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Bovine Enterokinase. Purification, Specificity, and Some Molecular Properties†

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ABSTRACT: Enterokinase has been isolated from the contents of bovine duodena and purified 1200-fold in 41% yield. The isolation procedure employed DEAE chromatography, affinity chromatography on immobilized *p*-aminobenzamidine, and gel filtration. The resultant pure enzyme exhibits a single band on sodium dodecyl sulfate gel electrophoresis corresponding to a molecular weight of 145 000. Two chains in the molecule, connected by disulfide linkages, have molecular weights of 57 000 and 82 000, respectively. The purified enzyme exhibits a restricted specificity which is directed toward the polyanionic amino acid sequence in the activation peptide of the zymogen substrate. Of the zymogens of the serine proteases tested, including several of those involved in blood coagulation, only native and guanidinated trypsinogen are activated by enterokinase, whereas acetylated trypsinogen is not. Partial heat denaturation of bovine enterokinase causes a differential response toward the activation of trypsinogen and the hydrolysis of benzoyl-L-arginine ethyl ester (BzArgOEt), further suggesting that secondary sites are important in the binding of trypsinogen. A sensitive assay for enterokinase (nanomole range) was developed using tritiated BzArgOEt.

Enterokinase (enteropeptidase, EC 3.4.4.8) occupies a key position in the utilization of dietary proteins. The enzyme initiates intraluminar digestion of proteins by the proteolytic conversion of trypsinogen to trypsin, which in turn activates the other pancreatic zymogens (Kunitz, 1939a,b; Hadorn et al., 1969). The proteolytic attack of enterokinase is directed exclusively toward the Lys_6-Ile_7 peptide bond of trypsinogen leaving all other lysine and arginine bonds in the molecule unaffected (Maroux et al., 1971). The resultant cleavage produces the simultaneous release of active trypsin and of the amino-terminal hexapeptide Val-$(Asp)_4$-Lys (Rovery et al., 1953; Davie and Neurath, 1955). This unique specificity exhibited by enterokinase is of interest as it relates to both the molecular basis of substrate recognition and the control of the digestive process.

Previous work, using synthetic peptide substrates, suggests that the polyaspartyl sequence in the activation peptide is important for optimal interaction of enterokinase and protein substrates (Maroux et al., 1971). This cluster of aspartyl residues is common to all trypsinogens thus far sequenced with two exceptions, both from the African lungfish (Reeck and Neurath, 1972; de Haën et al., 1975). In the investigation reported here, particular attention has been given to the significance of this unique sequence for the specificity of enterokinase. Since much of the critical work on pancreatic enzymes and zymogens has been done on bovine material, the present investigation has been directed toward the isolation of bovine enterokinase and the examination of some of its properties. We have also compared the bovine enzyme to that of porcine origin, purified and characterized by Desnuelle and co-workers (Maroux et al., 1971; Baratti et al., 1973).

Experimental Section

Materials

Bovine trypsinogen (once crystallized) and chymotrypsinogen A were obtained from Worthington Biochemical Corp. Bovine procarboxypeptidase B and plasminogen were purified in our laboratory by D. Grahn and Dr. M. A. Kerr, respectively. Dr. C. de Haën provided the lungfish proelastase B and lungfish trypsinogen B. Prothrombin and blood coagulation factor X were generous gifts from Dr. Earl W. Davie.

1-Guanyl-3,5-dimethylpyrazole nitrate was prepared by Dr. N. C. Robinson using the method of Bannard et al. (1958). Labeled benzoylarginine ethyl ester[1] (BzArgOEt) was synthesized in our laboratory by E. Fodor using a modification of

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[1] Abbreviations used are: BzArgOEt, benzoyl-L-arginine ethyl ester; TosArgOMe, N^{α}-tosyl-L-arginine methyl ester; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Mes, 2-(*N*-morpholino)ethanesulfonic acid; iPr_2P, diisopropylphosphoryl; iPr_2PF, diisopropyl phosphofluoridate; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

the method described by Roffman et al. (1970). The tritiated ester, labeled in the ethyl moiety, had a specific activity of 100 μCi/mmol.

Methods

Determination of Enzymatic Activities. Enterokinase activity was measured by its activating effect on bovine trypsinogen (Baratti et al., 1973). To 0.7 mL of 40 mM sodium succinate buffer (pH 5.65) up to 0.1 mL of enterokinase solution was added. The sample volume was increased to 0.9 mL with water and preincubated at 24 °C for 5 min. Subsequently, 0.1 mL of trypsinogen solution (1.0 mg/mL in 1 mM HCl, 7.0 mM in $CaCl_2$) was added to initiate activation. The reaction was terminated after 30 min at 24 °C by the addition of 50 μL of 1.0 N HCl. A 50- or 100-μL aliquot of the activated solution was assayed for trypsin activity using N^{α}-tosyl-L-arginine methyl ester (TosArgOMe) as the substrate (Hummel, 1959). The enterokinase unit is defined as the amount of enzyme which activates 1 nmol of trypsinogen in 30 min under the conditions of the assay.

