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Investigation on the precipitation, microenvironment and recovery of protein in CO_2-expanded reverse micellar solution

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Abstract

The effect of compressed CO_2 on the properties of protein (trypsin) in the reverse micelles of sodium bis(2-ethylhexyl)sulfosuccinate AOT/decane/water has been studied. UV-vis spectrum was used to determine the precipitation of trypsin in the reverse micelles, which reveals that trypsin can be precipitated from the reverse micelles by compressed CO_2 at suitable pressures, while the surfactant AOT remains in the solution. FT-IR spectroscopy was used to investigate the microenvironment of trypsin in the CO_2-expanded reverse micelles, which shows that the micropolarity inside the micelle cores is changed gradually with the CO_2 pressure. Using compressed CO_2 as antisolvent, the protein in the reverse micelles can be recovered, and trypsin nanoparticles with the size of less than 15 nm were obtained, which was characterized by transmission electron microscopy (TEM).

Keywords: Compressed CO_2; Transmission electron microscopy; Reverse micelles

1. Introduction

Reverse micelles are aggregation systems formed spontaneously in nonpolar solvent. The structure can be simply described as a shell formed by surfactant molecules with their polar headgroup inside, which surrounds a 'water pool' [1,2]. Hydrophilic molecules can be solubilized in the water pool. The ability of the reverse micelles to host smaller hydrophilic molecules such as proteins has been an interesting topic, and there have been many papers focused on the protein-containing reverse micelles [3–5]. The solubilization mechanism [6–10], the location [11,12], and the superactivity of protein [13–15], and the property changes of the reverse micelles [16–18] have been widely studied.

Carbon dioxide (CO_2) is an attractive solvent alternative for a variety of chemical and industrial processes, especially because it is plentiful and inexpensive, and has properties that are between those of many liquids and gases [19,20]. One of the useful techniques in material science is the gas antisolvent process [21]. The principle of this technique is that supercritical (SC) or compressed CO_2 is quite soluble in a number of organic solvents and expands them largely. Many solutes are soluble in organic solvents, but are not soluble in CO_2. Thus the CO_2 in organic solvents can act as an antisolvent to precipitate the solutes in the solvent. This technique has been used in different processes, such as extraction and fractionation [22,23], recrystallization of chemicals [24], micronization [25], and production of polymeric particles [26,27]. These methods have some potential advantages. For example, it is possible to remove the solvent and antisolvent completely from the products. Moreover, fine particles can be easily obtained because high supersaturation can be achieved in the solution, and the morphology of the products can be tuned by pressure of CO_2. Debenedetti et al. have used supercritical fluid (CO_2) antisolvent technique to produce protein particles, and microparticulate protein powders of about 1–5 μm have been obtained [28,29].

In previous work, we studied the effect of compressed CO_2 on the solubilization of BSA and lysozyme in reverse micelles [30], and it was revealed that these proteins could be precipitated from the reverse micelles under certain CO_2 pressure. In the present work, we furthered these studies to the protein of trypsin, and did some fundamental and application investigations on the CO_2-expanded protein-containing reverse micelles. The present work consists of three parts: (1) the precipitation of trypsin in the

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CO_2-expanded reverse micelles by UV-vis spectra; (2) the microenvironment changes of the protein in reverse micelles with pressure of CO_2 by FT-IR; (3) the recovery of protein particles from the revere micelles using compressed CO_2 as antisolvent. We believe that such studies can provide necessary understandings on the CO_2-expanded protein-containing reverse micelles and find potential advantages in a wide range of applications.

2. Experimental

2.1. Materials

Trypsin ($M_w = 24\,000$) was produced by Xinjiang Institute of Chemistry. The surfactant AOT was purchased from sigma with purity of 99%. The decane supplied by Tianjin Wendaxigui Chemical Plant was GR grade. Double distilled water was used, and D_2O was used in IR investigation. CO_2 (99.95%) was provided by Beijing Analysis Instrument Factory.

