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Site-specific degradation and transport of recombinant human epidermal growth factor (rhEGF) in the rat gastrointestinal mucosa

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Abstract

The purpose of the present study was to elucidate the site specificity of degradation of recombinant human epidermal growth factor (rhEGF) in the gastrointestinal mucosa and to screen absorption enhancers and enzyme inhibitors for the development of an rhEGF oral delivery system. The degradation of rhEGF after incubation with the rat mucosal sites was determined by measuring the disappearance of rhEGF as well as the appearance of metabolites by HPLC. Two degradation products of rhEGF, M-I and M-II, were detected. Comparing peak appearance order of rhEGF and its metabolites with the previous reports, M-I and M-II were estimated to be products by oxidation at the methionine residue, and by deamidation at the asparagine residue, respectively. The rhEGF degradation order was duodenum > ileum > stomach > jejunum > colon. rhEGF was rapidly degraded in the duodenum and the ileum, while relatively stable in the colon and jejunum mucosal sites. Sodium caprate slightly inhibited the rhEGF degradation, whereas STDHF or EDTA had no effect on its degradation in the jejunum mucosal sites. The degradation of rhEGF was inhibited by the addition of bestatin, sodium caprate or sodium salicylate in the duodenum mucosal sites. The transport of rhEGF across the gastrointestinal mucosa was investigated using [^{125}I]rhEGF. Possibly due to the strong barrier function of the membrane, the transported amount of [^{125}I]rhEGF across the intestinal mucosa was less than 3% up to 3 h. Moreover, the unlabeled rhEGF (16.7 or 50 μg/ml) had no significant effect on the tracer (0.29 μCi/ml rhEGF) penetration. Effects of various additives on the penetration of rhEGF across the colon were then investigated. Glyceryl palmitostearate, sodium caprate, sodium lauryl sulfate and Tween 80 had no significant effect on the [^{125}I]rhEGF penetration across the colon. Thus, low penetration of rhEGF with or without various additives might be responsible for the barrier function of the membrane rather than the enzymatic degradation.

Keywords: rhEGF; Degradation; Transport; Gastrointestinal mucosa; Enzyme inhibitors; Absorption enhancers

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1. Introduction

A large number of biologically active peptides have recently been evaluated for their therapeutic activity and proposed as candidates for drugs (Ferraiolo and Benet, 1985; Humphrey and Ringros, 1986). Human epidermal growth factor (hEGF) is a single-chain polypeptide containing 53 amino acid residues (MW = 6045) and three disulfide bridges (Senderoff et al., 1994). rhEGF stimulates the proliferation and differentiation of epithelial tissues such as the intestinal mucosa, corneal epithelial tissue, lung and trachea epithelia (Carpenter and Cohen, 1979). Moreover, rhEGF was able to inhibit gastric acid secretion (Bower et al., 1975; Elder et al., 1975; Gregory, 1975; Konturek et al., 1984; Carpenter and Zendegui, 1986) and to protect gastroduodenal mucosa against tissue injury induced by ulcerogenic agents (Gregory et al., 1978; Konturek et al., 1981a,b; Kirkegaard et al., 1983).

Recently many attempts have been made to develop rhEGF preparations not only for the treatment of skin and corneal injury but also for ulcer treatment. However, oral administration of peptides often results in very low bioavailability because of the extensive hydrolysis by digestive enzymes in the gastrointestinal tract. rhEGF degrades via oxidation at the methionine residue (Rao et al., 1986), deamidation at the asparagine residue (Ferraiolo and Benet, 1985), and succinimide formation at aspartic acid (Konturek et al., 1981a,b). The most prevalent chemical reaction for rhEGF degradation is deamidation at the asparagine residue. The asparagine residue is the most labile site in neutral or alkaline pH, high temperature and high ionic strength (Scotchler and Robinson, 1974). It was reported that deamidation was inhibited to a large extent by a surfactant such as Tween (Gonella et al., 1986; Son and Kwon, 1995). The physical instability of EGF came from polymerization of monomer into dimer and trimer by disulfide exchange (Brake et al., 1984). This aggregation could also be prevented by nonionic surfactants (Son and Kwon, 1995).

