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Short communication

Separation of microsomal cytochrome b_5 via phase separation in a mixed solution of Triton X-114 and charged dextran

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Abstract

The successful introduction of a charged dextran into the Triton X-114 phase separation system for the selective extraction of cytochrome b_5 (cyt. b_5) in liver microsomes is described. In the absence of charged dextran, 55% of total microsomal proteins and 84% of cyt. b_5 were extracted into the surfactant-rich phase. In the presence of anionic dextran sulfate, the extractability of total microsomal proteins was greatly reduced while that of cyt. b_5 was increased. After triplicate extraction, cyt. b_5 was purified more than 10-fold from microsomes with a recovery of 91% in the surfactant-rich phase. In view of its operational simplicity, this method provides a good means for the partial purification of cyt. b_5 prior to chromatographic separations.

Keywords: Cytochromes; Triton X-114; Dextrans

1. Introduction

Aqueous micellar solutions of Triton X-114 separate into two distinct phases above a certain temperature, 22~23°C, defined as the cloud point. One of the phases is a surfactant-rich phase and the other a surfactant-depleted phase (aqueous phase). If hydrophobic membrane proteins are present in such system, they often tend to partition into the surfactant-rich phase. The first use of such a temperature-induced phase separation (TIPS) method with Triton X-114 was conducted by Bordier [1] for separation of hydrophobic membrane proteins from hydrophilic proteins. Since then, a variety of hydrophobic membrane proteins have been processed by the TIPS method prior to chromatographic separations [2–5].

Aqueous polymer two-phase systems have also been widely used for separation of biomolecules, especially hydrophilic proteins [6,7]. In such systems, charged polymers are frequently exploited for controlling the distribution of proteins. Recently, a combination of micelles with the aqueous polymer two-phase system was introduced for the separation of hydrophilic proteins where the charged effect was also taken into account [8].

In our previous study [9,10], several water-soluble polymers were shown to induce phase separation in micellar solutions of alkylglucosides (AG) which do not normally exhibit temperature-dependent phase behavior. The surfactant-rich phase was a concen-

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trated AG solution, and the aqueous phase was a solution containing a water-soluble polymer. Among the water-soluble polymers tested, diethylaminoethyl–dextran (DEAE–Dx) served the dual purposes of inducing phase separation in the micellar solutions and of preventing the extraction of microsomal cytochrome b_5 (cyt. b_5) into the surfactant-rich phase at pH 7.4 [10]. In the polymer-induced phase separation methods with AG, the extractability of hydrophobic membrane proteins can be controlled by the use of charged polymer.

In this study, the extractability of membrane proteins in the TIPS methods with Triton X-114 by the addition of charged polymers is demonstrated. Cytochrome b_5 was partially purified from pig liver microsomes with the TIPS method containing charged dextran, dextran sulfate (Dx–S).

2. Experimental

2.1. Reagents

All the chemicals used were the same as those reported previously [10] except for dextran sulfate (Dx–S, average molecular mass 500 000) from Pharmacia Biotech (Uppsala, Sweden) and Triton X-114 from Wako Pure Chemical (Tokyo, Japan).

2.2. Solubilization and extraction of cytochrome b_5

Pig liver microsomes were prepared as previously described [11]. The microsomes (9.3 mg protein ml^{-1}) were solubilized with an aqueous buffer solution (pH 7.4) containing 2.0% (w/v) Triton X-114, 10 m*M* Tris–HCl, 1 m*M* EDTA, and 1 m*M* dithiothreitol for 1 h at 0°C. Insoluble materials were then removed by centrifugation at 105 000 *g* for 1 h. Supernatant solutions thus obtained were referred to as solubilized microsomes.

The solubilized microsomes were diluted ten times with 10 m*M* Tris–HCl buffer (pH 7.4) containing 2% (w/v) Triton X-114, and various concentrations of dextran. Then, a 2-ml portion of the solution was phase separated at 30°C for 10 min and centrifuged at 2000 *g* for 5 min. The extraction yields of cyt. b_5 and total proteins were determined by measuring their concentrations in the two phases.

2.3. Partial purification of cytochrome b_5

The same procedure as above was conducted without the use of dextran. An 0.8-ml portion of the surfactant-rich phase thus obtained was mixed with a 1.2 ml of 10 m*M* Tris–HCl buffer (pH 7.4) containing 0.1% (w/v) Triton X-114 and 0.2% (w/v) Dx–S. The solution was phase separated and centrifuged as described above. The surfactant-rich phase was then recovered and the phase separation was repeated once except for the addition of Triton X-114 at a 1.0% (w/v) level and sodium chloride (0.3 *M*). Finally, the concentrations of cyt. b_5 and total proteins in the surfactant-rich phase were determined.

