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Stability of neurotensin and acetylneurotensin 8–13 in brush-border membrane, cytosol, and homogenate of rat small intestine

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Abstract

The stability of neurotensin and acetylneurotensin 8–13 in small intestinal brush-border membrane, cytosol, and homogenate was studied. Proteolytic degradation of both compounds was pH dependent. At pH 7.5, rapid degradation was observed in brush-border membrane, cytosol, and homogenate. At pH 4.5, both compounds were stable in brush-border membrane, but were slowly degraded in mucosal homogenate of the proximal intestine. In general, acetylneurotensin 8–13 was more stable than neurotensin. Degradation of both compounds by $27000 \times g$ pellets, rich in brush-border membrane, was highest among the subcellular fractions. Studies using enzyme inhibitors suggested that both compounds were degraded by brush-border endopeptidase-24.11 and angiotensin-converting enzyme (ACE), and that endopeptidase-24.11 was the major enzyme degrading acetylneurotensin 8–13. At pH 4.5, degradation in homogenates was mainly due to serine proteases. In cytosol, degradation of both compounds was inhibited by a specific substrate of prolyl endopeptidase (EC 3.4.21.26) which is also called post-proline cleaving enzyme, an enzyme cleaving at the carboxyl end of proline. In summary, inhibitor studies suggest that both compounds are degraded at pH 7.5 by endopeptidase-24.11 and ACE in brush-border membrane, by activities of prolyl endopeptidase in cytosol, and at pH 4.5 by serine protease(s) in homogenate.

Keywords: Neurotensin; Acetylneurotensin 8–13; Endopeptidase-24.11; Angiotensin-converting enzyme; Post-proline cleaving enzyme; Intestinal mucosa; Proteolytic degradation

1. Introduction

Oral delivery is the long-term goal for peptide drugs. There are paracellular and transcellular pathways for intestinal absorption of large peptides; the former, selective for cations as well as excluding molecules with hydrodynamic radii greater than $4 \sim 8$ Å at the resting state (Pappenheimer et al., 1987), will be unlikely to allow penetration of polypeptides. The paracellular mechanism cannot explain the large differences in intestinal transport rates of cyclic peptides of oxytocin, vasopressin, and their analogues with similar molecular weights (Mol. Wt 1000) but varying hydrophobicity (Vilhardt et al., 1986). Cyclosporin (Mol. Wt 1203) has a better than 25% absorption in vivo (Lee, 1991), which is unlikely to be due to the paracellular pathway. Further, SMS 201–995, an octapeptide, was found to be absorbed transcellularly (Fricker et al., 1991). In-

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testinal absorption of insulin does induce decline of blood glucose levels (Kidron et al., 1982; Fujii et al., 1985). Although the mechanism of intestinal absorption of insulin is unknown, it is unlikely that insulin with a molecular weight of 6000 is absorbed paracellularly. From these observations, it is conceivable that transcellular absorption is not negligible. Intestinal absorption of potent peptide drugs containing more than 10 amino acids may not be as extensive as traditional drugs, but their oral low bioavailability, even if only 1%, can still achieve pharmacological efficacy. Low oral absorption of peptide drugs is acceptable as long as absorption is pharmacologically effective, but what is unacceptable is erratic absorption. Intracellular processing likely contributes to erratic absorption of bioactive peptides. Due to limited permeability of peptides and analogues through the cell membrane, intracellular degradation is particularly detrimental; for example, 50% of intracellular metabolism will reduce oral bioavailability from 1 to 0.5% if the fraction of dose absorbed is 1%. The change may not seem significant from the traditional point of view, since absorption is aldready low, but the resulting 50% decrease in pharmacological efficacy is tremendous. Therefore, to ensure reliable, acceptable oral bioavailability, it is necessary to inhibit intracellular degradation of peptide drugs, which may be due to random encounter or specific trafficking processes. Hence, an important step for achieving oral efficacy of peptide drugs is to understand how subcellular peptidases of intestinal enterocytes affect stability and availability of peptide drugs.

