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Hamid R. Rabie; Tamim Suyyagh; Juan H. Vera

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Reverse Micellar Extraction of Proteins Using Dioctyldimethyl Ammonium Chloride

HAMID R. RABIE, TAMIM SUYYAGH, and JUAN H. VERA*
DEPARTMENT OF CHEMICAL ENGINEERING
McGILL UNIVERSITY
MONTREAL, QUEBEC H3A 2A7, CANADA

ABSTRACT

The effects of pH, ionic strength, co-ion and counterion of the surfactant, alcohol concentration, and nature of the solvent on the reverse micellar extraction of albumin, α-chymotrypsin, and lysozyme, using dioctyldimethyl ammonium chloride (DODMAC), were determined. The nature of the solvent or the concentration of the alcohol had no significant effect on extraction. Extraction increased with pH and decreased with salt concentration. A broader range of pH at which proteins can be extracted was obtained with DODMAC compared with bis-2-(ethylhexyl) sulfosuccinate (AOT) and trioctylmethyl ammonium chloride (TOMAC). Negatively charged proteins are extracted from the aqueous phase by exchanging with the Cl^- counterion of DODMAC at the reverse micellar interface. The presence of counterions different from chloride in the system, which are introduced through the addition of a salt, has a significant effect on extraction. The highest extraction was obtained in the presence of F^- and the lowest in the presence of Br^-. The added counterions exchange with the chloride of the surfactant at the reverse micellar interface, thereby changing the nature of the surfactant. In turn, this change in the nature of the surfactant alters the extraction of negatively charged proteins.

INTRODUCTION

The separation of many proteins on a large scale is still performed by such batch-type processes as column chromatography, salt or solvent precipitation, and electrophoresis. There is a clear need for efficient, scalable bioseparation processes that can be operated on a continuous basis. Liq-

* To whom correspondence should be addressed.

uid–liquid extraction technology has been recognized as potentially useful for this purpose (1, 2). Two classes of two-phase extraction systems are suitable for protein recovery: 1) biphasic aqueous polymer systems and 2) systems in which an organic, reverse micellar solution is in equilibrium with a conjugated aqueous phase.

There are three experimental methods available to solubilize proteins in a reverse micellar phase: 1) the contact method, in which an aqueous protein solution is equilibrated with an organic reverse micellar solution; 2) the titration method, in which an aqueous protein solution is titrated into a surfactant–oil mixture; and 3) the solid–liquid extraction method, in which dry protein is added to an already prepared organic phase containing reverse micelles. Methods 1) and 3) are the only methods which can be used for purification and extraction purposes. Once proteins are extracted to the reverse micellar phase, they can be backextracted by contacting the organic phase, containing the proteins, with a fresh aqueous phase at a proper pH or at high ionic strength. Proteins extracted into the reverse micelles are not denatured (3). In the case of enzymes, they are sometimes found to be even more stable and more active in the reverse micelles than in the aqueous buffer (3). The present study deals only with the extraction of proteins using the contact method.

There are several experimental studies on the extraction of proteins with reverse micelles. Reverse micelles have been employed for the extraction and purification of several proteins, including amylases (4), proteases (5), lipases (6), and food proteins (7). Proteins have shown considerable response to complex feed mixtures, including whole and distributed cells (8), fermentation broth (9), and dried solids (10).

Based on experimental results, various groups have addressed the mechanism by which proteins are transferred from an aqueous phase to the organic phase via the use of ionic surfactants. They hypothesized that the main factors affecting the partitioning of proteins are electrostatic interactions and size exclusion phenomena (7, 11, 12). Size exclusion happens when the size of reverse micelles is reduced (for example, at higher ionic strength) and consequently the protein molecules of larger size are excluded.

These hypotheses are mainly based on two experimental evidences: 1) positively charged proteins are preferentially extracted into anionic surfactant systems, whereas negatively charged proteins are preferentially extracted into cationic surfactant systems; and 2) extraction decreases with ionic strength. The addition of salt, and consequently the increase of ionic strength, has been proposed to have two effects: 1) electrostatic interaction between charged protein molecules and charged surfactant head groups are decreased due to Debye screening, thus reducing extrac-

tion; and 2) due to shrinkage of the reverse micelles with the addition of salt, proteins are excluded from the reverse micelles (7, 13).

