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Transfer rate measurement of lysozyme by liquid-liquid extraction using reverse micelles with dense CO_2

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Abstract−Lysozyme was extracted from aqueous solution into *i*-octane using reverse micelles in the presence of pressurized CO_2. A squat vessel with two independent stirrers was used to measure the mass transfer of the lysozyme across a planar interface. Mass transfer coefficient, k_L of the lysozyme from the aqueous to the organic phase was measured at selected ionic strengths, pH, sodium bis(2-ethylhexyl) sulfosuccinate (AOT) surfactant concentrations, temperatures and pressurized CO_2. The mass transfer rate of lysozyme was higher in high temperature (318 K) and pressure (20 MPa). pH of 9 in aqueous phase showed highest mass transfer rate of lysozyme. The application of pressurized CO_2 markedly increased the mass transfer rate of lysozyme comparing to conventional non-pressurized system.

Key words: Lysozyme, Reverse Micelle, Mass Transfer, Pressurized CO_2, Sodium Bis(2-ethylhexyl) Sulfosuccinate (AOT)

INTRODUCTION

With recent developments in biotechnology, protein separation using reverse micelles is considered for commercial applications. The separation of proteins using reverse micelles is rather easy to scale up and can be operated continuously [1]. Organic solvents containing reverse micelles have great potential as novel media for bioseparation and biocatalysis [2,3]. In particular, protein extraction using reverse micelles has gained much attention because liquid-liquid extraction can be performed, which is especially attractive for use in large-scale, continuous processing [4,5].

In a reverse micellar extraction process, water soluble proteins are transferred from an aqueous phase to an organic one containing a surfactant, in the form of micelles. Mass transfer rates and other characteristics need quantitation to support design studies enabling commercial utilization of this technique. Some research works have been carried out on the interfacial transport of proteins across an aqueous-organic interface. Dekker et al. [6] investigated mass transfer rates in the extraction of α-amylase with a reverse micelle phase of the cationic surfactant trioctylmethylammonium chloride (TOMAC) in *i*-octane. Dungan et al. [7] studied the transport of α-chymotrypsin and cytochrome C between a bulk aqueous and an AOT-*i*-octane reverse micelle phase. The mass transfer processes in lysozyme extraction by AOT-*i*-octane reverse micelles were also studied by Lye et al. [8].

In this work lysozyme was selected as a model protein. Lysozyme is an industrially useful enzyme. Egg-white, which contains 3.5% (w/w) lysozyme, is a convenient source [9] and the anionic surfactant, AOT, used in this process, is known to form spherical nanometer-sized molecular aggregates in a variety of non-polar solvents. The work has been done under an atmosphere of high-pressure CO_2 which, by dissolving in the *i*-octane, caused expansion of the organic phase [10]. Carbon dioxide as a supercritical fluid has many excellent properties such as non-toxicity, non-explosiveness, ready availability and easy of removal from the extracted products [11]. In contrast to previous work using CO_2, a significant effect was observed on the rate of transport of protein into the reverse micellar system. The purpose of this study was to explore the recovery efficiency at different conditions by measuring the mass transfer rates of lysozyme from a bulk aqueous phase via reverse micelles to a bulk organic phase which had been expanded by pressurized CO_2.

EXPERIMENTAL

1. Materials

AOT (99%) was obtained from Aldrich Co. (USA), AR grade *i*-octane (99%) from Junsei Chemical Co. (Japan) and lysozyme (mucopeptide N-acetyl muramyl hydrolase, E. C. 3.2.1.17, molecular weight 14.3 kDa, pI 11.1) from hen egg-white obtained from Sigma Chemical Co. (USA). All other reagents were AR grade from Sigma.

Aqueous solutions were prepared by dissolving each of lysozyme (0.2 g/L) and KCl (0.1 M to 0.4 M, used to regulate ionic strengths) in Milli-Q water. Organic solutions were prepared by dissolving the desired amount of AOT in *i*-octane. The pH of aqueous solutions was adjusted within the range of 3.8 to 11.3 by addition of 0.1 M HCl or 0.1 M NaOH. The purity of CO_2 was 99%.

