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Purification and Further Characterization of Enteropeptidase from Porcine Duodenum[1]

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Enteropeptidase [EC 3.4.21.9] is a membrane-bound serine endopeptidase present in the duodenum that converts trypsinogen to trypsin. We previously cloned the cDNA of the porcine enzyme and deduced its entire amino acid sequence [M. Matsushima *et al.* (1994) *J. Biol. Chem.* 269, 19976-19982]. In the present study, we purified the porcine enzyme approximately 2,200-fold in a 12% yield from a duodenal mucosal extract to apparent homogeneity by an improved procedure comprising four steps of chromatography including benzamidine-Sepharose affinity chromatography. Lectin blotting analysis suggested that the enzyme is glycosylated mainly with *N*-linked carbohydrate chains of the tri- and/or tetraantennary complex type. The H and L chains of the enzyme were separated into two major bands upon SDS-PAGE under reducing conditions, suggesting that the enzyme mainly comprises two isoforms, a higher molecular weight form and a lower molecular weight form. The enzyme was also separated by lectin affinity chromatography into two major fractions, named isoforms I and II, which corresponded to the higher and lower molecular weight forms, respectively. These two isoforms appeared to be different only in the carbohydrate moiety, having essentially the same enzymatic properties. The enzyme was optimally active at pH 8.0 toward Gly-Asp-Asp-Asp-Asp-Lys-β-naphthylamide, and was inhibited strongly by various serine proteinase inhibitors. Furthermore, it was also strongly inhibited by E-64 [L-*trans*-epoxysuccinyl-leucylamide-(4-guanido)-butane], a cysteine proteinase inhibitor. Substrate specificity studies involving various synthetic peptides indicated that acidic residues at the P2, P3, and/or P4 positions are especially favorable for maximal activity, but are not absolutely necessary, at least in the cases of peptide substrates.

Key words: enteropeptidase, lectin blotting, porcine duodenum, purification, substrate specificity.

Enteropeptidase [EC 3.4.21.9] is known as a specific proteinase responsible for the initial conversion of trypsinogen to trypsin in the duodenum (*1, 2*). The resulting trypsin then activates several other zymogens secreted from the pancreas into the duodenum. Thus, it is a key enzyme in intra-intestinal digestion. A genetic deficiency of this enzyme, therefore, leads to malabsorption and malnutrition, particularly in infancy (*3, 4*).

A number of studies has been performed so far on the purification and characterization of this enzyme from various sources including human (*5*), bovine (*6-8*), and porcine (*9, 10*) duodena. Through these studies, the enzyme has been shown to be a membrane-bound, highly glycosylated serine endopeptidase with a rather restricted specificity, cleaving the Lys-X bond at the junction of the propeptide and trypsin moiety of trypsinogen, with a marked preference for acidic residues at the P2-P4 sites. The reason for this high specificity, however, was not explained clearly, mainly due to the lack of structural information on the enzyme. Therefore we undertook the isolation and analysis of a cDNA clone for the porcine enzyme and deduced its complete amino acid sequence,

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Abbreviations: Boc, *t*-butoxycarbonyl; Bz, benzoyl; Con A, concanavalin A (jack bean lectin); DSA, *Datura stramonium* lectin; DFP, diisopropylfluorophosphate; E-64, L-*trans*-epoxysuccinyl-leucylamide-(4-guanido)-butane; Gly-(Asp)$_4$-Lys-β-NA, Gly-Asp-Asp-Asp-Asp-Lys-β-naphthylamide; H chain, heavy chain; L chain, light chain; M chain, mini chain; MCA, 4-methylcoumaryl-7-amide; PBST, phosphate-buffered saline containing 0.05% Tween20; PNA, peanut lectin; RCA120, castor bean lectin; STI, soybean trypsin inhibitor; TPCK, *N*$^{\alpha}$-tosyl-L-phenylalanine chloromethyl ketone; WGA, wheat germ lectin.

which permitted us to explain the specificity based on a three-dimensional structure constructed by computor modeling (*10*). To date, the sequences of the bovine (*11, 12*), human (*12, 13*), and rat (*14*) enteropeptidases have also been determined. Furthermore, we found that the porcine enzyme is composed of three polypeptide chains: a 66-residue mini (M) chain, a 682-residue heavy (H) chain, and a 235-residue light (L) chain (*10*).

