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COMMUNICATION

Hemoglobin Extraction Using Cosurfactant-Free Nonionic Microemulsions

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ABSTRACT

Oil-continuous microemulsions provide an effective medium as a liquid membrane for extraction of proteins due to high solubilization and low miscibility in aqueous feed. Unlike the use of reverse micelles, this new method employs a preformulated cosurfactant-free nonionic microemulsion of high water content. The use of nonionic microemulsions for hemoglobin extraction demonstrates the potential for effective separation of proteins, amino acids, and other biochemicals. The extraction can be controlled by adjusting pH and microemulsion composition. It is enhanced by the addition of an appropriate liquid ion exchanger. The extraction results cannot be explained only in terms of ionic interactions.

INTRODUCTION

Biotechnology needs new and improved separation processes (1). The motivation for protein purification is to provide higher enzymatic activity or to produce a chemically pure product for use as a foodstuff or medicine. Among the many separation processes available, liquid–liquid extraction has recently gained renewed attention (2). Of particular importance is the aqueous two-phase partitioning technique (3). Two immiscible aqueous phases are generated by adding two incompatible but water-soluble polymers (or an electrolyte and a polymer) to an aqueous solution. The protein partitions between the two phases. The main advantage is the presence of water in both immiscible phases which maintains the native structural conformation of the proteins.

Hatton and coworkers (4–6) have pursued liquid–liquid extraction employing reverse micellar solutions. Previously, oil phases were avoided because they usually denature the protein. Reverse micelles can isolate the protein from the bulk oil phase as well as accommodate enough water to preserve its native conformation. Typically, the surfactants used are negatively charged. Therefore, proteins possessing a net positive charge (i.e., pH < p*I*) are effectively separated while proteins possessing a net negative charge are rejected due to electrostatic repulsion. The main advantage of the technique is the sharp separation obtained while maintaining the structural integrity of the protein.

Ayala et al. (7) solubilized pure proteins in nonionic reverse micelles in the presence of a cosurfactant. The activity of the extracted protein was dependent on the aqueous content. In contrast, Hilhorst et al. (8) reported that there was no relation between the aqueous content of the organic phase and the transfer properties of the surfactant. The extraction results were explained in terms of electrostatic interactions. The minimum concentration of an ionic surfactant (AOT) required to extract 100% protein (cytochrome c) was recently investigated (9).

Microemulsions are thermodynamically stable isotropic dispersions of two immiscible fluids, generally oil and water, containing one or more surfactants (10, 11). The phase behavior of microemulsions has been extensively studied, particularly for enhanced oil recovery (12). Lee and Biellmann (13) extracted β-hydroxysteroid dehydrogenase by treating whole cells with microemulsions. They reported that the enzyme activity was higher in nonionic microemulsions with high water content. The mechanism of amino acid extraction into microemulsion was elucidated by Adachi et al. (14). Wiencek and Qutubuddin (15–17) employed nonionic microemulsions as liquid membranes to separate organics and metal ions from aqueous solutions. The main advantage of using microemulsions as liquid membranes is their relatively high water content, which helps solubilization and maintains the structural integrity of proteins. The process involves four steps as depicted in Fig. 1 for the case of protein separation. A microemulsion is formulated in the first step by mixing oil and surfactant with an aqueous solution (e.g., NaCl solution). The saturated microemulsion is then contacted with the protein feed solution. The protein partitions into the microemulsion droplets, and separation is achieved. Next, the immiscible phases are allowed to disengage via gravity or centrifugation. Finally, the temperature is raised to separate most of the water along with the protein. The oil and surfactant mixture can be recycled to Step 1. Unlike a reverse micellar solution, the preformulated microemulsion is saturated with aqueous solution. A slight temperature

