Polymer-Induced Phase Separation in Aqueous Micellar Solutions of Alkylglucosides for Protein Extraction

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Aqueous micellar solutions of alkylglucosides were separated into two phases at 0°C upon addition of polyethylene glycol 6000 (PEG) or Dextran T-500 (Dextran). One was an aqueous phase in which hydrophilic proteins, cytochrome *c* and peroxidase (horseradish), were retained, and the other was a surfactant-rich phase into which hydrophobic membrane proteins, bacteriorhodopsin and cytochrome b_5, were extracted. A combination of octyl-β-D-thioglucoside (OTG) (or nonyl-β-D-glucoside (NG)) with PEG (or Dextran) was the best choice for extraction of hydrophobic proteins. Extraction yields (50 – 90%) and concentration factors (7 – 30) of the hydrophobic proteins were dependent on the types of nonionic surfactants and water-soluble polymers. Solubilization and phase separation in processing cell membranes could be made in a single step at 0°C. Hence the present method would be useful for the purification of thermolabile proteins.

Keywords Phase separation, alkylglucoside, polymer, protein extraction

Aqueous micellar solutions of alkylphenols with an appropriate oxyethylene head group separate into two phases above a certain temperature, called the cloud point.[1,2] One of the phases is a micelle-free aqueous phase, and other a surfactant-rich phase. This temperature-induced phase separation is used for extraction of metal chelates and aromatic hydrocarbons.[3–8]

Bordier[9] has extended the temperature-induced phase separation to the isolation of hydrophobic proteins[9–11], thus eliminating long and tedious procedures in processing cell membranes. By warming micellar solutions of Triton X-114 at 30°C, the proteins are extracted with high concentration factors into the surfactant-rich phase, while hydrophilic proteins remain in the aqueous phase.

However, some drawbacks still remain. Micellar solutions of Triton X-114, into which the proteins are solubilized, must be warmed typically at 30°C. This is unfavorable for thermolabile enzymes because of their deactivation.[12] Although a combined use of glycerol with Triton X-114 was successful in allowing a low-temperature phase separation in Triton X-114[11], the problem of denaturation still remained. This was ascribed to some oxidized impurities or phenolic compounds in Triton X-114.[11]

We were interested in phase diagrams for aqueous mixtures of octyl-β-D-glucoside and polyethylene glycol (PEG). The phase diagram indicates an upper consolute boundary separating a two-phase region from a lower aqueous micellar region.[13] In the former, one is an aqueous phase in which PEG is retained and the other a surfactant-rich phase. In contrast, a lower consolute boundary is present in a phase diagram for Triton X-114. Therefore, the phase separation in octyl-β-D-glucoside will be possible, upon addition of PEG, at a temperature much lower than 30°C for Triton X-114. In view of this, we have explored the possibility of using the polymer-induced phase separation for extraction of hydrophobic membrane proteins.

Experimental

Reagents

Octyl-β-D-glucoside (OG), octyl-β-D-thioglucoside (OTG), heptyl-β-D-thioglucoside (HTG), and sucrose monolaurate (SML) were obtained from Dojindo Laboratory (Kumamoto, Japan). Bacteriorhodopsin (*Halobacterium halobium*), cytochrome *c* (horse heart), peroxidase (horseradish), nonyl-β-D-glucoside (NG), and Ficoll type 400 (molecular weight 400000, Ficoll) were purchased from Sigma (St. Louis, MO). Polyethylene glycol 6000 (molecular weight: 7500, PEG), poly(vinylpyrrolidone) (PVP), methylcellulose 400 cp (MC), carboxymethylcellulose (CMC) were obtained from Wako Chemicals (Tokyo, Japan). Dextran T-500 (molecular weight: 460000, Dextran) and diethylamino-

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ethyl-Dextran (molecular weight: 460000, DEAE-Dextran) were from Pharmacia (Uppsala, Sweden). Cytochrome b_5 (rat liver microsome) was purified by chromatographic treatments (Whatman DE-52, DEAE-Sephadex A-50, and ω-amino-*n*-octyl Sepharose 4B).[14]

Procedures

Determination of phase separation temperature (Procedure 1). The phase separation temperature was determined by measuring temperatures required for clarification of an aqueous mixture (1.00 cm^3) of a surfactant and a water-soluble polymer upon heating or the onset of turbidity upon subsequent cooling of the aqueous solution in a 1.5 cm^3 sample tube.

