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Possible degradative process of cholecystokinin analogs in rabbit jejunum brush-border membrane vesicles

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Abstract

Our recent work on the intestinal metabolism and absorption of cholecystokinin analogs, sulfated C-terminal octapeptide (CCK8; Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe(NH_2) = $DY_{SO_3}HMGWMDF_{NH_2}$) and tetrapeptide (CCK4; Trp-Met-Asp-Phe(NH_2) = $WMDF_{NH_2}$), was extended to investigate the degradative process of these analogs using rabbit jejunum brush-border membrane vesicles and to find a better enzyme-inhibitor system for intestinal absorption of peptide drugs. Various enzyme inhibitors and a lower pH buffer were applied to discover the major enzyme(s) involved in each process. Metabolic pathways showing degradative processes were proposed for both analogs. The major cleavage site occurs at the W^1-M^2 for CCK4. At least three metabolic pathways occur independently for CCK8 and appear at peptides bonds between G^4-W^5, M^6-D^7, and D^7-$F^8_{NH_2}$. Many different enzymes of aminopeptidase, endopeptidase, angiotensin-converting enzyme, metalloenzyme, and others were involved in each process. Identification of more specific yet safe enzyme inhibitors and co-administration of various these inhibitors may lead to further enhancement in intestinal peptide absorption when administered orally.

Keywords: Degradative process; CCK analogs; Metabolism inhibition

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Introduction

Recently a successful strategy for oral peptide delivery employing a selective enzyme inhibitor and a specific absorption site was reported [1,2]. Following the co-administration of amastatin and thiorphan, which are known to be a selective enzyme inhibitor for aminopeptidase and endopeptidase, respectively, the absorption of sulfated cholecystokinin octapeptide (CCK8; Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe(NH_2) = $DY_{SO3H}MGWMDF_{NH_2}$) in the ileum was improved 3.5 times while that for [D-ala^2, D-leu^5] enkephalin (YdAGFdL) about 5 times. This absorption enhancement was significant considering the conventional understanding of the negligible oral absorption for large peptides [3]. These results imply that the intestinal metabolism is a major stumbling block for the oral absorption of a large peptide, CCK8. The greater than 1 dimensionless wall permeabilities (P*w) of CCK8 and enkephalin analogs were obtained in a rat jejunum single-pass perfusion system [1,4] and no permeability-limited absorption was seen with the compounds of the P*w values > 1 [5]. One could thus have a desire to improve the absolute bioavaliability of CCK8 (19%) and YdAGFdL (8.8%) by further controlling the metabolism [1,2]. The metabolites of CCK8 and CCK4, the C-terminal tetra peptide of CCK8 which is fully biologically active [6], in brush-border membrane vesicle (BBMV) systems were identified [1]. Further mechanistic analysis of CCK8 intestinal metabolism, as shown by Su and Amidon with Peptide-T [7], could add some insight into the peptide oral delivery strategies.

CCK8 is cleaved into several fragments by different enzymes and the degradation of various CCK analogs in the brain, stomach, and liver are reported elsewhere [8–22]. The peptide bonds between G^4-W^5, W^5-M^6, and D^7-$F_{NH_2}^8$ are hydrolyzed by enkephalinase (EC 3.4.24.11, also known to be neutral endopeptidase) [8–14]. Two endopeptidases related to the cleavage of M^3-G^4 and M^6-D^7 bonds include a serine peptidase and a thiol peptidase [13–15]. The cleavage between the W^5-M^6 becomes a minor cleavage site when sulfated CCK8 is a substrate [12]. The degradation of CCK4 (Trp-Met-Asp-Phe(NH_2) = $WMDF_{NH_2}$) in the brain has also been studied by several research groups [10,11,22]. An aminopeptidase can cleave the W^1-M^2 bond, whose enzymatic activity can be inhibited by puromycin and bestatin [8,10]. Enkephalinase (EC 3.4.24.11) can also hydrolyze the peptide bond between D^3-$F_{NH_2}^4$ of CCK4 [11,14]. One study done on the degradation of CCK8 in the gastric gland shows that the membrane-bound enkephalinase (EC 3.4.24.11) may be involved in the degradation of CCK8 at G^4-W^5 and D^7-$F_{NH_2}^8$ bonds in the stomach [18]. This enzyme can also hydrolyze CCK4 at the D^3-$F_{NH_2}^4$ bond [20]. Angiotensin-converting enzyme (ACE), a peptidyl-dipeptidase, can hydrolyze CCK8 to release the C-terminal dipeptide D-F_{NH_2} even though the C-terminal residue does not possess a free carboxyl group [21]. There is amidase activity that can release NH_3 from CCK4 and it is distributed in the liver and small intestinal mucus [22].

This variety in participating enzymes and cleavage sites of CCK analogs observed by various research groups could be in part due to differences in methods and species used. Less information is available regarding the hydrolysis of these analogs and degradation profiles of these metabolites in the intestine. Identifying all participating enzymes and characterizing the degradation kinetics of each cleavage might give a better selection of enzyme inhibitors to further improve the intestinal absorption of peptide drugs when administered orally. The purpose of this study was to elucidate the degradative process of both CCK8 and CCK4 in BBMV, and then to discover the major enzyme(s) that hydrolyze these peptides in the brush-border membrane for further development of the oral delivery strategy for peptide drugs.