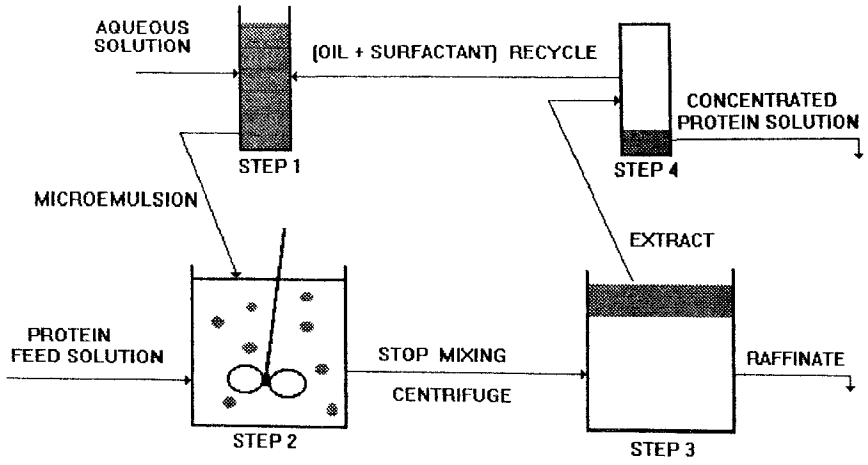

FIG. 1 A schematic of protein extraction and recovery using nonionic microemulsion.

increase may be used to strip the protein because the surfactant employed is a nonionic whose phase behavior is very sensitive to temperature. Of course, the temperature should not be excessive so as to prevent protein denaturation. It is also possible to use pH adjustment to transfer back the protein to an aqueous phase. In this communication the extraction of human hemoglobin using nonionic microemulsions is reported. Unlike previous work (7), a cosurfactant such as an alcohol is not used in formulating the microemulsion.

EXPERIMENTAL

Chemicals

The surfactant employed for this investigation is an industrial grade ethoxylated dinonyl phenol (Emery's Trycol 6985/DNP-8, Lot #Z-373-M). Tetradecane was employed as the oil phase and used as received from Humphrey Chemical Co. The water was distilled and deionized. Two ion exchangers were studied: benzoyl acetone, which was previously used as a cationic exchanger for the separation of Cu^{2+} using microemulsions (17), and Aliquat 336, which was used in amino acid separations (6). NaCl (Fisher Scientific), human hemoglobin (Sigma), benzoyl acetone (Sigma), and Aliquat 336 (Henkel) were all used as received.

Microemulsion Preparation

Three microemulsion formulations were employed as listed in Table 1. The surfactant was first dissolved in tetradecane. A small amount of liquid ion exchanger was added except for Formulation III. Microemulsions were prepared by equilibrating the oil phase with an equal volume of NaCl solution at 22°C for 1 day. The NaCl concentration was adjusted to allow significant water solubilization.

Protein Solutions

Human hemoglobin was used due to its ready availability and strong absorbance of visible light. It has a p*I* of approximately 7.0, a molecular weight of 64,500, and an absorption maximum around 360–412 nm depending on solution pH. Visible light absorption at 402 nm was used to measure hemoglobin concentration. The initial protein concentration was nominally 0.02% (w/w) in aqueous solutions. Solutions at nominal pHs of 3, 6, and 9 were prepared using potassium formate/formic acid (0.05 M), sodium chloride (0.1 M), and dibasic potassium phosphate (0.05 M), respectively.

Separation Experiments

Aliquots of protein solution (9 mL) were contacted with 1 mL of microemulsion in a test tube by gentle mixing for 10 minutes. The two immisible phases were disengaged by gravity. The phase behavior was observed under polarized light at different time intervals. The concentration of protein remaining in the bulk aqueous phase was measured by visible spectroscopy. The accuracy of the absorbance measurements (roughly ±5% of full scale) was affected by some light scattering caused by surfactant partitioning to the bulk aqueous phase. This effect was especially pronounced in microemulsion Formulation III. Therefore, the results were

TABLE 1
Microemulsion Formulations

Microemulsion identifier	Oil-phase concentration		Aqueous phase NaCl concentration (M)	Microemulsion aqueous content (% v/v)
	Surfactant (% w/w)	Ion exchanger (% w/w)		
I (Aliquat 336)	9.7	2.6	0.1	28
II (benzoyl acetone)	9.7	2.6	0.1	10
III (no ion exchanger)	10.0	—	1.0	4

corrected by subtracting a baseline absorbance from a hemoglobin-free sample. The pH of the aqueous feed solution was measured before and after extraction, and agreed to within a few tenths of a unit.

