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Liposomal Microcapsulation of Enzymes by Proliposome Method with Chitosan–Coating

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Liposomal microcapsules containing enzymes were prepared by proliposome method to improve a stability of enzymes under acidic conditions. Two model enzymes, β–galactosidase and alkaline phosphatase, were encapsulated by the method with soybean lecithins, and their encapsulation behaviors were investigated. The highest encapsulation efficiency was obtained by using 25 mg of the enzyme and 250 mg of soybean lecithin (β–galactosidase; 57.3%, alkaline phosphatase; 53.0%). However, the microcapsules did not exhibit the stabilizing effect for β–galactosidase at pH 3. Chitosan–coating was made for proliposome–capsules to keep enzymatic activity under acidic conditions. The microcapsules coated with >0.5% of chitosan 10 showed high stability at pH 3, and encapsulated β–galactosidase was retained 80.7% of its activity. Similarly, alkaline phosphatase was highly stabilized by the encapsulation with chitosan–coating. No decrease in the residual activity of encapsulated alkaline phosphatase was observed at pH 5 for 60 min, while uncapsulated one lost 53.3% of its activity. The activity of encapsulated alkaline phosphatase was kept 20.1 and 59.2% for 60 min at pH 3 and 4, respectively, while uncapsulated alkaline phosphatase was completely inactivated at these pHs.

INTRODUCTION

Microencapsulation techniques have been applied to stabilize the core material, to control the release of the core material, and to separate reactive or incompatible components of formulation. For example, in food industry, microencapsulation of oils and flavor compounds by spray drying was reported by many researchers (Kagami *et al.*, 2002; Kim *et al.*, 1996; Bhandari *et al.*, 1992; Soottitantawat *et al.*, 2002; Liu *et al.*, 2000). Encapsulation of probiotics for yogurt was reviewed by Krasaekoopt *et al.* (2003).

Liposomes, a type of microcapsule, are vesicles enclosing an aqueous solution with a membrane of phospholipids and have been extensively studied as carriers for drug or bioactive compounds. Various methods to prepare liposomes have been reported. Dehydration–rehydration (DR) method (Kirby and Gregoriadis, 1984) and Reverse–phase evaporation vesicle (REV) method (Szoka and Papahadjopoulos, 1978), and Proliposome method (Perrett *et al.*, 1991) were used for the encapsulation of water–soluble materials. In our previous study, poly–γ–glutamic acid (γ–PGA) from *Bacillus subtilis* (*natto*) was encapsulated in liposomes to reach the small intestine without degradation by gastric juice and to inhibit the formation of insoluble calcium phosphate in the intestinal tract (Ishikawa *et al.*, 2004). As a result, γ–PGA was effectively encapsulated in liposomes by

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DR method, and liposome-encapsulated γ-PGA showed 93.3% of intact inhibitory activity after the gastric juice treatment.

In food industry, there are many restrictions to prepare liposomes. Organic solvents, such as chloroform, diethyl ether, or methanol, cannot be used for the liposome preparation because organic solvents have a possible risk to human health. In addition, egg yolk lecithin is too expensive for large-scale production of microcapsules in food applications. Therefore, we prepared liposomal microcapsules with ethanol and soybean lecithin by using proliposome methods (Ishikawa *et al.*, 2004). The liposomes exhibited high encapsulation efficiency and high stability under acidic conditions. However, the effect of acidic pH on the function of encapsulated molecules has never been clear. In this study, the effect of acidic pH on two model enzymes (β-galactosidase and alkaline phosphatase) was investigated. In addition, chitosan-coating for the proliposome-capsules was made to improve the stability under acidic conditions.

MATERIALS AND METHODS

Materials

Beta-galactosidase (Lactase Y-AO, from *Aspergillus orizae*, EC 3.2.1.23) was purchased from Yakult Pharmaceutical Ind. Co. Ltd (Tokyo, Japan). Alkaline phosphatase (from porcine kidney, EC 3.1.3.1) was purchased from Sigma Chemical Co. (St. Louis, MO, U. S. A.). Soybean lecithin was purchased from Nacalai Tesque Co. (Tokyo, Japan). Chitosan 10, 100, and 500 were purchased from Wako Pure Chemicals Co. (Osaka, Japan). All other chemicals were obtained from Nacalai Tesque Co. and were of analytical reagent grade.

