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Stabilization of methionine enkephalin in various rabbit mucosal extracts by enzyme inhibitors

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Abstract

The inhibition of the enzymatic degradation of methionine enkephalin (Met-Enk) was investigated kinetically in nasal, rectal, and vaginal extracts of rabbits with and without inhibitors, such as puromycin (PM), amastatin (AM), thiorphan (TP), Na_2EDTA, and thimerosal (TM), alone or in combination, by analyzing the parent peptide and its hydrolytic fragments by HPLC. The effects of variation of pH in the nasal extracts, and the addition of 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CyD) on the stabilization of Met-Enk were also studied. The degradation of Met-Enk was found to be fastest at around pH 7, indicating that the activity of enkephalin-degrading enzymes is optimal at this pH. Addition of 2-HP-β-CyD (10%) to the nasal, rectal, and vaginal extracts was noted to reduce the first-order degradation rate constants for Met-Enk by 2.5–2.8-fold, compared to the control. AM alone inhibited the enzymatic degradation of Met-Enk with IC_{50} values of 3.5 and 0.22 μM for the rectal and vaginal extracts, respectively, whereas PM was found to be approx. 14.2- and 26.8-fold less potent than AM, respectively. The effects of both aminopeptidase inhibitors in the nasal extracts were smaller. Even at 50 μM, TP (a potent enkephalinase A inhibitor) alone revealed only a small increase of Met-Enk stability in the various mucosal extracts, however, EDTA (5 mM) was observed to inhibit enzymatic hydrolysis considerably by blocking both enkephalinase A and B and, to some extent, aminopeptidase. On the other hand, TM (0.05%) was found to be a new and potent inhibitor for enkephalinase B and aminopeptidases, which was more potent than AM (50 μM) in inhibiting the degradation of Met-Enk in various mucosal extracts. Furthermore, the addition of TM (0.01%) to a combination of AM (50 μM) and EDTA (5 mM) was observed to protect Met-Enk from enzymatic degradation in nasal, rectal, and vaginal extracts by more than 90%, after 24 h of incubation, by inhibiting almost completely all the enkephalin-degrading enzymes present in the incubation mixtures.

Keywords: Methionine enkephalin; Nasal extract; Rectal extract; Vaginal extract; Stabilization; Enzyme inhibitor; pH effect; Amastatin; Puromycin; Thiorphan; EDTA; 2-Hydroxypropyl-β-cyclodextrin; Composite effect

1. Introduction

In past years, there has been a remarkable increase in the understanding of mechanisms in-

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volved in the functions of enkephalins, and it has gained acceptance that the weak and short-lasting activity of these peptides is mainly due to its rapid enzymatic degradation in plasma, brain tissues and membranes (Schwartz et al., 1981; De La Baume et al., 1983; Bunnett, 1987). Several types of peptidases are known to be responsible for the degradation of natural enkephalins. Among them at least three types of enzymes seem to play a predominant role (Hersh, 1982; Malfroy and Schwartz, 1982; Van Amsterdam et al., 1983a; McDermott et al., 1985; Giros et al., 1986): an aminopeptidase which cleaves the Tyr-Gly bond, a dipeptidyl carboxypeptidase (also known as enkephalinase A) which hydrolyzes the Gly-Phe linkage, and a dipeptidyl aminopeptidase (also known as enkephalinase B) which breaks the Gly-Gly bond. These enzyme activities were also observed in the homogenates of various mucosal, eye, and stomach wall tissues (Dodda Kashi and Lee, 1986a,b; Bunnett et al., 1990).

As the peptidases hydrolyze enkephalins, the potent analgesic and antinociceptive actions of these opiate peptides are terminated with shortening in the duration of their actions. Many enzyme inhibition studies have focused on the objective of improving the physiological regulation of enkephalins. Inhibition of aminopeptidases has been studied by using puromycin, bestatin, amastatin, and actinonin (Aoyagi et al., 1978; Rich et al., 1984; Umezawa et al., 1985). Moreover, actinonin was observed to inhibit aminopeptidase as well as enkephalinase A and B. When given peripherally, actinonin was observed to potentiate the analgesic action of enkephalin (Hachisu et al., 1987). It has been reported that enkephalinase A is inhibited by thiorphan (Patey et al., 1981; Malfroy and Schwartz, 1982), phosphoramidon (Malfroy and Schwartz, 1982), phelorphan (Van Amsterdam et al., 1987, 1988), SCH 32615 (Chipkin et al., 1988), and small peptides (Fournie-Zaluski et al., 1979). Enkephalinase B has been reported to be present in high levels in the CNS, and to have a 3–4-fold higher affinity for enkephalins than other enkephalin-degrading enzymes (Van Buuren et al., 1985). Kelatorphan and its related inhibitors, which inhibit aminopeptidase as well as enkephalinase A and B, elicit a greater effect on the analgesic activity of enkephalins than a combination of inhibitors which inhibits only enkephalinase A or aminopeptidase (Bouboutou et al., 1984; Xie et al., 1989).

However, few reports have been published on investigation of the potential use of enzyme inhibitors to stabilize enkephalins during their transport through the various absorptive mucosal tissues (Hussain et al., 1989; Faraj et al., 1990a,b). Furthermore, the inhibition of peptidases involved in the degradation of enkephalins in the extracts of nasal, rectal, and vaginal mucosae has not been examined. Due to the very low bioavailability resulting from poor absorption and/or extensive degradation in the GI tract and liver, the use of these peptides as therapeutic agents has been limited to intravenous administration. To overcome this limitation, much attention has been focused on the systemic delivery of these peptides through the absorptive mucosae, such as the nasal, buccal, rectal, and vaginal mucosae, as a non-invasive route. However, one of the major problems in the transmucosal delivery of enkephalins is their rapid degradation by various peptidase at the site of administration. Recently, some aminopeptidase inhibitors, such as α-aminoboronic acid derivatives, bestatin, puromycin, and small peptides, have been used to stabilize externally administered leucine enkephalin in the nasal perfusate as well as in the nasal cavity (Hussain et al., 1989, 1990; Faraj et al., 1990a,b). However, data currently available indicate that total inhibition of the various enzyme activities to achieve a full stabilization of enkephalins in the brain and mucosal homogenates, and nasal perfusate has not yet been achieved. Moreover, the incubation time in most of the inhibition studies was less than 2 h, and the inhibitors used reduced the degradation rates only to some extent.

In our earlier paper (Chun and Chien, 1993), methionine enkephalin (Met-Enk) was found to degrade rapidly and extensively in the extracts of nasal, rectal, and vaginal mucosae of rabbits, and to form various hydrolytic fragments such as des-Tyr^1-methionine enkephalin [Des-(Tyr)-Met-Enk], Phe-Met, Tyr-Gly-Gly, Tyr-Gly, Tyr, and Phe. Kinetic analysis of the data has led to the

conclusion that aminopeptidases play a major role, while enkephalinase A and B contribute to some extent to the degradation of Met-Enk. This suggests that, to deliver Met-Enk through the mucosal routes, it is very important to protect Met-Enk from the degradation by these peptidases, if present, in the mucosal cavities and during the permeation through the mucosal membranes. Therefore, the present investigation was undertaken to study the feasibility of using enzyme inhibitors and/or their combinations to stabilize Met-Enk in various rabbit mucosal extracts over a period of up to 24 h.