There are some inconsistencies in the above hypotheses, and they indicate that the precise relations among different parameters affecting extraction are not yet clear. For example, it was found that size exclusion is more pronounced with sodium than with potassium or cesium in a bis-2-(ethylhexyl) sulfosuccinate (AOT) reverse micellar system (14). It is known, however, that AOT reverse micelles are significantly larger in the presence of sodium than in the presence of potassium or cesium (15).

Some authors have recently demonstrated that the extraction of a protein molecule into an organic phase containing ionic surfactants is due to direct interaction between the protein and individual surfactant molecules. Carlson and Nagarajan (16) speculated that the first step in extraction of a protein into the organic phase is the formation of a transferable complex between protein and surfactant molecules. Adachi and Harada (17) showed that extraction of cytochrome c by the contact method involves complexation between AOT and cytochrome c. Matsuura et al. (18) showed that insulin could be solubilized in 1-octanol by complexing the protein with the ionic surfactant SDS (sodium dodecyl sulfate). These studies provide further evidence that the mechanism of protein extraction into a reverse micellar organic phase is a complex phenomenon that has yet to be resolved.

Much of the previous work on protein extraction has focused on the use of AOT and trioctylmethyl ammonium chloride (TOMAC) (19), and little attention has been paid to develop new reverse micellar systems for this emerging technology. In addition, there are some experimentally proven limitations for these two surfactant systems. Each protein shows a characteristic pH range where solubilization occurs. This range is actually quite narrow (about 1–2 pH units) for most proteins in the case of AOT and also of TOMAC (13).

In the present study a new reverse micellar system using dioctyldimethyl ammonium chloride (DODMAC) is employed to extract proteins. The mechanism by which proteins are extracted into the reverse micelles is discussed.

MATERIALS AND METHODS

The commercial surfactant Bardac LF-80 was obtained from Lonza Inc. (Fair Lawn, NJ). This surfactant contains 80 wt% DODMAC in an ethanol-water solution. It was concentrated by vacuum evaporation at 40 mmHg for 10 hours. The purity of the concentrated surfactant was at least 99 wt% as determined by sodium lauryl sulfate titration with a Model 93-

42 Orion surfactant electrode (Orion Research Inc., Cambridge, MA). Reagent-grade isooctane, octane, nonane, decane, and decanol from Fisher Scientific (Montreal, QC), and Karl Fischer solvent from BDH Inc. (Toronto, ON) were used. Albumin (chicken egg), α-chymotrypsin (bovine pancreas), and lysozyme (chicken egg white) were obtained from Sigma (Saint Louis, MO) and used as received. The molecular weights (MW) and the isoelectric points (pI) of these proteins are: albumin (44,000/4.5), α-chymotrypsin (25,000/8.6), and lysozyme (14,300/11.0), where the first number in parentheses is the MW and the second one is the pI of the protein. All other chemicals were received from A&C American Chemicals Ltd. (Montreal, QC). Deionized water with an electrical conductivity lower than 0.8 μS/cm was used for all experiments.

The experimental procedure is shown schematically in Fig. 1. The initial organic phase was prepared by adding purified surfactant to decanol to obtain the desired molar ratio of decanol to DODMAC (2.5/1 in most experiments). Organic solvent was then added to make up the required volume. An aqueous electrolyte solution containing protein and salt was then contacted with the organic solution. The pH of the initial aqueous phase was adjusted by adding HCl or NaOH. The volume ratio of the two phases was set at unity. The phases were vigorously shaken for 2 hours at 23°C and then left to stand for 1 week at the same temperature. The phases were then separated for analysis. The settling time used here ensured equilibrium. Some samples were analyzed after 3 or 4 weeks, and the results of the analysis were unchanged.

The water content in the organic phase was measured by a Karl Fischer titrator Model 701 (Metrohm Ltd., Herisau, Switzerland). The pH of the aqueous phase was measured by a Model 691 pH meter (Metrohm, Ltd.). The concentrations of proteins in the aqueous phase were measured by a Cary 1/3 UV spectrophotometer (Varian Techtron Pty. Ltd., Victoria, Australia) at 280 nm (A_{280}). The concentration of protein solubilized in the organic phase was determined from analysis of the bulk aqueous phase.

