# Selective Solubilization of Microsomal Electron-Transfer Proteins with Alkylglucoside

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A series of alkylglucosides (AGs) was examined for the solubilization of four microsomal electron-transfer proteins (cytochrome P450 (P450), cytochrome $b_5$ ($b_5$), NADPH-cytochrome P450 reductase (fp2), and NADH-cytochrome $b_5$ reductase (fp1)) in rat liver microsomes. Among the four proteins, $b_5$ and fp2 were completely solubilized, and thus, almost recovered in the supernatant fraction after centrifugation, while P450 and fp1 were in the pellet. In particular, the solubilization yield of P450 was only about 10%. With a high ratio of alkylglucoside to membrane, along with a low ionic strength, a greater selectivity for $b_5$ and fp2 could be obtained. Such high selectivity was not observed by the use of sugar ester, bile salt, and poly(oxyethylene) alkylphenyl ether types of surfactants. After re-solubilization of the pellet with sodium cholate, the supernatant fraction contained P450 free from $b_5$ and the final recovery of P450 was about 40%. Thus, this two-step solubilization provides a simple method for the partial purification of P450 in microsomes.

**Keywords** Alkylglucoside, solubilization, electron-transfer protein, cytochrome $b_5$, cytochrome P450

The solubilization of membrane proteins from biological membranes is an essential step in the purification and subsequent structural and functional characterizations of membrane proteins. Surfactants have been widely used for the solubilization because of their ability to disrupt the membrane structure and to replace lipids surrounding membrane proteins.[1,2] For this purpose a number of surfactants are commercially available; however, a great concern is a choice of an appropriate surfactant which solubilizes the membrane proteins of interest without denaturation. Alkylglucosides (AGs) are nonionic surfactants having a hydrophilic glucose moiety. They have many advantageous characteristics for the purification of membrane proteins; the high critical micellar concentration, high optical transparency, and low denaturing character. Thus, AG has been widely used as a solubilizing agent for membrane proteins.[3-6].

We report here on the effect of AGs on the extent of the solubilization of the microsomal electron-transfer proteins, cytochrome P450 (P450), cytochrome $b_5$ ($b_5$), NADPH-cytochrome P450 reductase (fp2), and NADH-cytochrome $b_5$ reductase (fp1). In view of the important role of P450 and $b_5$ in mediating electrons in combination with fp1 and fp2 in microsomes, a number of methods have already been reported concerning the purification of P450 and $b_5$.[7-9] Imai *et al.*[10] proposed a chromatographic method with an amino-octyl Sepharose column; this method has been widely accepted as the first chromatographic step for the purification of P450.[7,9] Prior to column chromatographic separation, the electron-transfer proteins must be solubilized with a surfactant, for which sodium cholate and/or poly(oxyethylene)-type nonionic surfactant such as Triton X-100, Emulgen 911, or Nonidet P-40 are currently employed.[7,9] These surfactants solubilize the electron-transfer proteins well, however non-selectively. Regardless of this, little attention has been paid to the choice of the surfactants including AGs in view of the selectivity for the solubilization of these proteins. Thus, we tested a series of AGs in order to clarify their selective nature in the solubilization of electron-transfer proteins.

## Materials and Methods

### *Chemicals*

Octyl-β-D-glucoside (OG), heptyl-β-D-thioglucoside (HTG), and octyl-β-D-thioglucoside (OTG) were purchased from Dojindo Laboratory (Kumamoto, Japan). Nonyl-β-D-glucoside (NG), sodium taurodeoxycholate (STDC), and cytochrome *c* (cyt.*c*) (horse heart) were from Sigma Chemical Co. (St. Louis, MO). Sucrose monolaurate (SML), sucrose monocaprate (SMC), and sucrose monocholate (SMCh) were obtained from Kishida

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Chemical Co. (Osaka, Japan). Sodium cholate (SC), sodium deoxycholate (SDC), and sodium taurocholate (STC) were from Wako Pure Chemical (Tokyo, Japan). Triton X-100 (TX100) and Emulgen 911 (EM911) were from Nacalai Tesque (Kyoto, Japan) and Kao-Atlas Co. (Tokyo, Japan), respectively. All other chemicals used were of analytical or biochemical grade.

