# Effects of Various Protease Inhibitors on the Intestinal Absorption and Degradation of Insulin in Rats

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The effects of protease inhibitors on the intestinal absorption of insulin were investigated *in situ* in closed small and large intestinal loops in rats, and the stability of insulin was examined in homogenates of the small and large intestine. The intestinal absorption of insulin was evaluated by its hypoglycemic effect. When insulin alone was administered into small or large intestinal loops, no marked hypoglycemic response was observed in either region. Of the coadministered protease inhibitors, soybean trypsin inhibitor (1.5, 10 mg/ml) marginally promoted insulin absorption from the large intestine, whereas aprotinin (10 mg/ml) did to a moderate degree. However, a significant hypoglycemic effect was obtained following large intestinal administration of insulin with 20 mM of Na-glycocholate, camostat mesilate and bacitracin, when compared with the controls. In contrast, we found little hypoglycemic effect following small intestinal coadministration of insulin with these protease inhibitors. In the stability experiment, bacitracin, camostat mesilate and Na-glycocholate were effective in reducing insulin degradation in both small and large intestinal homogenates. It was found that the reduction in the proteolytic rate of insulin was related to the decrease in plasma glucose concentration by these protease inhibitors in the large intestine. These findings suggest that coadministration of protease inhibitors would be useful for improving the large intestinal absorption of insulin.

KEY WORDS: insulin; protease inhibitors; intestinal absorption; hypoglycemic effect; regional difference.

## INTRODUCTION

Generally, peptides and proteins, such as insulin and calcitonin, are administered parenterally because, with oral administration, they are degraded by the proteolytic enzymes in the gastrointestinal tract or are impermeable to the intestinal mucosa due to their hydrophilic characteristics and large molecular size (1). Various approaches such as alternative routes (2–7), absorption enhancers (8), protease inhibitors (1), chemical modification (9–11) and dosage forms (12), have been examined to overcome the delivery problems of these peptides and proteins via the gastrointestinal tract. Of these approaches, the use of protease inhibitors has been shown to improve both the small and large intestinal absorption of peptides. However, it remains to be determined whether the promoting effects of some protease inhibitors are better than others in a single study, and whether the promoting effects of these protease inhibitors are site-dependent. Recently, Morishita et al. (13) reported that the effect of aprotinin on the intestinal absorption of insulin was site-dependent, but they did not investigate whether aprotinin inhibited the degradation of insulin in the gastrointestinal tract.

In the present study, insulin was chosen as a model peptide and regional differences in the effects of various protease inhibitors on the small and large intestinal absorption of insulin were investigated in rats. We also examined the effects of these protease inhibitors on the stability of insulin in homogenates of the small and large intestinal mucosae.

## MATERIALS AND METHODS

### Materials

Insulin, Na-glycocholate and aprotinin were purchased from Sigma Chemical Co., (St Louis, MO). Bacitracin, soybean trypsin inhibitor and glucose B test Wako were obtained from Wako Pure Chemical Industries Co., (Osaka, Japan). Camostat mesilate was a gift from Ono Pharmaceutical Co. Ltd., (Osaka, Japan). All other chemicals and solvents were of reagent grade.

### Animal Experiments

Absorption experiments were performed by an *in situ* closed loop method (9,10). Male Wistar albino rats, weighing 240–300 g, were anesthetized with sodium pentobarbital (32 mg/kg body weight) injected intraperitoneally (i.p.). Animals were fasted for about 16 hr prior to experiments but allowed water ad libitum. The intestine was exposed through a midline abdominal incision, and a small or large intestinal loop was prepared by cannulation with silicone tubing (i.d., 3 mm: o.d., 5 mm) at the proximal and distal ends of the small or large intestine, respectively. Insulin was dissolved in isotonic phosphate buffer at pH 7.4 to a final concentration of 80 IU/kg body weight. The dosing solutions were added with protease inhibitors such as aprotinin (10 mg/ml), bacitracin (10 mM, 20 mM), soybean trypsin inhibitor (1.5 mg/ml, 10 mg/ml) or camostat mesilate (20 mM). The drug solution was warmed to 37°C and 5 or 2 ml was injected into the small or large intestinal loop without washing the luminal surface of the intestine, respectively. Insulin solution (0.1 IU/rat) was intravenously administered by bolus injection to calculate the pharmacological availability % (PA%) in each experiment.