2. Method

The extraction of lysozyme was performed in a liquid-liquid flat vessel designed by Lewis [12] using the high-pressure apparatus shown in Fig. 1. Mass transfer rates were measured in a stirred cy-

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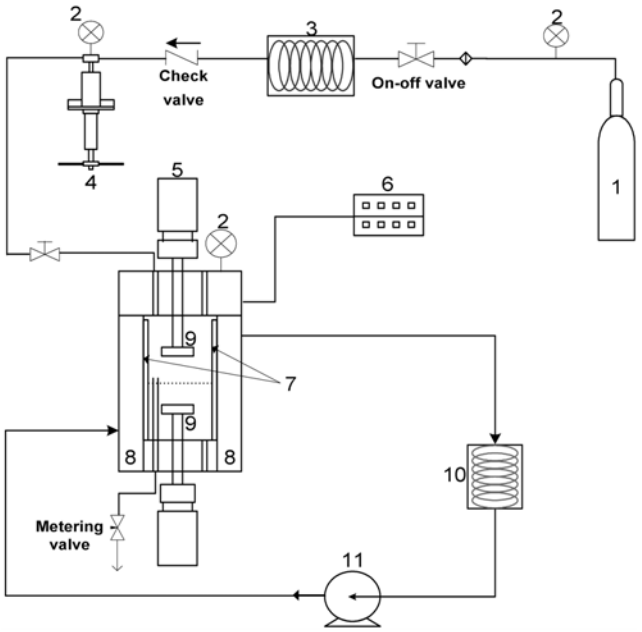

Fig. 1. Schematic diagram of lysozyme extraction by reverse micelle with pressurized CO_2.

1. CO_2 tank
2. Pressure gauge
3. Cooling bath
4. High pressure generator
5. High pressure reactor
6. Stirring speed & temperature controller
7. Baffle plates
8. Water jacket
9. Impeller
10. Heat exchanger
11. Pump

lindrical vessel of 0.06 m diameter and 0.14 m height containing 4 equi-spaced longitudinal baffles. The two co-axially mounted impellers were independently driven. A water jacket was used to control temperature. The vessel volume was 400 mL charged with 200 mL of aqueous solution followed by 100 mL of organic solutions (AOT in *i*-octane) with pressurized carbon dioxide. The organic phase was mixed with *i*-octane and liquefied carbon dioxide completely. The applied pressure of CO_2 was 10 to 20 MPa and controlled by an appropriate high-pressure regulator. Both phases were stirred by the impellers at the same speed of 170 rpm rotating in the same direction in order to cause minimal disturbance to the flat interface between the aqueous and the organic phases while experiments. 1 mL aliquots were withdrawn from the aqueous phase at 2 minute intervals for analysis. After each run the concentration of lysozyme was determined by UV spectrophotometry according to Lowry et al. [13]

MASS TRANSFER THEORY

In the mass transport process, protein transfer is envisaged to take place in three stages [6]. Initially the solute, which is dissolved in the aqueous phase, diffuses to the organic-water interface. This zone contains a high concentration of surfactant molecules which then capture the protein within reverse micelles (step 2). In step 3 the reverse micelles migrate into the bulk organic phase.

The mass transfer rate, R (mol/s), of lysozyme from aqueous to organic phase can be expressed by the following equation based on the two-film theory:

$$R=k_L a (C_{aq}-C_{aq,i}) \quad (1)$$

where, k_L (m/s) is the liquid phase mass transfer coefficient; a (m^2), the cross-sectional area of vessel; k_L a (m^3/s), the volumetric liquid phase mass transfer coefficient and C (mol/m^3), the concentration of protein in the relevant phase. Subscript aq refer to the aqueous and i to the interfacial zone.

If H is the distribution coefficient of protein between the two phases, then the phase equilibrium relationship is:

$$H=C_{org,i}/C_{aq,i} \quad (2)$$

where, subscript org refers to the organic phase.

