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Development of dried liposomes containing β-galactosidase for the digestion of lactose in milk

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Abstract

The hydrolyzed-lactose milk for lactase-deficient subjects has a sweeter taste than whole milk, and some subjects dislike its taste. In order to cope with this shortcoming, we examined whether β-galactosidase, which hydrolyzes lactose, added to the whole milk in the form of dried liposomes, would be able to digest lactose in milk following the lysis of liposomes in the presence of bile salts. Dried liposomes containing β-galactosidase were prepared in the presence of trehalose by the dehydration–rehydration vesicle method to overcome the instability of the conventional liposome suspension. The stability of liposomal membranes was evaluated by measuring the activity of entrapped β-galactosidase under various storage conditions. By treating liposomes with trehalose, which was found to prevent the fusion of liposomes and the leakage of entrapped drug, the entrapping efficiency increased up to fourfold. Over 95% of dried liposomes which had been stored at 17°C for 60 days were reconstituted to liposomes upon rehydration process. From the stability study, dried liposomes were found to retain 87% of β-galactosidase activity at 17°C after 60 days and to be more stable than the multilamellar vesicle suspension prepared without trehalose. The lysis study showed that dried liposomes were hardly lyzed in the simulated gastric fluid with pepsin, but lyzed immediately more than 90% in 0.01 M deoxycholic acid. Lactose hydrolysis in the presence of deoxycholic acid after the addition of dried liposome-entrapped β-galactosidase to whole milk was proportional to the quantity of entrapped β-galactosidase and the amount of dried liposomes added. These results demonstrate that β-galactosidase entrapped in liposome is stable and reconstituted mostly upon rehydration, and can digest lactose in milk after the efficient lysis of liposomes in the presence of bile salts. This study implies that β-galactosidase entrapped in liposome may be applied to whole milk for lactase-deficient subjects.

Keywords: Dried liposome; β-Galactosidase; Trehalose; Dehydration–rehydration vesicle method; Multilamellar vesicle; Lactose hydrolysis; Milk

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1. Introduction

Many people experience gastrointestinal disorders including abdominal distention, cramps, flatulence, and/or watery stools after the ingestion of milk or milk products (Launiala, 1968; Christopher and Bayless, 1971). It has been known that the majority of those people become lactase (β-galactosidase) deficient during the first or second decade of life (Simoons, 1970; Newcomer, 1978). Thus, most lactase deficient subjects are not able to digest milk sugar (lactose) well, discouraged from consuming milk, and may lose a major source of calcium and high-quality protein from their diets. For these lactose-intolerant subjects, hydrolyzed-lactose milk, cultured dairy products and sweet acidophilus milk that include microbial organisms producing β-galactosidase have been recommended as milk substitutes (Paige et al., 1975; Payne-Bose et al., 1977; McDonagh et al., 1987). However, the hydrolyzed-lactose milk has a sweeter taste than whole milk (Paige et al., 1975; Onwulata et al., 1989) since it is prehydrolyzed to its constituent monosaccharides of glucose and galactose by exogenous β-galactosidase, and some subjects dislike this taste. It was reported that yogurt was mostly effective to improve the absorption of lactose (Onwulata et al., 1989), but its taste is entirely different from milk. As far as the acidophilus milk is concerned, it is still controversial as to whether this milk is capable of improving the absorption of lactose (Savaiano et al., 1984). Although the encapsulation of β-galactosidase in liposome, which can segregate β-galactosidase from lactose in milk under storage conditions, has not been performed before, it may help overcome the shortcoming of the aforementioned milk substitutes. Liposome consisting of a lipid bilayer like biomembrane has great potential in pharmaceutical application as a drug delivery system (Kim et al., 1994; Kim and Han, 1995; Kim and Jeong, 1997). However, one of the difficulties in the practical applications of conventional liposome dispersion has been the lack of long-term stability. Many researchers have reported various methods being able to improve the stability of liposomes, but these methods have some defects (Payne et al., 1986): proliposome has the limitation to a hydrophobic drug content (Park et al., 1994), polymerization has the additional step for synthesizing polymerizable lipids (Johnston and Chapman, 1983), microencapsulation has to expose the drug content to organic solvents and the drug is leaked during preparation (Yeung and Nixon, 1988), freezing is not easy for storage and transportation (Higgins et al., 1986), and freeze-drying leaks entrapped drug after reconstitution (Crowe et al., 1986). However, the freeze-drying method called the dehydration–rehydration vesicle method has the following advantages over other methods; the entrapping efficiency is high, it is simple and easy to deal with the product, and it can be used for large scale production. In addition, the drug is not exposed to organic solvents so that it is useful to encapsulate the drug sensitive to organic solvents. Hence, among these methods, freeze-drying in the presence of carbohydrates, especially trehalose (Crowe and Crowe, 1988), may be suitable for encapsulating protein such as β-galactosidase in liposomes. Fusion and leakage of freeze-dried liposomes can be prevented by the addition of trehalose, which preserves a membrane structure in the dry state of anhydrobiotic organisms (Crowe et al., 1986). In this study, we established the preparation method of dried liposomes containing β-galactosidase, evaluated the reconstitution ability after rehydration process and the stability by measuring the activity of β-galactosidase under various storage conditions, and investigated further the lysis of the rehydrated liposomes in the presence of bile salts. Finally, the extent of lactose hydrolysis in whole milk was examined in the presence of bile salt by liposome-entrapped β-galactosidase.