2. Materials and methods

2.1. Materials

Met-Enk (Tyr-Gly-Gly-Phe-Met, as acetate salt), Des-(Tyr)-Met-Enk (as acetate salt), Phe-Met, Tyr-Gly, Tyr-Gly-Gly, Tyr, Phe, amastatin (as hydrochloride, AM), puromycin (PM), thiorphan (TP), and thimerosal (TM) were obtained from Sigma Chemical Co. (St. Louis, MO) and were used as received. Na_2EDTA and acetonitrile (HPLC grade) were purchased from Fisher Scientific Co. (Springfield, NJ). 2-Hydroxypropyl-β-cyclodextrin (2-HP-β-CyD) was kindly supplied by Pharmatec, Inc. (Alachua, FL). All other reagents were of analytical grade. Purified water (18 MΩ), freshly prepared by Nanopure System (Barnstead, Boston, MA), was used for preparation of the HPLC mobile phase and all the test solutions. Stock solutions of Met-Enk, its hydrolytic fragments, and enzyme inhibitors were prepared fresh daily.

2.2. HPLC assay of Met-Enk and its metabolites in incubation mixtures

Analytical methods used were similar to those described previously (Chun and Chien, 1993). All the assays were carried out using an HPLC system consisting of a Waters 590 pump, a WISP 710B injector, and a Kratos Spectroflow 783 detector. A stainless-steel column (4.6 × 150 mm) packed with Ultrasphere C8 (5 μm, Beckman Instruments, Irvine, CA) connected with a guard column (Ultrasphere C8, 4.6 × 45 mm) was used. The mobile phase used for the assay of Met-Enk, Des-(Tyr)-Met-Enk and Phe-Met was a 76:24 (v/v) mixture of phosphate buffer (0.05 M, pH 3.0 with phosphoric acid) and acetonitrile, which contains 0.1% heptanesulfonic acid sodium salt as an ion-pairing agent. An 88:12 (v/v) mixture of the same components was used to analyze the smaller hydrolytic fragments formed. The flow rate of the mobile phase used was 1.0 ml/min and detection was performed by measuring the UV absorbance at a wavelength of 214 nm.

2.3. Dissection of mucosal specimens

Female New Zealand White rabbits (Davidson Mill Breeding Labs, Jamesburg, NJ), weighing 2.5–3.5 kg each, were killed by i.v. injection of 1.0 ml of Beauthanasia D-Special Solution, which contains 390 mg/ml of pentobarbital sodium and 50 mg/ml of phenytoin sodium (Schering-Plough Corp., Kenilworth, NJ), into a marginal ear vein. Mucosae were excised immediately using a surgical blade (no. 10) in the following order: vaginal, rectal, and nasal mucosae. Each mucosal specimen was prepared according to the procedure described previously (Chun and Chien, 1993).

2.4. Preparation of mucosal extracts

Freshly excised mucosal specimens were each mounted over the cell opening (0.64 cm^2) of Valia-Chien permeation cells, with the mucosal epithelium facing the donor half-cell and the serosal side facing the receptor half-cell. Mucosal and serosal extracts were prepared in the same manner previously described (Chun and Chien, 1993). To minimize microbial growth during extraction and to investigate the effect of the antimicrobial agent on the degradation profile of Met-Enk, extraction of the mucosae was carried out using isotonic phosphate buffer with or without 0.01% of TM. All the extracts were used in the enzymatic degradation studies of Met-Enk within 1 h of the final extraction.

2.5. Inhibition studies of enzymatic degradation of Met-Enk in mucosal and serosal extracts by enzyme inhibitors

Concentrated stock solutions of Met-Enk, PM, AM, TP, TM and EDTA were prepared fresh in 0.9% saline solution. To 800 μl of each mucosal extract, 50 μl of Met-Enk stock solution (1000 μg/ml) and 50 μl of saline solution containing various inhibitors with varying concentrations were added. In the inhibition studies using a single inhibitor or combinations of two or three inhibitors, saline solution was added to maintain the final volume of the reaction mixture at 1000 μl. The mixture was incubated at 37° C in a shaking water bath with constant shaking at 60 oscillations per min. Aliquots (100 μl each) of the incubation mixture were periodically sampled over a period of 24 h, and immediately diluted with 400 μl of phosphate buffer (0.05 M, pH 2.2) to terminate the enzymatic activity. 20 μl of the solution were analyzed for Met-Enk and its metabolites by the HPLC method described above, and their concentrations in the incubation mixtures were determined using the calibration curve established for each component. All the inhibition studies were carried out, in triplicate, using the mucosal extracts obtained from three rabbits.

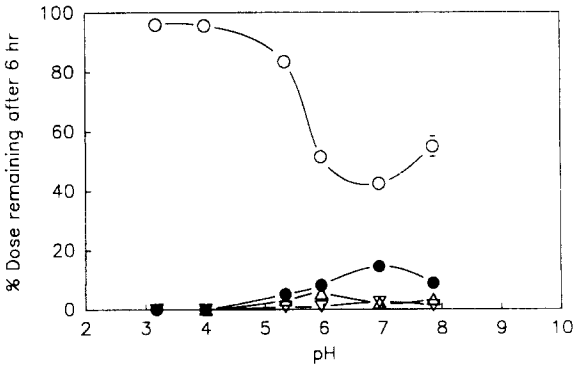

Fig. 1. Effect of pH on the degradation of Met-Enk and appearance of its hydrolytic fragments in nasal mucosa extracts at 37° C. Each data point represents the mean of three determinations. Met-Enk (○), Tyr (●), Tyr-Gly (▽) and Tyr-Gly-Gly (△).

2.6. Inhibition studies on enzymatic degradation of Met-Enk in mucosal extracts by 2-hydroxypropyl-β-cyclodextrin

To 900 μl of each mucosal extract containing 100 mg of 2-HP-β-CyD, 50 μl of Met-Enk stock solution (1000 μg/ml) and 50 μl of saline solution were added. The mixture was incubated at 37° C in a shaking water bath at 60 oscillations per min. Aliquots (100 μl each) of incubates were periodically sampled and treated in the same manner as in the procedure described above.

3. Results and discussion

3.1. Effect of pH of the extract on the degradation of Met-Enk

Nasal extracts (pH 8.0) were acidified stepwise using 10% phosphoric acid to vary the pH (7.9–3.19). Met-Enk (74 μM) was then added. These nasal extracts were incubated, in triplicate, at 37° C for 6 h. The results, shown in Fig. 1, suggest that Met-Enk is most susceptible to enzymatic degradation at pH 7.0 and the highest production of Tyr also occurs at this pH. As the pH increased or decreased from 7.0, the degradation of Met-Enk was markedly retarded. This observation implies that the enkephalin-degrading enzymes in the nasal extract are optimally active at around pH 7, which is in agreement with the report that aminopeptidases and enkephalinase A purified from brain have an optimal pH at pH 7.0 and 6.9, respectively (Schwartz et al., 1981; Van Amsterdam et al., 1983b). At pH 3.2–4.0, no hydrolytic fragments were detected, indicating the complete inactivation of enkephalin-degrading enzymes. Based on this finding, the pH of a Met-Enk formulation could be one of the important factors to be considered for the enzymatic stability of Met-Enk in the nasal cavity.