For some samples the concentration of protein in the organic phase was also measured with the same method to ensure no loss of the protein. When there was only two clear phases at equilibrium, a Winsor-type-II system, the protein mass balance closed within 2–6%. To ensure that the extinction coefficient did not vary significantly for proteins in water versus proteins in the reverse micellar solution, the absorptions of several reverse micellar solutions prepared by titration of concentrated protein aqueous solution into 0.1 M DODMAC and 0.25 M decanol in isooctane were measured. For overall protein concentrations ranging from 0.1 to 0.6 g/L, values of the extinction coefficient calculated were only a few percentage points (up to 3%) different from those observed in the aqueous phase.

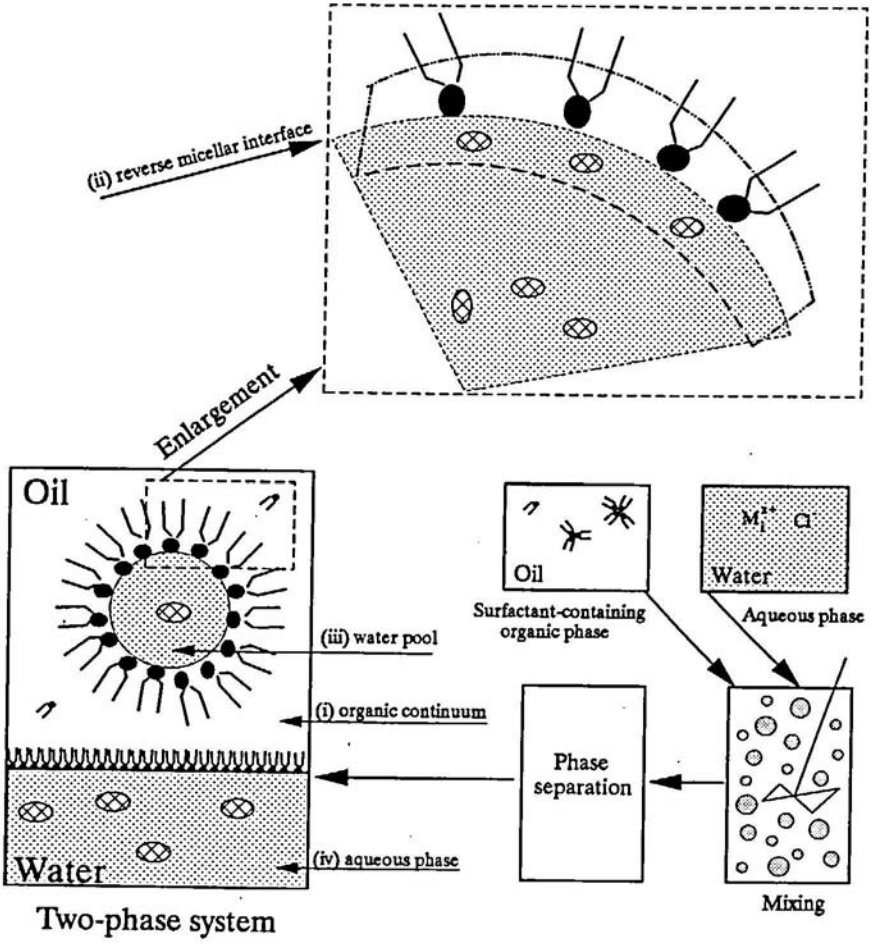

FIG. 1 Diagram of the experimental procedure and the solubilization sites, (solid dot with double legs) DODMAC surfactant, (cross-hatched oval) solute.

Göklen (20) made similar observations with cytochrome c in an AOT reverse micellar system.

At the stage when the two phases were separated for analysis, the separated aqueous phase for some samples was turbid to some extent even though the two phases were clear before phase separation. However, when this aqueous phase was left to stand undisturbed for sufficient time,

it became clear again. This is thought to be mainly due to the presence of small amounts of organic material left in the separated aqueous phase. When this phase was taken for UV analysis, it became turbid due to remixing during the analysis. The other reason for turbidity can be the presence of surfactant in the aqueous phase. As discussed by Rabie et al. (21), about 3–5% of the DODMAC surfactant remains in the aqueous phase at equilibrium. The solution turbidity creates a problem with the A_{280} method for protein analysis. This problem, however, was resolved as explained below.

