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**Research Papers**

# Novel oral microspheres of insulin with protease inhibitor protecting from enzymatic degradation

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## Summary

Eudragit L100 insulin microspheres (IMS) containing a protease inhibitor were prepared for an oral dosage form of insulin. The efficiencies of insulin incorporation of IMS containing trypsin inhibitor (TI), chymostatin (CS), Bowman-Birk inhibitor (BBI) and aprotinin (AP) amounted to approx. 80% and did not differ among the preparations. In a release study, the more than 90% of insulin remaining after treatment of simulated gastric fluid was released in a medium of simulated intestinal fluid during the initial 60 min. The protective efficiency of the various preparations toward three digestive enzymes (pepsin, trypsin and α-chymotrypsin) was investigated under the chosen experimental conditions. Strong insulin protective efficiency towards pepsinic degradation was observed in all preparations. The highest efficiencies towards each trypsinic and α-chymotrypsinic degradation were achieved with IMS containing TI and IMS containing CS, respectively. IMS containing AP was less resistant to trypsinic and α-chymotrypsinic degradation compared with IMS containing TI towards trypsin and IMS containing CS towards α-chymotrypsin, but was slightly more effective than IMS containing BBI towards both enzymes. On the other hand, complete insulin degradation after respective enzymatic incubation was observed in IMS without inhibitor. These results indicated that IMS containing protease inhibitor could be prepared and would be able to protect insulin from proteolytic degradation.

## Introduction

The treatment of diabetic patients with insulin requires parenteral injections. Since injections are poorly accepted by most patients, other routes for nonparenteral administration are highly desirable. This has led to many investigations of alternative ways such as oral (Fujii et al., 1985; Touitou and Rubinstein, 1986; Damgé et al., 1988), nasal (Moses et al., 1983; Nagai et al., 1984), buccal (Nagai, 1986), rectal (Nishihata et al., 1986), pulmonary (Wigley et al., 1971) and transdermal (Liu et al., 1988) routes for insulin delivery. However, the low biologic effect of these modes of administration restricts their practical use.

Oral administration of insulin is more convenient, provided that insulin is protected from proteolytic degradation in the gastrointestinal tract and that it is transported from the intestinal

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lumen to the blood compartment. Although there are various studies indicating that insulin can be absorbed in a physiologically active form from the intestine of rats (Kidron et al., 1982; Ziv et al., 1987; Schilling and Mitra, 1990), rabbits (Shichiri et al., 1973) and humans (Balsam et al., 1971), the extent of insulin absorption is extremely low.

It has been suggested that absorption of insulin may be enhanced by simultaneous administration of inhibitors of proteolytic enzymes (Kidron et al., 1982; Fujii et al., 1985; Ziv et al., 1987). Protection against proteolysis is believed to be the first step involved in keeping insulin intact in the intestinal lumen. To date, however, information as to whether protease inhibitors promote insulin absorption from the oral route is limited. In addition, only a few studies have been undertaken on the oral dosage forms of insulin with protease inhibitors such as enteric-coated gelatin capsules (Fujii et al., 1985) and water-in-oil microemulsion (Cho and Flynn, 1989).

The aim of our study is to investigate the possibility of developing an oral dosage form which is designed to deliver insulin into the intestine in the presence of protease inhibitor. Insulin and protease inhibitor were incorporated into a polymeric drug carrier in the form of microspheres. The merits of using microspheres are in the simplicity of preparation and the convenience of individualized dosage adjustment. Moreover, enteric-coated pellet formulations are less affected by the digestive state of the individual than are tablets (Bechgaard and Christensen, 1982; Bergdahl et al., 1983). In this study, the insulin incorporation efficiency of the microspheres, release profile and in vitro protective effects of the microspheres towards the degradation of insulin by three digestive enzymes (pepsin, $\alpha$-chymotrypsin and trypsin) were investigated.

## Materials and Methods

*Materials*

Crystalline bovine insulin (Zn-insulin, 24.4 U/mg), soybean trypsin inhibitor, aprotinin, Bowman-Birk inhibitor, pepsin (from porcine stomach mucosa, 2900 U/mg protein), $\alpha$-chymotrypsin (from bovine pancreas, 53 U/mg protein) and trypsin (from bovine pancreas, 14520 BAEE U/mg) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chymostatin was purchased from the Peptide Institute Inc. (Osaka, Japan). Gelatin was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Eudragit L100 was kindly supplied by Higuchi Co., Ltd (Tokyo, Japan). All other chemicals were obtained from commercial sources and were of analytical reagent grade.

