Preparation of Stable Liposomes Using Sucrose Density Gradient Centrifugation and Their Interaction with Insecticidal Cry1A Toxins of *Bacillus thuringiensis*

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Summary

Liposomes were constructed with phosphatidylcholine (PC) and fluorescence indicator, calcein, and purified using 9-40% discontinuous sucrose density gradients centrifugation (SDGC) or gel filtration column chromatography (GFCC). Stability and sensitivity of those liposomes to insecticidal Cry1A toxins of *Bacillus thuringiensis* were compared each other and one purified by GFCC was shown to leak calcein by 40% of total fluorescence contents during 96 hr storage. On the other hand, liposome purified by SDGC was more stable and almost no leakage observed during the same period. Cry1Ab of *B. thuringiensis* weakly reacted with the liposome purified by GFCC and 2.5% of total encapsulated fluorescence was released, but Cry1Aa and Cry1Ac did not. Contrarily, liposome purified by SDGC was highly reactive with Cry1Ab and 40% of the calcein was released and 15%-release by Cry1Aa and Cry1Ac were also observed. These data clearly indicated that liposomes purified by SDGC were more stable and highly reactive to Cry toxins compared to that purified by GFCC. These liposomes are useful tool to analyze the interaction between Cry1A toxins and lipid membranes

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Key words: Phosphatidylcholine (PC), Liposome, Insecticidal Cry1A toxins, *Bacillus thuringiensis*, calcein, interaction between liposome and Cry1Ab

Insecticidal Cry toxins produced by *B. thuringiensis* kill insect larvae with narrow specific spectrum and used as world-wide agent of insect pest control. However, the appearance of resistance insects has been a major threat to the use of Cry toxins. Cry toxins bind to brush border membrane (BBM) of insect larval midgut and form pore on the membrane that lead to insect death. Therefore, most common mechanism for resistance is reduced binding of Cry toxin to BBM[1,2,3]. On the other hand, resistance without reduced binding have also been reported in various insects[4, 5, 6]. We previously found that Cry1Ac bound to BBM proteins from midgut of Cry1Ac-susceptible and Cry1Ac-highly resistant *Plutella xylostella* larvae with almost identical binding kinetics[7]. Thus, the mechanisms of resistance seem to be complicated and the interaction between each Cry toxin and element of BBM should be analyzed precisely in native condition. But these analyses are not easy due to the complexity of BBM structures.

Artificial lipid membrane vesicle, liposome, is a useful tool to study the interaction with Cry toxins and several suggestive and informative observations have been reported. Sangadala *et al.* reconstituted liposome containing proteins, proteoliposome, with phosphatidylcholine (PC), 120 kDa aminopeptidase N (APN) and APN-like 65 kDa protein of *Manduca sexta*[8]. Release of entrapped $^{86}Rb^{+}$ from the proteoliposome was enhanced 1,000 fold compared to that of simple PC liposomes, suggesting the binding of APN with Cry1Ac and some important roles in an insecticidal activity of Cry1Ac. High pore-forming ability of α4-loop-α5 helix in domain I of Cry1Ac, supporting the umbrella model[9, 10, 11] was also shown by liposomes[12].

Recently we demonstrated that Cry1Ab destroyed PC/phosphatidylserine (PS)-Liposomes at 1.4 μM but Cry1Aa and Cry1Ac did not[13]. This suggested that Cry1Ab possessed unique mode of action different from Cry1Aa and Cry1Ac.

Kato *et al.*[13] separated liposomes from un-entrapped calcein with GFCC using Sephadex G-50. GFCC seemed to work well in separation of calcein from non reacted one, but the resulted liposome-suspension showed significant fluorescence back ground indicating leakage of calcein from those liposomes. This might be caused by the contamination of incompletely sealed or unstable liposomes that were disrupted easily. To obtain precise kinetics of the interaction, it is prerequisite to reconstruct liposome which is stable and does not release the entrapped calcein without interaction with Cry toxins. Here we described an improved method to prepare the liposome showing no leak during 4 days and we checked interaction of the liposome with Cry toxins.

MATERIALS AND METHODS

Materials

Egg yolk PC and fluorescence maker, calcein were purchased from Sigma Aldrich (St. Louis, MO, USA).