A differentiation is made between absorbance, which is due to the absorption of a photon at a given wavelength by a given molecule, and turbidity, which is a general phenomena due to the scattering of light in the visible range of wavelength by particles suspended in a medium of different refractive index [for general discussions of light scattering see, for example, Huglin (22) and Hiemenz (23)]. It was observed that the proteins studied here had negligible absorption at 310 nm, but that the turbidity measured with the spectrophotometer at this wavelength closely approximated the turbidity measured at 280 nm. Thus, during later experiments the transmitted intensities at both wavelengths were measured, and the value at 310 nm was subtracted from the value at 280 nm to reduce the error, due to turbidity, involved in the calculation of protein concentration. The validity of this modification was proven in practice by comparing the measured protein concentrations in synthesized protein solutions, one clear and one turbid, in the presence of organic materials.

For error analysis of each type of measurement, three replicates were performed for 15 different experimental conditions. The percentage of coefficient of variation, CV (%), for each type of measurement was obtained from the replicates for each experimental condition from the following equation (24):

$$\text{CV (\%)} = 100 \times \text{Standard Deviation/Mean} \qquad (1)$$

For water uptake the mean of the CV (%) values is 0.95 with the CV (%) values ranging from 0.36 to 2.66. Similar figures for protein concentration in the aqueous phase and in the organic phase are: (2.87, 0.50–3.47) and (3.64, 1.20–4.77), respectively.

SOLUBILIZATION REGIONS

There are essentially four distinct regions in reverse micellar systems where a solute can be solubilized. They are: (i) the organic continuum, (ii) the interface of the surfactant hydrophilic groups and the water pool in the reverse micelles, (iii) the water pool inside the reverse micelles,

and (iv) the excess aqueous phase. These regions are shown in Fig. 1. Salts and proteins are essentially insoluble in organic solvents. Therefore, only the latter three regions are considered in this study.

The reverse micellar interface is where the heads of surfactant molecules are placed shielding the water pool from contact with the organic solvent. The interface is considered here to be a uniform solubilization environment. As shown by Rabie and Vera (26–28), due to a strong electrostatic effect of the surfactant head groups, the interface is chemically active. Different solutes are able to react with the surfactant head groups and form different complexes. Thus, the nature of the solute at the interface is different from that in the water pool, where the solute is more likely to be in a state similar to that in the excess aqueous phase. The concentrations of different solutes in the water pool can be assumed to be the same as those in the excess aqueous phase (27–29).

RESULTS AND DISCUSSION

Unless specified, all the data reported here were obtained in Winsor-type-II systems. The data are reported as the overall percent extraction of protein to the organic phase, which is calculated from

$$\xi_P = 100 \times (N_P^0 - N_P)/N_P^0 \quad (2)$$

where N_P^0 is the initial number of moles of protein in the aqueous phase and N_P is the equilibrium number of moles of protein in the aqueous phase.

Effect of pH

Figure 2 shows the extraction of albumin, α-chymotrypsin, and lysozyme as a function of equilibrium pH. The dashed lines indicate the approximate isoelectric points of these proteins. Over a wide range of pH above the isoelectric point of the protein, almost 100% extraction was performed for α-chymotrypsin and lysozyme, whereas for albumin this figure was about 90%. The lower extraction of albumin can be attributed to the size exclusion effect. However, we emphasize in this work that size exclusion is not the mechanism responsible for the lower extraction of albumin but that this is due to the ion-exchange phenomenon as explained in the next section. Above the isoelectric point, where the protein is negatively charged, a reaction between the protein molecules and the positively charged surfactant head groups occurs. This favorable reaction can be obtained with anionic surfactants at pH values lower than the pI.

Previous work on TOMAC and AOT showed (13) that the maximum extraction of different proteins for TOMAC was 40–70% and about

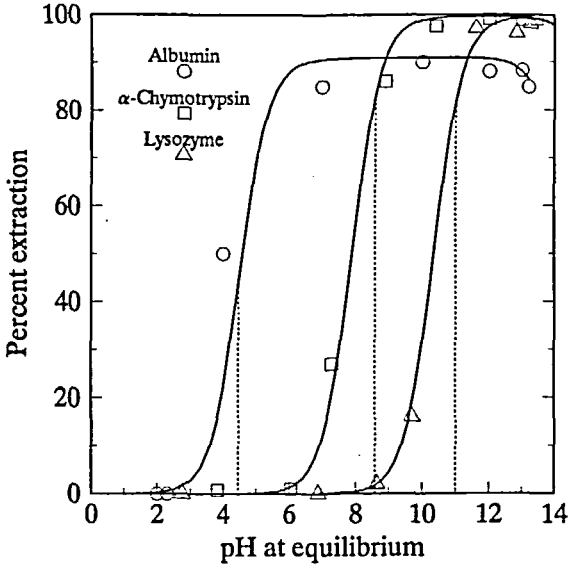

FIG. 2 Effect of pH on the extraction of proteins: initial organic phase, 100 mM DODMAC, 250 mM decanol in isooctane; initial aqueous phase, 0.5 g/L protein, 100 mM NaCl.

