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Interaction of Porphyran with a Hydrophobic Surface and Stabilization of Liposomes

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Three porphyran preparations with high emulsifying ability and varying molecular mass, 3,6-anhydrogalactose content, and sulfate content without any protenaceous component were prepared from dried nori processed from *Porphyra yezoensis*, a red alga. Each of these preparations was applied to demonstrate adsorption or binding to the surface of oil droplets. The decrease in porphyran concentration of the aqueous phase of O/W emulsions prepared with porphyran and with toluidine blue (TB)−porphyran complex formed by adding TB to the O/W emulsions indicated ready adsorption to the surface of oil droplets. The decrease in ζ-potential of the O/W emulsions suggested that the sulfate groups of the adsorbed porphyran were oriented toward the external aqueous phase. A biomolecular interaction analysis exhibited rapid binding of porphyran to C_{16}-alkane, probably through 3,6-anhydrogalactose. Porphyran-coated liposomes were tolerant to digestion with phospholipase D. The increased molecular weight of the porphyran preparations had an increased effect on these characteristics. The results of this study demonstrate that the emulsifying ability of porphyran is derived from the adequate adsorption to the surface of oil droplets and that porphyran could be effectively applied to stabilize liposomes.

KEYWORDS: Porphyran; liposomes; nori; ζ-potential; 3,6-anhydrogalactose; stabilization

INTRODUCTION

Dried nori is processed by roughly washing raw laver of *Porphyra yezoensis*, a red alga, with water, cutting it coarsely, and drying it with a desiccated automatic nori-drying machine. Dried nori usually contains about 11−13% water, about 29−36% proteinaceous components, about 39−40% carbohydrates (including 5−7% crude fiber), about 0.6−0.7% lipid, about 8−11% ash, and some vitamins (*1*, *2*).

Porphyran is known to originate from the cell wall and intercellular regions of the raw laver and to be closely related to agarose in its basic structure, altered 1,4-linked 3,6-anhydrogalactose units and 1,3-linked β-D-galactose units sometimes respectively occurring as the l-galactose-6-sulfate and 6-*O*-methyl derivative (*3*, *4*). However, it is very different from agarose in terms of its l-galactose-6-sulfate content. Although it has recently been reported that porphyran had some physiological functionalities (*5*−*8*), it has not received more general application, since porphyran has a relatively low viscosity and no gelling property due to its high sulfate content.

We have previously reported that porphyran prepared from low-quality dried nori by autoclaving the suspension, precipitating with ethanol, and separating by size-exclusion chromatography exhibited good emulsifying ability in terms of a high emulsifying activity index, emulsion stability, and particle size distribution of the oil droplets over a wide range of pH and temperature and also in the presence of sodium chloride (*9*). These features indicated the possibility of its use as a new polysaccharide surfactant. It is well-known that liposomes belong to a familiar case of emulsions and that liposomes are applied to numerous uses such as pharmaceuticals and cosmetics (*10*, *11*). The proper stabilization of liposomes with natural materials is thus considered to be the first consideration. In the present study, binding of the porphyran preparations without any proteinaceous component to the surface of oil droplets was investigated with respect to the adsorption to the surface of the oil droplets, the decrease in ζ-potential of the O/W emulsion, and binding to the hydrophobic surface analyzed by biomolecular interaction analyzer, and the stabilization of liposomes with porphyran was investigated with respect to the tolerance of porphyran-coated liposomes to digestion with phospholipase D.

