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Liquid-Liquid Extraction of Low Molecular-Weight Proteins by Selective Solubilization in Reversed Micelles

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ABSTRACT

The effects of pH and salt concentration on the solubilization of ribonuclease-a, cytochrome-c and lysozyme in Aerosol OT/isooctane reversed micelle solutions have been studied to explore the potential for employing this solvent system in the large-scale recovery and concentration of proteins using liquid extraction. For pH values below the isoelectric point, pI, of the protein, solubilization was high, probably owing to strong electrostatic interactions between the positively charged proteins and the anionic surfactant heads forming the inner micelle wall. Above the pI, the proteins could not be solubilized, probably because of unfavorable electrostatic repulsions between the like-charged proteins and surfactants. At low ionic strength and neutral pH, complete solubilization of the proteins was observed. As the ionic strength was increased, there was an abrupt decrease in solubilizing power of the reversed micelle solutions; the salt concentration at which this occurred was different for each protein. A mixture of the three proteins was cleanly resolved using a single extraction step and two stripping steps. The conditions in each step were selected according to the results of the single protein extraction studies.

INTRODUCTION

Efficient methods for the continuous, large-scale recovery of proteins from fermentation and cell-culture media are needed

if the promises afforded by recombinant DNA and genetic engineering techniques are to be realized. One such method would appear to be liquid-liquid extraction. It is only recently that this unit operation has been considered seriously as a viable approach for bioproduct separations and purification, primarily as a result of the extensive studies of Kula and co-workers in biphasic aqueous polymer systems (1,2). Their work relies on the uneven partitioning of many biopolymers between the two predominantly aqueous phases that result when two incompatible polymers are dissolved in water. It has been demonstrated that high selectivities and product recoveries can be obtained using such systems.

The use of organic solvents for protein extractions has not been considered, however. Except in some rare instances, hydrophilic proteins are insoluble in organics or are irreversibly denatured when forced into contact with these solvents. It has been proposed (3-5) that these limitations on the use of organic solvents can be circumvented by exploiting the unique power of certain surfactants to solubilize water and hydrophilic solutes in apolar media (6-10). This occurs through the formation of reversed micelles, or surfactant aggregates, containing small polar cores of solubilized water of macromolecular dimensions. The reversed micelles are able to host proteins in an aqueous environment, effectively shielding them from the hostile organic solvent in which they are solubilized, as illustrated in Figure 1. It has been shown that these proteins can be extracted from an aqueous phase into the organic solvent, and recovered in a second aqueous phase, by manipulation of the pH and ionic strength (3-5).

Van't Riet and Dekker (3) used trioctylmethyl ammonium chloride as a surfactant to solubilize α-amylase in isooctane, and recovered the enzyme by adjusting the pH of the aqueous stripping solution. They observed about a 20% loss in enzyme activity. The effect of manipulating ionic strength to solubilize and recover cytochrome-c in isooctane using the surfactant Aerosol OT (AOT) has been discussed by Goklen and Hatton (4,5), who demonstrated that increasing salt concentration inhibited the solubilizing powers of the surfactant-laden reversed micelle solutions. It was inferred that this was a result of the decrease in size of the micelles owing to partial electrostatic screening of the surfactant head group repulsions, and thus that the protein was rejected because of a size-exclusion effect. It was also argued that direct electrostatic interactions between the charged proteins and the surfactant head groups may provide a degree of selectivity for one protein over another, although it had not been shown at that time that this would indeed be the case.

In this paper we present new data on the solubilization of low molecular weight proteins to support our earlier conjectures. It is demonstrated that these reversed micelle processes exhibit a surprising degree of selectivity among proteins of similar size, and that these proteins may be separated from one another if care

