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The regional differences in the mucosal-cell lysosomal proteases within the rat small intestine

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Abstract

The specific aim of this study is to understand the distribution of lysosomal enzymes along the intestine. The rat was used as the animal model. Intestinal homogenates were prepared from the jejunum, jejunoileal junction, ileum, and caecum. Activities of lysosomal carboxypeptidase A, dipeptidyl peptidase II, prolyl carboxypeptidase, and cathepsin B in homogenates were studied at pH 4.5 using specific substrates. Prolyl carboxypeptidase, cathepsin B, and dipeptidyl peptidase II had the highest activities in the caecum; carboxypeptidase A had the highest activity in the jejunum. The ileum had the lowest activities of these four lysosomal proteases. In summary, except carboxypeptidase A, the distribution profiles of prolyl carboxypeptidase, cathepsin B, and dipeptidyl peptidase II are somewhat close. Carboxypeptidase A, prolyl carboxypeptidase, and dipeptidyl peptidase II are all serine proteases but do not share similar intestinal distribution profiles.

Key words: Intestinal lysosomal proteolytic activity; Cathepsin B; Carboxypeptidase A; Dipeptidyl peptidase II; Prolylcarboxypeptidase

1. Introduction

Recently, peptide and protein therapeutics have become more available because of the advancement of biotechnology. For example, antrial natriuretic factor, insulin, epidermal growth factor, interferon, macrophage colony stimulating factor, monoclonal antibodies, and adrenocorticotropic hormone are all very important protein therapeutics. Oral delivery of peptides and proteins is most convenient yet most challenging, since oral bioavailability is poor due to extensive enzymatic degradation and poor membrane permeability. The success in achieving oral efficacy will promote the use of this class of drugs to improve human health.

To achieve oral efficacy of peptide and protein drugs, it is essential to understand the cellular proteolytic features of enterocytes and the regional differences in enterocyte proteolytic activities within the small intestine. Protein molecules, which survive the lumenal proteolytic attack by pancreatic enzymes, can be absorbed into the intestinal absorptive cells in neonates and adult animals (Warshaw et al., 1971; Walker, 1981; Gonnella et al., 1987; O'Hagan et al., 1987; Marcon-Gentt et al., 1989). In neonates, following absorption by endocytosis, proteins are trans-

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ported to lysosomes and digested therein. In mature animals, absorption of proteins by endocytosis has been observed. Although absorption mechanisms in adult intestine are less well characterized, endocytotic uptake and subsequent degradation in lysosomes have been observed (Marcon-Genty et al., 1989).

The major subcellular localizations of proteolytic activities of enterocytes are the brush-border membrane, cytosol, and lysosomes (Bai, 1992; Dice, 1992). Brush-border and cytosolic proteases digest large peptides and di-/tripeptides. Lysosomes are usually involved in eliminating intracellular abnormal and unnecessary proteins (Dice, 1992). The ubiquitin-dependent pathway or specific amino acid sequences of proteins can induce intracellular trafficking events to translocate proteins to lysosomes. These phenomena are most established using mammalian cells, and have also been suggested in intact animals (Wing et al., 1991; Dice, 1992). Therefore, after being absorbed into enterocytes by any mechanism, proteins and large peptides may be targeted by lysosomal proteases via some mechanisms.

