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Extraction of Lysozyme Using Reverse Micelles and Pressurized Carbon Dioxide

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Abstract: Lysozyme was forward extracted in a reverse micellar process under conditions of varying ionic strength, pH, AOT level, and temperature both with and without high-pressure CO_2. It was found that high-pressure CO_2 reduced the extraction time under all conditions tested and increased the amount of protein transferred. Reasons for this are discussed. In addition, the CO_2 stripped lysozyme from the micelles efficiently, avoiding the need for the usual back extraction for purification.

Keywords: Lysozyme, reverse micelle, pressurized CO_2, *i*-octane

INTRODUCTION

With recent developments in biotechnology requiring the production of commercial quantities, there is the need for efficient manufacturing methods that can be readily scaled up, in particular for continuously separating and concentrating proteins. Organic solvents containing reversed micelles have been shown to have great potential as novel media in bioseparations and biocatalysis (1). Reverse micellar processes can be applied to protein extraction and purification and lend themselves to continuous liquid-liquid extraction and

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so are candidates for scale-up to large-scale processing. Compared with chromatographic separations, reversed micellar extraction has advantages of simplicity in scale-up, recovery, and process design.

Reverse micelles are spherical nanometer-sized molecular hydrophilic aggregates formed in a variety of nonpolar organic solvents (2). Surfactants are used to form these micelles. The lipophilic hydrocarbon tails of the surfactant molecules face the exterior of the aggregates while the head-groups containing the counterions are aligned to the interior of the aggregates. This permits the organic solvent to carry a relatively high load of polar "solute." Water can be captured in the reversed micellar mixture forming water pools. Certain biomolecules which are hydrophilic can then be dissolved in the water pools (3).

In conventional reversed micellar processes, proteins are first extracted from an aqueous broth into an organic phase to form the reversed micelles. The two bulk phases are then parted and a back extraction of the organic with another aqueous solution recovers the purified protein.

Several papers have been published on the transport properties of proteins between the bulk aqueous and organic phases. Dekker et al. (4) investigated mass transfer rates in the extraction of α-amylase with reversed micelles using the cationic surfactant trioctylmethylammonium chloride (TOMAC) in *i*-ocatane. The interfacial transport processes of α-chymotrypsin and cytochrome C between aqueous and an AOT-*i*-octane reversed micellar phase were studied by Dungan et al. (5). The mass transfer processes in lysozyme extraction by AOT-*i*-octane reversed micelles were also studied by Lye et al. (6). All these studies operated at essentially atmospheric pressure. We were intrigued by the possibility of carrying out similar extractions under high-pressure CO_2.

Under pressure and given suitable conditions, low molecular weight hydrocarbons can form gas hydrates. This is also the case with CO_2. When pressurized with water, CO_2-gas hydrate crystals may even precipitate, although the kinetics are slow. If this hydration were to occur within micelles the amount of water retained there may be reduced, enhancing the transfer of solute (protein). Hence, under pressurized CO_2, some or all of the biomolecules extracted into a reversed micellar phase may be easily recoverable without a back extraction.

For this investigation lysozyme, obtained from domestic hen egg albumin, was used as a model protein. Lysozyme is an industrially useful enzyme; egg white, which contains 3.5% w/w lysozyme, is a convenient source (7). The anionic surfactant, sodium bis(2-ethylhexyl) sulfosuccinate (AOT), was used as it is known to form spherical nanometer-sized molecular aggregates in a variety of nonpolar solvents. The hydrocarbon tails of the AOT molecules are directed outward from the micelle while the sulfonate head-groups with the sodium counterions are localized in the interior of the aggregate. The organic solvents were expanded by pressurized CO_2 (8) to form the lipophilic phase. The purpose of this study was therefore to observe the extraction

features of lysozyme from a bulk aqueous phase into reversed micelles and thence into a bulk organic phase under a pressurized CO_2 atmosphere and to compare those results with conventional (non-CO_2) extraction.

EXPERIMENTAL

Materials

Lysozyme from albumin (E. C. 3.2.1.17 from mucopeptide N-acetyl muramyl hydrolase, molecular weight 14300 Da, isoelectric point 11.1) was purchased from Sigma Chemical Co. (Product No. L-6876), sodium bis(2-ethylhexyl) sulfosuccinate (AOT surfactant) 99% was obtained from Aldrich Co. and AR grade 2,2,4-trimethylpentane(*i*-octane) 99% purity was obtained from Junsei Chemical Reagent Co. The liquid CO_2 purity was 99%. All other reagents were AR grade from Sigma Chemical Co.