Combining Eqs. (1) and (2) and introducing the molar flux, J (mol/s·m^2) of protein across the interface, we obtain [14,15]

$$J=R/a=(V/a)(-dC_{aq}/dt)=k_L\{C_{aq}-C_{org,i}/H\} \quad (3)$$

where, V (m^3) is the volume of the aqueous phase containing the 'dissolved' protein. Recognizing that the amount of protein at the interface will be small and assuming favorable equilibrium, then its concentration at the interface will also be small and negligible compared with that held in the bulk concentration (i.e., $C_{org,i}/H \ll C_{aq}$). The controlling step in the mass transfer was assumed to be in the aqueous phase film owing to its higher viscosity over the organic phase. Eq. (3) can then be integrated, with the following boundary conditions: t=0, $C_{aq}=C_o$ and t=t, $C_{aq}=C_{aq}$. Then

$$\ln(1-C_{org}/C_o)=-(a/V)k_L t \quad (4)$$

The value of the cross-sectional area, a, was 2.827×10^{-3} m^2 calculated by the vessel geometric dimension. The mass transfer coefficient, k_L for the process is obtained from the slope of the $-\ln(1-C_{org}/C_o)$ versus t curve [16].

RESULTS AND DISCUSSION

1. Effects of Ionic Strength on Mass Transfer Rate

Protein solubilization into the organic phase is governed mainly by electrostatic interactions between the protein and the polar head

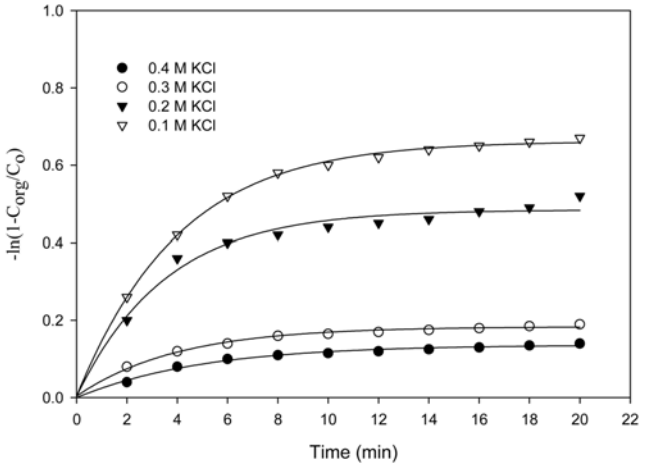

Fig. 2. Effect of KCl concentration on mass transfer rate of lysozyme by reverse micelle under pressurized CO_2 (lysozyme: 0.2 g/L, pH: 7, AOT: 20 mM, pressure: 15 MPa, temperature: 298 K).

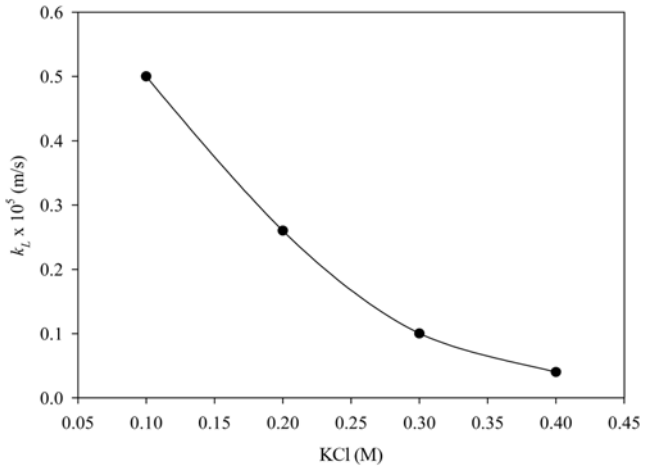

Fig. 3. Effect of ionic strength on mass transfer coefficient, k_L, of lysozyme (lysozyme: 0.2 g/L, pH: 7, AOT: 20 mM, pressure: 15 MPa, temperature: 298 K).