Measurement of phase volume (Procedure 2). An aqueous mixture of a surfactant and a polymer in a calibrated glass tube was kept at 0°C for a week.

Extraction of proteins (Procedure 3). In a 1.5 cm^3 tube, protein (0.2 mg) was solubilized at 0°C into a 0.800 cm^3 portion of an aqueous micellar solution (0.025 mol dm^{-3} potassium phosphate; pH 6.90) of 2.5 %(w/v) OTG (or NG). Then, 0.200 cm^3 of PEG (or Dextran) solution was added. The tube was centrifuged at 10000 rpm for 10 min at 0°C. Extraction yields (%) were calculated from absorbances at 540 nm for bacteriorhodopsin and at 400 – 420 nm for other proteins.

Stability of bacteriorhodopsin (Procedure 4). Bacteriorhodopsin (1.0 mg) was solubilized into an appropriate amount of a surfactant-rich phase by incubating for 5 h at 0°C. Then, the absorbance at 540 nm was measured with time: at 0°C for OTG and at 30°C for Triton X-114. The OTG-rich phase was obtained by the same method as Procedure 3, but the protein was omitted. The Triton X-114-rich phase was also prepared at 30°C[9] in a manner similar to Procedure 3.

Results and Discussion

Phase diagram of aqueous mixture of alkylglucoside and water-soluble polymer

We established phase diagrams for aqueous mixtures of a variety of nonionic surfactants with PEG. Figure 1 gives the plots of critical temperatures as a function of PEG concentration. A micellar solution is present in the region above the curves in Fig. 1, and the two phases are present in the region below the curves (upper consolute boundary). The critical temperatures increased with increasing PEG concentration. PEG induces the phase separation in the aqueous micellar solutions at temperatures lower than 30°C for the aqueous micellar solution of Triton X-114. Considerable amounts of PEG were required for HTG, OG, and SML, while very small amounts of PEG were more than adequate for OTG and NG. A combination of OTG or NG with PEG is the best choice.

This result is probably due to the greater hydrophobicity of OTG and NG than that of other surfactants. The hydrophobicity decreases in the order:

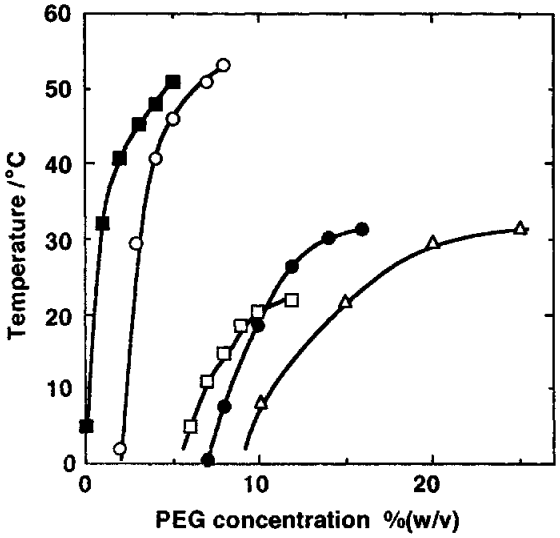

Fig. 1 Effect of PEG on phase separation in aqueous micellar solution of alkylglucoside. A two-phase region (aqueous and surfactant-rich phases) is below the curve, while a homogeneous region (micellar solution) is above the curve. Surfactant concentration: 2.00%(w/v). (■) OTG, (○) NG, (□) HTG, (●) OG, (△) SML.

