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Extraction of Concanavalin A with Affinity Reversed Micellar Systems

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ABSTRACT

An affinity reversed micellar system was used for extraction of concanavalin A (con A) with AOT and a biological detergent as the affinity cosurfactant. Six detergents with varying alkyl tail lengths and different head groups were compared for their extraction efficiency of con A. The effects of system parameters such as solution pH, cosurfactant concentration, and the presence of inhibitory saccharides on protein transfer were studied. For glucosides, protein transfer depends on the tail length of the cosurfactant, and the optimum length for maximum protein transfer is eight. A simple thermodynamic model for affinity partitioning of the protein between the two phases can fit the experimental results reasonably well. The estimated association constants for the protein–cosurfactant complex in the micellar phase are consistent with the literature values. For backward transfer of con A from a micellar phase with a stripping aqueous solution, 12.5% isopropyl alcohol addition to the aqueous phase can release the maximum amount of protein (88%). The reversed micelle still maintained its functional integrity after backward transfer, but loss of cosurfactant to the aqueous phase was significant, and reusability of the micellar phase was rather low. Using a more hydrophobic cosurfactant can only partially solve this problem, where spontaneous loss of cosurfactant to the phase interface during protein backward transfer still occurred.

Key Words. Reversed micelles; Concanavalin A; Affinity separation

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INTRODUCTION

Affinity-based methods for the purification of proteins are gaining increasing importance in downstream processing (1). This is primarily due to the exquisite selectivity provided by such affinity techniques, which can offer a highly purified product with a reduced number of processing steps. One of the affinity purification methods, affinity partitioning in an aqueous two-phase systems, has been studied extensively (2). Since the underlying mechanism of this process is liquid–liquid extraction, which has some special features and has been used in the chemical industry for decades, continuous operation and scale-up of this process will be much easier to achieve than using chromatographic separation processes.

A similar protein separation method using the same liquid–liquid extraction principle is the reversed micellar extraction system (3). Reversed micelles are water-in-oil microemulsion droplets stabilized by surfactants in organic solvents. The polar surfactant headgroups surround small water pools within which hydrophilic molecules, such as amino acids and proteins, can be solubilized. The attached hydrocarbon tails protrude into the surrounding organic solvent. Reversed micellar systems have been used in several liquid–liquid extraction processes for the purification of enzymes and shown to be feasible for whole broth processing (4, 5).

Although the results from reversed micellar extraction of proteins are promising, this process usually results in a low overall purification factor. This directly relate to the nature of the selection mechanism in reversed micellar extraction, which is based on electrostatic interactions between the protein and the charged surfactant headgroups lying inside the reversed micellar wall. To increase the selectivity for protein extraction in reversed micelles, affinity reversed micellar systems should be used (6, 7). This can be accomplished by adding a small concentration of an affinity cosurfactant, which has a hydrophilic ligand headgroup which the protein can recognize and bind, and an attached hydrophobic tail to impart surface activity to the protein–cosurfactant complex.

This study examines the extraction of concanavalin A (con A) into AOT/isooctane reversed micelles with a biological detergent as the affinity cosurfactant. Con A is an important plant lectin isolated from the jack bean plant (*Canavalin ensiformis*), which demonstrates specific binding sites for α-D-mannose or α-D-glucose. It has been utilized as an immunoabsorbent for blood typing (8) and as an affinity media in column chromatography (9). To optimize the extraction, the ligand headgroup and the alkyl tail length of the affinity cosurfactant were varied. Backward transfer of the protein and reusability of the affinity reversed micelles were also stud-

ied because they directly influence the yield and economics of this extraction process.

MATERIALS AND METHODS

Six biological detergents were used as the cosurfactant, and they were obtained from Sigma (St. Louis, Missouri, USA). They are hexyl-β-D-glucopyranoside (hexyl-glucoside), octyl-β-D-glucopyranoside (octyl-glucoside), nonyl-β-D-glucopyranoside (nonyl-glucoside), dodecyl-β-D-glucopyranoside (dodecyl-glucoside), octyl-β-D-thioglucopyranoside (octyl-thioglucoside), and dodecyl-β-D-maltoside (dodecyl-maltoside). Sodium di-(2-ethylhexyl) sulfosuccinate (AOT) and concanavalin A from jack bean (type IV) were also from Sigma. Isooctane (spectrophotometric grade), isopropyl alcohol, and ethyl acetate were obtained from Merck. All chemicals were reagent grade or of the highest grade commercially available.

All aqueous solutions were composed of 90% KCl solution and 10% potassium phosphate buffer at a total ionic strength of 0.1 M. Reversed micellar phases were 50 mM AOT and 2 mM cosurfactant in isooctane unless stated otherwise. In the absence of AOT, the ligand showed no observable solubility in isooctane.

For forward transfer of protein, 4 cm^3 aqueous phase containing 1 $g \cdot dm^{-3}$ con A was contacted with 4 cm^3 reversed micellar phase in a reagent bottle by stirring at 800 rpm with a Teflon-coated magnetic stirring bar for 5 minutes. The bottle was capped in order to prevent loss of the contents via splashing or evaporation, and it was set in a constant temperature water bath maintained at 25°C. Following extraction, the solution was poured into a test tube, corked, and centrifuged at 2000 rpm in a bench-top centrifuge for 15 minutes. The resultant two phases were separated and analyzed for protein concentration. Protein concentration was determined by UV absorption at 280 nm on a Shimadzu UV-160A spectrophotometer. The absorption at 310 nm was used as an indication of scattering from other sources and subtracted from the reading at 280 nm. Blanks were prepared for each sample from the same system but without protein. Transfer efficiency of protein was defined as the absorbance of the micellar phase divided by the initial absorbance of the aqueous phase. Partition coefficient was defined as the protein concentration in the reversed micellar phase divided by that in the aqueous phase.