Bovine enterokinase also hydrolyzes BzArgOEt. Using this substrate, an additional sensitive microassay for enterokinase was developed employing principles described by Roffman et al. (1970). Up to 0.1 mL of enterokinase solution was pipetted into a scintillation vial. To this aqueous solution was added 25 μL of 0.1 M piperazine-*N,N'*-bis(2-ethanesulfonic acid) buffer (Pipes, pH 7.25, 7.5 mM in $CaCl_2$) and water to a final volume of 0.125 mL. Subsequently, 25 μL of 0.58 mM tritiated substrate was added to the solution and the mixture was shaken in the presence of 10 mL of toluene-based scintillation fluid (Liquifluor, New England Nuclear Corp.). As the enzymatic hydrolysis proceeds the tritiated ethanol liberated is preferentially extracted by the toluene from the aqueous phase while the uncleaved substrate remains in the aqueous phase. The movement of tritium into the scintillant traces the progress of hydrolysis and the amount of radioactivity is determined in a liquid scintillation spectrometer.

Aminopeptidase activity was determined spectrophotometrically using leucine-β-naphthylamide as the substrate (Felgenhauer and Glenner, 1966).

Experimental conditions for evaluating the effect of enterokinase on modified trypsinogens were the same as those described above for bovine trypsinogen. The action of enterokinase on precursors of other enzymes was determined by incubating 30–50 μg of the zymogen with 1–20 μg of enterokinase in 1.0 mL of 0.1 M Tris (1.0 mM $CaCl_2$, pH 7.0–8.0 at 25 °C). Under comparable conditions, chymotrypsinogen (Wilcox, 1970), prothrombin (Kisiel and Hanahan, 1973), and factor X (Papahadjopoulos et al., 1964) were fully activated by 1 μg of trypsin within 30 min.

Preparation of Immobilized p-Aminobenzamidine. A coupling procedure similar to that described by Cuatrecasas et al. (1968) was used to prepare substituted agarose for affinity chromatography. All procedures were carried out in the cold room at 4 °C unless otherwise indicated. Sepharose 4B (100 mL of settled gel) was suspended in an equal volume of water and activated with 20 g of cyanogen bromide. The suspension was maintained at pH 11 by the addition of 10 N NaOH. Activated agarose was washed rapidly with 15 vol of 0.1 M sodium bicarbonate (pH 7.0) followed by 15 vol of water. The packed gel was suspended in 100 mL of 2.3 M ε-aminocaproic acid, the pH adjusted to 10.0, and the solution stirred overnight. Excess ε-aminocaproic acid was removed by washing the gel with 10 L of water followed by 250 mL of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (Mes) at pH 4.75. ε-Aminohexanoyl agarose was stored in Mes buffer containing 0.02% sodium azide.

The binding of *p*-aminobenzamidine to substituted agarose was performed by a minor modification of the method of Schmer (1972). To 60 mL of 0.1 M Mes (pH 4.75) was added 60 g of wet packed ε-aminohexanoyl agarose gel and 1.0 g of *p*-aminobenzamidine previously dissolved in 5 mL of Mes. The pH was readjusted to 4.75 under gentle stirring. Ten grams of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, dissolved in 20 mL of Mes, was then added dropwise to the gel suspension, maintaining a constant pH by titration with 1 N HCl in a Radiometer TTT-1 pH stat. The mixture was allowed to react overnight, and was then washed extensively with distilled water and 1 N HCl. The gel was subsequently equilibrated with 10 mM Tris-acetate, 0.2 M NaCl (pH 7.0).

Electrophoresis in sodium dodecyl sulfate-polyacrylamide gels was performed by a modification of the discontinuous system described by Davis (1964). The stacking gel (0.6 × 1 cm) contained 3% acrylamide while the gel used for separation (0.6 × 13 cm) contained 7% acrylamide and 0.2% *N,N'*-bismethyleneacrylamide. Enterokinase and reference samples (0.50–0.15 mL) were diluted 1:1 (v/v) into a preheated solution of 0.1 M Tris-HCl, 2% sodium dodecyl sulfate, 20% glycerol, 0.002% bromophenol blue (pH 6.8) and incubated for 15 min in a boiling water bath. In certain experiments β-mercaptoethanol, at a final concentration of 1%, was added to reduce the disulfide bridges of the proteins. Electrophoresis was carried out at 4 mA per gel until the bromophenol blue front had moved 11 cm (5–6 h). The gels were then removed, stained with 0.1% Coomassie Brilliant Blue, and destained (Vesterberg, 1971). The following proteins were used as references in establishing calibration curves for determining molecular weights: catalase (58 000), bovine serum albumin (68 000), phosphorylase B (100 000), and serum albumin dimer and trimer (136 000 and 204 000).