Preparation of liposomal microcapsules by proliposome method

Proliposome-microcapsules were prepared according to the method described by Perrett *et al.* (1991) and Rengel *et al.* (2002). Lecithin (25-500 mg) and ethanol (200 μL) was stirred and heated to 60 °C for 5 min. After cooling to room temperature, 500 μL of aqueous solution containing enzyme (25 mg) was added. The proliposome mixture was converted to a liposome suspension by dropwise-addition of 20 mL of 10 mM Tris-HCl buffer (pH 7.0) containing trehalose (1 g) under the controlled drop rate (1.5 ml/min) and stirred for 30 min. The prepared liposome suspension was left to hydrate at 2 °C. Proliposome-capsules were obtained by ultracentrifugation at 80,000×*g* for 45 min at 4 °C. Unencapsulated enzyme in supernatant of liposome suspension was measured by the Bio-Rad protein assay kit using bovine serum albumin (BSA) as a standard and encapsulation efficiency of enzyme was calculated by the following equation:

Encapsulation efficiency (%)
= {(added enzyme (mg) − enzyme in supernatant of liposome suspension (mg))
/ added enzyme (mg)} × 100

Chitosan coating of proliposome-microcapsules

Each chitosan was dissolved in 50 mM acetic acid solution, and then the pH of the solution was adjusted to 5.0 by the addition of 0.5 M Tris solution. Liposome solution (1 mL) was dropwise to the chitosan solution (4 mL). Final concentration of chitosan was

set at 0.1–1.0 wt %. After stirring of the mixed solution for 2 h, the solution was stored in a refrigerator overnight. Proliposome–capsules with chitosan coating were obtained by ultracentrifugation at 80,000×*g* for 45 min at 4 °C.

Stability test for uncapsulated and encapsulated enzymes

Uncapsulated (free) and encapsulated enzymes (*β*–galactosidase and alkaline phosphatase) were incubated in citrate buffer (pH 3, 4, and 5). After incubation, liposomes were degraded by the addition of 1% Triton X–100. The activity of each enzyme was assayed as follows.

Beta–galactosidase activity was assayed with lactose as a substrate. The solution (0.2 mL) containing *β*–galactosidase was added to 3.0 mL of 2% lactose solution (100 mM acetate buffer, pH 4.5). After the incubation at 37 °C for 15 min, the reaction was terminated by the addition of 0.1 mL of 1 M NaOH and neutralized by 0.1 mL of 1 M HCl. The amount of glucose formed during the reaction was determined with the glucose assay kit (glucose C–II test Wako, Wako Pure Chemicals Co., Osaka, Japan). One unit of enzyme activity was defined as the amounts of enzyme required to liberate 1 *μ*mole of glucose per minute from lactose under the assay conditions.

The activity of alkaline phosphatase was assayed with *p*–nitrophenyl phosphate (*p*NPP) as a substrate. An alkaline phosphatase solution (20 *μ*L) was reacted with 1 mM *p*–NPP and 1 mM $MgCl_2$ dissolved in 3.0 mL of Tris–HCl buffer (250 mM, pH 9.0). The increase in the absorbance at 410 nm (the formation of *p*–nitrophenol) was monitored. One unit was defined as the amount of enzyme that hydrolysed 1 *μ*mol of *p*NPP to *p*–nitrophenol in 1 minute at 37 °C.

Residual activity of each enzyme treated under acidic conditions was calculated as the following formula;

Residual activity (%)

=[(Activity of enzyme after the incubation under acidic conditions)/(Activity of untreated–enzyme)]×100

RESULTS AND DISCUSSION

Encapsulation efficiency of *β*–galactosidase by proliposome method

Encapsulation efficiency of *β*–galactosidase by proliposome method was investigated. The proliposome–capsules were prepared by using trehalose. Trehalose would stabilize liposomal membrane by the formation of hydrogen bonds with phosphate groups of phospholipids (Crowe *et al.*, 1986). In addition, encapsulation efficiency of *β*–galactosidase by DR method has been greatly improved by the addition of trehalose (Kim *et al.*, 1999). Similarly, in our previous study (Ishikawa *et al.*, 2004), encapsulation efficiency of BSA was increased by the addition of trehalose. Fig. 1 shows the encapsulation behavior of *β*–galactosidase by proliposome method with 250 mg of soybean lecithin and 1 g of trehalose. As a result, the maximal encapsulation efficiency (57.3%) was obtained by using 25 mg of *β*–galactosidase. Fig. 2 shows the encapsulation efficiency of *β*–galactosidase by proliposome method with various amounts of soybean lecithin. Encapsulation efficiency reached to a maximum at 250 mg of lecithin. Subsequently, the efficiency decreased markedly with increasing the amount of lecithin. Similar encapsulation