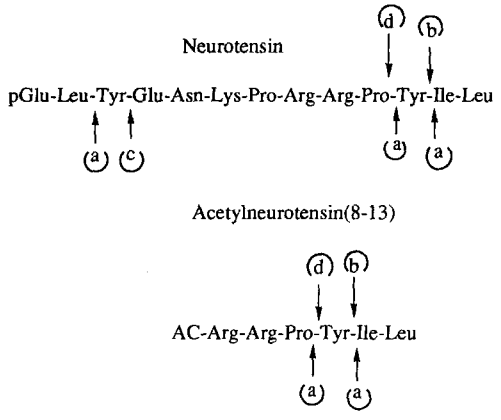
Our knowledge of peptidases of intestinal enterocytes all results from the research on digestion of oligopeptides, digestive products of proteins by pancreatic enzymes. Consequently, when it comes to oral delivery of bioactive peptides, our understanding of their proteolytic degradation in enterocytes is very limited. Though it is known that there are several exo- and endopeptidases in brush-border membranes capable of degrading polypeptides and di-/tripeptidases in cytosol (Bai and Amidon, 1992), it is unknown what proteolytic activities, in brush-border membrane and in cytosol, await bioactive peptides during absorption. In other words, it is unknown whether bioactive peptides larger than tetrapeptides are stable in cytosol and in other subcellular fractions. Since insulin B-chain containing 30 amino acids is a substrate for brush-border membrane endopeptidase-24.11 and endopeptidase-2 (Howell et al., 1992), it is expected that bioactive peptides of that size or smaller would be attacked by brush-border membrane peptidases. Degradation of DDAVP, a vasopressin analogue, by intestinal mucosal homogenate was suspected to be due to cytosolic post-proline cleaving enzyme (Lundin et al., 1989), suggesting that there are enzymes other than di-/tripeptidases in cytosol.

Neurotensin (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) stimulates secretion of pancreatic bicarbonate and inhibits gastric acid secretion (Blackburn et al., 1980). Moreover, it can be used to treat hypertension and hyperthermia (Fletcher et al., 1981). Acetylneurotensin 8–13 (Ac-Arg-Arg-Pro-Tyr-Ile-Leu) is the shortest analogue of neurotensin with its full pharmacological effects (Granier et al., 1982). According to the specificity of brush-border-membrane peptidases, these two peptides should be substrates for endopeptidase-24.11 and ACE since hydrophobic amino acids and the C-terminal leucine are in their structures (Turner, 1987; Bai and Amidon, 1992). Further, neurotensin will be a substrate for brush-border membrane endopeptidase-2 as well (Kenny and Ingram, 1987). The possible cleavage sites of these two compounds by peptidases in brush-border membrane are summarized in Scheme 1. These two compounds have proline residues in their sequences, hence they can be attacked by post-proline cleaving enzyme as well. These two compounds represent important model drugs for other peptides that also have the same cleavage signals for enterocyte peptidases.

2. Materials and methods

2.1. Materials

Dip-F (diisopropyl phosphofluoridate), E-64 (L-3-carboxy-*trans*-2,3-epoxypropionylleucylamido-



Scheme 1. a, endopeptidase-24.11; b, angiotensin converting enzyme; c, endopeptidase-2; d, prolyl endopeptidase (post-proline cleaving enzyume); AC, acetyl.

(4-guanidino)butane), DTT (dithiothreitol), EDTA, Tris base, Tris-HCl, neurotensin, acetyl-neurotensin 8–13, benzyloxycarbonyl-Gly-Pro-4-methylcoumaryl-7-amide, and pentobarbital were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine γ-globulin and dye reagent for the protein assay were purchased from Bio-Rad Lab. (Richmond, CA). Acetonitrile was of HPLC grade. All other chemical reagents and buffer components were of analytical grade.

2.2. Preparation of subcellular fractions

After the rat was killed by an overdose of pentobarbital sodium, its abdomen was opened by a mid-line incision and the small intestine was obtained. The small intestine was cut longitudinally to expose the mucosal surface and then mucosae were scraped off. Mucosal scrapping from the 12 rats was pooled and used for preparing subcellular fractions.