### Preparation of Mucosal Tissue Homogenates

Mucosal tissue homogenates were prepared as previously described (14). Briefly, twelve Wistar albino rats, weighing 240–300 g, were anesthetized with sodium pentobarbital (32 mg/kg body weight, i.p.). Animals were fasted for about 16 hr prior to experiments but allowed water ad libitum. After washing luminal surface with saline solution, small and large intestinal mucosae were removed by scraping the epithelial cell layers. These specimens were pooled by tissue type and stored at −80°C. Immediately before each

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experiment, specimens were thawed at room temperature, and then homogenated in 1–2 ml of isotonic phosphate buffer (pH 7.4) at 4°C using a polytron homogenizer. The homogenate was centrifuged at 5,000 × g in a refrigerated (4°C) centrifuge for 10 min to remove cellular and nuclear debris. The resulting supernatant was diluted with isotonic phosphate buffer to a protein concentration of 10 mg/ml, as determined by the Lowry method with bovine serum albumin as the standard (15).

### Degradation of Insulin in the Intestinal Mucosal Homogenates

The degradation of insulin was studied by incubating 240 μl of a tissue supernatant, which had been preincubated at 37°C for 15 min, with 360 μl of 0.02 mM insulin solution in the presence or absence of a given concentration of Na-glycocholate (20, 50 mM), soybean trypsin inhibitor (1.5, 10 mg/ml), camostat mesilate (20 mM), bacitracin (10, 20 mM), or aprotinin (10 mg/ml). At pre-determined times up until 120 min, 50 μl aliquots were withdrawn from the incubation mixture, to which 100 μl of 50% acetic acid was added, thereby terminating the reaction. The resulting mixture was centrifuged at 10,000 rpm for 5 min to remove the precipitated protein. These samples were analyzed by HPLC.

### Analytical Methods

The intestinal absorption of insulin was estimated by its hypoglycemic effect. For determination of glucose concentration in plasma, 200 μl blood samples were taken periodically from the left jugular vein after dosing, centrifuged at 5,000 rpm for 5 min, and the plasma samples were collected. The plasma glucose concentrations were determined by Glucose Test Wako (Wako Pure Chemical Industries, Co., Osaka, Japan). The decrement in plasma glucose level (D%) was calculated by a modification of the method of Hiral et al. (2) from the following equation:

$$D\% = \left(1 - \frac{AUC_{0\rightarrow 240}}{100\% \times 240\ \text{min}}\right) \times 100$$

Here, the area above 100% line was ruled out for calculating the $AUC_{0\rightarrow 240}$. The D% value was 4.4% when insulin solution (0.1 IU/rat) was intravenously administered by bolus injection.

The stability of insulin was assayed by reverse phase HPLC on a Vydac protein and peptide C18 column (150 × 4.6 mm, 5 μm). HPLC apparatus consisted of a Waters LC Module 1 and a Shimadzu model C-R4A integrator. The mobile phase was a mixture of acetonitrile and water containing 0.1% trifluoroacetic acid (TFA) adjusted to pH 3 with phosphoric acid. The proportion of acetonitrile in the mobile phase was increased linearly from 10 to 34% during the first 23 min, maintained at 34% for the next 11 min, and increased linearly to 40% for another 6 min. The flow rate was 1.1 ml/min. Insulin eluted from the column was detected by UV absorption monitored at 210 nm.

### Statistical Analyses

Results were expressed as the mean ± S.E. and statistical analyses were assessed using Student's *t*-test.

## RESULTS

### Effects of Various Protease Inhibitors on the Intestinal Absorption of Insulin

No significant changes in plasma glucose concentration were observed when isotonic phosphate buffer was administered to the small or large intestine (data not shown). Fig. 1 shows the time course of glucose concentration in plasma after large intestinal administration of insulin solution (20 IU/rat) in the absence or presence of various protease inhibitors. Table I summarizes the decrement in plasma glucose levels (D%) and pharmacological availability (PA%) of insu-