Materials and methods

Materials

Sulfated cholecystokinin-octapeptide (CCK8) and cholecystokinin-tetra peptide (CCK4) were purchased from Bachem Inc. (Torrance, CA). Amastatin ([(2S,3R)-3 amino 2-hydroxy-5-methyl hexanolyl] L-Val-L-Val-L-Asp), thiorphan (DL-3-mercapto-2-benzylpropanoylglycine), trifluoroacetic acid (TFA), hepes (N-[2-hydroxyethyl]piperazine-N′ [2-ethanesulfonic acid]), trizma (Tris[hydroxymethyl]aminomethane), mes (2-[N-morpholino]ethanesulfonic acid), alkaline phosphatase, p-nitrophenyl phosphate, and EDTA were obtained from Sigma Chemical Co. (St. Louis, MO). Five-methyl-5-phenylhydantoin was obtained from Aldrich Chemical Co. (Milwaukee, WI) and used for an internal standard. The Bio-Rad protein assay kit and bovine serum albumin were purchased from Bio-Rad (Richmond, CA). All other chemicals were either analytical or a HPLC grade. Deionized water was used throughout the study. Male New Zealand white rabbits weighing 1.8 ~ 2.2 kg were used to prepare brush-border membrane vesicles.

HPLC assay and buffer preparation

HPLC System I in the previous paper [1] was employed for the current study. To investigate pH effects on the degradation of these peptides in rabbit jejunum brush-border membrane vesicles (BBMV), different pH buffers were prepared as follows: 10 mM acetate/10 mM KOH buffer for pH at the range between 4 and 5, 10 mM mes/10 mM hepes for range of 5.5 to 6, 10 mM hepes/10 mM tris at the range of 6.5 to 7, and 10 mM tris/10 mM hepes for pH greater than 8.

Preparation of BBMV

Brush-border membrane vesicles from rabbit jejunum were prepared by the Ca precipitation method [23]. The purified BBMV were suspended in 25 mM hepes/tris buffer (pH 7.0) containing 100 mM mannitol and 100 mM KCl. The total protein concentration of the vesicles was measured by the Bradford method using a Bio-Rad protein assay kit and bovine serum albumin as standard [24]. The enrichment of vesicles was assessed by using alkaline-phosphatase as a marker, for which p-nitrophenyl phosphate was used as a substrate [25]. Subsequently, the vesicles were divided into several portions and stored in a freezer (− 80 °C) until used.

Metabolic study in BBMV

The detailed procedures described in our previous study [1,7] were used for the metabolism study in BBMV. To investigate the inhibitory effect of various enzyme inhibitors, 10 μl of vesicle and 10 μl of inhibitor solutions were pre-incubated at 25 °C for 20 minutes [26] and the BBMV was treated with the same procedure described elsewhere [1,7]. Another study was also performed in which a low pH (pH 4.0) was used under the same conditions. Inhibitors used were amastatin (aminopeptidase inhibitor), thiorphan (endopeptidase or enkephalinase inhibitor), captopril (ACE inhibitor), and EDTA (metalloenzyme inhibitor). Inhibitor concentrations were 100 mM for captopril and EDTA, 100 μM for amastatin and thiorphan in CCK8 study, and 10 μM of

amastatin and thiorphan in CCK4 study. Inhibitor concentrations were chosen to be able to measure the metabolites formed and to observe the metabolic time course. Less inhibitor concentrations were required for CCK4 suggesting less cleavage sites for each specific inhibitor.

Metabolites of the CCK analogs were identified using the HPLC and amino-acid analysis method [1] and formation of each metabolite at various time points over 60 min was evaluated as compared to the intact CCK4 and CCK8 in the absence and presence of various inhibitor treatments. The amount of metabolites was measured as a fraction relative (percent) to CCK8 or CCK4 assuming the same molar absorptivity (ε) of the parent drugs and its metabolites at the 210 nm UV wavelength. Therefore, the qualitative comparison of metabolites as to their parent compounds would be more appropriate for the metabolism study.

Results

HPLC assay method

The HPLC system employed for the current metabolism studies was found to be adequate to separate all metabolites and parent compounds as shown in the chromatograms of the previous paper [1]. The metabolites revealed from the CCK4 ($WMDF_{NH_2}$) metabolism study were M, F_{NH_2}, DF_{NH_2}, W, and MDF_{NH_2}, and from the CCK8 ($DY_{SO_3}HMGWMDF_{NH_2}$) metabolism study, $DY_{SO_3}HMG$, F_{NH_2}, $DY_{SO_3}HMGW$, W, MDF_{NH_2}, GWMD, $DY_{SO_3}HMGWM$, and $DY_{SO_3}HMGWMD$. Although both $DY_{SO_3}HMG$ and F_{NH_2}; $DY_{SO_3}HMGW$ and W have the same retention time in this HPLC gradient system (1), the data analysis was not interfered. Presence of various enzyme inhibitors did not also interfere with the metabolite peaks.