Each g of mucosal tissue was suspended in 10 ml of 50 mM Tris-HCl buffer (pH 7.5) and 125 mM NaCl and homogenized by a glass/Teflon Potter homogenizer in an ice bath with 10 strokes at a speed of 1140 rpm. $CaCl_2$ (1 M) was added to the mucosal homogenate to achieve a final concentration of 10 mM. Mucosal homogenate was subjected to a series of differential centrifugations at 4°C: 10 000 × g (20 min), 27 000 × g (30 min), and 100 000 × g (1 h) (Chowdhary et al., 1985; Bai, 1993). Protein concentrations were determined using the method of Bradford (1976) and γ-globulin as the protein standard. Enzyme markers for Golgi membrane (α-D-mannosidase), mitochondria (succinate dehydrogenase), and cytosol (lactate dehydrogenase) were assayed to confirm preparation of subcellular fractions (Miura et al., 1983; Moktari et al., 1986; Bai, 1993; Reim et al., 1993).

2.3. Preparation of brush-border membrane

A total of 12 rats were used to prepare brush-border membranes. The small intestine of each rat was divided into various segments. The first 8 cm of the rat small intestine was used 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, which is around 5–7 cm. The caecum was anatomically different from the small intestine and was clearly identified. Intestinal mucosa of each segment was scraped off; mucosal scrapings of each segment from 12 rats were pooled and suspended in a hypotonic solution (50 mM mannitol in the 2 mM pH 7.5 Hepes-Tris buffer), and then homogenized using a blender and a glass/Teflon potter homogenizer (Bai, 1993). Part of the mucosal homogenate was used for stability studies. Brush-border membrane was prepared from homogenate as reported previously (Kessler et al., 1978; Bai, 1993). Briefly, $CaCl_2$ (1 M) was added to the homogenate to achieve a final concentration of 10 mM and then the mixture was centrifuged at 3000 × 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 7.5) solution and homogenized with a glass/Teflon potter homogenizer. Then centrifugations at 3000 × g and 27 000 × g were repeated to obtain pure brush-border membrane. Protein concentrations were determined. Activity of alkaline phosphatase was determined to assess the purity of brush-border membranes (Bai, 1993).

2.4. Proteolysis

A 300 μl incubation mixture consisted of 50 mM Tris-HCl buffer (pH 7.5), 150 mM NaCl, 0.02 mM substrate, and 0.03–0.06 mg brush-border membrane proteins or 0.1–0.2 mg mucosal homogenate or subcellular proteins (Barelli et al., 1988; Bai, 1993). Proteolysis of neurotensin and acetylneurotensin 8–13 was performed at room temperature and reaction was terminated using 10% trichloroacetic acid. For each stability-evaluating experiment, there were three experimental and three control groups. In control, proteins in buffer were inactivated by 10% trichloroacetic acid and then mixed with substrates; during the experimental time frame, no hydrolysis of any peptide or analogue was observed.

2.5. Effects of enzyme inhibitors

Captopril and thiorphan are specific inhibitors of ACE and endopeptidase-24.11, respectively (Turner, 1987). Dip-F and E-64 are inhibitors of lysosomal serine and cysteine proteases, respectively (Barrett and McDonald, 1980; Kirschke and Barrett, 1987). These specific inhibitors were used to pinpoint the involvement of enterocyte peptidases in degradation of model peptides. The saturated solution of benzyloxycarbonyl-Gly-Pro-MCA, a specific substrate of cytosolic post-proline cleaving enzyme (Yoshimoto et al., 1982), in pH 7.5 Tris buffer was used to prepare the incubation mixture and its final concentration in the mixture was 50% of its solubility in Tris buffer. It was used to competitively inhibit degradation of neurotensin and acetylneurotensin 8–13 in cytosol. Experimental procedures were as described above. In experimental groups, the incubation mixture was the same as above except that an enzyme inhibitor was added; in control groups no inhibitor was added.