To study the effects of saccharides on con A forward transfer, the same transfer experiments were carried out as described above but with the aqueous phase containing different concentrations (5–100 $mmol \cdot dm^{-3}$) of glucose, fructose, galactose, or methyl-α-D-glucopyranoside.

For backward transfer of protein, 3.5 cm^3 of the micellar phase from the forward transfer was contacted with an equal volume of an aqueous phase containing 1 M KCl (0.01 M phosphate buffer at pH 7.77) under the same conditions as in the forward transfer. To study the influences of operating conditions on backward transfer, higher extraction temperatures (50 and 60°C), or additives in the aqueous phase [15% (v/v) ethyl acetate, 5–17.5% (v/v) isopropyl alcohol, and 1 $mol \cdot dm^{-3}$ glucose] were also used. The solution was mixed and analyzed as described above. The kinetics of backward transfer in the presence of glucose was studied by varying the mixing time from 10 to 80 minutes. Transfer efficiency of protein was defined as the absorbance of the aqueous phase divided by the initial absorbance of the micellar phase.

The partition coefficients of cosurfactant was determined by contacting an equal volume of a protein-free aqueous phase (pH 7.77) with a micellar phase containing 50 mM AOT and varying concentrations (2 to 8 $mmol \cdot dm^{-3}$) of cosurfactant in isooctane. The solution was stirred at 800 rpm for 5 minutes and centrifuged for phase separation. Cosurfactant concentration in the aqueous phase was determined as the total sugar concentration according to the phenol–sulfuric acid method (10). Cosurfactant concentration in the micellar phase was calculated from the mass balance.

All data reported are the mean value of triplicate determinations.

RESULTS AND DISCUSSION

Transfer of Con A with Different Cosurfactant

The extraction of con A was conducted in the absence and presence of an affinity cosurfactant for a variety of pHs. The cosurfactants have a carbohydrate moiety (glucose or mannose) to which con A can bind, and an alkyl tail of varying length. The results are shown in Table 1 as the percentage of protein extracted into the reversed micellar phase compared to the total protein initially present in the aqueous phase.

The percent transfer of con A without cosurfactant decreases with increasing pH, dropping significantly when pH is above 6.22 and approaching zero at pH 7.77. The effect of pH on the extraction of con A reflects both the influence of electrostatic interaction and size limitation. Con A associates as a dimer (molecular weight 53,000) at low pH (5 and below) and forms tetramer (molecular weight 106,000) at the physiological pH (11). Its isoelectric point is 8.35 (12). The electrostatic interaction between anionic surfactant AOT and con A is expected to be less favorably with an increase of pH. Also, the concomitant increase of the molecule size of con A can further hamper its transfer into reversed micelles.

TABLE 1
Percent Transfer of Con A with Different Cosurfactants[a]

Cosurfactant	pH				
	5.26	5.87	6.22	6.73	7.77
None	97.3	93.4	83.0	23.9	2.5
Heptyl-glucoside	98.8	90.7	82.2	70.6	57.3
Octyl-glucoside	96.7	93.1	88.8	82.3	66.0
Nonyl-glucoside	98.1	91.5	80.8	76.7	61.3
Dodecyl-glucoside	97.4	88.8	79.7	75.5	58.9
Octyl-thioglucoside	99.1	88.0	84.0	74.6	59.3
Dodecyl-maltoside	96.5	94.5	89.9	84.6	68.4

[a] Extraction was with 50 mmol·dm^{-3} AOT for 2 mmol·dm^{-3} cosurfactant in isooctane at the pH indicated

To extend the operating pH range over which con A can be extracted, the addition of a small concentration of affinity cosurfactant can substantially increase protein transfers at high pHs. The percent transfer still drops with increasing pH due to the same electrostatic and size effects. But ~60% con A can still be extracted into the micellar phase at pH 7.77. The enhancement of protein transfer is due to the additional driving force from protein–cosurfactant affinity interaction. To fully utilize the affinity effect, the affinity reversed micellar system should be operated around this pH, where maximum selectivity could be expected. Here, other proteins which cannot interact with the cosurfactant will usually show poor transfer with unfavorable electrostatic attraction. Nonetheless, the recovery of con A will be low with modest protein transfer efficiency, and multiple extraction steps may be needed to increase the yield.

Different cosurfactants show different effects on protein transfer. For β-glucosides with different alkyl lengths, there seems to be an optimum tail length for maximum protein transfer. Figure 1 compares transfer results for β-glucosides with increasing alkyl tail lengths at three pH values. The data shown were taken from Table 1. Octyl-glucoside gives the highest transfer at all pH values. As mentioned above, enhanced protein transfer in affinity reversed micellar systems is based on the increased surface activity of the protein–cosurfactant complex. But as shown in Fig. 1, cosurfactants with longer tail lengths do not lead to a higher extraction efficiency. The partition coefficient of glucosides is expected to increase with the tail length due to increased hydrophobicity. Experimentally determined partition coefficients, 5.58 and 66.3 for octyl- and dodecyl-gluco-