Stability

Both CCK8 and CCK4 were relatively stable in pH 4 either at room temperature for 24 hrs or in freezer-storage for 10 days or longer. CCK4 was lost in a higher pH buffer (pH 7.5 and pH 8) than in either an acidic or a neutral pH buffer, and that very little was degraded in an acidic solution. A similar trend was found for CCK8. The degradation of CCK8 in jejunal BBMV was not pH-dependent between pH 5 and pH 8, which correlates with the study by McDermott [14]. Therefore, a pH 7.0 buffer was chosen for the metabolic study of both CCK4 and CCK8 in BBMV.

CCK4 and CCK8 degradative process

Fig. 1a and 2a taken from the previous study [1] show the bi- or multi-phasic degradative curves of CCK4 and CCK8 in the absence and presence of various inhibitors and also in a pH 4 solution. CCK4 was apparently degraded faster than CCK8 showing more than 50% degradation within 2 min (Fig. 1a) while the half-disappearance time was approximately 5 min for CCK8 (Fig. 2a). The % inhibition of the inhibitors on degradation of CCK4 and CCK8 for 60 min in the BBMV is summarized in Table 1. More than 80% inhibition in metabolism was observed with amastatin for CCK4 while those

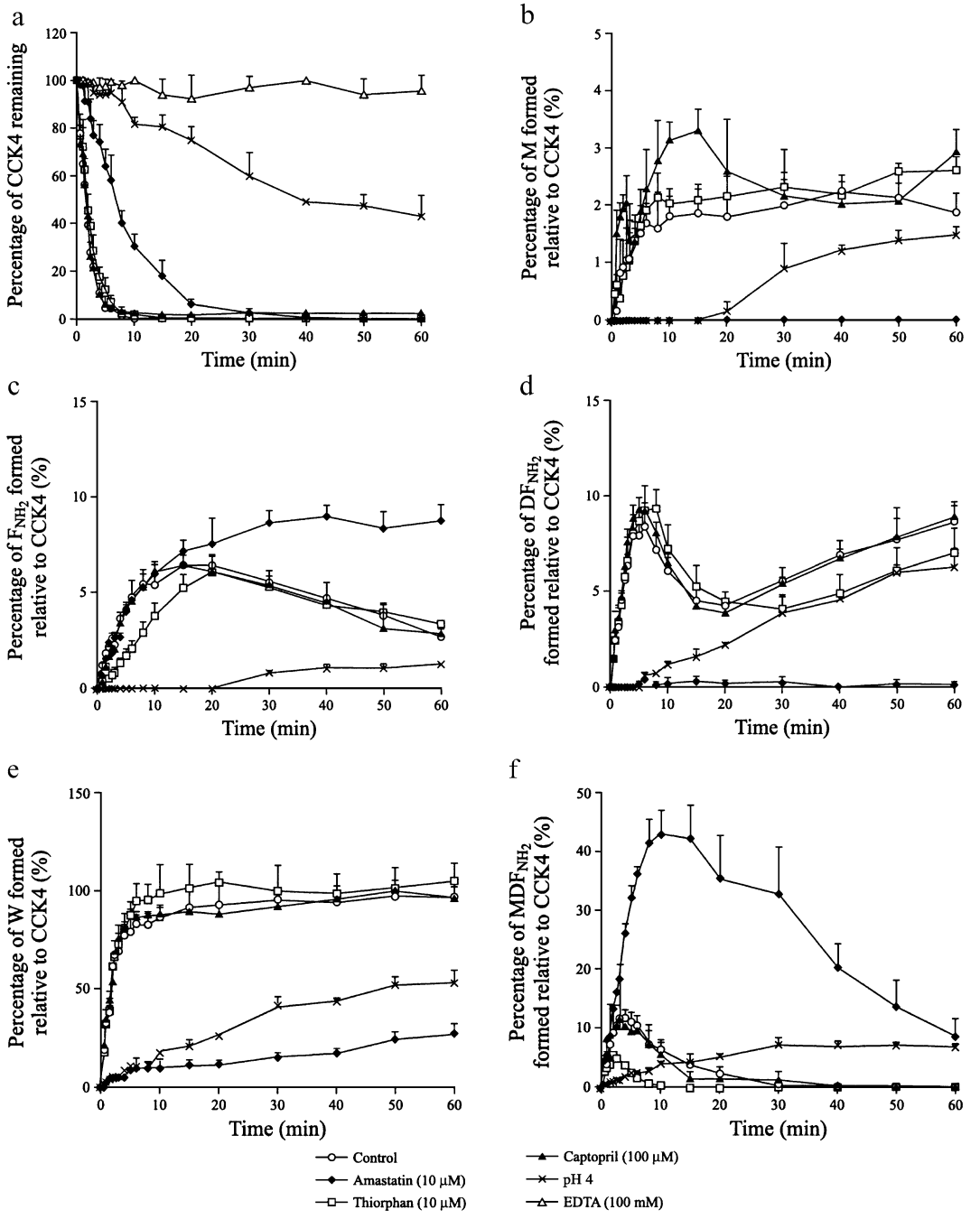


Fig. 1. Degradation curve of CCK4 (a), and percent of metabolites formed relative to CCK4 (b through f) in the absence and presence of various inhibitors and a lower pH in the rabbit jejunum BBMV. Values are mean (n = 3 ~ 5) ± SE. Fig. 1a is taken from the Ref. [1] with the authors' permission.

observed with thiorphan for CCK8. The pH 4 and EDTA treatments showed a marked inhibitory effect for both analogs.