During the studies on the porcine enzyme, we improved the purification procedure for the enzyme, and investigated some molecular and enzymatic properties of the purified enzyme which were not fully studied previously. Special attention was paid to its microheterogeneity and substrate specificity.

EXPERIMENTAL PROCEDURES

Materials—Fresh porcine duodena were obtained from a local slaughterhouse and stored frozen at −20°C. Bovine trypsinogen and Gly-Asp-Asp-Asp-Asp-β-naphthylamide [Gly-$(Asp)_4$-β-NA], aprotinin, soybean trypsin inhibitor (STI), benzamidine, diisopropylfluorophosphate (DFP), and N^{α}-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were purchased from Sigma. Benzoyl-L-arginine-4-methylcoumaryl-7-amide (Bz-Arg-MCA) and other MCA derivatives of peptides, L-*trans*-epoxysuccinyl-leucylamide-(4-guanido)-butane (E-64), antipain, bestatin, chymostatin, leupeptin, and pepstatin were from the Peptide Institute, Osaka. A protein assay kit was obtained from Bio-Rad. DEAE-cellulose (DE-52) and butyl Toyopearl 650S (prepacked column, 2×20 cm) were obtained from Whatman and Tosoh, Tokyo, respectively, and Sephacryl S-300 and benzamidine-Sepharose from Pharmacia. Peroxidase-labeled lectins, including those from *Dature stramonium* (DSA), peanut (PNA), castor bean (RCA120), jack bean (ConA), and wheat germ (WGA), and RCA120-agarose and WGA-agarose (Honen, Tokyo) were purchased from Seikagaku Kogyo, Tokyo.

Enzyme Assay—Throughout the purification, the enteropeptidase activity was mainly assayed essentially by the two-step procedure reported by Liepnieks and Light (*7*) with some modifications. The enzyme sample (10 μl) was mixed with 90 μl of 0.11 M sodium acetate buffer, pH 5.0, containing 56 mM calcium chloride, and the mixture was preincubated at 37°C for 2 min. Then, 10 μl of a 1 mg/ml bovine trypsinogen solution in 1 mM hydrochloric acid was added and the mixture was incubated at 37°C for 30 min. The reaction was terminated by the addition of 100 μl of 1 M hydrochloric acid. Five microliters of the reaction mixture was mixed with 500 μl of 0.1 M Tris-HCl buffer, pH 8.0, and then 5 μl of a 10 mM Bz-Arg-MCA solution in dimethylsulfoxide was added to this mixture. After 10-min incubation at 37°C, the reaction was terminated by the addition of 2.5 ml of 0.1 M sodium acetate buffer, pH 4.3, containing 0.1 M monochloroacetic acid. The fluorescence of the solution was measured with excitation at 370 nm and emission at 460 nm. One unit of the enteropeptidase activity (EKU) was defined as the activity producing 1 nmol of trypsin in 30 min at 37°C.

The activity of enteropeptidase toward various MCA-substrates was measured in the same way as in the second step described above using 5 μl of the enzyme solution. The activity toward Gly-$(Asp)_4$-Lys-β-NA was determined by essentially the same procedure as used for the assaying of proline-β-naphthylamidase (*15*). 125 μl of 0.1 M Tris-HCl buffer, pH 8.0, containing 2 mM substrate was mixed with 10 μl of enzyme, and the mixture was incubated at 37°C for 10 min. To this was added 375 μl of a coupling reagent prepared according to Barrett (*16*). The absorbance at 520 nm was measured against a control sample. To determine the k_{cat} and K_m values for Gly-$(Asp)_4$-β-NA and Z-Phe-Arg-MCA, the concentrations of the substrates were varied from 0.67 to 2.0 mM and 0.1 to 0.5 mM, respectively.