*Preparation of the microspheres*

In the present study, Eudragit L100, a pH-dependent copolymer which is soluble at pH 6 and above, was used for the preparation of insulin delivery formulations. As trypsin-chymotrypsin inhibitors, aprotinin (AP; Trautschold et al., 1967) and Bowman-Birk inhibitor (BBI; Birk, 1985) were used. As specific trypsin and chymotrypsin inhibitors, soybean trypsin inhibitor (TI; Birk, 1985) and chymostatin (CS; Umezawa et al., 1970) were used, respectively.

Insulin microspheres (IMS) containing protease inhibitor were prepared according to a previously reported method (Morishita et al., 1991). Briefly, weighed amounts of insulin with or without protease inhibitor were dissolved in 300 $\mu$l of 0.1 N HCl, and then ethanol and Eudragit L100 were added to the solution with stirring at 1200 rpm. The resultant solution was then poured into liquid paraffin. IMS were formed by the addition of a gelatin solution (0.5% w/w). It appeared to be likely that some drug existing on the surface of IMS would be dissolved and come into contact with pepsin in the stomach. Thus, the surface of IMS was further coated with Eudragit L100. All preparations were sized by sieving and the fraction ranging from 180 to 500 $\mu$m was used for the following experiments. The compositions of the microspheres are listed in Table 1. All batches were prepared at least three times.

*Determination of insulin incorporation efficiency of the microspheres*

This experiment was performed for all the preparations listed in Table 1. 20 mg of microspheres was completely dissolved in 10 ml of the

TABLE 1

*Composition [expressed as % (w/w)] of the microspheres*

| Ingredients | Insulin microspheres containing | | | | |
|---|---|---|---|---|---|
| | – | TI | CS | AP | BBI |
| Eudragit L100 | 98 | 97 | 97 | 97 | 97 |
| Insulin | 2 | 2 | 2 | 2 | 2 |
| Trypsin inhibitor (TI) | – | 1 | – | – | – |
| Chymostatin (CS) | – | – | 1 | – | – |
| Aprotinin (AP) | – | – | – | 1 | – |
| Bowman-Birk inhibitor (BBI) | – | – | – | – | 1 |

second fluid (pH 6.8) in the Japanese Pharmacopoeia XI (JP XI). The insulin incorporation efficiency of the products was evaluated according to the following equation:

insulin incorporation efficiency (%) = (assayed insulin amount)/(theoretical insulin amount) × 100.

*In vitro insulin release measurements*

A 300 mg sample of IMS without inhibitor was placed in a rotating basket (mesh size = 100 mesh) and introduced into a double-walled beaker (250 ml) containing 200 ml of the first fluid (pH 1.2, 37°C) in JP XI. The basket was then spun at 50 rpm for 1 h. After this first fluid treatment, the basket was washed with purified water and replaced in the same beaker containing 200 ml of the second fluid of JP XI (pH 6.8) at 37°C with constant stirring at 150 rpm. At appropriate intervals, 2-ml samples were taken from the medium and filtered using a Fine Filter F (10 $\mu$m, Ishikawa Manufactory Co., Ltd, Tochigi, Japan). 2 ml of fresh fluid was added to the beaker immediately after each sampling to maintain a constant volume.

*Enzymatic degradation study*

Pepsinic degradation experiments were performed for all the preparations. A 100 mg sample of the microspheres was incubated for 1 h at 37°C with reciprocal shaking (50 strokes/min) in 10 ml of pepsin solution (weight ratio of insulin to enzyme, 200:1; glycine buffer, pH 1.3). The microspheres were then collected by filtration and washed with purified water. The collected microspheres were then placed in 10 ml of phosphate buffer (pH 7.8) and dissolved completely. Similarly, incubation with non-incorporated insulin was conducted under the same experimental conditions.