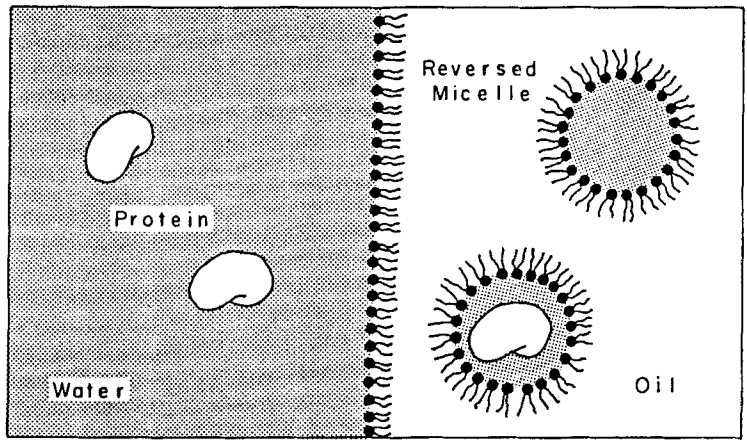

Figure 1. Protein solubilization in organic solvents by reversed micelles

is taken in the selection of the appropriate pH and ionic strength conditions for the individual extraction and stripping steps.

MATERIALS AND METHODS

In all the experiments reported here, we used the anionic surfactant sodium di-2-ethylhexyl sulfosuccinate, commonly known as Aerosol OT, or AOT. The AOT was obtained from Pfaltz and Bauer, Inc., of Stamford, CT. By the standard of other workers(11), this material was found to be relatively free of impurities, with a clean UV spectrum, and so was used without further purification. All experiments described here employed AOT in a 50mM solution in SpectrAR grade isooctane, obtained from Mallinckrodt, Inc. of Paris, KY. Salts were all reagent grade or better. Proteins were obtained from Sigma Chemical Co. of St. Louis, MO. Cytochrome-c was Sigma type III, from horse heart; lysozyme was Sigma grade I, from chicken egg white; ribonuclease-a was Sigma type II-A, from bovine pancreas.

Protein solubilization experiments were performed by contacting carefully measured amounts of aqueous and micellar solutions in a magnetically agitated beaker. Earlier results with cytochrome-c (4,5) indicated that the agitation rate and mixing time employed were more than adequate to ensure that the extent of protein transfer would be the maximum possible. In a typical experiment, 5ml of each phase was mixed in a 30ml beaker, for 5 minutes if protein was to be transferred from the aqueous to the micellar phase, or

15 minutes if the transfer was to be from the micellar to the aqueous phase. The resulting mixture was centrifuged at 2000rpm for 15 minutes, to obtain a distinct phase boundary. The phases were then separated and analyzed. The UV absorption at 280nm was measured on a Perkin-Elmer Lambda 3B spectrophotometer, as an indication of protein concentration. The percent of protein solubilized in the micellar solution was calculated from the ratio of the A_{280} in the organic phase to the A_{280} of the feed aqueous phase, both values corrected for their baseline readings. For the 1 mg/ml feed solution employed, this procedure essentially results in a calculation of the protein concentration in the organic phase, as it has been determined that the extinction coefficients for the protein in the two phases are equal. All experiments were performed at ambient temperature, 24 C±1.

Analytical separation and quantification of protein mixtures in aqueous solutions were made with a Perkin Elmer Series 4 HPLC system, with UV detection at 280nm. A Varian 30cmx4mm Protein C18 column was employed, with a gradient running from water with 0.1%TFA to 60% acetonitrile/40% water with 0.1%TFA over 15 minutes, maintaining a flowrate of 1ml/min.

RESULTS AND DISCUSSION

Effect of pH on Protein Solubilization

Solutions containing 1mg/ml of protein and 0.1M KCl were contacted with the micellar solution at various pH's. Modification of pH was obtained by direct addition of 0.1M HCl or NaOH solution to the protein solution. The pH values reported are those of the aqueous phase measured after contact with the micellar phase. Figure 2 shows the percent of protein transferred from the feed solution to the micellar solution with varying pH, for ribonuclease-a, cytochrome-c and lysozyme. At high pH values, little or no protein is solubilized. As the pH of the system is lowered below the isoelectric pH of the protein, which is given in Table 1, there is a rapid change in solubilization, such that almost 100% of the protein is solubilized at the lower pH values. For the proteins studied, this high level of solubilization was maintained over a wide range of pH values, although in the case of lysozyme and cytochrome-c, the percent solubilized declined again as pH was reduced below about 5 or 6.