Purification of Porcine Enteropeptidase—All the purification procedures were performed at 4°C.

Crude extract: After thawing the frozen duodena from 100 pigs overnight, they were freed from adipose and connective tissues, cut longitudinally and then washed roughly with water. Then they were squeezed with the fingers in 800 ml of 20 mM Tris-HCl buffer, pH 8.0 (buffer A). The solution was mixed with a one-ninth volume of 10% sodium deoxycholate and stirred for 1 h. After centrifugation at 9,000 rpm for 30 min, the supernatant was filtered through 2 sheets of gauze and then recentrifuged at 28,000 rpm for 60 min.

DEAE-cellulose chromatography: DEAE-cellulose (DE-52, 900 ml, wet weight) equilibrated with buffer A was added to the above supernatant and the mixture was kept overnight to adsorb the enzyme. The DEAE-cellulose was washed 3 times with buffer A and 3 times with 40 mM NaCl in the same buffer, and then packed into a glass column (7.6×24 cm). After washing the column with 10 liters of 40 mM NaCl in buffer A at the flow rate of 300 ml/h, it was eluted with 120 mM NaCl in the same buffer at the same flow rate and the active fractions were pooled.

Butyl-Toyopearl chromatography: The pooled fraction was brought to 25% saturation with ammonium sulfate and then stirred for 30 min. After centrifugation at 12,000 rpm for 20 min, the supernatant was applied to a prepacked butyl-Toyopearl column (2×20 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.9, containing 1 M ammonium sulfate at the flow rate of 1 ml/min. The column was washed with 400 ml of the same solvent, and then eluted with 800 ml of a linear gradient of ammonium sulfate, from 1 to 0 M, at the flow rate of 4 ml/min. The active fractions were pooled and concentrated with a Centricell.

Sephacryl S-300 chromatography: The enzyme solution obtained above was applied to a Sephacryl S-300 column (3.6×90 cm) equilibrated with buffer A containing 200 mM NaCl, and then eluted with the same solvent at the flow rate of 30 ml/h. The active fractions were pooled and concentrated with a Centricell.

Benzamidine-Sepharose chromatography: The enzyme solution was finally applied to a benzamidine-Sepharose column (0.9×25 cm) equilibrated with buffer A containing 200 mM NaCl and then eluted with the same solvent at the flow rate of 6 ml/h. The active fractions were pooled, concentrated and then used for characterization studies.

Determination of Protein Concentrations—Protein concentrations were estimated colorimetrically with a protein assay kit using mouse IgG as a standard protein unless otherwise specified.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)—SDS-PAGE was performed under reducing conditions essentially according to Laemmli (*17*) using SDS-PAG plates, 4/10 (Daiichi, Tokyo).

Lectin Blotting—After SDS-PAGE on a gradient gel (10–20% polyacrylamide concentration, Multigel, Daiichi) under reducing conditions, the enzyme protein was transferred to a nitrocellulose membrane (Immobilon NC, Millipore) in a wet type electric transfer apparatus with 25 mM Tris–190 mM glycine–20% methanol at 30 V for 2 h at 4°C. The membrane was preincubated with phosphate-buffered saline containing 0.05% Tween 20 (PBST) for 1 h at room temperature. Then, the membrane was incubated in PBST containing peroxidase-labeled lectins at proper concentrations for 1 h at room temperature according to the manufacturer's instructions. After washing 3 times with PBST, the membrane was stained in 50 ml of 20 mM Tris-HCl buffer, pH 7.5, containing 30 μl of 31% hydrogen peroxide and 10 ml of 4-chloro-1-naphthol (3 mg/ml in methanol). The reaction was terminated by washing with distilled water.