MATERIALS AND METHODS

Materials. Porphyran was prepared according to the method previously described (*9*). In brief, dried nori (80 g), which had been obtained by processing raw laver, *P. yezoensis*, a red alga cultivated in the Ariake Sea in Kyushu, Japan, was pulverized with a home-style mixer, immersed in 1600 mL of distilled water, and autoclaved at 120 °C for 30 min. The centrifuged supernatant (8000 rpm for 30 min) was fractionated with 67% ethanol, and the precipitate was recovered as the crude porphyran by centrifuging at 8000 rpm for 30 min. The crude porphyran was applied to a Toyopearl HW-65S column (104

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Table 1. Chemical Features of the Porphyran Preparations

porphyran preparation	total saccharide content (%)[a]	sulfate content (%)[b]	3,6-anhydro-galactose content (%)[c]	molecular mass (kDa)[d]
1	57.6	8.9	4.1	48
2	82.2	9.2	11.2	40
3	79.3	9.8	10.8	7

[a] Measured by the phenol–sulfuric acid method (*12*) with glucose as the standard. [b] Measured by the rhodizonate method (*17*) with sulfuric acid as the standard. [c] Measured by the resorecinol reaction (*18*) with fructose as the standard. [d] Evaluated by size-exclusion chromatogaraphy as the main component.

i.d. × 600 mm; Tosoh, Tokyo, Japan) and eluted with a 0.2 M phosphate buffer (pH 7.0) as the mobile phase at a flow rate of 10 mL/min. Three fractions containing saccharide without any proteinaceous components were collected, dialyzed against distilled water, and lyophilized to give porphyran preparations 1−3. The molecular mass, total saccharide content, sulfate content, and 3,6-anhydrogalactose content measured according to the respective analytical methods described later are summarized in **Table 1**. The other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Evaluation of the Sample Amount Adsorbed to Oil Droplets. Porphyran was dissolved in a 0.1 M citric acid−disodium hydrogenphosphate buffer (pH 7.0) at a concentration of 0.1% as saccharide. An O/W emulsion was prepared with 2 mL of the porphyran solution and 0.5 mL of corn oil by a Polytron PTA-7 homogenizer (Kinematica, Switzerland) run at 24 000 rpm for 1 min at 25 °C. Part of this emulsion (1 mL) was diluted with 2 mL of the fresh buffer and then centrifuged at 20 000 rpm for 30 min at 4 °C. The aqueous phase (1 mL) was filtered through a 0.5-μm Polyflon membrane filter (Advantec MFS, Tokyo, Japan). This operation was conducted twice to obtain a completely clear aqueous phase, before the saccharide concentration of the final aqueous phase was measured by the phenol−sulfuric acid method (*12*). The amount of a sample adsorbed to the oil droplets was estimated from the difference between the concentration of the initial and final aqueous phases.

Measurement of the Particle Size of Oil Droplets. The particle size distribution of each emulsion prepared with porphyran preparations was evaluated by using an SALD-2000J laser diffraction particle analyzer (Shimadzu, Kyoto, Japan) after the emulsion had been diluted with a 0.1% SDS solution according to the method previously described (*9*).

Evaluation of the Adsorption of Porphyran to Oil Droplets by Toluidine Blue (TB). TB recrystallized according to the method of Pal and Schubert (*13*) was used. The O/W emulsion prepared with each porphyran preparation was centrifuged at 1000 rpm for 10 min at 0 °C, and then 1 mL of the separated aqueous phase was filtered through a 0.5 μm Polyflon membrane filter (Advantec MFS, Tokyo, Japan) to obtain a clear aqueous phase. The recrystallized TB solution (4×10^{-4} M, 1 mL) was slowly added to 1 mL of the filtrate. After diluting the mixture four times with distilled water, the absorption spectrum was measured in a wavelength range of 400−700 nm. A control solution containing TB and porphyran with the same concentration as that of the emulsion was also prepared. The adsorption of porphyran to the oil droplets was estimated by the difference between the absorption spectrum of the diluted filtrate and that of the control solution diluted in the same manner.

Measurement of the ζ-Potential of the O/W emulsion. The O/W emulsion (30 μL) prepared with each porphyran preparation was diluted with 40 mL of a 0.1 M citric acid−phosphate buffer (pH 7.0), before the ζ-potential of the diluted emulsion was measured by an ELS-800 laser Doppler electrophoretic instrument (Otsuka Electric Co., Tokyo, Japan) at 25 °C.