2. Materials and methods

2.1. Materials

Egg phosphatidyl choline (EPC), cholesterol, β-galactosidase (BGase, 5–6 U/mg, 5000 units/6.7 mg), β-galactose dehydrogenase (26 U/ml), deoxycholic acid sodium salt, cholic acid sodium salt, *o*-nitrophenyl-β-D-galactoside (NPG) and trehalose were purchased from Sigma Chemical (St.

Louis, MO, USA). Nicotinamide-adenine dinucleotide (NAD^+) was obtained from P-L Biochemicals (Milwaukee, WI, USA). EPC was purified according to the method described previously with a minor modification (New, 1990). All other chemicals were of reagent grade and used without further purification.

2.2. Preparation of dried liposomes

The dehydration–rehydration vesicles entrapping β-galactosidase were prepared according to the method of Kim and Jeong (1997) with minor modifications.

Briefly, 100 mg of lipid mixture composed of chromatographically pure EPC and cholesterol (molar ratio 2:1) was dissolved in 1 ml of anhydrous chloroform. The chloroform was removed on a rotary evaporator (Büchi, Switzerland) under reduced pressure (360 mmHg) and then the dried lipid film formed was suspended in 1 ml of 10 mM phosphate buffer (pH 7.4). The suspension was sonicated using a probe type sonicator (Branson Cleaning Equipment, UK) at 24 μ for 30 min in an ice bath. The resultant suspension of small unilamellar vesicles (SUV) was centrifuged at $7500 \times g$ in a microcentrifuge (Sarstedt MH 2; Sarstedt, Germany) for 15 min at room temperature to remove trace of lipid aggregates which were not reconstituted to liposomes. One milliliter of the SUV suspension from the supernatant was mixed with an equal volume of phosphate buffer (10 mM, pH 7.4) containing β-galactosidase (500 μg/ml). After freezing at −196°C in a liquid nitrogen tank for 2 h, the preparation was lyophilized for at least 13 h under vacuum in a freeze-dryer (DURA-DRY Corrosion Resistant Freeze-dryer; FTS system, USA). Two milliliters of phosphate buffer (10 mM, pH 7.4) were added to the freeze-dried preparation and mixed by vortexing. The resultant dehydration–rehydration vesicles were centrifuged at $180\,000 \times g$ in an ultracentrifuge (MSE; Europa 65) for 20 min and the formed pellet of multilamellar vesicles (MLV) was resuspended in 1 ml of phosphate buffer (10 mM, pH 7.4). The MLV were then mixed with an equal volume of phosphate buffer (10 mM, pH 7.4) containing trehalose (4 g/g lipid) and lyophilized as already described. The final dried liposomes were flushed with nitrogen, sealed and stored in a refrigerator.