2.2. Preparation of protein-filled reverse micellar solution

The reverse micellar solution was prepared by dissolving AOT into decane in a flask and the required amount of water was added. Then the flask was shaken until the solution became transparent. The desired amount of protein was added into the reverse micellar solution, then shook it till transparent.

2.3. Phase behavior of reverse micellar solution in CO_2

The apparatus used to study the expansion curves and the cloud point pressure of the solution was the same as that used previously [31]. In the expansion experiment, suitable amount of reverse micellar solution was added into the view cell. The temperature of the water bath was controlled by a HAAKE D3 digital controller, and the accuracy of the temperature measurement was ± 0.1 °C. After the thermal equilibrium had been reached, CO_2 was charged into the cell to a suitable pressure. A magnetic stirrer was used to enhance the mixing of CO_2 and reverse micellar solution. The pressure and the volume at equilibrium condition were recorded. More CO_2 was added and the volume of the liquid phase at another pressure was determined. The volume expansion coefficients were calculated on the basis of the liquid volumes before and after dissolution of CO_2.

2.4. Precipitation of trypsin by UV-vis spectra

The UV spectrophotometer (TU-12010) was used to examine the precipitation of trypsin from the reverse micelles at different pressures. The UV sample cell was the same as that used previously [32]. The optical path length and the volume of the sample cell were 1.12 cm and 1.76 ml, respectively. A suitable amount of protein-filled reverse micellar solution was loaded into the cell. The temperature of the cell was maintained at 293.2 K. Then CO_2 was charged until the sample cell was full. The UV spectrum of the solution was obtained by repeated scans until it was unchanged.

2.5. High-pressure FT-IR spectra

The FT-IR spectroscopic apparatus consisted mainly of a gas cylinder, a high-pressure pump, an IR spectrometer (TENSOR 27) and a high-pressure IR sample cell. The high-pressure IR cell was similar to that used in UV, only ZnS windows (2.25 cm in diameter and 0.60 cm thick) was used. The internal volume and the path length of the cell were 1.78 ml and 1.566 cm, respectively. The temperature of the cell was stabilized at 293.2 K and CO_2 was compressed into the cell to the desired pressure. The IR spectrum was recorded after equilibrium had been reached. Each sample was recorded with 32 scans at resolution of 2 cm^{-1}.

2.6. Recovery of the protein and characterization

The desired amount of reverse micellar solution filled with protein was added into the cylinder-shaped autoclave of 120.0 ml. The temperature was controlled at 293.2 K. CO_2 was injected into the autoclave by a high-pressure pump until the desired pressure was reached, and the solution was stirred (120 r.p.m.) for 1 h. The stirring was stopped and the protein particles were precipitated from the solution and deposited at the bottom of the autoclave. The solution and CO_2 were released slowly. The deposits at the bottom of the autoclave were collected and washed several times using ethanol after the removal of the liquid solution. The size and shape of the obtained protein particles were determined by TEM with a HITACHI H-600A electron microscope. The maximum resolution was 0.5 nm.

3. Results and discussion

3.1. Phase behavior investigation of the reverse micelles in CO_2

The solution is expanded after dissolution of CO_2. The volume expansion coefficient ΔV ($\Delta V = (V - V_0)/V_0$, where V and V_0 are the volumes of the CO_2-saturated and CO_2-free reverse micellar solution) of the solution at different pressures is a very important parameter in the experiments. Fig. 1 shows the volume expansion curve of the solution ([AOT] = 100 mmol l^{-1}, $w_0 = 20$) versus the pressure at 293.2 K obtained in this work. The volume expansion coefficient increases with pressure. We also studied the effects of w_0 values (from 5 to 30) and protein concentrations (from 0 to 1.0 mg ml^{-1}) on the volume expansion. The results indicated that the effects of w_0 and protein concentration on the ΔV were not considerable. The determined volume

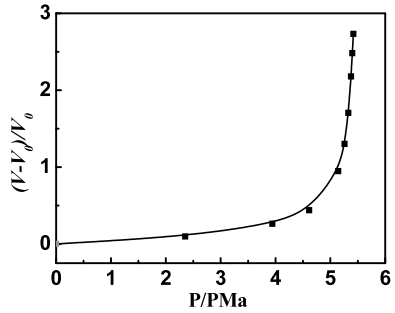

Fig. 1. Dependence of the volume expansion of AOT/decane solution ([AOT] = 100 mmol l^{-1}) on the pressure of CO_2 at 293.2 K.