To develop a rhEGF oral delivery system for the treatment of ulcer, the present study was undertaken to elucidate the site specificity of degradation of rhEGF in gastrointestinal mucosa and to screen the absorption accelerator and stabilizing agents which retard or inhibit hydrolysis (deamidation and peptide bond cleavage). Various additives, which are known as potential stabilizers or absorption enhancers for proteins and polypeptides (Hayakawa et al., 1992; Suda et al., 1976), were used to increase the stability and penetration of rhEGF in the gastrointestinal mucosa.

2. Materials and methods

2.1. Materials

Highly purified recombinant human epidermal growth factor (rhEGF, more than 99% purity) and [^{125}I]rhEGF prepared by genetic engineering was kindly provided by Daewoong Pharm. Co. (Seoul, South Korea). The specific activity of [^{125}I]rhEGF was 0.35016 μCi/μg. Sodium caprate, sodium salicylate, bestatin, soybean trypsin inhibitor (SBTI), bovine serum albumin (Sigma Chemical Co., St. Louis, MO), disodium ethylenediaminetetraacetic acid (Tokyo Chemical Industries, Tokyo, Japan), sodium tauro-24,25-dihydrofusidate (California Biotechnology Inc. Mountain View, CA) and trichloroacetic acid (Acros Organics, NJ) were used. Tris base, Tris–HCl, Tween 80, acetonitrile and methanol were purchased from Fisher Scientific Co. (Fair Lawn, NJ). All other reagents were analytical grade.

2.2. Animals

Male Sprague–Dawley rats (200–250 g) were purchased from Samyook Animal Company (Kyonggi-do, South Korea). The rats were housed under standard conditions in the animal unit of the College of Pharmacy, Chungbuk National University, where the room temperature 22 $\pm$ 1.0°C and the humidity 55 $\pm$ 5%, under a 12-h light–dark cycle (06:00 to 18:00, light period). Rats were deprived of food for about 12–16 h before experiments. Tap water was freely available.

2.3. HPLC analysis

The HPLC system consisted of model L-6000 and L-6200 programmable binary gradient pumps, a model L-4200 UV-Vis detector and model D-2500 integrator (Hitachi Co., Ltd.). Samples were injected into a 50-μl sample loop. Separation was achieved on a 10-μm reversed-phase C_{18} column (μ-Bondapak; 3.9×300 mm, 10 μm) and eluted by a gradient mobile phase: starting from 5 min of 93% system A (7% system B) followed by 27 min linear gradient to 58% system A (42% system B), and then continuation for 5 min before returning to 93% system A. The flow rate was 1.2 ml/min. Mobile phase A consisted of 90% 10 mM K_2HPO_4 (pH 6.5) and 10% acetonitrile. Mobile phase B consisted of 30% 10 mM K_2HPO_4 (pH 6.5) and 70% acetonitrile. Detection was monitored at 214 nm. Ibuprofen lysinate was used as the internal standard. The retention times of rhEGF and ibuprofen lysinate were 21 ± 0.7 and 24 ± 1.2 min, respectively (Fig. 1).

2.4. Enzymatic degradation of rhEGF in the gastrointestinal mucosal sites

rhEGF was dissolved in Tris–HCl buffer (50 mM, pH 7.9) containing 0.01% polysorbate 80. Polysorbate 80 was added to prevent surface adsorption of rhEGF (Kirkegaard et al., 1983). Artificial stomach solution (pH 1.2) and that of intestine (pH 6.8) were prepared on the basis of USP XXIII.

Rat gastrointestinal (GI) segments were excised and mounted in side-by-side diffusion cells (Precision Instrument Design, Los Altos, CA). One half ml of solution (pH 1.2 or 6.8 buffer) were added to the serosal side. An equal volume of the same solution containing rhEGF (100 μg/ml) was added to the mucosal side. The contents of each chamber were mixed by bubbling a 95% O_2–5% CO_2 mixture at the rate of three to four bubbles per second, and the temperature within each chamber was maintained at 37 ± 1°C by a circulating water bath. Periodically, up to 120 min, a 50-μl aliquot was taken from the mucosal side for analysis and replaced immediately by an equal volume of fresh buffer solution. Enzymatic reaction was terminated by adding acetonitrile (200 μl) containing internal standard (ibuprofen lysinate). After acetonitrile was evaporated with N_2 gas, 50 μl of the samples were injected to HPLC.