80–100% for AOT, both in a narrow pH range. The dramatic drop in extraction of proteins at favorable pH values, which is a characteristic of AOT and TOMAC, was not observed with DODMAC. This dramatic drop was found (13, 20, 25) to be due to the formation of a surfactant–protein complex which usually precipitates at the interface between the aqueous phase and the organic phase. No precipitation was observed for the results shown in Fig. 2.

Figure 3 presents the water uptake results for the data presented in Fig. 2. This figure also includes water uptake data for a system without protein and under the same conditions used in Fig. 2. As can be seen, the presence of protein did not have any significant effect on water uptake. This can be due to the fact that the concentrations of protein used in these experiments were rather small. At pH values greater than 12, water uptake increased with an increase of pH and then decreased at higher pH values. The appearance of a maximum in water uptake with the addition of salt, which is the result of competition between the ionic strength contribution and the counterion contribution, has been discussed elsewhere by Rabie et al. (21) and Rabie and Vera (30).

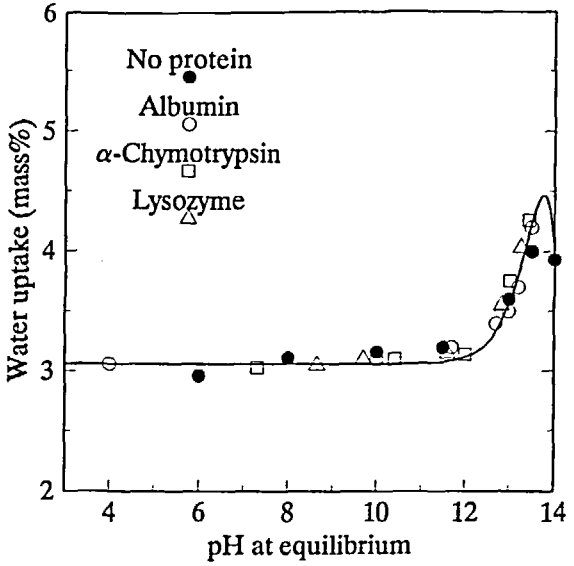

FIG. 3 Effect of pH on water uptake with and without the presence of proteins: initial organic phase, 100 mM DODMAC, 250 mM decanol in isooctane; initial aqueous phase, 0.5 g/L protein, 100 mM NaCl.

An interesting aspect of the results shown in Fig. 3 is that the water uptake increased significantly at pH values greater than 12. Unless there is a drastic change in the nature of the microemulsions for a fixed amount of surfactant, a larger water uptake is due to larger reverse micelles, which is the case for the results presented in Fig. 3. Therefore, if solubilization for albumin in the reverse micellar phase is limited by the size exclusion phenomenon, it would be expected to have a higher extraction at the higher water uptakes obtained in Fig. 3 for pH values greater than 12. However, no increase in the extraction of albumin was observed at pH values higher than 11, as shown in Fig. 2. Therefore, size exclusion is not the mechanism responsible for lower extraction of albumin compared to the other two proteins studied.

The results of Figs. 2 and 3 can be explained by ion exchange of the negatively charged proteins and the chloride of the surfactant at the reverse micellar interface. As mentioned earlier, the concentration of any solute in the water pool of the reverse micelles can be assumed to be the same as that in the excess aqueous phase. Therefore, water uptake has

no significant effect on the extent of the ion-exchange reaction, and consequently it has no significant effect on extraction. A slight decrease in the extraction of albumin at pH values greater than 12.5 was observed. This is due to further competition of the hydroxide anion with proteins to be exchanged with the chloride of the surfactant.