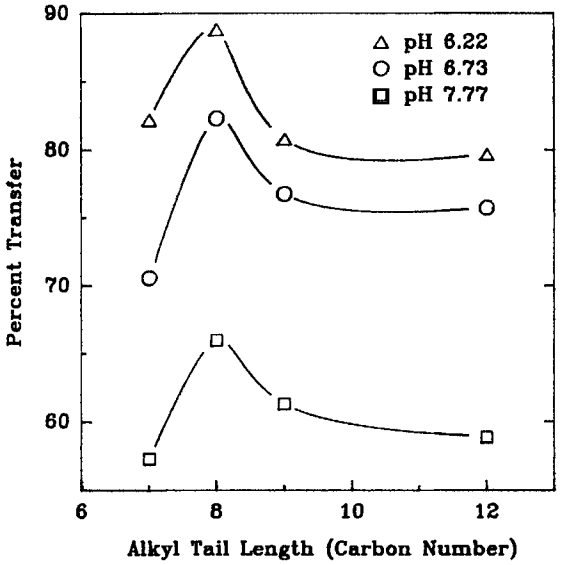

FIG. 1 The effect of alkyl length of glucosides as the cosurfactant on con A transfer to reversed micellar phase. 50 mmol·dm^{-3} AOT and 2 mmol·dm^{-3} cosurfactant.

side, respectively (see Fig. 2), support this statement. For a more hydrophilic cosurfactant (shorter tail), less cosurfactant can be found in the micellar phase, which will result in fewer proteins being transferred. On the other hand, for a more hydrophobic cosurfactant (longer tail), cosurfactant concentration in the aqueous phase will be low, resulting in fewer protein–cosurfactant complexes, and hence a lower overall partitioning into the micellar phase. The balance of both effects may determine the optimum alkyl tail lengths of glucosides for protein transfer.

For cosurfactants with the same alkyl tail length but a different headgroup, octyl-β-glucoside performs better than octyl-β-thioglucoside, and dodecyl-β-maltoside performs better than dodecyl-β-glucoside. This may relate to the strength of binding between con A and the cosurfactant.

Effects of Cosurfactant Concentration on Con A Transfer

The effects of cosurfactant (ligand) concentration on the partition coefficient of con A between the reversed micellar phase and the aqueous phase at a constant pH of 7.77 have been studied with four cosurfactants at 50 and 250 mmol·dm^{-3} AOT concentrations. Partition coefficients of con A

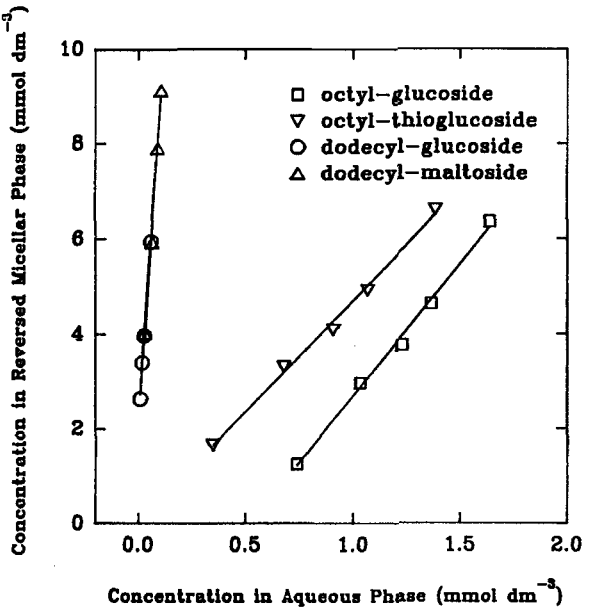

FIG. 2 Partitionings of cosurfactants between the reversed micellar phase (50 mmol·dm^{-3} AOT) and the aqueous phase at pH 7.77. The straight lines are from linear regression.

increase with cosurfactant concentration. The dependence exists in the form of a saturation curve as in affinity partitioning in aqueous two-phase systems. The partitioning of protein in affinity reversed micellar systems is similar to affinity partitioning in aqueous two-phase systems. Mathematical models developed previously can be used here to correlate the data. From thermodynamic analysis, the relations between several parameters influencing partition equilibrium have been developed by Flanagan and Barondes (13) and later modified by Cordes et al. (14). If the binding sites of con A are assumed to be identical and ligand binding is independent of the number of previously bound ligands, the protein partition coefficient (K_p) in the presence of the cosurfactant can be written as

$$K_p = K_{p0}\left[\frac{1 + L_T K_a^r}{1 + L_T K_a^a / K_L}\right]^n \tag{1}$$

where K_{p0} is the partition coefficient of protein in the absence of the cosurfactant, L_T is the total cosurfactant concentration in the micellar phase, K_L is the partition coefficient of the cosurfactant, n is the number of binding site of the protein, and K_a^r and K_a^a are the association constants

TABLE 2
Determination of Parameters with Partition Experiments[a]

Cosurfactant	K_L	K_A^r (mmol^{-1}·dm^3)	K_A^a (mmol^{-1}·dm^3)
Octyl-glucoside	5.58	3.12 × 10^3[b]	5.79 × 10^3
		3.81 × 10^3[c]	6.84 × 10^3
Octyl-thioglucoside	4.69	4.39 × 10^3	7.22 × 10^3
		4.44 × 10^3	7.23 × 10^3
Dodecyl-maltoside	67.0	1.58 × 10^4	ND[d]
		1.25 × 10^4	ND
Dodecyl-glucoside	66.3	4.50 × 10^3	ND
		5.49 × 10^3	ND

[a] K_L from experiments, K_A^r and K_A^a from regression of partition results.
[b] With 50 mmol·dm^{-3} AOT.
[c] With 250 mmol·dm^{-3} AOT.
[d] ND = not determined.