Differential distribution of membrane peptidases of intestinal enterocytes along the intestine has been observed in several species (Auricchio et al., 1978; Sterchi, 1981; Miura et al., 1983; Yoshioka et al., 1987; Bai, 1993). Nevertheless, nothing is known about the regional differences in the lysosomal proteolytic activities within the intestine. Since the lumenal concentrations of pancreatic enzymes in the distal intestine are lower, the distal jejunum and ileum may be more suitable for the delivery of protein-based drugs (Layer et al., 1990). The study of distribution of lysosomal proteases along the intestinal length will demonstrate whether lysosomal proteases of enterocytes in the distal jejunum and ileum have lower activities. There are four major classes of lysosomal proteases, including serine, cysteine, aspartic, and metallo-proteases (Kirschke et al., 1987). Among the lysosomal proteases, serine and cysteine proteases are most abundant and well characterized (Kirschke et al., 1987). This paper will focus on the distribution of serine proteases (carboxypeptidase A, dipeptidyl peptidase II, prolyl carboxypeptidase) and cysteine protease (cathepsin B), since specific substrates for these enzymes are commercially available and these enzymes are the only lysosomal proteases of which distinct and substrate specificities are better characterized (Kirschke, 1987). Cathepsin B has an extended active site containing up to seven individual subsites. Little relationship has been observed between the specificities of its cleavage of polypeptides and synthetic short peptides. For the cleavage of polypeptidases, in general, the amino acid in P1 varies greatly; glycine, arginine, and hydrophobic amino acids can be found in P2. Synthetic substrates which contain a pair of basic amino acids are selective for cathepsin B. Dipeptidyl peptidase II removes N-terminal dipeptides from unblocked oligopeptides and prefers peptide bonds having Ala or Pro in the P1 position. Carboxypeptidase A removes amino acids sequentially from the unsubstituted C-termini of polypeptides. Prolyl carboxypeptidase releases C-terminal amino acids adjacent to proline from polypeptides.

2. Materials and methods

2.1. Materials

Dip-F (diisopropyl phosphofluoridate) and E-64 (L-3-carboxy-*trans*-2,3-epoxypropionylleucylamido-(4-guanidino)butane), DTT (dithiothreitol), EDTA, Tris base, Tris-HCl, soybean trypsin inhibitor, Gly-Pro-*p*-nitroanilide, benzoxycarbonyl-Glu-Tyr, benzyloxycarbonyl-Arg-Arg-MCA (MCA, 4-methyl coumaryl-7-amide), benzyloxycarbonyl-Pro-Phe, Lys-Ala-MCA, urethane, 7-amino-4-methylcoumarin, Lys-Ala, benzoxycarbonyl-Glu, Leu-β-naphthylamide, and pentobarbital were obtained from Sigma Chemical Co. (St. Louis, MO). Soybean trypsin inhibitor has the potency of 1 mg being able to inhibit 1–3 mg of trypsin with activity of approx. 10000 BAEE (*N*-α-benzoyl-L-arginine ethyl ester) units per mg protein. Bovine γ-globulin and dye reagent for the protein assay were obtained from Bio-Rad Laboratories (Richmond, CA). Acetonitrile was of HPLC grade. All other chemical reagents and buffer components were of analytical grade.

2.2. Animals

Male Sprague-Dawley rats weighing 250–300 g were used as the animal model.

2.3. Preparation of intestinal enterocyte homogenates

The first 8 cm of the rat small intestine was regarded as the duodenum, the next 35 cm as the jejunum, the last 25 cm of the small intestine proximal to the ileocecal junction as the ileum, and the segment between the jejunum and the ileum as the jejunoileal junction (Bai, 1993). The caecum was anatomically different from the intestine and was clearly identified. The intestinal mucosa of the desired segment was scraped off, suspended in a hypotonic solution (50 mM mannitol in 2 mM Tris-HCl buffer (pH 7.5)), and then homogenized using a blender and a glass/Teflon potter homogenizer. Homogenates were thus obtained and used as the enzyme source after protein concentrations were determined. Protein concentrations were determined according to Bradford (1976) using γ-globulin as the protein standard. Homogenates were prepared from 13 rats.

