Influence of Liposomes on Tryptic Digestion of Insulin

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The influence of phosphatidylcholine liposomes on the tryptic digestion of insulin was studied to obtain basic information on the interaction between liposomes and peptides or proteins. Protection of insulin from tryptic digestion by liposomes depended on the encapsulation efficiency and the method of preparation of liposomes. REV (reverse phase evaporation vesicles) were most effective for the protection of insulin from tryptic digestion. Tryptic digestion of insulin was accelerated by negatively-charged liposomes (containing 10% phosphatidylserine, phosphatidylinositol, or phosphatidic acid) or by neutral empty liposomes, this digestion being enhanced by progressively smaller liposome size (from 2 to 0.1 μm) in the case of neutral empty liposomes. These results suggest that this enhancement had occurred on the surface of the empty neutral or negatively-charged liposomes. Positively-charged empty liposomes (containing 10% of stearylamine) weakly suppressed the tryptic digestion of insulin. The size of the positively-charged liposomes increased after the addition of insulin, this size increase suggesting that these liposomes were fused by insulin. Presumably, insulin was protected by the lipids around the insulin molecule after the insulin-induced fusion or aggregation of positively-charged liposomes

Keywords liposome; insulin; trypsin; digestion; HPLC

Introduction

Liposomes, which have been investigated as carriers of various drugs,[1,2] can protect the encapsulated drugs from digestion by enzymes in the body. This characteristic is particularly significant when peptides or proteins are encapsulated in liposomes. Many studies of liposome-encapsulated insulin have been reported.[3-10] In these studies, liposomes were used to protect insulin from digestive enzymes after oral administration. The delivery of insulin liposomes to the liver has also been used after intravenous administration.[11]

Successful results have been achieved with oral administration of insulin liposomes.[3-5] Weingaten *et al.*[8] reported that the insulin liposome was useful for absorption from the buccal route, but that it was inactive when it was introduced by a strictly intragastric route. The oral administration of insulin has never been established. Weingarten *et al.*[12] reported that the encapsulation of insulin into liposomes protected it from digestive enzymes such as pepsin, α-chymotrypsin, and trypsin. Wiessner *et al.*[13] reported that insulin was bound to the surface of liposomes and that this could lead to overestimation of the real amount of insulin encapsulated in liposomes. Apparently the position of the peptides in liposomes affects the efficacy of peptides as drug carriers.

When liposomes are used clinically, they rapidly disappear from the blood after intravenous administration, one reason for this phenomenon being the disruption of the liposomes caused by liposome/plasma protein interaction. It is well known that lipid transfer[14-18] from liposomes to high density lipoprotein induces the leakage[18] of drugs from liposomes. It has been reported that low density lipoprotein, serum albumin, immunoglobulin, and interferon interact with liposomes.[18-23] Understanding the interaction between liposomes and plasma proteins is very important for the clinical application of insulin liposomes. In this study, we examined the influence of liposomes on the tryptic digestion of insulin in an attempt to obtain some basic information regarding liposome/protein interaction. We found that the tryptic digestion of insulin was accelerated by the empty liposomes.

Materials and Methods

Materials Bovine insulin was purchased from Sigma Ltd., porcine trypsin (2.5% solution) from Gibco Ltd., and soybean phosphatidylcholine (PC: Epikuron 200) was purchased from Nikko Chemicals. Phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylserine (PS), and sodium glycocholate were purchased from Sigma Ltd. Stearylamine (StA) and TritonX-100 were purchased from Wako Pure Chemical Ltd. All materials were used without any purification.

Tryptic Digestion of Insulin Two mg of insulin was dissolved in 1 ml of 0.02 N HCl, and the solution was diluted to 0.4 mg/ml of insulin with 0.05 M, pH 7 phosphate buffer solution. This solution was the stock solution. Tryptic digestion of insulin was performed under the following conditions:

Insulin, 1.7×10^{-5} M; trypsin, 4.3×10^{-6} M, phospholipids, 0—5.1×10^{-2} M, in 0.01 M, pH 7 phosphate buffer, at 37 °C.