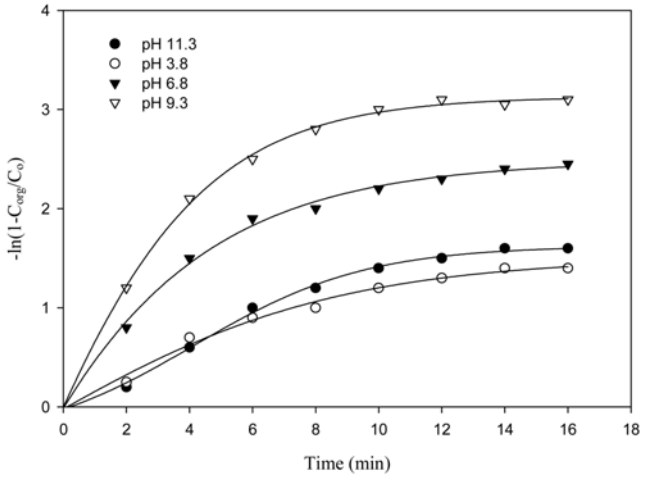

Fig. 4. Effect of pH on mass transfer rate of lysozyme by reverse micelle under pressurized CO_2 (lysozyme: 0.2 g/L, KCl: 0.1 M, AOT: 20 mM, pressure: 15 MPa, temperature: 298 K).

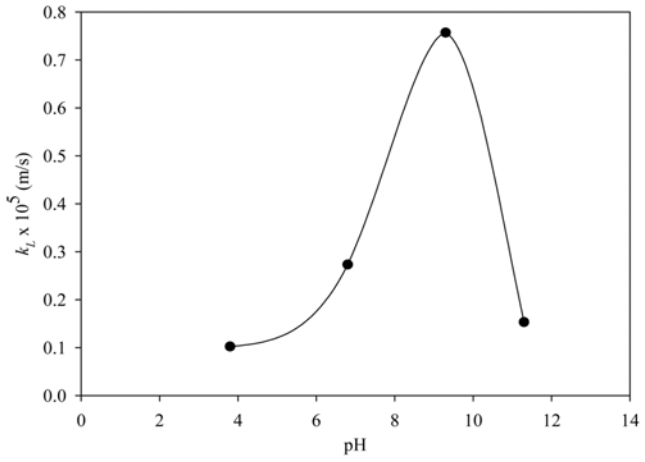

Fig. 5. Effect of pH on mass transfer coefficient, k_L, of lysozyme (lysozyme: 0.2 g/L, KCl: 0.1 M, AOT: 20 mM, pressure: 15 MPa, temperature: 298 K).

group of the AOT. The electrostatic interaction is affected by the ionic strength of the aqueous solutions. The influence of ionic strength is explained using the theory of "Debye screening" which states