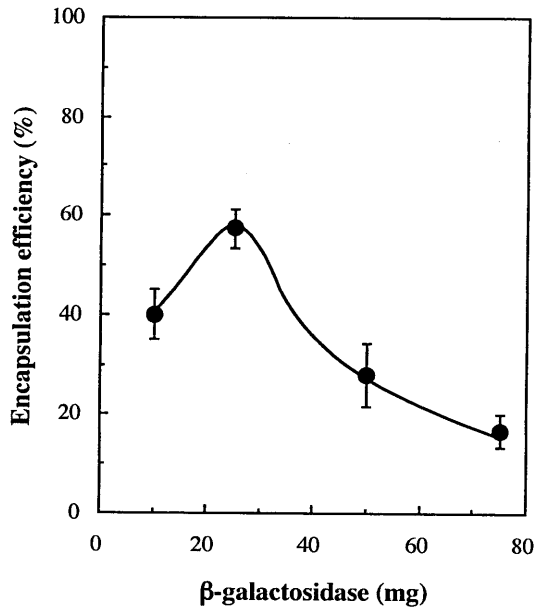

Fig. 1. Encapsulation efficiency of β–galactosidase by proliposome method. Each encapsulation was carried out by using 250 mg of soybean lecithin.

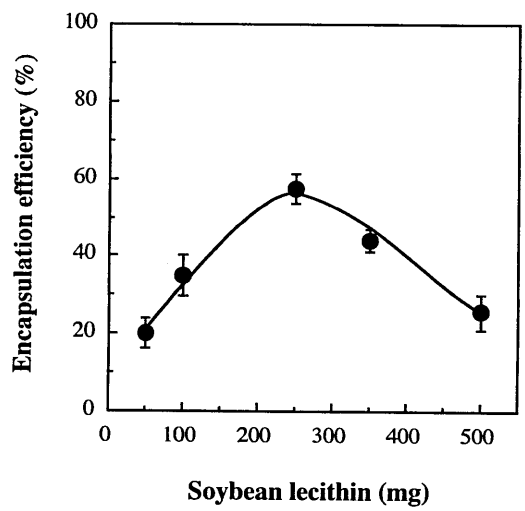

Fig. 2. Effect of amounts of soybean lecithin on encapsulation efficiency of proliposome capsules. Each encapsulation was carried out by using 25 mg of β–galactosidase.

behaviors were observed for the encapsulation of alkaline phosphatase and the maximal efficiency was 53.0% by using 250 mg of lecithin (Data not shown).

β-galactosidase was encapsulated in lecithin–cholesterol liposomes prepared by DR and REV methods (Matsuzaki *et al.*, 1989). In both methods, high encapsulation efficiency was not obtained (DR method; 34.7%, REV method; 31.8%). Kim *et al.* (1999) investigated the encapsulation of β-galactosidase by DR method. The maximum efficiency was nearly equal to that of our proliposome method. However, their encapsulated amount (ca. 300 μg/100 mg–lecithin) was much less than that of our proliposome method (14 mg/250 mg–lecithin). In addition, their DR–capsules were prepared by using chloroform as a solvent. The capsules have a possible risk for human health. In the present study, the enzymes were efficiently encapsulated by proliposome method without using chloroform. Thus, the microcapsules are suitable for food application.

Acid resistance of β-galactosidase encapsulated by proliposome method with and without chitosan–coating

Acid resistance of encapsulated β-galactosidase by proliposome method was determined by the incubation with citrate buffer (pH 3) at 37°C for 60 min. Residual activities of encapsulated and uncapsulated (free) β-galactosidase after the incubation were shown in Fig. 3. Unfortunately, the microcapsules did not exhibit the stabilizing effect for β-galactosidase. Residual activity of encapsulated β-galactosidase was only 10%–higher than that of uncapsulated one. Therefore, we tried to stabilize proliposome–capsules with chitosan–coating. Chitosan is a hydrophilic, biocompatible, and biodegradable polymer of