At appropriate intervals, samples were collected and stored on ice. A 0.5 ml aliquot of 5% Triton X-100 and 0.5 ml of 0.2% sodium glycocholate were added to 1 ml of sample; these samples were immediately analyzed by high performance liquid chromatography (HPLC), Triton X-100 was used as a detergent for liposomes. Sodium glycocholate was used for preventing insulin adsorption onto the container and also as the internal standard of HPLC analysis.

Preparation of Liposomes

Hydration Method (HY-Method) Multi-lamellar vesicles (MLV) were prepared as described by Bangham *et al.*[24] Lipids were dissolved in ethanol or chloroform, and the solvent was then dried in a vacuum in a glass test tube. Insulin stock solution was diluted two-fold with distilled water. After the addition of 0.5 ml of the insulin solution (200 μg/ml), lipid films were hydrated with repeated vortex mixing. The liposome suspension was then sonicated for 2 min with a bath-type sonicator (Iuchi, Ultrasonic cleaner VS-200).

Reverse-Phase Evaporation Vesicles (REV-Method) REV were prepared as described by Szoka *et al.*[25] Lipids were dissolved in diethylether, and then 0.5 ml of insulin solution (200 μg/ml) was added to the lipid solution. A water in oil (W/O) emulsion was obtained with repeated vortex mixing and 5 min sonication, using the bath-type sonicator. The solvent was dried in a vacuum in a glass test tube; the emulsion then changed into the gel form. Phase inversion occurred after the ether was completely removed from the gel, and REV were then obtained.

Freeze-Thawing Method (FT-Method) FT-Liposomes were prepared as described by Pick *et al.*[26] MLV containing insulin were prepared. Liposome suspension was frozen at −80 °C, and thawed at 25 °C. These freeze-thawing treatments were performed three times.

Preparation of Empty Liposomes MLV were prepared as described by Bangham *et al.*[24] LUVET (large unilamellar vesicles by extrusion techniques, vesicles have unilamellar or oligolamellar) were prepared from

MLV. MLV suspension was passed ten times through a polycarbonate membrane filter (Nucleopore, pore size 0.1—3.0 μm, Nomura Micro Sciences Ltd.) to size the liposomes, as described by Hope *et al.*[27]

Determination of Encapsulated Insulin in Liposomes by Using Resin Treatment Encapsulated insulin was determined as described by Sammons.[28] Insulin (100 μg) was added to a glass vial containing 100 mg of anion exchange resin (BioRad AG1-X2) which had been washed with distilled water gently for 1 min using a Rotator II apparatus (Taitec Ltd.). before use. This suspension was transferred into a minicolumn (Poly-Pep Chromatography Columns, Bio Rad) and this column was then placed into the test tube. After centrifugation (2000 rpm, 5 min), the elution solution in the test tube was collected. A 0.5 ml aliquot of 5% Triton X-100, as a liposome solubilizer, and 0.5 ml of 0.2% sodium glycocholate, as the internal standard, were added to 1 ml of the elution solution. HPLC analysis was performed immediately.

HPLC Analysis The HPLC system consisted of a Hitachi 655-15 liquid chromatograph, a Hitachi variable wavelength UV monitor, and a Shimadzu CR 2AX chromatopac. The column was 3.9 × 150 mm, Waters Nova-Pak C18, 4 μm. The mobile phase was a mixture of acetonitrile and 0.01 M phosphoric acid–sodium perchlorate solution (1 : 2). Triton X-100 was added to the mobile phase (0.01 w/v%). Detection of insulin was carried out by using sodium glycocholate as the internal standard, at 210 nm.

Measurement of Particle Size The mean size of liposomes was determined by using a dynamic laser light scattering instrument (model DLS-700; Ohtsuka Electronics Ltd.).