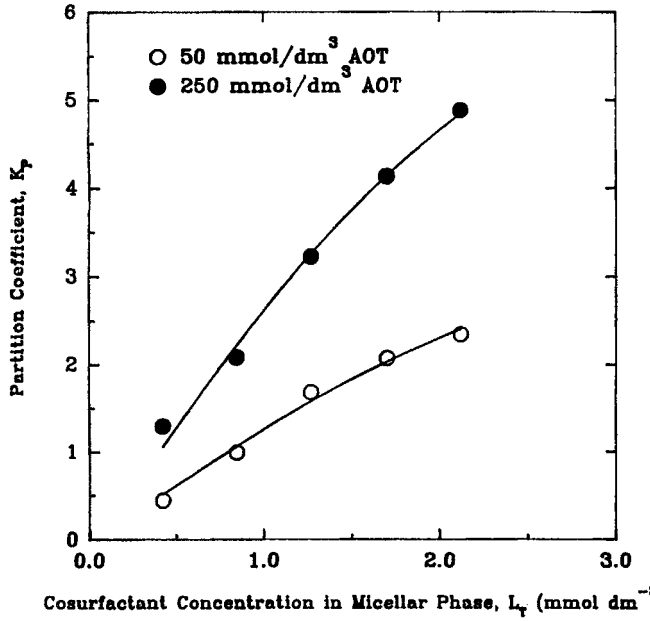

FIG. 3 The effect of the cosurfactant concentration on the partition coefficient of con A at pH 7.77. Octyl-glucoside is the cosurfactant. The lines are from model predictions.

of the protein–cosurfactant complex in the reversed micellar and the aqueous phase, respectively.

The parameter K_L was obtained in the absence of protein by measuring the equilibrium cosurfactant concentration in each phase, as shown in Fig. 2. The values of K_L calculated from the slopes of the linear regression lines in the figure are listed in Table 2. The parameter K_{p0} was measured in the absence of cosurfactant, and the values are 0.075 and 0.12 for 50 and 250 mmol·dm^{-3} AOT concentration, respectively. For con A, the number of binding site n is 4 at pH 7.77. The association constant in each phase, K_A^r and K_A^a, can be estimated by fitting Eq. (1) to the experiment data using nonlinear least-squares regression analysis. The results are shown in Figs. 3 to 6 with the parameters listed in Table 2. For dodecyl-glucoside or dodecyl-maltoside, K_A^a was not determined since the cosurfactant is retained predominantly in the micellar phase in view of the large K_L value, whence the denominator in Eq. (1) can be simplified to 1.

The model predictions agreed reasonably well with the partition results. The association constants from data regression need to be compared with literature values. The protein, the cosurfactant, and the protein–cosurfac-

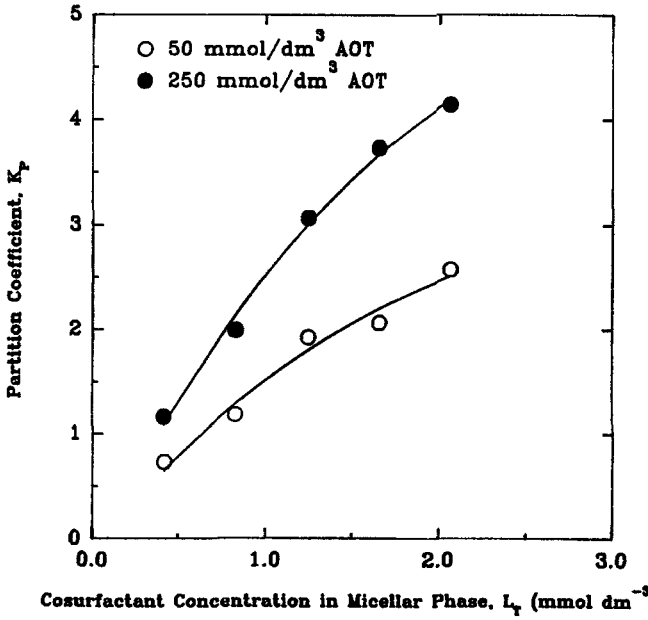

FIG. 4 The effect of the cosurfactant concentration on the partition coefficient of con A at pH 7.77. Octyl-thioglucoside is the cosurfactant. The lines are from model predictions.

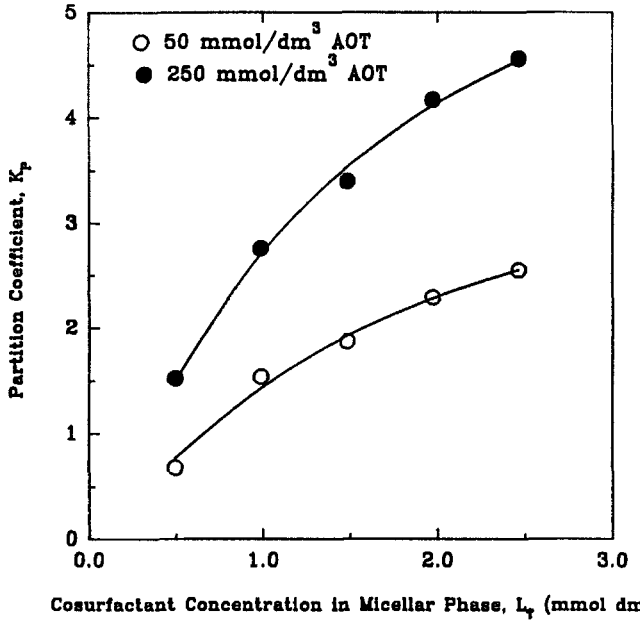

FIG. 5 The effect of the cosurfactant concentration on the partition coefficient of con A at pH 7.77. Dodecyl-glucoside is the cosurfactant. The lines are from model predictions.