Separation of Enteropeptidase Isoforms by Lectin Chromatography—A column of RCA120-agarose (2 ml) and a column of WGA-agarose (2 ml) were tandemly connected in that order and equilibrated with 20 mM Tris-HCl, pH 8.0, containing 0.2 M NaCl. A portion of the enzyme solution obtained on benzamidine-Sepharose chromatography was applied to the first column and eluted with the same solvent, the enzyme being bound to either of the columns. Subsequently, the enzyme bound to the first column was eluted with 0.2 M *N*-acetylglucosamine, and that bound to the second column with 0.2 M lactose in the same solvent.

RESULTS AND DISCUSSION

Purification of Porcine Enteropeptidase—By the procedure described in the preceding section porcine enteropeptidase was purified approximately 2,200-fold in a yield of 12%, as summarized in Table I. The results were much better than those we previously obtained (*10*), apparently due to improvement of the chromatographic conditions. In the current procedure, the step of affinity chromatography on benzamidine-Sepharose was especially effective. A typical chromatogram of the enzyme on benzamidine-Sepharose is shown in Fig. 1a. The enzyme rather weakly bound to benzamidine-Sepharose under the conditions used, and eluted from the column with 200 mM NaCl as a rather broad peak. The activity and protein peaks overlapped nearly completely, and the specific activity was essentially the same among the active fractions collected (data not shown). The SDS-PAGE pattern under reducing conditions of every other fraction is shown in Fig. 1b. As reported previously (*10*), the enzyme protein in each fraction was composed of heavy (H), light (L), and mini (M) chains, as judged on SDS-PAGE under reducing conditions; however, the M chain (16–19 kDa) was hardly detectable under the present staining conditions. As can be seen from the figure, the molecular weights of the H and L chains appeared to gradually decrease from 158K to 145K and 50K to 46K, respectively, as the fraction number increased. Since the specific activity (*i.e.*, activity/protein) of each fraction was essentially the same, this difference may chiefly reflect the difference in the molecular weight of the carbohydrate moiety. The order of elution may be partly explained by the molecular shieving effect of Sepharose CL-6B to which benzamidine was bound. This type of heterogeneity of enteropeptidase has not been reported previously. Porcine enteropeptidase was previously purified by Baratti *et al.* (*9*) using a six-step procedure including DEAE-cellulose chromatography, and gel filtration on Sephadex G-100 and G-200. The purification factor was reported to be about 1000-fold; however, the activity and