2.3. Measurement of β-galactosidase activity

The entrapping efficiency and the stability of liposomal membrane were determined by measuring the activity of β-galactosidase in liposomal pellet and supernatant. The final dried liposomes (0.5 g) were rehydrated with 4 ml of phosphate buffer (10 mM, pH 7.4) and centrifuged at $180\,000 \times g$ for 20 min. The activity of unentrapped β-galactosidase in the liposomal pellet was then measured. For the measurement of the activity of β-galactosidase in the liposomal pellet, the pellet was resuspended with 2 ml of phosphate buffer (10 mM, pH 7.4). Of this solution, 0.5 ml was added to the mixture of 2 ml of propylalcohol and 4 ml of phosphate buffer followed by vortexing. Upon addition of propylalcohol, the liposomal membranes were disrupted sufficiently to allow β-galactosidase to be released to the dispersion medium, and the clear solution was formed. The activity of β-galactosidase in this solution was then measured with the substrate–enzyme reaction. Of the sample containing β-galactosidase, 0.5 ml was reacted with 2.3 mM of NPG and 1 mM of $MgCl_2$ dissolved in 2.5 ml of phosphate buffer (10 mM, pH 7.4). After the incubation for 30 min at room temperature, the enzymatic reaction was stopped by adding 0.25 ml of 2 M Na_2CO_3 solution. The absorbance of the reaction mixture was then measured spectrophotometrically at 405 nm.

2.4. Quantitative measurement of phosphatidyl choline and galactose

The reconstitution of liposomes after rehydration of dried liposomes was examined by measuring the concentration of phosphatidyl choline (PC) using the Stewart assay (New, 1990) with a minor modification. Before the assay, phospholipids in liposomes were extracted by the Bligh–Dyer method (New, 1990).

The extent of lactose hydrolysis by β-galactosidase was determined from the amount of galactose produced as follows:

$$\text{Lactose} + H_2O \xrightarrow{\beta-\text{galactosidase}} \beta\text{-D-galactose} + \text{D-glucose} \quad (1)$$

$$\beta\text{-D-galactose} + NAD^+ \xrightarrow{\beta-\text{galactosedehydrogenase}} \beta\text{-galacto-}\gamma\text{-lactone} + NADH + H^+ \quad (2)$$

The sample dissolved in 1 ml of phosphate buffer (0.5 M, pH 8.6) was mixed with 0.1 ml of NAD^+ solution (8.7 mg/ml) and 0.02 ml β-galactose dehydrogenase (5 mg/ml). After the mixture was incubated at 37°C for 30 min and left at room temperature for 15 min, the absorbance was measured at 339 nm.

2.5. Measurement of osmotically induced turbidity

Glucose solution (50 mM) containing 2 mM EDTA and 10 mM phosphate buffer (pH 7.4) was added to SUV suspension, MLV and rehydrated liposomes. After the rehydration of dried liposomes, 0.5 ml of dispersed liposome was mixed with glucose solution of various concentrations to give a desired concentration gradient across the lipid bilayer membranes. C_{in}/C_{out}, the ratio of glucose concentration entrapped in liposome to that of external medium, varied from 0.4 to 4. After the incubation for 1 h, the turbidity of mixture was measured at 450 nm.