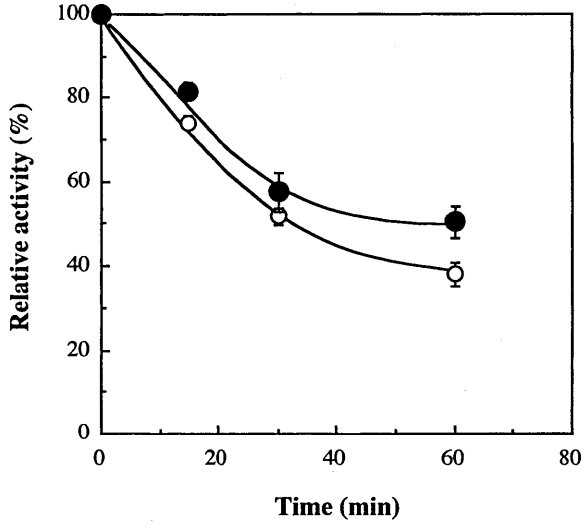

Fig. 3. Residual activities of uncapsulated (free) and encapsulated β-galactosidase treated at pH 3 and 37°C for 60 min. Encapsulation was carried out by proliposome method with 250 mg of lecithin.
Uncapsulated (○); encapsulated (●).

low toxicity. Because of its bioadhesive and permeation enhancing properties, chitosan has received substantial attention in drug delivery system (Henriksen *et al.*, 1994). Fig. 4 shows residual activities of β-galactosidase encapsulated with and without chitosan-coating after the incubation at pH 3 and 37°C. Chitosan 10, 100, and 500 were used in this study. The viscosities of 0.5% solution of chitosan 10, 100, and 500 were 5–20, 50–150, and 300–700 cps, respectively, and all chitosans were deacetylated >80%. Proliposome capsules were coated with 0.5% chitosan. As a result, acid resistance of encapsulated β-galactosidase was greatly improved, and the highest resistance was achieved by using chitosan 10 with lowest molecular mass. Encapsulated β-galactosidase coated with chitosan 10 retained 80.7% of its original activity even after the incubation at pH 3 for 60 min. Fig. 5 shows the acid resistance of encapsulated β-galactosidase coated with various concentrations of chitosan 10. Residual activity of encapsulated β-galactosidase increased with increasing the chitosan concentration. At the concentration higher than 0.5%, 80% of the activity was obtained even after the incubation at pH 3 for 60 min.

Acid resistance of β-galactosidase encapsulated by DR and REV methods were investigated by Matsuzaki *et al.*(1989). It was shown that β-galactosidase would have acid resistance by encapsulating in lecithin-cholesterol liposomes. However, cholesterol-free liposomes exhibited little stability against acidic buffer solution (pH 3). Thus, it has been

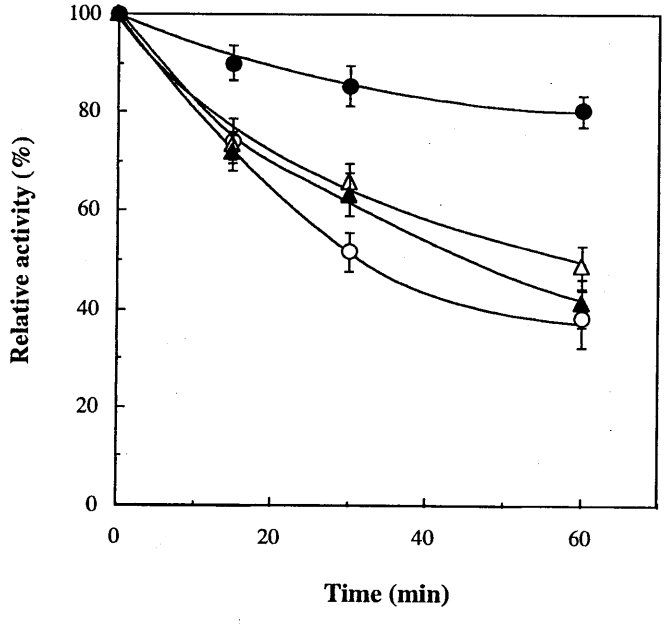

Fig. 4. Effect of chitosan-coating on the acid resistance of β-galactosidase encapsulated by proliposome method. Each stability test was carried out at pH 3 and 37°C for 60 min.
Un-coated (○); Chitosan 10 (●); Chitosan 100 (△); Chitosan 500 (▲).