The protein net charge changes from negative to positive as the pH of the system is lowered past the protein's pI. Thus, these data indicate that for the case of AOT micellar solutions, it is necessary that the proteins have a positive charge for significant solubilization to occur. AOT is an anionic surfactant, and the reversed micelle will therefore have a surface of negative charge surrounding its polar core. Thus it appears that solubilization requires an attractive electrostatic interaction between the micelle and the protein (4,5). The decrease of solubilization for cytochrome-c

TABLE 1
Protein Properties

	Molecular Weight	Isoelectric Point	Ref.
Ribonuclease-a	13,683	7.8	14,15
Cytochrome-c	12,384	10.6	16,17
Lysozyme	14,300	11.1	14,18

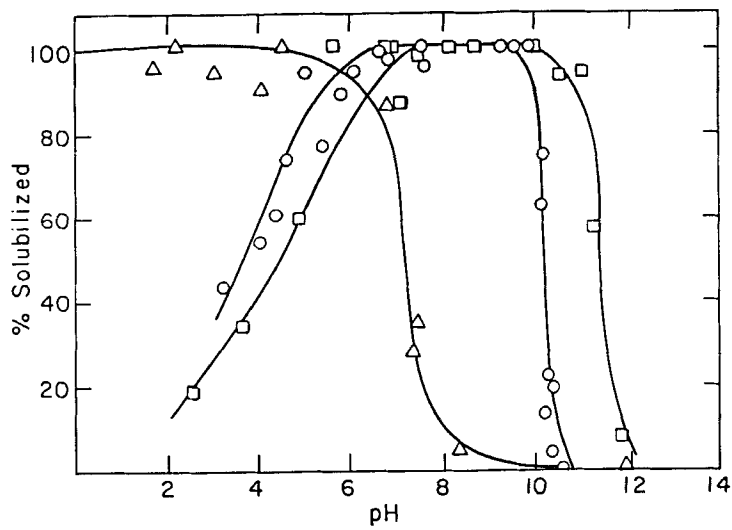

Figure 2. Effect of pH on protein solubilization.
(O) = cytochrome-c, (□) = lysozyme,
(Δ) = ribonuclease-a

and lysozyme observed at very low pH is not consistent with this hypothesis, however, and it is not yet clear why this should be so. It is possible that some pH denaturation of these proteins has occurred (12), and that the unfolded proteins are resistant to solubilization by the reversed micelles. Future studies will address this issue.

Effect of Ionic Strength on Solubilization

Solutions containing 1mg/ml of protein and varying concentrations of KCl were contacted with the micellar solution, without control of the system pH. The resulting system pH's for ribonuclease-a, cytochrome-c and lysozyme in 0.1M KCl solutions were 6.75, 7.12, and 6.74, respectively. Some pH shift can occur with varying salt concentration, but this effect was found to be relatively small; given the broad range of pH which yields high solubilization (Figure 2), this should not affect our results. Figure 3 shows the percent of protein transferred with changing salt concentration of the feed for the three proteins.

All three proteins were completely solubilized in the micellar phase at low ionic strengths(0.1M), with no solubilization at high ionic strengths(1.0M). All three also exhibited sharp transitions, in which the decrease in solubilization was pronounced over a fairly narrow range of [KCl]. These transitions occurred in the region of 0.3M KCl for cytochrome-c, 0.45M KCl for ribonuclease-a, and 0.6M KCl for lysozyme.