Results

Influence of Method of Preparation of Liposomes on Encapsulation Efficiency and Tryptic Digestion of Insulin The encapsulation efficiency of insulin and residual insulin after 1-h tryptic digestion in the case of the HY-method are shown in Fig. 1A. This low encapsulation efficiency has been demonstrated previously.[4,7] The encapsulation efficiency value was about 20% when 51 mM PC was used. Protection of insulin from tryptic digestion by entrapping it inside liposomes was not effective at that concentration. The encapsulation efficiency of insulin and residual insulin after 1-h tryptic digestion in the case of the REV-method are shown in Fig. 1B. The encapsulation efficiency was dependent on the amount of PC. When 51 mM PC was used, the encapsulation efficiency was 40%. In this case, nearly all the encapsulated insulin was protected from tryptic digestion. The encapsulation efficiency of insulin and residual insulin after 1-h tryptic digestion in the case of the FT-method are shown in Fig. 1C. Similarly to the results for the REV-method, the encapsulation efficiency of insulin was dependent on the amount of PC. When 51 mM PC was used, the encapsulation efficiency was about 50% and about 35% of the insulin was protected from tryptic digestion.

Table I shows residual insulin after 30- and 60-min tryptic digestion by the HY-method, using 5.1 mM PC. In the case of insulin not incorporated in liposomes (free insulin, below), 18.7% and 8.3% of insulin remained after 30- and 60-min tryptic digestion, respectively. On the other hand, residual insulin after 30- and 60-min tryptic digestion in liposomes prepared by the HY-method was 11.3% and 5.1%, respectively. Liposomal insulin in liposomes prepared by the HY-method was relatively easily degraded by trypsin compared to free insulin.

TABLE I. Degradation of Free and Liposomal (HY-Method) Insulin by Trypsin without the Removal of Free Insulin

Time (min)	Free insulin (%)	Liposomal insulin (%)
		HY-method (PC 5.1 mM)
30	18.7	11.3
60	8.3	5.1

Data presented are those for residual insulin.

Influence of Empty Liposomes on the Tryptic Digestion of Insulin The influence of empty liposomes on the tryptic

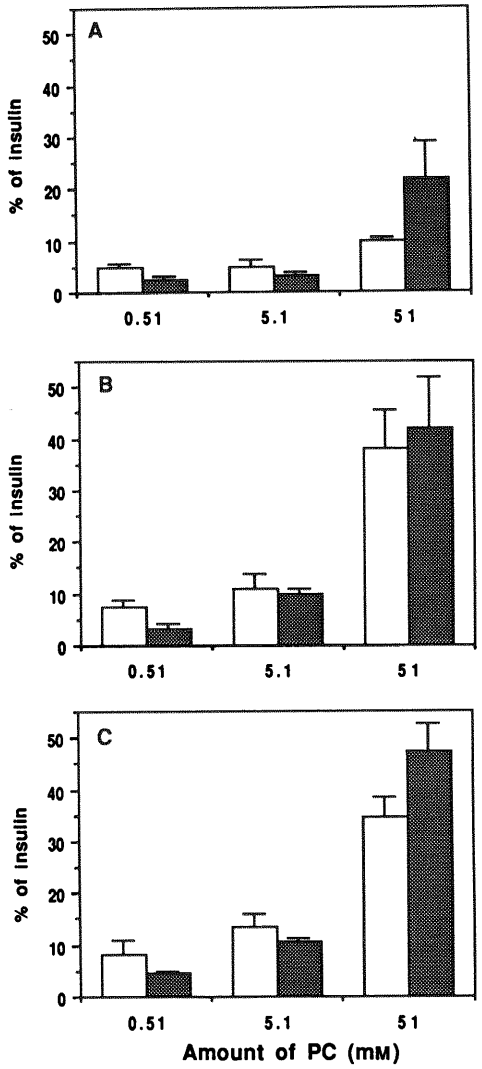

Fig. 1. Residual Insulin after 1-h Degradation by Trypsin without Removing Free Insulin; Encapsulation Efficiency of Insulin in Liposomes

A, HY-method; B, REV-method; C, FT-method. Data are presented as the mean ± S.D. of three experiments. □, residual insulin; ▩, encapsulation efficiency.