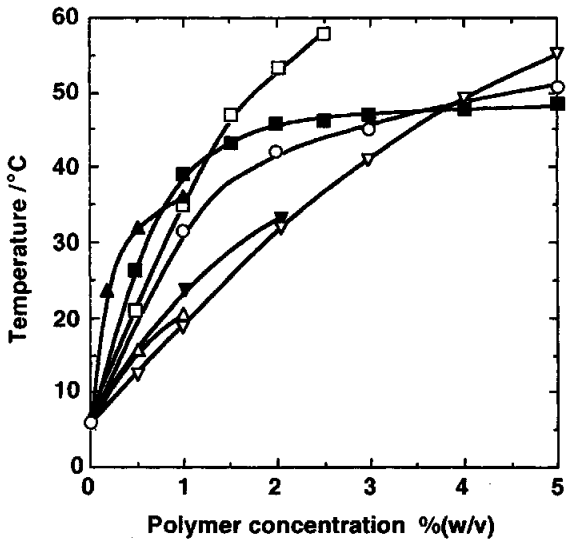

Fig. 2 Effect of water-soluble polymers on phase separation in aqueous OTG solution (2.00%(w/v)). The curves separate the two regions as in Fig. 1. (○) PEG, (■) Dextran, (□) DEAE-Dextran, (▼) PVP, (▲) MC, (△) CMC, (▽) Ficoll.

OTG>NG>HTG>OG>SML. It seems that the greater the hydrophobicity, the smaller the dehydration energy. Hence, a hydrophobic surfactant having a long alkyl substituent will require smaller amounts of PEG than a hydrophilic surfactant having a short alkyl substituent or a large sugar group. This is consistent with the results in Fig. 1.

Then, we have examined the effect of other water-soluble polymers on the phase separation in OTG. Figure 2 shows the critical temperature as a function of a

polymer concentration. All the polymers tested increased the temperature with an increase in the polymer concentration. Charged polymers, DEAE-Dextran and CMC, also facilitated the phase separation in OTG.

The critical temperature was dependent on the nature of the polymers used. Of the polymers, MC, DEAE-Dextran and PEG increased the critical temperature above room temperature at concentrations below 1%(w/v) and then leveled off at higher concentrations. In contrast, Dextran increased the critical temperature over the whole concentration range tested. Other polymers such as PVP and CMC were inferior to those described above. Ficoll is a spherical polymer having almost the same monomer structure and molecular weight as those of Dextran. However, Ficoll was less effective than Dextran.

Among the polymers, the most effective was MC, as the amount required was minimal. However, the aqueous MC solution is highly viscous, thus precluding its further use in the present study. PEG and Dextran did not cause any troubles in their handling. As the phase separation could be simply carried out by centrifuging for 10 min at 4000 rpm and 0°C, the use of PEG or Dextran is recommended.

Salt effect on phase separation

Next, the effect of potassium chloride on the phase separation in OTG was examined (Figs. 3 and 4). In the absence of PEG, two distinct curves (A and B) separating two two-phase regions from a homogeneous aqueous micellar region were found in the phase diagram (Fig. 3). An aqueous micellar solution separated into two phases below the lower curve A (upper consolute boundary) and in addition, also separated into two phases above the upper curve B (lower consolute boundary).

Along the lower curve A, the (first) critical temperature decreased with an increase in salt concentration, the phase separation in OTG being reduced by the salt. On the other hand, the (second) critical temperature represented by the upper curve B remarkably decreased with increasing salt concentration. The salt strongly induces the phase separation in OTG. Thus this salt effect on the second critical temperature is different from that on the first critical temperature.

The salt effect on the first critical temperature in Fig. 3 is probably due to a salting-in effect, as occurs for OTG and zwitterionic surfactants. The latter often display the upper consolute boundary[15], but the phase separation temperature increased with the increase in the salt concentration.[16] This is interpreted in terms of a salting-out effect. On the other hand, the salt effect on the second critical temperature for OTG is ascribable to the salting-out effect as is observed in polyoxyethylated nonionic surfactants. Their micellar solutions also display a lower consolute boundary, where the addition of salts results in a salting-out effect or a decrease in the cloud point *via* dehydration.[17-19]

In the presence of PEG (Fig. 4), the first critical temperature increased over the whole concentration range, while the second critical temperature was not observed. With increasing concentration of potassium chloride, the first critical temperature increased in the presence of 2%(w/v) PEG while it decreased in the presence of high concentration (5%(w/v) and 20%(w/v)) of PEG. Unfortunately, the mechanistic details on this salt effect are not yet clear. The salt effect seems to be very complicated, because the salt also induces the phase separation in an aqueous PEG solution.[20] Many interactions may play significant roles in the phase separation.

