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Solubilization by Triton X-100 makes possible complete recovery of lipids from liposomes in enzymatic assay

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Abstract

We have developed an accurate and sensitive method for enzymatically determining phosphatidylcholine (PC) and cholesterol (CHOL) in liposomes. Solubilizing liposomes with a high concentration (80%) of Triton X-100 at 65°C for 5 min led to the complete recovery of the lipids by current assay using commercial kits. The method had good linearity in a range of 0.004–0.4 μmol PC. Using this method, PC and CHOL were completely recovered from various liposomes. We conclude that PC and CHOL in liposomes can be determined accurately and sensitively by this method.

Key words: Liposome; Solubilization; Phosphatidylcholine; Cholesterol; Enzymatic method; Triton X-100

1. Introduction

Liposomes have been extensively studied as drug carriers for chemotherapy [1] or gene therapy [2] and as biomembrane models [3]. The accurate determination of lipids in liposomes is necessary for reproducibility and validity of preparations and experiments.

There are many methods to determine phospholipids and other lipids in liposomes [4–7]. In general, the phosphorus assay [4], based on the colorimetric determination of a complex of ammonium molybdate with inorganic phosphate yielded by acid digestion of liposome, appears to be the most favored [5]. The method has high sensitivity, but it requires strong acids and heavy metals [6]. It is complicated and time-consuming [5,6] and cannot be applied to phospholipids in the presence of other phosphoric compounds. Unlike such a chemical method, the enzymatic method using phospholipase or cholesterol oxidase is simple, rapid and specific for lipids such as phosphatidylcholine and cholesterol [6,7], and can be used to determine these lipids in serum. But in some cases, the recovery of lipids and reproducibility are low compared with chemical methods such as the phosphorus assay.

A nonionic detergent, Triton X-100, is widely used for the solubilization of biomembranes [8] and liposomes [9]. Mixed micelles of phospholipids and Triton X-100 cause an increase in the specific activity of phospholipases toward phospholipids [10,11]. This indicates that solubilizing liposomes with a detergent such as Triton X-100 would increase the recovery of lipids in the enzymatic assay.

This paper reports that accurate and sensitive determination of lipids in various liposomes using the enzymatic method is possible by pretreatment with a high concentration (80%) of Triton X-100 at 65°C.

2. Materials and methods

2.1. Chemicals

Distearoylphosphatidylcholine (DSPC) was purchased from Sigma (St. Louis, MO, USA). Dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphos-

Abbreviations: MLV, multilamellar vesicle; SUV, small unilamellar vesicle; PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; EPC, egg phosphatidylcholine; HEPC, hydrogenated egg phosphatidylcholine; CHOL, cholesterol; DCP, dicetyl phosphate; DAOS, 3,5-dimethoxy-*N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)aniline sodium; Mes, 4-morpholineethanesulfonic acid.

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phatidylcholine (DPPC) were from Nippon Oil and Fats (Tokyo, Japan). Egg phosphatidylcholine (EPC) and hydrogenated egg phosphatidylcholine (HEPC) were from Asahi Chemical (Tokyo, Japan). Cholesterol (CHOL) and Triton X-100 were from Wako Pure Chemicals (Osaka, Japan). Dicetyl phosphate (DCP) was from Nacalai Tesque (Kyoto, Japan). Other chemicals used were of reagent grade.

2.2. Preparation of liposomes

Multilamellar vesicles (MLVs) were prepared by vortexing dried lipid films in saline. For the preparation of small unilamellar vesicles (SUVs), MLVs were sonicated for 60 min with a probe-type sonicator (model US-600, Nihonseiki Kaisha, Osaka, Japan). The concentration of total lipids was usually 40 mM.

2.3. Kits for enzymatic assay of lipids

Phospholipids C-test Wako and Free Cholesterol E-test Wako (Wako Pure Chemicals, Osaka, Japan) were used as kits for enzymatic assay of PC and CHOL, respectively. The enzymatic reagent of the PC assay kit contains 0.47 units phospholipase D (from *Streptomyces* species), 2.0 units choline oxidase (from *Arthrobacter* species), 4.2 units peroxidase (from Horseradish), 3.9 units ascorbate oxidase (from cabbage), 0.77 μmol 3,5-dimethoxy-*N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)aniline sodium (DAOS) and 0.24 μmol 4-aminoantipyrine in 1 ml of 50 mM Good's buffer (pH 7.5). The enzymatic reagent of the CHOL assay kit contains 0.29 units cholesterol oxidase (from *Streptomyces* species), 4.7 units peroxidase (from Horseradish), 4.4 units ascorbate oxidase (from cabbage), 0.98 μmol DAOS and 0.19 μmol 4-aminoantipyrine in 1 ml of 50 mM Mes buffer (pH 6.1). The principles of these assays are to determine a blue dye (λ_{max} = 600 nm) due to the oxidative coupling of hydrogen peroxide with 4-aminoantipyrine and DAOS by peroxidase [6]. PC is hydrolyzed by phospholipase D and choline is liberated. Choline is oxidized by choline oxidase to betaine with the simultaneous production of hydrogen peroxide. CHOL is oxidized by cholesterol oxidase to obtain hydrogen peroxide.