3.2. Effect of aminopeptidase inhibitors on the degradation of Met-Enk in various mucosal extracts

Met-Enk (74 μM) was incubated for 90 min in various mucosal extracts containing varying con-

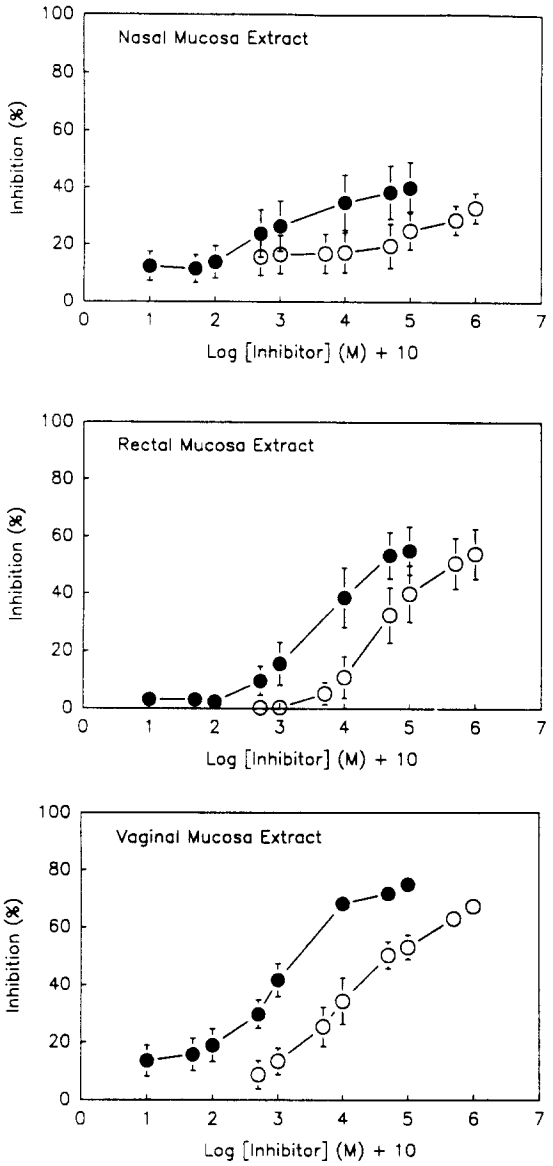

Fig. 2. Effect of PM (○) and AM (●), the aminopeptidase inhibitors, on the inhibition of Met-Enk degradation in nasal, rectal, and vaginal mucosa extracts. Each point indicates the mean (±SE) of the data from three rabbits.

centrations of AM and PM, the aminopeptidase inhibitors. The enzymatic hydrolysis of Met-Enk was found to be inhibited, in a concentration-dependent manner by either PM or AM (Fig. 2). However, the inhibition vs concentration profile varied from one mucosa to another. AM appeared to be more effective than PM in the inhibition of Met-Enk degradation. The IC_{50} values (the concentrations required to achieve 50% inhibition of Met-Enk degradation) were determined from the plots. The values for AM and PM were 49.9 and 3.5 μM, respectively, for the rectal mucosal extract, and 5.9 and 0.22 μM, respectively, for the vaginal mucosal extract. In the nasal mucosal extract, however, the IC_{50} values could not be determined, since maximal inhibition was about 32 and 40%, respectively, at 100 μM PM and 10 μM AM, respectively. For the nasal, rectal, and vaginal extracts, the maximal inhibition was found to be about 40, 55 and 80%, respectively. PM (an inhibitor for aminopeptidase B and N, but not for leucine aminopeptidase) was noted to be less effective than AM (an inhibitor for aminopeptidase A and leucine aminopeptidase). AM (IC_{50} = 0.01 μM) was reported to be a stronger inhibitor than bestatin (IC_{50} = 0.2 μM), and to be 100-fold more potent than PM (Barclay and Phillipps, 1980).

Fig. 3 shows the extent of inhibition of Met-Enk degradation in mucosal and serosal extracts of nasal, rectal, and vaginal membranes by AM (10 μM). The results suggest that there is no significant difference in the extent of inhibition between mucosal and serosal extracts. In the absence of AM or other inhibitors after 90 min, 42.4, 84.4, and 52.8% of Tyr was formed in nasal, rectal, and vaginal mucosal extracts, respectively,

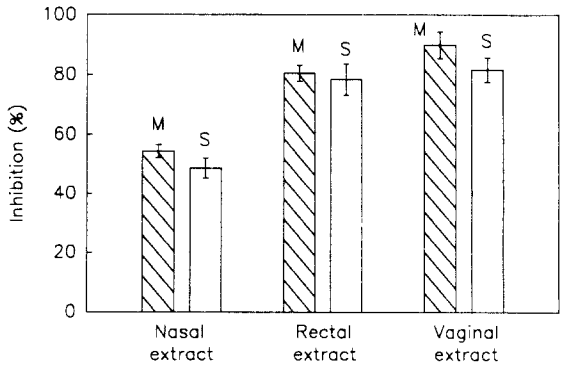

Fig. 3. Effect of AM (10 μM) on the inhibition of degradation of Met-Enk in mucosal (M) and serosal (S) extracts of nasal, rectal, and vaginal mucosae after incubation at 37° C for 30 min (mean ± SE, n = 3).

but only 20.3, 24.1, and 9.0% of Tyr was detected, respectively, in the presence of AM (10 μM). Similar results were also obtained for the serosal extracts.

Fig. 4 demonstrates the degradation profiles of Met-Enk in nasal, rectal, and vaginal mucosal extracts in the presence of different concentrations of AM, compared to those in the absence of this inhibitor. It was found that the enzymatic degradation of Met-Enk was slowed by increasing the concentration of AM in all the mucosal extracts. For example, Met-Enk found intact in the rectal mucosal extract in the presence of 0.1, 1.0, and 10 μM AM was 17.5, 42.8, and 55.6%, respectively, as compared to only 1.3% in the absence of this inhibitor after 90 min incubation. Table 1 summarizes the first-order rate constants for the degradation of Met-Enk in the absence and presence of AM. The data demonstrate that apparent rate constant decreases proportionally with increasing concentration of AM. In the presence of AM at 10 μM, the degradation rate of Met-Enk is reduced 1.5-, 5.1-, and 6.5-fold in nasal, rectal, and vaginal mucosal extracts, respectively, compared with that in the absence of this enzyme inhibitor.

It should be pointed out that the inhibition of Met-Enk degradation by AM in the nasal extract is smaller than that in the rectal or vaginal extracts. The difference could be due to the difference in the relative contribution of aminopeptidases, enkephalinase A and B, to the degradation in various mucosal extracts, as pointed out in the previous paper (Chun and Chien, 1993). It has been reported that, even in the presence of 0.1 μM boroleucine, which has been reported to be an excellent inhibitor for aminopeptidase, 14% of leucine enkephalin (100 μM) was degraded in the nasal perfusate after 90 min of incubation (Hussain et al., 1989). Based on the results outlined above, it is thus considered that the inhibition of aminopeptidase alone is not sufficient to give a total stabilization of Met-Enk in various mucosal extracts.

