Chapter 2

Stabilization of Enzymes Through Encapsulation in Liposomes

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Abstract

Phospholipid vesicle (liposome) offers an aqueous compartment surrounded by lipid bilayer membranes. Various enzyme molecules were reported to be encapsulated in liposomes. The liposomal enzyme shows peculiar catalytic activity and selectivity to the substrate in the bulk liquid, which are predominantly derived from the substrate permeation resistance through the membrane. We reported that the quaternary structure of bovine liver catalase and alcohol dehydrogenase was stabilized in liposomes through their interaction with lipid membranes. The method and condition for preparing the enzyme-containing liposomes with well-defined size, lipid composition, and enzyme content are of particular importance, because these properties dominate the catalytic performance and stability of the liposomal enzymes.

Key words: Liposomes, Phospholipid vesicles, Lipid bilayer membranes, Enzyme encapsulation, Membrane permeability, Enzyme structure, Enzyme reactivity, Bovine liver catalase

1. Introduction

The liposomal aqueous phase is isolated from the bulk liquid by the semipermeable lipid bilayer membranes, which means chemical reactions can be induced inside enzyme-containing liposomes by adding membrane-permeable substrate to the bulk liquid. In the liposomal system, the enzyme molecules are confined without chemical modification, which is advantageous to preserve the inherent enzyme affinity to the cofactor and substrate molecules. So far, various liposome-encapsulated enzymes have been prepared and characterized for developing diagnostic and biosensing materials, functional drugs, and biocompatible catalysts (1, 2). The reactivity of liposomal enzymes was extensively examined mainly focusing on the membrane permeation of the substrate molecules as a rate-controlling step of the liposomal reaction (3, 4). For example, sodium

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cholate is a useful modulator of the liposome membranes. Incorporation of sublytic concentrations of cholate in the membranes induced permeation of substrate and as a result the rate of liposomal enzyme reaction increased (4). An excess amount of cholate causes complete solubilization of liposome membrane, which is utilized for determining the total amount and inherent activity of the enzyme encapsulated in liposomes. On the other hand, the stability of enzyme activity in liposomes is relatively unknown. We recently reported that the thermostability of bovine liver catalase and yeast alcohol dehydrogenase considerably increased through encapsulation of each enzyme in liposomes composed of POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) (5–7). Furthermore, the liposomal glucose oxidase system was shown to be applicable as a stable catalyst for the prolonged oxidation of glucose in the gas–liquid flow in a bubble column reactor (8–10). In the liposomal system, the aggregate formation among the partially denatured enzyme molecules was indicated to be depressed through the interaction of the enzyme with lipid membranes (11). This chapter describes the preparation, reactivity, and stability of the enzyme-containing liposomes with various sizes and enzyme contents using the catalase as a model enzyme. The preparation and analytical methods described are basically applicable to liposomes containing other water-soluble enzymes. To prepare stable and reactive liposomal enzyme systems, the enzyme content in liposomes and the lipid composition need to be changed and optimized considering the characteristics of each enzyme employed and the permeability of its substrate through the liposome membranes.

2. Materials

2.1. Preparation of Catalase-Containing Liposomes

1. Phospholipid: POPC (>99%, $M_r = 760.1$, main phase transition temperature T_m of -2.5 ± 2.4°C (12)) (Avanti Polar Lipids, Inc., Alabaster, AL).
2. Chloroform (>99%). Diethylether (>99.5%).
3. Ethanol (>99.5%).
4. Dry ice (solid CO_2).
5. Rotary evaporator (REN-1, Iwaki Co., Ltd., Japan) with an aspirator (ASP-13, Iwaki Co., Ltd.).
6. Freeze-dryer (FRD-50 M, Asahi Techno Glass Corp., Funabashi, Japan) with a vacuum pump (GLD-051, ULVAC, Inc., Chigasaki, Japan).
7. Enzyme: bovine liver catalase (EC 1.11.1.6, ca. 10,000 U/mg, $M_r = 240{,}000$) (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