Preparation of Modified Trypsinogens. Trypsinogen (1.5×10^{-4} M) was guanidinated with 0.75 M 1-guanyl-3,5-dimethylpyrazole nitrate using the method described by Robinson et al. (1973). After 68 h of incubation at 4 °C, guanidination was complete and the modified zymogen could no longer be activated by trypsin. Quantitative conversion of lysine residues to homoarginine in the modified zymogen was established by amino acid analysis.

Trypsinogen (4.0×10^{-4} M) was acetylated with acetic anhydride using the method of Frankel-Conrat et al. (1949). Since the acetylated trypsinogen could still be partially activated (17.5% of potential activity of the native zymogen) the modified zymogen was subjected to a second acetylation which abolished any observable activation by trypsin.

Estimation of Protein Content. Protein determinations were carried out by the microbiuret method of Zamenhof (1957). A stock solution of bovine serum albumin was used in preparing standards and each sample was dialyzed against distilled water before analysis.

Amino Acid Analysis. Amino acid analyses were performed on a Durrum (Model D-500) amino acid analyzer according to the method of Spackman et al. (1958). Samples were hydrolyzed in 6 N HCl at 110 °C for 24, 48, and 96 h in evacuated tubes. The values for serine and threonine were determined by extrapolation to zero-time hydrolysis. Valine and isoleucine values were calculated from the 96-h hydrolysis. Half-cystine was evaluated as cysteic acid after performic acid oxidation. Tryptophan was determined colorimetrically by the method of Bencze and Schmid (1957).

Carbohydrate Composition. Neutral sugar content was

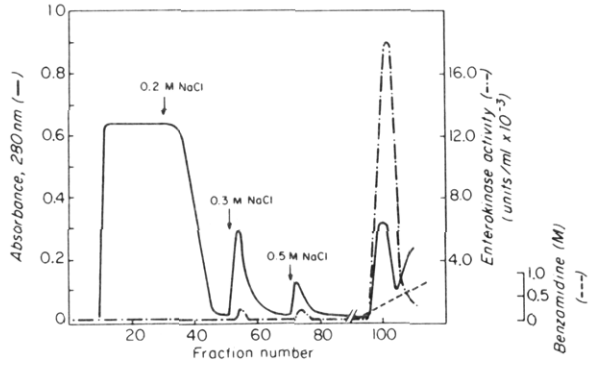

FIGURE 1: Chromatography at 4 °C of bovine enterokinase on benzamidine-Sepharose (1.6 × 12.0 cm). The column was equilibrated with 0.01 M Tris-acetate buffer, 0.2 M NaCl, pH 7.0. After sample application, the column was washed with 500 mL of equilibration buffer containing successively 0.2, 0.3, and 0.5 M NaCl. The column was then developed by application of 500 mL of a linear gradient of benzamidine (0.0–0.7 M). Fractions of 25 mL were collected at a flow rate of 20 mL/h upon the application of salt solutions. Five-milliliter fractions were collected at a rate of 6 mL/h during the gradient elution. Fractions containing benzamidine were dialyzed for 4 days against 3 × 40 L of distilled water before absorbance and enterokinase activity were measured.

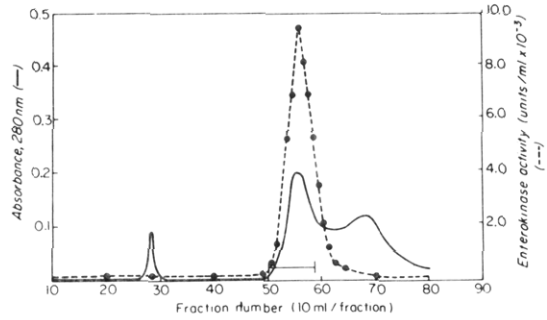

FIGURE 2: Gel filtration at 4 °C of bovine enterokinase. The column of Sepharose 6B (2.6 × 160 cm) was equilibrated with 0.01 M Tris-acetate buffer, 0.5 M NaCl, pH 7.0. The lyophilized eluate from the affinity column was dissolved in 4.0 mL of the same buffer and applied to the column. The filtration rate was 36 mL/h. Fractions of 10 mL were collected, assayed for enterokinase, and pooled as indicated by the solid bar.