Aqueous solutions were prepared by dissolving lysozyme in distilled water and adding KCl to regulate the ionic strength. pH was adjusted within the range 3 to 12 by addition of 0.1 M HCl or NaOH. Organic solutions were prepared by dissolving the desired amounts of AOT in *i*-octane.

Procedure

For each run the vessel was charged with 200 mL of aqueous solution followed by 100 mL of organic. CO_2 was then introduced until the desired pressure was reached within the range of 3.4 to 20.4 MPa. At low pressures, CO_2 formed a third gaseous phase above the two liquids; at higher pressures, it dissolved and distributed itself between the organic and aqueous phases. Both liquid phases were stirred for 90 min at the same speed of 170 rpm and in the same direction to maintain a smooth interface. At 10-min intervals, 0.5 mL aliquots were withdrawn from the (lower) aqueous phase for analysis. Experimental conditions are shown in Table 1. Lysozyme concentration was determined by Lowry assay with UV-vis spectrophotometry.

Table 1. Experimental conditions for reversed micellar extraction under pressurized CO_2

Condition	Range
KCl concentration (M)	0.1–0.4
pH	3–12
AOT concentration (mM)	20–100
CO_2 pressure (MPa)	3.4–20.4
Temperature (°C)	25–35

The amount of lysozyme (expressed as %) transferred from the aqueous to the organic phase was defined by Eq. (1).

$$\text{Transfer } (\%) = \frac{C_{org} V_{org}}{C_{aq} V_{aq}} \times 100 \quad (1)$$

The water content, W_o, in the reverse micelles was measured by Karl-Fischer titration. The radius of the reversed micelles was determined using Eq. (2) based on that of Gölken and Hatton (9) and modified to include a correction for the volume of the surfactant shell:

$$R_s = 3(V_w \cdot W_o + V_s)/f_s \quad (2)$$

Apparatus

The extraction was performed using the high-pressure system depicted in Fig. 1. The cylindrical vessel (i.d. 60 mm, height 140 mm) contained 4 equispaced longitudinal baffles. Two co-axially mounted impellers were driven by independent magnetic drives. In all experiments, the impellers rotated in the same direction. A water jacket maintained the temperature constant in the range of 25 to 35°C. CO_2 was delivered from a cylinder and

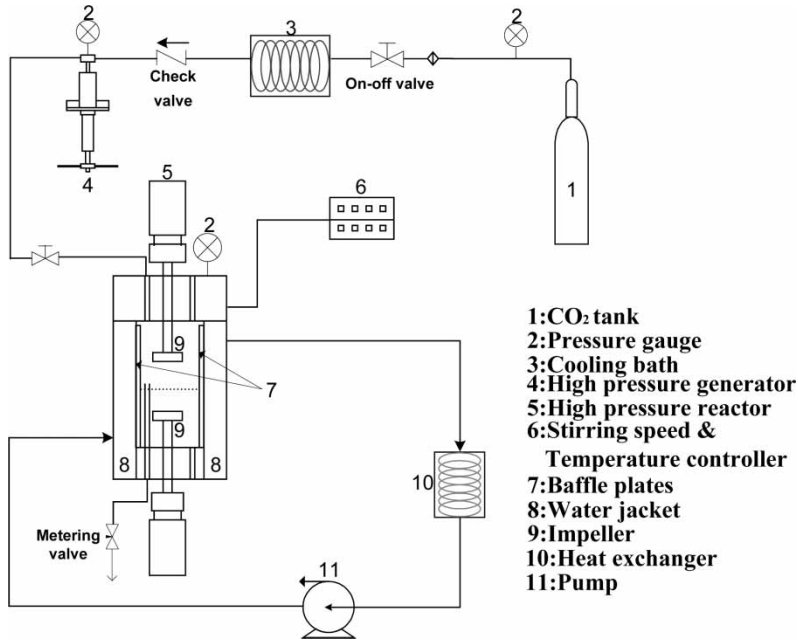

Figure 1. Schematic diagram of test apparatus.

pressurized by a high-pressure piston generator. Figure 2 shows details of the extraction vessel. The interfacial contact area between bulk phases was $2.827 \times 10^{-3}\,m^2$.