2.4. Enzymatic assay for lipids

Liposomes (0.02 ml) were mixed vigorously with Triton X-100 aqueous solution (0.08 ml) and incubated at room temperature (about 23°C) or 37–65°C for 0–5 min. The concentrations of liposome and Triton X-100 in the mixture were 0.08–8 mM as total lipids and 0–80% (v/v), respectively. The mixture was incubated with the enzymatic reagent (4.9 ml) for 37°C for 5 min. The final concentrations of lipids and Triton X-100 in the reaction mixture were 0.0016–0.16 mM and 0–1.6%, respectively. Within this range, the detergent had no influence on the enzymatic activity. After incubation, the reaction mixture was cooled on ice and centrifuged at 18800 × *g* for 10 min at 4°C to separate the unreacted vesicles. Absorbance at 600 nm of the supernatant was detected with a spectrophotometer (U-3410, Hitachi, Tokyo, Japan) against a blank. Calibration curves were obtained with the standard solutions of the assay kits. The recovery of PC and CHOL from liposomes in the enzymatic assay was expressed as the percentage of the content of each as determined by chemical assay.

2.5. Chemical assay for lipids

The concentration of phospholipids in liposomes was determined by a phosphorus assay using the Phospholipids test Wako (Wako Pure Chemicals, Osaka, Japan), which is based on the colorimetric determination of a complex of ammonium molybdate with inorganic phosphate yielded by acid digestion of liposomes. PC concentration was estimated by multiplying the total phosphate concentration by the mole fraction of PC in total phospholipids. CHOL concentration in liposomes was determined according to the method of Franey and Amador [12].

3. Results

3.1. Effects of pretreatment with Triton X-100 at room temperature on PC recovery

The recovery of PC when 8 mM MLVs composed of DSPC and CHOL (1:1) (DSPC/CHOL (1:1)-MLVs) were pretreated with 0–80% of Triton X-100 at room temperature is shown in Fig. 1A. The final concentration of the detergent in the reaction mixture was above its critical micelle concentration (0.02% [13]) to prevent re-transition from micelles to vesicles. For no pretreatment with Triton X-100 using a commercial kit, the recovery of PC was only 6%. It was less than 20% when liposomes were pretreated with 4–48% Triton X-100. It increased to 40–60% by pretreatment with Triton X-100 at a concentration higher than 50%. Fig. 1B shows the recovery of PC when the concentration of liposomes decreased from 8 to 0.08 mM at a fixed concentration (80%) of Triton X-100 used in the pretreatment of them. The recovery of PC remained about 50% over the liposomal concentration examined. Throughout the experiment, pellets of vesicles were observed after centrifugation at 18800 × *g* for 10 min. This indicates that some PC molecules in lipid bilayer were not hydrolyzed by phospholipase D in the reagent under such conditions. The enzymatic reagent in the

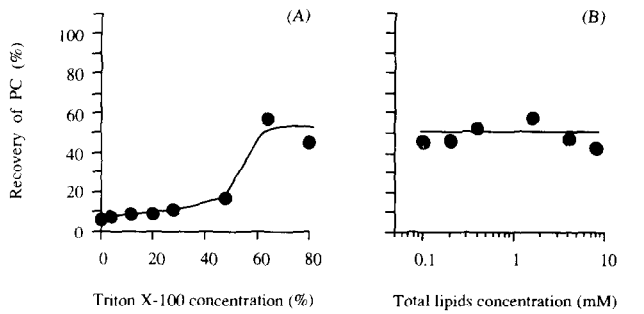

Fig. 1. Recovery of PC from DSPC/CHOL (1:1)-MLV pretreated with various concentrations of Triton X-100 at room temperature by the enzymatic method. (A) After pretreatment of 8 mM of MLVs with 0–80% Triton X-100 at room temperature, the final concentrations of lipids and detergent were adjusted to 0.16 mM and 0–1.6%, respectively, by the enzymatic reagent. The mixture was incubated at 37°C for 5 min. (B) After pretreatment of 0.08–8 mM of MLVs with 80% Triton X-100 at room temperature, the final concentrations of lipids and detergent were adjusted to 0.0016–0.16 mM and 0–1.6%, respectively, by the enzymatic agent. The mixture was incubated at 37°C for 5 min. Recovery of PC was determined as described in Materials and methods.