2.5. Stabilizing effects of various additives on the rhEGF degradation

After 1.5 ml of buffer solution (pH 1.2 or 6.8) was added to the serosal side, an equal volume of the same solution containing rhEGF (100 μg/ml) with or without 500 μM bestatin, 0.5% SBTI, 1% EDTA, 0.5% sodium caprate, 1% sodium salicylate or 1% STDHF was added to the mucosal side. rhEGF degradation in the mucosal side was measured in the same way as described above.

2.6. Transport of rhEGF across the gastrointestinal mucosa

rhEGF penetration across the GI mucosal membranes was investigated using the same diffusion cells as those of degradation study. GI segments were excised and mounted in the side-by-side diffusion cells. One half ml of buffer solution (pH 1.2 or 6.8) was added to the serosal side. An equal volume of the same solution containing of [^{125}I]EGF (0.436 μCi) was added to the mucosal side. The contents of each chamber were mixed by bubbling a 95% O_2–5% CO_2 mixture at a rate of three to four bubbles per second, and the temperature within each chamber was maintained at 37 ± 1°C by a circulating water bath. Periodically, up to 4 h, a 50-μl aliquot was taken from the serosal side for analysis and replaced immediately by an equal volume of fresh buffer solution.

Total radioactivity of the sample was counted using a γ-counter (Packard Cobra II, Canberra Co. Meriden, CT). To determine real radioactivity from protein fraction, 50 μl of 1% bovine serum albumin were added to the sample, and then 500 μl of 15% trichloroacetic acid were mixed. After enough vortexing and centrifuging at 12000 rpm for 4 min, its TCA-precipitable

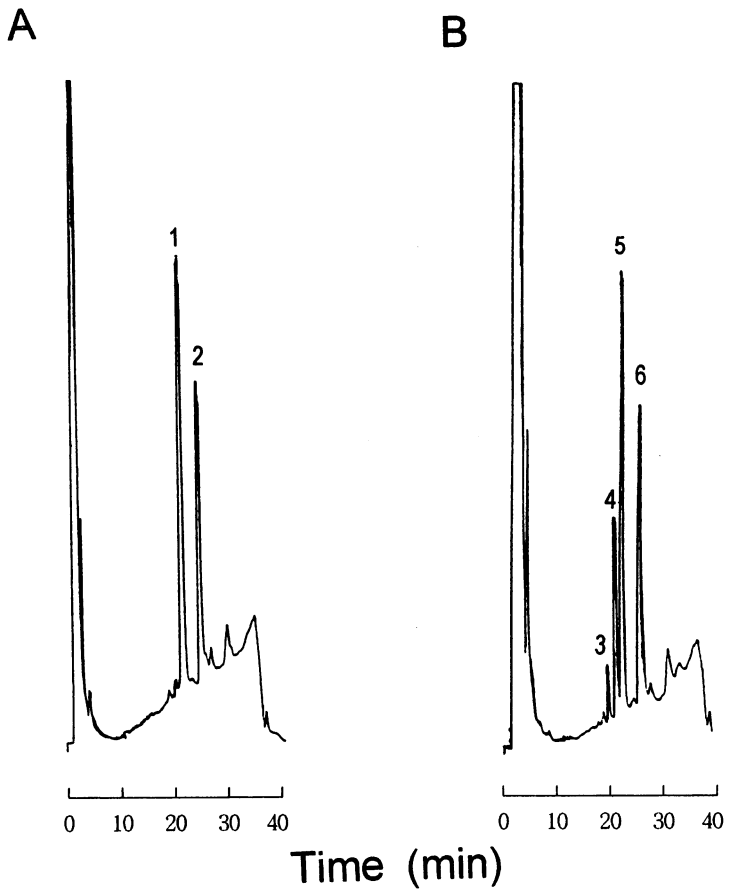

Fig. 1. Chromatograms of rhEGF and its metabolites. (A) rhEGF in PBS. The retention time of rhEGF (1) and internal standard (2) were 20.1 and 24.2 min, respectively. (B) rhEGF and its metabolites in the duodenum mucosal sites. M-I (3; 18.5 min), M-II (4; 19.8 min), rhEGF (5; 20.1 min) and internal standard (6; 24.2 min).