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Cry toxins

B. thuringiensis sotto T84A1, a gift of Professor M. Ohba, Kyushu University, and *B. thuringiensis kurstaki* HD-73 were used to prepare Cry1Aa and Cry1Ac, respectively. Cry1Ab was purified from *Escherichia coli* JM109 harboring *pYD4.0* containing genetically engineered *cry1Ab* given by Professor K. Kanda, Saga University[14]. Solubilization, activation and purification of Cry toxins were done as described[15].

Preparation of PC-Liposomes

Large unilamellar vesicle (LUV) of liposome was prepared as described[16,17] with some modifications. Two mg of egg yolk PC was dissolved in 2 ml of chloroform and the chloroform was then removed to generate thin film of PC using circulating aspirator (WJ-15, SHIBATA SCIENTIFIC TECHNOLOGY LTD., Tokyo, Japan). The film was suspended with 1 ml of 100 mM calcein in 0.3 M NaOH containing 100 mM NaCl and 50 mM Tris-HCl, pH 8.3 by sonication for 10 min with 30 sec interval after every 30 sec performance. This, PC suspension, was allowed to stand for 30 min at room temperature. In these steps, PC forms multilamellar vesicle (MLV) followed by formation of small unilamellar vesicle (SUV) of liposome. LUV of PC-Liposome was generated by the repetition of freeze and thaw of SUV using liquid nitrogen at room temperature.

Generally, these are right protocols to generate LUV of liposome, however to make stable proteoliposomes with BBM proteins, further modifications were required. Thus, we combined the method making LUV with the one constructing proteoliposome[17,18]. Triton X-100 containing BBM proteins was added to the LUV of PC-Liposome at 0.12% (final concentration) and incubated for 1 hr at 4 °C. Then, Triton X-100 in the suspension was removed thoroughly using Bio-beads SM-2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The LUV of PC-Proteoliposome containing calcein constructed was used in following experiments.

Purification of liposomes

Liposomes containing calcein were separated from un-entrapped calcein using GFCC or SDGC. GFCC was done with Sephacryl S-300 HR column (0.5 cmϕ x 50 cm *h*) equilibrated with 50 mM Tris-HCl, pH 8.3 containing 100 mM NaCl and each 0.7 ml fraction was collected at flow rate of 10 ml/hr. Discontinuous SDGC was done with sucrose gradient made by piling of sucrose layer of 37.0, 32.5, 29.5, 25.1, 21.0, 17.2, 13.4 and 9.0% (w/w) sucrose solutions. Liposome suspension of 700 μl was mixed with 1.3 ml of 64% (w/w) sucrose to make liposome suspension in ~40% sucrose aqueous solution and the suspension was placed at the bottom of sucrose gradient. After a centrifugation at 160,000 x *g* for 3 hr at 4 °C, the fractions showing orange color of calcein were collected.

Measurement of fluorescence

Fluorescence intensity of calcein was measured by fluorescence spectrophotometer (F3010, Hitachi, Ltd., Tokyo, Japan) with excitation at 490 nm and emission at 520 nm[12].

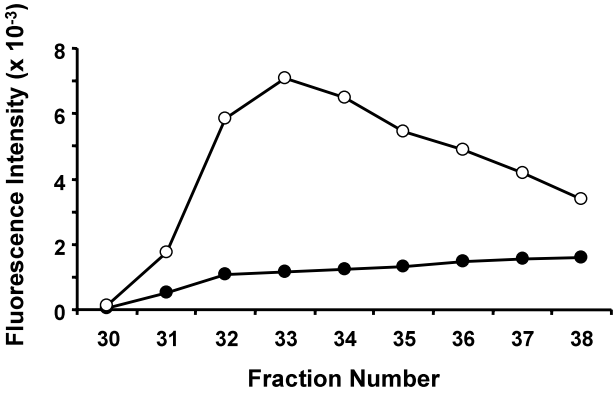

Fig. 1. Fractionation of liposomes with gel filtration column chromatography (GFCC). PC-Liposomes containing calcein were separated from un-entrapped calcein using Sephacryl S-300 HR column (0.5 cmϕ x 50 cm *h*) equilibrated with 50 mM Tris-HCl, pH 8.3, containing 100 mM NaCl. Fluorescence intensity in each fraction was measured before (●) and after (○) the disruption of the liposomes with Triton X-100 at a final concentration of 1%.

