1 μ mol of *p*-nitroaniline from the nitroanilide in 1 min under the assay conditions. One unit of trypsin inhibitor is the amount that suppressed the activity of trypsin by one unit.

Inhibitory activities against trypsin and chymotrypsin were also measured by the caseinolytic method (Sudhaker Prabhu & Pattabiraman, 1980). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as standard.

Purification of enterokinase inhibitor from kidney bean

All operations were performed at 4°C unless other-

wise stated, and the inhibitory assay was done with bovine enterokinase and bovine trypsinogen.

Kidney-bean seed powder (10 g) was stirred with 100 ml of 25 mM-Tris/HCl buffer, pH 8.0, for 2 h. The homogenate was centrifuged at 12000 *g* for 20 min at 4°C. The supernatant ('crude extract') was warmed to 30°C and the solution was adjusted to pH 4.0 with 1.0 M-HCl, stirred for 1 h at 4°C, and centrifuged at 12000 *g* for 20 min at 4°C. The pH of the solution was adjusted to 7.5 with 1.0 M-NaOH. This fraction was dialysed against 100 vol. of 10 mM-Tris/HCl buffer, pH 7.5, for 24 h with two changes of the dialysis medium, at 8 and 16 h. The dialysed solution was centrifuged at 10000 *g* at 4°C for 10 min.

DEAE-cellulose chromatography. The clear supernatant ('acid fraction', volume 75 ml) was passed through a DEAE-cellulose column (2.5 cm $\times$ 20.5 cm, bed volume 100 ml) equilibrated with 10 mM-Tris/HCl buffer, pH 7.5, at a flow rate of 30 ml/h; the column was washed with equilibration buffer at a flow rate of 40 ml/h, and 10 ml fractions were collected. After washing with 400 ml of the equilibration buffer, the column was eluted with 300 ml of 75 mM-NaCl in the equilibration buffer, followed by elution with 200 ml of 200 mM-NaCl in the buffer. The fractions that showed enterokinase-inhibitory activity and no antitryptic activity (fractions 60–70; Fig. 1) were pooled and concentrated by ultrafiltration with Millipore immersible CX filters. The product was dialysed against 100 vol. of 10 mM-Tris/HCl buffer, pH 7.5, containing 100 mM-NaCl, for 8 h.

Sephadex G-200 chromatography. The DEAE-cellulose fraction (volume 10 ml) was loaded on a Sephadex G-200 column (2.5 cm $\times$ 40.8 cm, bed volume 200 ml) equilibrated with 10 mM-Tris/HCl buffer, pH 7.5, containing 100 mM-NaCl. The column was eluted with the equilibration buffer at a flow rate of 15 ml/h; 10 ml fractions were collected. The fractions that showed enterokinase-inhibitory activity and no antitryptic activity (fractions 7–13, Fig. 2) were pooled and dialysed against 100 vol. of 10 mM-Tris/acetate buffer, pH 5.0, for 16 h, with one change of the dialysis medium at 8 h.

CM-cellulose chromatography. The Sephadex fraction (volume 62 ml) was applied to a CM-cellulose column (2.5 cm $\times$ 20.5 cm, bed volume 100 ml) equilibrated with 10.0 mM-Tris/acetate buffer, pH 5.0, at a flow rate of 30 ml/h. The column was eluted successively with 240 ml of equilibration buffer, 200 ml of 70.0 mM-NaCl in the equilibration buffer and 500 ml of 250.0 mM-NaCl in the equilibration buffer, at the same flow rate; 10 ml fractions were collected. This step helped in removal of three minor contaminating protein fractions. The 'active' fractions (nos. 68–90) were pooled and concentrated by ultrafiltration. This fraction was used for studies of inhibitor properties.

Fig. 1. Chromatography of acid-fraction on DEAE-cellulose

For details see the Experimental section. Δ , Protein; $\bullet$, enterokinase-inhibitory activity; $\circ$, trypsin inhibition.

Fig. 2. Chromatography of DEAE-cellulose fraction on Sephadex G-200

For details see the Experimental section. Δ , Protein; $\bullet$, enterokinase-inhibitory activity.