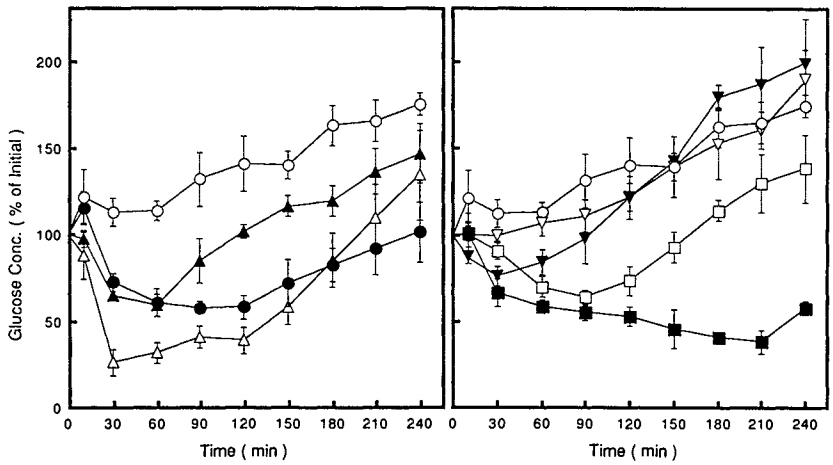


Fig. 1 Concentration-time profile of glucose in plasma after large intestinal administration of insulin in the presence of various protease inhibitors. (○) control, (△) 20 mM Na-glycocholate, (▲) 10 mM bacitracin, (●) 20 mM bacitracin, (□) 10 mg/ml aprotinin, (■) 20 mM camostat mesilate, (▽) 1.5 mg/ml soybean trypsin inhibitor, (▼) 10 mg/ml soybean trypsin inhibitor. The glucose concentrations were expressed as percentage values of that at time zero. Each point represents the mean ± S.E. of 4 rats.

Table I. Effects of Various Protease Inhibitors on the Small and Large Intestinal Absorption of Insulin

| Protease inhibitors | Conc. | Small intestine D% | Small intestine PA%[c] | Large intestine D% | Large intestine PA%[c] |
|---|---|---|---|---|---|
| Control | — | 0.0 | 0.0 | 0.0 | 0.0 |
| Na-GC[a] | 20 mM | 2.3 ± 1.1 | 0.3 | 45 ± 4.1** | 5.1 |
| | 50 mM | 2.9 ± 1.2 | 0.3 | — | — |
| Aprotinin | 10 mg/ml | 0.2 ± 0.2 | 0.0 | 14 ± 2.4* | 1.6 |
| Camostat | 20 mM | 1.2 ± 0.9 | 0.1 | 45 ± 4.9* | 5.1 |
| STI[b] | 1.5 mg/ml | — | — | 1.0 ± 0.4 | 0.1 |
| | 10 mg/ml | 0.8 ± 0.3 | 0.1 | 6.2 ± 2.2 | 0.7 |
| Bacitracin | 10 mM | — | — | 12 ± 1.9* | 1.3 |
| | 20 mM | 0.0 | 0.0 | 31 ± 2.0** | 3.5 |

[a] Na-glycocholate.
[b] Soybean trypsin inhibitor.
[c] Pharmacological availability % = D% G.I./D% I.V. × Dose I.V./Dose G.I. × 100.
The D% values are expressed as the mean ± S.E. of 4 rats. (**) $p < 0.01$, (*) $p < 0.05$, compared with the control.

lin following small or large intestinal administration with these protease inhibitors. When insulin alone was administered to the small intestine, no remarkable hypoglycemic response was noted during the 4 hrs of observation. Furthermore, in the presence of all these inhibitors, no significant hypoglycemic effect was observed following small intestinal administration of insulin (data not shown). In contrast, a marked decrease in plasma glucose levels was observed after large intestinal coadministration of insulin with 20 mM of Na-glycocholate, camostat mesilate or bacitracin as compared with insulin alone, whereas a slight hypoglycemic effect was observed with aprotinin (10 mg/ml) or bacitracin (10 mM). However, no significant hypoglycemic effect was observed following large intestinal administration of insulin with soybean trypsin inhibitor or with no protease inhibitors. Overall, these protease inhibitors were more effective in enhancing insulin absorption from the large intestine than from the small intestine, and Na-glycocholate, camostat mesilate and bacitracin showed the greatest enhancing effects.

### Stability of Insulin in the Gastrointestinal Homogenates

The effects of various protease inhibitors on the degradation of insulin in homogenates of the small or large intestinal mucosae are shown in Fig. 2. Insulin disappearance followed first order kinetics. Na-glycocholate (20 mM, 50 mM), camostat mesilate (20 mM) and bacitracin (10 mM, 20 mM) were effective in reducing the degradation of insulin in both homogenates. Table II summarizes effects of protease inhibitors on the half life of insulin hydrolysis in homogenates of the small and large intestine. The half-life values were calculated from the first order rate constants obtained from the semilogarithmic plots shown in Fig. 2. The rank order of effectiveness for decreasing insulin hydrolysis in homogenate of the small intestine was 50 mM Na-glycocholate > 20 mM bacitracin > 20 mM Na-glycocholate > 20 mM camostat mesilate > 10 mg/ml aprotinin > 10 mg/ml soybean trypsin inhibitor. In the large intestinal homogenate, all protease inhibitors, with the exception of 1.5 mg/ml soybean trypsin inhibitor, were effective in reducing the degradation of insulin, and the maximum reduction in proteolytic rate of insulin was seen in the presence of 20 mM bacitracin (Table II).