[^{125}I]rhEGF radioactivity was measured using a γ-counter.

Enhancing effects of various additives on the rhEGF penetration across colonic mucosa were investigated. For this purpose, 1.5 ml of [^{125}I]EGF solution (0.436 μCi) containing 1% sodium laurylsulfate, 1% glyceryl palmitostearate, 1% sodium caprate or 1% Tween 80 were added to the mucosal side. Furthermore, unlabeled rhEGF (25 μg, 75 μg) was added to [^{125}I]EGF solution (0.436 μCi) in the donor side to investigate the effect of the rhEGF receptor binding on the permeation. rhEGF transport from the mucosal to the serosal side was determined in the same way as described above.

3. Results and discussion

3.1. Enzymatic degradation of rhEGF in the stomach, duodenum, jejunum, ileum and colon mucosal sites

The degradation of rhEGF after incubation with the stomach, duodenum, jejunum, ileum and colon mucosal sites was determined by measuring the disappearance of rhEGF as well as the appearance of metabolites by HPLC. Two degradation products of rhEGF were detected (Fig. 1). For the sake of convenience, the two degradation products were tentatively named M-I and M-II, respectively.

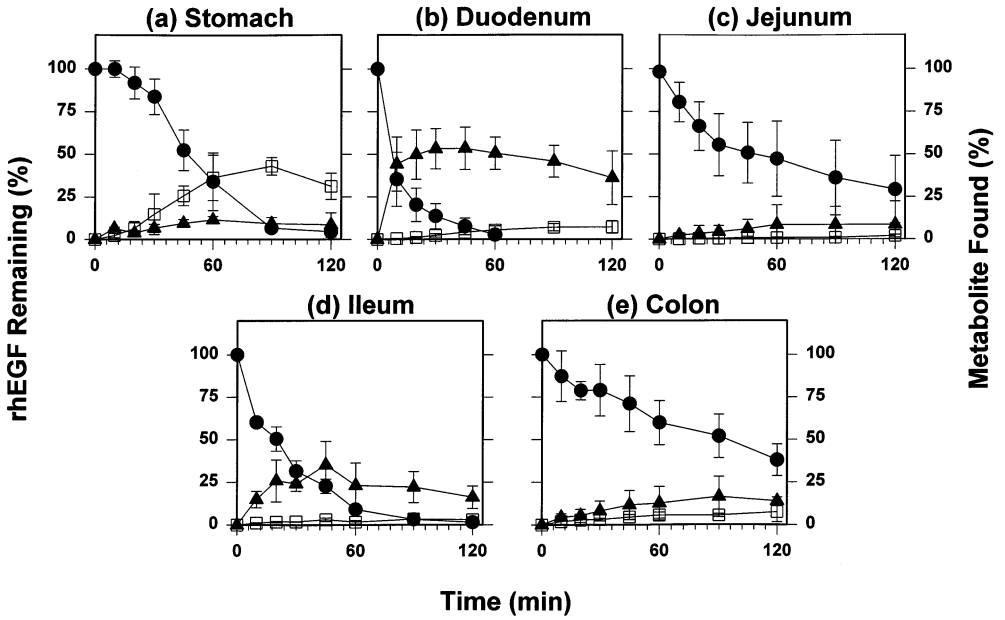

Fig. 2. Degradation of rhEGF and the formation of metabolites in the gastrointestinal mucosal sites using side-by-side diffusion cells. Each point represents the mean ± S.E. of three different experiments. Key: (●) rhEGF; (□) M-I; (▲) M-II.

The rhEGF degradation order was duodenum > ileum > stomach > jejunum > colon (Fig. 2). The degradation half-lives calculated from the disappearance curves are summarized in Table 1. rhEGF was rapidly degraded in the duodenum and its half-life was less than 10 min, while it was relatively stable in the colon and jejunum mucosal sites. Interestingly, the degradation of rhEGF in the ileum, a lower part of the small intestine, is more pronounced than that in the jejunum, an upper part of the small intestine, perhaps due to enrichment of proteolytic enzymes in the lower small intestinal segments. As a result, the colon or jejunum appeared to be a preferred route for rhEGF, on the basis of only presystemic metabolism.

