Distribution of Brush-Border Membrane Peptidases Along the Rat Intestine

Jane P. F. Bai[1]

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The longitudinal distribution of brush-border endopeptidase-24.11, endopeptidase-2, aminopeptidase W, angiotensin-converting enzyme (ACE), dipeptidyl peptidase IV (DPP IV), carboxypeptidase P, and aminopeptidase P in the rat intestine was determined. The jejunum has the highest activities of endopeptidase-24.11 and ACE while the ileum has the highest activities of aminopeptidase W and carboxypeptidase P, and the jejunoileal junction has the highest activity of aminopeptidase P. The jejunum and ileum have similar activities of DPP IV. The profiles of differential hydrolysis of neurotensin and acetylneurotensin (8–13) along the intestine agree with distribution of endopeptidase-24.11 and ACE, suggesting that amino acid sequences of peptides and the substrate specificity of enzymes will determine site-dependent hydrolysis. There is substantial similarity in the intestinal distribution of peptidases in the human, rat, and rabbit.

KEY WORDS: brush-border peptidases; intestinal distribution; neurotensin; acetylneurotensin (8–13).

INTRODUCTION

A major problem in oral delivery of peptide drugs is extensive lumenal degradation. Effects of enhancers, formulations, and enzyme inhibitors on intestinal absorption seemed to show site-dependence (1–4). To understand what influences site-dependent absorption and enhancement, it is necessary to determine the contribution of different factors, including membrane permeability, enzymatic activities, and action of enhancers.

There are nine major brush-border peptidases, i.e., aminopeptidase N, aminopeptidase A, carboxypeptidase P, DPP IV, endopeptidase-24.11, ACE, aminopeptidase P, aminopeptidase W, and endopeptidase-2 (5–7). Endopeptidase-24.11 and ACE have similar distribution profiles in the rat and rabbit intestines (8). Without considering the caecum, intestinal distribution of DPP IV was also similar in the rat and rabbit with its activity being high at the distal end. Further, activities of aminopeptidase N, aminopeptidase A, and carboxypeptidase P increased distally in the rabbit intestine (9). Activities of brush-border aminopeptidase A, aminopeptidase N, DPP IV, and carboxypeptidase P also increased distally in the human intestine (10,11). Hence, rabbit and human intestines have similar distributions of some peptidases while rat and rabbit intestines are similar in others.

The activities of aminopeptidase N, ACE, and total activities of neutral endopeptidases along the rat intestine increased toward the mid intestine and then decreased distally whereas DPP IV activity decreased slightly toward the distal end (12–14). The distribution of aminopeptidase N in the rat and rabbit intestines was different (9,12). Rats are the animal model most often used to evaluate intestinal absorption of peptide drugs. The distribution of aminopeptidase P, aminopeptidase W, and endopeptidase-2, carboxypeptidase P in the rat intestine, has not been reported. Further, caecal peptidase activities have not been compared with small intestinal activities. In this study, the distribution of seven major brush-border membrane peptidases, i.e. carboxypeptidase P, DPP IV, endopeptidase-24.11, ACE, aminopeptidase P, aminopeptidase W, and endopeptidase-2, along the rat intestine is determined.

MATERIAL AND METHOD

Material

Benzyloxycarbonyl-Pro-Ala, benzyloxycarbonyl-Pro, Ala, Gly-Pro-p-nitroanilide, Glu-Trp, insulin B-chain, benzoyl-Gly-His-Leu, [D-Ala^2, Leu^5]-enkephalinamide, p-nitrophenyl phosphate, hippuric acid, His-Leu, p-nitroaniline, 1,10-phenanthroline, phosphoramidon, $MnCl_2$, Tris, Tyr-D-Ala-Gly, and pentobarbital were obtained from Sigma Chemical Co. (St. Louis, MO). Arg-Pro-Pro was from Bachem Bioscience Inc. (Philadelphia). Cilastatin was a gift from Dr. Helmut Krop (Merck Sharp & Dohme Research Laboratories). Bovine γ-globulin and dye reagent for the protein assay were obtained from Bio-Rad Lab. (Richmond, CA). All other chemical reagents and buffer components were of analytic grade. Acetonitrile was of HPLC grade. All the chemicals were used as obtained.