Determination of M_r

The M_r of the purified inhibitor (CM-cellulose fraction) was determined by electrophoresis on polyacrylamide gel (10% acrylamide) in the presence of

SDS as described by Weber *et al.* (1972). The M_r was also determined by gel chromatography on Sephadex G-200, with 10.0 mM-Tris/HCl buffer, pH 7.5, containing 100.0 mM-NaCl as eluent. The protein samples in 0.5 ml were applied to a column of Sephadex (0.9 cm $\times$ 62.8 cm, bed volume 40 ml); 1 ml fractions were collected at a flow rate of 6 ml/h and the protein content was measured.

pH stability of the inhibitor

The inhibitor (44 μ g of protein) was incubated at 4°C in 1 ml of 0.1 M buffers of different pH values (HCl/KCl buffer, pH 2.0; acetate buffer, pH 4.0; Tris/acetate buffer, pH 5.0, 6.0 and 7.0; Tris/HCl buffer, pH 8.0, 9.0 and 10.0; 0.1 M-NaOH, pH 12.0); 25 μ l samples were drawn at different intervals of time and assayed for the residual inhibitory activity against bovine enterokinase with appropriate controls.

Action of proteinases on the inhibitor

Inhibitor protein (100 μ g) was incubated at pH 2.0 (0.1 M-HCl/KCl buffer) with crystalline pig pepsin (100 μ g of protein) in a total volume of 1.0 ml at 37°C. Samples (20 μ l) were withdrawn at different intervals of time and assayed for residual inhibitory activity against bovine enterokinase with appropriate controls.

The inhibitor (2 μ g of protein) in 0.1 ml of 10 mM-Tris/HCl buffer, pH 7.5, was incubated with 2.0 μ g of Pronase in 0.1 ml of Tris/HCl buffer, pH 7.5, for

different intervals of time and assayed for inhibitory activity against bovine enterokinase with appropriate controls.

Chemical modification of the inhibitor

Arginine residues were modified by treatment with cyclohexane-1,2-dione (Abe *et al.*, 1978) and with ninhydrin (Chaplin, 1976). Modification of lysine residues was performed with 2,4,6-trinitrobenzenesulphonic acid (Haynes *et al.*, 1967). Thiol groups were modified by 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959).

The inhibitor protein (200 µg) was treated with 2.0 mg of the various modifiers in the presence of 100 µmol of borate buffer, at pH 9.0 for cyclohexane-1,2-dione and ninhydrin, and pH 7.6 for 2,4,6-trinitrobenzenesulphonic acid and 5,5'-dithiobis-(2-nitrobenzoic acid), in a total volume of 10 ml at 30°C. Samples (2 ml) were withdrawn at different intervals of time (up to 32 h) and dialysed against 100 vol. of 10 mM-acetate buffer, pH 5.0, for 6 h at 4°C.

The residual inhibitory activity of the dialysed samples was measured against bovine enterokinase. Controls without modifiers were run simultaneously. In another set of experiments the inhibitor protein in water was heated at 80°C for 5 min before addition of the modifiers. All other details were the same as described above.

Reduction and alkylation

The inhibitor (30 µg of protein) was reduced in the presence and absence of urea and alkylated by the method of Crestfield *et al.* (1963).

Identification and quantification of carbohydrate moieties in the purified inhibitor

The total carbohydrate content of the inhibitor was measured by the phenol/H₂SO₄ method of Dubois *et al.* (1956), with glucose as standard. Uronic acid was measured by the modified carbazole method of Bitter & Muir (1962). Reducing sugars were measured by the method of Nelson (1944).

Glucosamine was determined by the method of Levvy & McAllan (1959).

Inhibitor protein (20 mg) was hydrolysed with 8.0 ml of 1 M-H₂SO₄ in a sealed tube, for 10 h at 105°C. The hydrolysate was neutralized with solid BaCO₃, and the BaSO₄ formed was separated by centrifugation at 10000 g for 10 min. The supernatant was separated into the acid, neutral and basic fractions by coupled column chromatography as described by Spiro (1960). The individual sugars in the neutral fraction were identified and quantified by descending paper chromatography for 96 h, on quantitative Whatman no. 1 filter paper in butanol/ethanol/water (10:1:2, by vol.). The sugars were detected with the AgNO₃ spray of Trevelyan *et al.* (1950). The individual sugar fractions from the chromatogram were also eluted with water and quantified by Nelson's (1944) method for reducing sugar, with appropriate standards.

Determination of the N-terminus of the inhibitor

The inhibitor was dansylated as described by Boulton & Bush (1964), and the dansyl compound was subjected to t.l.c. on silica gel G in the solvent systems diethyl ether/methanol/acetic acid (100:50:1, by vol.) and chloroform/methanol/acetic acid (15:4:1, by vol.).