tant complex are believed to exist in the water pools of the reversed micelles, as protein or cosurfactant itself is not soluble in isooctane. Thus it is more accurate to use the volume of the water pool in reversed micelles to calculate concentrations of those compounds, and not the total volume of the micellar phase. Since, on average, the solubilized water represented 4% of the total micellar phase volume in the present system (6), the effective K_A^r based on the water pool volume will be about 25 times smaller than the value reported in Table 2. This gives average K_A^r values of 139, 177, 200, and 566 $mol^{-1} \cdot dm^3$ for octyl-glucoside, octyl-thioglucoside, dodecyl-glucoside, and dodecyl-maltoside, respectively. The corrected K_A^r values for glucosides are in line with the measured values for the association constants of con A and methyl-β-glucoside, which are in the area of 167 $mmol^{-1} \cdot dm^3$ (15). No literature value is available for binding between con A and maltoside, but con A is known to bind stronger to mannose than to glucose (15), and maltose is a disaccharide of mannose. That the association constant of maltoside and con A is about three times that of glucoside is therefore reasonable. The K_A^a value is generally an order of magnitude higher than the value reported in the literature. The validity

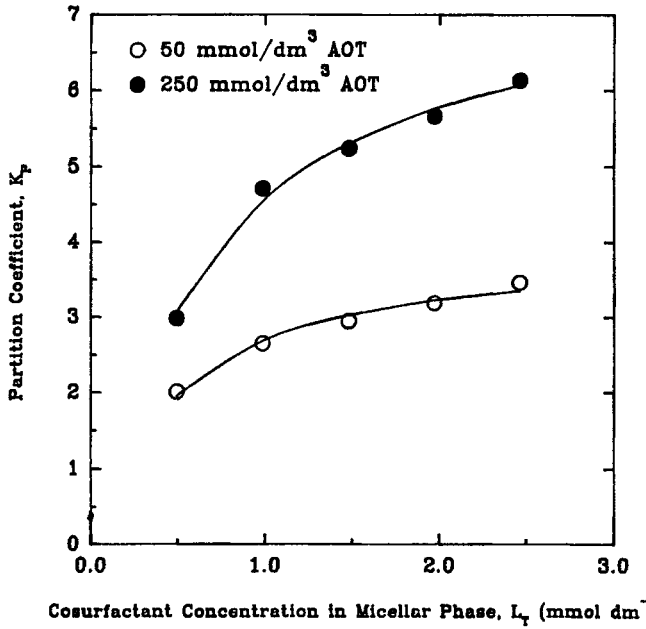

FIG. 6 The effect of the cosurfactant concentration on the partition coefficient of con A at pH 7.77. Dodecyl-maltoside is the cosurfactant. The lines are from model predictions.

of the assumption that con A has independent binding sites with the same binding constant, and that some cosurfactant will exist in the interface, which will overestimate K_L, may be responsible for this.

Inhibition of Con A Transfer

The association of protein and ligand is a competitive process, with ligands of higher affinity displacing those with weaker interactions. The addition of a hydrophilic, no surface-active substance as the free ligand to the aqueous phase will result in some protein to bind the free ligand, reducing the number of protein–cosurfactant complexes. This should reduce con A transfer into the reversed micellar phase. Experiments were conducted with 2 mmol·dm^{-3} octyl-glucoside as the cosurfactant and with different saccharides added to the aqueous phase before contacting with the reversed micellar phase.

Figure 7 shows the results of protein transfer. The control experiment gave approximate 65% transfer at zero carbohydrate concentration under the experiment conditions (pH 7.77). The saccharides have different ef-

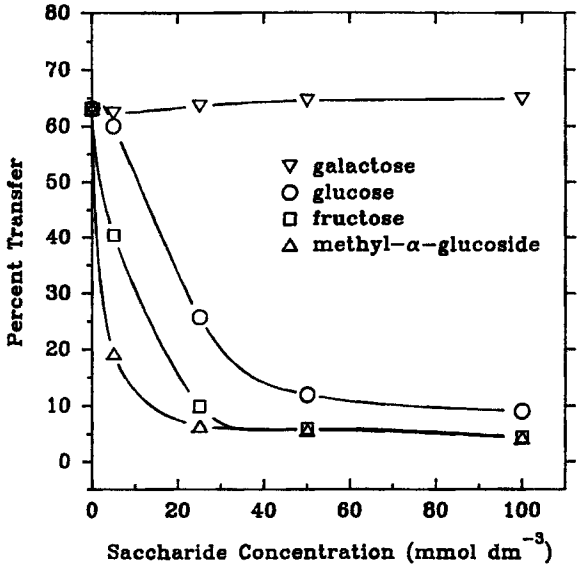

FIG. 7 The inhibition of con A transfer to the reversed micellar phase by saccharides in the aqueous phase at pH 7.77. 50 mmol·dm^{-3} AOT and 2 mmol·dm^{-3} octyl-glucoside.

fects on protein transfer: galactose does not influence the protein transfer, while glucose, fructose, and methyl-glucoside compete with the cosurfactant headgroup for con A binding sites and thus can prevent protein transfer, but to a different degree. The addition of 50 mmol·dm^{-3} fructose or methyl-α-D-glucoside results in a 15-fold drop in protein transfer, and the percent transfer is close to that in the absence of the cosurfactant. The association constants between the saccharides and con A have been reported, and the rise in order is expected from the extent of inhibition. Con A has virtually no affinity for galactose ($K_A < 10$ mol^{-1}·dm^3), moderate affinity for glucose ($K_A = 590$ mol^{-1}·dm^3) and fructose ($K_A = 1400$ mol^{-1}·dm^3), and high affinity for methyl-α-D-glucoside ($K_A = 5000$ mmol^{-1}·dm^3) (16). Those results suggest that one means of backextracting the protein may be to employ the saccharide to reduce the number of protein–cosurfactant complexes formed, thus decreasing the protein partition coefficient.

