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Solubilization of Genistein in Phospholipid Vesicles and Their Atioxidant Capacity

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Abstract: Water-insoluble genistein was solubilized in aqueous medium by using phospholipid vesicles composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dioleoyl-*sn*-glycero-phosphocholine (DOPC) with 0-30% cholesterol. For each vesicle, the maximum solubilization amount of genistein was investigated by X-ray scattering measurement. In addition, the antioxidant capacity of the solubilized genistein was evaluated by the ABTS assay. Genistein was found to be solubilized by 10-20% and 40-50% of the vesicle concentrations of pure DPPC and DOPC respectively. The maximum solubilization amount of genistein decreased to 0-10% and 20-30% when 30% of cholesterol is present in the respective vesicles. Cholesterol is solubilized in a hydrophobic core whereas genistein is solubilized in the polar head region or in the polar-apolar interface. The overlapping of solubilizing sites affected the solubilization of genistein when cholesterol was present in the vesicles. Moreover, the lamellar interval was largely affected by cholesterol in compared to the little impact of genistein because the later can indirectly affect the acyl chains. Genistein solubilized in DOPC showed the same degree of antioxidant capacity as that of vesicle-free genistein system. On the other hand, genistein solubilized in DPPC had lower antioxidant activity than the former systems. The distinction of antioxidant activity at different systems probably related to the difference of accessibility of ABTS radical cation to solubilized genistein through different vesicles. Finally, cholesterol-free DOPC vesicles were found to be the best solubilizer for genistein among the investigated systems.

Key words: phospholipid vesicles, solubilization of genistein, antioxidant capacity, X-ray scattering measurement

1 Introduction

Genistein is one of the isoflavones found in soy and soy products[1,2]. It has extraordinary advantages for its antioxidant[1-3], anticancer[1,2,4] and estrogen effects[5,6]. Three hydroxyl groups in the aromatic rings of genistein contribute to its excellent antioxidant capacity[3]. Moreover, genistein can inhibit the induction of stress proteins on tumor cells and retard the action of tyrosine kinase. This ultimately contributes to its anticarcinogenic benifits[4,6]. Genistein is also a kind of phytoestrogen contained in plants and is capable of showing estrogen effect. The structure of 17β-estradiol is similar to genistein therefore has the ability to bind to the estrogen receptor. Furthermore, genistein acts as a weak agonist or antagonist of estradiol depending its amount[5,6]. Despite having greater beneficial effects on the human body, genistein is hard to use because of its poor water-solubility[1]. Therefore, it requires a novel solubilization treatment when genistein is intended to use in the aqueous systems. In this study, phospholipid, DPPC and DOPC vesicles were utilized to solubilize genistein in the aqueous medium.

Phospholipids are constituents of cell membranes[7]. The lipid bilayer exhibits a change of phases with respect to the "main phase transition temperature". Below this temperature, the bilayer remains as a gel-phase which has a high order of acyl chain and lower diffusion mobility. On the other hand, above the main phase transition temperature, the bilayer becomes a liquid crystal which has a lower order of acyl chain and high diffusion mobility[8]. The bilayer properties also fluctuate depending on the concentration of cholesterol[7-12]. Cholesterol cannot form a bilayer by itself but dissolves in the phospholipid bilayer and modulates bilayer properties[11]. Cholesterol exhibits distinct properties depending on gel phase or liquid crystal phases. In the gel phase, cholesterol decreases the order of the acyl chain and increases the permeability[8,10-12] while in the

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liquid crystal phase, it does the opposite[7−9, 12]. Cholesterol-containing bilayer shows a liquid-ordered phase. The liquid-ordered phase has a high order of acyl chain and high diffusion mobility[8, 10, 11]. Hydrophobic interaction plays an important role when cholesterols reside in the bilayer and shows diverse characteristics[13]. Previous studies on the mixed-system of phospholipids and genistein have been reported mainly for the prevention of oxidation in the phospholipids[14, 15]. This study utilizes phospholipid vesicles to solubilize genistein with a possibility of using genistein in an aqueous solution which will introduce a new technique to the large-scale application of this significantly important material in the biological system. Maximum solubilization of genistein into phospholipid vesicles and the influence of genistein on the vesicle structure, in particular the lamellar intervals were examined by X-ray scattering technique. Finally, the antioxidant capacity of solubilized genistein was evaluated by ABTS assay.