2.6. Stability of dried liposomes and β-galactosidase

Dried liposomes prepared with trehalose (4 g/g lipid) were stored in sealed bottles at 17 and 37°C for 30 or 60 days. After the rehydration of dried liposomes, the activity of β-galactosidase, and the PC content in pellet and supernatant were monitored every 10 or 15 days. To compare the stability of MLV with that of dried liposomes, MLV before adding trehalose were dispersed with 4 ml of phosphate buffer (pH 7.4) and stored in sealed bottles at 37°C for 30 days. Every 10 days, they were taken out and centrifuged at 180 000 × *g* for 20 min, and the activity of β-galactosidase and the PC content in pellet and supernatant were measured.

2.7. Lysis of liposomes

Lysis of liposomes in simulated gastric fluid (pH 1.2) with pepsin (USP XXII) and in bile salt solution was investigated. Final dried liposomes (0.5 g) were rehydrated with 4 ml of phosphate buffer (pH 7.4) and centrifuged at 180 000 × *g* for 20 min. The pellet was resuspended with 2 ml of phosphate buffer. Of the resuspended liposome, 0.2 ml was incubated at 37°C for 1 h in bile salt (deoxycholic acid, cholic acid) solutions with various concentrations. The percent lysis of liposome was assessed by measuring the turbidity at 450 nm. The time-dependent lysis of rehydrated liposomes was also investigated. Resuspended liposomes (0.2 ml) were incubated at 37°C for 60 min in simulated gastric fluid with pepsin, 0.01 and 0.02 M deoxycholic acid solutions, and then turbidity was measured spectrophotometrically at 450 nm at designed time intervals.

2.8. Hydrolysis of lactose by liposomal β-galactosidase in milk

The hydrolysis of lactose in milk solution by liposome-entrapped β-galactosidase was examined. The liposome-entrapped β-galactosidase was rehydrated with 3 ml of phosphate buffer (pH 7.4). Of the rehydrated liposomes, 0.5 ml and 0.15 ml of 0.2 M deoxycholic acid were added to 2 ml of 136 mM lactose solution or whole milk. This mixture was incubated at 37°C for designated time intervals, and β-galactosidase was removed with deproteinizing agent (Carrez's solution I, II) to protect further hydrolysis of lactose (Bergmeyer et al., 1983). The galactose produced from the lactose in milk was determined by the reactivity toward β-galactose dehydrogenase in the presence of NAD^+ solution as already described. The hydrolysis of lactose was represented by the percentage of galactose concentration to lactose concentration of the initial reaction mixture. To determine the hydrolytic activity of liposome-entrapped β-galactosidase under the storage condition of milk, the percent hydrolysis of lactose was also measured without deoxycholic acid at 4°C for 7 days.

3. Results and discussion

3.1. Osmotic behavior of liposomes

The rehydrated liposomes respond to osmotic gradient as ideal osmometers (Yoshikawa et al., 1983; Kim et al., 1993). When the rehydrated liposomes act as perfect osmometers, the linear relationship between $(1/A)^{1.5}$ and C_{in}/C_{out} can be derived as

$$(1/A)^{1.5} = \{V_{act}(C_{in}/C_{out}) + V_{dead}\}\ 1/k \quad (3)$$

where A is the absorbance at a given wavelength, k represents a constant, V_{act} and V_{dead} denote the osmotically active and inactive volume of liposomes, respectively, and C_{in}/C_{out} is the ratio of solute concentrations in the inner to those in the outer parts of liposomes.

Fig. 1 shows the good linear relationship between C_{in}/C_{out} and $(1/A)^{1.5}$, implying that the liposomal membrane behaves as an osmometer and has a barrier function. The linearity was observed between 0.4 and 1.5 of C_{in}/C_{out} values. Under more hypotonic conditions, the plot deviated from the linear relationship since the liposomes were lyzed, the solute leaked, and the liposomal membrane lost their barrier function. Under hypertonic condition with C_{in}/C_{out} less than 0.4, the liposomes might have shrunk, thus showing the deviation from the linearity.

