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Youn-Ok Shin[a]; Eva Rodil[ab]; Juan H. Vera[a]
[a] Department of Chemical Engineering, McGill University, Montreal, Quebec, Canada [b] Department of Chemical Engineering, University of Santiago de Compostela, Santiago de Compostela, Spain

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Precipitation and Recovery of Cytochrome c and Hemoglobin Using AOT and Acetone

Youn-Ok Shin,[1] Eva Rodil,[1,2] and Juan H. Vera[1,*]

[1]Department of Chemical Engineering, McGill University, Montreal, Quebec, Canada
[2]Department of Chemical Engineering, University of Santiago de Compostela, Santiago de Compostela, Spain

ABSTRACT

The precipitation of two transport proteins, cytochrome c and hemoglobin, was carried out by using di-(2-ethylhexyl) sulfosuccinate, known as aerosol-OT (AOT), as a precipitating agent. The percent precipitation was 100% when the molar ratio between AOT and the targeted protein was 11 for cytochrome c and 30 for hemoglobin. By using acetone as a polar solvent, the maximum recovery obtained was 98% for cytochrome c and 40% for hemoglobin. The surfactant contamination in the recovered product was below the detection limit. The required usage of surfactant to purify 1 mole of targeted protein was many orders of magnitude smaller than that required for a reverse micellar extraction when using AOT.

*Correspondence: Professor Juan H. Vera, Department of Chemical Engineering, McGill University, 3610 University Street, Montreal, Quebec H3A 2B2, Canada; Fax: 514 398 6678; E-mail: juan.vera@mcgill.ca.

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Advantages of the suggested precipitation method over the reverse micellar extraction method for protein purification are discussed.

Key Words: Cytochrome; Hemoglobin; AOT; Precipitation; Recovery.

INTRODUCTION

According to their biological functions, proteins may be classified as enzymes, hormones, immune proteins, or transport proteins.[1] Many of these proteins are important components in the food, textile, and/or pharmaceutical industries. The cost of proteins often is determined by their purification cost.[2] The use of surfactants forming reverse micelles has been suggested as a method for large-scale extraction of proteins from dilute aqueous phases.[3,4] Proteins entrapped in the small water pool of reverse micelles maintain their stability in an organic environment for many days,[5] suggesting the possible use of this technique for the purification of proteins from an aqueous solution.[6] The most common surfactants used in the studies of reverse micellar extraction are di-(2-ethylhexyl) sulfosuccinate [Aerosol-OT (AOT)], as an anionic surfactant,[5,7–9] and trioctylmethyl ammonium chloride (TOMAC) or cetyltrimethylammonium bromide as cationic surfactants.[10,11] Enzymes often have been used in the study of the mass transfer kinetics, into and out of the reverse micellar phase, with special attention to the quality of the final product after the process.[7,10–14] The transport proteins cytochrome c and hemoglobin also are commonly used in studies of reverse micellar extraction.[15–17] Cytochrome c is an electron carrier for the oxidizing end of cytochrome oxidase,[18] and hemoglobin is responsible for the oxygen transport from the lung to all body tissues.[19]

A study of the solubilization mechanism of cytochrome c shows that this protein first forms an ion-pair complex with the surfactant at a characteristic surfactant-to-protein ratio.[20] However, this ion-pair complex does not have sufficient hydrophobicity to be solubilized into an organic phase, and it precipitates at the aqueous–organic interface. The study[20] concluded that, for cytochrome c to be solubilized into a reverse micellar phase, additional surfactant is required to provide with a hydrophobic surface around the protein. A similar conclusion was reached in a study of the solubilization mechanism of α-chymotrypsin.[21] In this latter study, the interface mass transfer kinetics was found to be an important factor governing the formation of a white precipitate at the interface.