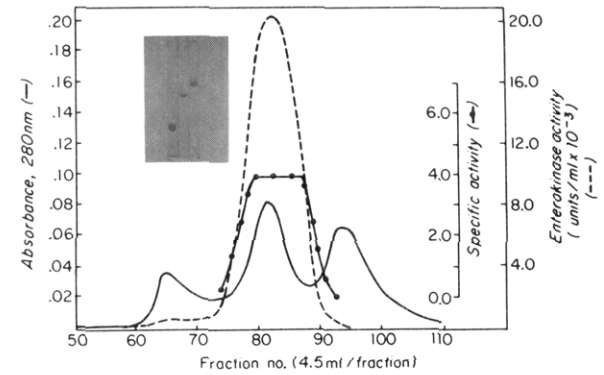

FIGURE 3: Gel filtration at 4 °C of bovine enterokinase. The column of Sephadex G-200 (2.6 × 90 cm) was equilibrated with 0.01 M Tris-acetate buffer, 0.5 M NaCl, pH 7.0. The lyophilized eluate from the Sepharose 6B column was dissolved in 3.0 mL of the equilibration buffer and applied to the column. The filtration rate was 19 mL/h. Fractions of 4.5 mL were collected, assayed for enterokinase, and active fractions were pooled. The specific activity of fractions is indicated (●) in units of enterokinase activity ($\times 10^{-5}$) per unit of absorbance at 280 nm. The inset shows the sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of bovine enterokinase (right), phosphorylase B (center), and catalase (left). The discontinuous system employed a stacking gel (3.5% acrylamide) at pH 6.8 and a separation gel (7.0% acrylamide) at pH 8.3. Electrophoresis was performed at 3 mA/gel for 5 h.

determined by the phenol-sulfuric acid method of DuBois et al. (1956), employing a 1:1 mixture of glucose and galactose as a reference standard. Amino sugar was determined by analysis on a Durrum D-500 analyzer according to Spackman et al. (1958).

Amino-Terminal Sequence Analysis. Amino-terminal sequence was determined by automated Edman degradations using a Beckman Sequencer Model 890A (Hermodson et al., 1972).

Results

Purification of Bovine Enterokinase. The duodena and proximal 10 ft of small intestine from 3 cattle were excised immediately following the death of the animals. Bovine intestinal content (1.7 L) was collected and chilled with ice. The intestines were washed once with 500 mL of ice water and the wash was added to the content. The contents were then diluted with 3 vol of ice water and the mixture was filtered through 8 layers of cotton gauze. The filtrate was adjusted to pH 6.0 by dropwise addition of 2% acetic acid. Insoluble material was removed by centrifugation for 40 min at 7500*g* in a Sorvall refrigerated centrifuge, Model RC-3, and 6 L of clear supernatant was obtained. Further purification steps were performed at 4 °C.

Three liters of pH 6.0 supernatant was charged onto a column of DEAE-cellulose (4.8 × 50 cm) preequilibrated with 10 mM Tris-acetate buffer containing 50 mM NaCl (pH 6.0). The column was washed with 2.5 L of buffer, and the protein eluted by applying a linear concentration gradient of NaCl (50 to 500 mM). Under these conditions, the enterokinase activity was eluted by 180–200 mM NaCl. Fractions containing enterokinase activity were pooled to give 1.8 L of eluate.

The eluate was pumped in 0.5-L aliquots directly onto a column (1.6 × 12 cm) of matrix-bound *p*-aminobenzamidine (Anderson et al., 1975; Grant and Hermon-Taylor, 1975). After loading, the column was washed with 10 mM Tris-acetate buffer (pH 6.0) containing successively 0.2, 0.3, and 0.5 M NaCl. The enzyme was eluted by applying 0.5 L of equilibration buffer containing 0.2 M NaCl and a linear concentration gradient (0 to 0.7 M) of benzamidine. As indicated in Figure 1, a small amount of enterokinase activity emerged during the application of salt solutions, whereas the bulk of activity was eluted by 0.25–0.3 M benzamidine. Fractions containing benzamidine were assayed for enterokinase activity after extensive dialysis against distilled water. Active fractions were pooled and lyophilized to give 18.5 mg of a white powder per 0.5 L of the material eluted from the DEAE-cellulose column.

Purification was completed by successive gel filtration steps on Sepharose 6B and Sephadex G-200. The lyophilized eluate from the affinity column was dissolved in 5 mL of 10 mM Tris-acetate buffer (pH 7.0) containing 0.5 M NaCl and the solution was filtered through a column of Sepharose 6B (2.6 × 160 cm) equilibrated with the same buffer. This step served to remove the remaining benzamidine inhibitor from enterokinase eluted from the affinity column. The elution profile (Figure 2) shows enterokinase to be largely separated from other contaminants. Active fractions, indicated by the horizontal bar, were pooled, dialyzed against water, and lyophilized. At this stage the enzyme has been purified 1000-fold. However, a low level of contaminating aminopeptidase activity still remained.