*Preparation and solubilization of rat liver microsomes*

Phenobarbital-treated rat liver microsomes were prepared by a previously described method[11] and stored at -80°C until use. Solubilization of the microsomes was carried out in aqueous micellar solutions at fixed and various concentrations of each surfactant containing potassium phosphate (pH 7.4), 1 mM EDTA, and 20%(v/v) glycerol. The whole mixture thus obtained was incubated on ice for 1 h and centrifuged (Hitachi 55P-72 ultracentrifuge) at 105000×*g* for 1 h at 4°C. The resulting supernatant solution was referred to as the solubilization fraction (the first fraction).

A two-step solubilization was carried out as follows. The microsomes (3.5 mg protein/ml) were solubilized with 1.5%(w/v) OTG containing 10 mM potassium phosphate (pH 7.4), 1 mM EDTA, 20%(v/v) glycerol. The solution was incubated and centrifuged as mentioned above. The supernatant was collected as the first fraction. Then, the insoluble microsomal fraction, recovered as a pellet, was suspended for washing once with 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 20%(v/v) glycerol, and then centrifuged. The resulting pellet was resuspended in an appropriate amount of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.6%(w/v) sodium cholate, 1 mM EDTA, and 20%(v/v) glycerol, to give a total protein concentration of 2.5 mg/ml. After incubation on ice for 1 h, the whole solution was recentrifuged at 105000×*g* for 1 h. The resulting supernatant solution was referred to the second fraction. The residual pellet was also recovered.

*Analytical methods*

Cytochrome P450 was determined by measuring the CO differential spectrum for the reduced form of P450, the extinction coefficient of which is 91 $mM^{-1}$ $cm^{-1}$ ($\Delta A_{450\text{-}490nm}$).[12,13] Cytochrome $b_5$ was determined from the differential spectrum between its reduced and oxidized forms using a coefficient of 185 $mM^{-1}$ $cm^{-1}$ ($\Delta A_{424\text{-}409nm}$).[14] The NADH-cytochrome $b_5$ reductase (fp1) and NADPH-cytochrome P450 reductase (fp2) activities were evaluated as ferricyanide and cytochrome *c* reductase activities by measuring at 420 and 550 nm, respectively.[14,15] The former extinction coefficient is 1.02 and the latter 21.1 $mM^{-1}$ $cm^{-1}$. The protein concentration was determined by the bicinchoninic acid method[16] in which bovine serum albumin was used as a standard.

The solubilization yields were calculated from the content or the activity of the respective proteins in the microsomes and from those in the first or second supernatant fractions.

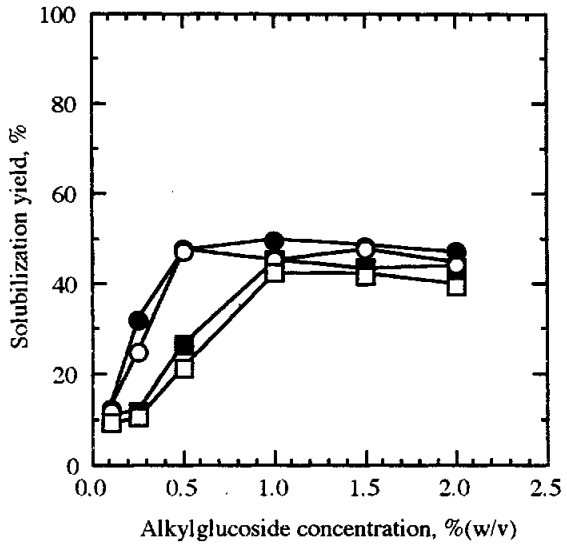


Fig. 1 Effect of the alkylglucoside concentration on the solubilization of the microsomal protein. The experiment was carried out at a protein concentration of 3.5 mg/ml and at various concentrations of HTG (□), OTG (○), OG (■), or NG (●) with 10 mM potassium phosphate (pH 7.4), 1 mM EDTA, and 20%(v/v) glycerol.

## Results and Discussion

*Suitability of alkylglucosides for the solubilization of microsomal proteins*

In order to demonstrate the effectiveness of alkylglucosides (AGs) for solubilizing microsomal proteins, we tested a series of AGs under various conditions. Figure 1 shows the dependence of the AG concentrations on the solubilization yields of microsomal proteins. The yields were drastically increased around the cmc of each AG and then leveling off. At a level of 1.5%(w/v) AGs the yields were about 40-50%. The yields were hardly dependent on the alkyl chain length or the ether bond. We also tested the effect of the solubilization time on the yield. Solubilization was quickly completed within 15 min, independent of the kind of AGs used. Thus, the AGs appear to have sufficient ability to solubilize the microsomal proteins.