The extraction of hemoglobin with common commercial surfactants, such as AOT or TOMAC, was found to be unsatisfactory due to the precipitation of hemoglobin at the aqueous-organic interface. Thus, a new surfactant, dioleyl

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phosphoric acid (DOLPA), was synthesized and used to form a reverse micellar phase for the extraction of hemoglobin.[17]

Recently, the reverse micellar extraction of lysozyme was described in terms of chemical equilibrium rather than phase equilibrium.[22] Based on this observation, a new method of purifying proteins by using a surfactant was proposed.[23] In this latter work, instead of using a reverse micellar phase for protein extraction, the surfactant AOT was directly added to the lysozyme solution to form an insoluble lysozyme–AOT complex. The precipitated protein was then recovered from the insoluble complex by contacting the complex with acetone. This polar solvent dissociates the complex to yield a solid protein product and a solution containing the surfactant. Despite the common belief that the ionic surfactants are denaturing agents for proteins[24] and that the water pool is essential to maintain the protein stability,[25] the lysozyme recovered from a lysozyme–AOT complex kept its original activity, and it was free of surfactant and free from other contaminating proteins.[23]

The objective of this work is to study the use of AOT as a precipitating ligand for two transport proteins, cytochrome c and hemoglobin. The effect of pH and salt concentration on the precipitation efficiency is reported.

MATERIALS AND METHODS

Reagents

Cytochrome c from horse heart type III (p*I* 10.6; 12,384 Da), hemoglobin from bovine blood (pI 6.8; 64,500 Da), and the anionic surfactant di-2-ethylhexyl sodium sulfosuccinate ($C_{20}H_{37}O_7Na$), abbreviated as AOT (99% purity), were purchased from Sigma (Oakville, ON). The high-performance liquid chromatography grade organic solvents, acetonitrile and triflouric acid, and a reagent grade acetone were purchased from Fisher Scientific (Montreal, QC). Sodium phosphate, dibasic, and potassium phosphate, monobasic, were obtained from A and C American Chemicals Ltd. (Montreal, QC). The deionized water used for the preparation of the aqueous phase was obtained by passing distilled water through ion-exchange columns type Easy pure replacement filters (RF), Compact Ultrapure Water System, Barnstead Thermoline, Dubuque, Iowa.

Precipitation of Proteins

The initial aqueous phase used contained from 0.2 to 1 g/L protein. This corresponds to 0.011–0.071 mmol/L of cytochrome c or

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0.003–0.019 mmol/L hemoglobin. The pH of the aqueous phase was adjusted using 1 N HCl or NaOH. For some of hemoglobin experiments, 0.25 M sodium phosphate or potassium phosphate buffer solutions were used to adjust the pH of the initial hemoglobin aqueous solution. The pH of the solutions was measured using an accupHast reference/pH probe, model 13-620-116, purchased from Fisher Scientific (Montreal, QC), filled with a saturated potassium chloride solution (SP 138-500). An OAKTON pH/mV Benchtop Meter, model WD-35616-00, was used to monitor pH measurements.

An aqueous phase containing 5 g/L AOT was prepared without any salt addition or pH adjustment. A volume of 0.3 mL of the AOT solution was directly added to the 10 mL of a protein-containing aqueous solution. No micelles were formed in the protein solution, since the initial AOT concentration was less than the critical micellar concentration of AOT in water: 4.1 mM at 25°C.[26] Upon the addition of the AOT, an insoluble protein–AOT complex was formed instantaneously. The mixture was vortexed for 5 sec and centrifuged for 1–2 min. The supernatant liquid was removed and analyzed for protein content. The precipitated protein–AOT complex was washed with distilled water and centrifuged. The supernatant was removed, and the solid was used for the protein recovery process.

Recovery of the Proteins

For the recovery of the proteins, 10 mL acetone was added to the test tube containing either the precipitated cytochrome c or hemoglobin, complexed with AOT. After 5 sec of vortexing, the solid dissolved in the acetone phase. This solution was left to set for 5 min, and the recovered protein precipitated as a solid while AOT remained in solution. For the samples prepared without any salt during the precipitation process, a small amount of NaCl solution (normally less than 10 μL of 0.1 M NaCl) was required to neutralize the charges of dissociated protein and the surfactant. The surfactant-free protein was then precipitated out from the acetone phase within 2 min after the acetone addition. This solid protein recovered was washed with acetone to further remove any residual surfactant. The qualitative and quantitative analyses of the final product were then carried out by dissolving the recovered-solid protein into a fresh aqueous phase.