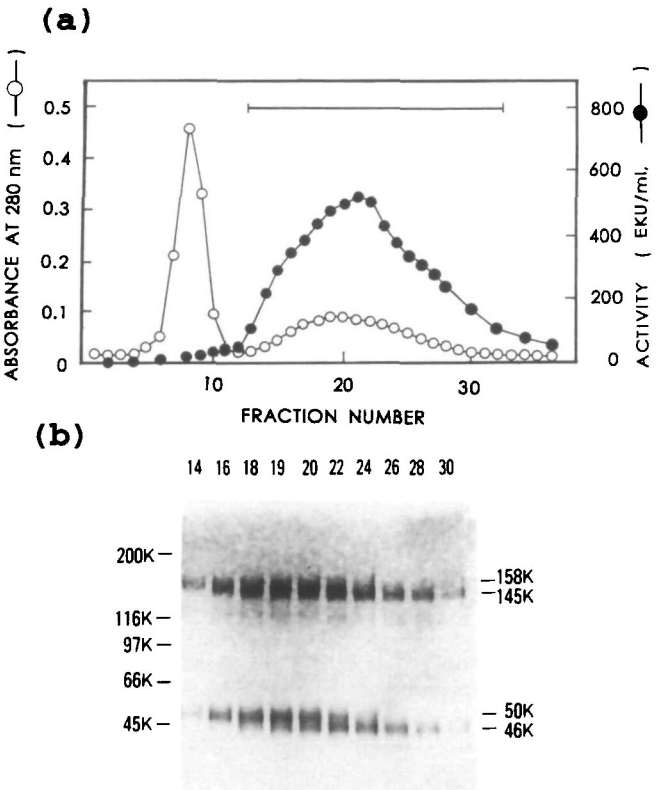

Fig. 1. **Purification of porcine enteropeptidase.** (a) Chromatography on benzamidine-Sepharose. The active fraction obtained on Sephacryl S-200 chromatography was fractionated on a column (0.9 × 25 cm) of benzamidine-Sepharose equilibrated and eluted with 20 mM Tris-HCl buffer, pH 8.0, containing 200 mM NaCl. Flow rate, 6 ml/h; fraction size, 2.0 ml. The fractions under the bar were pooled. (b) SDS-PAGE. The active fractions were analyzed by SDS-PAGE under reducing conditions on a 4–10% gradient gel. The protein was stained with Coomassie Brilliant Blue.

TABLE I. **Purification of porcine enteropeptidase.** The duodena from 100 pigs were used.

	Total protein (mg)	Total activity (EKU)	Specific activity (EKU/mg)	Purification (-fold)	Yield (%)
Crude extract	12,600	103,000	8.2	1	100
DE-52	850	48,100	56.6	6.9	46.7
Butyl-Toyopearl	97.1	29,100	300	36.6	28.3
Sephacryl S-200	15.0	17,300	1,150	140	16.8
Benzamidine-Sepharose	0.7	12,400	1,770	2,160	12.0

protein peaks still failed to overlap on the final chromatography on Sephadex G-200. Thus, the present four-step purification procedure including affinity chromatography on benzamidine-Sepharose seems to be superior to the previously used method.

Analysis of the Carbohydrate Moiety by Lectin Blotting and Separation of Two Enteropeptidase Isoforms—To examine the nature of the carbohydrate moieties, we performed lectin-blotting analysis of the peptide chains separated by SDS-PAGE of the pooled enzyme fraction, the results for the H and L chains being shown in Fig. 2.

All peptide bands showed a positive reaction with *Datura stramonium* lectin (DSA), which is known to bind to *N*-linked carbohydrate chains of the tri- and/or tetraantennary complex types. On the other hand, no peptide bands showed a positive reaction with peanut lectin (PNA), which is known to have affinity toward galactose and to strongly bind to Galβ1→3GalNAc in *O*-linked carbohydrates. Furthermore, neither peptide band showed a positive reaction with jack bean lectin (ConA), which is known to have affinity toward α-D-mannose and α-D-glucose, and to bind strongly to *N*-linked carbohydrate chains of the high-mannose, biantennary complex, and hybrid types.

Thus, porcine enteropeptidase is deduced to contain *N*-linked carbohydrate chains of the tri- and/or tetraantennary complex types as the major components of the carbohydrate moiety, and that it contains apparently little *O*-linked carbohydrate chains and *N*-linked carbohydrate chains of the high mannose, biantennary complex, and hybrid types. The suggested low content of *O*-linked carbohydrates is rather unexpected since the H chain of the enzyme was shown to have a Ser/Thr-rich sequence which could be a potential *O*-glycosylation sites (*10*).

Furthermore, only the lower molecular weight bands for both H and L chains showed a positive reaction with castor bean lectin (RCA120), which is known to have affinity toward galactose and to bind strongly to Galβ1→4GlcNAc. The staining with RCA120 became stronger after acid treatment, suggesting the removal of some terminal sialic acid. On the other hand, only the higher molecular weight bands for both H and L chains showed a positive reaction with wheat germ lectin (WGA), which is known to have affinity toward *N*-acetylglucosamine and to bind strongly to GlcNAcβ1→4Manβ1→4GlcNAcβ1→4GlcNAc-Asn, and the staining did not change upon acid treatment. These results suggest that there are apparently two major isoforms of enteropeptidase, differing in the carbohydrate moiety, *i.e.*, a higher-molecular-weight form with affinity toward WGA (isoform I) and a lower-molecular-weight form with affinity toward RCA120 (isoform II). These two isoforms, therefore, could be separated by sequential lectin affinity chromatography. When a sample was first applied to an RCA120 column, only isoform II was bound to the column, isoform I passing through the column and subsequently binding to a WGA column (data not shown). As for the M chain, however, none of the lectins examined showed a positive reaction (data not shown). These results are consistent with the amino acid sequence of porcine enteropeptidase, which has several potential *N*-glycosylation sites in its H and L chains, but not in its M chain (*10*).