Evaluation of the Hydrophobic Affinity. The interaction between porphyran and the hydrophobic surface of a hydrophobic cuvette with a hexadecyl group as the ligand of the resonant layer corresponding to palmitic acid residue was examined by an IAsys Plus biomolecular interaction analyzer (Affinity Sensors Co., Cambridge, UK) according to the method previously described (*14*). A porphyran solution (200 μL) at a concentration from 6.25×10^{-3} to 0.10% (from 2.47 to 39.5 μM for porphyran preparation 1; from 1.55 to 25.0 μM for porphyran preparation 2; and from 4.64 to 143 μM for porphyran preparation 3) in a 10 mM phosphate buffer (pH 7.0) was applied to a hydrophobic cuvette that had been equilibrated with the same buffer, and the association with the hydrophobic surface was monitored by the resonant angle of laser light at 670 nm. After the detected binding curve had been equilibrated, the sample was dissociated with 200 μL of 50 mM NaOH, and the cuvette was washed with a 10 mM phosphate buffer (pH 7.0)−10 mM NaOH (1:1, v/v) containing 2.5% SDS and 0.05% Tween 20. The pseudo-first-order rate constant (K_{on}) was obtained from the single-exponential equation describing the association data for each sample concentration by FASTfit analytical software. Using a near-least-squares regression, the association rate constant (K_{ass}) and dissociation rate constant (K_{diss}) were respectively derived from the gradient and intercept of the plot of K_{on} against the sample concentration (association plot). The dissociation constant ($K_D = K_{diss}/K_{ass}$) was then obtained.

Table 2. Adsorption to Oil Droplets Evaluated by Measuring the Residual Porphyran Concentration of the Aqueous Phase of an O/W Emulsion and the Average Diameter of Oil Droplets

	porphyran preparation		
	1	2	3
initial amount of porphyran (mg)	1.67	1.63	1.59
residual porphyran in the aqueous phase (mg)	1.37	1.37	1.36
adsorbed porphyran (mg)	0.30	0.26	0.23
adsorption (%)[a]	18.0	16.0	14.5
average oil droplet diameter (10^{-6} m)	16.1	11.4	11.2
total surface area of oil droplets (10^{-1} m^2)[b]	9.02	1.34	1.32
adsorbed porphyran rate (10^{-3} g/m^2)[c]	3.3	1.9	1.7

[a] Percentage of adsorbed porphyran to the initial amount of porphyran. [b] The surface area of an oil droplet × (oil volume (0.5 mL)/oil droplet volume). [c] Adsorbed porphyran (g)/the total surface area of oil droplets (m^2).

Coating of Calcein-Encapsulated Liposome with Porphyran. Calcein-encapsulated liposomes (multilamellar vesicles) were prepared according to the method of Kawaguchi and Matsuoka (*15*). Egg phosphatidyl choline and cholesterol were each dissolved in chloroform/methanol (8:2, v/v) to give a concentration of 5 μmol/mL. After transferring 9 mL of each solution to an eggplant-shaped flask, the solvent was removed at 30 °C with a rotary evaporator. After adding 4 mL of 4×10^{-4} M calcein to the flask, the thin film was swollen while shaking, and then a calcein-encapsulated liposome suspension was prepared by agitating it with a vortex mixer. The nonencapsulated calcein was removed by size-exclusion chromatography as follows. The sample solution was applied to a Toyopearl HW-40F column (10 i.d × 250 mm; Tosoh, Tokyo, Japan) that had been equilibrated with a 0.1 M phosphate buffer (pH 7.0) and eluted at a flow rate of 0.6 mL/min with the same buffer. The eluate was divided into 1-mL fractions, and the fractions containing the calcein-encapsulated liposomes detected by the absorbance at 600 nm were collected. The concentration of the calcein-encapsulated liposomes of the obtained fraction was adjusted to 0.8 mol/mL on the basis of phosphorus concentration with a 0.1 M phosphate buffer (pH 7.0), this being measured by a phospholipid test kit (Wako, Osaka, Japan), and the obtained suspension was kept in a refrigerator until needed. Porphyran-coated liposomes were prepared by adding porphyran to the calcein-encapsulated liposome suspension to give a 1 or 3 mg/mL final concentration and then stirring the suspension for 1 h at room temperature.