Backward Transfer of Con A

The backward transfer of con A from the micellar phase to an aqueous stripping solution was studied next. In contrast to the large number of

studies investigating forward transfer, the release and recovery of proteins from reversed micelles has received little attention in the literature. Most of the available studies assume that conditions which normally prevent protein uptake in the forward transfer experiments would promote their release in the backward transfer experiments. However, in most reported attempts to release the solubilized proteins from the reversed micelles, low yields were obtained. Current understanding of the backward transfer process in reversed micellar system suggests that, in many cases, recovery can be obtained by altering the pH of the extraction buffer and increasing the salt concentration (17). The addition of disrupting agents such as ethyl acetate (18) or ethanol (19) could be used to achieve dewatering of the reversed micelles, with subsequent protein rejection. Temperature increases have been shown to reduce the water content of a cationic–nonionic reversed micellar phase, with excellent recovery of the product amylase (20). The addition of isopropyl alcohol to the aqueous phase was found to be necessary for releasing acid proteases from AOT reversed micelles (21). However, no data are available for backward transfer in affinity reversed micellar systems.

Several methods were compared for their effectiveness in releasing con A in the absence or presence of 2 mmol·dm^{-3} octyl-glucoside. The results are reported in Fig. 8 for experiments conducted at pH 7.77. KCl (1 M) cannot release con A from reversed micelles with the cosurfactant. Only less than 20% con A was released in the system without the cosurfactant, although forward transfer of con A at the same pH is close to 0 (Table 1). This indicates that conditions (high pH and high salt concentration) which prevent the forward transfer of con A cannot result in good backward transfer efficiency. Higher temperatures can promote the release of con A (50–70%) from reversed micelles with or without the cosurfactant, and the efficiency is higher for the latter system. The addition of 15% (v/v) polar and water-immiscible ethyl acetate to the aqueous stripping solution can functionally disrupt the reversed micellar structure, leading to repulsion of the protein. About 75% of the protein can be recovered regardless of the presence of the cosurfactant.

The addition of isopropyl alcohol to the aqueous phase represents the best method for protein recovery, which at 12.5% (v/v) gives transfer efficiency close to 90%. Since isopropyl alcohol is a cosurfactant itself, it will influence the phase diagram of the water/oil two-phase system and lead to water and protein rejection from reversed micelles. The effect of isopropyl alcohol concentration in the aqueous solution on con A release is shown in Fig. 9. The optimum concentration was 12.5% (v/v). Backward transfer at a higher temperature (50°C) showed a similar dependence on isopropyl alcohol concentration and could not further increase the transfer

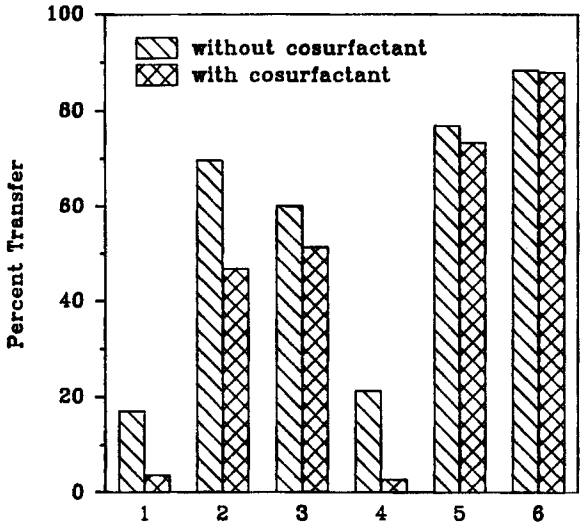

FIG. 8 The effect of different operation conditions for backward transfer of con A. Forward transfer is with 50 mmol·dm^{-3} AOT and with or without 2 mmol·dm^{-3} octyl-glucoside. All backward transfers were carried out with an aqueous phase containing 1 mol·dm^{-3} KCl at pH 7.77. 1, 25°C; 2, 50°C; 3, 60°C; 4, 1 mol·dm^{-3} glucose; 5, 15% (v/v) ethyl acetate; 6, 12.5% (v/v) isopropyl alcohol.

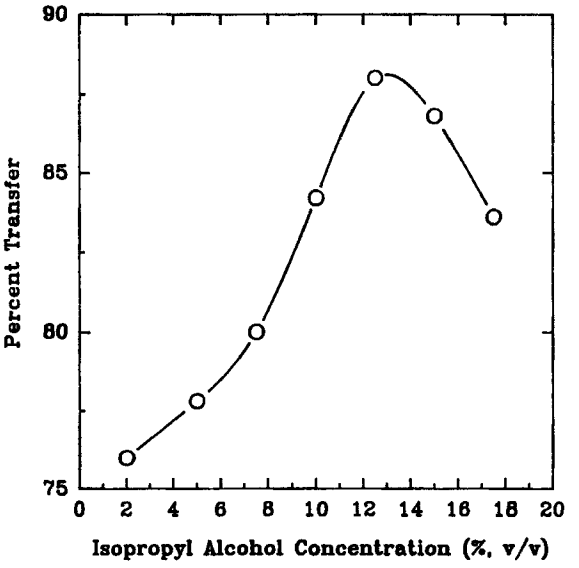

FIG. 9 The effect of isopropyl alcohol concentration on backward transfer of con A. Forward transfer is with 50 mmol·dm^{-3} AOT and 2 mmol·dm^{-3} octyl-glucoside. Backward transfers were carried out with an aqueous phase containing 1 mol·dm^{-3} KCl at pH 7.77.

efficiency (data not shown). Carlson and Nagarajan also found 10–15% isopropyl alcohol was necessary for pepsin release from AOT reversed micelles (21). The released con A was reextracted with an affinity reversed micellar phase at pH 7.77, and the protein transfer was no different from that when the protein was extracted from a fresh protein solution under the same conditions. It can be concluded that no damage occurred to the binding sites of con A during its backward transfer with isopropyl alcohol.