However, it is very interesting to observe that the competition between hydroxide and negatively charged proteins was not significant for lysozyme and α-chymotrypsin at the pH values studied, and for albumin at pH values between the pI and 12.5. This is also in total agreement with the ion-exchange hypothesis. The charge number of proteins increases with the difference between the pH and the pI (pH − pI) (31). Proteins with a higher charge number have a greater tendency to react with the surfactant due to the stronger electrostatic effect. Similar behavior was observed for the extraction of different cations with the AOT surfactant (26). This competition of hydroxide at the higher pH values is compensated for by the greater tendency of the protein to be extracted at these pH values due to its higher negative charge.

Effects of Salt Type and Concentration

Figure 4 shows the effect of NaCl concentration on the extraction of different proteins. The extraction decreased with the addition of salt for any protein. The addition of NaCl provides more chloride which competes with the protein molecules for reaction with the surfactant head groups. Lysozyme showed the highest extraction, even at high salt concentrations, and albumin showed the lowest. As was shown in Fig. 2, albumin had the lowest extraction among the three proteins studied.

These results tend to support the size exclusion hypothesis. However, they can also be explained by ion exchange. These proteins have different structures, and therefore they have different equilibrium constants in the ion-exchange reaction. It can be postulated that a protein with a lower molecular weight has a higher tendency to react with the surfactant, and thus it has a higher equilibrium constant in the ion-exchange reaction. This argument does not necessarily ignore the size exclusion effect. Both factors, ion exchange and size exclusion, can play a major role in protein extraction. However, as discussed in the previous section, ion exchange, not size exclusion, is the main factor affecting extraction in the present study.

The effect of salt type can be studied through changes in the anion of a salt or through changes in the cation of a salt. Since the surfactant studied here is a cationic surfactant, anions are the counterions and cations are the co-ions of the surfactant. The co-ion effect is presented in Table

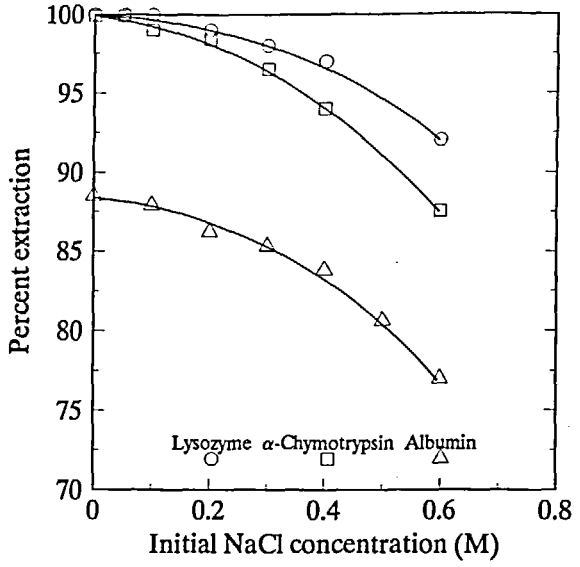

FIG. 4 Effect of NaCl concentration on the extraction of proteins: initial organic phase, 100 mM DODMAC, 250 mM decanol in isooctane; initial aqueous phase, 0.5 g/L protein, pH 13.

1. In this table the extraction results for albumin and α-chymotrypsin using the chloride salts of sodium, potassium, and cesium are presented. As shown in this table, the results for different co-ions were essentially the same. This is in total agreement with the ion-exchange hypotheses

TABLE 1
The Effect of Co-ion on the Percent Extraction of Albumin and α-Chymotrypsin: Initial Organic Phase, 125 mM DODMAC, 250 mM Decanol in Isooctane; Initial Aqueous Phase, 0.5 g/L Protein, pH 13

Salt	Albumin			α-Chymotrypsin		
(mM)	NaCl	KCl	CsCl	NaCl	KCl	CsCl
100	88.5	88.0	89.0	99.5	100	99.0
300	86.0	85.3	86.5	97.0	98.0	98.0
400	85.0	84.0	84.0	95.3	94.5	95.0
500	82.0	81.9	81.5	93.0	93.0	91.8
600	79.0	78.8	80.0	91.0	90.0	89.5

since the co-ions are not the exchangeable ions with the surfactant counterion. The co-ions were found to have no significant effect on ion distribution in reverse micellar systems (26, 27) or on water uptake of reverse micelles (21, 30). The effect of counterions on the extraction of proteins is presented in Figs. 5 and 6.