Time-courses of CCK4 metabolites in the presence of various enzyme inhibitors

To identify participating enzymes in each pathway, the effects of enzyme inhibitors on the degradation of CCK4 and the formation of metabolites were evaluated and the results are shown in Fig. 1b through Fig. 1f. The formation of M (Fig. 1b) increased during the first 8 minutes and then stayed constant. PH 4 solution inhibited the formation of M up to 20 ~ 30 min and amastatin can completely inhibit its

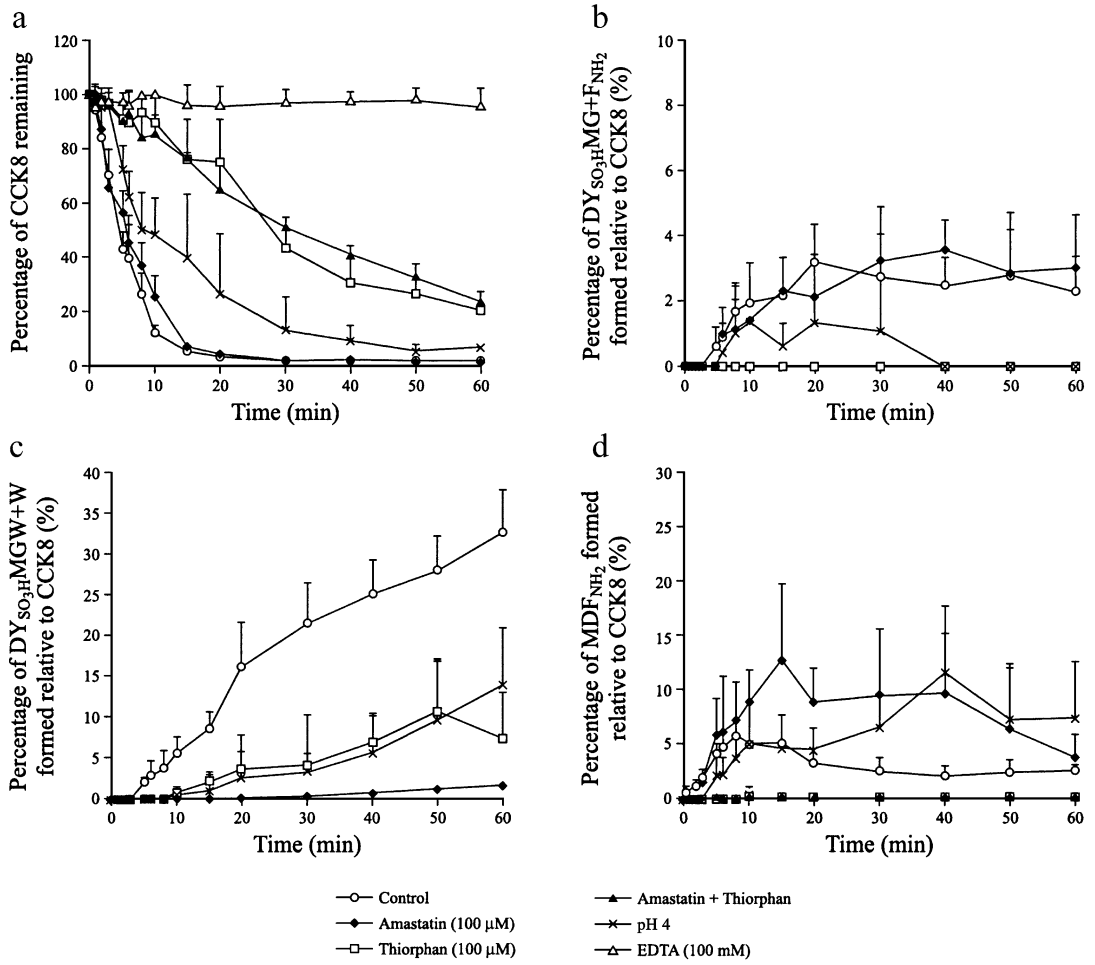


Fig. 2. Degradation curve of CCK8 (a), and percent of metabolites formed relative to CCK8 (b through g) in the absence and presence of various inhibitors and a lower pH in the rabbit jejunum BBMV. Values are mean (n = 3 ~ 5) ± SE. Fig. 2a is taken from the Ref. [1] with the authors' permission.