So far, not much information has been obtained on the carbohydrates of enteropeptidase except for a few reports. Previously, the carbohydrate content of porcine enteropeptidase was reported to be 37% (w/w) (neutral sugars, 20%;

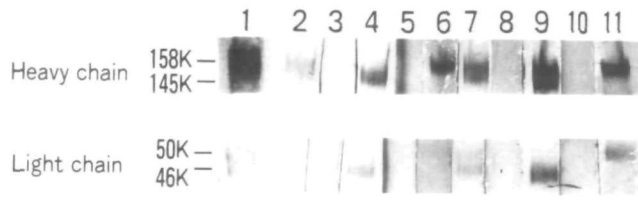

Fig. 2. **Lectin staining of the H and L chain bands separated on SDS-PAGE of the pooled enteropeptidase fraction.** Lane 1, Coomassie Brilliant Blue staining. Lanes 2–6, lectin staining before acid treatment. Lanes 7–11, lectin staining after acid treatment. The lectins used were DSA (lanes 2 and 7), PNA (lanes 3 and 8), RCA120 (lanes 4 and 9), ConA (lanes 5 and 10), and WGA (lanes 6 and 11).

TABLE II. **Effects of various reagents on the activity of the porcine enteropeptidase isoforms.** The activity was determined with Gly-(Asp)$_4$-Lys-β-NA as the substrate.

Reagent	Concentration (mM)	Activity (%)	
		Isoform 1	Isoform 2
DFP	1	0	0
Benzamidine	0.1	31.1	31.4
	1	18.4	4.1
E-64	0.1	14.8	15.4
Aprotinin	0.01	6.4	8.1
Leupeptin	0.01	19.1	12.9
Antipain	0.1	10.3	17.4
Chymostatin	1	81.5	77.2
Pepstatin	0.1	102	104
Bestatin	1	98.0	100
STI	0.005	102	111
TPCK	1	96.0	112
PCMB	1	99.0	116
2-Mercaptoethanol	1	108	101
EDTA	1	81.7	60.1
$MgCl_2$	1	127	111
$CaCl_2$	1	114	103
$MnCl_2$	1	104	108
$CoCl_2$	1	133	119
$HgCl_2$	1	159	154

TABLE III. **Activity of the enteropeptidase isoforms toward various peptide MCA substrates.**

Substrate	Activity			
	Isoform 1		Isoform 2	
	nmol min mg protein	(%)	nmol min mg protein	(%)
Z-Phe-Arg-MCA	5,110	(100)	5,870	(100)
Boc-Leu-Ser-Thr-Arg-MCA	527	(10.3)	634	(10.8)
Boc-Gln-Ala-Arg-MCA	323	(6.3)	423	(7.2)
Boc-Leu-Thr-Arg-MCA	241	(4.7)	338	(5.8)
Boc-Val-Leu-Lys-MCA	198	(3.9)	320	(5.5)
Boc-Phe-Ser-Arg-MCA	178	(3.5)	251	(5.3)
Boc-Gln-Gly-Arg-MCA	66.6	(1.3)	72.6	(1.2)
Boc-Val-Pro-Arg-MCA	50.6	(1.0)	57.4	(1.0)
Boc-Leu-Gly-Arg-MCA	48.0	(0.9)	41.3	(0.7)
Boc-Gln-Arg-Arg-MCA	30.7	(0.6)	24.2	(0.4)
Boc-Glu-Lys-Lys-MCA	29.5	(0.6)	8.8	(0.1)
Bz-Arg-MCA	21.5	(0.4)	27.8	(0.5)
Boc-Gly-Arg-Arg-MCA	20		20	
Boc-Leu-Arg-Arg-MCA	20		20	
Boc-Gly-Lys-Arg-MCA	20		20	
Boc-Leu-Lys-Arg-MCA	20		20	
Arg-MCA	20		20	