## DISCUSSION

In the present study, we observed that coadministration of insulin with a variety of protease inhibitors were more effective in improving the insulin absorption in the large intestine than in the small intestine. This result was inconsistent with those of previous reports by Kidron et al. (16) and Morishita et al. (13), who observed that insulin coadministered with aprotinin or soybean trypsin inhibitor into the ileum reduced plasma glucose concentrations, while no ef-

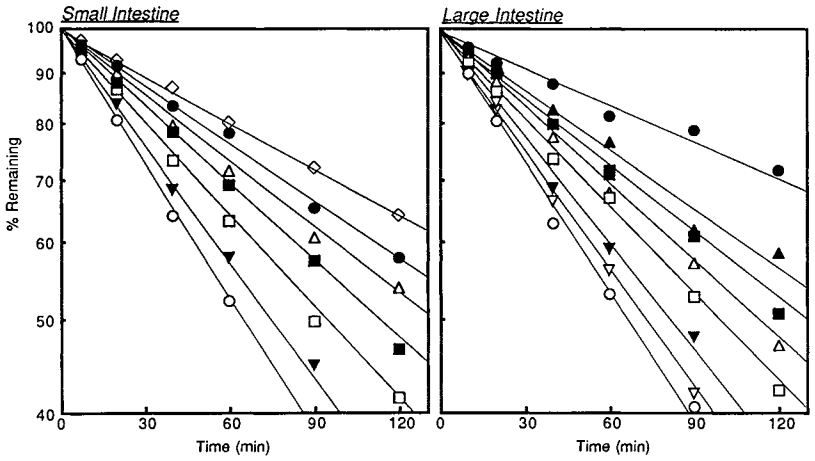


Fig. 2 Effects of various protease inhibitors on the degradation of insulin in the small and large intestinal homogenates. Keys: (○) control, (△) 20 mM Na-glycocholate, (◇) 50 mM Na-glycocholate, (□) 10 mg/ml aprotinin, (■) 20 mM camostat mesilate, (▽) 1.5 mg/ml soybean trypsin inhibitor, (▼) 10 mg/ml soybean trypsin inhibitor, (▲) 10 mM bacitracin, (●) 20 mM bacitracin. Each point represents the mean of 3 experiments.

Table II. Effects of Protease Inhibitors on Half-Lives of Insulin Hydrolysis in Homogenates of the Small and Large Intestine

| | | Small intestine | | Large intestine | |
|---|---|---|---|---|---|
| | | $T_{1/2}$ (min) | ratio | $T_{1/2}$ (min) | ratio |
| Control | | 66 ± 3.8 | 1.0 | 71 ± 2.6 | 1.0 |
| Na-GC[a] | (20 mM) | 140 ± 4.0*** | 2.1 | 110 ± 2.3*** | 1.5 |
| | (50 mM) | 190 ± 6.5*** | 2.9 | — | — |
| Aprotinin | (10 mg/ml) | 98 ± 5.7 | 1.5 | 100 ± 4.7* | 1.4 |
| Camostat | (20 mM) | 110 ± 2.2*** | 1.7 | 140 ± 6.6** | 2.0 |
| STI[b] | (1.5 mg/ml) | — | — | 82 ± 9.1 | 1.2 |
| | (10 mg/ml) | 74 ± 4.6 | 1.1 | 90 ± 1.0* | 1.3 |
| Bacitracin | (10 mM) | — | — | 170 ± 8.9** | 2.4 |
| | (20 mM) | 170 ± 3.2*** | 2.6 | 300 ± 4.2*** | 4.2 |

[a] Na-glycocholate.
[b] Soybean trypsin inhibitor.
The $T_{1/2}$ values are expressed as the mean ± S.E. of 3 experiments.
(***)$p < 0.001$, (**) $p < 0.01$, (*) $p < 0.05$, compared with the control.

fect was observed in the colon. This discrepancy may be attributed to the presence or absence of luminal enzymes during the absorption experiments. That is, in our experiment, the luminal surface was not washed with a saline solution and the large amounts of luminal enzymes responsible for insulin proteolysis may be present in the small intestinal loops. In contrast, a washing step was included in the previous reports and most of the luminal enzymes were washed away from the gut. Indeed, our pilot study indicated that after washing the luminal surface of the small intestine, a significant hypoglycemic response was observed in the presence of these protease inhibitors.