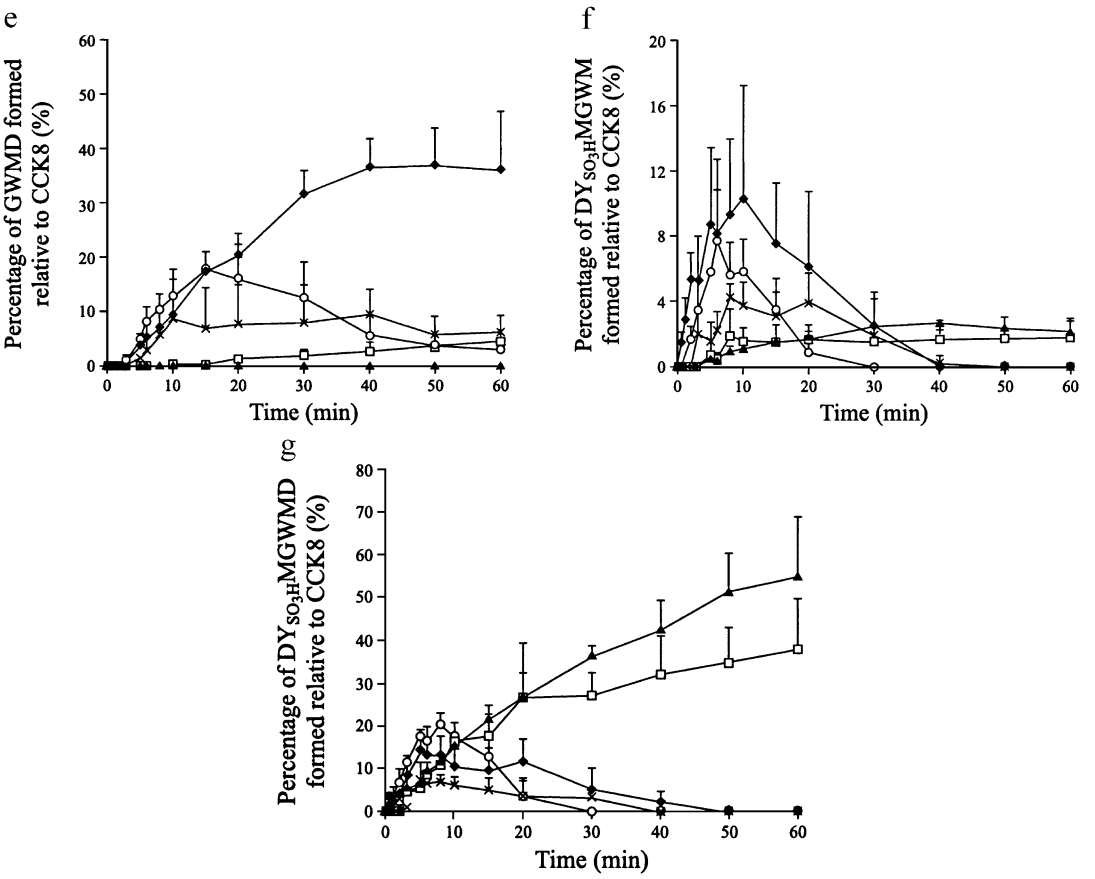


Fig. 2 (*continued*).

formation. Both thiorphan and captopril did not show marked inhibitory effects. The formation of F_{NH_2} (Fig. 1c) increased gradually during the initial 15 minutes, and then decreased. One could explain this phenomenon by BBMV uptake or possible further metabolism of F_{NH_2} by other enzymes in BBMV. The addition of amastatin improved its production while a lower pH buffer reduced its formation. The generation of DF_{NH_2} (Fig. 1d) increased rapidly for 6 minutes, then decreased slowly, then rose gradually. Amastatin could inhibit its formation to a great extent at a concentration of 10 μM; while a lower pH buffer reduced its formation. The formation of W (Fig. 1e) showed a similar trend as that of M. Neither thiorphan nor captopril showed marked effects on its generation. However, amastatin and pH 4 buffer could suppress its formation to a great extent. The amount of MDF_{NH_2} (Fig. 1f) increased rapidly for the first 4 minutes, then decreased afterwards, and none could be detected after 30 minutes. Amastatin showed marked effects where three and a half times as much of this metabolite was detected after 10 minutes of incubation and then decreased slowly. Scheme 1 summarizes the hypothesized metabolic pathway of CCK4 according to the time course studies of the intact peptide and each metabolite as depicted in Fig. 1a–1f.