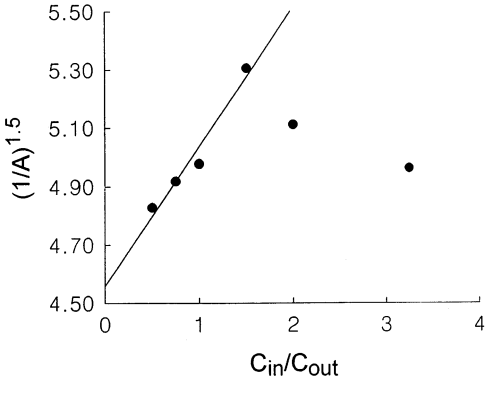

Fig. 1. Osmotic behavior of liposomes.

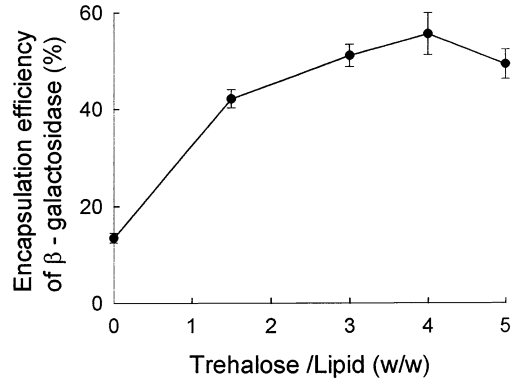

Fig. 2. The effect of trehalose on the encapsulation efficiency of β-galactosidase in liposomes. Each point represents the mean ± SD ($n = 5$).

3.2. Effect of trehalose on the encapsulation efficiency

The effect of trehalose on the activity of β-galactosidase in rehydrated liposomal pellets is shown in Fig. 2. The activity of β-galactosidase in liposomal pellet increased up to fourfold with increasing amount of trehalose compared with that without trehalose. The activity showed the maximum value at trehalose concentration of 4 g/g lipid, although there was no significant difference in the activities among trehalose concentrations of 3, 4, and 5 g/g. This means that the amount of β-galactosidase entrapped in liposomes increased with trehalose up to fourfold compared with that without trehalose. This result indicates that trehalose may serve as a protectant against the damage of liposome during the dehydration and rehydration processes, preventing the leakage of entrapped β-galactosidase (Crowe et al., 1986).

3.3. Stability of dried liposomes and β-galactosidase

The following aspects concerning the stability of dried liposomes were examined; the reconstitution of liposomes after rehydration of dried liposomes, and the extent of retained β-galactosidase activity. The reconstitution of liposomes after rehydration of dried liposomes was assessed by measuring the percent change of PC content in

Table 1
The reconstitution of liposomes after rehydration of dried liposomes[a]

Storage condition	Reconstitution (%)
4°C, 60 days	98.3 ± 1.2
17°C, 60 days	92.9 ± 4.3
37°C, 60 days	68.7 ± 8.9
50°C, 60 days	31.6 ± 11.4

[a] Each value represents the mean ± SD ($n = 5$).

pellets. As summarized in Table 1, when the dried liposomes were stored at 4 and 17°C for 60 days, 98.3 and 92.9% of dried liposomes were reconstituted, respectively. This indicates that the dried liposomes are very stable at low temperature.

During the storage of dried liposomes at 17°C up to 60 days and 37°C up to 30 days, over 95% of the initial β-galactosidase activity was restored after rehydration (Table 2). Moreover, 87% of β-galactosidase activity was retained in liposome stored at 17°C for 60 days and 70% retained, stored at 37°C for 30 days. The supernatant, consisting of lipid aggregates which were not reconstituted, and leaked β-galactosidase from liposomes stored at 17°C for 60 days and 37°C for 30 days, contained 8 and 25% of β-galactosidase activity, respectively. Although the dried liposome itself was somewhat unstable at 37°C, it is concluded that, overall, β-galactosidase in dried state is stable.