2.4. Lysosomal enzyme activities

Enzyme activities were assayed as described by Ekstrom et al. (1991) and methods indicated in the literature with minor modification (Ekstrom et al., 1991; McDonald et al., 1986). The 300 μl incubation mixture consisted of 50 mM acetate/NaOH buffer (pH 4.5), 125 mM NaCl, a substrate, and 1–5 mg homogenate proteins. The pH of the final mixture was 4.5. The reaction mixture was incubated at 37°C. Periodically, samples were taken and reaction was terminated using 10% trichloroacetic acid. In the control group, 10% trichloroacetic acid was mixed with the incubation mixture before the substrate was added. Lysosomal proteolytic activities in enterocyte homogenates were determined using the specific substrate of each protease. Specific substrates of the lysosomal enzymes and their concentrations were as follows: (1) serine proteases: 0.04 mM Lys-Ala-MCA for dipeptidyl aminopeptidase II, 0.017 mM benzoxycarbonyl-Glu-Tyr for carboxypeptidase A, 0.016 mM benzyloxycarbonyl-Pro-Phe for prolylcarboxypeptidase; (2) cysteine protease: 0.004 mM benzyloxycarbonyl-Arg-Arg-MCA for cathepsin B (Taylor et al., 1974; Barrett and McDonald et al., 1980; Barrett et al., 1981; McDonald et al., 1986; Mentlein et al., 1989). Dithioerythritol (2 mM) and 2 mM EDTA (2 mM) were used to activate cathepsin B. Moreover, soybean trypsin inhibitor at 1 mg/ml was used to eliminate trace activity of trypsin (Boyer, 1971). Initial hydrolysis rates were estimated from the first 15–20% degradation using linear regression. Hydrolysis rates are expressed as μmol/min per g protein. After normalization for the protein concentration, the average hydrolysis rate was obtained from three experimental groups. All the data reported represent Mean $\pm$ SE.

2.5. Effects of enzyme inhibitors and pH

Effects of specific inhibitors and pH on the lysosomal proteolytic activities were used to further demonstrate that hydrolysis of a specific substrate was due to a certain lysosomal protease. Dip-F and E-64 are specific inhibitors of lysosomal serine and cysteine proteinases, respectively (Kirschke et al., 1987). Dip-F was used at 15 and 30 mM and E-64 at 30 μM. Experimental procedures were as described above except that inhibitors were added to the incubation mixture and 50 mM Tris-HCl was used for the pH 7.5 studies. For the inhibitor studies, the control groups did not contain any inhibitor; and for the pH-dependent studies, pH 4.5 buffer was used for the control group and pH 7.5 buffer for the experimental group.

2.6. Activities of brush-border membrane peptidases

Mucosal homogenates were prepared as described above. Brush-border membranes were then prepared from the homogenates as reported previously (Bai, 1993). $CaCl_2$ (1 M) was added to the homogenates to achieve a final concentration of 10 mM and then the mixture was centrifuged at $3000 \times g$ for 15 min. The supernatant was

collected and centrifuged at 27000 × *g* for 30 min to obtain pellets. The pellets were resuspended in a 125 mM NaCl, 50 mM Tris-HCl (pH 7.5) solution and homogenized with a glass/Teflon potter homogenizer. Then centrifugations at 3000 × *g* and 27000 × *g* were repeated to obtain pure brush-border membrane. Protein concentrations were determined. The activity of alkaline phosphatase was determined to assess the purity of brush-border membranes (Miura et al., 1983). Activities of brush-border membrane aminopeptidase M and dipeptidyl peptidase IV (DPP IV) were examined using 0.1 mM Leu-β-naphthylamide and 0.45 mM Gly-Pro-*p*-nitroanilide at pH 4.5, respectively (Bai et al., 1992; Bai, 1993). Incubation procedures were as described above.

2.7. Assay methods

The HPLC system consisted of an SIL autoinjector, LC-600 pump, SPD-6A UV spectrophotometric detector, and CR 601 recorder (Shimadzu Corp., Kyoto, Japan). Samples of lysosomal enzyme substrates were assayed using a C8 Beckman column (5 μ, 4.6 mm × 15 cm) and mobile phases of 0.05% TFA (trifluoroacetic acid) and acetonitrile (ACN). A UV wavelength of 220 nm was used for all the substrates. Lys-Ala-MCA was eluted at 8 min and benzoxycarbonyl-Arg-Arg-MCA (CBZ-Arg-Arg-MCA) at 6 min by 0.05% TFA and ACN in the volume ratio 75:25. Benzoxycarbonyl-Glu-Tyr (CBZ-Glu-Tyr) was eluted at 7 min and benzyloxycarbonyl-Pro-Phe (CBZ-Pro-Phe) at 6 min by 0.05% TFA and ACN in the volume ratio 65:35. Gly-Pro-*p*-nitroanilide was eluted at 12 min using the same column with 0.01 M NaH_2PO_4 (pH 3) and ACN in the volume ratio of 80:20. Leu-β-naphthylamide was eluted at 10 min using a strong cation-exchange column (Partisil 10 SCX, Whatman) with 0.005 M $Na(NH_4)_2PO_4$ (pH 2.5) and ACN in the volume ratio of 90:10. In the HPLC assay, standard solutions of known concentrations of each substrate were used to establish the standard curves and also used as the indicator to check the precision of HPLC assay periodically. All the products, inhibitors, and activators were separated from the parent compound for the study of each substrate.