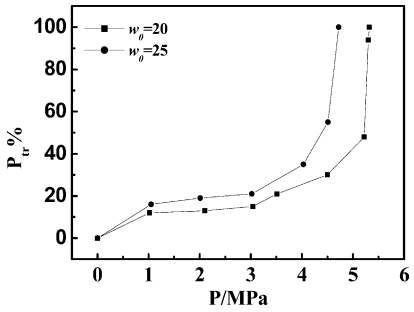

Fig. 3. Variation of the precipitation ratio of trypsin (P_{tr} %) with pressure at 293.2 K.

expansion allowed us to determine how much CO_2-free reverse micellar solutions should be loaded into sample cell for the UV and IR investigations. The solution became cloudy at the cloud point pressure, where the surfactant began to precipitate. The cloud point pressure of the AOT/water/decane micellar solutions determined is 5.18 MPa at 293.2 K, with a precision of ±0.03 MPa.

3.2. Precipitation of protein from reverse micelles by UV spectra

The protein solubilized in the reverse micelles do not precipitate in the absence of CO_2. However, the protein particles can be precipitated by dissolution of antisolvent CO_2 in the micellar solution, which is known by our UV study. As examples, Fig. 2 illustrates the UV spectra of the protein-filled reverse micelles ($w_0 = 25$, $T = 293.2$ K) at different pressures. For all the experiments, the UV cell was full of solution after CO_2 was added and the concentrations of protein and AOT in the solution were 0.5 mg ml^{-1} and 100 mmol l^{-1} if precipitation does not occur. The two absorbencies around 225 and 272 nm belong to AOT and trypsin, respectively. Extinction coefficient of protein changes with the environment more or less. To a first approximation, we neglect the effect of the dissolved CO_2 on the extinction coefficient. As can be seen, the intensity of the band at 272 nm decreases with pressure, while that for AOT remains unchanged in a certain pressure range. This indicates that the protein particles can be precipitated from the reverse micelles, and the surfactant remains in the organic continuous phase. Some AOT is precipitated as the pressure exceeds the cloud point pressure (5.18 MPa), which is easy to understand because AOT begins to precipitate at cloud point pressure.

Our experiments show that at a fixed w_0 the absorbance of protein nanoparticles is a linear function of protein concentration as the concentration is lower than 1.0 mg ml^{-1}. Thus the precipitation percentage of protein can be calculated from the absorbance and the working curve. Fig. 3 shows the dependence of precipitation percentage of protein (the mass of the precipitated protein divided by the total mass of protein) on CO_2 pressure at $w_0 = 20$ and 25. It is shown that more protein particles are precipitated at the higher pressures, and it is more difficult to precipitate the protein particles at the lower w_0. This can be interpreted by the stronger interaction of particles with the matrices at a lower value of w_0 (smaller micellar core radius). At 4.71 MPa, which is still lower than the cloud point pressure of the micellar solution, the precipitation percentage of protein particles ($w_0 = 25$) can reach to 100%, while the absorbance of AOT remains unchanged. This indicates that at suitable pressure, protein particles in the reverse micelles can be precipitated completely, leaving the surfactants in organic continuous phase.

3.3. FT-IR spectra of the protein-filled reverse micellar solution in the presence of CO_2

The concentration of AOT and the protein in all CO_2-expanded solutions are 100 mmol l^{-1} and 0.5 mg ml^{-1}, respectively, if precipitation does not occur. Fig. 4 gives the

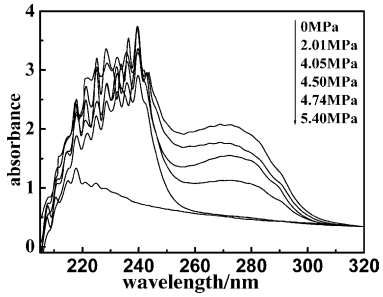

Fig. 2. UV spectra of the protein-filled reverse micelles ([AOT] = 100 mmol l^{-1}, $w_0 = 25$, [trypsin] = 0.5 mg ml^{-1}) at 293.2 K and different pressures.