2 Materials and Methods

2.1 Materials

Phospholipids, DPPC and DOPC, were purchased from NOF Corporation (Tokyo, Japan). Cholesterol was purchased from Wako Pure Chemical Industry (Osaka, Japan). Genistein was purchased from Tokyo Chemical Industry (Tokyo, Japan). In the ABTS method, we used 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid ammonium salt) (ABTS) and peroxodisulfate purchased from Tokyo Chemical Industry (Tokyo, Japan) and Wako Pure Chemical Industry (Osaka, Japan) respectively. Phosphate buffer solution (0.1 M, pH = 7.4) was purchased from Nacalai Tesque (Kyoto, Japan). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was employed as a standard antioxdation agent and purchased from Tokyo Chemical Industry (Tokyo Japan).

2.2 X-ray Scattering

2.2.1 Preparation of multi-lamellar vesicles for X-ray scattering

An appropriate amount of DPPC, DOPC or binary lipid mixtures of DPPC and DOPC with cholesterol was solubilized in methanol in a test tube. Genistein (with various amounts) was put in the methanol solution simultaneously. After that, methanol was completely removed under the reduced pressure by means of a rotary evaporator and drying in an oven at 50℃. A mixed lipid film was formed on the inner wall of the test tube and then distilled water was added to it. The lipid dispersions were vortexed sufficiently at 50℃ and finally, the multi-lamellar vesicle (MLV) dispersions were obtained. Final concentration of lipid was 1.4 mM and concentrations of genistein were prepared as 0 to 0.7 mM. Therefore the molar ratio of genistein and total lipids correspond to 0 to 0.5.

2.2.2 X-ray scattering measurement

MLV dispersions were centrifuged at 15000 rpm for 20 minutes and the precipitates were transferred to capillaries. X-ray scattering measurement was performed by Nanoviewer (Rigaku; Japan). Characteristic X-ray of copper (CuKα; λ = 1.5418 Å) was used for this measurement. Camera length was 700 mm and 85 mm for small-angle X-ray scattering (SAXS) and wide-angle X-ray scattering (WAXS) respectively. The measurement performed at about 25℃. The X-ray irradiated imaging plate was read by RAXIA-Di (Rigaku) and processed by 2DP (Rigaku). The lamellar intervals were obtained from the first diffraction peak.

2.3 Antioxidant capacity measurement

2.3.1 Preparation of small uni-lamellar vesicles for ABTS assay

Several kinds of vesicle dispersion containing genistein were prepared as MLV by the similar procedure mentioned in 2.2.1. However, for the ABTS assay the concentration of lipid was changed to 14 mM, ten times higher than that for X-ray scattering measurement. Concentrations of genistein were set from 0 to 1 mM. These concentrations were under the solubility of genistein in lipid vesicles. The MLV dispersions were sonicated for 10 minutes at 50℃ and they were used as small uni-lamellar vesicle (SUV) dispersions before the measurement of antioxidation capacity.