3. Results

In general, the cytosolic pH is around 7.2 and intralysosomal pH is below 6 (Kirschke et al., 1987; Bai et al., 1992). Lysosomal proteases have high activities at acidic pH. Usually, pH 5 or 5.5 is used for lysosomal enzymatic assays (Taylor et al., 1974; Barrett and McDonald et al., 1980; Barrett et al., 1981; McDonald et al., 1986; Mentlein et al., 1989). The pH of 4.5 was chosen so that the activities of cytosolic and membrane peptidases, of which the optimum pH is 7 or above, could be negligible. Since, in homogenates, there were activities of brush-border membrane peptidases (dipeptidyl peptidase IV and aminopeptidase M) which might attack the substrate of lysosomal dipeptidyl peptidase II, brush-border membranes were prepared to confirm that these enzymes were not active at pH 4.5. Brush-border membrane was purified around 12-fold. No activities of brush-border membrane dipeptidyl peptidase IV and aminopeptidase M were detected at pH 4.5.

Table 1
Distribution of mucosal-cell lysosomal proteolytic activities along the small intestine

Enzyme	Jejunum	Jejunoileal junction	Ileum	Caecum
Prolyl carboxypeptidase	0.118 (0.090)	0.291 (0.010)	0.181 (0.020)	0.358 (0.020)
Cathepsin B	0.007 (0.001)	0.004 (0.001)	0.004 (0.001)	0.015 (0.001)
DPP II	0.110 (0.010)	0.130 (0.004)	0.115 (0.004)	0.347 (0.040)
Carboxypeptidase A	2.162 (0.300)	0.582 (0.020)	0.288 (0.020)	0.197 (0.010)

The reported values represent mean ± S.E.; enzyme activities were measured at pH 4.5 ($n = 3$) and are expressed as μmol/min per g protein.

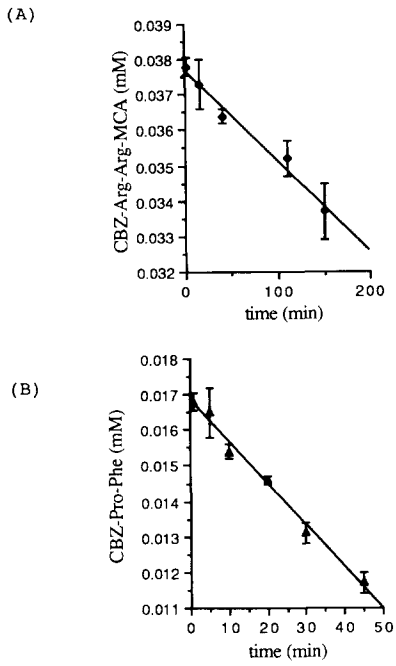

Fig. 1. Concentration/time profiles of CBZ-Arg-Arg-MCA (♦) and CBZ-Pro-Phe (▲) in the jejunal homogenate at pH 4.5.

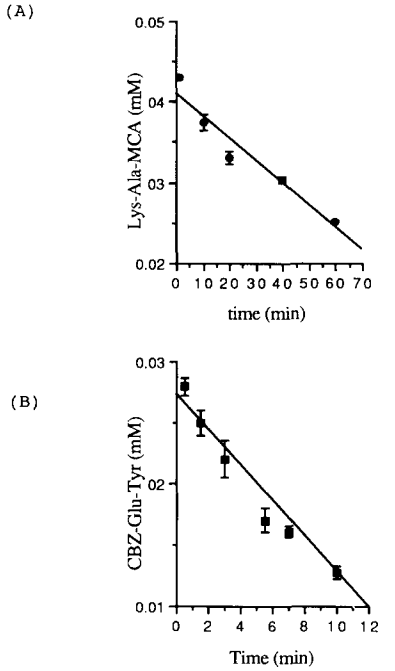

Fig. 2. Concentration/time profiles of Lys-Ala-MCA (●) and CBZ-Pro-Phe (■) in the jejunal homogenate at pH 4.5.