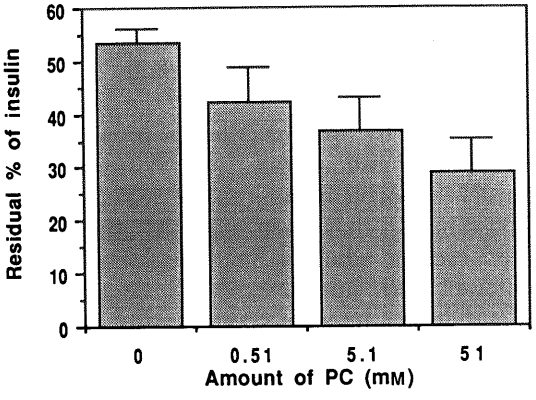

Fig. 2. Influence of Empty Liposomes on 15 min Degradation of Insulin by Trypsin

Data presented are the mean ± S.D. of three or seven (5.1 mM PC) experiments.

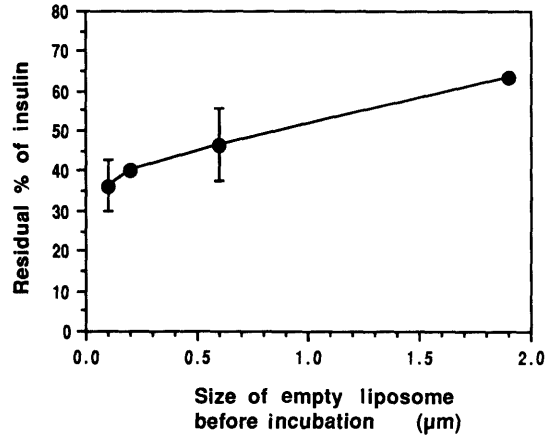

Fig. 3. Influence of Liposomal Size on 15 min Degradation of Insulin by Trypsin

PC, 5.1 mM; insulin, 1.7×10^{-5} M; trypsin, 4.3×10^{-6} M at 37 °C, in 0.01 M, pH 7 phosphate buffer.

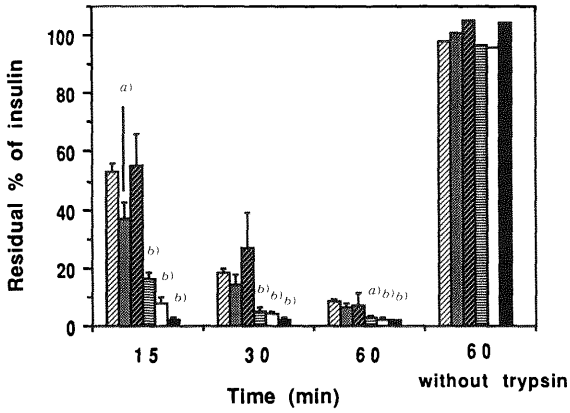

Fig. 4. Influence of Lipid Charge of Empty Liposomes on Degradation of Insulin by Trypsin

Data presented are the mean ± S.D. of three or seven (PC) experiments. Significant differences from free insulin are according to Student's *t*-test; *a*) $p < 0.01$, *b*) $p < 0.001$. Lipids; 5.1 mM. ▨, free insulin; ▩, PC; ▨, PC/StA (9/1); ▤, PC/PS (9/1); □, PC/PI (9/1); ■, PC/PA (9/1).

digestion of insulin is shown in Fig. 2. Residual insulin after 15-min tryptic digestion decreased depending on the amount of empty liposomes, suggesting that this digestion was enhanced by empty liposomes.

Figure 3 shows the influence of the empty liposome particle size before incubation with insulin and trypsin. PC 5.1 mM was used for empty liposomes. Residual insulin decreased with decreasing liposome size from 2 to 0.1 μm.

These results suggest that the tryptic digestion of insulin is enhanced with increasing liposome surface area.

Influence of Lipid Charge of Empty Liposomes on the Tryptic Digestion of Insulin The influence of the lipid charge of empty liposomes on the tryptic digestion of insulin is shown in Fig. 4. The total amount of lipid used for empty liposomes was 5.1 mM. When positively-charged empty liposomes made by including StA were used, the tryptic digestion of insulin was similar to the control (without empty liposomes) or suppressed. On the other hand, the tryptic digestion on insulin was enhanced by negatively-charged empty liposomes made by including PS, PI, or PA. Degradation of insulin was not observed during incubation with empty liposomes only. The enhancement effect of neutral empty liposomes was weaker than that of