RESULTS AND DISCUSSION

As shown in Fig. 3, where the lysozyme remaining in the aqueous phase is plotted against time, lysozyme was extracted into the reverse micelles rapidly during the first 30 to 40 min; after which time the transfer rate decreased to a negligible value. The extraction rate was noticeably higher at the higher CO_2 pressure. This behavior contrasts with previous studies (10, 11) where, in the absence of CO_2, the steady increase in protein transfer persisted for over 90 min. At 3.4 MPa, 25°C, the CO_2 was gaseous and formed a third upper layer which was not well mixed with the other (organic and aqueous) layers. At 6.8 MPa and 25°C however, the CO_2 was liquid and dissolved fully and mainly into the organic layer. The higher concentraton of dissolved CO_2 enabled more intimate contact of it with the reversed micelles, which clearly influenced the forward extraction rate.

Since the kinetics of hydrate formation is relatively slow, this rate increase is thought to be due to a reduction in interfacial tension between the aqueous and organic phases from the dissolved CO_2. Chun and Wilkinson (13) have measured interfacial tension in high-pressure carbon dioxide-water-organic

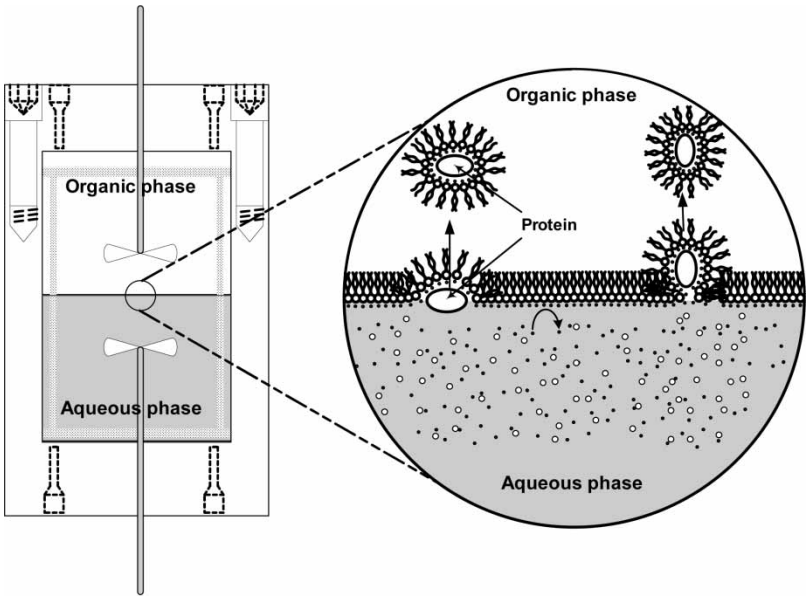

Figure 2. Vessel detail and schematic of protein transfer using reversed micelles.

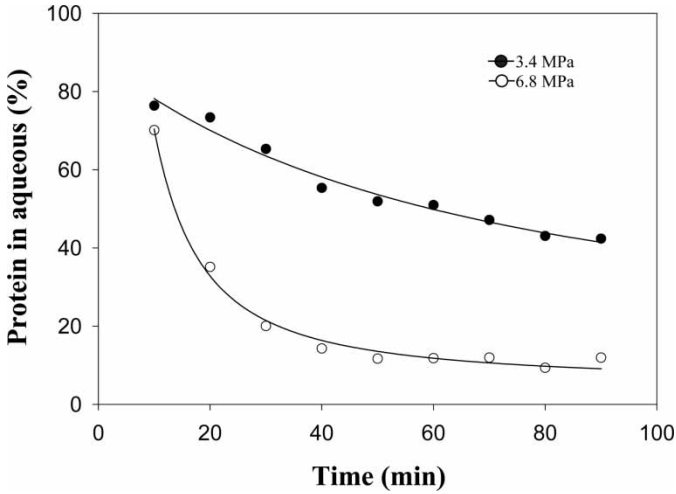

Figure 3. Time vs. protein retained in aqueous phase (%) at 3.4 and 6.8 MPa CO_2 (lysozyme 0.2 g/L, pH 7, KCl 0.1 M, AOT 20 mM, 25°C).

mixtures and reported that interfacial tension (IFT) was strongly affected by system properties, particularly near the critical point of either phase, and displayed markedly reduced IFT as the carbon dioxide rich phase approached its dew point. In supercritical fluid conditions it is also known that solutes have elevated transfer rates due to the unique properties of the dense but highly compressible solvent; properties such as high diffusivity, low viscosity, and low interfacial tension (14). The subject of mass transfer rates under CO_2 is explored further in another paper currently under preparation.