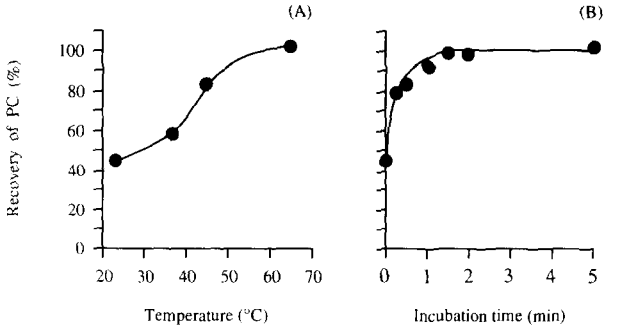

Fig. 2. Effects of incubation temperature and time with 80% Triton X-100 on the recovery of PC from DSPC/CHOL (1:1)-MLV by the enzymatic method. (A) After pretreatment of 8 mM of MLVs with 80% Triton X-100 at various temperatures for 5 min, the final concentrations of lipids and detergent were adjusted to 0.16 mM and 1.6%, respectively, by the enzymatic reagent. The mixture was incubated at 37°C for 5 min. (B) After pretreatment of 8 mM of MLVs with 80% Triton X-100 at 65°C for 0–5 min, the mixture was incubated with the enzymatic reagent under the same conditions as above. Recovery of PC was determined as described in Materials and methods.

assay kit contained a Triton X-100-like detergent to solubilize lipoproteins in serum, but this detergent had almost no effect on the recovery of PC from MLVs in a preliminary experiment using a detergent-free enzymatic reagent (data not shown).

3.2. Combined effects of pretreating with Triton X-100 and heating on PC recovery

Temperature may affect recovery of PC because it can influence the state of liposomal membranes such as fluidity, as well as the solubilizing ability of detergent. Fig. 2A shows the effects of temperature during pretreatment with 80% Triton X-100 on recovery of PC from DSPC/CHOL (1:1)-MLVs. The recovery of PC increased with temperature and reached 100% at 65°C. At this temperature, a series of enzymatic reactions was proceeded to completion by pretreating with 80% Triton X-100 at least for 2 min (Fig. 2B). The pretreating temperature was chosen considering the phase transition temperature of PC. No clouding was observed during pretreatment with Triton X-100 at 65°C.

We performed two experiments to determine the optimal concentration of Triton X-100 for liposomal pretreatment with heating (Fig. 3A and B). Fig. 3A shows the recovery of PC when 8 mM DSPC/CHOL (1:1)-MLVs were pretreated with various concentrations of Triton X-100 at 65°C for 5 min. The complete recovery of PC required more than 30% Triton X-100. Fig. 3B shows the effects of Triton X-100 concentration and incubation temperature on the recovery of PC from DMPC/CHOL (1:1)-, DPPC/CHOL (1:1)- and DSPC/CHOL (1:1)-MLVs. In this experiment, the molar ratio of detergent/lipids was constant to prevent differences in solubilizing ability of the detergent. The ratio was about 160, corresponding to the value when 8 mM liposomes were pretreated with 80% Triton X-100. The complete recovery of PC required more than 20% Triton X-100 in pretreatment of DSPC/CHOL (1:1)-MLVs at 65°C. On heating DMPC/CHOL (1:1)- and