The regional difference in the effects of protease inhibitors on the intestinal absorption of insulin may be explained by the elevated activities of the proteolytic enzymes responsible for insulin hydrolysis in the small intestine compared with the large intestine. Alternatively, there may be differences in the types of proteolytic enzymes involved in insulin hydrolysis in the intestinal fluids or mucosae between the small and large intestine. However, in our stability experiments, these protease inhibitors effectively reduced insulin degradation in both small and large intestinal homogenates, and no site-dependent effects were noted for the reduction in insulin degradation by the protease inhibitors in mucosal homogenates between the small and large intestine. This positive result of protease inhibitors in the stability experiment was not in agreement with the results of the in vivo small intestinal absorption studies. Presumably, insulin may be degraded not only in homogenate of the small intestinal mucosa but also in the small intestinal fluids, and the observed regional differences in the intestinal absorption of insulin may be due to the instability of insulin in the small intestinal fluids. This idea was supported by our preliminary study that insulin was extremely unstable in the luminal mucosal fluids rather than in homogenate of the small intestine (the half-life for insulin proteolysis was 11.5 min in the small intestinal fluids.).

On the other hand, in the large intestine, a reduction in the proteolytic rate and corresponding decrease in plasma glucose concentration was observed in the presence of protease inhibitors, and a significant reduction was observed in both the proteolytic rate and hypoglycemic effect in the presence of bacitracin and camostat mesilate. However, in the presence of Na-glycocholate, no clear relationship was obtained between in vivo absorption and in vitro stability study in the large intestinal tract. That is, the proteolytic rate of insulin in homogenates of the large intestine was slightly reduced with Na-glycocholate, although we found a predominant hypoglycemic effect following large intestinal administration of insulin with Na-glycocholate. This discrepancy may be attributed to both absorption enhancing (17) and protease inhibitory actions (14) of Na-glycocholate. Indeed, Gordon et al. (18) suggested that bile acids interact with cell membranes to form a reverse micelle which acts as a channel to increase permeation by the test solution. Further, leucine aminopeptidase activity has been reported to be inhibited by various bile salts in the nasal homogenates of rats (2). Furthermore, our previous reports demonstrated that the hydrolysis of insulin in the various mucosal homogenates of rats was inhibited by Na-glycocholate (14), and that this bile salt inhibits the association of insulin (19). From these findings, it is suggested that Na-glycocholate not only increases the large intestinal absorption of insulin by reducing the degradation of insulin in the large intestine, but may also enhance the membrane permeability of insulin by acting the epithelial membrane and by reducing insulin association.

Bacitracin, a cyclic polypeptide antibiotic obtained from *Bacillus Licheniformis*, has been used to inhibit the degradation of various peptides and proteins, and to increase their absorption from various absorptive mucosae (7,20,21). This previous finding correlated well with the present observation that the large intestinal absorption of insulin was improved by coadministration with bacitracin by inhibiting its degradation in the large intestinal mucosae.

Camostat mesilate inhibits the activities of aminopeptidase and proteases such as trypsin, plasmin and kallikrein. Morimoto et al. (22) reported that this compound enhanced nasal absorption of vasopressin and was slightly absorbed through the nasal mucosa. Therefore, our present result suggests that camostat mesilate may have inhibitory effects on the activities of proteolytic enzymes and increases the large intestinal absorption of insulin.

The reason for the lack of a marked increase in the large intestinal absorption of insulin on coadministration with soybean trypsin inhibitor is not clearly understood, although the hydrolysis of insulin was reduced by this protease inhibitor. We speculate that the concentration of the protease inhibitor may have been too low to elicit a reduction in insulin degradation *in vivo* in the gastrointestinal tract.

At present, we have not examined the type of proteolytic enzymes responsible for the hydrolysis of insulin in the gastrointestinal tract. However, it was assumed that there exist aminopeptidase-like and trypsin-like proteases which may play an important role on the degradation of insulin in the gastrointestinal homogenates, because bacitracin and Na-glycocholate inhibit the activities of aminopeptidase (21), while the activities of trypsin and chymotrypsin are reduced by the addition of aprotinin and soybean trypsin inhibitor (23).

In conclusion, the present study demonstrated that the effects of protease inhibitors on the insulin absorption from the gut are site-dependent. Protease inhibitors such as Na-glycocholate, camostat mesilate and bacitracin were effective for improving the large intestinal absorption of insulin, and insulin hydrolysis was clearly inhibited by coincubation of these protease inhibitors in the intestinal homogenates. Additional studies of local toxicity and irritation of these protease inhibitors are required for their clinical application.

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