2.6. Assay

The HPLC system consisted of an SIL autoinjector, an LC-600 pump, an SPD-6A UV spectrophotometric detector, and a CR 601 recorder (Shimadzu Corp., Kyoto, Japan). Samples of enzyme substrates were assayed using a C8 Beckman column (5 μm, 4.6 mm × 15 cm) and mobile phases of 0.01 M NaH_2PO_4 (pH 3) and acetonitrile in a volume ratio either of 80:20 or 85:15. A UV wavelength of 220 nm was used. Neurotensin and acetylneurotensin 8–13 were assayed using a C18 Beckman column (5 μm, 4.6 mm × 15 cm) and the mobile phase of 0.005% trifluoroacetic acid and acetonitrile in a volume ratio of 75:25. Neurotensin eluted at 6 min at a flow rate of 1.5 ml/min, and acetylneurotensin 8–13 eluted at 6 min at a flow rate of 1 ml/min.

3. Results and discussion

3.1. The rationale of using rats as the animal model

The rat has been used as the animal model for evaluating drug absorption and nutrient digestion and absorption for more than two decades. The gastrointestinal physiology of the rat is similar to that of the human: brush-border membrane peptide and amino acid transporters exists in both species (Matthews and Payne, 1980; Bai and

Table 1
Characteristics of subcellular fractions

Enzyme	10000 × g pellets (rich in intracellular organelles)	27000 × g pellets (rich in brush-border)	100000 × g pellets (microsomes)	100000 × g supernatant (cytosol)
LDH	17 ± 0.4%	1.3 ± 0.09%	0.5 ± 0.1%	81 ± 4%
SH	82 ± 2%	5.6 ± 0.6%	2.3 ± 0.07%	10 ± 0.9%
α-D-Mannosidase	91 ± 3%	7 ± 0.8%	2.3 ± 0%	0 ± 0%

LDH and SH represent lactate dehydrogenase and succinate dehydrogenase, respectively. Each result was the average of three experiments (mean ± SD).

Amidon, 1992); the rat and human intestine has a similar distribution profile of each of aminopeptidase N and aminopeptidase A, carboxypeptidase, and dipeptidyl peptidase IV (Auricchio et al., 1978; Skobjerg et al., 1981; Sterich 1981; Miura et al., 1983); and all endo- and exopeptidases found on the brush-border membrane of rats are also present on the human intestinal apical membrane, suggested by the work using human colonic cell lines, Caco-2 and HT-29 (Bai and Amidon, 1992; Howell et al., 1992). Such overwhelming evidence of the striking physiological similarity between the human and rat intestine suggests that the rats would be an ideal model for evaluating the stability of neurotensin and acetylneurotensin 8–13 in subcellular fractions of enterocytes.

3.2. Characteristics of subcellular fractions and brush-border membrane

In the 27000 × *g* and 100000 × *g* pellets, lactate dehydrogenase (cytosolic marker) activities were negligible (Table 1), while the 100000 × *g* supernatant had the highest specific and total lactate dehydrogenase activities. The 10000 × *g* pellet had the highest specific activities of succinate dehydrogenase (mitochondria marker) and α-D-mannosidase (Golgi membrane marker). Moreover, this fraction had the highest percentages of both enzyme activities. Activities of α-D-mannosidase in the 100000 × *g* pellet and cytosol were negligible. Ratios of alkaline phosphatase activity in individual subcellular fractions prepared from the whole intestine to that in homogenate were 0.8 for the 10000 × *g* pellet, 12.6 for 27000 × *g* pellet, 6.8 for 100000 × *g* pellet, and 0.1 for the 100000 × *g* supernatant. This suggests that the 27000 × *g* pellet were rich in brush-border membrane. Overall, the 10000 × *g* pellet were rich in mitochondria and Golgi apparatus, the 27000 × *g* pellet were rich in brush-border membrane, and the 100000 × *g* supernatant was rich in cytosol. The brush-border membrane was 14-fold purified as suggested by the enrichment of alkaline phosphatase activity in this preparation.