Compared with dried liposomes, the conventional MLV suspension was significantly less stable. Only 40% of the initial activity was maintained in MLV stored at 37°C for 30 days as shown in Table 2. The leakage of β-galactosidase from MLV increased up to 33%. The leaked β-galactosidase might have lost its activity in the medium, thus the total β-galactosidase activity decreased to about 80%.

3.4. Lysis of liposome

The lysis extent of liposome in simulated gastric fluid (pH 1.2) with pepsin (USP XXII) and bile salt solution was examined. Bile salts form molecular aggregates called micelles above a critical concentration of about 0.002 M, and are necessary for the normal intestinal absorption of dietary fats via a micellar transport mechanism (Greenberger and Isselbacher, 1994). The concentrations of cholic acid and deoxycholic acid in bile within the gallbladder are about 0.049 and 0.036 M, respectively (Guyton, 1981; Malet and Soloway, 1988). When incubated in the presence of 0.049 M cholic acid and 0.036 M deoxycholic acid at 37°C for 1 h, liposomes were lysed about 50 and 97%, respectively (Fig. 3). Thus, it is expected that the liposomes are easily lysed in physiological concentration of bile salts.

Table 2
The activity of β-galactosidase entrapped in dried liposomes or in MLV suspension stored at various temperatures[a]

Storage temperature (°C)		β-galactosidase activity (%)			
		0 day	15 days	30 days	60 days
Dried liposome					
17°C	Total	100	97 ± 2.3	99 ± 1.9	95 ± 6.1
	Liposome	0	96 ± 5.7	95 ± 4.8	87 ± 5.2
	Leaked	0	3 ± 1.2	4 ± 1.8	8 ± 2.2
37°C	Total	100	96 ± 3.7	95 ± 5.7	
	Liposome	0	89 ± 2.3	70 ± 4.7	
	Leaked	0	7 ± 2.5	25 ± 3.5	
MLV suspension					
37°C	Total	100	90 ± 4.1	80 ± 3.7	
	MLV	0	74 ± 2.0	40 ± 2.6	
	Leaked	0	16 ± 2.8	33 ± 1.9	

[a] Each value represents the mean ± SD ($n = 5$).

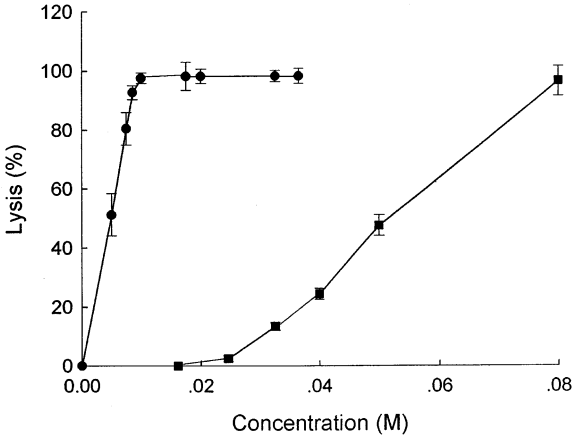

Fig. 3. The percent lysis profiles of liposomes in bile salt solution as a function of bile salt concentration at 37°c for 1 h. (●) Deoxycholic acid; (■) cholic acid. Each point represents the mean ± SD ($n = 5$).

The percent lysis in a simulated gastric fluid with pepsin was 5.9% at 1 h after the incubation, while the lysis in bile salt solutions was over 85% within 5–10 min as shown in Fig. 4. Therefore, the liposomes may be stable when they stay in the stomach and lysed rapidly after they go down to the upper part of small intestine where the bile is excreted. Additionally, since over 90% of the liposomes are not lyzed in a simulated gastric fluid