Preparation of Brush-Border Membranes

Young male Sprague-Dawley rats, 300 g, were used as the animal model. The first 8 cm of rat small intestine was used as the duodenum, the next 35 cm as the jejunum, the last 25 cm proximal to the ileocecal junction as the ileum, and the segment between the jejunum and the ileum as the jejunoileal junction. The caecum was anatomically different from the small intestine and was clearly identified. Brush-border membranes were prepared using the method by Kessler et al., 1978 (15). Briefly, the intestinal mucosa of each segment was scraped off, suspended in a hypotonic solution (50 mM mannitol in the 2 mM pH 7.5 Hepes/Tris buffer), and homogenized using a blender and a glass/Teflon potter homogenizer. $CaCl_2$ (1 M) was added to the homogenates to achieve a final concentration of 10 mM and then the mixture was centrifuged at 3,000 g for 15 min. The supernatant was collected and centrifuged at 27,000 g for 30 min to obtain pellets. Pellets were resuspended in a 150 mM NaCl, 10 mM Tris/HCl (pH 6.8) buffer and homogenized with a glass/Teflon potter homogenizer. Then centrifugations at 3000 g and 27,000 g were repeated to obtain pure brush-border membranes. Protein concentrations were determined using the Bradford method and γ-globulin as the protein standard (16). Activity of alkaline phosphatase was determined to assess the purity of brush border membranes (9).

[1] College of Pharmacy, University of Minnesota, 308 Harvard St. S. E., Minneapolis, Minnesota 55455.

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On the average, brush-border alkaline phosphatase was 12.5 (±1.2) times enriched.

Enzyme assay

The incubation mixture (300 μl) consisted of 10 mM pH 6.8 Tris/HCl buffer, NaCl (150 mM), a substrate, and brush-border membrane protein (0.03–0.006 mg) or homogenate protein (0.1–2 mg). Benzyloxycarbonyl-Pro-Ala was used as the substrate for carboxypeptidase P, Arg-Pro-Pro for aminopeptidase P, Gly-Pro-p-nitroanilide for DPP IV, N-benzoyl-Gly-His-Leu for ACE, benzyloxycarbonyl-Pro-Ala for carboxypeptidase P, insulin B-chain for endopeptidase-2, and [D-Ala^2, Leu^5]-enkephalinamide for endopeptidase-24.11 (6,9,14,17–19). To assay aminopeptidase W activity, 0.05 mM cilastatin and 5 mM 1,10-phenanthroline were included in the reaction mixture to eliminate activities of aminopeptidase N, aminopeptidase A, and microsomal dipeptidase (7). Phosphoramidon of 0.02 mM was used to eliminate endopeptidase-24.11 activity so that endopeptidase-2 activity could be determined properly (6,20). Kenny et al., suggested that 1 μM phosphoramidon completely eliminated endopeptidase-24.11 activity against insulin B-chain (20). In this study, it was observed that an increase of phosphoramidon concentration from 0.002 mM to 0.06 mM did not significantly reduce activity degrading insulin B-chain; and there was no significant difference in insulin B-chain degradation in the presence of phosphoramidon of 0.002 to 0.06 mM. $MnCl_2$ of 3 mM was included in the assay of aminopeptidase P activity (19).

The final substrate concentration in the incubation mixture was 0.5 mM for Glu-Trp, 0.45 mM for Gly-Pro-p-nitroanilide, 0.018 mM for [D-Ala^2, Leu^5]-enkephalinamide, 0.1 mM for N-benzoyl-Gly-His-Leu, 0.2 mM for aminopeptidase P, 0.02 mM insulin B-chain, and 0.027 mM benzyloxycarbonyl-Pro-Ala. The concentration of individual substrate was below the reported concentrations or below or close to its Km (7,9,14,17,18,20,21). The concentration of each substrate was the same for both homogenate and brush-border membrane studies for all the segments. This will ensure that results can be compared since hydrolysis rate per g of protein depends on the substrate concentration. Activities of all the enzymes were studied at 37° C except those of DPP IV, ACE, and endopeptidase-24.11 at room temperature, because activities of these three enzymes were suggested to be high by others (9,13,14). Aliquots were taken periodically and 10% trichloroacetic acid or 0.1 N HCl was added to stop enzymatic degradation. For each study, there were three experimental and three control groups. In the control group, brush-border membrane or homogenate proteins in the buffer were denatured using trichloroacetic acid or deactivated using 0.1 N HCl before each substrate was added; and no hydrolysis was observed for all the substrates within the experimental time frame. For each experimental group, the initial hydrolysis rate was obtained from the first 10% to 20% of proteolysis. After being normalized for the protein concentration, average hydrolysis rate was obtained by averaging results from three experimental groups. Enzyme activity is expressed as the amount of substrate hydrolyzed per g of protein per min.

Assay methods

HPLC systems consisted of SIL autoinjector, LC-600 pump, SPD-6A UV spectrophotometric detector, and CR 601 recorder (Shimadzu Corporation, Kyoto, Japan), reversed-phase columns of a C_8 (Altex Ultrasphere-ODS, 5μ, 4.6 mm × 15 cm) (Beckman Instrument, Berkley, CA), a C_{18} (Vydac 5μ, 4.6 mm × 25 cm) (Vydac, Hesperia, CA), or a strong cation-exchange column (Partisil 10 SCX, 25 cm, Whatman, Maidstone, U.K.). HPLC methods, including mobile phase, column, flow rate, UV wavelength are listed in Table I.