The proteins must be prevented from denaturing while being solubilized. Thus, the stability of the proteins in the presence of OTG was investigated. Table 1 shows the apparent contents of $b_5$ and P450, and the activities of fp1 and fp2 in microsomes with and without OTG. The content of $b_5$ in the presence of OTG was slightly larger than that in the absence of OTG. This fact is probably due to the decrease in turbidity of the microsomal suspension by the addition of OTG. The activities of fp1 and fp2 remained almost unchanged. It is evident that these three proteins did not undergo denaturation in the presence of OTG. However, the content of P450 was drastically decreased. From a spectrometric observation, P450 has been shown to change to its denatured form, P420, in the

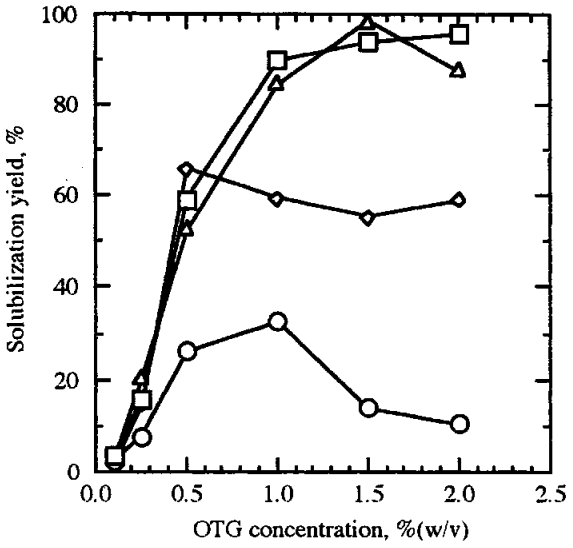


Fig. 2 Effect of the OTG concentration on the solubilization of the microsomal electron-transfer proteins. (□), cytochrome $b_5$; (○), cytochrome P450; (◇), NADH-cytochrome $b_5$ reductase; (△), NADPH-cytochrome P450 reductase. Conditions as given in Fig. 1.

Table 1 Stability of the microsomal electron-transfer proteins in an OTG micellar solution

| | $b_5$ | P450 | fp1 | fp2 |
|---|---|---|---|---|
| without OTG | 100 | 100 | 100 | 100 |
| with 1.5% OTG | | | | |
| 30 min | 108 | 63 | 100 | 102 |
| 60 min | 107 | 64 | 98 | 98 |
| 180 min | 106 | 68 | 97 | 105 |

Solution contains 10 mM potassium phosphate buffer (pH 7.4), 3.5 mg/ml protein, 1 mM EDTA, and 20%(v/v) glycerol.

presence of OTG (data not shown). Thus, it can be concluded that alkylglucosides are useful for the solubilization of $b_5$, fp1, and fp2.

*Selectivity in the solubilization of the microsomal electron-transfer proteins with alkylglucoside*

Subsequently, we tested the solubilization of $b_5$, P450, fp1, and fp2. Figure 2 shows the dependence of the solubilization yields on the alkylglucoside concentration. Similar to the result in Fig. 1, the solubilization yield of each protein was increased around the cmc of OTG. While $b_5$ and fp2 were completely solubilized at 1.5%(w/v) of OTG, fp1 was only partially solubilized. In the solubilization of P450, the yield was a maximum at 1.0%(w/v) of OTG and then decreased with an increasing concentration of OTG. The extent of solubilization of P450 was only 11% at 1.5%(w/v) OTG. This result clearly indicates the selective nature of OTG for $b_5$ and fp2.

The selectivity was affected by both the contents of the membrane constituents and the ionic strength in aqueous micellar solutions. Figures 3 and 4 show the effects of the total protein contents and phosphate concentrations, respectively, on the solubilization of the electron-transfer proteins at a level of 1.5%(w/v) OTG. The total protein