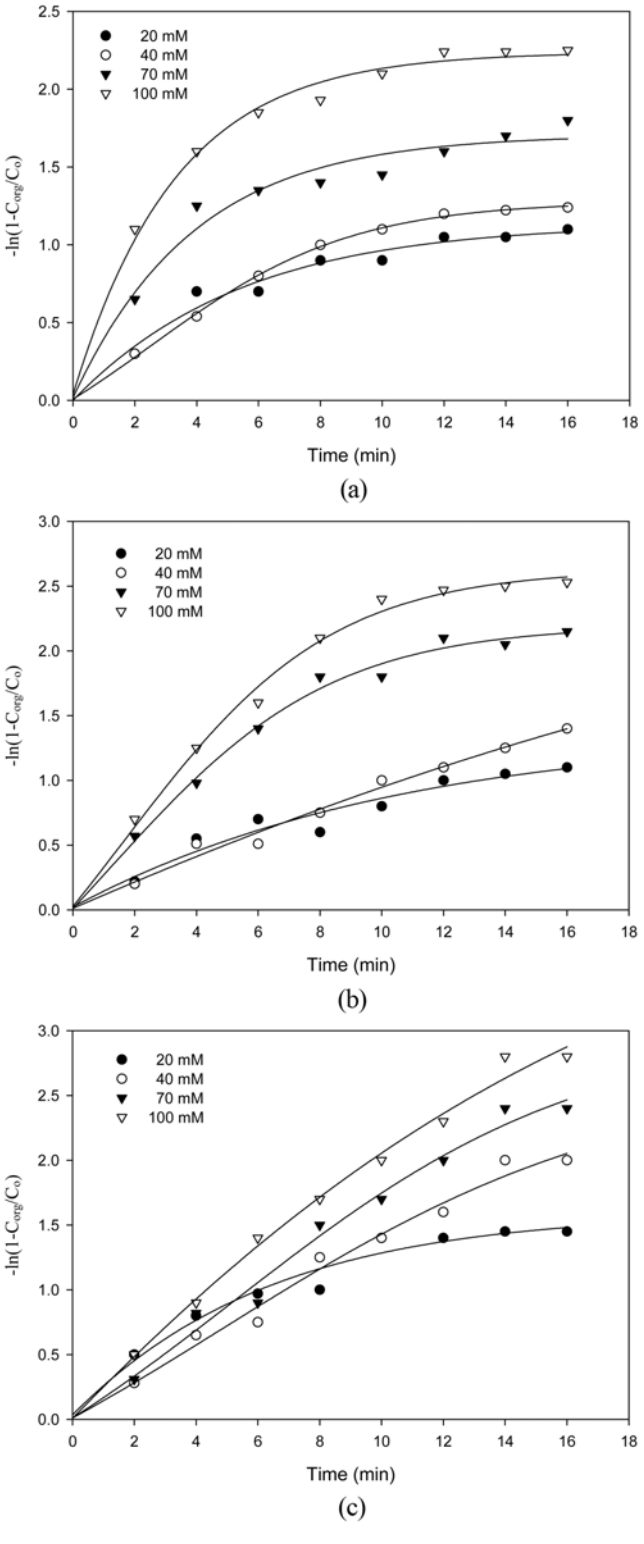

Fig. 6. Effect of AOT concentration on mass transfer rate of lysozyme by reverse micelle under pressurized CO_2 (lysozyme: 0.2 g/L, pH: 7, KCl: 0.1 M, temperature: 298 K). (a) 10 MPa, (b) 15 MPa (c) 20 MPa.

that the range an electric field extends into an electrolyte solution decrease with increasing ionic strength [1].

Figs. 2 and 3 show the mass transfer rates of lysozyme at various ionic strengths (expressed as KCl concentration). It was found that the solubility of protein into the reverse micelle was reduced by increasing KCl concentration of the aqueous solution, and the mass transfer rate of lysozyme was decreased. The addition of KCl in the system caused a significant reduction of electrostatic attraction between the lysozyme molecules and the polar head group of AOT. Similar results were found in alkaline protease separation using reverse micelles [17].

2. Effects of pH on Mass Transfer Rate

The mass transfer rates of lysozyme at various pH of the aqueous solutions are given in Figs. 4 and 5. Results for the transferred lysozyme in the reverse micellar systems have been 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. Lysozyme was solubilized in anionic reverse micelles. Lysozyme possessed a net positive charge at pH values below their isoelectric point (pI). It was also found that the largest mass transfer coefficient, k_L was at pH 9.3, which is the nearest the pI of lysozyme. When the pH of aqueous solution crossed the value of pI, the mass transfer rate of lysozyme was decreased. The electrostatic repulsions between the negative charges of the protein and polar groups of AOT act as a suppression in the extraction, resulting in lower mass transfer rate. Similar results were found in protein extraction by reverse micelle [18,19]. Lye et al. [19] measured the volumetric liquid phase mass transfer coefficients, k_L a, at 0.2 M KCl and different pH conditions with the well mixed systems. It was around 0.3×10^{-7} m^3/s to 43.4×10^{-7} m^3/s, which provided higher value than the flat interface system.

3. Effects of AOT Concentration in Pressurized CO_2

Figs. 6 and 7 show the rate of protein transfer into the reverse micellar phase in the presence of CO_2 at selected anionic surfactant concentrations with pressure. The amount of lysozyme dissolved increased with the increasing surfactant concentration in pressurized CO_2. The lysozyme transfer rate also increased at high pressure. The critical micelle concentration (CMC) in this work was 100 mM AOT. When the concentration of surfactants is higher than the CMC, surfactants form micelles-spherical or rod-like aggregates