3.3. Proteolytic degradation by subcellular fractions

Subcellular proteolytic degradation of neurotensin and acetylneurotensin 8–13 was studied at pH 7.5, since the physiological pH of cytoplasm is 7.2 and the same pH was use for all subcellular fractions to ensure that comparison of activity would be adequate. Further, though the microclimate pH is slightly acidic, brush-border membrane peptidases have high activity at high pH; studying brush-border degradation at pH 7.5 would make detection of degradation easier.

In terms of proteolytic activities that degraded neurotensin and acetylneurotensin 8–13, the 27000 × *g* pellet had the highest degradative activity per g of protein, followed by 100000 × *g* pellet, then by 10000 × *g* pellet, and by cytosol (Table 2). When the total amount of proteins in each subcellular fraction was considered, both the 10000 × *g* pellet and cytosol had comparable activities that degraded acetylneurotensin 8–13 and neurotensin. Apparently, the brush-border membrane is the major metabolic barrier that will limit absorption of these two peptides.

Table 2
Small intestinal subcellular distribution of proteolytic activities degrading neurotensin and its analogue

Compounds	10000 × *g* pellet (rich in intracellular organelles)	27000 × *g* pellet (rich in brush-border)	100000 × *g* pellet (microsomes)	100000 × *g* supernatant (cytosol)
Acetyl-NT (8–13)	0.67 (0.01)	2.23 (0.03)	1.82 (0.06)	0.68 (0.05)
NT	0.76 (0.02)	4.38 (0.31)	3.29 (0.07)	0.85 (0.02)

Results (μmol/min per g protein) represent mean ± SD ($n = 3$). Acetyl-NT (8–13) and NT respresent acetylneurotensin 8–13 and neurotensin, respectively. Adapted from Bai (1994).

Table 3
Effects of pH on acetylneurotensin 8–13 and neurotensin hydrolysis by rat intestinal brush border membranes

Intestinal segment	Acetyl neurotensin 8–13		Neurotensin	
	pH 7.5	pH 4.5	pH 7.5	pH 4.5
Duodenum	2.6 ± 0.2 [a]	0 [b]	5.3 ± 0.2 [a]	0 [b]
Jejunum	5.5 ± 0.3 [a]	0 [b]	9.3 ± 0.1 [a]	0 [b]
Jenuoileal junction	2.7 ± 0.3 [a]	0 [b]	5.0 ± 0.2 [a]	0 [b]
Ileum	1.5 ± 0.1 [a]	0 [b]	2.5 ± 0.2 [a]	0 [b]
Caecum	0.3 ± 0.1 [a]	0 [b]	0.7 ± 0.0 [a]	0 [b]

Hydrolysis rate expressed as μmol/min per g protein. Mean ± SE ($n = 3$).
[a] Adapted from Bai and Chang (1993).
[b] No significant hydrolysis was observed after 90 min incubation at a protein concentration of 0.25 mg/ml at room temperature.

Table 4
Effects of pH on acetylneurotensin 8–13 and neurotensin hydrolysis by rat intestinal homogenates

Intestinal segment	Acetyl neurotensin 8–13		Neurotensin	
	pH 7.5	pH 4.5	pH 7.5	pH 4.5
Duodenum	3.1 ± 0.4 [a]	0.2 ± 0.0 [a]	5.4 ± 0.2 [a]	0.3 ± 0.0 [a]
Jejunum	0.6 ± 0.0 [a]	0 [b]	1.3 ± 0.2 [a]	0.1 ± 0.0 [a]
Jejunoileal junction	1.0 ± 0.4 [a]	0 [b]	0.9 ± 0.0 [a]	0 [b]
Ileum	0.8 ± 0.4 [a]	0 [b]	0.6 ± 0.0 [a]	0 [b]
Caecum	0.3 ± 0.1 [a]	0 [b]	0.5 ± 0.1 [a]	0 [b]

[a] Hydrolysis rate expressed as μmol/min per protein. Mean ± SE ($n = 3$).
[b] No signficant hydrolysis was observed after 90 min incubation at a protein concentration of 0.25 mg/ml at room temperature.