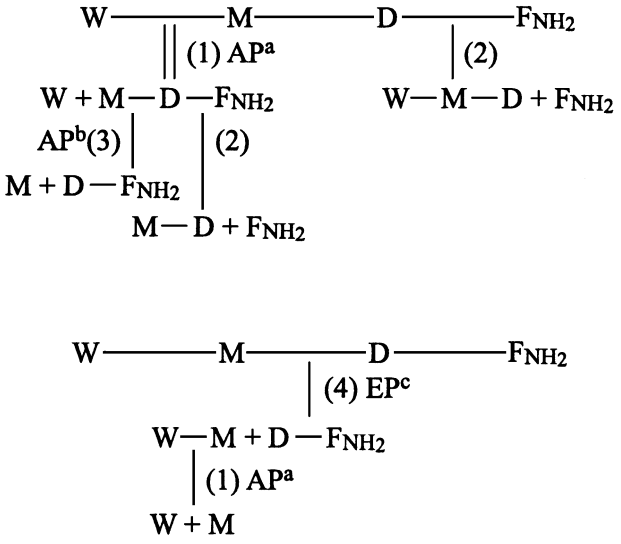
Table 1
Percent inhibition of CCK4 and CCK8 metabolism under various treatments in the rabbit jejunum BBMV

Inhibitor	% inhibition	
	CCK4	CCK8
None	0	0
Amastatin*	84	25
Thiorphan*	26	85
Captopril†	23	–
pH 4	98	72
Amastatin + Thiorphan**	–	88
EDTA††	~ 100	~ 100

* The concentrations of 10 μM and 100 μM were used in the BBMV study of CCK4 and CCK8, respectively.
** A concentration of 100 μM of each inhibitors was added.
† A concentration of 100 μM was applied.
†† A concentration of 100 mM was added.

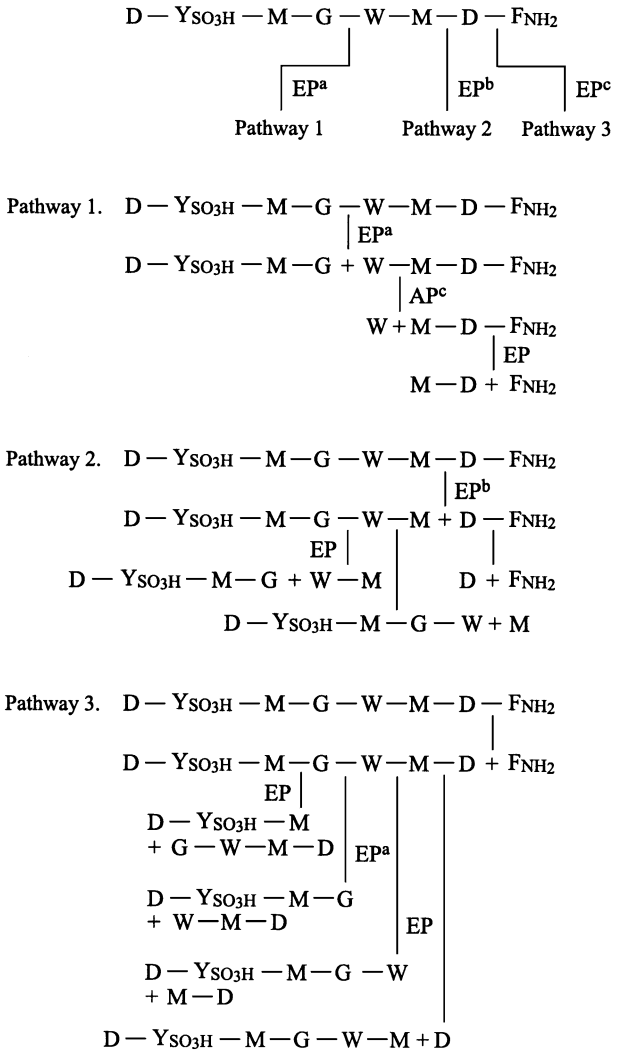
Time-courses of CCK8 metabolites in the presence of various enzyme inhibitors

The same methodology was applied to investigate the metabolic pathways of CCK8 in rabbit jejunal BBMV. The concentration changes of each metabolite relative to CCK8 in the time-dependent study are drawn in Fig. 2b through Fig. 2g. The formation of both $DY_{SO_3}HMG$ and F_{NH_2} (Fig. 2b) was completely inhibited by thiorphan and EDTA at a concentration of 100 μM and 100 mM, respectively. Their formation was detected after 5 minutes and increased linearly during the first 15 minutes, then



Scheme 1. Proposed metabolic pathways of CCK4 in the rabbit jejunum BBMV; [a] aminopeptidases: more than one aminopeptidase is involved, [b] amastatin-sensitive aminopeptidase, [c] endopeptidase(s), ‖ indicates a faster degradation rate than |.

stayed constant. The addition of amastatin did not affect its generation greatly, while a lower-pH buffer could reduce its formation after 10 minutes. In contrast, the formations of $DY_{SO_3}HMGW$ and W (Fig. 2c) were markedly inhibited by amastatin and totally inhibited by a combination of amastatin and thiorphan at a concentration of 100 μM of each; while either a pH 4 buffer or thiorphan could only suppress the enzymatic activity to a lesser extent. Their generation was not detected until 5 minutes had passed, and increased linearly at two different rates (one for the first 20 minutes; the other between 20 and 60 minutes). Another metabolite, MDF_{NH_2} was generated gradually and linearly for the first 10 minutes; then decreased slowly (Fig. 2d). Its formation was completely suppressed by