2.4. Protein assay

Cytochrome b_5 was determined on the basis of the differential spectrum between the reduced and the oxidized forms, for which NADH was employed as a reducing agent [12]. Total proteins were analyzed by the bicinchoninic acid method [13].

3. Results and discussion

3.1. Phase separation temperature

Fig. 1 shows the cloud point of the solubilized microsomes containing 2% (w/v) Triton X-114 as a function of DEAE–Dx and Dx–S concentrations. It is evident that the solubilized solution separates into two phases when kept at 30°C. The phase separation temperature tends to decrease with an increase in dextran concentrations. Both of the dextrans were exclusively distributed into the aqueous phase while the concentration of Triton X-114 in the aqueous phase was decreased with increasing dextran concentrations (data are not shown).

3.2. Extraction of cytochrome b_5 and total proteins from microsomes

In Fig. 2, the extraction yields of cyt. b_5 and total microsomal proteins as a function of charged dextran concentrations are presented. In the absence of dextran, total microsomal proteins and cyt. b_5 were

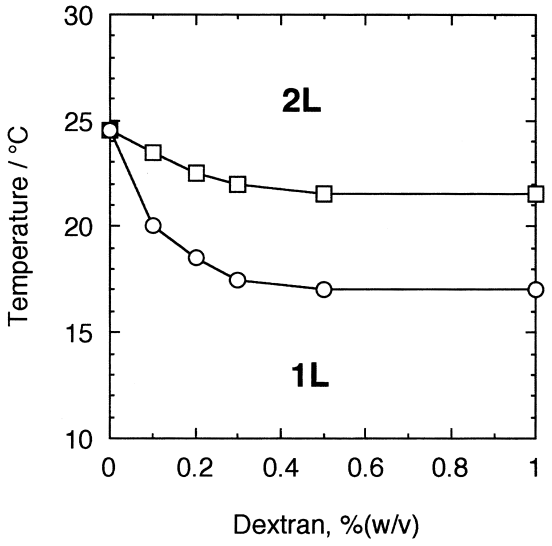
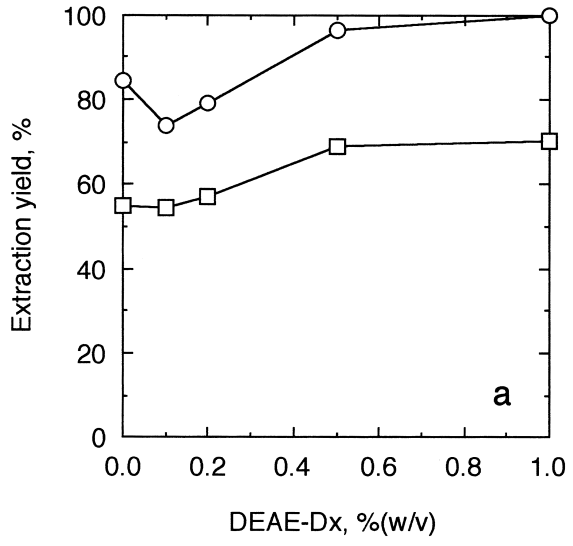


Fig. 1. Effect of dextran sulfate (Dx–S) and diethylaminoethyl–dextran (DEAE–Dx) on cloud point temperature in micellar solutions of 2% (w/v) Triton X-114 solubilizing 0.78 mg microsomal protein/ml. 1L refers to the single isotropic phase and 2L means the presence of two isotropic phases. The cloud points were determined by measuring the temperature required for the onset of turbidity in the mixed solutions of Triton X-114-solubilized microsomes and dextran containing 10 m*M* Tris–HCl (pH 7.4) upon heating. (○) Dx–S; (□) DEAE–Dx.



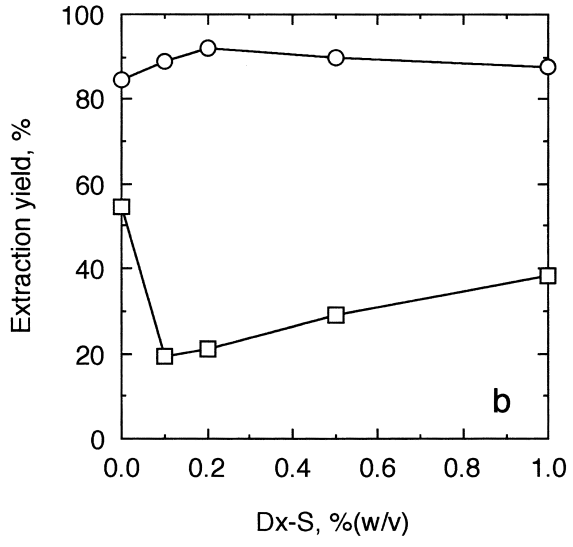


Fig. 2. Extraction of cytochrome b_5 and total proteins from the solubilized pig liver microsomes as a function of the concentration of diethylaminoethyl–dextran (a) or dextran sulfate (b). The solubilized microsomal solutions contain 2.0% (w/v) Triton X-114, 0.78 mg protein/ml (including 0.39 μ*M* cytochrome b_5), 10 m*M* Tris–HCl (pH 7.4) and various concentrations of dextrans. Phase separation and extraction were made at 30°C. (○) Cytochrome b_5; (□) total proteins.