Treatment of the Porphyran-Coated Liposomes with Phospholipase D. The porphyran-coated liposome suspension (2 mL) was treated with phospholipase D (EC 3.1.4.4; 40 units/50 μL; Asahi Kasei Industries, Tokyo, Japan) at room temperature for 40 min, and the fluorescence intensity was measured at a wavelength of 518 nm with excitation at a wavelength of 490 nm. After 40 min, 50 μL of 10% Triton X-100 was added to release the whole calcein by solubilizing the liposomes. The ratio of calcein released with phospholipase D is represented as the percentage of the intensity of fluorescence after 40 min to that of the whole calcein.

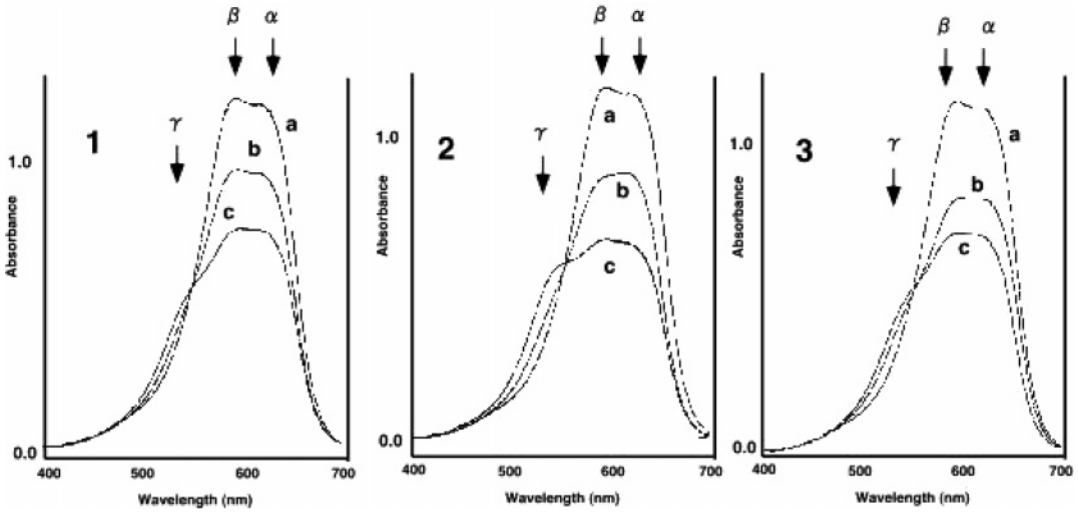

Figure 1. Comparative absorption spectra of the toluidine blue (TB) solution (**a**), the TB–aqueous phase (**b**) of the O/W emulsion prepared with porphyran, and the TB–porphyran solution (**c**). 1, porphyran preparation 1; 2, porphyran preparation 2; 3, porphyran preparation 3. The amounts of TB and porphyran used were the same among **a**, **b**, and **c**.

Table 3. ζ-Potential of the O/W Emulsion Samples Prepared with Porphyran[a]

porphyran preparation	ζ-potential (mV)
1	−33.46
2	−35.07
3	−25.17
control	−23.36

[a] The O/W emulsion [corn oil:0.1% porphyran solution, 20:80 (v/v)] was diluted 1500-fold with a 10 mM phosphate buffer (pH 7.0), and the ζ-potential was measured at 25 °C.

Chemical Analysis. The total saccharide content, protein content, sulfate content, and anhydrosaccharide content were respectively measured by the phenol−sulfuric acid method (*10*) with galactose used as the standard, the Lowry method (*16*) with bovine serum albumin used as the standard, the rhodizonate method (*17*) with sulfuric acid used as the standard, and the resorcinol method (*18*) with fructose used as the standard.