Scheme 2. Proposed metabolic pathways of CCK8 in the rabbit jejunum BBMV; [a] enkephalinase (EC 3.4.24.11), [b] endopeptidases (including enkephalinase), [c] aminopeptidase.

thiorphan and a mixture of amastatin and thiorphan. However, a greater amount of this metabolite was detected after 15 and 40 minutes when amastatin and a pH 4 buffer were used, respectively. The fraction of GWMD increased until 15 minutes had passed and then decreased (Fig. 2e). Its formation was totally inhibited by a combination of thiorphan and amastatin; it could be detected after 15 minutes only in a small amount when thiorphan alone was added. The application of a pH 4 buffer could inhibit the enzymatic activity in a lesser degree. In contrast, the amount of GWMD increased steadily when amastatin was added (Fig. 2e). The yield of the metabolite DY_{SO_3}HMGWM reached a maximum during the initial 6 minutes, subsequently declined, and could not be detected at all after 30 minutes (Fig. 2f). All of the enzyme inhibitors and an acidic buffer showed complicated effects on the formation of this fragment. The effects of both amastatin and pH 4 buffer showed a similar trend, but more was formed with the addition of amastatin, whereas less was formed in pH 4 buffer. A concentration of 100 μM thiorphan could markedly suppress the generation, where only 14% of this metabolite could be detected at 5 minutes, then it doubled and then remained constant. The addition of both amastatin and thiorphan did not exhibit much difference compared to when only thiorphan was added. DY_{SO_3}HMGWMD was produced rapidly during the initial 8 minutes, then decreased when CCK8 was incubated in pH 7 buffer (Fig. 2g), which showed a similar trend to that of DY_{SO_3}HMGWM. However, the effects of enzyme inhibitors were quite different; the addition of thiorphan caused a marked increase in the amount of this metabolite detected during the first 15 minutes, then a steady increment was obtained afterward. When both amastatin and thiorphan were added together, no apparent difference was observed at 20 minutes, then more of this fragment was found. Both a pH 4 buffer and amastatin suppressed the enzymatic activities and reduced its production (unpublished data). Based on these degradative curves of CCK8 and each metabolite as shown in Fig. 2a through 2g, Scheme 2 was derived and 3 possible pathways for CCK8 metabolism in BBMV were proposed.

Discussion

In this study, the metabolic pathways of both CCK4 ($WMDF_{NH_2}$) and CCK8 (DY_{SO_3}HMG $WMDF_{NH_2}$) show some degree of similarity, in which the subsequent degradation was similar after CCK8 was cleaved at the G^4-W^5 bond. The further cleavage in $WMDF_{NH_2}$ also resembled that of CCK4.

From the CCK4 study, the addition of 10 μM thiorphan, an enkephalinase inhibitor, does not show a marked change in the amount of CCK4 and all of its metabolites although Najdovski [12] and Rose [15] discovered that this enzyme could hydrolyze the peptide bond between D-F_{NH_2} where purified enkephalinase was used in their studies. The addition of 100 μM captopril does not improve the recovery of intact CCK4 indicating that both enkephalinase and ACE do not influence the degradation of CCK4 which is different from the findings of Dubreuil et al. ([21], to be further discussed later). When 10 μM amastatin was added, a marked increase in the amount of CCK4 remaining was obtained and it showed an inhibitory effect of 84%. However, W could be detected even though amastatin was added; therefore, it is possible that more than one enzyme is involved in step 1 of Scheme 1 and further metabolism of W is processed resulting in very low recovery of W (<5% in Fig. 1e). The increment of the amount of F_{NH_2} after 16 minutes also demonstrates that step 2 becomes dominant when amastatin is added. This is due to the inhibition of enzymatic activity at step 3 and partially at

step 1 (Scheme 1). From the 100 μM EDTA study, MDF_{NH_2} was detected but not WMD, which suggests that the catalytic rate of step 1 is faster than that of step 2 in Scheme 1. Moreover, step 1 and step 2 are parallel processes. The enzymes involved in step 2 are neither the enkephalinase nor the ACE. An endopeptidase other than enkephalinase or ACE might be related to the formation of DF_{NH_2}; however, this step was not noticeable compared to the other steps in Scheme 1. Hence, it can be concluded that the major cleavage is at the W-M bond, which is mainly hydrolyzed by an amastatin-sensitive aminopeptidase.