M-II was the main degradation product of rhEGF in the duodenum, jejunum, ileum and colon mucosal sites (Fig. 2). Son and Kwon (1995) reported that the most predominant degradation reaction of rhEGF is deamidation at the asparagine residue. The deamidation reaction was found to be favored at pH > 6 (Senderoff et al., 1994). It was, therefore, assumed that M-II might be produced by deamidation at the asparagine residue. On the other hand, M-I was detected in a small amount in all intestinal mucosal sites. By contrast, M-I was the major degradation product in the stomach mucosal sites (Fig. 2). Comparing peak appearance order of rhEGF and its metabolites with the results of Senderoff et al. (1994), M-I was estimated to be produced by oxidation at the methionine residue.

The degradation of rhEGF in buffer solution (pH 1.2) without mucosa was then determined. rhEGF was slightly degraded, but no metabolites were detected (Fig. 3). Over 80% of rhEGF was intact until 1.5 h incubation in the artificial stomach solution, indicating that rhEGF metabolism in stomach mucosa may be attributed to enzymatic degradation.

3.2. Penetration of rhEGF across the intestinal mucosa

The transport of rhEGF across the intestinal mucosa was investigated using [^{125}I]rhEGF. Fig. 4 shows the transport profiles of [^{125}I]rhEGF (0.29 μCi/ml) through the duodenum, jejunum, ileum and colon membrane. Possibly due to the strong

Table 1
Half-life and percentage of remaining rhEGF after degradation in the gastrointestinal mucosal sites[a]

Gastrointestine	% remaining				Half-life (min)
	10 min	20 min	30 min	60 min	
Stomach	94.7 ± 4.81	90.9 ± 9.32	83.9 ± 10.4	36.5 ± 16.9	48.5 ± 10.1
Duodenum	36.5 ± 15.9	22.3 ± 9.86	15.2 ± 7.39	5.01 ± 2.32	8.92 ± 2.11
Jejunum	80.2 ± 11.5	67.0 ± 14.2	57.1 ± 18.3	48.1 ± 22.1	53.1 ± 9.32
Ileum	60.1 ± 0.72	52.1 ± 7.12	31.3 ± 6.21	11.4 ± 2.32	22.0 ± 4.75
Colon	86.6 ± 14.9	80.2 ± 5.37	79.0 ± 15.2	60.5 ± 12.9	100 ± 13.4

[a] The values represent the mean ± S.E. of three different experiments.

barrier function of the membrane, the transported amount of [^{125}I]rhEGF across the intestinal mucosa was less than 3% up to 3 h. The colon showed a little higher permeability compared with the other intestinal segments. This higher permeability in the colon may be attributed to less enzymatic degradation compared to the other intestinal segments (Fig. 2). We therefore determined the penetrated radioactivity across the colon after the addition of [^{125}I]rhEGF (0.29 μCi/ml) in the presence or absence of rhEGF (16.7 or 50 μg/ml). The unlabeled rhEGF had no significant effect on the tracer penetration (Fig. 5).

Recently, specific receptors for EGF in the rat intestinal microvillus membrane were reported (Gonella et al., 1984; Tompson, 1986). EGF proved to be transported by a specific receptor-mediated endocytosis in intestinal epithelial cells of suckling rats (Rao et al., 1986). Very low penetration of rhEGF in the present study might be responsible for the strong barrier function of the membrane, although its specific receptors are present in the membrane surface. These results were supported by the fact that [^{125}I]rhEGF was taken by absorptive villus cells, but its binding and uptake by crypt cells were minimal (Gonella et al., 1986).