Assay

The concentration of protein and AOT in aqueous phase was determined using an Agilent HPLC 1100 with a Zorbax 300SB-C8 (4.6 mm inner diameter

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[ID] × 150 mm length) as a column. The mobile phase consisted of two solvents with different polarities: the solvent A containing 5% acetonitrile and 0.1% triflouric acid in water, and the solvent B containing 5% water and 0.085% triflouric acid in acetonitrile. The mobile phase was set at an initial composition of 90% solvent A and 10% solvent B with a solvent gradient of 40% solvent A and 60% solvent B in 20 min. To avoid any impurities that may damage the column, HPLC-grade solvents were used to prepare the mobile phase, and the samples were filtered before being injected to the column. The flow rate through the column was set at 1.0 mL/min, and the temperature of the column was set at 25°C. The sample volume was set at 5 μL, and the wavelength of the detector was set at 210 nm.

RESULTS AND DISCUSSION

Effect of pH on the Precipitation of Cytochrome c and Hemoglobin

The precipitation behavior of cytochrome c and hemoglobin was studied as a function of pH by using 0.3 g/L protein solution, either cytochrome c or hemoglobin. A volume of 0.3 mL of AOT solution was added to yield the AOT concentration of 0.33 mM in the initial protein solution. The molar ratio between AOT and the protein was 14 for cytochrome c and 70 for hemoglobin. The pH of the solution was adjusted by using 1 N HCl or NaOH. The percent precipitation of protein, either cytochrome c or hemoglobin was calculated as

$$\%\text{precipitation} = \left(\frac{1 - C_{P^e} \cdot V_e}{C_{P^i} \cdot V_i}\right) \times 100 \quad (1)$$

where C_{P^i} refers to the concentration of protein, either cytochrome c or hemoglobin, in the initial aqueous solution before the addition of AOT, and C_{P^e} refers to the equilibrium concentration of protein remaining in the aqueous phase after being contacted with AOT. The notations, V_i and V_e indicate the volume of aqueous phase, initially and after the addition of AOT, respectively. The concentrations used in Eq. (1) are expressed in mmol/L.

Figure 1 shows the percent precipitation of protein as a function of the initial pH of the protein solution. The pH change before and after the AOT was added was less than 0.2 pH units. A complete precipitation of cytochrome c was obtained at a pH range between 4.5 and 6.8. An increase in pH from 7 to 8 resulted in a dramatic decrease in the formation of a cytochrome c–AOT complex, and the precipitation was near zero percent. In comparison, the

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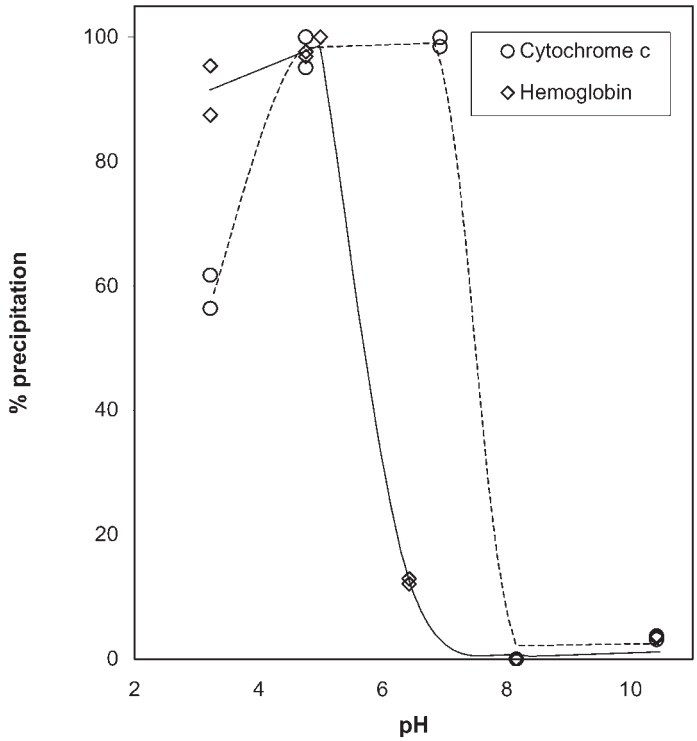