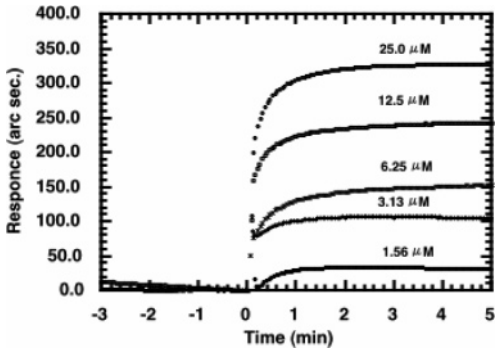

Figure 2. Individual binding curve for porphyran preparation 2 to the C_{16}-alkane surface evaluated by an IAsys biomolecular interaction analyzer. A sample solution (200 μL) at a concentration range from 1.55 to 25.0 μM in a 10 mM phosphate buffer (pH 7.0) was applied to a hydrophobic cuvette equilibrated with the same buffer, and the association with the hydrophobic surface was monitored by the resonant angle of laser light at 670 nm.

RESULTS AND DISCUSSION

Adsorption to the Surface of Oil Droplets. It has previously been shown that the three porphyran preparations used here could form stable O/W emulsions (*9*). The adsorption of porphyran to the surface of oil droplets was thus evaluated by measuring the remaining total saccharide concentration of the aqueous phase of the O/W emulsion prepared with porphyran. Consequently, about 14−18% of the added porphyran was adsorbed to the surface of the oil droplets just after emulsification (**Table 2**), and the porphyran preparations with higher molecular mass showed higher adsorption. The adsorbed amount per the surface area of oil droplets was estimated to be $(1.7-3.3) \times 10^{-3}$ g/m^2 from the average diameter of the oil droplets. However, the adsorbed amount decreased to about 3−10% 2 h after emulsification due to slow coalescence of the oil droplets.

It is known that TB shows a metachromagy shift resulting from forming a complex with an acidic polysaccharide. The absorption spectrum of TB shows the α peak at 633 nm and the β peak at 598 nm, being previously assigned to the monomer and dimer of TB, and in the presence of the acidic polysaccharide, the γ peak of the metachromatic band appeared at 550 nm due to the formed acidic polysaccharide−TB complex (*19*). The absorption spectrum of a TB solution containing the same amount of porphyran as that initially used in preparing the emulsion was measured. The spectrum of each porphyran preparation showed markedly suppressed α and β peaks and the appearance of a γ peak as compared with that of the TB solution itself (**Figure 1**). However, the spectrum of the aqueous phase of the emulsion prepared with porphyran showed a decrease in the γ peak and increases in the α and β peaks as compared with the porphyran−TB solution. The decrease in the γ peak, representing a decrease in the porphyran−TB complex, indicates a decrease in the free porphyran concentration of the water phase of the emulsion due to the adequate adsorption of porphyran to the surface of the oil droplets, resulting in a corresponding increase in free TB.

The ζ-potential of the emulsions prepared with the porphyran preparations was also measured. The ζ-potential of the control emulsion without porphyran was about −23 mV, whereas that of the emulsions prepared with porphyran was in the range of about −25 to −35 mV (**Table 3**). A decreased ζ-potential indicates adequate adsorption of porphyran with an anionic charge to the surface of oil droplets and orientation of the sulfate groups of the adsorbed porphyran toward the external aqueous phase. It is concluded from these results that the outstanding emulsifying ability of porphyran is not caused by inhibiting the

Table 4. Kinetic Constants for Binding of Porphyran to the C_{16}-Alkane Surface Evaluated by an IAsys Biomolecular Interaction Analyzer

porphyran preparation	K_{ass}[a] (M^{-1} s^{-1})	K_{diss}[b] (s^{-1})	K_D[c] (M)
1	7.55×10^3	1.75×10^{-2}	2.36×10^{-6}
2	3.30×10^3	2.04×10^{-2}	6.19×10^{-6}
3	5.05×10^2	$4.25 \times 10\text{-}^2$	8.95×10^{-5}
1,6-β-D-anhydrogalactose	2.41×10	1.62×10^{-2}	6.67×10^{-4}

[a] K_{ass} (association rate constant) was obtained from the association plot as the gradient. [b] K_{diss} (dissociation rate constant) was obtained from the association plot as the extrapolated intercept on the *Y*-axis. [c] K_D (dissociation constant) was found from K_{diss}/K_{ass}.