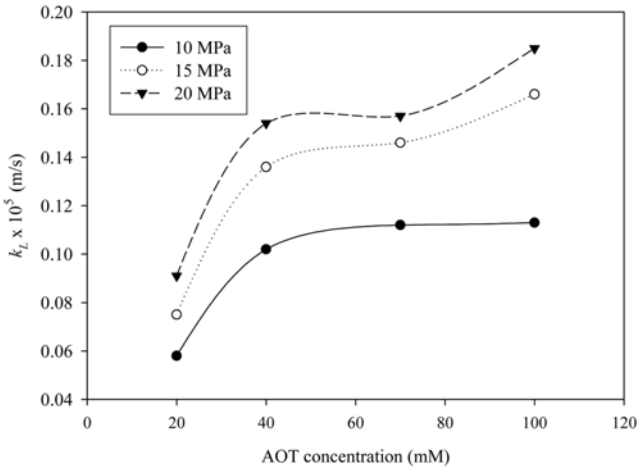

Fig. 7. Effect of AOT concentration with pressure on mass transfer coefficient, k_L, of lysozyme (lysozyme: 0.2 g/L, KCl: 0.1 M, pH: 7, temperature: 298 K).

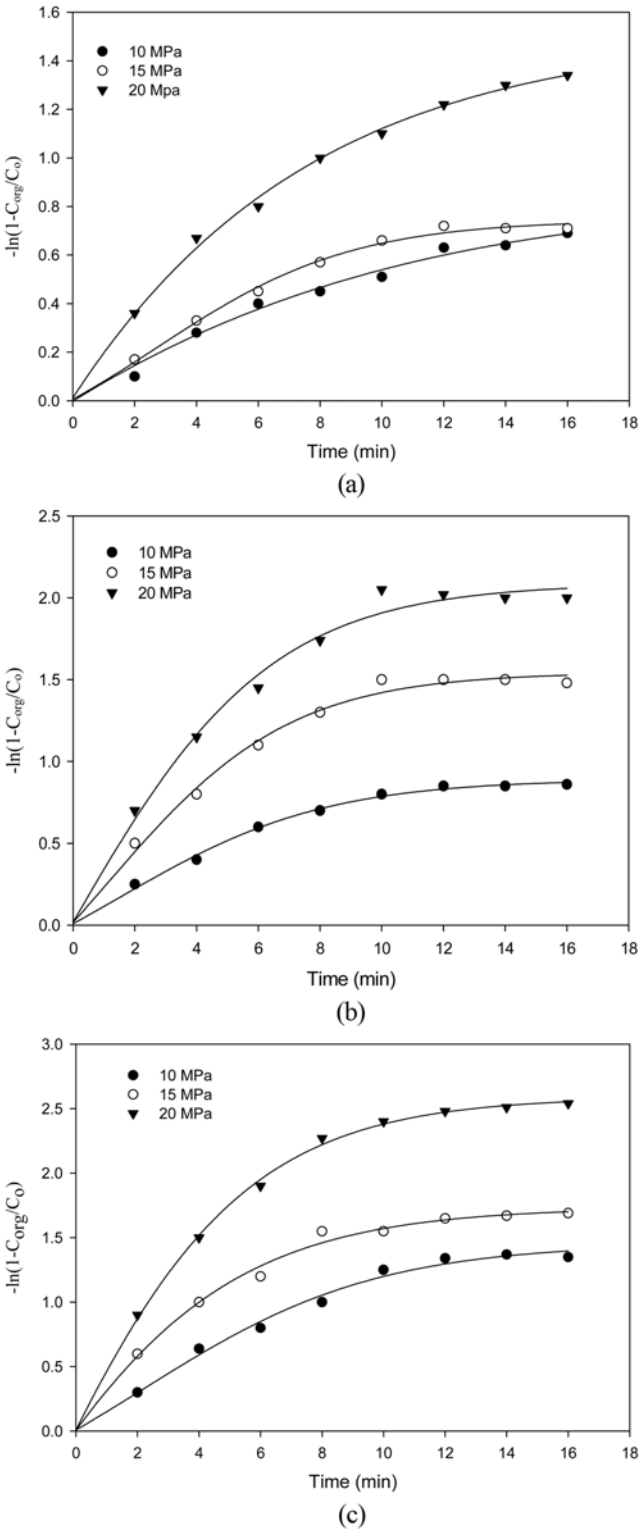

Fig. 8. Effect of temperature with pressure on mass transfer rate of lysozyme by reverse micelle under pressurized CO_2 (lysozyme: 0.2 g/L, KCl: 0.1 M, pH: 7, AOT: 20 mM). (a) 298 K (b) 308 K (c) 318 K.