Table 2
Carboxypeptidase A activity in the intestinal mucosal-cell homogenates and membranes

Intestinal segment	Crude homogenates	Membranes
Jejunum	2.16 ± 0.30	0.02 ± 0.0
Ileum	0.29 ± 0.02	0.01 ± 0.0

Enzyme activity was measured at pH 4.5; the reported values represent mean ± S.E. ($n = 3$) and are expressed as μmol/min per g protein.

As shown in Fig. 1a and b, the concentrations of CBZ-Pro-Phe and CBZ-Arg-Arg-MCA decreased linearly with time as enzymatic hydrolysis proceeded. The concentrations of CBZ-Glu-Tyr and Lys-Ala-MCA decreased linearly with time as hydrolysis proceeded, as demonstrated in Fig. 2a and b. These results suggest that initial hydrolysis rates of those five substrates were obtained appropriately. The activities of lysosomal cysteine and serine proteases along the intestinal length

Table 3
Effects of pH and inhibitor on activities of lysosomal enzymes

Enzyme	pH 4.5	pH 7.5	pH 4.5 15 mM Dip-F	pH 4.5 30 mM Dip-F
Carboxypeptidase A	2.160 (0.300)	0.160 (0.024) [a]	0.620 (0.010) [c]	0.840 (0.035) [a]
Prolylcarboxypeptidase	0.118 (0.010)	0.073 (0.008) [b]	0 [a]	–
DPP II	0.130 (0.004)	–	0.004 (0.002) *	

Jejunal homogenates were used for carboxypeptidase A and prolylcarboxypeptidase, and the jejunoileal junction homogenates were used for DPPII. Enzyme activity is expressed as μmol/min per g protein; the reported values represent mean ± S.E. ($n = 3$). All the statistical differences were compared with the pH 4.5 study for each enzyme.
[a] $p < 0.005$;
[b] $p < 0.025$;
[c] $p < 0.001$.

Table 4
Effect of E-64 on activity of cathepsin-B

Condition	Enzyme activity (μmol/min per g protein) (mean ± SE)
pH 4.5, 1 mg/ml trypsin inhibitor	0.007 ± 0.001
pH 4.5, 1 mg/ml trypsin inhibitor, 30 uM E-64	0.001 ± 0.000 [a]

2 mM EDTA and 2 mM DTT were used to activate cathepsin B in the homogenates ($n = 3$); enzyme activity was measured at pH 4.5.
[a] $p < 0.005$.

are summarized in Table 1. Dipeptidyl peptidase II had the highest activity in the caecum and was around 3-fold lower in the rest of the small intestine. From the jejunum to the ileum, this enzyme had similar activity. The rank order of carboxypeptidase A activity was jejunum > jejunoileal junction > ileum > caecum. Prolyl carboxypeptidase had the highest activity in the caecum, followed by the jejunoileal junction, and then the jejunum and ileum. Cathepsin B had the highest activity in the caecum, followed by the jejunum and then the jejunoileal junction and ileum.

Lysosomal carboxypeptidase A activity in the jejunal and ileal homogenates was more than 25-fold higher than in the jejunal and ileal brush-border membranes, as shown in Table 2. The effects of inhibitors and pH on lysosomal enzyme activities are summarized in Table 3. Carboxypeptidase A activity decreased around 14-fold as pH increased to 7.5 and was also significantly inhibited by 15 and 30 mM Dip-F. Dipeptidyl peptidase II activity was reduced 18-fold by 30 mM Dip-F. Prolyl carboxypeptidase activity was reduced significantly as pH increased to pH 7.5 and was completely inhibited by 15 mM Dip-F. Cathepsin B activity was significantly reduced by 30 μM E-64, as listed in Table 4.