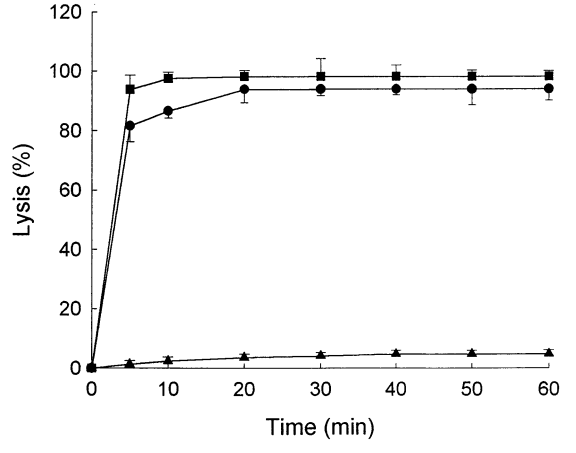

Fig. 4. The percent lysis profiles of liposomes in simulated gastric fluid and deoxycholic acid solution as a function of time at 37°C. (●) 0.01 M Deoxycholic acid; (■) 0.02 M deoxycholic acid; (▲) simulated gastric fluid with pepsin. Each point represents the mean ± SD ($n = 5$).

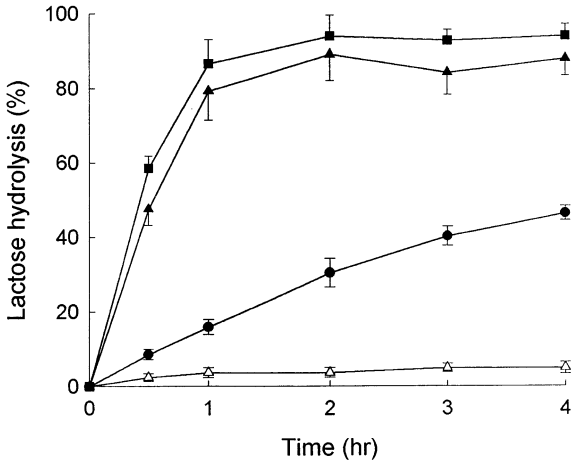

Fig. 5. The percent lactose hydrolysis by the liposome-entrapped β-galactosidase with bile salt in lactose solution and whole milk. (△) 0 U β-galactosidase, lactose solution; (●) 2000 U β-galactosidase, lactose solution; (■) 5000 U β-galactosidase, lactose solution; (▲) 5000 U β-galactosidase, whole milk. Each point represents the mean ± SD ($n = 5$).

with pepsin, we expect that β-galactosidase entrapped in the liposomes may maintain its activity in the stomach, and be released from the liposomes into the intestine.

3.5. Enzymatic hydrolysis of lactose

The enzymatic hydrolysis of lactose by the liposome-entrapped β-galactosidase with/without bile salt, deoxycholic acid was examined in lactose solution and whole milk as shown in Fig. 5. The enzymatic hydrolysis of lactose without bile salt hardly occurred at 37°C ($< 5\%$). On the other hand, the percent hydrolysis of lactose with bile salt increased proportionally to the amount of β-galactosidase entrapped in the liposomes. The lactose both in phosphate buffer (pH 7.4) and whole milk was hydrolyzed about 80% within 1 h by the liposomes entrapping 5000 U of β-galactosidase.

4. Conclusion

In conclusion, the dried liposome-entrapped β-galactosidase prepared by the dehydration–rehy-

dration vesicle method was reconstituted over 95% after the rehydration. Trehalose used as a cryoprotectant increased the encapsulation efficiency by preventing the fusion of liposomes and the leakage of the entrapped β-galactosidase. The dried liposomes were found to be more stable than MLV suspension in maintaining the activity of β-galactosidase and not to affect the activity of entrapped β-galactosidase, thus functioning as a stable enzyme-carrier at 4 and 17°C. In addition, they were hardly lysed in simulated gastric fluid, while they were rapidly lysed in bile salt solution. Furthermore, the dried liposomes entrapping 5000 U of β-galactosidase hydrolyzed over 80% of lactose in both lactose solution and whole milk within 1 h. Further work needs to be performed to ascertain the effect of β-galactosidase entrapped in liposomes on the digestion of whole milk in the complex system of an intact gastrointestinal tract.

Acknowledgements

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