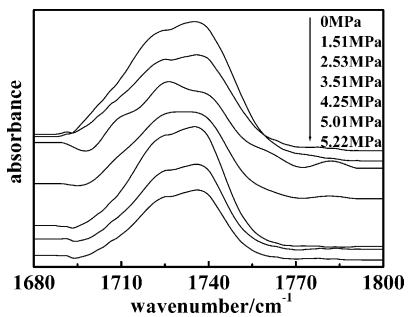

Fig. 4. IR spectra of carbonyl stretching vibration of AOT in reverse micelles ($w_0 = 20$) at 293.2 K and different pressures.

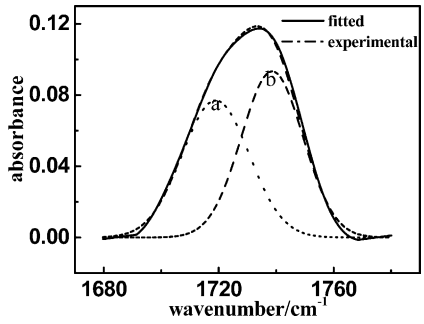

Fig. 5. Fitted carbonyl stretch peak of AOT in the CO_2-free reverse micelles (AOT = 100 mmol l^{-1}, $w_0 = 20$) at 293.2 K.

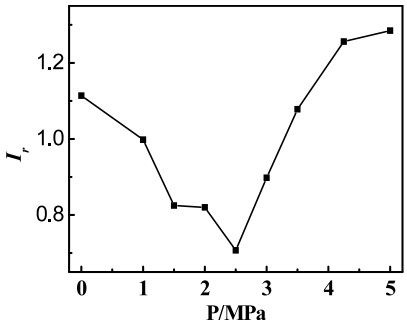

Fig. 6. Variation of intensity ratio ($I_r = I_{1740}/I_{1721}$) as a function of CO_2 pressure for reverse micelles ([AOT] = 100 mmol l^{-1}, $w_0 = 20$, [Trypsin] = 0.5 mg ml^{-1}) at 293.2 K.

IR spectra of carbonyl stretching vibration υ(C = O) of AOT situated around 1730 cm^{-1}, and it gives some microstructure information on the CO_2-expanded reverse micelles [33]. The broad peak with an asymmetric shape shown in Fig. 4 suggests that the band is a fusion of peaks corresponding to carbonyl groups in different microenvironments [33]. Gaussion curve fitting program is used to treat the peaks. As an example, Fig. 5 shows the experimental and fitted carbonyl stretch peak of AOT in the CO_2-free reverse micelles (AOT = 100 mmol l^{-1}, $w_0 = 20$) at 293.2 K. And the two peaks centered at 1740 ± 2 and 1721 ± 2 cm^{-1} are obtained, which corresponds to *trans* and *gauche* configurations of the rotational isomerism of AOT [33,34]. The intensity ratio ($I_r = I_{1740}/I_{1721}$) shows the relative quantities of *trans*- and *gauche*-like isomers of AOT [34]. Their intensity ratio ($I_r = I_{1740}/I_{1721}$) is closely related to the polarity of the water core, i.e. the ratio decreases in a more polar environment because more conformations change from *gauche* to *trans* [35,36]. The intensity ratios I_r of the protein-containing reverse micelles ([AOT] = 100 mmol l^{-1}, $w_0 = 20$) are given in Fig. 6 as a function of pressure. From Fig. 6, it can be seen that the intensity ratio decreases with the increasing pressure until it reaches to a minimum at 2.53 MPa, and then the intensity ratio (I_r) increases gradually with the increasing pressure. CO_2 dissolves in the 'water pool' of reverse micelles can be ionized ($H_2O + CO_2 = H^+ + HCO_3^{-1}$) and makes the polarity of the micelle core increased. However, in this work, the existence of the protein in the micelle cores results in the obvious increase of the polarity in the reverse micelles, which is confirmed by our experimental results, i.e. the I_r value of the protein-containing revere micelles (1.12) is smaller than that of the protein-free reverse micelles (1.48) without the addition of CO_2. As we know from UV-vis studies, when CO_2 is added to the micellar solutions, the protein can be precipitated from the reverse micelles, which decreases the polarity inside micellar cores. However, the precipitation percentage of protein from the reverse micelles is relatively small at low pressures (shown in Fig. 3). Thus it is expected that the effect of CO_2 may play a dominant role on the polarity of the reverse micelles at low pressures, which results in the increased polarity and corresponding decreased I_r. At higher pressures, the precipitation percentage of the protein is increased largely, and its effect plays the dominant role on the polarity of the reverse