RESULTS AND DISCUSSION

The primary objective of this study was to demonstrate that proteins like hemoglobin can be extracted into a cosurfactant-free nonionic microemulsion. Figure 2 shows the effect of pH on the extent of hemoglobin separation for the three microemulsions at different pH. The maximum extraction is observed at pH 9 using Microemulsion I, which contains cationic Aliquat 336. On the basis of ionic interactions only, one would expect good extraction at high pH where the protein is negatively charged, and no extraction below the p*I* since it would display a net positive charge. Using Microemulsion I, more than 90% extraction was achieved at pH 9, and about 50% at pH 6. Microemulsion I was unstable at pH 3 and extraction was not possible. At both pH 9 and 6 less extraction was achieved with Microemulsion II containing benzoyl acetone. Some partitioning of hemoglobin is observed even in the absence of an ion exchanger (Microemulsion III). Surprisingly, Microemulsions I and III show good extraction

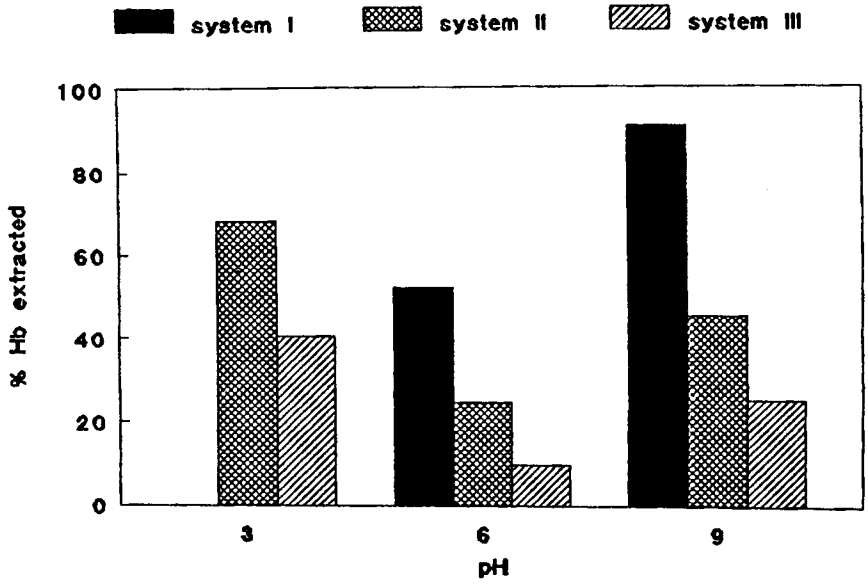

FIG. 2 Effect of pH on hemoglobin extraction for different microemulsion formulations.

at pH 3 where the protein exhibits a positive charge. The interactions of the protein with the nonionic surfactant and benzoyl acetone need further investigation. Where the electrostatic interactions are weak or absent, as for Microemulsions II and III, the water content plays a dominant role. Microemulsion II, which has a higher water content (Table 1), shows higher extraction. This is consistent with the protein partitioning to the aqueous core of the microemulsion droplets.

The ion exchanger used in the microemulsion affects the kinetics of phase separation after extraction. When 1 mL of the microemulsion was contacted with 9 mL of aqueous protein buffer, System II containing benzoyl acetone separated faster than System I containing Aliquat 336. At the start of phase separation, the bulk aqueous phase was translucent due to the presence of surfactant and oil globules. With time, the aqueous phase became clear due to the transfer of surfactant, protein, and oil to the organic phase on the top. With benzoyl acetone, the phase separation was within 5 minutes at all pH. Unlike benzoyl acetone, Aliquat 336 is surface active in addition to being an ion exchanger. pH also affects the dynamics of phase separation for Aliquat 336. At pH 9 the phase separation occurred over 2 weeks while at pH 6 it was completed in about 10 minutes. Microemulsion I lost its integrity at pH 3, and no phase separation was observed even after 3 months. These preliminary results indicate the importance of the interactions between the surfactant, the protein, and the ion exchanger not only on the extent of extraction but also on the dynamics of phase separation.

CONCLUSIONS

A preliminary investigation of the extraction of hemoglobin using co-surfactant-free nonionic microemulsions as a liquid membrane indicates the potential for effective separation of proteins. The extent of extraction depends strongly on the pH of the aqueous solution and the microemulsion composition. The type of liquid ion exchanger used to enhance the extraction affects the dynamics of phase separation. The extraction results cannot be solely explained by ionic interactions, and further study is warranted.

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