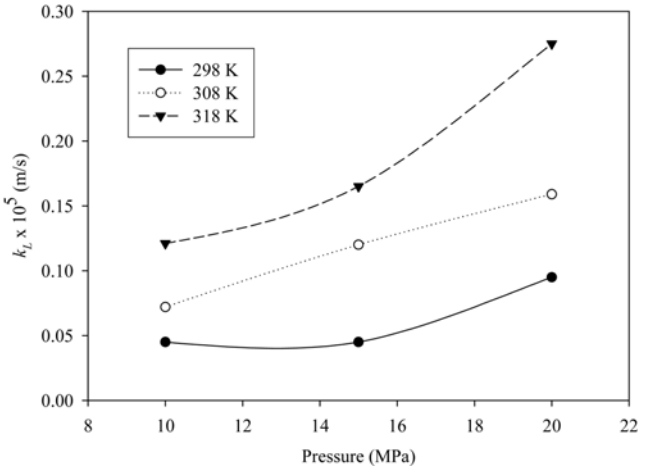

Fig. 9. Effect of pressure and temperature on mass transfer coefficient, k_L, of lysozyme (lysozyme: 0.2 g/L, KCl: 0.1 M, pH: 7, AOT: 20 mM).

[20]. It was difficult to measure the mass transfer rate beyond this concentration due to coagulation.

4. Effect of Pressure and Temperature on Mass Transfer Rate of Lysozyme

The effects of temperature and pressure on protein transfer are shown in Figs. 8 and 9. The highest amount of lysozyme was solubilized at high pressure, 20 MPa. The take-up of CO_2 into the water pools would be enhanced through ionization (i.e., $H_2O+CO_2=H^++HCO_3^-$). Thus at the high pressure, lysozyme was able to transfer into the reverse micelles of the organic phase more readily. In pressurized CO_2 atmosphere, the mass transfer rate was increased markedly. The influence of temperature on mass transfer of lysozyme was measured from 298 to 318 K. The range was deliberately limited since the enzymic activity of lysozyme is substantially lost above 318 K. It was found that the highest amount of lysozyme was solubilized at high temperature. The mass transfer rate of lysozyme was increased with increasing temperature.

5. Measurement of the Mass Transfer Rate in Conventional (Atmospheric Pressure) and Pressurized CO_2

The mass transfer rate of lysozyme in conventional process (without CO_2) and pressurized CO_2 are given in Fig. 10. The mass transfer rate of lysozyme in pressurized CO_2 was much higher than that of the conventional process. Without pressurized CO_2, the transfer rate of lysozyme was uniform with time. On the other hand, in pressurized CO_2, lysozyme transferred more rapidly for the first 40 min