3.3. Inhibition of Met-Enk degradation by 2-hydroxypropyl-β-cyclodextrin

Met-Enk (74 μM) was incubated in the nasal, rectal, and vaginal mucosal extracts containing 10% 2-HP-β-CyD at 37° C over a period of 6 h. The effect of 2-HP-β-CyD on the first-order rate constants obtained from the degradation kinetics profiles is summarized in Table 2. In the presence of 2-HP-β-CyD, the degradation rate constant decreased more than 2.5-fold in all three mucosal extracts. It is interesting to note that 10% 2-HP-β-CyD was more effective than 10 μM AM in stabilizing Met-Enk in the nasal extract. Furthermore, the degradation rate constant of Met-Enk in the nasal mucosal extract was found to decrease upon increasing the concentration of 2-HP-β-CyD: the first-order rate constants (mean ± SE, $n = 3$) in the presence of 0, 5, 10, and 20% 2-HP-β-CyD were 0.86 (±0.18), 0.49 (±0.11), 0.31 (±0.09) and 0.23 (±0.08) h^{-1}, respectively. This inhibition of degradation could be due to the formation of an occlusion-type complex, which

Table 1
Apparent first-order rate constants for the degradation of Met-Enk [a] in various rabbit mucosal extracts as a function of amastatin concentrations

Extract	Site extracted	First-order rate constant (h^{-1}) (±SE) [b] at			
		0 μM	0.1 μM	1.0 μM	10.0 μM
Nasal	mucosal	0.86 (±0.18)	0.59 (±0.14)	0.53 (±0.15)	0.46 (±0.11)
	serosal	1.01 (±0.34)	0.66 (±0.16)	0.52 (±0.12)	0.45 (±0.10)
Rectal	mucosal	3.11 (+0.51)	1.31 (+0.31)	0.70 (±0.11)	0.48 (±0.09)
	serosal	3.28 (±0.55)	1.41 (±0.28)	0.73 (±0.14)	0.50 (±0.11)
Vaginal	mucosal	1.23 (±0.38)	0.52 (±0.11)	0.35 (±0.08)	0.24 (±0.04)
	serosal	1.46 (±0.39)	0.70 (±0.16)	0.43 (±0.09)	0.31 (±0.07)

[a] Concentration = 74×10^{-6} M.
[b] Expressed as the means (±SE) of the results obtained from three rabbits.

encapsulates Met-Enk within and thus reduces its degradation.

Recent studies have shown that aromatic amino acids, such as tyrosine and tryptophan, are capable of interacting with α- and β-CyDs in a thermodynamically favorable manner (Matsuyama et al., 1987); it was also suggested that CyDs may well have an application in protecting peptides from enzymatic hydrolysis. Even though β-CyD has been shown to have a complexing capacity with a wide range of guest molecules, its use in higher concentrations is limited because of its low solubility (1.85 g/ml). Therefore, chemically modified CyDs, such as 2-HP-β-CyD, have been suggested to solubilize and stabilize various peptides and proteins (Brewster et al., 1989). HP-β-CyDs are amorphous isomeric mixtures, which have been demonstrated to have a complexing ability and to be innocuous even when administered intravenously. The effect of 2-HP-β-CyD complexation on the biological activity of interleukin-2 was investigated, and the peptide was shown to retain 100% of its biological potency in the presence of 2-HP-β-CyD (Brewster et al., 1989). Similar studies have also been performed on tumor necrosis factor and macrophage colony stimulating factor. The solutions prepared, using 2-HP-β-CyD, for both biologicals were clear, and retained 100% of their biological activity. Therefore, the use of 2-HP-β-CyD in protein and peptide formulations is a potentially valuable method for the solubilization and stabilization of many peptides and proteins against chemical and enzymatic degradation.

3.4. Effect of thimerosal on the inhibition of Met-Enk degradation

TM has been used as a bacteriostatic and fungistatic in several topical pharmaceutical products. In this investigation, some turbidity was noted during the extraction of mucosal membranes. This turbidity may be attributed to the proliferation of microbes in the extracts. Therefore, TM (0.01%) was added to the extracting medium (isotonic phosphate buffer) in order to prevent microbial growth. The degradation of Met-Enk and its hydrolytic fragments [Des-(Tyr)-Met-Enk, Phe-Met, Tyr-Gly, and Tyr-Gly-Gly] using the same molar concentration (74 μM) in the various mucosal extracts with and without 0.01% TM was investigated.

Fig. 5 shows the inhibitory effect of TM on the degradation of Met-Enk in various mucosal extracts after 90 min incubation. The inhibition of enzymatic degradation of Met-Enk by TM was greatest (75.7 $\pm$ 1.8%) in the nasal mucosal extract, and almost the same in the rectal (54.9 $\pm$ 11.5%) and vaginal mucosal extracts (55.1 $\pm$ 5.7%). This can be explained by the fact that the relative contribution of enkephalinase B activity to the degradation of Met-Enk is considerably higher in the nasal extract than other mucosal extracts, although aminopeptidases are still the dominating ones (Chun and Chien, 1993), and its activity could be completely blocked by the presence of TM. The complete inhibition of enkephalinase B is demonstrated by no formation of Tyr-Gly fragment in the presence of TM, as

Table 2
Effect of 2-hydroxypropyl-β-cyclodextrin on the first-order rate constants for the degradation of Met-Enk in various rabbit mucosal extracts

Mucosal extract	First-order rate contant [a] (h^{-1}) ($\pm$SE)		k_0/k_{CyD} [c]
	No 2-HP-β-CyD (k_0)	With 2-HP-β-Cyd [b] (k_{CyD})	
Nasal	0.86 ($\pm$0.18)	0.31 ($\pm$0.05)	2.77
Rectal	3.11 ($\pm$0.51)	1.13 ($\pm$0.21)	2.75
Vaginal	1.23 ($\pm$0.38)	0.49 ($\pm$0.10)	2.51

[a] Expressed as the means ($\pm$SE) of the results obtained from three rabbits.
[b] 10% of 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CyD).
[c] Ratio of the first-order rate constant for the degradation of Met-Enk without 2-HP-β-CyD (k_0) over that with 2-HP-β-CyD (k_{CyD}).

Table 3
Effect of thimerosal (TM) [a] on the first-order rate constants for the degradation of Met-Enk and its primary hydrolytic fragments in various rabbit mucosal extracts

Peptide [a]	First-order rate constant [c] (h^{-1}) (±SD) ($\times 10^2$)					
	Nasal extract [d]		Rectal extract [d]		Vaginal extract [d]	
	No TM	With TM	No TM	With TM	No TM	With TM
Met-Enk	85.55 (±16.68)	10.16 (±0.31)	149.89 (±37.44)	17.33 (±8.44)	45.31 (±8.88)	10.10 (±2.29)
Des-(Tyr)-Met-Enk	306.06 (±9.72)	4.03 (±0.28)	175.20 (±2.76)	1.92 (±0.15)	34.08 (±0.12)	1.91 (±0.19)
Phe-Met	199.74 (±7.38)	1.11 (±0.22)	216.42 (±22.26)	2.42 (±0.70)	78.84 (±1.08)	6.68 (±2.17)
Tyr-Gly	18.30 (±1.02)	1.01 (±0.27)	14.70 (±0.04)	1.08 (±0.54)	14.04 (±0.54)	1.09 (±0.15)
Tyr-Gly-Gly	120.66 (±22.56)	1.69 (±0.30)	249.30 (±30.66)	1.68 (±0.24)	28.50 (±4.98)	2.05 (±0.30)

[a] Thimerosal (TM) concentration = 0.01%.
[b] The initial concentration of Met-Enk and its hydrolytic fragments was constant (74 μM).
[c] Data expressed as the means (±SD) of three determinations using the respective extracts.
[d] Combined mixture of mucosal and serosal extracts obtained from each mucosal membrane.

shown in Table 3. The first-order rate constants obtained from the degradation profiles are summarized in Table 4. The data indicate that with addition of TM, the rate constants for the degradation of both Met-Enk and its hydrolytic fragments in all the mucosal extracts are markedly reduced.