TABLE I: Balance Sheet of the Purification of Enterokinase from 1 L of Bovine Intestinal Content.[a]

	Enterokinase units ($\times 10^5$)	Yield (%)	Sp act. (units/mg)	Purification (fold)
pH 6.0 supernatant	9.35	100	34	1
DEAE-cellulose chromatography	8.2	87	669	19.7
Affinity chromatography on benzamidine-Sepharose	6.6	70	15 400	453
Gel filtration on Sepharose 6B	5.2	56	35 800	1050
Gel filtration on Sephadex G-200	3.8	41	40 800	1200

[a] One enterokinase unit is defined as the amount of enzyme activating 1 nmol of trypsinogen within 30 min under the conditions of the assay (25 °C, pH 5.6, 0.001 M $CaCl_2$).

TABLE II: Amino Acid and Carbohydrate Composition of Bovine Enterokinase.

Components	No. of residues	Integral values
Alanine	43.8	44
Arginine	15.2	15
Aspartic/NH_2	75.4	75
Half-cysteine[a]	20.3	20
Glutamic/NH_2	85.8	86
Glycine	63.0	63
Histidine	28.6	29
Isoleucine[b]	32.2	32
Leucine[b]	37.8	38
Lysine	25.4	25
Methionine	7.1	7
Phenylalanine	28.5	29
Proline	62.3	62
Serine[c]	82.7	83
Threonine[c]	76.8	77
Tryptophan[d]	9.9	10
Tyrosine	25.2	25
Valine	51.9	52
Total no. residues		772
Carbohydrate (% by weight)		
Neutral sugars[e] 16%		
Amino sugars[f] 13%		
Total sugar by weight 29.5%		
Molecular weight by composition, 141 000		
Molecular weight by sodium dodecyl sulfate gel electrophoresis, 145 000		

[a] Determined as cysteic acid after performic acid oxidation. [b] Highest value after 96-h hydrolysis. [c] After extrapolation to zero time of results obtained after 24-, 48-, and 96-h hydrolysis. [d] Determined by spectrophotometric assay on the intact protein. [e] Hexose determined by phenol-sulfuric acid method. [f] Amino sugars including glucosamine and galactosamine were determined by analysis on an amino acid analyzer.

The lyophilized powder was dissolved in 5 mL of 10 mM Tris-acetate buffer (0.5 M NaCl, pH 7.0) and filtered through a 2.6 × 100 cm Sephadex G-200 column, equilibrated with the same buffer. As indicated in Figure 3, enterokinase activity was separated from the remaining contaminating protein at this step. The small amount of contaminating aminopeptidase still present was eluted following enterokinase. Peak fractions containing enterokinase showed constant specific activity toward trypsinogen and the pooled fractions gave a single band upon disc electrophoresis in a sodium dodecyl sulfate-polyacrylamide gel system (Figure 3). These data suggest that the enterokinase was homogeneous. A balance sheet of the procedure is presented in Table I which shows a purification of 1200-fold as compared to pH 6.0 supernatant starting material, with a concomitant yield of 41%.

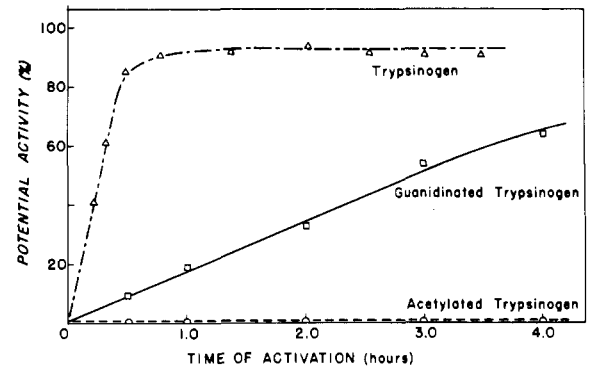

FIGURE 4: Progress of the activation of acetylated (○), guanidinated (□), and native (△) trypsinogen by enterokinase at 25 °C in 0.04 M sodium succinate buffer (pH 5.6). Aliquots of 0.1 mL were withdrawn at the times indicated and the amount of trypsin or modified trypsin was determined as indicated under Methods.

Determination of Molecular Weights. When the purified enzyme was subjected to electrophoresis on polyacrylamide gels, both before and after treatment with 1% sodium dodecyl sulfate, only a single band was observed. A semilog plot of molecular weight vs. relative mobility in the sodium dodecyl sulfate gel yielded a molecular weight of 145 000 for enterokinase.

When the enzyme was incubated with sodium dodecyl sulfate containing β-mercaptoethanol three bands were observed upon gel electrophoresis. One band had the same mobility as unreduced enterokinase and the other two bands corresponding to the individual polypeptide chains exhibited mobilities equivalent to molecular weight values of 57 000 and 82 000.