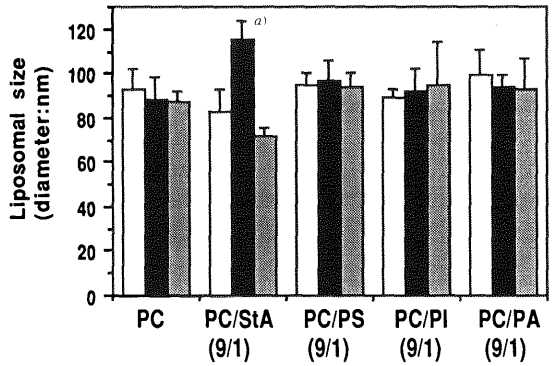

Fig. 5. Change of Liposomal Size after Addition of Insulin or Trypsin to Empty Liposomes

Data presented are the mean ± S.D. of three experiments. Significant differences from liposomes only are according to Student's *t*-test; *a*) $p < 0.05$. Lipids; 5.1 mM. □, liposome only; ■, after addition of insulin; ▨, after addition of trypsin.

negatively-charged empty liposomes.

Change of Liposomal Size Changes in liposomal size after the addition of insulin or trypsin to empty liposomes are shown in Fig. 5. The size of the StA liposome increased after the addition of insulin. This phenomenon occurred when liposomes were fused or aggregated by insulin. The size of neutral and negatively-charged liposomes was not changed by the addition of insulin or trypsin.

Discussion

The encapsulation efficiency of insulin and the protection of insulin from tryptic digestion was increased as the amount of the lipid liposome was increased. This finding has previously been reported by Weingarten *et al.*[12] Insulin encapsulated in liposomes made by the HY-method was not entirely protected from tryptic digestion (Fig. 1A). On the other hand, insulin encapsulated in liposomes made by the REV-method was almost 100% protected (Fig. 1B). The FT-method was more effective than the HY-method, but was less effective than the REV-method (Fig. 1C). Generally, the REV-method is suitable for encapsulating water-soluble drugs into liposomes.[29] The entire amount of insulin required can be encapsulated in the inner space of the liposome by the REV-method. In the case of free insulin, the residual insulin after 1-h tryptic digestion was 8%. If all of the encapsulated insulin was protected from tryptic digestion, the residual insulin was the total of encapsulation efficiency and 8%. Nevertheless, the residual insulin was equivalent to the encapsulation efficiency in the case of the REV-method. This phenomenon may be related to the enhancement of the tryptic digestion of insulin on the surface of liposomes, as described below.

On the other hand, one can speculate that insulin molecules were adsorbed onto the surface of the liposome or that a part of the insulin molecule may project from the surface of the liposome in the case of the HY- and FT-methods.

In our experiment, the amount of observed insulin on the surface, which could not be removed by gel filtration, as shown by Wiessner,[13] could not be estimated by determining the encapsulating efficiency using an anion exchange resin. The anion exchange resin cannot remove the encapsulated insulin, or the part of the encapsulated insulin that projects from the liposome surface. The residual

insulin after the tryptic digestion was less than the encapsulation efficiency when 51 mM PC was used (Fig. 1), since, in this case, part of the insulin projecting from the liposome surface might be degraded by trypsin. Trypsin cleaves the peptide at the C-terminal side of Lys or Arg. Insulin has B22-Arg and B29-Lys and B29-Lys exists in a position away from the α-helix in the insulin B chain, as described by Stenz.[30] It is well known that the α-helix can easily bind the lipid membrane.[31-33] Pocker *et al.*[34] reported that the proportion of α-helix in the insulin molecule was 42%. It is possible that the α-helix of insulin is incorporated into the liposome and that other parts of the insulin molecule, that is, B26—30, may project from the liposome surface. When only a small amount of insulin was encapsulated in liposomes by the HY-method, the tryptic digestion of insulin was enhanced, compared to that of free insulin (Table I). These phenomena cannot be explained by the low encapsulation efficiency of insulin in the liposome and may be due to the influence of empty liposomes on the tryptic digestion of insulin.