For CCK8, at least three metabolic pathways are involved independently as shown in Scheme 2. One pathway is that CCK8 is hydrolyzed by enkephalinase (EC 3.4.24.11) at the G^4-W^5 bond. Another pathway is the cleavage at D^7-$F_{NH_2}^8$, in which enkephalinase could be partially involved. Yet another pathway is at the peptide bond between M^6-D^7, and enzymes other than enkephalinase participate. Moreover, thiorphan displays 85% inhibition (Table 1) and the half-disappearance time of CCK8 rose to 24 minutes (Fig 2a). It can also completely abolish the formation of $DY_{SO_3}HMG$, F_{NH_2}, and MDF_{NH_2}, and excessively inhibit the generation of GWMD. This suggests that the peptide bonds between M^3-G^4, G^4-W^5, W^5-M^6, and D^7-$F_{NH_2}^8$ might be sensitive to enkephalinase. Our results resemble those of the studies on CCK8 in the brain [8–11], in the stomach [18], and in the kidney [12]. However, other studies show that two different endopeptidases were involved in breaking the M^3-G^4 bond of CCK8 in the brain: thiol peptidase and serine peptidase [27]. The generation of a combination of $DY_{SO_3}HMGW$ and W was reduced extensively when thiorphan was added, while it increased steadily in pH 7 buffer. This implies that the fraction detected later could be W. Its formation was affected by thiorphan partially because the cleavage between G^4-W^5 was inhibited. When thiorphan was applied, it inhibited the breakage of several peptide bonds; therefore, the time-curve of the metabolite $DY_{SO_3}HMGWM$ increased slowly and steadily to a lesser extent, but did not decline. It is possible that an enzyme(s) other than enkephalinase might hydrolyze this peptide bond and release DF_{NH_2}. The same trend could also be observed with the generation of $DY_{SO_3}HMGWMD$. Hence, it is concluded that enkephalinase (EC 3.4.24.11) is the major enzyme, which hydrolyzes CCK8 at different peptide bonds in BBMV. The amastatin-sensitive aminopeptidase could also influence its metabolism in the subsequent steps but not the major ones. According to other research, the primary site of cleavage of CCK8 was at the D^7-$F_{NH_2}^8$ bond, while further degradation occurred mainly at the G^4-W^5 bond [18,20,21]. However, a study done by Roques and his colleagues [28] suggested that enkephalinase was not a critical enzyme involved in the metabolism of CCK8 using slices from rat cortex. When both thiorphan and amastatin were added together, the amount of CCK8 remaining did not differ notably from when only thiorphan was added. This also shows that enkephalinase plays an important role in its hydrolysis. No distinctive change was noticed in the fraction of the following fragments: $DY_{SO_3}HMG$, F_{NH_2}, MDF_{NH_2}, and $DY_{SO_3}HMGWM$. Moreover, the combined use of two inhibitors could multiply the inhibitory effect to completely inhibit the formations of $DY_{SO_3}HMGW$, W, and GWMD.

The C-terminal amide derivatives of both CCK4 and CCK8 were poor substrates for carboxypeptidase A activity, the enzyme that releases C-terminal amino acids from peptides with a free-carboxyl group [22]. ACE (EC 3.4.15.1) is a metalloenzyme which is present in the GI tract and can release C-terminal dipeptide by cleaving the peptide bond. A study by Dubreuil and his colleagues [21] showed that purified rabbit lung ACE could break the peptide bond between M-D and release DF_{NH_2}, where ACE activity is enhanced in the presence of chloride ion (300 mM NaCl). In our study, the addition of captopril did not improve the recovery of intact CCK4 in rabbit BBMV during the first 10 minutes of incubation either with or without the presence of 300 mM NaCl. The discrepancy may be due to different sources of ACE and methods used. Only purified

rabbit lung ACE was used in Dubreuil's study, whereas the current study used rabbit jejunal BBMV containing different kinds of enzymes.

As expected, an acidic condition could reduce enzyme activities drastically and an inhibitory effect of 98% was observed (Table 1 and Fig 1a). Furthermore, the metabolites are not the hydrolytic products caused by the lower pH buffer. The pH-dependent study shows that most enzymatic activities are reduced under an acidic condition. Therefore, all CCK8 and its metabolites were found to be decreased except the fragment of MDF_{NH_2}, which increased after 20 minutes when CCK8 was incubated in pH 4 buffer. The study of CCK4 also showed similar results. The cause remains uncertain. No metabolites were detected when 100 mM EDTA was added indicating the metalloenzyme inhibitor with chelating ability can completely suppress all the enzymatic activities related to the hydrolysis of CCK4 in rabbit BBMV (Fig. 1a). However, high concentration EDTA may influence the morphology of the intestinal membrane.

In conclusion, the present study demonstrates that the CCK analogs were degraded in various ways in the rabbit BBMV indicating participation of many different enzymes of aminopeptidase, endopeptidase, ACE, metalloenzyme, and etc. However, there seems to be a major or dominant metabolic pathway(s) for each CCK analogs. In addition, our findings suggest that the blocking (inhibiting) the major (or dominant) metabolic pathway employing a specific inhibitor could result a marked inhibitory effect in the intestinal peptide metabolism. Identification of more specific and potent yet safe enzyme inhibitors for the enzyme(s) involved in the major cleavage site(s) or the development of 'an inhibitor cocktail' for various enzymes may result in a better system to block intestinal metabolism of peptide drugs when administered orally. Therefore, continuous efforts should be put on to characterize the intestinal metabolism of peptide drugs, to search for enzyme inhibitors, and to improve peptide oral absorption.

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