For example, with the addition of 0.01% TM, the rate constants decreased 8.4-, 8.6-, and 4.5-fold in the nasal, rectal, and vaginal extracts, respectively. The results indicate that TM at a concentration of 0.01% is more effective in inhibiting the degradation of Met-Enk than 10 μM AM. Furthermore, the degradation rate constants of Des-(Tyr)-Met-Enk, which is a hydrolytic fragment of Met-Enk with the Tyr-Gly bond already cleaved, were remarkably reduced 75.9-, 91.1-, and 17.9-fold in the nasal, rectal, and vaginal extracts, respectively, with the addition of TM. Moreover, the first-order rate constants for the degradation of Phe-Met, which is a hydrolytic fragment with the Gly-Phe bond cleaved, were decreased to an even greater extent, by as much as 179.3-, 89.4-, and 11.8-fold in the nasal, rectal, and vaginal extracts, respectively. The results indicate that dipeptidyl carboxypeptidase and carboxypeptidase activities, which are known to be responsible for the cleavage of Des-(Tyr)-Met-Enk and Phe-Met, respectively, were strongly inhibited by TM. On the other hand, the degradation rate constants of Tyr-Gly-Gly and Tyr-Gly fragments in the presence of TM were decreased

Table 4
Inhibitory effect of thimerosal [a] on the degradation of Met-Enk [b] and formation of various hydrolytic fragments in various rabbit mucosal extracts [c]

Mucosal extract	% Met-Enk remaining [d]		% hydrolytic fragment found [d]					
	No TM	With TM	Tyr		Tyr-Gly		Tyr-Gly-Gly	
			No TM	With TM	No TM	With TM	No TM	With TM
Nasal	1.7 (±1.0)	72.0 (±0.8)	74.2 (±2.5)	12.7 (±0.9)	19.4 (±0.6)	ND [e]	2.5 (±0.2)	13.5 (±0.4)
Rectal	ND	48.4 (±6.7)	92.7 (±0.2)	39.3 (±8.8)	5.8 (±0.3)	ND	ND	8.9 (±0.1)
Vaginal	27.7 (±3.3)	68.5 (±2.9)	18.1 (±2.3)	30.8 (±2.8)	7.9 (±0.3)	ND	ND	1.6 (±0.1)

[a] Thimerosal (TM) at 0.01%.
[b] Met-Enk at 7.4×10^{-5} M.
[c] Combined mixture of mucosal and serosal extracts obtained from each mucosal membrane, which was incubated at 37° C for 3 h.
[d] Data expressed as the means (±SE) of three determinations using the respective extracts.
[e] Not detected.

18.1- and 15.7-fold, respectively, in the nasal extract; 13.6- and 148.5-fold, respectively, in the rectal extract; and 12.9- and 13.9-fold, respectively, in the vaginal extract. These results demonstrate that TM strongly inhibits the activities of aminopeptidases and dipeptidyl carboxypeptidase.

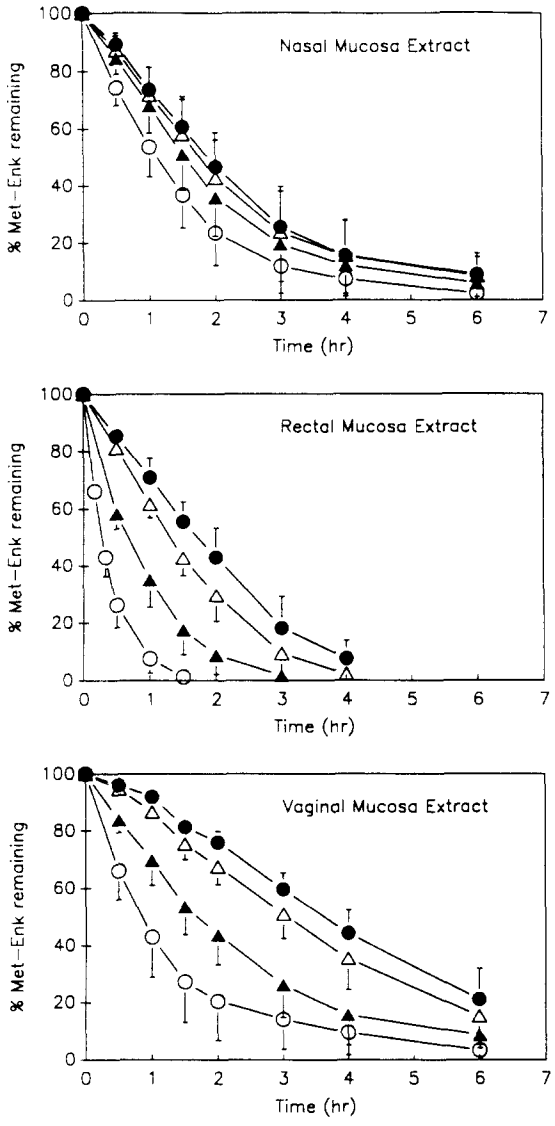

Fig. 4. Degradation profiles of Met-Enk in nasal, rectal, and vaginal mucosa extracts at 37°C in the absence (○) and presence of AM at concentrations of 0.1 (▲), 1.0 (△) and 10 μM (●). Each data point represents the mean of three determinations.

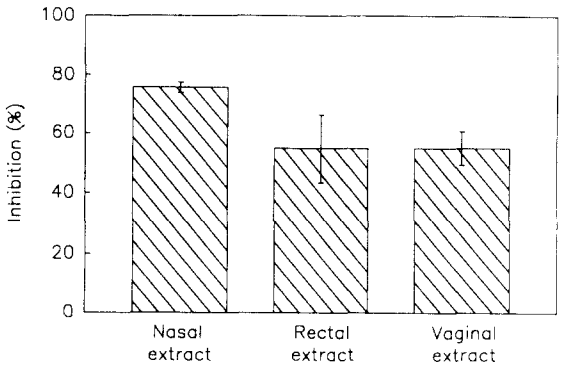

Fig. 5. Inhibitory effect of TM (0.01%) on the degradation of Met-Enk in various rabbit mucosal extracts after 90 min incubation (mean ± SE, $n = 3$).