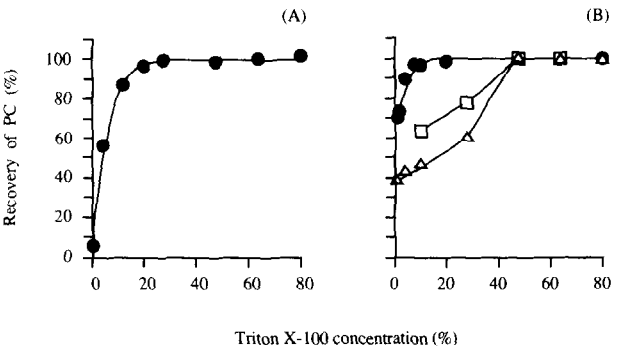

Fig. 3. (A) Recovery of PC from DSCP/CHOL (1:1)-MLV pretreated with various concentrations of Triton X-100 at 65°C by the enzymatic method. After pretreatment of 8 mM of MLVs with 0–80% Triton X-100 at 65°C for 5 min, the mixture was incubated with the enzymatic reagent under the same conditions as in Fig. 1A. Recovery of PC was determined as described in Materials and methods. (B) Recovery of PC from PC/CHOL (1:1)-MLVs pretreated with various concentrations of Triton X-100 at constant Triton X-100/lipids concentration ratio by the enzymatic method. DMPC/CHOL-MLV (□) or DPPC/CHOL-MLV (△) and DSPC/CHOL-MLV (●) were pretreated with various concentrations of Triton X-100 at 45 and 65°C for 5 min, respectively. The ratio corresponded to the value when 8 mM of MLVs were pretreated with 80% Triton X-100 and was about 160 in a molar ratio. Each mixture was incubated with the enzymatic reagent at 37°C for 5 min. Recovery of PC was determined as described in Materials and methods.

Table 1
Recovery of PC from various liposomes pretreated with 80% Triton X-100 at 65°C by the enzymatic method

Lipid composition (in a molar ratio)	Vesicle size	Recovery of PC (%)	
		no pretreatment	pretreatment
DMPC/CHOL = 1:1	MLV	17.27	101.18
DPPC/CHOL = 1:1	MLV	7.19	98.62
DSPC/CHOL = 1:1	MLV	5.56	101.86
DMPC/DCP = 9:1	MLV	96.18	98.30
DPPC/DCP = 9:1	MLV	73.64	100.16
DSPC/DCP = 9:1	MLV	8.70	101.30
DSPC/CHOL/DCP = 7:2:1	MLV	11.52	97.80
DSPC/CHOL/DCP = 5:4:1	MLV	28.46	100.56
HEPC/CHOL/DCP = 5:4:1 [a]	MLV	10.73	98.07
EPC/CHOL/DCP = 5:4:1 [a]	MLV	78.95	103.20
DSPC/CHOL/DCP = 5:4:1	SUV	92.14	98.57

After 8 mM of liposomes were pretreated with 80% Triton X-100 at 65°C for 5 min, the mixture was incubated with the enzymatic reagent under the same conditions as in Fig. 2A. Recovery of PC was determined as described in Materials and methods. Each value represents the mean of one to three experiment(s).
[a] MLVs were pretreated with Triton X-100 at 60°C for 5 min.

DPPC/CHOL (1:1)-MLVs at 45°C, it was necessary for the complete recovery of PC to pretreat with Triton X-100 at more than 50%.

PC was accurately determined in the enzymatic assay when DSPC/CHOL (1:1)-MLVs were pretreated with 80% Triton X-100 at 65°C for 5 min in a concentration range of lipids from 0.08 to 8 mM, corresponding to a DSPC range from 0.004 to 0.4 μmol.

3.3. Determination of PC and CHOL in various liposomes

Recovery of PC from various liposomes in the enzymatic assay after pretreatment with 80% Triton X-100 at 65°C for 5 min is summarized in Table 1. For no pretreatment, i.e., current assay, PC recovery was influenced by lipid composition and vesicle size, and was less than 100% for all liposomes. However, pretreatment with 80% Triton X-100 at 65°C for 5 min gave complete recovery.

Table 2
Recovery of CHOL from various MLVs pretreated with 80% Triton X-100 at 65°C by the enzymatic method

MLV	Recovery of CHOL (%)	
	no pretreatment	pretreatment
DMPC/CHOL = 1:1	29.12	99.50
DPPC/CHOL = 1:1	7.95	100.13
DSPC/CHOL = 1:1	6.15	97.19

After 8 mM of MLVs were pretreated with 80% Triton X-100 at 65°C for 5 min, the mixture was incubated with the enzymatic reagent under the same conditions as in Fig. 2A. Recovery of CHOL was determined as described in Materials and Methods. Each value represents the mean of three experiments.

CHOL is used for the preparation of liposomes, as well as PC. We tested whether CHOL could be accurately determined by enzymatic assay after pretreatment of liposomes with Triton X-100 under the same conditions (Table 2). Like PC, no pretreatment with Triton X-100 gave incomplete recovery of CHOL, whereas CHOL was completely recovered when the liposomes were pretreated with 80% Triton X-100 at 65°C for 5 min.