2.3.2 ABTS assay

Antioxidant capacity was evaluated by ABTS assay[16]. Equal volume of 7 mM ABTS aqueous solution and 2.45 mM potassium peroxodisulfate aqueous solution were mixed and placed in the dark for over 16 hours. As a result, stable ABTS radical cation was obtained. The solution was diluted with 0.1 M phosphate buffer solution (pH 7.4) so that the absorbance at 735 nm was 0.70 ± 0.02 at 30℃. The final volume of the solution was 1980 μL. Absorbance was measured 1 minute after dilution and it was taken as 0-minute data. Two minutes after the dilution, 20 μL of the vesicle dispersion or genistein (in methanol) was added. Absorbance was measured every minute for a total time of 6 minutes after the genistein addition and they were taken as 1-6 minutes data. The percent inhibition was determined from the following equation.

$$\% \text{ Inhibition} = \frac{A_0 - A}{A} \times 100 \quad (1)$$

where A_0 is absorbance at 735 nm after 6 minutes in the absence of genistein, A is absorbance at 735 nm after 6 minutes in the presence of genistein. The percent inhibition was plotted as a function of the concentration of genistein. IC50, the concentration at which the percentage inhibition was 50%, was determined. Trolox equivalent antioxidant capacity (TEAC) was calculated from the following equation,

$$\text{TEAC} = \frac{\text{IC50 of trolox}}{\text{IC50 of genistein}} \quad (2)$$

IC50 of trolox was 8.8 μM.

3 Results and discussion

3.1 Maximum amount of solubilization

From SAXS data, the peaks originated from the lamellar structure were detected in all measured systems. Therefore it was confirmed that MLV was formed in all systems. WAXS profiles of MLV dispersions with several concentrations of genistein are shown in Fig. 1 for DPPC and DOPC systems, respectively. The peaks shown at around $q = 1.5$ Å^{-1} are caused by a lipid bilayer and become obscure with increasing cholesterol concentration. The peaks originated from a crystal of excess genistein molecules are clearly distinguishable from that from MLV and they appeared at around $q = 1.1$ and 1.8 Å^{-1}. Thus, an appearance of these peaks can be used as an index of decision for maximum solubility of genistein in the MLVs. It was seen from the results of Figs. 1(a) to 1(c) that the solubility of genistein