A more useful approach for backward transfer will involve dissociating protein from cosurfactant headgroups, as is commonly done in the elution step of affinity chromatography. Elution of a bound substance from con A-Sepharose is usually achieved with a gradient of methyl-α-D-mannoside or methyl-α-D-glucoside (22). Many substances desorb at 0.1 to 0.2 $mol \cdot dm^{-3}$ sugar concentration. Desorption occurs as glucose or mannose competes for the carbohydrate binding sites on con A. The same strategy was tested for releasing con A from affinity reversed micelles by adding glucose. However, the results in Fig. 8 do not agree with what was expected. The addition of 1 $mol \cdot dm^{-3}$ glucose had no additional effect beyond that of 1 $mol \cdot dm^{-3}$ KCl for releasing con A. Previously, glucose was shown to inhibit con A transfer (Fig. 7) by occupying the binding sites on con A and preventing cosurfactant binding. However, for con A–cosurfactant complexes already residing in reversed micelles, it seems that even a high concentration of glucose cannot displace con A from the cosurfactant and subsequently lead to protein release. The rate of releasing con A from the affinity reversed micellar phase was then studied. The amount of protein released was measured as a function of time, as shown in Fig. 10. The results indicate that protein release began immediately after contact with the glucose-containing aqueous solution, but it needs 60 minutes to reach the final equilibrium value (62%). A similar time dependence and a final equilibrium value of 64% were observed using 1 $mol \cdot dm^{-3}$ methyl-α-D-glucoside. No increase of the release of con A was observed over the same time period if no glucose was added. We may conclude from the observations that the rate of dissociation of con A from the cosurfactant is slow, hence the backward transfer of protein is controlled by the kinetics of con A release from the protein–cosurfactant complex.

Based on the above discussion, it appears that the addition of 12.5% isopropyl alcohol to an aqueous stripping solution with 1 M KCl is the best method for recovering con A in the affinity reversed micellar system.

Reusability of Affinity Reversed Micelles

An important economic factor to be considered in using the affinity reversed micellar system for con A purification is the reusability of the

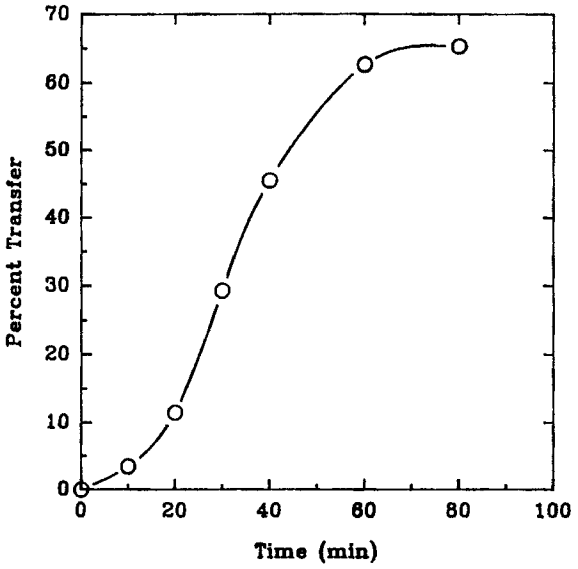

FIG. 10 Recovery of protein from the reversed micellar phase during backward transfer of con A as a function of time. Backward transfers were carried out with an aqueous phase containing 1 mol·dm ^{3} KCl and 1 mol·dm^{-3} glucose at pH 7.77.

reversed micellar phase. The reversed micellar phase can be reused for further extraction if isopropyl alcohol addition does not destroy the functional integrity of AOT reversed micelles. This was proved experimentally by carrying out two protein transfer studies with the same micellar phase containing 50 mmol·dm^{-3} AOT and 2 mmol·dm^{-3} octyl-glucoside. The micellar phase from the first backward transfer was contacted with a new aqueous solution of con A at pH 5.26, after which the micellar phase was extracted as before with an aqueous phase containing 12.5% isopropyl alcohol to complete the second transfer. The percent transfers of protein for the second forward and backward transfers are 90.3 and 87.1%, respectively, comparable to those observed during the first transfer. The slight decrease during the second forward transfer might result from the loss of AOT at a high KCl concentration during backward transfer (23).

The above experiments indicate that a spent micellar phase can maintain its protein extraction capability at pH 5.3, where the electrostatic attraction force from anionic AOT headgroups is the main transfer mechanism. To prove the reusability of the cosurfactant, the second forward transfer was conducted at pH 7.77, where protein transfer depends mainly on affinity effects. A very low transfer efficiency (2.5%) was observed at this

high pH, indicating that cosurfactant molecules might be lost from the micellar phase after backward transfer and that retaining the cosurfactant with reversed micelles presents a major problem. Using 1 mol·dm^{-3} glucose or methyl-α-glucoside for backward transfer would still lead to the same problem, where transfer efficiencies during the second forward transfer are 15 and 16%, respectively.