In our previous paper (Chun and Chien, 1993), we demonstrated the pathways for the degradation of several primary hydrolytic fragments of Met-Enk in the various mucosal extracts: Des-(Tyr)-Met-Enk is cleaved at the Gly-Phe bond, forming Phe-Met; and further cleavage of Phe-Met yields Phe; degradation of Tyr-Gly-Gly also forms Tyr but without the detection of Tyr-Gly; and cleavage of Tyr-Gly fragment also forms Tyr. It has been reported that dipeptidyl carboxypeptidase and aminopeptidases are the primary enzymes responsible for the breakdown of Des-(Tyr)-Met-Enk and Tyr-Gly-Gly, respectively (De La Baume et al., 1983; Dodda-Kashi and Lee, 1986a).

On the other hand, it is surprising to note that the highest concentration of Tyr-Gly formed from the enzymatic degradation of Met-Enk is only 19.4% of the full formation (74 μM) of Tyr-Gly in the nasal extract (3 h after incubation), 9.5% in the rectal extract (2 h), and 7.9% in the vaginal extract (3 h) in the absence of TM, while no Tyr-Gly fragment was detected in any of the mucosal extracts even over the incubation period of 6 h in the presence of TM (Table 4). This complete inhibition of the formation of Tyr-Gly fragment suggests that TM completely inhibits the activity of dipeptidyl aminopeptidase (enkephalinase B).

The formation of Tyr, which is one of the major metabolites of Met-Enk in all the mucosal

extracts, was observed to rapidly reach a peak concentration at first and then slowly decrease when there was no TM in the incubation mixture. In the presence of TM, on the other hand, the concentration of Tyr was almost linearly accumulated. This result suggests that TM could probably also inhibit the activity of tyrosine oxidase which may be present in the rabbit mucosal extracts.

The appearance of a considerable amount of Tyr-Gly-Gly from the Met-Enk incubation mixture in all the mucosal extracts in the presence of TM could be the result of strong inhibition of aminopeptidase activity by TM, since, in the absence of TM, the Tyr-Gly-Gly fragment was found to be more rapidly degraded in the nasal and rectal extracts than Met-Enk itself (Table 3). It was reported earlier that the accumulation of this fragment is not observed in the rectal and vaginal extracts (Chun and Chien, 1993). This finding suggests the potential use of TM as a new, strong composite inhibitor for aminopeptidases and enkephalinase B.

3.5. Effect of combination of peptidase inhibitors on Met-Enk degradation in the various mucosal extracts

It is difficult to totally protect Met-Enk from the enzymatic degradation with a single inhibitor alone, such as PM, AM, or TM at the concentrations used. Although aminopeptidase and enkephalinase B activities have been shown to be effectively inhibited by AM and TM, enkephalinase A is still active in the various mucosal extracts, since, in the presence of TM, the Tyr-Gly-Gly fragment was found to be present in the nasal, rectal, and vaginal extracts at concentrations of 25.4, 17.1 and 1.9% of its full appearance after 6 h, respectively. Without the presence of TM, Tyr-Gly-Gly was not detectable, since this fragment was rapidly further degraded immediately after formation in the mucosal extracts, with the exception of nasal extract. The detection of the Tyr-Gly-Gly fragment in the nasal extract suggests significant enkephalinase A activity in the nasal extract.

Therefore, in order to inhibit this enkephalinase A activity present in the extracts, TP (a selective enkephalinase A inhibitor) and EDTA (a chelating agent) were also selected for study. TP has been reported to be an effective and selective inhibitor for enkephalinase A at an IC_{50} of 4 nM, but not for enkephalinase B, while EDTA inhibits both enkephalinases A and B at 1 mM (Van Amsterdam et al., 1983a,b). Enkephalinases A and B, as well as aminopeptidases, are known to be metalloenzymes (Schnebli et al., 1979; Schwartz et al., 1981; Malfroy and Schwartz, 1982; Van Amsterdam et al., 1983a,b).

Based on the concentration-dependent effect of TM and EDTA on Met-Enk degradation, Met-Enk (74 μM) was added to nasal extract containing different concentrations of TM and EDTA and incubated at 37° C for 90 min. In the presence of TM at 0.01, 0.05, and 0.1%, the degree of inhibition of Met-Enk degradation achieved was found to be 46.1, 45.7, and 46.3%, respectively, indicating that maximal inhibition had already been reached at 0.01% of TM. Although TM has been shown to strongly inhibit the activities of aminopeptidases and enkephalinase B, it is not certain from this experiment whether TM inhibits enkephalinase A. On the other hand, the data in Fig. 6 show that the extent of inhibition of Met-Enk degradation increases as the concentration of EDTA is increased. From Fig. 6, the IC_{50} value of EDTA was determined to be 1.17 mM, and that the maximal inhibition can be achieved at concentrations above 0.5 mM. A trace amount

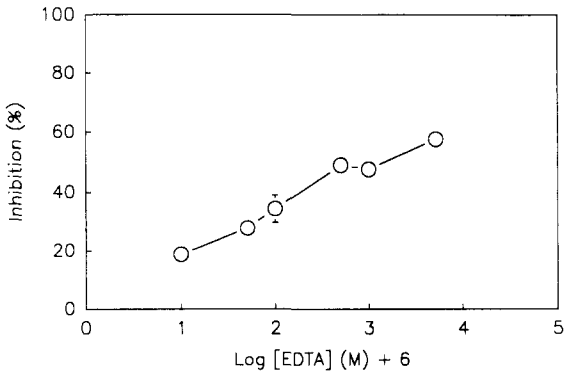

Fig. 6. Effect of EDTA, a chelating agent, on the inhibition of Met-Enk degradation in the nasal extract (mean ± SE, $n = 3$).

Table 5
Effect of various enzyme inhibitors on the first-order rate constants for the degradation of Met-Enk in nasal extracts

Inhibitor	First-order rate constant [a] (h^{-1}) (±SE) ($\times 10^2$)
No inhibitor	48.35 (±16.84)
(A) TM (0.05%)	11.46 (±1.01)
(B) AM (50 μM)	23.65 (±9.76)
(C) TP (50 μM)	42.15 (±16.78)
(D) EDTA (5 mM)	7.74 (±1.29)
(B)+(C)	24.93 (±8.32)
(B)+(D)	4.64 (±1.14)
(A)+(B)+(D)	0.88 (±0.11)

[a] 37° C data obtained from the nasal extracts of three rabbits and expressed as mean (±SE).

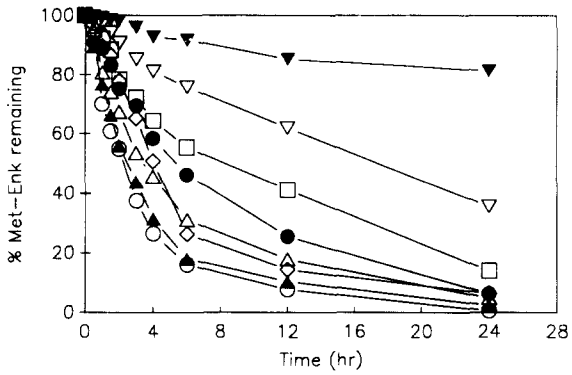

Fig. 7. Degradation profiles of Met-Enk in nasal mucosa extracts at 37° C in the absence (○), and presence of TM (0.05%) alone (●), AM (50 μM) alone (△), TP (50 μM) alone (▲), EDTA (5 mM) alone (□), AM (50 μM)/TP (50 μM) (◇), AM (50 μM)/EDTA(5 mM) (▽), and TM (0.05%)/AM (50 μM)/EDTA (5 mM) (▼). Each data point represents the mean of the data obtained from three rabbits.

of Tyr-Gly fragment was found in the presence of 5 mM EDTA. The Tyr-Gly-Gly fragment was not detected with an EDTA concentration of 0.1 mM. These results suggest that EDTA is a more effective inhibitor for enkephalinase A than for enkephalinase B. On the other hand, TP did not show a significant inhibitory effect on the degradation of Met-Enk in the nasal extract, as shown in Table 5.