Amino Acid and Carbohydrate Composition. The amino acid and carbohydrate compositions of bovine enterokinase are shown in Table II. Carbohydrate was estimated to comprise 29.5% of the total weight of protein and to be approximately equally divided between neutral (16%) and amino (13%) sugars. Sialic acid content was not determined.

Activation of Native and Modified Trypsinogens. Native trypsinogen (0.1 mg/mL) is readily activated by bovine enterokinase. As shown in Figure 4, at pH 5.6 and 25 °C, the formation of active trypsin is a linear function of time, up to 30–40 min. (When the trypsinogen concentration exceeds 1 mg/mL, the rate is nonlinear.) The specific activity of the trypsin formed upon maximal activation was 90% of the potential activity of the trypsinogen sample.

N-Acetylated trypsinogen could not be activated by enterokinase even after 50 h of incubation. However, guanidinated

TABLE III: Kinetic Parameters of Enterokinase- and Trypsin-Catalyzed Activations of Trypsinogen and Guanidinated Trypsinogen.

Zymogen	Bovine enterokinase		Trypsin[a]	
	K_m (M)	k_{cat} (s^{-1})	K_m (M)	k_{cat} (s^{-1})
Bovine trypsinogen	7×10^{-6}	1.48	4×10^{-4}	2.5×10^{-3}
Bovine guanidinated trypsinogen	1×10^{-6}	5×10^{-3}		0

[a] Data of Abita et al. (1969).

		Reference to Sequence
ACTIVATED BY ENTEROKINASE		
Bovine Trypsinogen	-ASP-ASP-ASP-ASP-LYS↓ILE-VAL-	Davie and Neurath (1955)
Lungfish Trypsinogen B	-ILE-GLU-GLU-ASP-LYS↓ILE-VAL-	Hermodson, et al. (1971)
Guanidinated Trypsinogen	-ASP-ASP-ASP-ASP-HAR↓ILE-VAL-	Robinson, et al. (1973)
NOT ACTIVATED BY ENTEROKINASE		
Acetylated Trypsinogen	-ASP-ASP-ASP-ASP-LYS↓ILE-VAL-	Robinson, et al. (1973)
Lungfish Proelastase B	-GLU-GLU-MET-GLU-ARG↓VAL-VAL-	deHaen and Gertler (1974)
Prothrombin	-TYR-ILE-GLU-GLY-ARG↓ILE-VAL-	Magnusson (1971)
Chymotrypsinogen A	-SER-GLY-LEU-SER-ARG↓ILE-VAL-	Brown and Hartley (1966)
Coagulation Factor X	-SER-GLN-VAL-VAL-ARG↓ILE-VAL-	Titani, et al. (1975b)
Plasminogen	-LYS-CYS-PRO-GLY-ARG↓VAL-VAL-	Wiman and Wallen (1975)
Procarboxypeptidase B	-ARG↓THR-THR-	Cox, et al. (1962); Titani, et al. (1975a)
PARTIALLY ACTIVATED BY ENTEROKINASE		
Prococoonase[a]	-ASP-ASP-GLY-GLY-LYS↓ILE-VAL-	Felsted, et al. (1973)

FIGURE 5: Activity of bovine enterokinase toward zymogens of several proteolytic enzymes. The dashed line indicates the site of cleavage in normal activation. Activation was by porcine enterokinase.

trypsinogen was activated, although at the much slower rate of 8.6% of that of native zymogen (Figure 4). Seventy percent of the maximal potential activity was attained after 4–5 h of incubation of the guanidinated zymogen with enterokinase. This level of activity corresponds to that observed by Robinson et al. (1973), when guanidinated trypsinogen was treated with an acid protease from *Aspergillus oryzae*.

A comparison of the kinetic parameters of the activation by enterokinase and by trypsin of native and guanidinated trypsinogens is presented in Table III. The activation rate was measured at various substrate concentrations and the values of k_{cat} and K_m were derived from Lineweaver–Burk plots. As can be seen the K_m values for activation of native and guanidinated trypsinogen by enterokinase are of the same order of magnitude, i.e. 0.007 and 0.001 mM, respectively. The k_{cat} values, however, differ by 3 orders of magnitude, i.e. 1.48 s^{-1} for native trypsinogen and 5×10^{-3} s^{-1} for guanidinated trypsinogen. The values obtained by Abita et al. (1969), for the trypsin-catalyzed activation of trypsinogen, are included in Table III for comparative purposes.