Generally, calcein at high concentration, like one in the liposome we made, is in self-quenched, but when once is diluted, emits fluorescence. Disruption of liposome by Cry1A toxins at 1.6 μM was monitored every 30 sec for 30 min with fluctuation of fluorescence intensity. The amount of fluorescence released was expressed as a percentage of the maximum fluorescence intensity achieved by the addition of Triton X-100 (florescence recovery). In control experiment, only the buffer (100 mM NaCl, 50 mM Tris-HCl, pH 8.3) was added.

RESULTS AND DISCUSSION

GFCC of PC-Liposomes

Liposome suspension was fractionated using Sephacryl S-300 HR column, 1% of Triton X-100 (final concentration) was added to aliquots of each fraction, and the fluorescence released from disrupted liposomes was measured. The fluorescence was rapidly increased from fraction 31 and the maximum intensity, 7090 was obtained at fraction 33 (Fig. 1). Fraction 33 was corresponded to void volume of the column, thus the results indicated that the majority of the liposomes was the vesicle with large diameter.

Although the fluorescence of calcein released by treatment with Triton X-100 was decreased at fractions 35-38, the fluorescence intensity of PC-Liposome suspension in the same fractions even slightly increased (Fig. 1). As described in materials and methods, LUV of PC-Liposome was constructed with MLV and SUV of PC-Lliposome. Probably the fractions of 32-34 contained LUV liposomes, whereas the fractions 35-38 might contain liposomes of MLV and SUV. The fractions 32-34 were collected as PC-Liposome and used

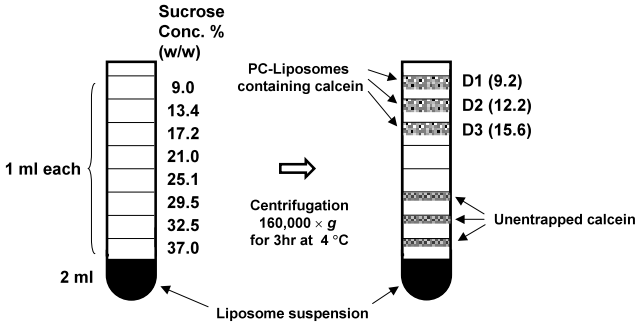

Fig. 2. Schematic diagram of the purification of PC-Liposomes with discontinuous sucrose density gradient centrifugation (SDGC). Two ml of liposome suspension in ~40% sucrose solution was placed at bottom of a centrifuge tube containing layered sucrose solution as shown and centrifuged at 160,000 x *g* for 3 hr at 4 °C. Bands, D1, D2 and D3 containing PC-Liposome with entrapped calcein and bands containing un-entrapped calcein were resulted.

for the following experiments.

Fractionation of PC liposomes in a sucrose density gradient, SDGC

PC-Liposomes were fractionated by the discontinuous SDGC with 9 to 37% (w/w) sucrose aqueous solution. After the centrifugation, 3 bands (D1, D2 and D3) showing thin orange color of calcein were detected at the sucrose layer of 9.2, 12.2 and 15.6%, respectively (Fig. 2). These distinguish bands were also observed when continuous sucrose density gradient centrifugation was employed (data not shown). Fluorescence intensity of the liposomes in D1, D2 and D3 fractions were measured and shown in Fig. 3 with the intensity of the fluorescence obtained by Triton X-100-disruption.

Nothing was clear about size of the liposomes in these 3 fractions, but it was thought to be possibly heterogeneous due to contamination of different types of liposome such as LUV, SUV and MLV. D1, D2 and D3 were collected individually and used for the following experiments.