8. Tris buffer: 50 mM Tris (2-amino-2-hydroxymethyl-1, 3-propanediol)–HCl, pH 7.4, containing 0.1 M sodium chloride.
9. Small-volume extrusion device Liposofast™ and its stabilizer (Avestin Inc., Ottawa, Canada) (13).
10. Polycarbonate membranes for sizing liposomes (Avestin, Inc., 19 mm in membrane diameter and 30, 50, or 100 nm in the nominal mean pore diameter).
11. Gel beads for gel permeation chromatography (GPC): sepharose 4B suspended in ethanol/water (GE Healthcare UK Ltd., Buckinghamshire, England).
12. Glass column with a stopcock for the GPC, 1.0 (id) × 35 cm, 20 mL in packed gel bed volume.
13. Enzyme kit for the quantification of POPC (Phospholipid C-Test Wako, Wako Pure Chemical Industries, Ltd.).
14. UV/visible spectrophotometer (Ubest V-550DS, JASCO, Tokyo, Japan) equipped with a perche-type temperature controller (EHC-477S, JASCO).

2.2. Measurement of Enzyme Activity of Catalase-Containing Liposomes

1. 0.3 M sodium cholate (Wako Pure Chemical Industries, Ltd.) in the Tris buffer.
2. Substrate of catalase: hydrogen peroxide (H_2O_2) solution (Wako Pure Chemical Industries, Ltd.).

3. Methods

3.1. Preparation of Catalase-Containing Liposomes

1. Weigh 50 mg of POPC powder (see Note 1).
2. Dissolve 50 mg of POPC in 4 mL of chloroform in a 100-mL round-bottom flask in a draft chamber.
3. Remove the solvent from the flask by using the rotary evaporator under reduced pressure in a draft chamber to form a lipid film on the inner wall of the flask.
4. Dissolve the lipid film in 4 mL of diethylether and remove the solvent as described above. Repeat this procedure once more.
5. Dry the lipid film formed in the flask by using the freeze-dryer connected to the vacuum pump for 2 h in the dark to remove the residual organic solvents molecules in the lipid layers. Keep the inner pressure of the flask <10 Pa throughout the dry process.
6. Dissolve the catalase in 2.0 mL of the Tris buffer in a glass test tube at the enzyme concentrations of 1.3–80 mg/mL (see Note 2).

7. Hydrate the dry lipid film with 2.0 mL of the enzyme-containing Tris buffer solution with gentle shaking to induce the formation of multilamellar vesicles (MLVs) (see Note 3).
8. Freeze the MLVs suspension with a refrigerant (dry ice/ethanol, −80°C) in the Dewar flask for 5 min and then thaw it in a water bath thermostatted at 35°C with gentle shaking. Repeat this freezing and thawing treatment seven times to transform a fraction of the small vesicles into the larger MLVs (see Note 4).
9. Extrude the MLVs suspension through a polycarbonate membrane with nominal mean pore diameter of 100, 50, or 30 nm 11 times using the extruding devise to obtain the catalase-containing unilamellar liposomes (see Note 5).
10. Pass the catalase-containing liposome suspension through the GPC column with the Tris buffer as an eluent collecting at 1.0-mL fraction volumes in order to separate the catalase containing liposomes from free (nonentrapped) enzyme (see Note 6).
11. Measure the concentration of POPC in the liposome-containing fractions obtained by the chromatographic separation using the enzyme kit (see Note 7).
12. Store the enzyme-containing liposome suspension in a capped plastic tube in the dark at 4°C before use (see Note 8).

3.2. Measurement of Enzyme Activity of Catalase-Containing Liposomes (See Tables 1 and 2)

1. Prepare 100 mM H_2O_2 in the Tris buffer solution considering the molar extinction coefficient of H_2O_2 at 240 nm ε_{240} of 39.4 $M^{-1}\,cm^{-1}$ (see Note 9).
2. Add the catalase-containing liposome suspension to the Tris buffer solution containing H_2O_2 to give the intrinsic catalase and initial H_2O_2 concentrations of 0.1–0.2 μg/mL (about 1.0–2.0 U/mL) and 10 mM, respectively, and the total volume of 3.0 mL in a quartz cuvette in order to initiate the liposomal catalase-catalyzed decomposition of H_2O_2 (see Note 10).
3. Measure the time course of H_2O_2 decomposition at 25 ± 0.3°C for 60 s based on the decrease in absorbance at 240 nm using the spectrophotometer. The decomposition rate of H_2O_2 is taken as the enzyme activity of liposomal catalase.
4. Measure the intrinsic enzyme activity of the liposomal catalase in the same way as above except that the reaction solution contains 40 mM sodium cholate for complete solubilization of liposome membranes (see Note 11).
5. Calculate the activity efficiency *E* of the liposomal catalase as its observed activity measured in the absence of cholate relative to the intrinsic one (see Note 12).