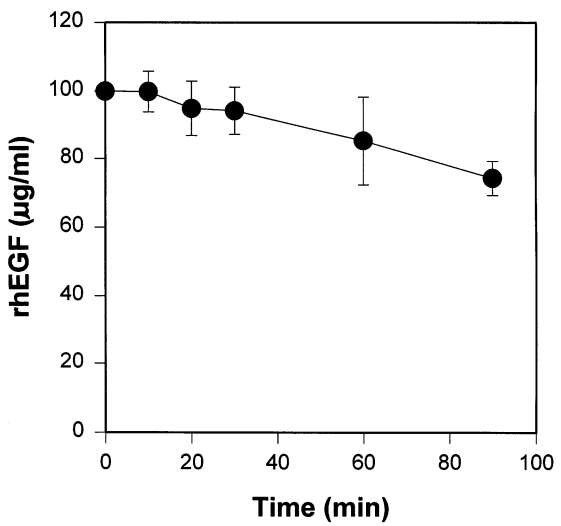

Fig. 3. Degradation profiles of rhEGF in artificial gastric acid (pH 1.2).

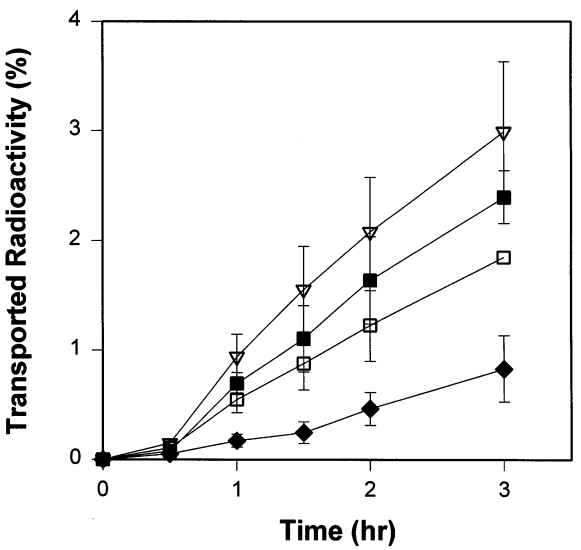

Fig. 4. Penetration of [^{125}I]rhEGF across the intestinal mucosa. [^{125}I]rhEGF was detected in the serosal side. Initial concentration of [^{125}I]rhEGF was 0.29 μCi/ml. Key: (■) duodenum; (◆) jejunum; (□) ileum; (▽) colon.

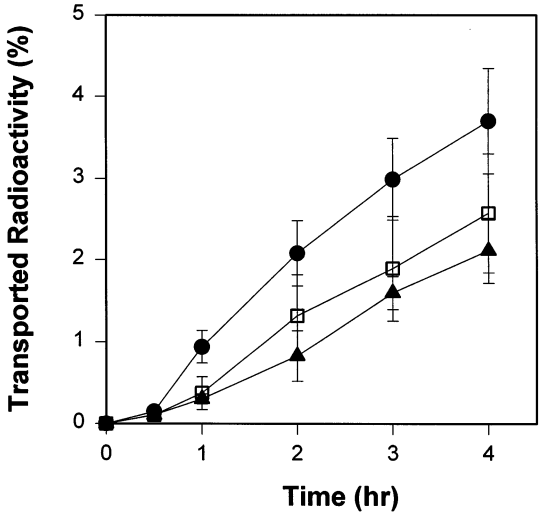

Fig. 5. Penetration of [^{125}I]rhEGF across the colon. Transported radioactivity in the serosal side was determined after the addition of [^{125}I]rhEGF (0.29 μCi/ml) in the presence or absence of rhEGF (16.7 or 50.0 μg/ml). Key: (●) [^{125}I]rhEGF 0.29 μCi/ml; (□) [^{125}I]rhEGF 0.29 μCi/ml + rhEGF 16.7 μg/ml; (▲) [^{125}I]rhEGF 0.29 μCi/ml + rhEGF 50.0 μg/ml.