Evidence that trypsinogen is cleaved by bovine enterokinase exclusively at the Lys_6-Ile_7 peptide bond was obtained as follows. Trypsinogen was first treated exhaustively with diisopropyl phosphofluoridate (Morgan et al., 1972) to preclude any autocatalytic cleavage by trypsin. The resultant iPr_2P trypsinogen was incubated with enterokinase under activating conditions and subjected to amino-terminal sequence analysis. A major sequence, Ile-Val-Gly-Gly-Tyr-Thr-, corresponding to the amino-terminal sequence of trypsin was observed. A very low amount of Val-Asp-Asp-Asp- was also seen, representing less than 5% uncleaved trypsinogen. No other sequences were observed. These findings confirm those of Maroux et al. (1971) for porcine enterokinase.

Activity of Enterokinase toward Other Zymogens. Further evidence for the importance of the $(Asp)_4Lys$ sequence in substrate recognition was obtained by determining the action of enterokinase toward several other native or denatured proteins. Lungfish trypsinogen, in which two of the aspartic acid residues of the activation peptide are replaced by glutamic acid, is activated by enterokinase (Figure 5). However, in lungfish proelastase, interruption of the acidic sequence by a methionine (i.e., Glu-Glu-Met-Glu-Arg) prevents activation by enterokinase. As indicated in Figure 6, no enterokinase activity was observed toward the zymogens of other serine proteases, i.e., bovine chymotrypsinogen, procarboxypeptidase B, prothrombin, or blood coagulation factor X.

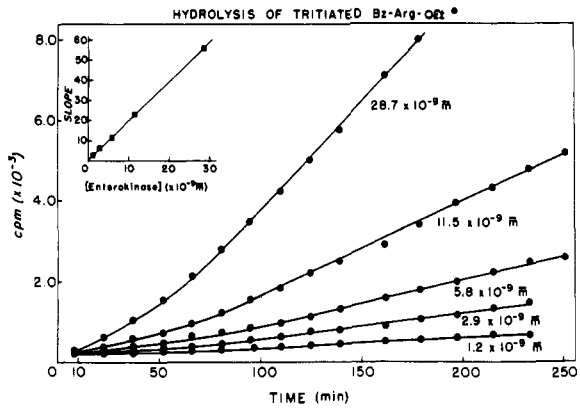

FIGURE 6: Progress curves showing the amount of radioactivity released from tritiated benzoylarginine ethyl ester by the indicated concentrations of enterokinase. The inset illustrates the relationship between concentrations of enzyme and the rate of appearance of radioactivity after a steady-state condition has been established in the assay.

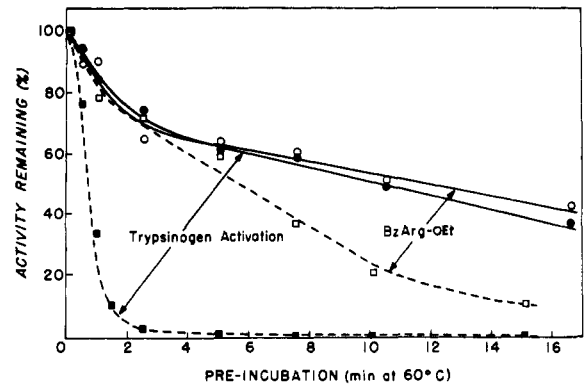

FIGURE 7: The heat stability of enterokinase in the presence (—) and absence (- - -) of 4 mM Ca^{2+}. The enzyme was incubated at 60 °C in 72 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 5.6), and then assayed either by trypsinogen activation (solid symbols) or by activity toward benzoylarginine ethyl ester (open symbols).

Secondary Substrate Recognition Site. Since enterokinase is a trypsin-like enzyme which appears to require also the presence of a unique tetrapeptide sequence in the substrate, experiments were carried out to determine if the catalytic activity toward model substrates for trypsin could be separated from the activity toward the natural substrate, trypsinogen. To facilitate these investigations a sensitive esterase assay for enterokinase was first developed, capable of measuring nanomole quantities of enterokinase. This assay is described in the Methods section and in Figure 6. The curves represent the radioactivity counted in the toluene phase as a function of time of incubation for increasing levels of enterokinase. A linear relationship exists between concentration of enterokinase in the assay and the observed rate of hydrolysis of tritiated BzArgOEt (inset, Figure 6).

Barns and Elmslie (1974) have reported a selective and preferential inactivation of the activity toward trypsinogen as compared to the activity toward the ester by porcine enterokinase. Utilizing the more sensitive esterase assay just described, we have extended such measurements to the bovine enzyme. In these experiments, enterokinase was incubated at 60 °C for varying lengths of time before adding the substrate. The results shown in Figure 7 indicate complete destruction of enterokinase activity toward trypsinogen upon preincubation at 60 °C for 2 min. In contrast, the esterase activity was more stable, retaining 40% of its initial value after 8 min at 60 °C. Significantly, the presence of 4 mM $CaCl_2$ during the preincubation stabilized both activities (Figure 7).