Several factors may be responsible for the loss of cosurfactant, but the major one may be partitioning of cosurfactant to the aqueous phase, since the partition coefficient of octyl-glucoside is low. Octyl-glucosides of 27.6 and 1.5% were found in the aqueous phase after the forward and backward transfers, respectively. Therefore, a more suitable candidate of cosurfactant should be one with a high partition coefficient. Also, the presence of high salt and isopropyl alcohol concentrations in the water pools of a spent micellar phase may also prevent protein transfer.

To solve those problems, experiments were carried out with 2 mmol·dm^{-3} dodecyl-maltoside which is almost insoluble in water (K_L = 67). Cosurfactants of 4.1 and 1.5% were found in the aqueous phase after the forward and backward transfers, respectively. The spent micellar phase was treated by equilibrating it with a fresh aqueous solution without protein (pH 7.77) before it was reused to reduce the salt and isopropyl alcohol concentrations. The second forward transfer with this micellar phase at pH 7.77 resulted in 40% transfer efficiency, which is much higher than those observed previously but still below the value (68.4%) of a corresponding new micellar phase.

The recovery of hydrophilic solutes from AOT reversed micelles was shown to be an interfacial process governed by the coalescence of the reversed micelles with the macroscopic interface (24). The reversed micellar AOT shell has to merge with the AOT monolayer of the macroscopic interface by a surfactant layer fusion step. The cosurfactant can remain in the interface and be lost during this step, which will not be detected by measuring the cosurfactant concentration in the aqueous solution alone. This argument is supported by comparing the values of cosurfactant loss to the aqueous phase during backward transfer (both 1.5%) with those during forward transfer (27.6 and 4.1%) for the two cosurfactants. Therefore, the spontaneous loss of dodecyl-maltoside to the phase interface during backward transfer, which results in a lower protein partition coefficient, sets a limit on the reusability of the affinity reversed micellar system.

One way to circumvent this difficulty and to recycle AOT and the cosurfactant may be by using a spent micellar solution with a new micellar solution for extraction. Protein extraction has been carried out in this way with a micellar phase containing an increasing volume fraction of the new

solution. The percent transfer of con A slowly increases with the volume fraction of the new solution (data not shown), and could approach 66% when three-fourths of the total volume is the new solution, which represents 97% of the original extraction efficiency.

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REFERENCES

1. J. P. Chen, *J. Ferment. Bioeng.*, *70*, 199 (1990).
2. M.-R. Kula, K. H. Kroner, and H. Hustedt, *Adv. Biochem. Eng.*, *24*, 73 (1982).
3. K. E. Goklen and T. A. Hatton, *Biotechnol. Prog.*, *1*, 69 (1985).
4. M. Dekker, K. Van't Riet, and S. R. Weiers, *Chem. Eng. J.*, *33B*, 27 (1986).
5. R. S. Rahaman, J. Y. Chee, J. M. S. Cabral, and T. A. Hatton, *Biotechnol. Prog.*, *4*, 218 (1988).
6. J. M. Woll, T. A. Hatton, and M. L. Yarmush, *Ibid.*, *5*, 57 (1989).
7. R. W. Coughlin and J. B. Baclaki, *Ibid.*, *6*, 307 (1990).
8. N. Sharon and H. Lis, *Science*, *177*, 949 (1972).
9. P. Franchimont, J. C. Hendrick, and A. M. Reuter, in *Principles of Competitive Protein-Binding Assays* (W. D. Odell and P. Franchimont, Eds.), Wiley, New York, 1983, p. 33.
10. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Roberts, and F. Smith, *Anal. Chem.*, *28*, 350 (1956).
11. A. Salahuddin, R. Begum, and B. K. Averill, *Biochem. J.*, *220*, 639 (1984).
12. L. Bhattacharyya and C. F. Brewer, *J. Chromatogr.*, *502*, 131 (1990).
13. S. D. Flanagan and S. H. Barondes, *J. Biol. Chem.*, *250*, 1484 (1975).
14. A. Cordes, J. Flossdorf, and M.-R. Kula, *Biotechnol. Bioeng.*, *30*, 514 (1987).
15. L. L. So and I. J. Goldstein, *Biochim. Biophys. Acta*, *165*, 398 (1968).
16. L. L. So and I. J. Goldstein, *J. Immunol.*, *99*, 158 (1967).
17. G. Marcozzi, N. Correa, P. L. Luisi, and M. Caselli, *Biotechnol. Bioeng.*, *38*, 1239 (1991).
18. J. M. Woll, A. S. Dillon, R. S. Rahaman, and T. A. Hatton, in *Protein Purification: Micro to Macro* (R. Burgess, Ed.), Liss, New York, 1987, p. 117.
19. M. R. Aries-Barros and J. M. S. Cabral, *Biotechnol. Bioeng.*, *38*, 1302 (1991).
20. M. Dekker, K. Van't Riet, J. J. Vander Pol, J. W. A. Baltussen, R. Hilhorst, and B. H. Bijsterbosch, *Chem. Eng. J.*, *46B*, 69 (1991).
21. A. Carlson and R. Nagarajan, *Biotechnol. Prog.*, *8*, 85 (1992).
22. C. Sutton, in *Protein Purification Methods—A Practical Approach* (E. L. V. Harris and S. Angal, Eds.), IRL Press, Oxford, 1989, p. 268.
23. R. Aveyard, B. P. Binks, S. Clark, and J. Mead, *J. Chem. Soc., Faraday Trans. I*, *82*, 125 (1986).
24. T. E. Bausch, P. K. Plucinski, and W. Nitsch, *J. Colloid Interface Sci.*, *150*, 226 (1992).

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