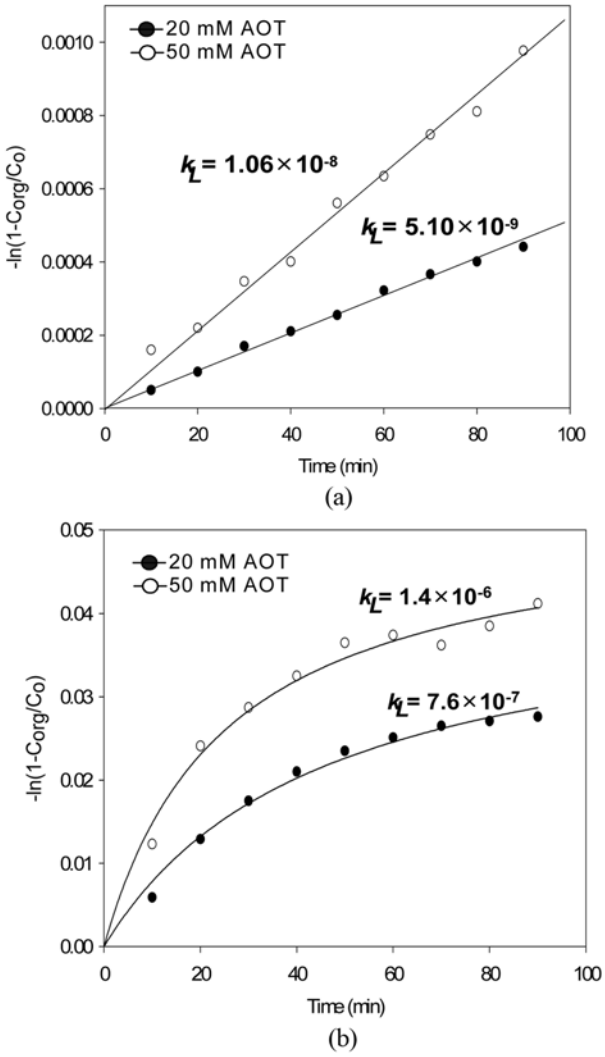

Fig. 10. Mass transfer rate of lysozyme by reverse micelle (lysozyme: 0.2 g/L, KCl: 0.1 M, pH: 7, AOT: 20 mM, temperature: 298 K). (a) Conventional (0.1 MPa) (b) Pressurized CO_2 (15 MPa).

Table 1. Mass transfer coefficients of lysozyme in atmospheric and pressurized conditions

		$k_L \times 10^6$ (m/s)		$k_L \cdot a \times 10^8$ (m^3/s)		Conditions
		Atmospheric pressure (0.1 MPa)	Pressurized CO_2 (15 MPa)	Atmospheric pressure (0.1 MPa)	Pressurized CO_2 (15 MPa)	
KCl (M)	0.1	0.0127	5.03	0.035	1.422	Lysozyme 0.2 g/L, pH 7,
	0.2	0.0182	2.65	0.051	0.749	AOT 20 mM, 298 K
	0.3	0.0178	1.20	0.050	0.339	
pH	5	0.0055	1.02	0.016	0.288	Lysozyme 0.2 g/L, KCl 0.1 M,
	7	0.0073	2.73	0.021	0.770	AOT 20 mM, 298 K
	9	0.0107	7.57	0.030	2.140	
AOT (mM)	20	0.0051	0.76	0.014	0.215	Lysozyme 0.2 g/L, pH 7,
	50	0.0106	1.42	0.030	0.400	KCl 0.1 M, 298 K

and then the transfer rate was decreased. Table 1 shows a comparison of mass transfer coefficients between the conventional and pressurized processes (15 MPa). The k_L values are about 70 to 700 times greater under the pressurized conditions. CO_2 reduces the interfacial tension in aqueous-organic systems [21]. This suggests that at least one effect of the pressurized CO_2 was to decrease the interfacial tension, resulting in higher k_L values. Asai et al. [22] and Park et al. [23] measured mass transfer coefficients of different solutes such as caproic acid, I_2 and cyclohexanol etc. at different conditions using an agitated vessel based on the Lewis system [12] with a flat interface between the aqueous and organic phases. They used the cross-sectional area of the vessel to calculate mass transfer coefficients. The values of the mass transfer coefficients were lower than the system mixed with pressurized carbon dioxide in the organic phase. The interfacial tension between the aqueous and the organic phases depends on the physical properties of the solutions. The organic phase mixed with pressurized carbon dioxide may decrease the interfacial tension which occurs at a high transfer rate of the solute [24].

CONCLUSIONS

The transport of lysozyme between aqueous solution and AOT-*i*-octane solution was studied under high pressure of CO_2 using reverse micelles. The mass transfer rates were influenced by various ionic strengths, pH, AOT concentrations and temperatures. The mass transfer behavior was similar to the conventional process. However, the transfer rate of lysozyme was higher in pressurized CO_2 than atmospheric conditions. The values of k_L obtained from conventional process and pressurized process were around 0.0051×10^{-6} m/s to 0.0182×10^{-6} m/s and 0.76×10^{-6} m/s to 7.57×10^{-6} m/s, respectively. It was more effective to extract and separate protein using reverse micelle with pressurized CO_2. A model can be developed for estimating mass transfer coefficients based on data obtained from an agitated vessel with a flat interface of two-phase system in which the organic phase contains complete mixtures with liquefied solvent.

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