Figure 1. Effect of pH on the precipitation of cytochrome c (○) and hemoglobin (◇); initial aqueous phase, 0.3 g/L either cytochrome c or hemoglobin, pH adjusted by using HCl or NaOH, 0.33 mM AOT.

precipitation of hemoglobin was about 90% or higher, in the pH range between 3 and 5. At pH = 6.4, about 13% precipitation of hemoglobin was obtained. At pH higher than 7, the precipitation was 0%. The results observed in Fig. 1 agree with the electrostatic interaction theory, which considers that the driving force for the removal of proteins from an aqueous phase is the interaction of protein with an oppositely charged surfactant head group.[4,7,11,27,28]

Precipitation and Recovery of Cytochrome c

The usage of AOT for the precipitation of cytochrome c was examined using an initial aqueous solution containing from 0.011 to 0.071 mmol/L

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cytochrome c. The natural pH of this protein solution is in a range of 6.1–6.4. Triplicate samples were used in the experiments, and the errors in the pH measurement were within 0.1 pH units. Since, as shown in Fig. 1, the precipitation of cytochrome c at this pH range was 100%, no pH adjustment was made to the initial cytochrome c solution. A fixed amount of AOT was added to the cytochrome c solutions of varying protein concentration. Figure 2 shows the percent precipitation of cytochrome c as a function of the molar ratio between AOT and the cytochrome c, denoted by *R*. As more AOT was added, the percent mass of precipitated cytochrome c increased. A 100% precipitation was obtained when the molar ratio between AOT and cytochrome c was 11. Below this molar ratio, the concentration of AOT in the remaining aqueous solution was below the detection limit of the HPLC,

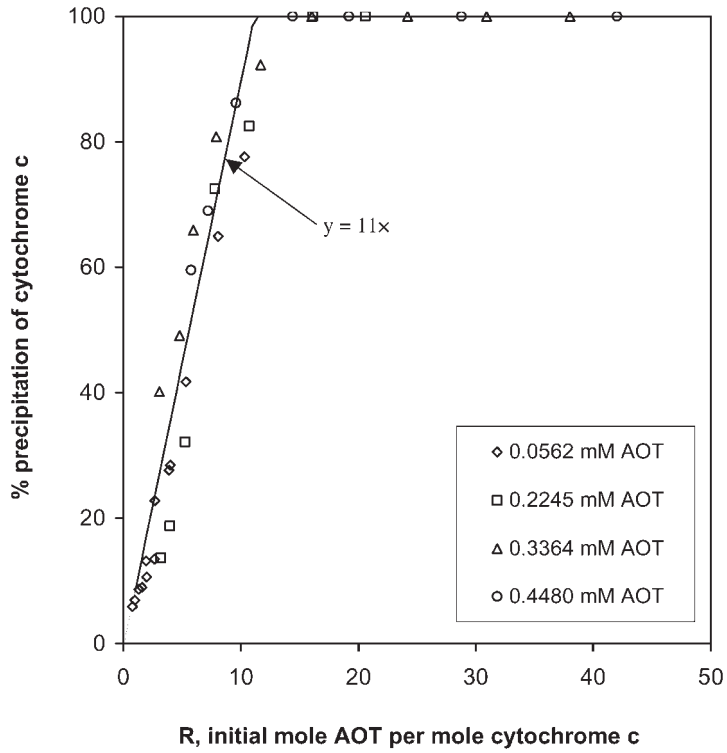

Figure 2. Percent precipitation of cytochrome c as a function of *R*, molar ratio between AOT and cytochrome c: initial aqueous phase, 0.1–1.0 g/L cytochrome c, 0.33 mM AOT.