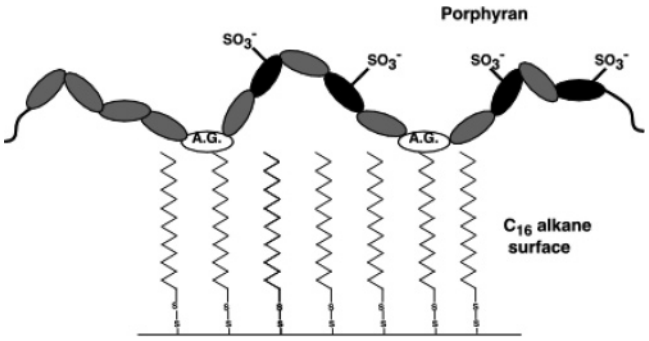

Figure 3. Postulated binding manner of porphyran to the hydrophobic surface: (gray ovals) galactose, (ovals labeled A.G.) anhydrogalactose, (black ovals) galactose-6-sulfate.

coalescence of oil droplets with its thickening property but by protecting the oil droplets from rapid coalescence by its hydrophilicity and by the electrostatic repulsion among its sulfate groups due to adequate adsorption to the surface of the oil droplets.

Binding to the Hydrophobic Surface. The adsorption of porphyran to the surface of the oil droplets resulted from binding between porphyran and a hydrophobic moiety, such as the fatty acid residue of the lipid. The affinity of porphyran to a C_{16}-alkane surface, as a model for the lipid component of the oil droplet surface, was thus evaluated by an IAsys biomolecular interaction analyzer to obtain the binding kinetic constants. The response resulting from the binding of porphyran to the C_{16}-alkane surface was detected just after adding each porphyran sample, and reached equilibrium after 3−5 min. The increase in response depended on the sample concentration, with typical results being shown in **Figure 2** for porphyran preparation 2. It is thus concluded that porphyran could rapidly bind to C_{16}-alkane of the hydrophobic surface. The association plot (K_{on} vs sample concentration) for each porphyran preparation could be resolved by a linear regression curve with a high correlation coefficient within the range $r = 0.971-0.997$, enabling the association rate constant (K_{ass}) and the dissociation rate constant (K_{diss}) to be derived, and the dissociation constant ($K_D = K_{diss}/K_{ass}$) was obtained. Porphyran preparations 1 and 2 showed a K_D of 10^{-6} M, while the porphyran preparation 3 showed a K_D of 10^{-5} M (**Table 4**). The porphyran sample with high molecular mass is thought to have shown higher binding affinity to the lipid than that with low molecular mass, probably due to many binding sites in that with high molecular mass.

The previous study showed the adequate correlation between the particle size of the oil droplets and the 3,6-anhydrogalactose content, suggesting the substantial contribution of 3,6-anhydrogalactose to the emulsification with porphyran (*9*). Since 3,6-anhydrogalactose probably has higher hydrophobicity than other monosaccharide components of porphyran, it is possible that porphyran is anchored with 3,6-anhydrogalactose to the surface of the oil droplets. In addition, 6-*O*-methyl-D-galactose may take part in the hydrophobic affinity of porphyran. The affinity of 1,6-anhydro-β-D-galactose to the C_{16}-alkane surface as an analogue of 3,6-anhydrogalactose was thus investigated by IAsys, because there was little 3,6-anhydrogalactose. The results showed that 1,6-anhydrogalactose could also readily bind to the C_{16}-alkane surface, as shown with the K_D value of 10^{-4} M. The ready binding of 1,6-anhydrogalactose strongly suggests that 3,6-anhydrogalactose is the anchor for porphyran to the surface of the oil droplets. The 3,6-anhydrogalactose content and sulfate content per total galactose content for porphyran preparation 2 could be from the analytical data calculated to be about 13.7% and 11.2%, respectively (**Table 1**). These figures indicate about 2 and 3 residues, respectively, for 3,6-anhydrogalactose and galactose-6-sulfate per 15 total galactose residues. If 3,6-anhydrogalactose is assumed to be the anchor for porphyran to the C_{16}-alkane surface, a possible binding mechanism to the hydrophobic surface could involve the presence of a galactose-6-sulfate residue every two or three residues (*4*, *17*) and its orientation toward the external aqueous phase (**Figure 3**), as indicated by the decreased ζ-potential of the oil droplets.