Results and discussion

The data on the isolation of a specific enterokinase inhibitor are summarized in Table 1. All the observed enterokinase-inhibitory activity and antitryptic activity in the acid fraction were bound to DEAE-cellulose. The enterokinase inhibitor, devoid of antitryptic activity, was eluted from the column with 10 mM-Tris/HCl buffer, pH 7.5, containing 75 mM-NaCl (Fig. 1). The trypsin inhibitor was eluted from the column at a higher salt concentration. The sudden fall in total enterokinase-inhibitory activity during this stage is due to the separation of a trypsin inhibitor. The enterokinase inhibitor was further purified by gel chromatography on Sephadex G-200 and by chromatography on CM-cellulose. The

Table 1. *Purification of enterokinase inhibitor from kidney bean*

For full details see the text. The enterokinase inhibitor units were calculated with bovine trypsinogen and bovine enterokinase

Fraction	Total protein (mg)	Enterokinase inhibitor (units)	Trypsin inhibitor (units)	Specific activity of enterokinase inhibitor (units/mg)	Yield (%)
Crude extract	1039.0	546.7	121.5	0.526	100.0
Acid fraction	230.0	472.0	91.7	2.050	86.3
DEAE-cellulose fraction	96.8	231.0	0.0	2.390	42.3
Sephadex fraction	56.7	187.0	0.0	3.300	34.2
CM-cellulose fraction	41.4	165.0	0.0	4.000	30.2

purified inhibitor did not affect the esterolytic activity of enterokinase on benzoylarginine ethyl ester.

The inhibitor moved as a single protein band during SDS/polyacrylamide-gel electrophoresis (Fig. 3). *N*-Terminal analysis identified aspartic acid in that position. The M_r of the inhibitor was found to be 31 000 by SDS/polyacrylamide-gel electrophoresis; however, gel chromatography on Sephadex G-200 led to a calculated M_r of 60 000. This suggests that the inhibitor exists as a dimer of probably identical polypeptide chains. Treatment with mercaptoethanol in the presence and the absence of urea did not diminish the inhibitory activity. However, alkylation of the inhibitor after reduction (in the presence of urea) resulted in complete loss of inhibitory activity.

The inhibitor was found to be a glycoprotein containing 9.6% carbohydrate (expressed as glucose), on the basis of the phenol/ H_2SO_4 reaction.

Fig. 3. Polyacrylamide-gel electrophoresis of inhibitor in the presence of SDS

The electrophoresis was performed on polyacrylamide gel (10% acrylamide) in the presence of 0.1% SDS in both gel and electrode buffers. The inhibitor was treated with 1% SDS and 1% β -mercaptoethanol for 2 h at 37°C at pH 7.2 and subjected to electrophoresis for 10 h with a current of 7 mA/tube. The protein band was stained with Coomassie Brilliant Blue R-250 (0.25%) in methanol/acetic acid/water (5:1:5, by vol.).

Fig. 4. Effect of pH on inhibitory activity of purified inhibitor towards bovine enterokinase

The enterokinase-inhibitory assay and enterokinase assay were carried out in the presence of 40 μ mol of buffer of different pH values (Tris/acetate, pH 4.0, 4.5, 5.0, 5.5, 6.0; Tris/HCl, pH 7.0, 7.5 and 8.0). All other assay conditions were as described in the Experimental section. ●, Bovine enterokinase activity; ○, enterokinase inhibition.

Fig. 5. Gel chromatography of the inhibitor (●), bovine trypsinogen (○) and the mixture (Δ)

The Sephadex G-200 column (0.9 cm $\times$ 62.5 cm, bed volume 40 ml) was equilibrated with 10 mM-Tris/HCl buffer, pH 7.5, containing 100 mM NaCl at 4°C. The inhibitor protein (600 μ g) or bovine trypsinogen (700 μ g of protein) or the mixture (1300 μ g of protein) in a volume of 1.0 ml was applied to the column; 1 ml fractions were collected at a flow rate of 6 ml/h and the fractions were assayed for protein.

The individual monosaccharides identified were mannose (5%), glucose (3.2%), glucosamine (1.35%) and glucuronic acid (0.85%). In addition, another reducing sugar with an R_{fucose} value of 0.13 during chromatography was present to the extent of 1.35%. However, this moiety could not be characterized. Xylose, fucose and arabinose were found to be absent.