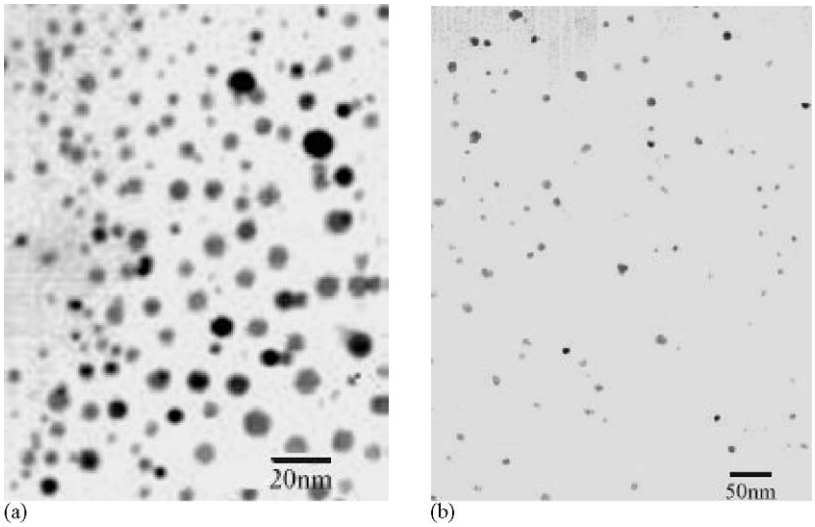

Fig. 7. TEM photographs of protein recovered from the reverse micelles with [trypsin] = 0.5 mg ml^{-1} (a) and [trypsin] = 0.8 mg ml^{-1} (b).

micelles. Thus I_r value increases largely corresponding to the decreased polarity of the reverse micelles.

3.4. Morphologies of the recovered protein nanoparticles

Fig. 7(a) and (b) illustrate the TEM photographs of protein recovered from the reverse micelles ($w_0 = 20$) with [trypsin] = 0.5 and 0.8 mg ml^{-1} at 293.2 K, respectively. The operation pressure is 5.02 MPa. It is estimated from TEM photographs that the particle size at [trypsin] = 0.5 mg ml^{-1} is in the range of 3–10 nm, and is 5–14 nm at [trypsin] = 0.8 mg ml^{-1}. It means that the particle size is larger at the larger concentration of trypsin. This maybe dues to the reduced aggregation behavior of the nanoparticles at lower concentration. The protein particles produced in this work are much smaller than those obtained by other methods [28,29].

4. Conclusion

In summary, this work investigates the effect of compressed CO_2 on the precipitation and microenvironment of the protein in the reverse micelles by phase behavior, UV-vis spectra, and FT-IR spectra. It is revealed that the proteins can be precipitated from the reverse micelles by the compressed CO_2, accompanied with the changed micropolarity inside the micelle cores. Using this concept, the protein confined in the reverse micelles could be recovered by compressed CO_2, and well dispersed protein nanoparticles with the diameter of about 10 nm were obtained. This method has many potential advantages for applications such as simple, timesaving, and the solutions can be recycled.

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