Table 1
Characteristics of catalase-containing liposomes (5)

Liposomal catalase[a]	Concentration of catalase in hydration step C_0 [mg/mL]	Concentration of catalase in liposome C_{in} [mg/mL]	Mean number of catalase molecules in a liposome N [–]	Activity efficiency of liposomal catalase E [–]
CAL_{30}	1.3	66[b]	0.032 ± 0.008	0.59 ± 0.01
CAL_{50}	1.3	9.9[b]	0.15 ± 0.02	0.54 ± 0.06
CAL_{100}–I	1.3	0.96[b]	0.64 ± 0.06	0.57 ± 0.04
CAL_{100}–II	20	4.9 ± 0.9	5.2 ± 0.9	0.40 ± 0.04
CAL_{100}–III	80	16 ± 0.9	17 ± 0.9	0.34 ± 0.03

[a]CALs mean catalase-containing liposomes with the subscripts standing for the mean diameter of liposomes approximately equal to the nominal pore size used in the extrusion step
[b]C_{in} values were calculated considering that each liposome contain one catalase molecule (reproduced from ref. 5 with modification with permission from Elsevier, Inc., Amsterdam)

Table 2
Characteristics of various enzyme-containing liposomes reported

Enzyme encapsulated in liposomes	Concentration of enzyme in hydration step C_0 [mg/mL]	Concentration of enzyme in liposome C_{in} [mg/mL]	References
α-Chymotrypsin from *bovine pancreas*	20	7.1	(16)
Glucose oxidase from *Aspergillus niger*	1.3	0.78	(7)
Proteinase K from *Tritirachium album*	2.0	0.95	(4)
Yeast alcohol dehydrogenase	5.0	3.3	(6)

The C_{in} values are calculated assuming that the diameter D of the enzyme-containing liposomes is 115 (11) or 100 nm

6. Calculate the catalase concentration in liposomes C_{in} and the number of biologically active catalase molecules per liposome N (see Note 13).

3.3. Stability of Liposomal Catalase Activity (See Figs. 1 and 2)

The stabilities of free and liposomal catalase at 55°C are shown in Figs. 1 and 2, respectively (5). It is clearly seen in Fig. 1 that the thermal stability of free catalase is dependent on its concentration. The higher the enzyme concentration employed, the higher its thermal stability at the free catalase concentration range of 0.25 μg/mL to 5.0 mg/mL. This means that at the enzyme

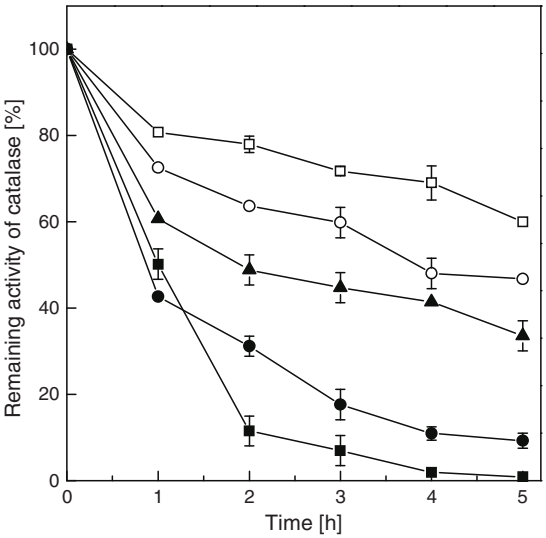

Fig. 1. Time courses of remaining activity of free catalase at enzyme concentrations of 16 mg/mL (*closed circles*), 5.0 mg/mL (*open squares*), 0.1 mg/mL (*open circles*), 5.0 μg/mL (*closed triangles*), and 0.25 μg/mL (*closed squares*) in 50 mM Tris–HCl/100 mM NaCl buffer (pH 7.4) at 55°C (reproduced from ref. 5 with permission from Elsevier, Inc., Amsterdam).