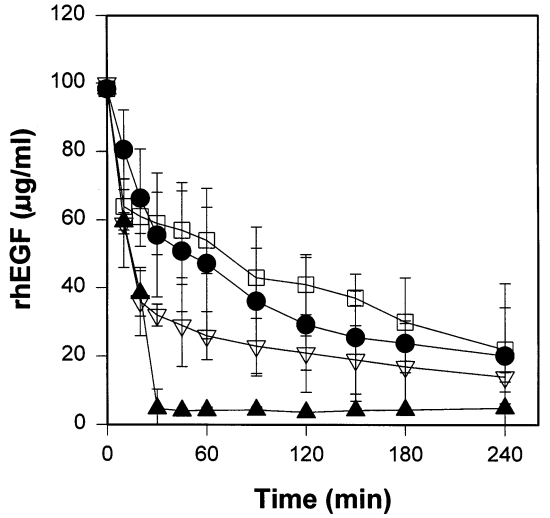

Fig. 6. Effects of sodium caprate, EDTA and STDHF on the degradation of rhEGF in the jejunum. Key: (●) control (no additives); (□) + 0.5% sodium caprate; (▽) + 1% STDHF; (▲) + 1% EDTA.

3.3. Effects of various additives on the rhEGF degradation and penetration in the intestinal mucosal sites

Fig. 6 shows the effects of sodium caprate, STDHF or EDTA on the rhEGF degradation in jejunum mucosal sites. Sodium caprate slightly inhibited the rhEGF degradation, whereas STDHF or EDTA had no effect on its degradation. Unexpectedly, both EDTA and STDHF accelerated the degradation of rhEGF, producing unknown metabolic products (data not shown). These three additives, sodium caprate, STDHF and EDTA, were proved to inhibit the degradation of luteinizing hormone-releasing hormone (LHRH) in vaginal mucosa in the our previous report (Han et al., 1995). This different finding should not be surprising, since rhEGF is a polypeptide containing many (53) amino acid residues compared to LHRH.

We therefore investigated the effects of the other stabilizing additives such as sodium salicylate, bestatin and SBTI, as well as sodium caprate, on the rhEGF degradation in the duedenum, the most susceptible intestinal segment to degradation. The degradation of rhEGF was inhibited by the addition of bestatin or sodium salicylate in the duodenum mucosal sites (Fig. 7). Bestatin and sodium salicylate raised the half-life of rhEGF by

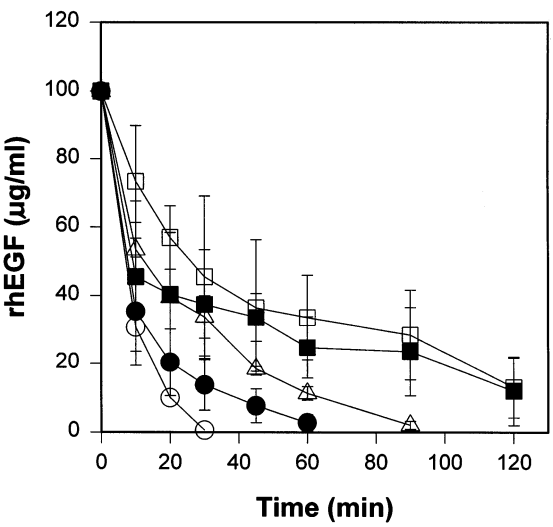

Fig. 7. Effects of sodium caprate, sodium salicylate, STBI and bestatin on the degradation of rhEGF in the duodenum. Key: (●) control (no additives); (■) + 1% sodium caprate; (□) + 500 μM bestatin; (△) + 1% sodium salicylate; (○) + 0.5% SBTI.

about 3 and 1.5 times, respectively. Sodium caprate also showed a stabilizing effect on the rhEGF degradation. The effect of sodium caprate was greater in the duodenum than in the jejunum (Figs. 6 and 7). By contrast, SBTI had no effect on the degradation. The addition of three additives, bestatin, sodium salicylate and sodium caprate, abolished M-I product which is produced by the oxidation at methionine residue of rhEGF.

On the other hand, glyceryl palmitostearate, sodium lauryl sulfate and Tween 80, three known membrane penetration enhancers, had no significant effect on the [^{125}I]rhEGF penetration across the colon (data was not shown). The penetration of [^{125}I]rhEGF was also not changed by the addition of sodium caprate which inhibited the rhEGF degradation in the intestinal mucosa (Figs. 6 and 7). The intact rhEGF was over 40% of the initial amount by 2 h in the colon mucosal sites (Fig. 2). Thus, low penetration of rhEGF with or without various additives might be due to the barrier function of the membrane rather than the enzymatic degradation.

Acknowledgements

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