We investigated the influence of empty liposomes by adding a reaction solution of insulin and trypsin. The tryptic digestion of insulin was enhanced by increasing the amount of empty liposomes, as shown in Fig. 2. Further the ratio of residual insulin was decreased by the size of the empty liposome, as shown in Fig. 3. Reduction of liposome size increases the surface area of liposomes when the same amount of lipid is used. These results suggest that the interaction of insulin and trypsin on the surface of empty liposomes enhanced the tryptic digestion of insulin.

The influence of the electrical charge of the empty liposomes was investigated. Liposomes that containing 10 mol percentage of StA suppressed the tryptic digestion as shown in Fig. 4. These results are similar to the protection of insulin from enzymes produced by insulin adsorption onto liposomes containing StA (Weingarten *et al.*[12]). Insulin is negatively charged in a neutral pH solution because of its isoelectric point (pI = 5.5) and negatively-charged insulin can easily bind with positively-charged StA liposomes. Wiessner reported that insulin was tightly adsorbed on liposomes containing StA.[13] The size of empty liposomes containing StA increased after mixing with insulin, as shown in Fig. 5, suggesting that insulin induced the fusion or the aggregation of empty liposomes containing StA, similar to results reported by Wiessner[35] and Lai.[36] This fusion results in the protection of insulin from tryptic digestion as a lipid barrier then surrounds the insulin molecule.

Tryptic digestion of insulin was strongly enhanced by the addition of empty liposomes containing PS, PI, or PA, being negatively-charged lipids, as shown in Fig. 4. The size of negatively-charged liposomes was not changed by the addition of insulin (Fig. 5). Farias *et al.*[37] reported that negatively-charged liposomes were fused by insulin and that a large amount of insulin was bound on the surface of these negatively-charged liposome in acidic solution. They also reported that binding of insulin to the surface of liposomes reduced the fluidity of liposomes; these phenomena were observed in neutral pH solution, but the interactions were weaker than in acidic solution. These results suggest that insulin cannot bind strongly to the surface of negatively-charged liposomes, although it may be attracted to the surface of these liposomes by electrostatic or hydrogen bonding interaction between insulin and phosphate, hydroxyl, carboxyl, or amine groups in the molecules of PA, PI, or PS.

Tocanne *et al.*[38] reported the ionization state of various phospholipids in their review; PA bore one negative charge at around pH 4, two negative charges above pH 8, and approximately one and half negative charges around pH 7. PI bore one negative charge above pH 3. PS bore two negative charges and a positive charge between pH 5 and pH 7.4 approximately, while PI and PS had a similar negative charge around pH 7. However, PI has hydroxyl groups that can play a role in hydrogen bonding to insulin and trypsin. These ionic states and hydroxyl groups of the negatively-charged phospholipids produced enhancement of the tryptic digestion of insulin to different degrees in the three types of empty liposomes (order of enhancement; PA > PI > PS).

Trypsin in pH 7 solution is positively charged, because the isoelectric point of trypsin is 10.1—10.8. Positively-charged trypsin seemed to bind the negatively-charged liposome tightly. However, the size of negatively-charged empty liposomes was not changed after addition of trypsin, suggesting that trypsin can bind with negatively-charged liposomes but that is does not induce the fusion or the aggregation of liposomes.

We concluded that weak interactions among negatively-charged empty liposomes, insulin, and trypsin enhanced the tryptic digestion of insulin on the surface area of liposomes. The mechanism of enhancement of tryptic digestion on the surface of neutral liposomes may be similar to that in negatively-charged liposomes. The present findings suggest that the proteolysis of an unencapsulated drug will be enhanced by empty liposomes after administration of the liposomal drug, without the removal of free drug that either leaked from liposomes after administration or was unencapsulated in liposomes. This phenomenon may be important in the evaluation of the efficacy of liposomal insulin.

Our results could be useful and important in the investigation of interactions between liposomes and serum proteins, and could provide a technique for investigation of the stabilization of liposomes in the blood. Rosing[39] reported that prothrombin was activated by the association of prothrombin and factor Xa on the surface of positively-charged membranes containing StA. Our results indicate that relatively nonspecific reactions may be important in enzymatic reactions on the surface of platelet membranes.

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