Since the solubilization of these proteins appears to be dependent on an attractive electrostatic interaction between the protein and the micelle inner charged double layer, an explanation of these data could be advanced based on the Debye screening ability of electrolyte solutions. The extent to which an electrostatic potential set up by a charged surface can extend into an electrolyte solution varies inversely with the ionic strength of the solution. This is characterized by the inverse Debye length, given by (13)

$$\kappa = \{8\pi e^2 N_a I/1000\varepsilon kT\}^{1/2}$$

where κ^{-1} is the Debye length in angstroms, e is the unit of electronic charge, N_a is Avagadro's number, ε is the dielectric constant of the medium, k is the Boltzmann constant, T is the absolute temperature and I is the ionic strength of the solution, equivalent to its concentration in molarity for monovalent salts. Increasing the ionic strength decreases the Debye length, decreases the range over which an electric field extends from a charged surface, and thus decreases the strength of interaction between two charged objects at a fixed separation. Increases in the ionic strength of the protein feed solution can therefore be expected to reduce the magnitude of interaction between the protein and micelle. The fact that the different proteins exhibit solubilization transitions at different salt concentrations probably reflects differences

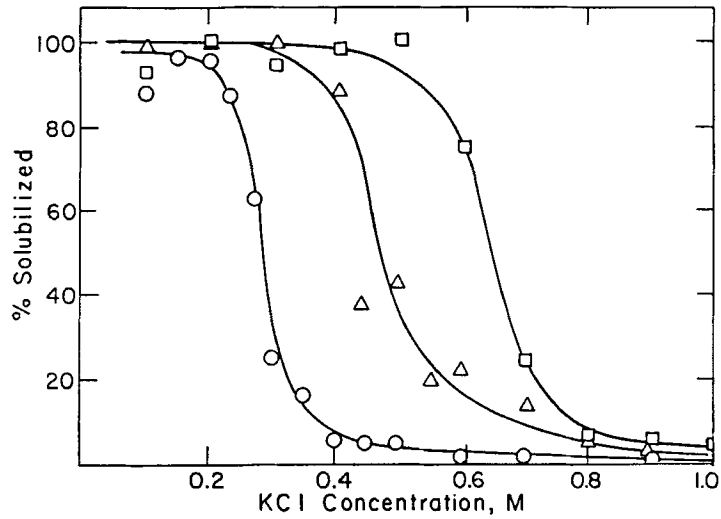

Figure 3. Effect of ionic strength on protein solubilization. (O) = cytochrome-c, (□) = lysozyme, (Δ) = ribonuclease-a

in the manner in which charge is distributed over their surfaces, or differences in their relative hydrophobicities.

Selective Separation of a Protein Mixture

The results of the preceding sections have demonstrated that proteins of similar molecular weight have different solubilization characteristics which depend on their charge-pH functionality(pI), and other, less well understood factors. We have used this knowledge of the solubilization behavior of single proteins to separate a protein mixture by selective solubilization in a micellar solution. A feed mixture was prepared containing 0.33mg/ml of each of the proteins ribonuclease-a, cytochrome-c and lysozyme. The technique employed to isolate these proteins in separate aqueous phases is shown in Figure 4. The feed solution and each resulting aqueous solution was analyzed by reverse phase chromatography; the chromatograms of the solutions are given in Figure 5.

As ribonuclease does not solubilize at pH=9, while the other proteins do, the feed was adjusted to this pH with 0.1M HCl (Figure 5a). On contacting this solution with the micellar phase, we obtained a complete solubilization of cytochrome-c and lysozyme, with almost all the ribonuclease remaining in the aqueous raffinate