RESULTS AND DISCUSSION

Activity of individual peptidase in the homogenate was 6 to 108 times lower than in the brush-border membrane, depending on whether each substrate was exclusively degraded by a specific brush-border membrane peptidase in the homogenate. Activities of endopeptidase-24.11, ACE, and DPP IV in the brush-border membrane were 9 to 108 times higher than in the homogenate; and for other peptidases, the

Table I. HPLC Methods

Compound	Column & flow rate	Mobile phase	λ(nm)	Retension time (min)
Glu-Trp	Cation-exchange 1.5 ml/min	pH2.5 0.005M $(NH_4)H_2PO_4$:ACN =90:10	220	10.3
Insulin B chain	C_{18} RP 0.8 ml/min	0.05%TFA:ACN =65:35	220	6
[D-Ala^2]-Leu-enkephalinamide	C_8 RP 1 ml/min	pH3 0.01M $(NH_4)H_2PO_4$:ACN =80:20	220	7.3
Gly-Pro-p-nitroanilide	C_8 RP 1 ml/min	pH2.5 0.02M NaH_2PO_4:ACN =85:15	280	14
Hippuryl-His-Leu	C_8 RP 1 ml/min	pH2.5 0.01M NaH_2PO_4:ACN =80:20	220	13
Arg-Pro-Pro	Cation-exchange 1 ml/min	pH2.5 0.05M $(NH_4)H_2PO_4$:ACN =90:10	220	8
N-CBZ-Pro-Ala	C_8 RP 1 ml/min	0.05%TFA:ACN =75:25	220	8

difference between the brush-border membrane and homogenate activities was lower.

Both endopeptidase-24.11 and endopeptidase-2 can hydrolyze insulin B-chain; however, the former is sensitive to inhibition by phosphoramidon while the latter is not (20). Enkephalins are resistant to endopeptidase-2 (20), therefore, degradation of [D-Ala^2, Leu^5]-enkephalinamide will only reflect activity of endopeptidase-24.11. Activities of endopeptidase-24.11 and endopeptidase-2 are summarized in Table II, the former having the rank order of jejunum > duodenum ~ jejunoileal junction ~ ileum > caecum while the latter ileum > jejunoileal junction ~ jejunum ~ duodenum > caecum. Endopeptidase-24.11 had the highest activity in the jejunum whereas endopeptidase-2 in the ileum. Since [D-Ala^2, Leu^5]-enkephalinamide is not substrate for endopeptidase-2, and endopeptidase-24.11 can not degrade insulin B-chain in the presence of 0.02 mM phosphoramidon, the results should truly project intestinal distribution of both enzymes. Both enzymes have similar substrate specificity, and the bonds cleaved are those involving a hydrophobic amino acid residue (21). In light of their distribution in the rat intestine complementing each other, proteins involving hydrophobic amino acid residues will be assuredly completely digested in the small intestine. The caecum had the lowest activities of both enzymes. Even though distribution of activities against insulin B-chain has been reported, it only represents distribution of total activities of neutral endopeptidases (13). Total activities increased to reach the highest in the jejunum and then decreased distally, agreeing with the observed longitudinal distribution of endopeptidase-24.11 but not that of endopeptidase-2. Likely, degradation of insulin B-chain was dominated by endopeptidase-24.11 activity.

Distribution profiles of ACE and carboxypeptidase P are summarized in Table II, activities of the former being jejunum > duodenum ~ jejunoileal junction > ileum > caecum and those of the latter ileum ~ caecum ~ jejunoileal junction ~ duodenum ~ jejunum. ACE releases dipeptides from the C-terminus of polypeptides with a carboxy-terminal proline while carboxypeptidase P releases the carboxy-terminal amino-acid residue from polypeptides with proline as the penultimate residue. ACE activity was the highest in the jejunum and carboxypeptidase P activity the highest in the ileum. Specifically degrading polypeptides with proline near or at the carboxy terminus, these two enzymes seemed to have complementary distribution profiles. However, in the distal region, the decline of ACE was more dramatic than the increase of carboxypeptidase P. Distribution of ACE activity in the rat intestine is similar to that reported by Yoshioka et al., (14). Distribution of carboxypeptidase P is similar in human, rat, and rabbit intestines with a rather flat distribution profile and the highest activity in the ileum (9,11). The caecum had the lowest activities of carboxypeptidase P and ACE.