Stability of liposomes

Leakage of calcein from the liposomes purified with GFCC and SDGC cited above was monitored. Liposome suspensions were stored for 96 hr at 4 °C and fluorescence intensity of the suspension was determined (Fig. 4). In the suspension of liposomes purified with GFCC, fluorescence intensity was 1,200 at right after the purification but after the storage, it was increased to 3,400. Thus, net amount of fluorescence was evaluated as 2,200 and this was corresponded to 40% of the total calcein entrapped, 5,300, into the liposome (Fig. 4, columns of GFCC). In the liposome purified with SDGC, total entrapped fluorescence was roughly equal each other and corresponded to that of liposome purified with GFCC. After 96-hr storage, however,

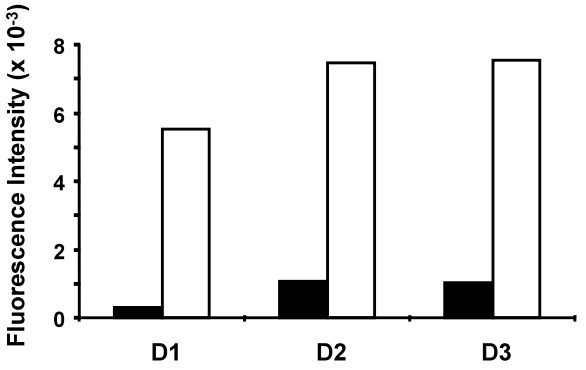

Fig. 3. Fluorescence intensity of PC-Liposome fractions purified by SDGC. Fluorescence intensity of D1, D2 and D3 fractions shown in Fig. 2 were measured before (■) and after (□) disruption with Triton X-100 as shown in Fig 1.

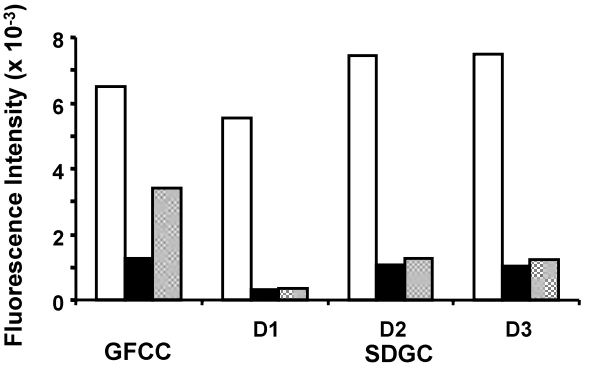

Fig. 4. Comparison of stability of PC-Liposomes purified by GFCC and SDGC. Fluorescence intensity of PC-Liposome suspensions was measured after 96-hr-storage (▩) and compared with that right after the purification (■). The total fluorescence intensity of entrapped was also measured after the disruption of the liposome with 1% Triton X-100 (□).

fluorescence of the liposome suspension did not increase in all three samples, D1, D2 and D3 (Fig. 4, columns of D1, D2, D3). Fluorescence intensity of liposome suspension of D1 measured right after the purification was significantly low compared to that of other three samples. It was not clear that why liposomes purified by GFCC showed significant leakage but liposomes purified by SDGC not. Nonetheless, it was guessed that the contamination of liposomes which were incompletely sealed might be taking place in GFCC-purified fraction, but might not in the fraction separated by SDGC. If this is the case, osmotic pressure given by sucrose solution during SDGC experiments might work to result the well sealed liposomes.