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indicating that all the AOT was precipitated with the protein. The slope of the line in Fig. 2 is 11, indicating that the precipitation of cytochrome c occurs when 11 moles of AOT associate with 1 mole of protein. Protein molecules combined with less than 11 molecules of AOT do not precipitate. At pH 6, cytochrome c has an overall surface charge of about +11 at the pH range of the solution used,[29] indicating that the AOT required to form 1 mole of water-insoluble cytochrome c–AOT complex is exactly the number of moles required to neutralize the surface charge of the cytochrome c.

Once precipitated, the insoluble cytochrome c–AOT complex was separated from the aqueous phase by centrifugation and dissolved in acetone to recover solid cytochrome c free of surfactant. The efficiency of this recovery process was calculated as

$$\%\text{recovery} = \left(\frac{C_{P^f} \cdot V_f}{C_{P^i} \cdot V_i - C_{P^e} \cdot V_e} \right) \times 100 \tag{2}$$

where C_{P^f} and V_f denote the concentration of protein and the volume of the final aqueous solution used to solubilize the recovered solid cytochrome c. The other symbols are those defined for Eq. (1). The percent recovery of the solid cytochrome c obtained from the cytochrome c–AOT complex was 88 ± 10%. The large standard deviation on the percent recovery was due to the mass loss at the washing in the recovery process as well as the processing time. As the samples are small, a small loss during washing represents a major change in the results. The percent recovery of cytochrome c was a strong function of the processing time used for the precipitation and the recovery process. As soon as the insoluble cytochrome c–AOT complex was formed, the precipitate was collected and dissolved in acetone. When the solid cytochrome c stays in the acetone phase for more than 10 min, the recovered solid showed the different conformational stability, which was monitored using HPLC. The cytochrome c recovered after 30 min showed a severely distorted peak shape, and the percent recovery was calculated to be 0%. When the precipitation and the recovery were conducted within 10 min, the recovered cytochrome c showed the original peak shape, and the percent recovery was about 95%. The AOT concentration in the aqueous solution containing the recovered cytochrome c was below the detection limit of the HPLC.

In comparison, a previous study[16] of the reverse micellar extraction of cytochrome c into an AOT reverse micellar phase showed that a minimum of 260 moles of AOT was required to purify 1 mole of cytochrome c. In the present study, 11 moles of AOT were sufficient to purify 1 mole of cytochrome c. The recovery stage of the reverse micellar extraction method required the addition of a counterionic surfactant[8] or alcohol[30] to

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obtain the same efficiency as the results reported here. Furthermore, the precipitation method described in this study is advantageous since it does not require an organic phase such as isooctane to form reverse micellar phases. The organic phase is expensive and unsafe for industrial applications.[31]

Figure 3 shows the effect of the salt concentration on the precipitation of cytochrome c measured at pH 6. As the salt concentration increases, the percent precipitation of cytochrome c decreased. At 0.1 M NaCl, 100% precipitation was obtained at a molar ratio of 20 between AOT and cytochrome c. An increase in the salt concentration further decreased the precipitation efficiency, and, at 0.6 M NaCl, about 60% precipitation of cytochrome c was obtained at a molar ratio of 50 between AOT and the protein. The increase in the salt concentration results in the competition of chloride ion with the negative-charged head group of AOT to bind with the