The effect of time of preincubation of the inhibitor with enterokinase indicated that 15 min preincubation was essential to elicit maximal inhibition. The inhibitory activity was linear with respect to concentration up to 40%. A maximum of 65% inhibition could be obtained at higher inhibitor concentrations. Heat treatment at 90°C for 60 min resulted in the loss of only 30% of the activity, whereas at 100°C nearly 60% of the activity was destroyed. Autoclaving the inhibitor solution at 15 lb/in² (100 kPa) pressure for 15 min completely abolished its action. The inhibitor was stable to exposure to the pH range 2–10 for 34 h at 4°C. However, at pH 12.0 there was a gradual loss of activity, resulting in its complete abolition in 12 h. The interaction between

enterokinase and inhibitor was maximal in the pH range 5.0–7.0 (Fig. 4). At higher pH values there was a decrease of the potency. Treatment of the inhibitor with crystalline pepsin resulted in a gradual decrease of its anti-enterokinase action; 70% of the activity was lost after pepsin treatment for 90 min. However, treatment with Pronase for 6 h did not affect its action.

Treatment of the inhibitor with cyclohexane-1,2-dione or ninhydrin for 32 h resulted in a loss of 81% or 67% of the activity respectively. Heat treatment of the inhibitor for 5 min at 80°C before addition of the modifiers hastened the process of modification, and inactivation was complete in both cases. These data suggest that arginine groups are essential for the interaction of the inhibitor with enterokinase. Treatment with 5,5'-dithiobis-(2-nitrobenzoic acid) did not result in the loss of inhibitory action. Amino groups were not found to be essential for the action of the inhibitor, since prolonged treatment with 2,4,6-trinitrobenzenesulphonic acid did not result in any loss of the activity of the inhibitor.

Fig. 6. Studies on complex-formation between the inhibitor and bovine enterokinase on a Sephadex G-200 column (a) The column (0.9 cm $\times$ 62.5 cm, bed volume 40 ml) was equilibrated with 10 mM-Tris/HCl buffer, pH 7.5, containing 100 mM-NaCl at 4°C. The inhibitor protein (800 μ g) or bovine enterokinase (2400 μ g of protein) in 1 ml was subjected to chromatography. For the complex-formation 2400 μ g of bovine enterokinase protein was mixed with 800 μ g of inhibitor protein in a volume of 1.0 ml and left for 30 min at 4°C. A precipitate formed, and the mixture was centrifuged at 2500 g for 10 min. The clear supernatant was subjected to chromatography; 1 ml fractions were collected at a flow rate of 6.0 ml/h, and each fraction was assayed for protein. ●, Bovine enterokinase; △, inhibitor; ○, mixture. (b) The precipitate of enzyme-inhibitor complex obtained in the above experiment was solubilized with 10 mM-Tris/acetate buffer, pH 5.0, containing 0.1% SDS and 100 mM-NaCl at 25°C. A 1 ml portion of this solution (250 μ g of protein) was subjected to chromatography on a Sephadex G-200 column (0.9 cm $\times$ 62.5 cm, bed volume 40 ml) equilibrated with 10 mM-Tris/acetate buffer, pH 5.0, containing 0.1% SDS and 100 mM-NaCl at 25°C; 1 ml fractions were collected at a flow rate of 6.0 ml/h, and each fraction was assayed for protein.

Table 2. Action of inhibitor on different enterokinases

The assay system was the same as that described in the Experimental section, except that different amounts of enterokinase preparations, to give comparable activity of 20 munits, were used.

Enterokinase	Inhibitor protein (μ g)	Inhibition (munits)
Pig	1.0	8.14
Bovine	2.0	7.77
Human	20.0	7.40
Dog	500.0	1.11

The potency of the purified inhibitor on different enterokinase preparations is shown in Table 2. Pig enterokinase was most powerfully inhibited, followed by the bovine enzyme. The action on human enzyme was 20 times less than that on the pig enterokinase. Dog enterokinase was found to very weakly inhibited. At 50 μ g concentration, the inhibitor had no action on trypsin or chymotrypsin.

The inhibition of enterokinase activity observed is not due to the complexing of trypsinogen with the seed protein. When a mixture of trypsinogen and inhibitor was subjected to chromatography on Sephadex G-200, two distinct peaks corresponding to the two proteins were observed, and there was no evidence for the formation of a complex (Fig. 5). When a mixture of bovine enterokinase and the inhibitor was subjected to gel chromatography, the inhibitor peak disappeared (Fig. 6) and all the protein was eluted in the void-volume region. However, when high concentrations of the inhibitor and the enzyme were mixed for these studies, most of the complex was immediately precipitated. When this precipitate was treated with 0.1% SDS in 10 mM-Tris/acetate buffer, pH 5.0, containing 100 mM-NaCl and rechromatographed on Sephadex G-200, the inhibitor was eluted in the expected region (Fig. 6).

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