Note

Substrate Specificities of Porcine and Bovine Enteropeptidases toward the Peptide Val-(Asp)$_4$-Lys-Ile-Val-Gly and Its Analogs

Yong-Tae Kim,$^1,2$ Wataru Nishii,$^{1,3}$ Masashi Matsushima,$^4$ Hideshi Inoue,$^3$ Hisashi Ito,$^2$ Sun Joo Park,$^{1,5}$ and Kenji Takahashi$^{1,†}$

$^1$Protein Research Group, RIKEN Genomic Sciences Center, Tsurumi, Yokohama, Kanagawa 230-0045, Japan
$^2$Department of Chemistry, Aoyama Gakuin University, Sagamihara, Kanagawa 229-8558, Japan
$^3$School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-0392, Japan
$^4$Department of Internal Medicine, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan
$^5$Department of Biochemistry, Institute of Medical Science, University of Tokyo, Tokyo 108-8539, Japan

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The substrate specificities of porcine and bovine enteropeptidases were investigated using the peptide Val-(Asp)$_4$-Lys-Ile-Val-Gly and its various analogs with mutations in the (Asp)$_4$-Lys sequence as substrates. The results indicated that in addition to P1 Lys, P2 Asp in the substrates is most important, that P3 Asp is additionally important, and that P5 Asp contributes somewhat to the susceptibility, and that P4 Asp is the least important. These results were essentially identical as between porcine and bovine enteropeptidases.

Key words: enteropeptidase; substrate specificity; synthetic peptide substrates; trypsinogen activation peptide

Enteropeptidase is a membrane-bound serine endopeptidase capable of converting trypsinogen to trypsin in the duodenum.$^1$ Mammalian trypsinogens have a highly conserved N-terminal activation peptide possessing the common motif Asp-Asp-Asp-Asp-Lys, and specific hydrolysis by enteropeptidase occurs after the Lys residue. Thus, this peptide moiety constitutes the substrate P5-P1 sites for the enzyme. Previous specificity studies using synthetic peptides and protein substrates have indicated that acidic residues are required for recognition and cleavage by enteropeptidase.$^2$–$^8$ In these studies, however, the relative importance of the respective Asp residues was not quantitatively or systematically analyzed, although P2 and P3 Asps were indicated to be most important, in addition to P1 Lys. The crystal structure of the recombinant bovine enteropeptidase light chain coupled with an inhibitor, an analog of the trypsinogen activation peptide, was elucidated.$^9$ The results indicate that the Asp residues at positions P2-P4 of the inhibitor interact with the N$^\text{ε}$ atom of Lys99 (chymotrypsin numbering), which is consistent with those obtained from the specificity studies. However, to determine the relative importance of these residues more clearly, it was thought to be desirable to perform further quantitative studies using systematically designed substrates.

In the present study, we investigated the action of porcine and bovine enteropeptidases on the peptide Val-Asp-Asp-Asp-Asp-Lys-Ile-Val-Gly (VDDDDKIVG) based on the N-terminal sequence of porcine trypsinogen and a series of its analogs in which one or more of the amino acid residues in the DDDDK sequence were systematically replaced with other amino acids, especially Ala.

Fresh porcine and bovine duodenal segments were obtained from a local slaughterhouse (Tokyo) and stored frozen at $-20^\circ\text{C}$ in a deep-freezer. Bovine trypsinogen was purchased from Sigma (St. Louis, Mo). Benzoyl-L-arginine-4-methylcoumaryl-7-amide (Bz-Arg-MCA) was from the Peptide Institute (Osaka, Japan). A protein assay kit was obtained from Bio-Rad (Hercules, CA). DEAE-cellulose (DE-52) and butyl-Toyopearl 650S (a prepacked column, 2 × 20 cm) were obtained from Whatman (Tokyo) and Tosoh (Tokyo), respectively, and Sephacryl S-300 and benzamidine-Sepharose from GE Healthcare (Buckinghamshire, UK). Other reagents used were of analytical grade.

Through purification, the enteropeptidase activity was determined essentially by the trypsinogen activation assay described previously.$^6,10$ 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-µg/ml bovine trypsinogen solution in 1 mM hydrochloric acid was added, and the mixture was

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$^†$ To whom correspondence should be addressed. Kenji Takahashi, Fax: +81-49-297-8168; E-mail: kenjitak@ls.toyaku.ac.jp; Sun Joo Park, Fax: +81-78-382-5419; E-mail: parksj@med.kobe-u.ac.jp
incubated at 37 °C for 30 min. The reaction was terminated by the addition of 100 μl of 1 M hydrochloric acid. Five μl 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 of 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 synthetic peptides was measured as follows: The reaction was carried out at 37 °C for 15 min in a 0.1 ml mixture containing 100 mM Tris–HCl (pH 8.0), 50 mM calcium chloride, 0.8 mM substrate, and 5 μl of the enzyme. The reaction was initiated by the addition of substrate, and was terminated with 0.1 ml of 0.1% trifluoroacetic acid. The reaction mixture was centrifuged at 3,000 × g for 10 min, and the supernatant was analyzed by HPLC using a C18 column (3.5 × 250 mm) with a linear gradient of acetonitrile (0–40% in 22 min) in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. The column effluent was identified and quantitated by amino acid analysis using a Waters AccQTag system (Milford, MA) after acid hydrolysis (6 N HCl, 110 °C, 24 h). To determine the kcat and Km values, the substrate concentration was varied from 0.3 to 4.0 mM.