Comparison of intestinal distribution of DPP IV and aminopeptidase P is summarized in Table II. Distribution of aminopeptidase P was jejunoileal junction ~ ileum > caecum ~ duodenum ~ jejunum, that of DPP IV being ileum ~ jejunum > jejunoileal junction > duodenum > caecum. The caecum had the lowest activities of DPP IV and aminopeptidase P. In summary, the distal intestine had high activities of these two enzymes. Distribution of DPP IV in the rat intestine is in general similar to that reported by Miura et al. (12). The difference is that Miura observed a slight decrease toward the distal end while this study suggested that activity remained high in the ileum (13). Without considering the caecum, DPP IV has a similar distribution in both rabbit and rat intestines (9). DPP IV activity is similar in the human, rabbit, and rat intestines with high activity at the distal end (9,11,13).

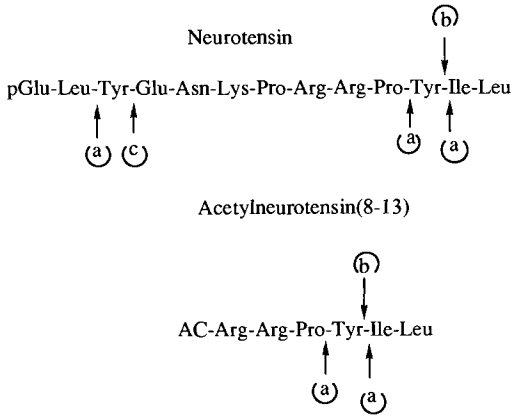
Aminopeptidase W had the distribution of ileum ~ jejunoileal junction > jejunum > duodenum ~ caecum in rat intestine (Table II). Aminopeptidase W degrades dipeptides and large peptides, and the former is degraded more quickly than the latter (8). This enzyme permits a wide range of amino-acid residues in the P1 position, but favors amino-acid residues with bulky side chains in the P1 position. Activities of each of carboxypeptidase P, aminopeptidase P, and aminopeptidase W in the caecum and duodenum are comparable.

The ileum has the highest or second highest activities of endopeptidase-2, DPP IV, carboxypeptidase P, aminopeptidase P, and aminopeptidase W while the jejunum has the highest activities of endopeptidase-24.11 and ACE. Regarding activities of endopeptidase-24.11 and ACE, the ileum has the lowest proteolytic activities. Even though the ileum may have lower pancreatic enzyme, its gut-wall proteolytic activities are high. In summary, the rabbit, rat, and human intestines have similar distribution of aminopeptidases N &

Table II. Distribution of proteolytic enzymes along the rat intestine.

	Enzyme activity (μmole/min g protein)				
	Duodenum	Jejunum	Jejunoileal junction	Ileum	Caecum
Endopeptidase-24.11[a]	1.2 (0.1)	3.2 (0.2)	1.2 (0.03)	1.1 (0.1)	0.41 (0.07)
Endopeptidase-2	8.9 (1.4)	9.3 (1.0)	9.6 (0.5)	11 (2)	2.7 (1.0)
ACE[a]	2.1 (0.1)	6.5 (0.5)	1.8 (0.6)	0.83 (0.2)	0.12 (0.02)
Carboxypeptidase P	0.06 (0.01)	0.06 (0.0)	0.07 (0.02)	0.11 (0.02)	0.09 (0.02)
DPP IV[a]	45 (1)	140 (15)	100 (7)	140 (10)	2.7 (0.9)
Aminopeptidase P	20 (4)	19 (3)	37 (8)	31 (6)	23 (6)
Aminopeptidase W	14 (2)	26 (4)	64 (9)	77 (7)	13 (3)

ACE and DPP IV represent angiotensin-converting enzyme and dipeptidyl peptidase IV, respectively.
N = 3, Mean ± S.D.
Incubation temperature was 37 °C except those marked by a superscript a- incubation was at room temperature.



a: endopeptidase-24.11
b: angiotensin converting enzyme
c: endopeptidase-2

Scheme I.

A, carboxypeptidase P, and DPP IV while the rat and rabbit intestines have similar distribution of ACE and endopeptidase-24.11, suggesting that distribution of brush-border peptidases in these three species may have a high degree of similarity.

According to the substrate specificity of brush-border peptidases and amino acid sequences of peptides, it is proposed, in Scheme I, that neurotensin is subjected to brush-border endopeptidase-24.11, ACE, and endopeptidase-2 while acetylneurotensin (8–13) is only subjected to the former two enzymes (5,23,24). Contribution of endopeptidase-2 is likely less than the other two. Hydrolysis of neurotensin and acetylneurotensin (8–13) was shown to be dominated by activities of endopeptidase-24.11 and ACE (22). Profiles of differential hydrolysis of neurotensin and acetylneurotensin (8–13) closely followed the distribution profiles of endopeptidase-24.11 and ACE: jejunum > duodenum ~ jejunoileal junction > ileum > caecum, implying that site-dependent degradation of polypeptides is attributed to differential distribution of peptidases. This sheds light on the influence of regional differences in the intestinal activities of peptidases on site-dependent stability, availability of polypeptides.

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