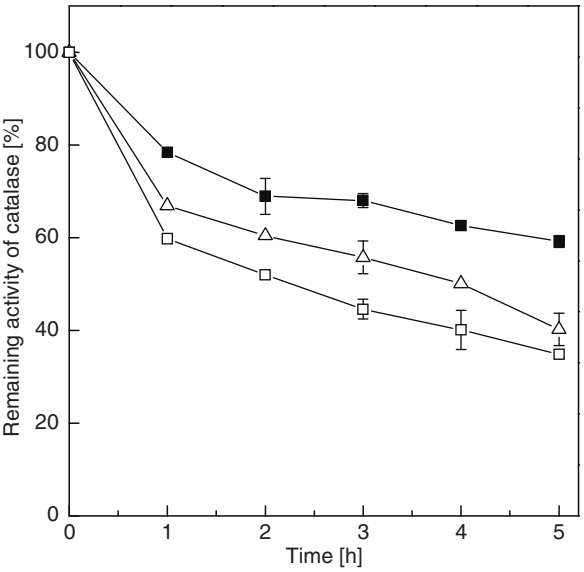

Fig. 2. Time courses of remaining activity of catalase in CAL_{100}-I (*open squares*), CAL_{100}-II (*closed squares*), and CAL_{100}-III (*open triangles*) at fixed POPC concentration of 2.8 mM in Tris buffer (pH 7.4) at 55°C. The overall catalase concentrations were 5.9, 52, and 140 μg/mL for suspensions of CAL_{100}-I, CAL_{100}-II, and CAL_{100}-III, respectively. For detailed characteristics of CAL_{100}, see Table 1 (reproduced from ref. 5 with permission from Elsevier, Inc., Amsterdam).

concentrations, dissociation of the enzyme into its subunits dominates the enzyme deactivation observed. On the other hand, the catalase at the highest concentration of 16 mg/mL shows lower stability than that at 5.0 μg/mL. For the 16 mg/mL of catalase, the formation of irreversible intermolecular aggregates is facilitated among the conformationally altered enzyme molecules. Figure 2 shows the stability of liposomal catalase systems with different liposomal enzyme concentrations C_{in} at 55°C. The liposomal catalase with C_{in} of 4.9 mg/mL (CAL_{100}-II) shows the highest thermal stability. Quite importantly, the thermal stability of the liposomal catalase with C_{in} of 16 mg/mL (CAL_{100}-III) is much higher than that of free enzyme at the identical concentration (see Fig. 1). This is because the formation of enzyme aggregates is prevented in the liposomal aqueous phase through the interaction of the inner surface of the liposome membrane with the encapsulated enzyme molecules (5). The liposomal system therefore can be a functional carrier that has the stabilization effect on the structure and activity of the liposome-encapsulated catalase molecules.

4. Notes

1. Unsaturated lipid POPC is hygroscopic and thus should be treated under dry atmosphere.
2. Water should be sterilized and deionized using a water purification system (Elix 3UV, Millipore Corp., Billerica, MA). The minimum resistance to the water is 18 MΩcm.
3. Avoid vigorous mixing using a vortex mixer to minimize possible conformational change of the enzyme through its adsorption to the gas–liquid interface. The freezing and thawing treatments (see below) with occasional gentle shaking induce complete removal of the lipid film from the wall of the flask.
4. For preparing the refrigerant, the dry ice is crashed into small pieces with a hammer and mixed with ethanol in the Dewar flask. Check the effects of the repetitive freezing and thawing treatments on the enzyme activity. When saturated lipids with high T_m are employed instead of POPC, the thawing temperature should be carefully controlled to minimize the thermal deactivation or partial denaturation of the enzyme molecules.
5. The bubbles formed in the syringes of the extruder should be carefully removed before passing the MLV suspension through the membrane. The commercially available stabilizer for the extruder is recommended to be used. For preparing the liposomes with mean diameter of about 30 or 50 nm, the MLVs are extruded through the pores with the diameter of

100 nm and then the membranes with smaller pores are employed for further reduction in the size of liposomes.