Porcine and bovine enteropeptidases were purified by successive column chromatographies on DEAE-cellulose, butyl-Toyopearl, Sephacryl S-300, and benzamidine-Sepharose as described previously.6) To analyze the contribution to the activity of the respective residues at the five positions in the DDDDK motif, 20 different substrate peptides were synthesized by the solid-phase peptide synthesis method.11) Cleavage of the peptide from the Wang resin and removal of all of the side-chain protecting groups were achieved in trifluoroacetic acid solution. The crude peptide was purified by reversed-phase high performance liquid chromatography in a Shimadzu HPLC apparatus (Kyoto, Japan) on a C18 semipreparative column with gradients of acetonitrile in 0.1% trifluoroacetic acid. Peptide homogeneity and identity were analyzed by analytical HPLC, mass spectroscopy, and amino acid analysis.

Protein concentration was estimated colorimetrically using a protein assay kit (Bio-Rad) and mouse IgG as the standard.12) Molecular weight determination was performed by mass spectrometry using a JEOL (JMS-SX102/JMA-DA6000) mass spectrometer (JEOL, Tokyo). Amino acid analysis was performed with a Waters AccQTag amino acid analysis system after acid hydrolysis in 6 N HCl at 110 °C for 24 h.

The porcine and bovine enzymes purified in this study had specific activities of 1,490 and 1,310 EKU/mg respectively. These values are comparable to those reported previously for purified porcine enzyme.6) Porcine enteropeptidase showed pH optima at 8.0–8.5 for VDDDDKIVG and 8.5–9.0 for VDDDAKIVG, and hence the subsequent assays with synthetic peptide substrates were performed at pH 8.0.

Table 1 lists the relative activities and kinetic parameters of the purified porcine and bovine enzymes for 20 synthetic peptides, S01–S20, including the original peptide S01, VDDDDKIVG. The relative activities were similar between the two enzymes. In the first four analog peptides, S02–S05, the effect of replacement was especially striking with peptide S02. The relative activities decreased 80–84%. The decrease in susceptibility of S02 was mainly due to a marked increase in the Km value; the kcat value increased 4.7-fold, while the kcat/Km value decreased by 7%, resulting in an 84% decrease in the kcat/Km value. Thus the replacement of P2 Asp with Ala mainly affected the Km value. In S03, the relative activities were 10–20% reduced. Due to this replacement, both kcat and Km value decreased by 71–72%, resulting in almost no change in the kcat/Km value. On the other hand, in S04 and S05, the relative activities were 10–20% elevated. In this replacement, the kcat values decreased by 27% and 11%, while the Km values decreased by 57% and 43%, and hence the kcat/Km values increased by 71% and 56% for S04 and S05 respectively. Thus the substitution at the P4 or P5 position affected the Km value more than the kcat value. These results indicate that P2 Asp is most important for the substrate of enteropeptidase, and that P3 Asp additionally contributes to susceptibility, whereas P4 and P5 Asps are not as important.

S06 was not hydrolyzed at all, consistently with the results obtained with S02 and S03. The relative activities and kinetic parameters for S07 were similar to those for S02, suggesting unimportance of P4 Asp. On the other hand, the relative activities toward S08 were much lower than those toward S07. Thus P5 Asp appears to contribute somewhat to susceptibility to the enzyme. The relative activities toward S09 were comparable with those toward S03, again suggesting unimportance of P4 Asp. S10 was less susceptible to the enzyme than S09, also indicating some contribution of P5 Asp to susceptibility. The relative activities and kinetic parameters for S11 were similar to those for S04 and S05, consistently with the foregoing results.

S12 was very weakly hydrolyzed, although it was expected not to be hydrolyzed from the results for S06. P4 Ala appears to be somewhat more favorable than P4 Asp to the enzyme. The relative activities and kinetic parameters for S13 were similar to those for S02 and S07, indicating the importance of P2 Asp, but not of P4 and P5 Asps. S14 was not hydrolyzed, as expected from the results for S06. The results obtained for S15 were similar to those obtained for S03, demonstrating the major importance of P2 Asp. S16 was not hydrolyzed at all, as expected.
S17 showed about half-reduced susceptibility, and Asp appears to have been preferred to Glu for the most effective binding to the active site of enteropeptidase. Among S18–S20, only S20 was well hydrolyzed, the relative activity being about 30% higher and the catalytic efficiency nearly three times higher than those of S01. Arg appears to fit better than Lys to the active site of the enzyme. These results are consistent with those reported recently for bovine enteropeptidase, in which the efficiency of hydrolysis of the synthetic substrate APFD$_4$KIVGG was increased nearly two-fold by replacement of P1 Lys with Arg. \(^8\)

According to X-ray crystallographic studies of the recombinant bovine enteropeptidase light chain coupled with an analog of the trypsinogen activation peptide, \(^9\) the main-chain atoms of P2 Asp make four close contacts with the enzyme light chain, and its carboxylate side-chain makes two H-bonds with the N/C16 atom of Lys99; P3 Asp makes half as many contacts, including an H-bond with the hydroxyl group of Tyr 174; P4 Asp makes only one H-bond between its carboxylate group and the N/C16 atom of Lys99, and P5 Asp and P6 Val are disordered. The results obtained in the present study are largely consistent with the above crystal structure.

The present results are also consistent with those of a previous study of porcine enteropeptidase using various model peptides as substrates. \(^3\) Although P4 Asp in the synthetic peptide was found to be unimportant to susceptibility in the present study, its functional role, especially in the trypsinogen molecule, cannot be completely excluded. On the other hand, the Asp/Ala substitution at the P4 position appeared greatly to decrease the $K_m$ value, thus promoting susceptibility, although the reason for this is not clear at present. Hence, taking this into consideration together with the preference for Arg over Lys at the P1 position, VDADDRIVG might be a better substrate for enteropeptidase than VDDDDKIVG, and might provide more efficient cleavage sites for the enzyme in recombinant protein methodology.

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### References