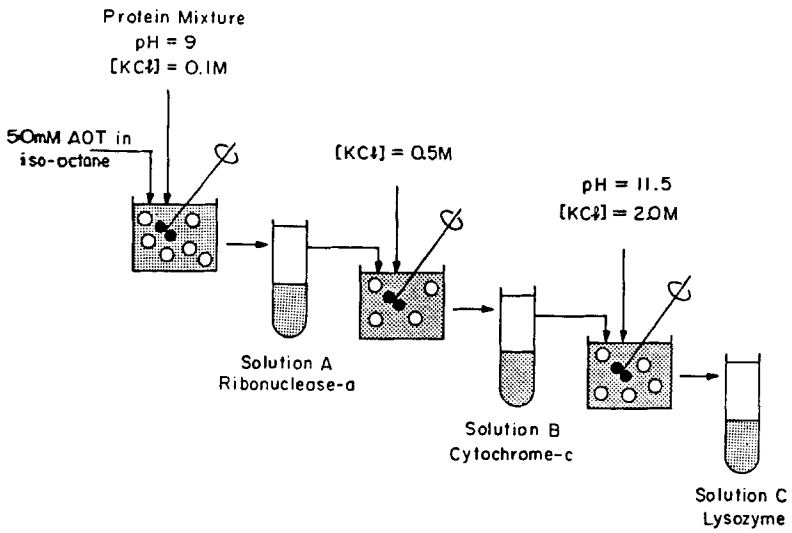

Figure 4. Experimental procedure for the separation of a ternary protein mixture

(Figure 5b). The organic phase, now loaded with cytochrome-c and lysozyme, was next contacted with 0.5M KCl to recover cytochrome-c in the aqueous phase, along with trace ribonuclease (Figure 5c). Finally, the organic phase, containing only lysozyme, was subjected to a 2.0M KCl solution, adjusted to pH=11.5. This resulted in transfer of the lysozyme to the aqueous phase (Figure 5d), completing our separation.

These results demonstrate the efficacy of resolving this protein mixture using the reversed micelle solutions by manipulating the aqueous phase pH and ionic strength in accordance with the solubilization behavior exhibited by the individual proteins in a separate series of experiments. The results are encouraging and are cause for optimism that other, more complex protein mixtures may be resolved in this way.

CONCLUSION

The controlled solubilization of proteins in organic solvents via reversed micelles appears to have application in the development of new liquid extraction processes for the efficient recovery and concentration of selected proteins from fermentation and cell culture media, as has been indicated by the experimental results presented

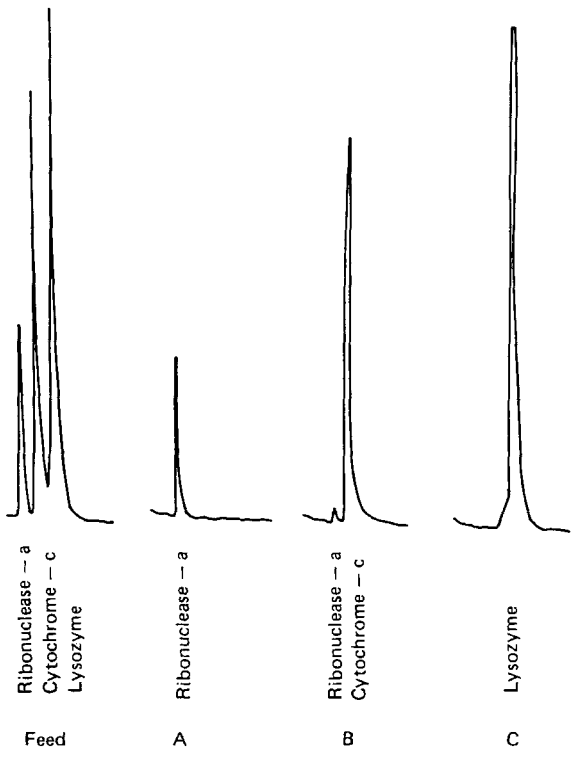

Figure 5. HPLC analyses of aqueous solutions obtained in the separation of a ternary protein mixture

in this paper. We have demonstrated that with judicious control of pH and ionic strength, we can solubilize proteins selectively in isooctane solutions using Aerosol OT reversed micelles to host the proteins. Indeed, sufficient differences exist in the responses of the three proteins examined to changes in these parameters that a mixture of the proteins could be resolved with surprising ease.

Perhaps the most important consideration in the design of new separations based on this technique would be the electrostatic interactions between the charged proteins and the ionic surfactant head layer comprising the micelle wall. Clearly, the protein charge should be opposite in polarity from that of the surfactant. This

can be controlled either by adjusting the pH to alter the protein charge, or by using cationic instead of anionic surfactants. The very marked differences in the solubilization of like-sized proteins as ionic strength is increased indicate that the relative hydrophobicities of the different proteins to be separated can also be exploited in formulating an appropriate separation scheme for protein mixtures. We are currently investigating this aspect from a more fundamental point of view, and anticipate the development of a strong theoretical framework on which to base our future experimental efforts.

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