6. The GPC column is prepared by packing sepharose 4B gel beads into the glass column. Avoid the formation of bubbles in the gel bed. The ethanol originally contained in the gel suspension should be exhaustively removed by eluting the Tris buffer solution through the column to eliminate the effect of ethanol on the enzyme conformation. Using a transparent glass column is advantageous to visualize elution and separation behaviors of slightly colored enzymes including catalase and turbid liposomes. The sample volume applied to the GPC column is <1.0 mL. Elution of liposomes and free (nonentrapped) enzyme molecules is quantitatively confirmed by measuring the optical density at 400 nm and the enzyme activity, respectively, of each fraction collected. For obtaining the enzyme-containing liposomes with narrow size distribution, the liposome-containing fractions should not be mixed together.
7. This assay is applicable to the determination of phospholipids containing choline group such as POPC. The POPC concentration in an enzyme-containing liposome suspension is selectively measured by quantifying the H_2O_2 produced from the lipid by a series of reactions catalyzed by phospholipase D, choline oxidase, and peroxidase. The effect of catalase activity derived from the catalase-containing liposomes is negligible in the quantification of the H_2O_2 produced because the enzyme concentration in the assay solution is low enough.
8. The deactivation of catalase molecules encapsulated in liposomes at the number of enzyme molecules per liposome of 5.2 is negligible at least for 22 days (5). The storage stability of the liposomal catalase decreases with decreasing the liposomal enzyme concentration (9). On the other hand, the stability of liposomal enzyme is practically unaffected by the liposome concentration.
9. Since decomposition of H_2O_2 slowly occurs during its storage, the 100 mM H_2O_2 solution should be freshly prepared each day.
10. The catalase-catalyzed decomposition of H_2O_2 yields oxygen and water. The stoichiometric equation is $H_2O_2 \rightarrow (1/2)\,O_2 + H_2O$.
11. Effects of cholate and cholate/lipid mixed micelles on the enzyme activity measurement should be checked. The activity of bovine liver catalase is practically unaffected by cholate up to 40 mM. The minimal cholate concentration required for complete solubilization of liposomes is generally dependent on the lipid concentration in the system and the lipid–water

partitioning behavior of cholate as previously reported (3, 4). Triton X-100 (*tert*-octylphenoxypolyethoxyethanol) can be alternatively used for solubilization of liposome membranes.

12. The observed activity of liposomal enzyme is smaller than the intrinsic one because of the permeation resistance of lipid membranes to the substrate (H_2O_2) molecules as shown in Table 1. The reactivity of other liposomal enzymes is controlled by modulating the preformed enzyme-containing liposomes with sublytic concentrations of detergents such as sodium cholate and Triton X-100 (4) and the channel-forming protein (10). Mechanical stresses such as liquid shear and gas–liquid flow were also shown to be effective for increasing the permeability of liposome membranes to the dye molecules with low molecular mass (14). To obtain reliable E value in the presence of the membrane modulators, the leakage of enzyme molecules from the liposome interior to the bulk liquid should be minimized.
13. To determine the POPC liposome concentration, the number of lipid molecules per liposome is calculated on the basis of the assumptions that the bilayer membrane thickness t is 37 Å, the mean head group area A is 72 Å^2 (15), and liposomes are spherical with their diameter of D. The number of lipid molecules per liposome n can be calculated as $n=(4\pi/A)\{D^2/4+(D/2-t)^2\}$. The liposome concentration C_v is then determined as $C_v=C_T/n$, where C_T stands for the lipid concentration measured. The mean number of active enzyme molecules per liposome N could be calculated as the concentration of active enzyme relative to that of liposomes C_v determined as described above. As shown in Table 1, the catalase concentration in liposomes C_{in} is definitely dependent on that in the Tris buffer C_0 employed in the lipid hydration (step 7 in the Subheading 3.3.1). In the table, the N values are less than unity for the catalase-containing liposomes prepared at C_0 of 1.3 mg/mL. This means that the liposomal enzyme suspensions prepared at the above condition are the mixture of catalase-containing liposomes and empty (enzyme-free) ones. For the liposomal catalase with $N>1$, the C_{in} values are smaller than C_0. For other liposomal enzyme systems reported, the C_{in} values are consistently smaller than the C_0 ones, see Table 2 for details.

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