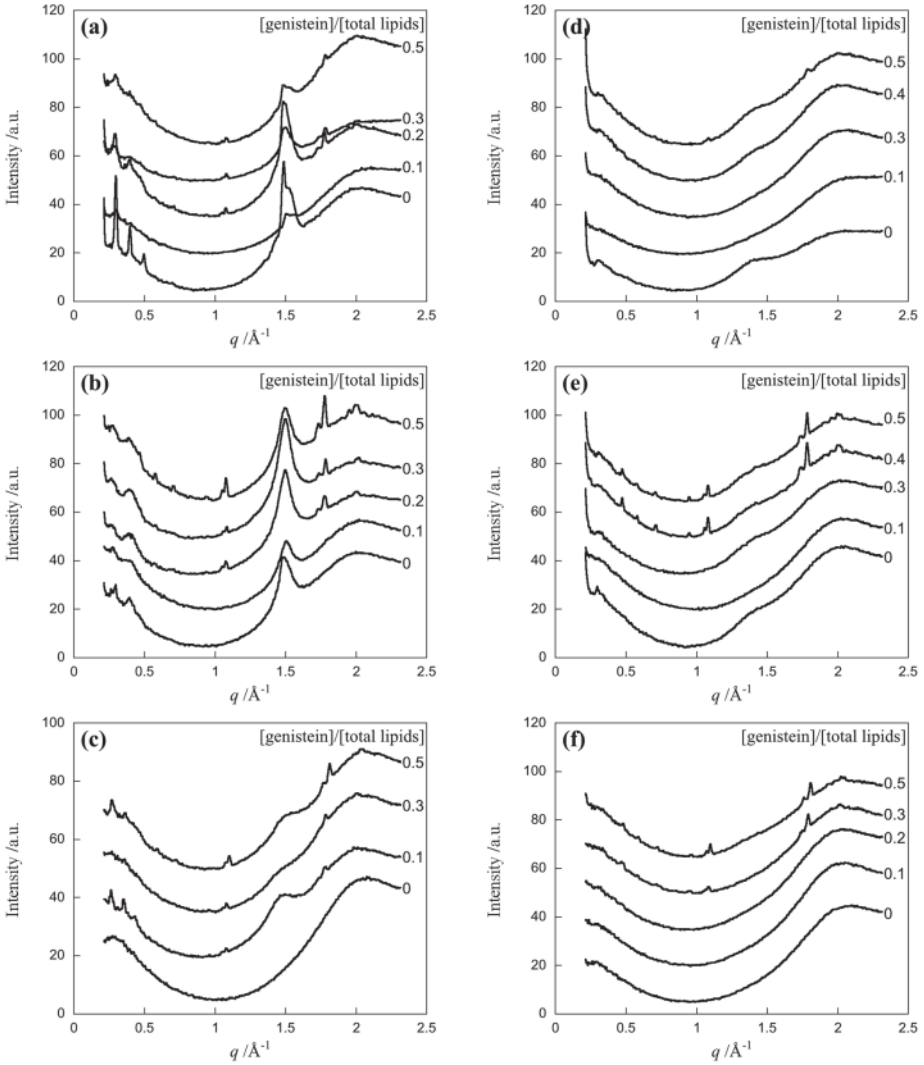

Fig. 1 WAXS profiles of MLV of DPPC; (a) without cholesterol, (b) with 10% cholesterol, (c) with 30% cholesterol, and those of DOPC; (d) without cholesterol, (e) with 10% cholesterol, (f) with 30% cholesterol. The ratio[Genistein]/[total lipids]is shown on the right side of the graph. All measurements were performed at about 25℃.

in the MLV of DPPC was relatively small, about 10% against the amount of the total lipid molecules. Especially the MLV of DPPC with 30% cholesterol cannot hold even 10% of genistein molecules. In contrast, the solubility of genistein in the MLV of DOPC was more than 40% against the amount of DOPC molecules and it decreased with increasing concentration of cholesterol in the MLV as shown in Figs. 1(d) to 1(f).

It can be concluded that MLV of DPPC has a less soluble amount of genistein than that of DOPC. The higher fluidity of the DOPC membrane in a liquid crystalline phase must contribute to the higher solubilization capacity of genistein compared with the DPPC membrane in a gel phase below 41.4℃. By the way, that of DOPC is −22℃. In addition, DOPC molecules occupy a larger area in the bilayer than DPPC[9]. Therefore, MLV of DOPC has enough space where a greater number of genistein molecules are trapped as shown in Fig. 1(a) and 1(d): more than 10% in MLV of DPPC and more than 40% in MLV of DOPC, respectively. The solubility of genistein in MLVs showed a tendency to decrease with increasing composition of cholesterol in the both MLV systems. This result suggests that because of the similarity of molecular shape and size between cholesterol and genistein, the solubilization sites of the two molecules in the phospholipid bilayer are overlapped with each other. As a result, added cholesterol molecules might expel the genistein molecules.

3.2 Effect of genistein on the lamellar interval of MLV concerned

Genistein is solubilized into the polar head and in the polar-apolar interfacial regions of bilayer of phospholipid vesicle[2, 17]. By expecting any structural change of vesicle by the inclusion of genistein, the lamellar intervals were investigated by SAXS measurements. Figure 2 shows the obtained lamellar interval against the molar ratio of genistein and total lipids for several MLV systems. It is seen that drastic variation of the interval by addition of cholesterol occurs in the DPPC system. A significant increase of the interval between 0 and 10% cholesterol composition in the DPPC system seems to be due to the "tilt to untilt transition" of DPPC membrane[18]. It is supposed that the decrease between 10 and 30% cholesterol composition is caused by a phase transition from gel to liquid-ordered phases. On the other hand, the addition of cholesterol to DOPC vesicles improves the order of the acyl chains of DOPC in the bilayer. Therefore, it is considered that the lamellar interval increases gradually with increasing cholesterol composition for the DOPC-MLV systems.