4. Discussion

Since the substrate used is specific, the only possible contribution to lysosomal carboxypeptidase A activity is from pancreatic carboxypeptidase. Pancreatic and lysosomal carboxypeptidases A are both serine preotease and share similar substrate specificity, however, they have different optimum pH (Boyer, 1971; Kirschke et al., 1987). The former has higher activity at alkaline pH while that of the latter occurs at acidic pH. In jejunal homogenates, there was only 7% residual activity at pH 7.5 compared to pH 4.5, suggesting there was minimal contamination of pancreatic enzyme in homogenate preparation. Dip-F at high concentration (serine protease inhibitor) was not able to completely inhibit carboxypeptidase A activity and there was around 28% residual activity of lysosomal carboxypeptidase A in the presence of 15 mM Dip-F. It is possible that a higher concentration of Dip-F is required to achieve complete inhibition of carboxypeptidase A activity, since the extent of inhibition depends on the K_m of substrate, K_i of inhibitor, and concentrations of both substrate and inhibitor. Prolyl carboxypeptidase activity was also lower at pH 7.5 compared to pH 4.5. It is unknown why, at pH 7.5, prolyl carboxypeptidase still had 62% residual activity. At pH 4.5, its activity was completely eliminated by 15 mM Dip-F.

Soybean trypsin inhibitor at 1 mg/ml was used in the assay of cathepsin B activity, since benzyloxycarbonyl-Arg-Arg-MCA could also be a substrate of trypsin (Kirschke et al., 1987). Although both trypsin and cathepsin B can attack benzyloxycarbonyl-Arg-Arg-MCA, they are different types of proteases. Trypsin is a serine protease sensitive to Dip-F (serine protease inhibitor), while cathepsin B is a cysteine protease sensitive to E-64 (cysteine protease inhibitor) (Boyer, 1971; Kirschke et al., 1987). At pH 4.5, cathepsin B activity in the duodenal homogenate was 0.02 (0.002) μmol/min per g in the presence of 1 mg/ml soybean trypsin inhibitor and 0.016 (0.003) without inhibitor. At pH 4.5, more than 85% inhibition of cathepsin B activity by 30 μM E-64 in the presence of 1 mg/ml trypsin soybean inhibitor indicates that proteolysis of benzyloxycarbonyl-Arg-Arg-MCA was mainly due to cathepsin B activity. It was reported that aminopeptidase M had no activity against Lys-Ala-MCA, the substrate of dipeptidyl peptidase II (Boyer, 1971).

Moreover, activities of two brush-border membrane peptidases (aminopeptidase M and dipeptidyl peptidase IV) were not detectable at pH 4.5 so that their contributions to dipeptidyl peptidase II activity were negligible.

The concentration of each lysosomal specific substrate, which was used to characterize the activity of individual proteases, was either well below the concentration used by other laboratories or the K_m of individual substrate (Tayleor and Tappel, 1974; Barrett and McDonald et al., 1980; Barrett et al., 1981; Mentlein et al., 1989). The K_m is the Michaelis constant for an individual enzymatic reaction, and it is the concentration at which the hydrolysis rate is one half of the maximum rate (V_{max}). Therefore, the distribution profiles of these lysosomal enzyme activities truly reflect the regional differences of their activities within the intestine.

The two serine proteases, prolyl carboxypeptidase and dipeptidyl peptidase II, have the highest activity in the caecum while it is lower in the rest of segments. For prolyl carboxypeptidase, the jejunoileal junction had slightly lower activity than the caecum and the jejunum and ileum had the lowest. The activity of dipeptidyl peptidase II was similar from the jejunum to the ileum. Carboxypeptidase A, though a serine protease, had a different distribution pattern along the intestine from that of DPP II and prolyl carboxypeptidase. Cathepsin B, a cysteine protease, showed a slow decrease in activity from the jejunum to the ileum and had the highest activity in the caecum. The results do not suggest similar distribution profiles among the serine proteases studied. It is not clear why the caecum has the highest activities of dipeptidyl peptidase II, prolyl carboxypeptidase, and cathepsin B. The caecal brush-border membrane has lower proteolytic activities than the proximal intestinal membranes (Bai, 1993). High lysosomal proteolytic activity may ensure that caecal enterocytes are well defended and protected and that intact toxic proteins will not be smuggled intact into the body via the caecum. Overall, the results indicate that, in general, the ileum has similar lysosomal proteolytic activities to that of the proximal intestine except carboxypeptidase A.

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