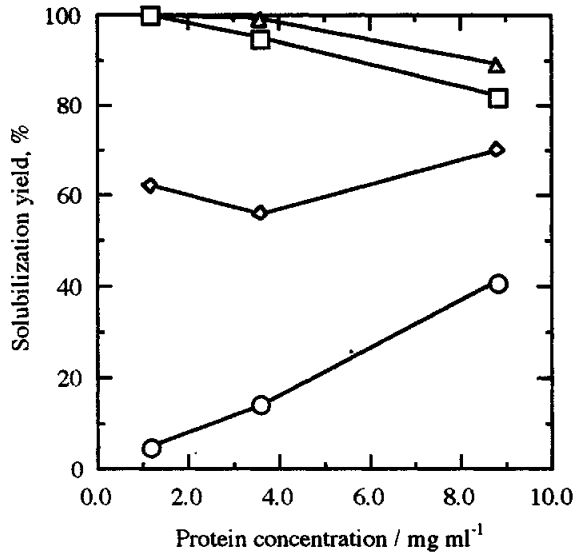


Fig. 3 Effect of the microsomal protein concentration on the solubilization of the microsomal electron-transfer proteins. (□), cytochrome $b_5$; (○), cytochrome P450; (◇), NADH-cytochrome $b_5$ reductase; (△), NADPH-cytochrome P450 reductase. The experiment was carried out at 1.5%(w/v) OTG with 10 mM potassium phosphate (pH 7.4), 1 mM EDTA, and 20%(v/v) glycerol.

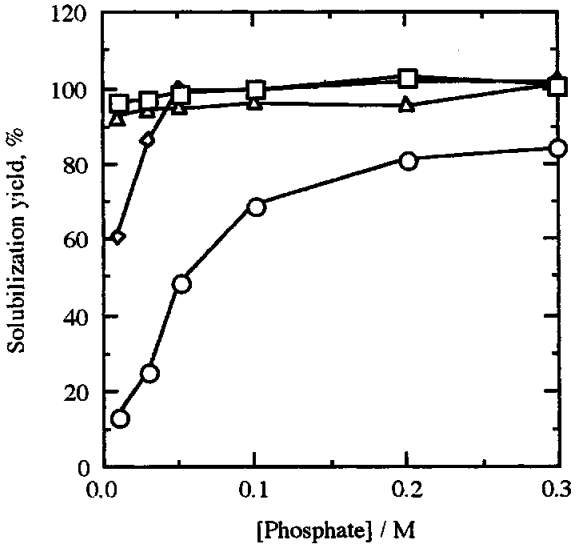


Fig. 4 Effect of the phosphate concentration on the solubilization of the microsomal electron-transfer proteins. (□), cytochrome $b_5$; (○), cytochrome P450; (◇), NADH-cytochrome $b_5$ reductase; (△), NADPH-cytochrome P450 reductase. The experiment was carried out at a protein concentration of 3.5 mg/ml and at 1.5%(w/v) OTG with 1 mM EDTA and 20%(v/v) glycerol.

Table 2 Solubilization yields of microsomal proteins with various surfactants

| Surfactant | $b_5$ | P450 | fp1 | fp2 | Total protein |
|---|---|---|---|---|---|
| (alkylglucoside) | | | | | |
| 1.5% HTG | 93.6 | 11.5 | 37.9 | 95.5 | 42.3 |
| 1.5% OTG | 94.6 | 11.2 | 55.5 | 98.8 | 47.8 |
| 1.5% OG | 93.5 | 17.7 | 39.0 | 97.6 | 43.8 |
| 1.0% NG | 91.4 | 24.1 | 58.9 | 94.5 | 42.9 |
| (sugar ester) | | | | | |
| 1.0% SMC | 90.0 | 40.3 | 54.2 | 102 | 46.7 |
| 0.5% SML | 86.5 | 49.3 | 76.3 | 97.7 | 48.5 |
| 1.5% SMCh | 88.6 | 28.4 | 28.1 | 93.8 | 40.2 |
| (bile salt) | | | | | |
| 1.5% SC | 64.0 | 76.1 | 60.6 | 65.8 | 60.4 |
| 1.0% SDC | 109 | 79.6 | 97.1 | 101 | 82.4 |
| 1.5% STC | 87.1 | 77.8 | 85.2 | 88.5 | 68.8 |
| 1.0% STDC | 104 | 89.5 | 96.1 | 104 | 85.5 |
| (polyoxyethylene alkyl ether) | | | | | |
| 0.5% TX100 | 95.9 | 56.5 | 70.4 | 102 | 55.0 |
| 0.2% EM911 | 74.6 | 46.5 | 30.9 | 79.0 | 48.1 |

Solutions contain 10 mM potassium phosphate buffer (pH 7.4), 3.5 mg/ml protein, 1 mM EDTA, and 20%(v/v) glycerol.