The significant effect of genistein on the lamellar intervals was hardly observed for all MLV systems except for the 10% cholesterol of DPPC system where two types of phases coexisted. As shown in Fig. 3, two lamellar peaks at around 84 Å and 64 Å, respectively were observed in the

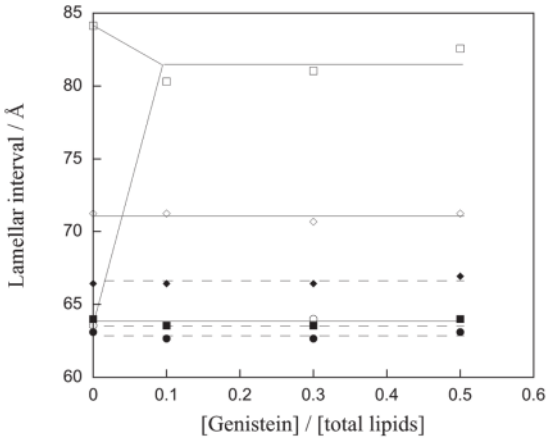

Fig. 2 Lamellar intervals obtained from SAXS measurement at about 25℃ for DPPC systems (solid lines); without cholesterol (open circle), 10% cholesterol (open square), 30% cholesterol (open diamond), and those for DOPC systems (broken lines); without cholesterol (filled circle), 10% cholesterol (filled square) and 30% cholesterol (filled diamond).

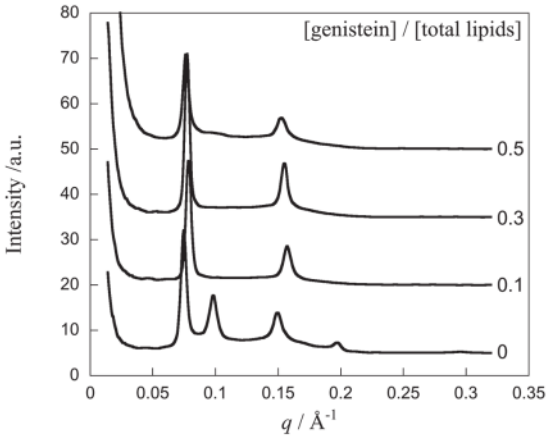

Fig. 3 SAXS profile of MLV for DPPC with 10% cholesterol system.

genistein absent system. We consider that cholesterol molecules expel genistein molecules. It is reasonable to suppose, therefore, that genistein molecules might be more solubilized in gel phase in where the population of cholesterol is relatively poor than in liquid-ordered phase. Thus the two phases became to be similar each other, the two peaks were converged at 81 Å by addition of genistein, and then the lamellar interval was not varied by increasing genistein ratio. As mentioned in the previous section, it is supposed that both cholesterol and genistein occupy the

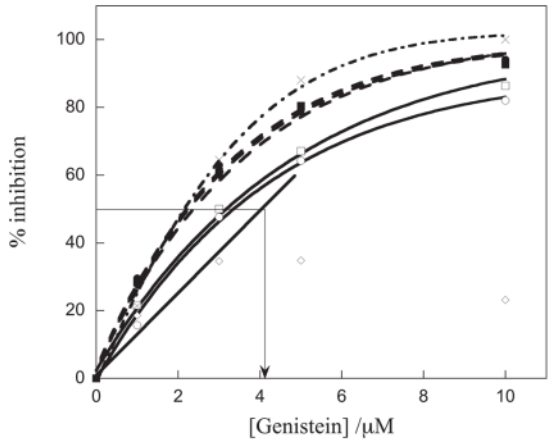

Fig. 4 Percent of inhibition vs genistein concentration curves; solubilized in methanol (cross and dashed line); DPPC systems (solid line) without cholesterol (open circle), 10% cholesterol (open square), 30% cholesterol (open diamond); DOPC systems (broken line) without cholesterol (filled circle), 10% cholesterol (filled square), 30% cholesterol (filled diamond).

similar site in the phospholipid bilayer. However, their influence on the lamellar structure of phospholipid vesicles might be different to a certain degree. It has been reported that cholesterol molecules affect the acyl chains of lipids by hydrophobic interaction in the hydrophobic core of bilayer[12], while genistein molecules interact with phosphate and ester groups of lipids via hydrogen bond[19]. These differences seem to lead to the dissimilar effect on the lamellar interval of phospholipid vesicles.