Protein solubilization into reverse micelles under conventional conditions has been shown to be governed by electrostatic interaction between the protein and the polar head group of the surfactant AOT. This electrostatic interaction is modified by the ionic strength of the aqueous solutions through Debye screening (12). Figure 4 shows the lysozyme transfer vs. different KCl concentrations and CO_2 pressures, and as expected, increased ionic strength reduced the amount transferred. However, under CO_2 the amount transferred increased significantly.

Figure 5 shows the effect of pH on protein transfer which reached a maximum near its isoelectric point and decreased at higher and lower pH. Results are interpreted in terms of electrostatic interactions between charged amino acid residues on the protein surface and the electrical double-layer created by the surfactant head group. Thus, lysozyme preferentially dissolved in the anionic reversed micelles when it possessed a net positive charge, i.e., at pH values below the protein's isoelectric point (pI). Shin et al. (15) reported that the net surface charge of lysozyme lies between +9 and +11 in the pH range between 5.8 and 4.2.

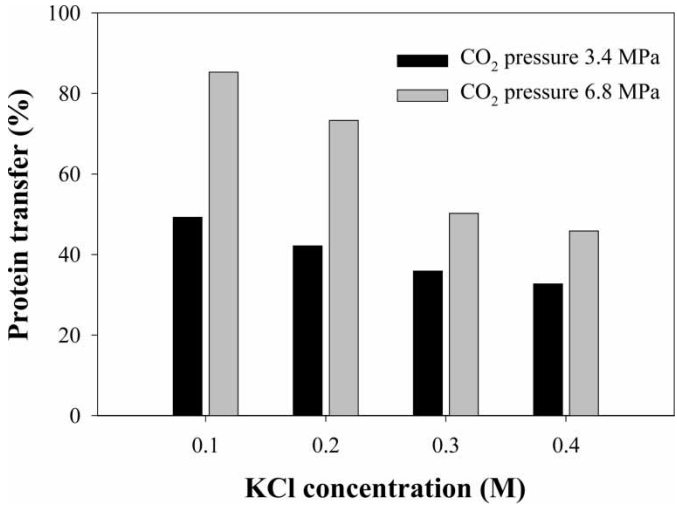

Figure 4. The amount of lysozyme transferred [Eq. (1)] into the organic phase at selected ionic strengths (lysozyme 0.2 g/L, pH 7, AOT 20 mM, 25°C).

Above the lysozyme isoelectric point, the transfer rate decreased due to repulsion between the same-charged protein and surfactant. Luisi et al. (3) has suggested that at extreme pH, competition for OH^- with perhaps denaturation and changes in ionization state also affects protein uptake.

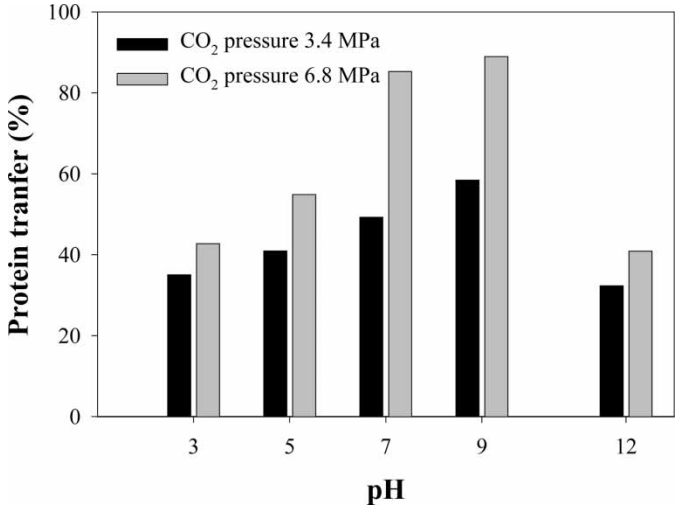

Figure 5. The amount of lysozyme transferred [Eq. (1)] vs. pH (lysozyme 0.2 g/L, KCl 0.1 M, AOT 20 mM, 25°C).

Again the influence of high-pressure CO_2 is apparent in increasing the amount of protein transferred.