Discussion

The success of the present procedure for the isolation of bovine enterokinase from the contents of duodena depends on affinity chromatography involving the highly effective inhibitor, *p*-aminobenzamidine (Geratz, 1967), coupled to substituted agarose. This step alone increased the specific activity of the enzyme 22-fold, with only a small loss of yield. The final purification of the enzyme was 1200-fold and the final yield 41%.

The purified enzyme has a molecular weight of 145 000, as measured by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate, and contains about 30% carbohydrate (w/w). Neutral sugars comprise 16% and amino sugars 13%. The protein molecule contains two polypeptide chains held together by one or more disulfide bond(s). In most of these respects bovine enterokinase resembles the enzyme previously isolated by Maroux et al. (1971) from the brush border of porcine intestines, and further characterized by Baratti et al. (1973). The light chains of the bovine and porcine enzymes have similar molecular weights (57 000 and 62 000, respectively) whereas the heavy chain of the porcine enzyme is larger (82 000 as compared to 134 000). As a result, the molecular weight of the porcine enzyme exceeds significantly that of bovine origin. It remains to be determined whether these differences are related to the different physiological origins (intestinal mucosa vs. intestinal contents). The amino acid composition of the bovine enzyme reveals a high percentage of dicarboxylic acids (20%) and a relatively low content of aromatic residues (8%), resulting in a low extinction coefficient of $E_{1cm}^{1\%} = 8.8$. The protein contains but 7 methionine residues among a total of 772 and 20 half-cystine residues.

The specificity of enterokinase is uniquely dependent on the presence of two or more acidic residues preceding the susceptible lysyl bond in the activation peptide of trypsinogen of various species (Davie and Neurath, 1955; Maroux et al., 1971; Baratti et al., 1973; Baratti and Maroux, 1976). Although some flexibility in this region of the activation peptide is permissible, as evidenced by the variability in the sequences of trypsinogen of various species (Reeck and Neurath, 1972; Bricteux-Gregoire et al., 1971), a positively charged amino acid residue in position 7 is essential for the activation of bovine trypsinogen by enterokinase. Thus, N^{ϵ}-acetylated trypsinogen is not activated, whereas N^{ϵ}-guanidinated trypsinogen (in which the lysine residues are converted to homoarginine) is activated. In fact, comparison of the kinetic parameters, k_{cat} and K_m, indicates that enterokinase binds the guanidinated zymogen more tightly than the native one but that hydrolysis proceeds more slowly. Prococoonase, containing the sequence -Asp-Asp-Gly-Gly-Lys-, has been reported to undergo slow activation by porcine enterokinase (Felsted et al., 1973) and preliminary experiments indicate that bovine enterokinase can cleave certain proteins containing amino acid sequences similar to that of the activation peptide of bovine trypsinogen (L. E. Anderson and R. Fukura, unpublished observations).

Enterokinase is a serine protease related to trypsin. It is inhibited by iPr_2PF and it catalyzes the hydrolysis of BzArgOEt. Baratti and Maroux (1976) have recently reported that the light chain of the porcine enzyme contains both the serine and histidine residues essential for catalytic activity. However, there are reasons to assume that BzArgOEt is not a complete substrate for the enzyme and that not all of the functional groups required for the activation of trypsinogen participate

in the hydrolysis of this ester substrate. The microassay using tritiated BzArgOEt is two orders of magnitude more sensitive than the colorimetric assays recently described (Hesford et al., 1976; Baratti and Maroux, 1976) and allows nanomolar quantities of enterokinase to be routinely determined with great precision. When this assay was applied to measurements of the effects of partial heat denaturation of enterokinase on the activation of bovine trypsinogen, it was found that the esterase activity was far less sensitive to denaturation than was the activation of the zymogen (Figure 7). These observations are in full accord with those of Barns and Elmslie (1974) on porcine enterokinase and suggest that a subsite, important for the binding of trypsinogen, is more labile than the sites required for the binding and hydrolysis of BzArgOEt. A recent report by Baratti and Maroux (1976) suggests that the polyaspartyl sequence of the activation peptide of trypsinogen is bound to a cluster of lysine residues on the enzyme since acetylation of porcine enterokinase abolishes 98% of the activity toward trypsinogen, whereas the esterase activity remains essentially intact. It is possible that the binding site for trypsinogen is located on one chain and the functional groups required for the hydrolysis of BzArgOEt on the other chain.

Since enterokinase shares with other regulatory proteases, notably those of the coagulation cascade (Davie and Fujikawa, 1975), a two-chain structure, it seems inviting to consider that its biosynthetic precursor is a zymogen containing a single polypeptide chain which loses an internal peptide segment upon activation by a proteolytic enzyme. Alternatively, one may argue that the narrow and unique specificity of this enzyme precludes the need for an inactive precursor form. This problem is now being investigated in this laboratory.

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