For the trypsinic and $\alpha$-chymotrypsinic degradation study, the respective enzymatic solutions (weight ratio of insulin to each enzyme, 1:0.5, 1:1, 1:2; phosphate buffer, pH 7.8) were used. A 20 mg sample of IMS containing TI, BBI and AP was incubated for 3 h at 37°C with reciprocal shaking (90 strokes/min) in 5 ml of trypsin solution. IMS containing CS, BBI and AP was similarly incubated in $\alpha$-chymotrypsin solution. A 100 $\mu$l sample of Bowman-Birk inhibitor solution in phosphate buffer at pH 7.8 (10% w/v) was added to terminate the enzymatic reaction.

For control experiments, all preparations were incubated in enzyme-free solution under the same conditions. The protective efficiency of the preparation towards each enzymatic degradation was expressed as the relative percentage of the residual insulin amount to the control.

*Analytical method*

Determination of insulin was performed by an enzyme immunoassay (EIA) using the Insulin EIA Kit (Dainabot Co., Ltd, Tokyo, Japan).

## Results and Discussion

*Insulin incorporation efficiency*

Table 2 lists the insulin incorporation efficiencies of the preparations. As indicated, approx. 80% insulin incorporation efficiency was attained

TABLE 2

*Insulin incorporation efficiency (%) of the microspheres*

| | Insulin microspheres containing | | | | |
|---|---|---|---|---|---|
| | – | TI | CS | AP | BBI |
| Incorporation efficiency | 78.5 ± 1.9 | 77.3 ± 1.3 | 75.2 ± 9.3 | 78.1 ± 7.8 | 78.6 ± 2.9 |

Each value represents the mean ± S.D. of five determinations.

by all preparations. Moreover, there was no difference among the preparations.

*Release of insulin from the microspheres*

The release of insulin from the microspheres is illustrated in Fig. 1. It was assumed that a number of drug molecules existing on the surface of the microspheres were released in acidic solution, and therefore that a small amount of drug was lost during the treatment with acid. The remaining insulin amount in the microspheres could be determined from the concentration at the plateau phase in the JP XI second fluid release profiles. The percentage of insulin released at 120 and 180 min reached 94.3 ± 4.2 and 94.0 ± 5.9% (mean ± S.D.), respectively. Therefore, more than 90% of the incorporated insulin could be retained after the treatment with acidic solution.

It could be observed that the time required for 90% of the remaining insulin amount to be released in the second fluid is relatively short, approx. 30–60 min. This suggests that the preparations can release considerable amounts of insulin rapidly at pH 6.8, the value corresponding to upper-intestinal regions.

*Protective efficiency of the preparations towards enzymatic degradation*

It is well known that insulin is easily destroyed and inactivated by digestive enzymes such as pepsin, trypsin and chymotrypsin. Many investigations have thereby failed to reduce the blood glucose level following the oral administration of free insulin alone (Patel and Lyman, 1976; Aungst et al., 1988; Damgé et al., 1988). Thus, in order to enhance insulin oral bioavailability, preparations which circumvent these enzymatic barriers are required. To assess the protective effect of the preparations towards enzymatic degradation, various preparations were incubated in the presence of pepsin, trypsin or α-chymotrypsin.

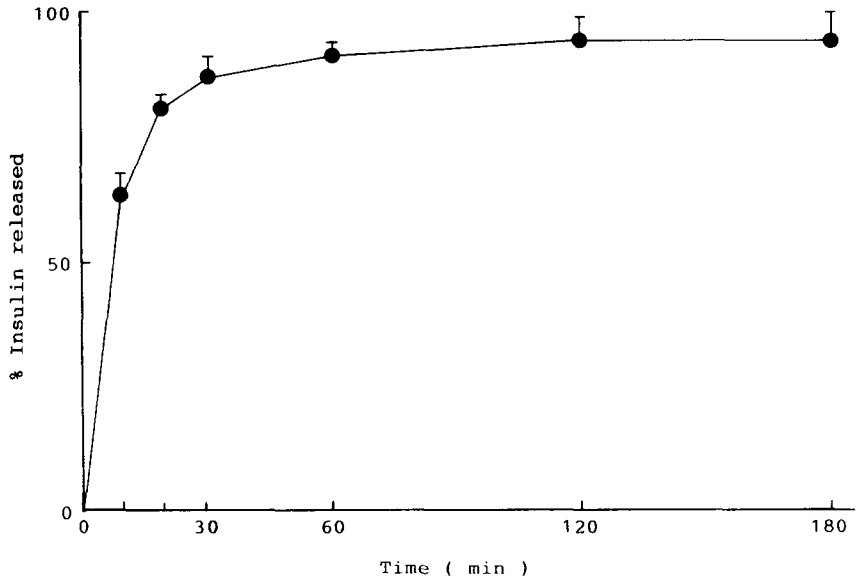


Fig. 1. Release behavior of insulin from the microspheres in the JP XI 2nd fluid after treatment with the JP XI 1st fluid for 1 h. Each point represents the mean ± S.D. of three experiments.