Albumin extraction data as a function of salt concentration for the sodium salts of fluoride, chloride, and bromide are presented in Fig. 5. The counterion of a salt had a remarkable effect on albumin extraction. The extraction decreased with salt concentration for any salt. The highest extraction was obtained with fluoride and the lowest was obtained with bromide. Similar results obtained for α-chymotrypsin are shown in Fig. 6.

The sharp decrease in protein extraction in the presence of bromide, shown in Figs. 5 and 6, at salt concentrations higher than 0.1 M is due to precipitation of a protein–surfactant complex at the aqueous–organic interface. Both aqueous and organic phases were analyzed under this condition. The protein concentration in the aqueous phase was almost zero, and the data shown in Figs. 5 and 6 for bromide for concentrations higher

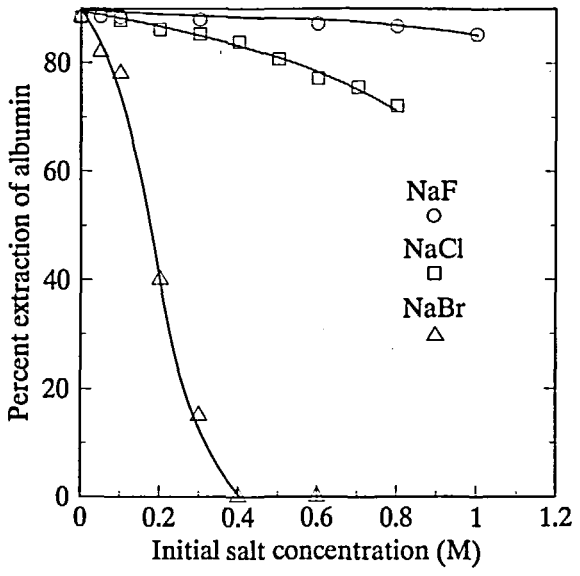

FIG. 5 Effect of counterions on the extraction of albumin: initial organic phase, 100 mM DODMAC, 250 mM decanol in isooctane; initial aqueous phase, 0.5 g/L protein, pH 13.

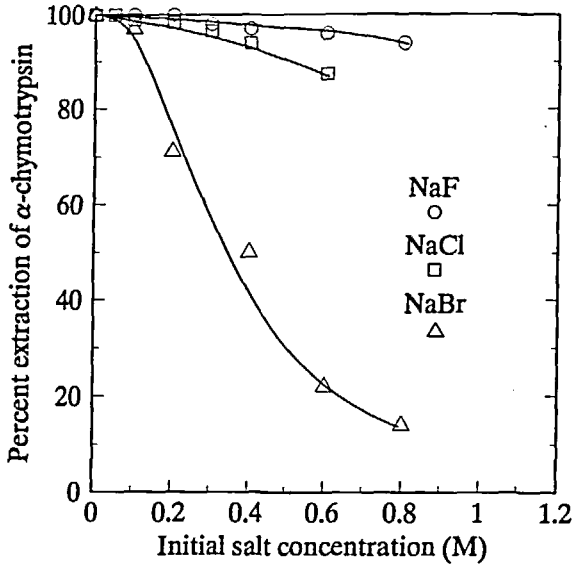

FIG. 6 Effect of counterions on the extraction of α-chymotrypsin: initial organic phase, 100 mM DODMAC, 250 mM decanol in isooctane; initial aqueous phase, 0.5 g/L protein, pH 13.

than 0.1 M are based on organic-phase analysis. Therefore, the missing protein remained at the aqueous–organic interface.

The extraction of both proteins, shown in Figs. 5 and 6, correlates with the type of anion of the salt used in the aqueous phase according to the Hofmeister or lyotropic series of anions: $F^- < Cl^- < Br^-$. Going from F^- to Br^-, the extraction decreased. These results are in agreement with the ion-exchange hypotheses.

According to Rabie et al. (21), the equilibrium constants of ion-exchange reactions between different anions and the chloride of the DODMAC surfactant also follow the lyotropic series of anions. This means that fluoride has the lowest equilibrium constant, and thus has the lowest tendency to be exchanged with the surfactant counterion, and bromide has the highest equilibrium constant, and thus the highest tendency for exchange. Therefore, the competition between fluoride and the protein for reaction with the surfactant head groups is less than that for bromide, resulting in a higher extraction in the presence of fluoride. It is very interesting to note that similar observations were made by Krei and Hustedt (32) who studied

TABLE 2
Effect of Decanol Concentration on the Percent Extraction of Albumin and α-Chymotrypsin: Initial Organic Phase, 100 mM DODMAC in Isooctane; Initial Aqueous Phase, 0.5 g/L Protein, pH 13

Decanol (mM)	Albumin	α-Chymotrypsin		
	100 mM NaCl	100 mM NaCl	400 mM NaCl	700 mM NaCl
200	86	98	91	81
250	88	99	94	78
300	86	100	95	80
350	89	100	91	78

the extraction of proteins (e.g., α-amylase) with the cationic surfactant CTAB (cetyltrimethyl ammonium bromide) in isooctane.