Stabilization of Liposomes with Porphyran. Each of the three kinds of porphyran was added to calsein-encapsulated lipospmes while stirring to coat their surface. The porphyran-coated liposomes were digested with phospholipase D to evaluate the stabilization with porphyran by measuring the amount of calcein released from the liposomes. Noncoated liposomes rapidly released calcein with phospholipase D, and the released amount reached about 73% of the total encapsulated

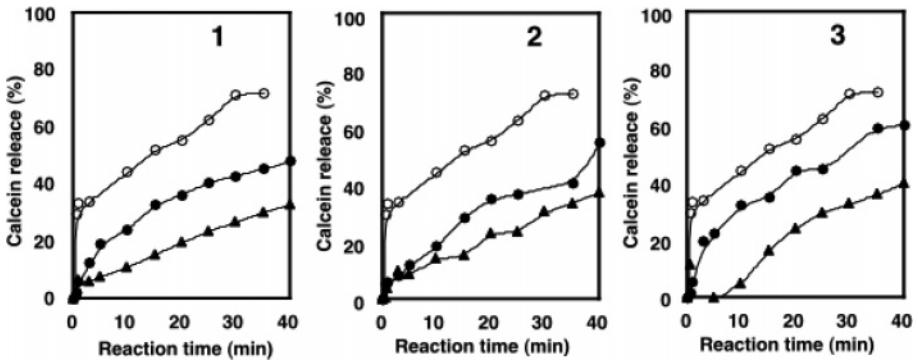

Figure 4. Release of calcein from the porphyran-coated liposomes by phospholipase D. Phospholipase D (40 U/50 μL) was added to each liposome suspension (2 mL) encapsulating calcein, and the fluorescence was measured with excitation at 490 nm and emission at 518 nm. The released calcein is represented as the percentage of each fluorescence intensity to the total fluorescence intensity by adding 50 μL of 10% Triton-X. ●, coated with porphyran at 1 mg/mL; ▲, coated with porphyran at 3 mg/mL; ○, noncoated.

calsein after 30 min (**Figure 4**). On the other hand, the initial release rate of calcein from the liposomes coated with each type of porphyran was much less (about 48−61% for coating at 1 mg/mL or 32−40% for 3 mg/mL), the total released calcein virtually ceasing after 40 min. The higher the molecular weight of the porphyran sample, the lower the amount of released calcein, probably due to more effective tolerance to phospholipase D with more bulky steric hindrance. It is considered from these results that the whole surface of the liposomes was effectively encapsulated by porphyran, resulting in much greater stabilization to phospholipase D.

Concluding Remarks. The decrease in porphyran concentration of the aqueous phase of O/W emulsions prepared with porphyran, together with the concentration of the toluidine blue−porphyran complex, formed by adding TB to the O/W emulsions, and the ζ-potential of the O/W emulsions indicated the ready adsorption to the surface of oil droplets and the orientation of the sulfate groups toward the external aqueous phase. The results of the biomolecular interaction analysis exhibited the rapid binding of porphyran to C_{16}-alkane. The higher the molecular weight of the porphyran preparation, the greater was the effect on these characeristics. These results demonstrate that the outstanding emulsifying ability of porphyran is due to adequate adsorption to the surface of oil droplets to protect coalescence and that porphyran could be effectively applied to stabilize liposomes.

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