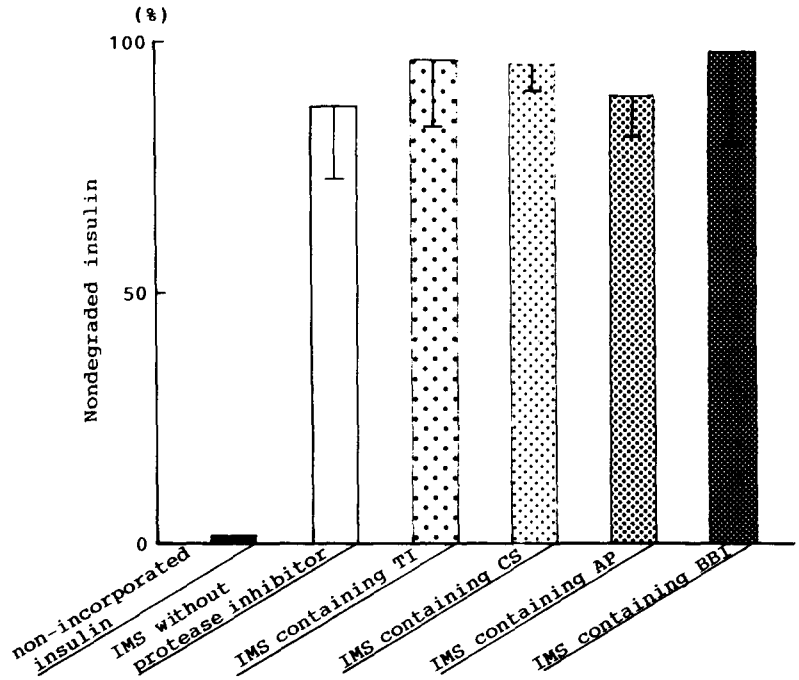


Fig. 2. Protection of insulin towards pepsinic degradation. Each bar represents the mean ± S.D. of three to four experiments.

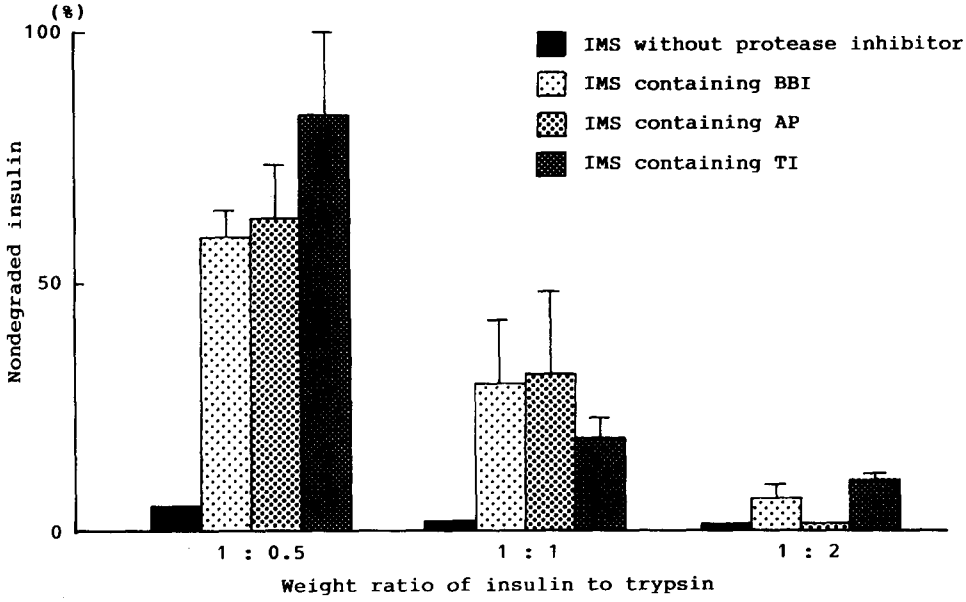


Fig. 3. Protection of insulin towards trypsinic degradation. Each bar represents the mean ± S.D. of five experiments.