Effect of Alcohol Concentration and of the Nature of the Solvent

Table 2 shows the effect of decanol concentration on protein extraction for different proteins and different NaCl concentrations. The water uptake

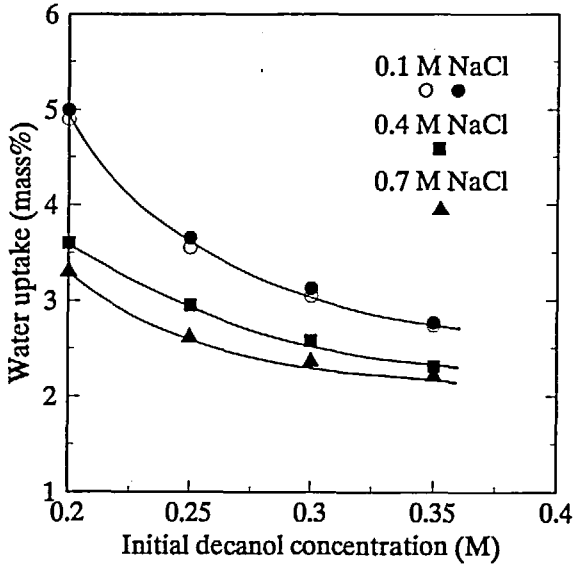

FIG. 7 Water uptake as a function of decanol concentration for different proteins and different salt concentrations: initial organic phase, 100 mM DODMAC in isooctane; initial aqueous phase, 0.5 g/L protein, pH 13. Open symbols refer to albumin and closed symbols refer to α-chymotrypsin.

TABLE 3
Effect of the Nature of the Solvent on the Percent Extraction of Albumin and α-Chymotrypsin: Initial Organic Phase, 100 mM DODMAC, 250 mM Decanol; Initial Aqueous Phase, 0.5 g/L protein, 100 mM NaCl, pH 13

	Albumin		α-Chymotrypsin	
Nature of solvent	Percent extraction	Water uptake (mass%)	Percent extraction	Water uptake (mass%)
Isooctane	88.0	3.76	97.5	3.80
Octane	89.0	3.95	99.5	3.91
Nonane	88.5	4.02	96.5	3.99
Decane	86.5	4.11	99.5	4.16

data for the results presented in Table 2 are shown in Fig. 7. Table 3 presents the experimental data of the percent extraction of albumin and α-chymotrypsin along with the water uptake data for four different solvents: isooctane, octane, nonane, and decane. Fluctuations of the protein extraction results in Tables 2 and 3, and of the water uptake in Fig. 7 for 0.1 M NaCl, are within experimental error. Therefore, neither the alcohol concentration nor the nature of the solvent had any significant effect on the extraction of the proteins studied. This agrees with the ion-exchange hypotheses.

As shown in Table 3, water uptake is higher for less penetrating solvents. Similar results were obtained for other surfactant systems (33). The water uptake also decreased with an increase in alcohol concentration. The alcohol effect, however, is strongly influenced by the method by which the reverse micelles are formed, and by the salt concentration. Detailed discussions are available elsewhere (33–37).

CONCLUSIONS

The extraction of proteins with reverse micelles of a cationic surfactant (DODMAC) was investigated. The effects of pH, protein type, salt type and concentration, alcohol concentration, and nature of solvent were studied. The extraction of proteins with DODMAC was found not to be a function of the alcohol concentration, nature of solvent, or of the cation of a salt. However, it increased with pH and it decreased with salt concentration. The extraction of proteins with DODMAC was found to be a strong function of the nature of the anion of a salt. Negatively charged proteins are extracted from an aqueous phase by exchanging the chloride counterion of DODMAC at the reverse micellar interface. The effect of

anions on extraction is due to competition between the anions and the negatively charged proteins acting as counterions of the surfactant.

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