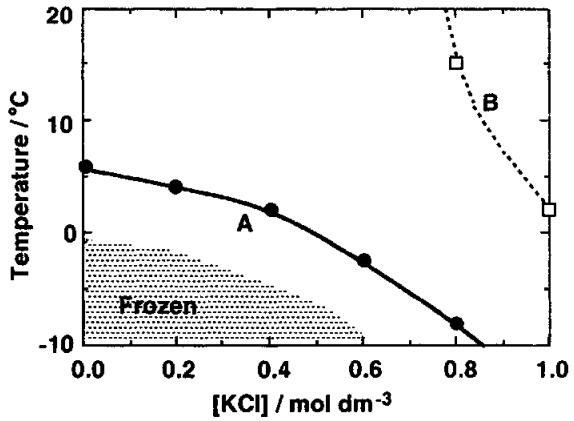

Fig. 3 Effect of potassium chloride on phase separation in OTG. Two two-phase regions are below the solid curve A and above the broken curve B, and a homogeneous region is between the curves.

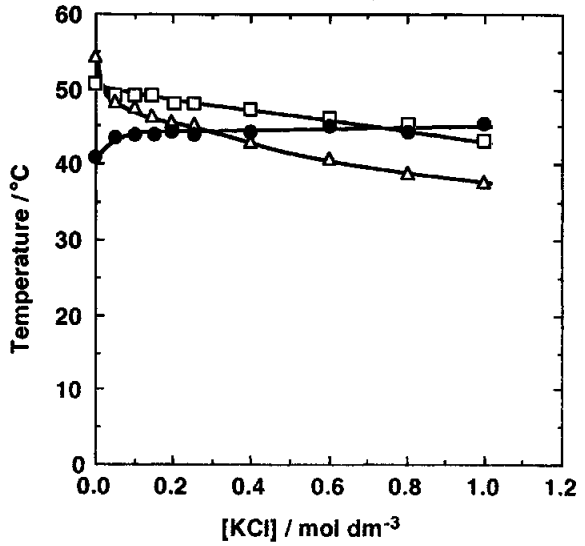

Fig. 4 Effect of potassium chloride on phase separation in aqueous mixture of OTG and PEG. The curves separate the two regions as in Fig. 1. Concentration of PEG %(w/v): (●) 2, (□) 5, (△) 20.

Extraction of proteins

Table 1 lists the volume fractions of the two phases, extraction yields (%) of proteins, and their concentration factors. Above 50% of hydrophobic proteins, bacteriorhodopsin and cytochrome b_5, are extracted into small

Table 1 Volume fraction of aqueous (AP) and surfactant-rich phases (SRP), extraction yield (%) of proteins, and their concentration factors at 0°C

Composition of solution	Volume fraction, %		Extraction yield, %				Concentration factor	
	AP	SRP	BR	b_5	PO	C	BR	b_5
2.0% (w/v) OTG 2.0% (w/v) PEG	95.6	4.4	64	94	4	3	13.1	21.3
2.0% (w/v) OTG 2.0% (w/v) Dx	92.7	7.3	52	87	1	3	7.1	11.9
2.0% (w/v) NG 4.0% (w/v) PEG	97.5	2.5	52	80	6	2	20.9	31.6

Solutions contain potassium phosphate (0.025 mol dm^{-3}, pH=6.90). Dx: Dextran, BR: bacteriorhodopsin, b_5: cytochrome b_5, PO: peroxidase, C: cytochrome *c*.

volume of the surfactant-rich phase. Thus, their high concentration factors are obtained. On the other hand, hydrophilic proteins, cytochrome *c* and peroxidase, are retained in aqueous phase. The polymer-induced phase separation in OTG and NG could be useful for the isolation of hydrophobic membrane proteins.