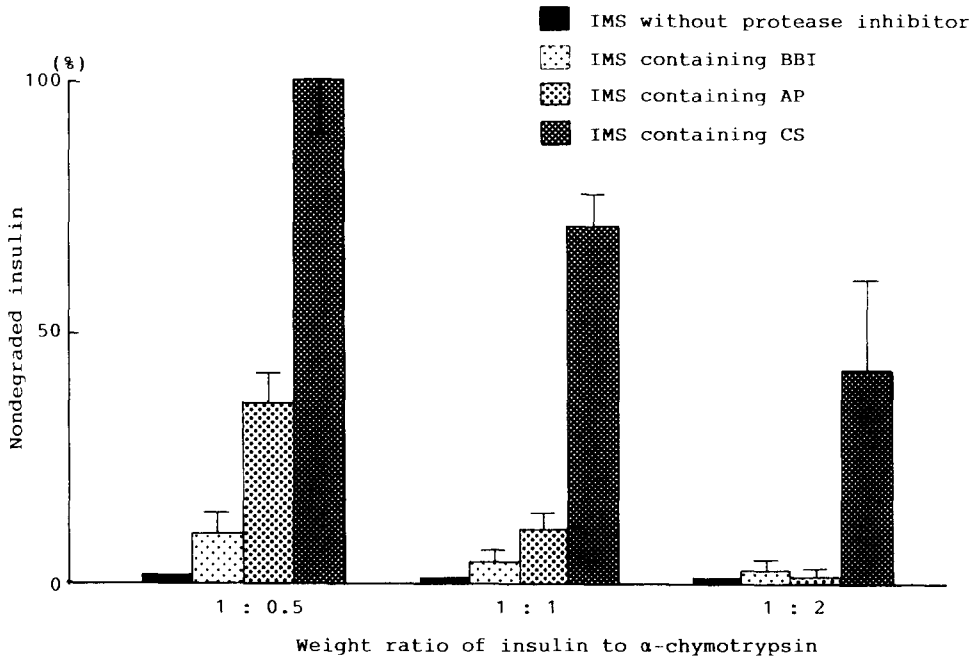


Fig. 4. Protection of insulin towards $\alpha$-chymotrypsinic degradation. Each bar represents the mean $\pm$ S.D. of five experiments.

The results from the pepsinic degradation study are shown in Fig. 2. Non-incorporated insulin was found to be completely degraded during incubation in pepsin solution. On the other hand, for all preparations, insulin loss was scarcely observed when the microspheres were exposed to pepsin solution. The amount of insulin existing on the surface of the microspheres was presumably very low due to coatings on the particles with insulin-free Eudragit L100. This may be largely responsible for the considerable amount of insulin remaining after the acid treatment, irrespective of the existence of pepsin (Figs 1 and 2).

The results from trypsinic and $\alpha$-chymotrypsinic degradation studies are shown in Figs 3 and 4, respectively. After the respective enzymatic incubations, complete insulin degradation was observed in IMS without protease inhibitor. In general, the protective efficiencies of the preparations towards trypsinic and $\alpha$-chymotrypsinic degradation decreased with increasing enzyme concentration. At the lowest trypsin concentration in the incubation medium, the highest degree of insulin protective efficiency was observed with IMS containing TI (Fig. 3). The protective efficiencies of IMS containing both BBI and AP were similar although slightly less than that of IMS containing TI. In the $\alpha$-chymotrypsinic degradation study, IMS containing CS provided the highest insulin protective efficiency at all enzyme concentrations (Fig. 4). In contrast, IMS containing AP or BBI were less resistant to $\alpha$-chymotrypsinic digestion. In both enzymatic degradation studies, IMS containing AP were slightly more effective at protecting insulin when compared with IMS containing BBI.

In conclusion, our results demonstrate that insulin and various protease inhibitors can be incorporated with an enteric-coating polymer, Eudragit L100. These microspheres were able to protect insulin from proteolytic degradation under the chosen experimental conditions. The dosage forms reported here can be considered as a new tool for the development of alternatives to prevailing insulin parenteral therapy.

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