3.4. Site dependency of proteolytic degradation

Further studies using mucosal homogenate and purified brush-border membrane were performed in order to delineate whether degradation of neurotensin and acetylneurotensin 8–13 within the intestine showed any site dependency. The hydrolysis of neurotensin and acetylneurotensin 8–13 by brush-border membrane at pH 7.5 is summarized in Table 3. These two compounds had similar profiles of differential hydrolysis within the intestine and showed the order of jejunum > duodenum > jejunoileal junction > ileum > caecum. The lack of hydrolysis of these two compounds at pH 4.5 is consistent with the low activities of brush-border membrane enzymes at acidic pH. Degradation of neurotensin and acetylneurotensin 8–13 by homogenate is shown in Table 4. In mucosal homogenate, neurotensin hydrolysis at pH 7.5 showed the order of duodenum > jejunoileal junction > ileum > jejunum > caecum while that of acetylneurotensin 8–13 was duodenum > jejunum > jejunoileal junction > ileum > caecum. Profiles of differential hydrolysis by mucosal homogenate were different from those by brush-border membrane for both compounds. At pH 4.5, acetylneurotensin 8–13 was slowly degraded by duodenal mucosal homogenate, and was stable in mucosal homogenate of other segments. Although at pH 4.5 neurotensin was slowly degraded by duodenal and jejunal mucosal ho-

Table 5
Inhibitor effects on acetylneurotensin 8–13 and neurotensin proteolysis by rat jejunum at room temperature

	Acetyl neurotensin 8–13 (duodenal homogenate)		Neurotensin (jejunal homogenate)	
	Homogenate	BBM [c]	Homogenate	BBM [c]
Blank	0.9 ± 0.0	5.2 ± 0.6	1.1 ± 0.3	10.6 ± 0.6
Captopril (30 μM)	0.5 ± 0.0 ($p < 0.005$)	3.5 ± 0.0 ($p < 0.025$)	0.7 ± 0.1	5.7 ± 0.5 ($p < 0.01$)
Thiorphan (30 μM)	0.5 ± 0.0 ($p < 0.005$)	0 [b] ($p < 0.005$)	0.5 ± 0.1	4.8 ± 0.5 ($p < 0.01$)

[a] Hydrolysis rate expressed as μmol/min per g protein.
[b] No significant proteolysis was observed within 90 min.
[c] BBM, brush border membrane. BBM data adapted from Bai (1993).
[d] pH was 7.5. Mean ± SE ($n = 3$).

mogenate, it resisted peptidases in other mucosal homogenate.

At pH 4.5, proteolytic activities of homogenate will mainly come from lysosomal enzymes, since cytosolic and brush-border membrane peptidases are active at alkaline pH and lysosomal enzymes are active at acidic pH (Kirschke and Barrett, 1987; Bai and Amidon, 1992). It is likely that the duodenum possessed higher lysosomal proteolytic activities instead of having contamination of pancreatic enzymes since at pH 4.5 pancreatic peptidases exhibit negligible activities (Boyer, 1971; Kirschke and Barrett, 1987).

3.5. Degradation by peptidases in brush-border membrane

As listed in Table 5, captopril and thiorphan, specific inhibitors of ACE and endopeptidase-24.11, respectively (Turner, 1987), significantly inhibited hydrolysis of acetylneurotensin 8–13 by jejunal homogenate and brush-border membrane. These results suggested that both endopeptidase-24.11 and ACE of enterocytes were involved in initiating degradation of acetylneurotensin 8–13, and complete inhibition by thiorphan suggested that endopeptidase-24.11 was the major enzyme. Thiorphan and captopril also significantly inhibited hydrolysis of neurotensin by jejunal brush-border membrane. Though these two inhibitors reduced degradation of neurotensin in mucosal homogenate, the extent of inhibition did not show any statistical significance due to high data variability. Incomplete inhibition by either thiorphan or captopril suggested neither endopeptidase-24.11 nor ACE was dominant in degrading neurotensin in mucosal homogenate. It is likely that endopeptidase-24.11 was more important in degrading neurotensin since thiorphan had a slightly greater inhibitory effect than captopril.