As previously discussed, the use of any single inhibitor alone is not sufficient to achieve total stabilization of Met-Enk, even though it may have strong inhibitory action against one or two enkephalin-degrading enzymes in various rabbit mucosal extracts. This finding led us to extend our investigation to study the inhibitory effect of various combinations of inhibitors. The results compared in Fig. 7 indicate that the degradation of Met-Enk in the nasal mucosal extracts was inhibited to varying degrees in the presence of various peptidase inhibitors, alone or in combination, during 24 h of incubation at 37° C. In the presence of 0.05% TM, 50 μM AM, and 5 mM EDTA, 80.9% of Met-Enk was found to stay intact after 24 h, indicating that a remarkable stabilization of Met-Enk had been achieved in the nasal extract. The stabilization of Met-Enk in the nasal extract was found to follow the rank

Table 6
Effect of various enzyme inhibitors on the degradation of Met-Enk [a] and the formation of various hydrolytic fragments in nasal extracts [b]

Inhibitor	% Met-Enk remaining	% hydrolytic fragment found [c]			Total (%)
		Tyr	Tyr-Gly	Tyr-Gly-Gly	
No inhibitor	37.5 (±11.5)	39.4 (±11.1)	12.2 (±4.8)	6.0 (±2.0)	95.1
(A) TM (0.05%)	69.4 (±4.6)	14.1 (±5.0)	ND [d]	17.3 (±2.4)	100.8
(B) AM (50 μM)	54.6 (±11.2)	14.1 (±2.7)	11.9 (±4.1)	10.1 (±1.4)	90.7
(C) TM (50 μM)	44.0 (±13.6)	37.0 (±14.9)	12.9 (±3.6)	0.4 (±0.3)	94.3
(D) EDTA (5 μM)	71.9 (±6.8)	17.7 (±4.0)	1.5 (±0.8)	0.7 (±0.5)	91.8
(B) + (C)	65.1 (±6.4)	13.6 (±2.2)	14.2 (±5.2)	0.7 (±0.5)	93.7
(B) + (D)	84.4 (±4.0)	7.4 (±2.9)	1.4 (±0.6)	0.4 (±0.3)	93.6
(A) + (B) + (D)	95.5 (±1.5)	ND	0.7 (±0.5)	0.3 (±0.2)	96.5

[a] Met-Enk at 7.4×10^{-5} M.
[b] Combined mixture of mucosal and serosal extracts obtained from the nasal membrane, which was incubated at 37° C for 3 h.
[c] Data expressed as the means (±SE) of three determinations from three rabbits.
[d] Not detected.

Table 7
Effect of various enzyme inhibitors on the first-order rate constants for the degradation of Met-Enk in various rabbit mucosal extracts [a]

Inhibitor	First-order rate constant [b] (h^{-1}) ($\pm$SE) ($\times 10^2$)					
	Nasal extract		Rectal extract		Vaginal extract	
	Mucosal	Serosal	Mucosal	Serosal	Mucosal	Serosal
No inhibitor	85.50 ($\pm$18.22)	100.74 ($\pm$33.53)	311.16 ($\pm$51.37)	327.72 ($\pm$54.73)	123.00 ($\pm$37.62)	146.46 ($\pm$38.76)
(A) TM (0.01%)	13.87 ($\pm$4.09)	10.40 ($\pm$2.94)	46.30 ($\pm$3.41)	26.92 ($\pm$6.70)	12.21 ($\pm$4.24)	10.92 ($\pm$3.35)
(A) + (B) AM (50 μM)	10.11 ($\pm$3.78)	7.83 ($\pm$1.94)	9.77 ($\pm$0.93)	1.84 ($\pm$0.36)	1.27 ($\pm$0.33)	0.99 ($\pm$0.11)
(A) + (C) TP (50 μM)	7.47 ($\pm$2.71)	6.85 ($\pm$2.23)	17.87 ($\pm$3.98)	6.57 ($\pm$2.03)	4.80 ($\pm$1.93)	5.03 ($\pm$1.26)
(A) + (D) EDTA (5 mM)	0.88 ($\pm$0.06)	1.02 ($\pm$0.07)	0.66 ($\pm$0.02)	0.45 ($\pm$0.11)	0.66 ($\pm$0.13)	0.43 ($\pm$0.02)
(A) + (B) + (C)	0.66 ($\pm$0.03)	0.85 ($\pm$0.02)	2.67 ($\pm$1.44)	0.46 ($\pm$0.04)	0.53 ($\pm$0.02)	0.52 ($\pm$0.01)
(A) + (B) + (D)	0.26 ($\pm$0.05)	0.28 ($\pm$0.03)	0.38 ($\pm$0.09)	0.37 ($\pm$0.03)	0.27 ($\pm$0.10)	0.36 ($\pm$0.03)
k_0/k_{min} [c]	332.7	359.8	816.7	893.0	452.2	402.4

[a] Extracted using isotonic phophate buffer containing 0.01% TM from the beginning.
[b] Data expressed as the means ($\pm$SE) of the data obtained from three rabbits.
[c] Ratios of the first-order rate constant for the degradation of Met-Enk without inhibitor (k_0) over that with combined inhibitors, (A) + (B) + (D).

order of TM/AM/EDTA > AM/EDTA > EDTA > TM > AM/TP > AM > TP > none at the concentrations used. TP alone was least effective in stabilizing Met-Enk, and even when it was combined with AM, its stabilizing effect was low. This result could be attributed to the fact that TP has an insufficient duration of action (Roques et al., 1980). The first-order rate constants for the degradation of Met-Enk degradation are summarized in Table 5. In the presence of TM/AM/EDTA combination, the degradation rate constant decreased 55.1-fold, as compared to that of the control.

In order to evaluate the contribution of each inhibitor in the combination to the protection of Met-Enk, results on the concentrations of Tyr, Tyr-Gly and Tyr-Gly-Gly fragments found after 3-h incubations were summarized and are listed in Table 6. Addition of 0.05% TM alone was noted to reduce the amount of Tyr to approx. 36% of the control, but no Tyr-Gly was detected, as previously described. On the other hand, a significant increase of Tyr-Gly-Gly was observed. The effect of 50 μM AM alone on the formation of Tyr and Tyr-Gly-Gly was similar to that of 0.05% TM alone, but AM did not affect the formation of Tyr-Gly. The observation suggests that AM is not effective in the inhibition of enkephalinase B activity. Addition of 50 μM TP alone was noted to reduce the formation of Tyr-Gly-Gly, which reached 6% of the control, but did not affect the formation of Tyr and Tyr-Gly. However, the addition of 5 mM EDTA alone reduced the formation of Tyr, Tyr-Gly, and Tyr-Gly-Gly to about 45, 13, and 12% of the control, respectively. The results suggest that EDTA inhibits aminopeptidases as well as enkephalinase A and B, which are known to be metallopeptidases and require Zn to be active (Schwartz et al., 1981), by chelating Zn and other metallic ions.