amino sugars, 15%; and sialic acid, 2%) (*9*), which is consistent with the results obtained above. On the other hand, human enteropeptidase was reported to contain 57% (w/w) carbohydrate (L-fucose 21%, D-mannose 13%, D-galactose 13%, D-GlcNAc 10%, and no sialic acid) (*5*), and to be separated into one major and two minor fractions on lectin affinity chromatography with *Helix pomatia* lectin, which is specific for *N*-acetyl-D-galactosamine, and *Lotus tetragonolobus* lectin, which is specific for L-fucose (*5*). Although the information is rather limited, the above results seem to indicate that the carbohydrate moiety is considerably different between the porcine and human enzymes.

Enzymatic Properties of Enteropeptidase—Various enzymatic properties were examined separately with isoforms I and II; however, no significant difference was found between them. Therefore, the difference is thought to be present only in the carbohydrate moieties. The enzyme exhibited the optimal activity at pH 8.0, 50% activity at pH 6.4 and 9.0, and practically no activity at pHs below 5.0 with Gly-(Asp)$_4$-Lys-β-NA as the substrate. The enzyme was strongly inhibited by DFP, aprotinin, leupeptin, antipain, and benzamidine (Table II), which are typical inhibitors of trypsin-type serine proteases. It is noteworthy that aprotinin (bovine pancreatic trypsin inhibitor) was previously reported to inhibit bovine enteropeptidase (*7*), but not to inhibit porcine or human enteropeptidase (*1, 17*). The reason for this discrepancy is not certain at present. Interestingly, E-64, a cysteine protease inhibitor, was found to strongly inhibit the enzyme. To our knowledge, this is the first time that E-64 was found to strongly inhibit a trypsin-type serine endopeptidase. The mechanism of this inhibition remains to be elucidated, but the guanidinium group in the inhibitor may be partly responsible for this inhibition since the enzyme prefers Arg (or Lys) at the P1 position of substrates. Among the metal salts examined, $HgCl_2$ appeared to significantly activate the enzyme. The reason for this activation is also not certain at present.

Table III summarizes the activity of the enzyme toward various synthetic substrates. In addition to the native substrate, trypsinogen, and the typical synthetic substrate, Gly-(Asp)$_4$-Lys-β-NA, which includes part of the propeptide sequence of trypsinogen, the enzyme was found to hydrolyze various peptide MCA substrates at varying rates. Unexpectedly, Z-Phe-Arg-MCA was hydrolyzed most rapidly among the peptide MCA substrates examined even though it lacks an acidic residue at the P2 position, which was deduced to be important for the cleavage specificity of enteropeptidase (*1, 18*). The k_{cat}, K_m, and k_{cat}/K_m values at pH 8.0 for Gly-(Asp)$_4$-Lys-β-NA and Z-Phe-Arg-MCA were determined to be 520 s^{-1}, 1.12 mM and 4.64×10^5 $M^{-1} \cdot s^{-1}$, and 263 s^{-1}, 1.24 mM and 2.12×10^5 $M^{-1} \cdot s^{-1}$, respectively. Thus, the former exhibits over 2 times higher catalytic efficiency than the latter. Furthermore, Gly-Asp-Asp-Asp-Asp-Lys-Ile-Val-Gly and Z-Phe-Arg-Ile-Val-Gly were synthesized, and the rates of hydrolysis by enteropeptidase of the Lys-Ile bond in the former and the Arg-Ile bond in the latter were determined. The results indicated that the former peptide was hydrolyzed about 6 times as rapidly as the latter one. This is consistent with the cleavage specificity of enteropeptidase toward trypsinogen. Taken together, it is suggested that the interaction of enteropeptidase with some part of the trypsinogen molecule other than the propeptide region is also important for the high specificity of enteropeptidase toward trypsinogen among various proteins.

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