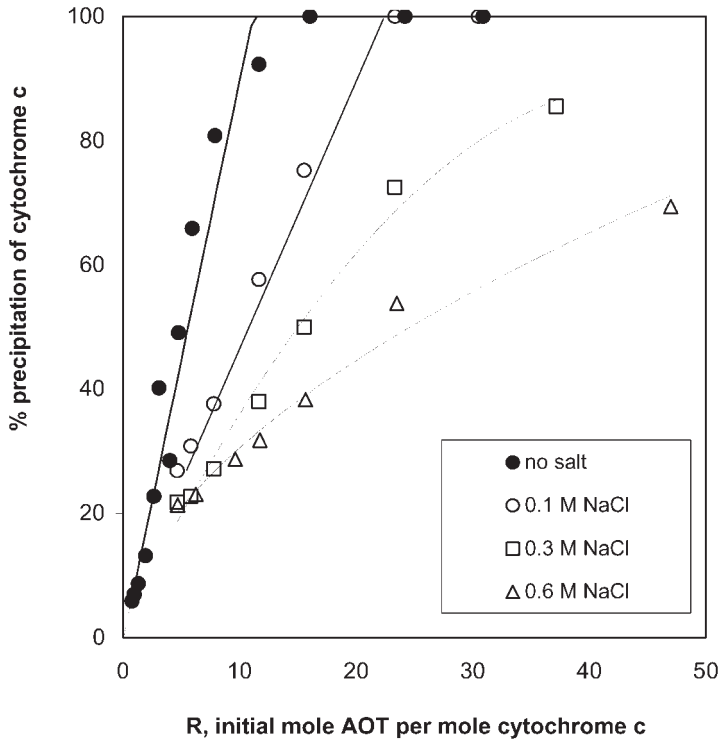

Figure 3. Effect of salt on the precipitation of cytochrome c: initial aqueous phase, 0.1–1.0 g/L cytochrome c, 0.33 mM AOT.

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cytochrome c, resulting in the decrease in the formation of a cytochrome c–AOT complex.

Precipitation and Recovery of Hemoglobin

The initial aqueous solution containing 0.003–0.019 mmol/L hemoglobin was prepared without pH or salt concentration adjustment. The natural pH of the hemoglobin solution was found to be in a range from 6.0 to 6.33, when using triplicate samples. The precipitation of hemoglobin in this pH range was about 13%, as shown in Fig. 1. Thus, the pH of the hemoglobin solution was adjusted to pH = 5.0 by using a potassium phosphate and sodium phosphate buffer solutions. The concentration of phosphate was 0.01 M in the initial hemoglobin solution. The change in pH after the AOT was added was less than 0.1 pH units. Figure 4 shows the percent precipitation of hemoglobin, calculated by using Eq. (1). As the molar ratio between AOT and hemoglobin increased, the percent precipitation of the protein increased. At $R = 30$ or higher, a precipitation efficiency of 100% was obtained. When R was lower than this value, the formation of a hemoglobin–AOT complex decreased, and the concentration of AOT in the aqueous solution was below the detection limit of the HPLC, indicating that all the AOT precipitated with hemoglobin at a molar ratio of 30 between AOT and the protein. Once precipitated, the hemoglobin was recovered from the complex using acetone as a polar solvent to separate the protein from the surfactant. Using Eq. (2), the percent recovery of hemoglobin from the insoluble complex was calculated to be 38 ± 3. The concentration of AOT in the recovered hemoglobin solution was below the detection limit, indicating that the recovered protein was free of surfactant.

In comparison, the extraction of hemoglobin into a reverse micellar system was successful when using a newly developed surfactant, DOLPA.[17] The hemoglobin precipitated at the aqueous–organic interface by using AOT reverse micellar phase was considered to be a loss in that work.[17] The minimum usage of DOLPA to extract hemoglobin was 13,000 moles DOLPA per 1 mole of hemoglobin. Furthermore, the reverse micellar extraction method for hemoglobin using a DOLPA reverse micellar system required long processing times, about 60 h for the extraction and recovery steps. An addition of alcohol during the back extraction process was necessary to remove the hemoglobin from the reverse micellar phase into a fresh aqueous phase to obtain a similar recovery efficiency as this work.[17]

The effect of salt on the precipitation of hemoglobin with AOT is shown in Fig. 5. Unlike cytochrome c, the presence of salt did not have a noticeable effect on the percent precipitation of hemoglobin. The percent precipitation