Additionally, the polymer-induced phase separation will be superior to the temperature-induced phase separation in Triton X-114 when the surfactants are to be removed by dialysis. The critical micelle concentration of OTG is 0.009 mol dm^{-3} and that of Triton X-114 is 2×10^{-4} mol dm^{-3},[21,22] and hence OTG can be dialyzed more rapidly than Triton X-114.[23] A simple expedient of dialyzing a micellar solution will also be effective for isolation of the proteins from surfactants.

Stability of bacteriorhodopsin

Another advantage of polymer-induced phase separation will be in the stability of hydrophobic proteins in the surfactant-rich phase at 0°C. In testing the stability of the proteins, bacteriorhodopsin is a good choice, since its denaturation can be monitored by measuring absorbance at 540 nm. The absorbance decreases with denaturation, whereas that of lower wavelength than 540 nm increases.[24,25] The stability was examined according to Procedure 4. Figure 5 shows the results on the stability of bacteriorhodopsin in the Triton X-114-rich phase (9.6%(w/v) Triton X-114) and in the OTG phase (16.0%(w/v) OTG). In the former the phase separation occurred made at 30°C and in the latter at 0°C.

The absorbance in Triton X-114 decreased rapidly, indicating considerable denaturation with time. On the other hand, the absorbance in the OTG-rich phase decreased very slowly. Evidently, bacteriorhodopsin in the latter phase is more stable than in the former phase. The difference in the stability is attributable to the difference in the temperature for the phase separation.

In this respect, the polymer-induced phase separation at 0°C is very promising, because of the occurrence of minimum denaturation even in a highly concentrated solution of OTG. Denaturation of membrane proteins is, in general, dependent on the nature of surfactants and on their concentrations. Therefore, the use of a water-soluble polymer will extend the range of choice of a nonionic surfactant, independently of its cloud point, which is appropriate with respect to the denaturation. Of course, the polymer-induced phase separation in PONPE-7.5 and Triton X-114 at 0°C can also be achieved upon addition of PEG.

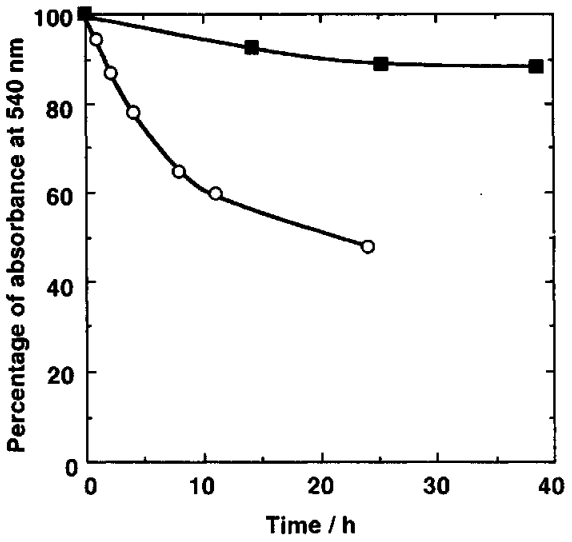

Fig. 5 Stability of bacteriorhodopsin in surfactant-rich phase. (○): absorbance in Triton X-114 surfactant-rich phase at 30°C; (■): absorbance in OTG-rich phase at 0°C. The absorbance was measured after complete solubilization of bacteriorhodopsin in the respective surfactant-rich phase at 0°C. Concentration of bacteriorhodopsin is 0.83 mg cm^{-3}.

In conclusion, the polymer-induced phase separation would provide a new route for extraction of thermolabile proteins. Solubilization and phase separation in processing the proteins with a surfactant can be made in a single step at 0°C with the least possibility of denaturation. Thus, PEG and Dextran would be very useful for extending the range of choice of a surfactant, independently of its cloud point.

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