It has been shown that endopeptidase-2 has weak activity degrading neurotensin and this enzyme has a substrate specificity similar to that of endopeptidase-24.11 (Kenny and Ingram, 1987). Since there are no specific substrates or inhibitors of enodopeptidase-2, studies using competitive inhibitors were not pursued to verify the involvement of endopeptidase-2.

Table 6
Inhibitor effects on acetylneurotensin 8–13 and neurotensin proteolysis at pH 7.5 by rat jejunum at room temperature

	Acetyl neurotensin 8–13 (duodenal homogenate)	Neurotensin (jejunal homogenate)
Blank	0.19 ± 0.00	0.15 ± 0.04
Dip-F [b] (30 mM)	0 [c]	0 [c]
E-64 [b] (30 μM)	0.20 ± 0.00	0.15 ± 0.10

[a] Hydrolysis rate expressed as μmol/min per g protein. Mean $\pm$SE ($n = 3$). Incubation was at pH 7.5.
[b] Dip-F (diisopropyl phosphofluoridate) is a serine proteinase inhibitor; E-64 is a cysteine proteinase inhibitor.
[c] No significant proteolysis was observed within 1 h incubation.

3.6. Degradation by peptidases in mucosal homogenates at pH 4.5

Dip-F (a serine protease inhibitor) completely abolished degradation of both compounds but E-64 (a cysteine protease inhibitor) had no inhibitory effect (Table 6). Apparently, lysosomal serine proteases were the major enzymes that metabolized these compounds in mucosal homogenate at pH 4.5 while cysteine proteases had no degradative activities. Since there are many serine proteases in lysosomes, it is unknown which will degrade neurotensin and acetylneurotensin 8–13. The significance of lysosomal peptidases in limiting absorption of these two compounds is unknown, since it is unclear whether they will be translocated to lysosomes during transport through enterocytes.

3.7. Degradation in cytosol

The effects of benzyloxycarbonyl-Gly-Pro-MCA on degradation of neurotensin and acetylneurotensin 8–13 in cytosol at pH 7.5 are summarized in Table 7. Benzyloxycarbonyl-Gly-Pro-MCA, a specific post-proline cleaving enzyme, significantly inhibited cytosolic degradation of neurotensin and acetylneurotensin 8–13. Though post-proline cleaving enzyme has not been demonstrated to exist in enterocytes, its existence has been suggested by the observation of degradation of DDAVP and inhibition of DDAVP

Table 7
Effects of CBZ-Gly-Pro-MCA on acetylneurotensin 8–13 and neurotensin by cytosol of enterocytes

	Acetylneurotensin 8–13	Neurotensin
Blank	0.86 ± 0.02	1.18 ± 0.02
CBZ-Gly-Pro-MCA	0.18 (0.01) ($p < 0.01$)	0.76 (0.04) ($p < 0.05$)

[a] Hydrolysis rate expressed as μmolmin per protein.
[b] Incubation was at pH 7.5 and room temperature.
[c] The final concentration of CBZ-Gly-Pro-MCA (benzyloxycarbonyl-Gly-Pro-4-methyl-coumaryl-7-amide) in the incubation mixture was 50% of its solubility in pH 7.5 Tris buffer.

degradation by oxytocin and analogues in intestinal homogenate (Lundin et al., 1989). The results suggested that these two compounds were likely attacked by activity of post-proline cleaving enzyme in cytosol.

4. Conclusion

The brush-border membrane of enterocytes is the major metabolic barrier that will limit intact absorption of neurotensin and acetylnerotensin 8–13. Further, endopeptidase-24.11 and ACE are two key peptidases that are involved in their degradation by brush-border membranes. Cytosolic degradation of these two compounds is not negligible and is likely due to activity of post-proline cleaving enzyme. Although much more stable than neurotensin, acetylneurotensin 8–13 is degraded in brush-border membrane, cytosol, and homogenate. In order to promote therapeutic usefulness of neurotensin and acetylneurotensin 8–13, design of stable analogues that resist brush-border and cytosol peptidases should be pursued.

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