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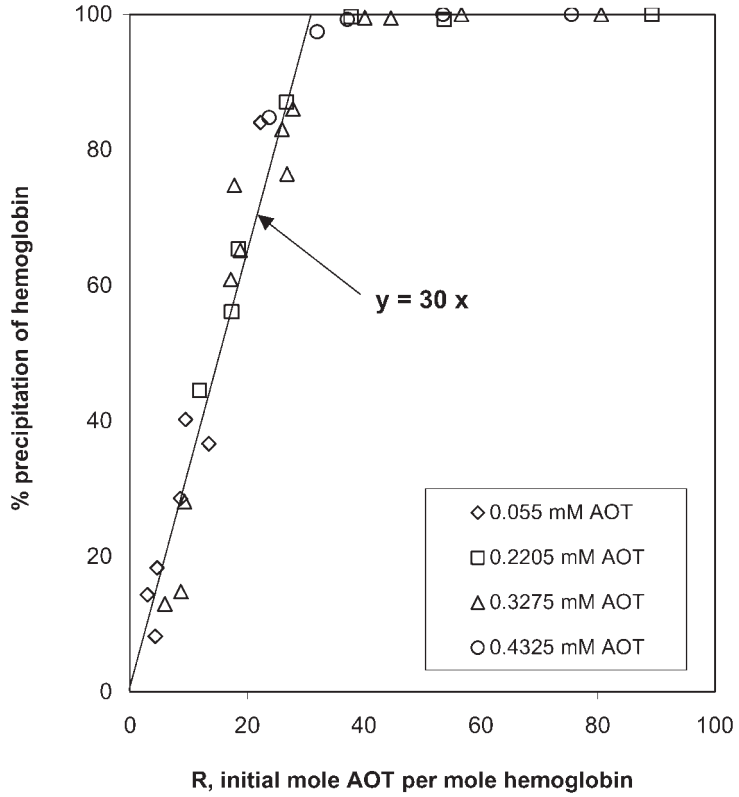

Figure 4. Percent precipitation of hemoglobin as a function of R, molar ratio between AOT and hemoglobin: initial aqueous phase, 0.1–1.0 g/L hemoglobin, no salt added.

of hemoglobin was 100% at $R = 30$ in a salt concentration range from 0 to 1 M NaCl.

CONCLUSIONS

The precipitation and recovery of two transport proteins, cytochrome c and hemoglobin, was successfully achieved using AOT as a precipitating ligand and acetone as a polar solvent. This method has clear advantages over a reverse micellar extraction method. Less usage of surfactant and shorter processing time are required to achieve a similar purification efficiency as that

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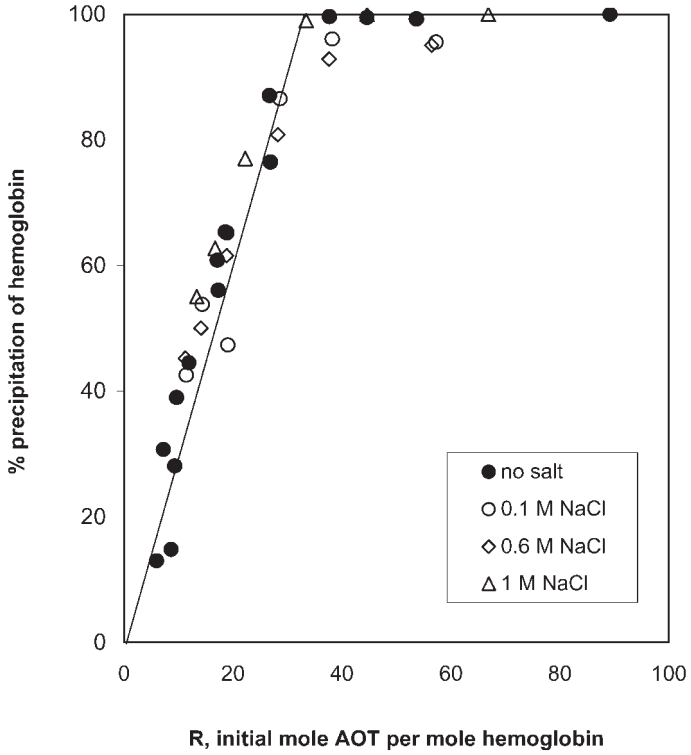

Figure 5. Effect of salt on the precipitation of hemoglobin: initial aqueous phase, 0.1–1.0 g/L hemoglobin, 0.22 mM AOT.

obtained with the reverse micellar extraction method. By adding AOT directly to the aqueous phase, the use of an organic phase, such as isooctane, to form a reverse micellar phase was eliminated.

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