extracted into the surfactant-rich phase with yields of 55% and 84%, respectively. Cytochrome b_5 is negatively charged at pH 7.4 [10] and, thus, the extractability could be reduced by the use of cationic DEAE–Dx. Indeed, the extraction of cyt. b_5 was slightly reduced at 0.1% (w/v) DEAE–Dx and then increased with increasing the DEAE–Dx concentration (Fig. 2a). The decrease in the yield of cyt. b_5 should be due to the electrostatic attraction with DEAE–Dx in the aqueous phase, and the following increase due to the steric repulsion with DEAE–Dx. Difference in the extraction yield between cyt. b_5 and total proteins was reduced at 0.1% (w/v) DEAE–Dx. This is unfavorable for the separation of cyt. b_5 from microsomes. On the other hand, by the addition of Dx–S, the extraction of cyt. b_5 was only slightly enhanced (yield>90%), while that of total proteins was markedly reduced. The extraction yield of the latter was 20% in a Dx–S concentration range from 0.1 to 0.2% (w/v). In the absence of Dx–S, the extraction yield of total proteins was 55%. Thus a combined use of Triton X-114 with Dx–S provides a good means for selective isolation of cyt. b_5 from other microsomal proteins. The reduced extraction of

total proteins in the presence of Dx–S is ascribed to the average net charge of the total proteins which is different from that of cyt. b_5. The electrostatic interaction with charged polymer and the hydrophobic interaction with Triton X-114 appear to play dominant roles in controlling the extraction behavior of cyt. b_5 and other microsomal proteins in this phase separation system.

3.3. Purification of cytochrome b_5

According to successive Procedures 1 and 3, cyt. b_5 was isolated from pig liver microsomes. In Procedure 3 the first phase separation without the use of Dx–S was made for isolation of microsomal hydrophobic proteins from hydrophilic proteins which were possibly solubilized in Procedure 1. The second phase separation was for the selective isolation of cyt. b_5 from other hydrophobic proteins. The third was a washing step for the surfactant-rich phase involving cyt. b_5, where sodium chloride was added to prevent a loss of cyt. b_5 into the aqueous phase. As is seen in Table 1, the results are satisfactory. The overall yield of cyt. b_5 was 91% and the purification factor was 10.6. Additionally, no denaturation of cyt. b_5 in the extraction processes was observed. From these results, a combination of Triton X-114 with Dx–S seems to be applicable to the purification of cyt. b_5 prior to chromatographic separations, due to operational simplicity and practicality.

The purification of membrane proteins including cyt. b_5 has been conducted in combination with several chromatographic methods such as size-exclusion, ion-exchange, hydrophobic, and affinity, after solubilization and prefractionation. In the purification of cyt. b_5, DEAE–cellulose is usually used as the first column [14–16]. In this step, the yield and the purification factor obtained are approximately 30–60% and 2–15, respectively, which are largely dependent on the starting materials. In our preliminary experiments, 10-fold purification with a yield of 35% was obtained via DEAE–cellulose for the same starting material as was used in this study. The present method is superior to the first chromatographic method in the purification of cyt. b_5. Moreover, in the large-scale purification, chromatographic methods are time-consuming, and frequently, one column operation needs more than 1 week, while the present method can be conducted within a few hours. Thus, the present method can be exchangeable for the first column, thus providing the effective purification of cyt. b_5.

In this work, by the addition of charged polymer, the extractability of membrane proteins was successfully controlled in the TIPS method with Triton X-114, as was shown for the partial purification of microsomal cyt. b_5. This method can be applied to the separation of various membrane proteins having different net charges by the use of various types of polymers.

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Table 1
Partial purification of cytochrome b_5 from pig liver microsomes

Fraction	Total protein (mg)	Total content (nmol)	Specific content (nmol/mg)	Recovery (%)	Purification (fold)
Microsomes	1.86[a]	0.78[a]	0.42	100	1
Solubilized microsomes	1.56	0.78	0.50	100	1.2
Surfactant-rich phase[b]	0.16	0.71	4.44	91	10.6

[a] Values for a starting volume of 0.2 ml microsomes.
[b] Surfactant-rich phase was obtained after triplicate phase separation (see Procedure 3).

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