3.3 Antioxidant capacity

The percent inhibition obtained by the ABTS assay were plotted against the concentration of genistein for each vesicular system (Fig. 4). Since the values of percent inhibition were beyond 50% (except for 30% cholesterol) of DPPC system, the IC50 values were estimated by the interpolation for each experimental result. Based on the previous section, the solubilization capacity for genistein in 30% cholesterol of DPPC system was the minimum. Therefore, the IC50 value of the 30% cholesterol-DPPC system was estimated by extrapolation only using a few concentration points of genistein lower than 3 μM.

The determined IC50 and TEAC values are shown in Table 1. IC50 and TEAC values of genistein dissolved in methanol were 2.33 μM and 3.78, respectively. TEAC of genistein solubilized in DOPC vesicles were around 3.7 which is almost the same as that in methanol (mono-dispersed state). It is found that genistein molecules in DOPC vesicles have the same capacity as an antioxidant with that in mono dispersed state, whereas that was slightly lower in DPPC vesicles. Therefore, we consider that the difference is from the gaps of reaction rate and collision frequency of genistein and ABTS radical cation. ABTS radical cation might penetrate into DOPC vesicles and react with solubilized genistein molecules more easily than DPPC because of the greater fluidity of DOPC membrane. Interestingly, the antioxidation activity of genistein was found to be different in DPPC and DOPC when cholesterol is present in the vesicles. The addition of cholesterol into DPPC vesicles reduced the TEAC of genistein except for 30% cholesterol system. On the other hand, the inclusion of cholesterol into DOPC vesicles increased the TEAC of genistein slightly. It is well known that membrane fluidity of DPPC vesicle increases with increasing cholesterol density, conversely the packing of DOPC vesicle increases with the addition of cholesterol. It can be concluded that the antioxidation capacity of genistein solubilized into the vesicular system is strongly influenced by the membrane fluidity. Although it has been reported that genistein alter the membrane fluidity[19], it is considered that there are little significant impact on determining antioxidantion capacity compared with cholesterol.

4 Conclusion

The maximum solubilization, lamellar interval, and antioxidant capacity of solubilized genistein in DPPC or DOPC vesicles were investigated. DOPC vesicles were able to sol-

Table 1 IC 50 (μM) and TEAC (μM Trolox Equivalent of genistein). IC 50 of trolox is 8.8 μM. The data in parentheses are obtained by extrapolation of the experimental results.

	in MeOH		in DPPC vesicle		in DOPC vesicle	
	IC 50	TEAC	IC 50	TEAC	IC 50	TEAC
	2.33	3.78	–	–	–	–
0% cholesterol	–	–	3.15	2.79	2.29	3.84
10% cholesterol	–	–	2.82	3.12	2.37	3.71
30% cholesterol	–	–	(4.08)	(2.15)	2.41	3.65

ubilize more genistein than DPPC vesicles. The solubilization of genistein was inhibited by cholesterol in both the phospholipid vesicles due to overlapping of the solubilization sites. Cholesterol affected the lamellar interval in a larger way than genistein because of its greater influence on the acyl chain. Due to greater accessibility of ABTS radical cation to solubilized genistein through the DOPC vesicle, genistein solubilized in DOPC was found to be more antioxidant than solubilized in DPPC vesicle. Among the target vesicles, cholesterol-free DOPC vesicle was found to be the best solubilizer for genistein.

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