The combination of AM and TP, 50 μM each, was observed to decrease the formation of Tyr and Tyr-Gly-Gly to 34.6 and 12.1% of their respective controls, respectively, but did not affect the quantity of Tyr-Gly formed. The addition of 50 μM AM and 5 mM EDTA to the nasal extract markedly reduced the formation of Tyr, Tyr-Gly, and Tyr-Gly-Gly to 18.8, 11.5 and 6.0% of their respective controls, respectively. Furthermore, in the presence of the combination of 0.05% TM, 50 μM AM, and 5 mM EDTA, Tyr could not be detected, and the formation of Tyr-Gly and Tyr-Gly-Gly was found to be less than 1.0%. This observation suggests that all the enkephalin-degrading enzymes were almost completely inhibited by the additive and composite effects of the combination of three inhibitors. It should be

pointed out that the addition of TM to a mixture of AM and EDTA played a crucial role in enhancing the stability of Met-Enk against the enzymatic degradation in the nasal extract, with as much as 80.9% of Met-Enk found intact after 24 h, compared to only 35.2% with the combination of AM and EDTA for the same duration.

To study the mode of addition of TM on the stabilization of Met-Enk in various mucosal extracts, TM was also added to the extracting medium prior to the extraction of the nasal, rectal, and vaginal membranes. Met-Enk (74 μM) was incorporated into each of the mucosal extracts and incubated, in the absence or presence of single or combined inhibitors, at 37° C over a period of 24 h. Samples were taken periodically and analyzed by HPLC. The effect of various inhibitors, alone or in combination, on the degradation profiles of Met-Enk in various mucosal extracts is compared in Fig. 8. With the incorporation of the AM (50 μM)/EDTA (5 mM) combination into the extracts where TM (0.01%) was added before the extraction, 92.9, 90.6, and 93.2% of Met-Enk, remained intact in the nasal, rectal, and vaginal mucosal extracts, respectively, after 24 h. Similar profiles were also obtained for the serosal extracts under the same conditions (profiles not shown). The addition of TM prior to extraction appeared to be slightly more effective in the stabilization of Met-Enk than that after extraction, since the quantities of Met-Enk staying intact in the nasal extract after 24 h incubation were 11.55 and 6.34% when TM was added at 0.01 and 0.05%, respectively, before and after extraction. This difference in residual concentration may be due to the retardation of microbial growth during the extraction, which may affect the degradation of Met-Enk. It was observed earlier in the presence of 50 μM AM and 50 μM TP that only 26.3% of Met-Enk remained intact in the nasal extract after 6 h (Fig. 7). The presence of 0.01% TM in the AM/TP combination remarkably enhanced the stability of Met-Enk against enzymatic degradation in all the extracts regardless of mucosal and serosal extracts (the amount of Met-Enk staying intact after 24 h was 83.8, 60.4, and 86.9% for the nasal, rectal, and vaginal extracts, respectively). The combination

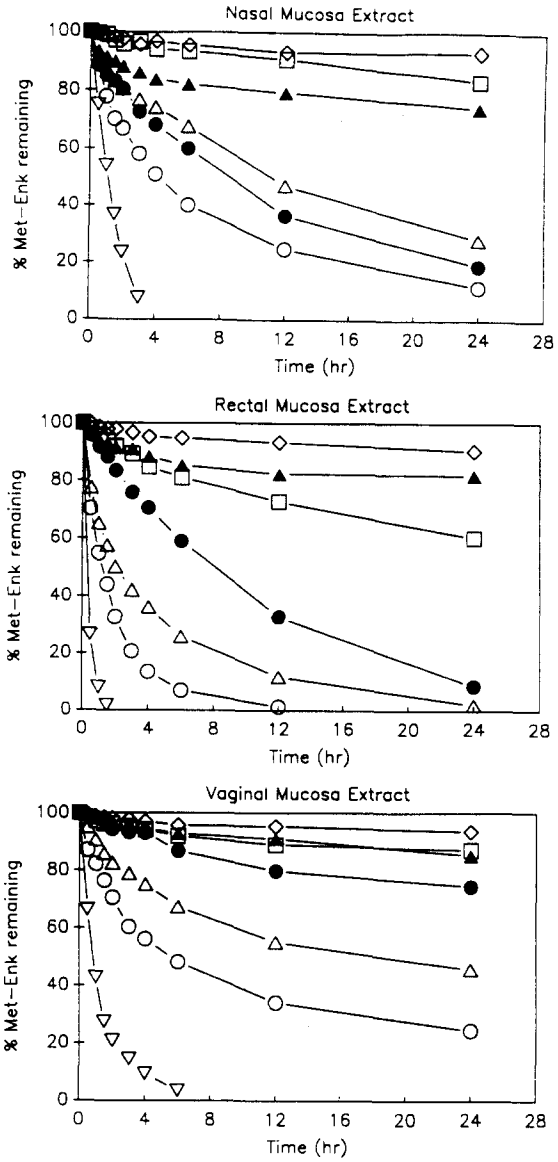

Fig. 8. Degradation profiles of Met-Enk in nasal, rectal, and vaginal mucosa extracts at 37° C in the absence (▽) and presence of TM (0.01%) alone (○), TM (0.01%)/AM (50 μM) (●), TM (0.01%)/TP (50 μM) (△), TM (0.01%)/EDTA (5 mM) (▲), TM (0.01%)/AM (50 μM)/TP (50 μM) (□), and TM (0.01%)/AM (50 μM)/EDTA (5 mM) (◇). Each data point represents the mean of the data obtained from three rabbits.

of 0.01% TM with 5 mM EDTA considerably improved the stability of Met-Enk, since 74.2, 82.0, and 85.1% of the initial Met-Enk concentra-

tions were found intact in the nasal, rectal, and vaginal extracts, respectively, even after 24 h. With a combination of AM (or TP) with TM, the stability of Met-Enk was also considerably enhanced in all the extracts as compared to that when AM or TP was used alone.

The first-order rate constants calculated from the degradation profiles of Met-Enk (Fig. 8) are summarized in Table 7. It is interesting to note that with the use of TM/AM/EDTA combination, the degradation rate constant of Met-Enk was reduced 332.7-, 816.7-, and 452.2-fold in the nasal, rectal, and vaginal extracts, respectively, as compared to those without addition of any inhibitor. This remarkable reduction in Met-Enk degradation is mainly a result of the potent enzyme inhibition properties of TM and its additive effects to those of other inhibitors.

In conclusion, the combinations of inhibitors, such as TM/AM/EDTA and TM/AM/TP, which can simultaneously inhibit the enzymes responsible for the degradation of enkephalin in the nasal, rectal, and vaginal extracts, are useful in the potential delivery of Met-Enk through the mucosae. Furthermore, in the pharmacological and clinical studies on the mechanism of action and behavioral effects of the enkephalinergic peptides, Met-Enk can be fully protected from unwanted enzymatic degradation over a period of 24 h. Judging from the composite effects of these inhibitors, TM might be a suitable adjuvant for the possible clinical use of Met-Enk as a new analgesic and psycho-active drug.

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