Figure 6 shows the protein transferred at several surfactant concentrations under CO_2. At 3.4 MPa the higher the AOT concentration the more lysozyme was transferred. This trend is consistent with conventional non-CO_2 extraction; however the degree of increase in the presence of CO_2 was less than that normally observed. Increasing the CO_2 pressure to 6.8 MPa increased the transfer at low [AOT] more than at high. In fact at this pressure, there was little effect of surfactant concentration on protein transfer. CO_2 had the greatest influence on protein transfer at low AOT concentrations, with a much reduced improvement as [AOT] increased. In this work it was not possible to extend readings above 100 mM AOT due to protein coagulation.

When CO_2 is dissolved under pressure, it causes expansion of both orgainc and aqueous liquids. The solubility of CO_2 increases with pressure over and above the Henry's law value due, in aqueous, to ionization (e.g., $H_2O + CO_2 = H^+ + HCO_3^-$) and in the organic to nonideal solution behavior. Figure 7 demonstrates this effect clearly. At low pressure, CO_2 density is low (see Table 2) and the degree of fluid expansion is small. As pressure increased, density increased, and more lysozyme was taken up by the reversed micellar phase reaching a maximum at around 10.2 MPa. Further increase in pressure gave little further increase in CO_2 density and the transfer of protein was observed to decrease. This can be explained by competition between increased solubility due to increased CO_2 density and reduced transport properties due to increased pressure recalling that enhancement of transport properties is greatest close to the critical point region. This behavior was the case at both 0.2 and 0.4 g/L lysozyme.

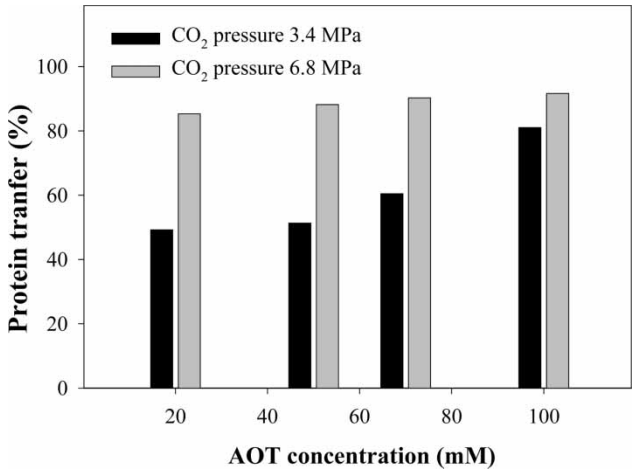

Figure 6. The amount of lysozyme transferred [Eq. (1)] at various surfactant concentrations (lysozyme 0.2 g/L, KCl 0.1 M, pH 7, 25°C).

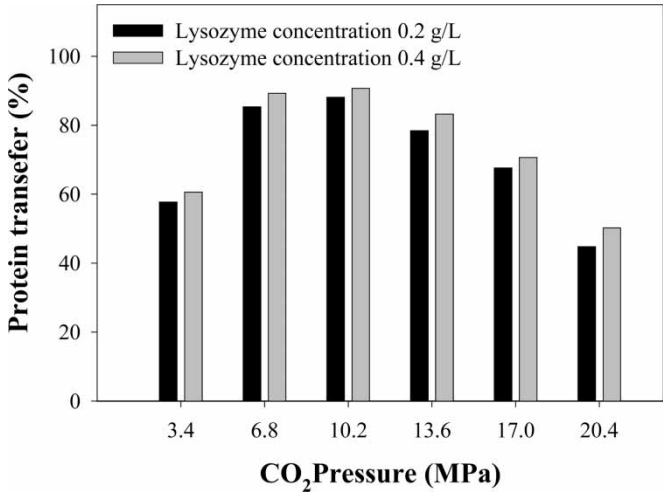

Figure 7. The amount of lysozyme transferred [Eq. (1)] to the organic phase in the presence of CO_2 at various pressures (KCl 0.1 M, pH 7, AOT 20 mM, 25°C).

Lysozyme is an enzyme whose activity is lost above 35°C. Accordingly, temperature was kept below 35°C. As shown in Fig. 8 temperature had no noticeable effect within the range 25–35°C.

The water content of the micellar organic phase is a significant parameter affecting mass transfer of protein and enzymatic activity. Figure 9 shows the water content, W_o, of the organic phase with and without pressurized CO_2 and at different ionic strengths in the aqueous phase. When CO_2 was used, W_o increased significantly, suggesting that the reversed micelles formed more readily under pressurized CO_2. This in turn resulted in an increased amount of lysozyme to be transferred from aqueous to organic phase.