4. Discussion

The specific activities of various phospholipases such as phospholipase A_2, phospholipase C and phospholipase D toward phospholipids have been reported to increase in mixed micelles of phospholipids and Triton X-100 [10,11]. This suggests that solubilizing liposomes with this detergent may increase the recovery of lipids. Alonso et al. [13] have reported that the solubilizing reaction of Triton X-100 for MLVs is much slower than that for SUVs and that it progresses in a timescale of hours. A high concentration of detergent and/or high reaction temperature may thus be necessary for enhancing the solubilization of MLVs by the detergent.

In this study, DSPC/CHOL (1:1)-MLVs were used mainly to clarify the effects of Triton X-100 concentration and pretreating temperature on PC recovery. The reasons are as follows. DSPC was the least reactive in the current enzymatic assay as shown in Table 1; the incorporation of CHOL into lipid membranes inhibits the solubilization of vesicles by Triton X-100 [14] and if pretreatment by detergent allows the complete recovery of PC in this MLV, the same should be possible for any type of liposome.

The results in Figs. 1 and 3B indicate that PC recovery from DSPC/CHOL (1:1)-MLVs remarkably increases by pretreatment with more than 50% Triton X-100. At more than 50%, Triton X-100 is in the reverse micellar phase [15]. A change in liposomal structure due to phase inversion may enhance liposomal solubilization.

As shown in Fig. 2, the effect of Triton X-100 increased with temperature and became maximal at 65°C. An increase in temperature may enhance the membrane fluidity of liposomes and the solubilizing ability of Triton X-100. In general, membrane fluidity increases with temperature [16]. DSPC has the highest phase-transition temperature (58°C [17]) among PCs used in this study and thus lipid bilayers of all liposomes are in a liquid-crystal state at 65°C. The insertion of detergent into liposomal membranes and its lateral diffusion therein may be facilitated under these conditions. The ability of a nonionic detergent such as Triton X-100 to solubilize lipid components increases as the temperature increases to near its cloud point

[18]. At 65°C, Triton X-100 shows very high solubilizing ability because its cloud point is 67°C [19]. This increase may maximize the solubilization of liposomes together with increased membrane fluidity.

The increase in solubilizing ability of Triton X-100 is considered to contribute more to the increase in PC recovery than that of membrane fluidity in liposomes, since the complete recovery of PC from DMPC/CHOL (1:1)- or DPPC/CHOL (1:1)-MLVs required pretreatment with more than 50% Triton X-100 at 45°C which is higher than the phase-transition temperatures of DMPC and DPPC (23 and 41°C [17], respectively) (Fig. 3B). Therefore, if a detergent has higher solubilizing ability, PC may be completely recovered either at a lower temperature or at a lower concentration of the detergent.

The solubilization of liposomes by a detergent proceeds in three steps: equilibrium distribution of the detergent between the lipid and water phases, coexistence of mixed bilayers and mixed micelles with detergents and transformation of all of the bilayers into mixed micelles with the detergents [20,21]. A high concentration of detergent and high pretreating temperature would enhance the liposomal transition from lamellar to micellar structure. The changes in liposomal structures induced by pretreatment with Triton X-100 were monitored using resonance energy transfer [22] (data not shown). The results showed that the recovery of PC corresponded to the state of the lamellar-to-micellar transition.

The complete recovery implies that all PC molecules are hydrolyzed by phospholipase D. In liposomes, it should take a long time for PC molecules in the inner layer to be hydrolyzed by the enzyme due to the lamellar structure. The packing between PC molecules may interfere with interfacial reactions between PC molecules and phospholipase D. In mixed micelles, PC molecules may be more sensitive to the enzyme because of the increase in their number and surface area compared with liposomes [23]. Triton X-100 may facilitate the adsorption of phospholipase D on the surface of micelles due to decreased packing or clustering between PC molecules [10,11] and increase the reactivity of the enzyme to PC molecules by reducing the activation energy of the enzyme at the interface [11]. The increased reactivity of cholesterol oxidase to CHOL in the mixed micelles may also be interpreted in the same way.

The enzymatic method proposed in this study has good linearity in a wide range from 0.004 to 0.4 μmol PC. The determination range of the phosphorus assay is about 0.02–0.1 μmol as PC [4]. The sensitivity of the enzymatic method is not lower than that of the phosphorus assay. PC and CHOL were completely recovered from various liposomes using the present enzymatic method (Tables 1 and 2). We thus conclude that PC and CHOL in various liposomes can be accurately and sensitively determined by the enzymatic method. In addition, it is expected that PC and CHOL in cell membranes such as RBC ghosts will be determined using this method.

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