Table 3 Two-step solubilization of $b_5$ and P450

| Fraction | Recovery, % | | |
|---|---|---|---|
| | $b_5$ | P450 | Total protein |
| Ms | 100 | 100 | 100 |
| S1 | 95 | 11 | 48 |
| P1 | nd | 57 | 46 |
| S2 | nd | 43 | 31 |
| P2 | nd | 7 | 10 |

nd: not detected

concentration was used as an index of the contents of the membrane constituents. In Fig. 3, the solubilization yields of $b_5$ and fp2 were reduced by increasing total protein concentration, while those of P450 and fp1 were increased. In Fig. 4, with an increase in the phosphate concentration, the solubilization yields of $b_5$ and fp2 remained unchanged, while those of P450 and fp1 were increased. The lower the concentration ratio between the membrane constituents and OTG or the lower the salt concentration, the higher the selective solubilization for $b_5$ and fp2 was obtained.

The changes in the selectivity are probably due to the nature of the soluble mixed micelles of OTG and lipids. In the solubilization process, membrane proteins are distributed into the soluble mixed micelles, and complexes are formed. It is generally accepted that in a lower ratio of surfactant to membrane constituents, large lipid-rich mixed micelles are formed, and in the higher ratio, small OTG-rich micelles are formed. In fact, the turbidity of the solubilized microsomes decreased along with an increase in the ratio of surfactant to membrane constituents (data not shown). P450 and fp1 might be distributed into a lipid-rich phase, lipid-rich micelles or insoluble membrane residues, while $b_5$ and fp2 into OTG-rich micelles because of the lower hydrophobicity of $b_5$ and fp2 than that of P450 and fp1. Hence, the solubilization yields of P450 and fp1 were increased with increasing the ratio of the protein (lipid-rich fraction) to OTG, and thus the selectivity was reduced.

The results concerning solubilization with HTG, OG and NG are listed in Table 2. Furthermore, in order to compare the selectivity with other surfactants, sugar ester, bile salt, and poly(oxyethylene) alkylphenyl ether types of surfactants were also tested.

HTG, OG, and NG selectively solubilized $b_5$ and fp2 as well as OTG. SMC and TX100 also solubilized $b_5$ and fp2, the extent of which was more than 90%. SML, SMCh, and EM911 are also good alternatives for $b_5$ and fp2. However, their solubilization yields with P450 were about 30 - 60%, indicating poor selectivity, which is inferior to alkylglucosides.

Bile salts (SC, SDC, STC, and STDC) are the most effective for solubilizing P450 among the surfactants tested. However, $b_5$, fp1, and fp2 were also solubilized with bile salts to the same degree. For example, SC solubilized the proteins to an extent of about 60 - 76%. Such non-selectivity can not be observed in the solubilization with other types of surfactants. This fact is attributable to the nature of bile salts which can form unique mixed micelles with lipids at high ratios of lipid-to-bile salt.[17] In a mixed micelle the lipid bilayer maintains its structure and bile salts surround the bilayer structure with a side-to-side hydrophobic interaction. Membrane proteins would be solubilized with bile salts, being submerged in a lipid bilayer. Thus, bile salts can be regarded to be non-specific solubilizing agents for microsomal electron-transfer proteins.

*Simple separation of P450 from $b_5$*

Differential solubilization is frequently effective for the fractionation of membrane proteins.[18,19] The present experimental results provide a new route for the differential solubilization of P450 and $b_5$ by the use of two different surfactants. Thus, in a subsequent study, the high solubilization selectivity of OTG for the microsomal proteins was exploited to develop a simple method for the separation of P450 from $b_5$. The method includes a two-step solubilization; firstly, $b_5$ was solubilized from the microsomes by the use of OTG, and secondly, the resulting pellet was treated again with sodium cholate to solubilize P450.

Table 3 shows the results concerning the recovery of the proteins in the two fractions obtained by the two-step solubilization. In the first solubilized fraction, S1, almost all the $b_5$ (95%) was solubilized, as described above. Thus, in the second solubilized fraction, S2 and in the insoluble pellet, P, $b_5$ was not detected. On the other hand, only 11% of P450 was solubilized in the S1 fraction. However, in the S2 fraction, the recovery of P450 was over 40% when cholate, the best solubilizing agent for P450, was

employed. Moreover, the S1 fraction contained an additional 25% of P450 in its denatured form, P420, whereas no P420 was detected in the S2 fraction. Thus, in the S2 fraction P450 are completely separated from $b_5$ and P420.

In this study, it appears that the nonionic surfactants, alkylglucosides, are a good choice for the selective solubilization of $b_5$ and fp2 from the microsomal electron-transfer proteins. Separation of P450 from $b_5$ was simply achieved by the re-solubilization of the residual pellet with cholate after the solubilization with OTG. Thus, the use of alkylglucosides in the solubilization of the microsomal electron-transfer proteins could be an effective means for purifying these proteins.

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