Table 2. Densities of pure CO_2 at 25°C at selected pressures. Critical point of CO_2, $p_c = 73.9$ MPa, $T_c = 304.2$ K

Pressure, MPa	Density, kg/m^3
3.4	75
6.8	732
10.2	820
13.6	863
17.0	893
20.4	917

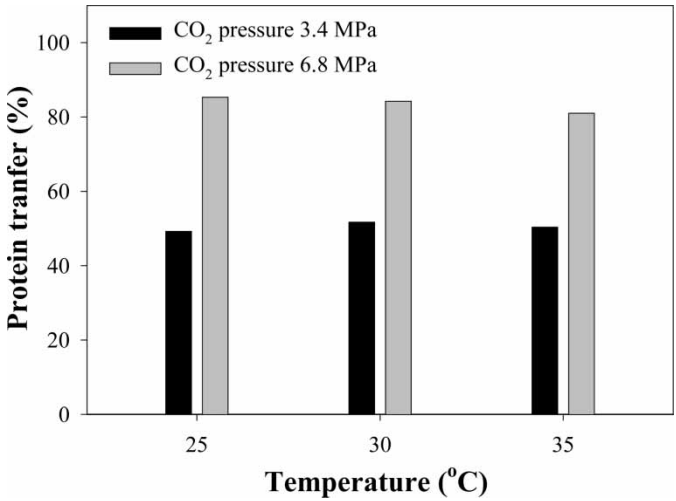

Figure 8. Lysozyme transferred [Eq. (1)] at various pressures and temperatures (lysozyme 0.2 g/L, KCl 0.1 M, pH 7, AOT 20 mM).

An interesting corollary of the faster and increased rejection of water, as exemplified in Fig. 3, is the reduced need for a back extraction for the recovery of protein. This is an attractive possibility that deserves further examination.

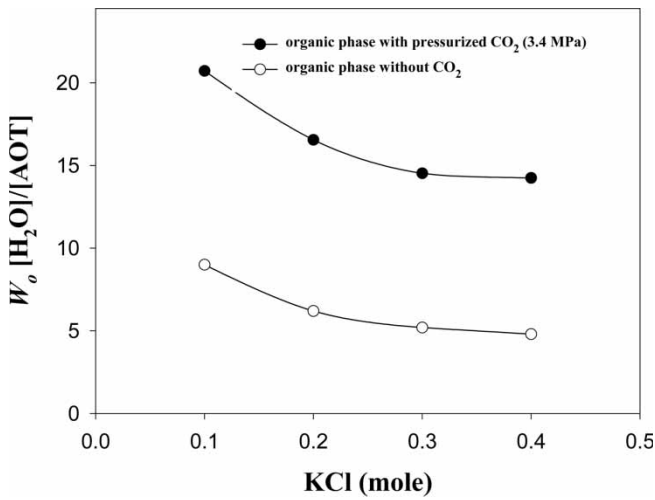

Figure 9. Comparison of W_o values in the organic phase both with and without CO_2 (lysozyme 0.2 g/L, KCl 0.1 M, pH 7, AOT 20 mM, 25°C).

CONCLUSION

The transport of lysozyme between aqueous and AOT-*i*-octane organic phases using reverse micelles at various ionic strengths, pHs, surfactant concentrations, pressures, and temperatures was examined in the presence of pressurized CO_2. In the absence of CO_2 the results agreed with previous similar studies. Under pressurized CO_2, however, lysozyme transfer was enhanced markedly with increased initial transfer rates and higher overall transfers. The increase in rate is attributed to a reduction in surface tension under high-pressure CO_2 and the increased total transfer to modification of the solubility of protein in the presence of dissolved CO_2.

The improvement in protein transfer with CO_2 was most pronounced at low [AOT] with reduced improvement as [AOT] increased. Protein transfer increased with pressure and went through a maximum at around 10.2 MPa. Competition between decreased transport properties and increased solubility are thought responsible for the optimum.

W_o increased at high surfactant concentration and high pressure, due to the increased solubilization of water in the now highly compressible organic phase.

NOMENCLATURE

C_{aq}, C_{org}	lysozyme concentrations of aqueous and organic phases, mol/L
V_{aq}, V_{org}	volumes of aqueous and organic solutions, m^3
R_s	outer radius of reversed micelle, m
V_w	molecular volume of water in the micelle, m^3
V_s	molecular volume of surfactant in the shell, m^3
W_o	molarity of water in the organic phase, mol/L
f_s	surface coverage of the surfactant head group (i.e., inner surface area of outer surfactant shell), m^2

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