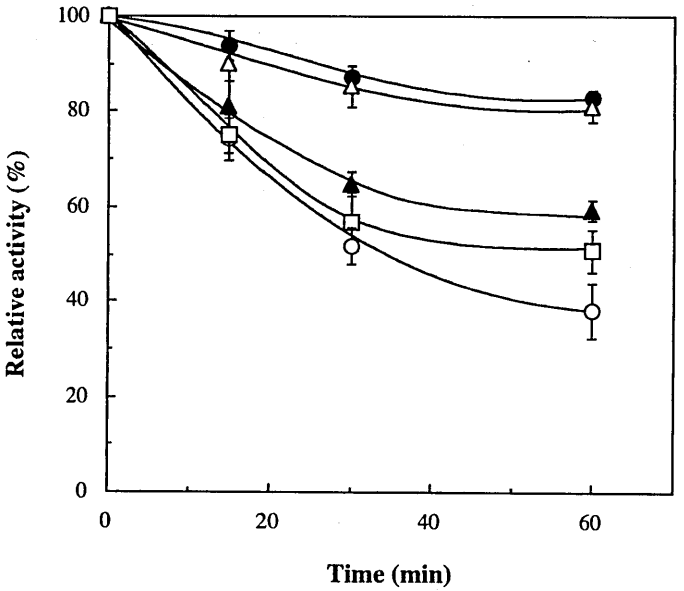

Fig. 5. Effect of chitosan concentration on the acid resistance of encapsulated β-galactosidase coated with chitosan. Each stability test was carried out at pH 3 and 37 °C for 60 min. Chitosan concentration: 0% (Un-coated) (○); 0.1% (□); 0.2% (▲); 0.5% (△); 1.0% (●).

concluded that cholesterol is necessary to stabilize DR- and REV-liposomes. In the present study, encapsulated β-galactosidase exhibited high resistance to acidic conditions without cholesterol. Chitosan-coating was extremely suitable for enzyme encapsulation as well as the use of cholesterol.

Acid resistance of alkaline phosphatase encapsulated by proliposome method with chitosan-coating

To improve stability under acidic conditions, alkaline phosphatase was encapsulated by proliposome method. The proliposome-capsules were coated with 0.5% of chitosan 10. Fig. 6 represents the residual activities of uncapsulated and encapsulated alkaline phosphatase after the incubation at pH 3, 4, and 5. Acid resistance of alkaline phosphatase was greatly improved by the encapsulation. After the incubation at pH 5 for 60 min, no decrease in the residual activity of encapsulated alkaline phosphatase was observed, while uncapsulated one lost 53.3% of its activity. The activity of encapsulated alkaline phosphatase was kept 20.1 and 59.2% for 60 min at pH 3 and 4, respectively, while uncapsulated alkaline phosphatase was completely inactivated at these pHs. These results suggest that encapsulation by proliposome method with chitosan-coating would be effective for the stabilization of bioactive compounds (functional food components) which are liable under acidic conditions.

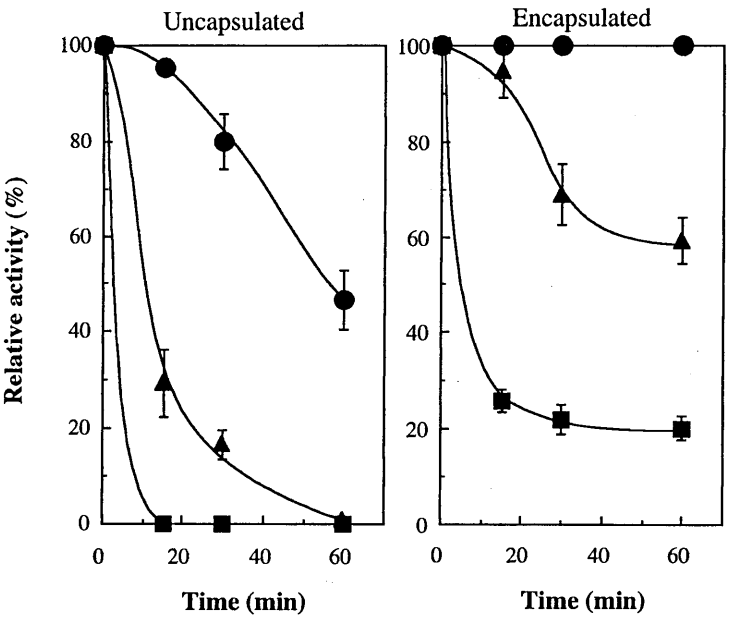

Fig. 6. pH–Stability of uncapsulated and encapsulated alkaline phosphatase. Encapsulation was carried out by proliposome method, and proliposome–capsules were coated with 0.5% chitosan 10.
pH 5 (●); pH 4 (▲); pH 3 (■).

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