Interaction between PC-Liposomes and Cry1A toxins

Ability of Cry1Aa, Cry1Ab and Cry1Ac to form pores

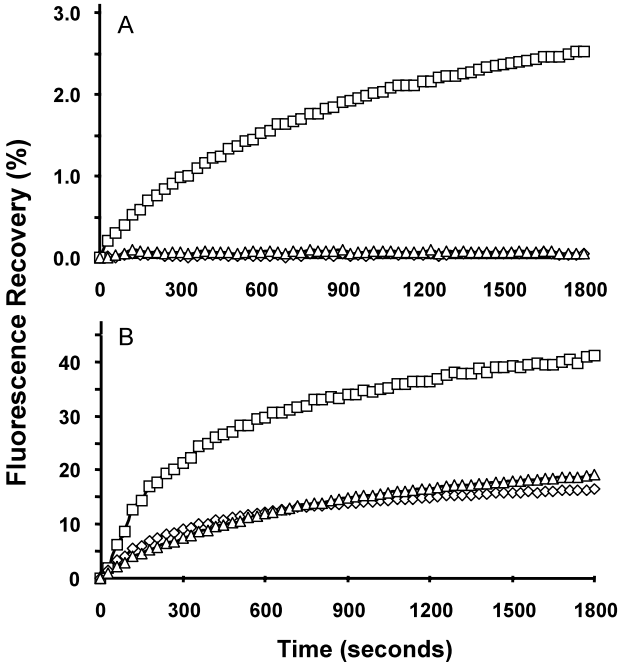

Fig. 5. Pore formation by Cry1A toxins on PC-Liposome purified with GFCC (Panel A) and SDGC (Panel B). Cry1Aa (◇), Cry1Ab (□) and Cry1Ac (△) at 1.6 μM were reacted with liposomes purified by GFCC or SDGC and amount of fluorescence released was expressed as a percentage of the maximum fluorescence intensity achieved by the addition of Trition X-100 (% of florescence recovery). In control experiment, only the buffer, 50 mM Tris-HCl, pH 8.3 containing 100 mM NaCl, was added.

on PC-Liposomes were estimated by calcein release assay. Cry1Ab formed pores at 1.6 μM on PC-Liposomes purified by GFCC and it led 2.5% release of fluorescence (Fig. 5, panel A) but Cry1Aa or Cry1Ac did not. On the other hand, when the D2 liposome fraction purified with SDGC was reacted with Cry1Ab, fluorescence intensity increased by 40% (Fig. 5, panel B). D1 and D3 were also showed almost the same increase of fluorescence on the reaction with Cry1Ab (data not shown). Cry1Aa and Cry1Ac also resulted relatively high levels of calcein release by 15% (Fig. 5 panel B).

Generally, in gel filtration chromatography, sample molecules are fractionated by size and shape, therefore there are some possibilities that the liposomes fraction may contain various liposomes such as incompletely sealed, sealed but without calcein, or even large sized lipid debris. Low stability of the liposomes purified by GFCC is matched with this hypothesis. Cry1A toxins may target not only right liposome but also liposomes without calcein or lipid debris and these heterogeneities may cause low efficiency of calcein release. Liposomes in D1, D2 and D3 were supposed to contain different type of vesicles in size but not in specific gravity. Since no significant difference was observed in increase of fluorescence among them on reaction with respective Cry1A toxins, the liposomes which had homogeneous sensitivity toward respective those toxins must resulted by the fractionation according to their specific gravity.

Liposomes purified by SDGC were more stable and highly reactive to Cry1Ab toxins compared to that purified by GFCC. Our data shown here are preliminary and further analyses are necessary to clarify availability of these techniques for reconstruction of proteoliposome with BBM proteins, however, we believe that we have established fundamental technology to prepare not only proteoliposome but also other various liposomes such as containing specific lipids.

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ショ糖密度勾配遠心法を用いた安定リポソームの作製とCry1A毒素との反応性の評価

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要　約

ホスファチジルコリン（PC）を用いてリポソームを構築し、内部空間に蛍光色素、カルセインを封入した。PCリポソームをショ糖密度勾配遠心法（SDGC）あるいはゲルろ過カラムクロマトグラフィー（GFCC）で精製し、各々の精製PCリポソームについて、溶液中での安定性と*Bacillus thuringiensis*のCry1A殺虫毒素との反応性を比較解析した。GFCCで精製したPCリポソームは不安定で、96時間保存すると内包カルセインの40%がリポソームの外に漏出した。一方、SDGC精製PCリポソームは安定で96時間保存後もほとんどカルセインの漏出を示さなかった。殺虫毒素Cry1AbはGFCC精製PCリポソーム膜に小孔形成を示したが、カルセインの漏出は2.5%にとどまり、小孔形成率は非常に低いと思われた。またCry1AaとCry1Acは共に小孔を形成しなかった。一方、SDGC精製PCリポソームはCry1Abと反応し、40%のカルセイン漏出が検出された。Cry1AaとCry1Acとの反応に際しても15%のカルセイン漏出が検出された。これらの実験結果は、SDGC精製PCリポソームの方がGFCC精製PCリポソームよりも安定で、Cry1A毒素との反応性も高いことを示し、比重に基づきリポソームを精製することが均一な安定リポソームを得るうえで有効であることを示した。SDGC精製PCリポソームはCry1A毒素と脂質膜との相互作用を解析する上で有効である。

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キーワード：ホスファチジルコリン（PC）、リポソーム、Cry1A殺虫トキシン、*Bacillus thuringiensis*、蛍光色素カルセイン、リポソームとCry1Abトキシンとの相互作用

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