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Protein separation and enrichment by counter-current chromatography using reverse micelle solvent systems

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Abstract

A protein mixture consisting of myoglobin, cytochrome *c*, and lysozyme was separated by high-speed counter-current chromatography using a two-phase aqueous/reverse micelle-containing organic solvent system. About 50% stationary phase retention ratio was obtained in most chromatographic experiments. Separations were manipulated mainly by pH gradients that controlled the electrostatic interactions between the protein molecules and reverse micelles. Separations were further improved by incorporating an ionic strength gradient along with the pH gradient. Control of ionic strength in the aqueous solution helped fine-tune protein partitioning between the stationary and mobile phases. Although non-specific protein interactions affected baseline resolution, recovery of cytochrome *c* and lysozyme reached 90% and 82%. Furthermore, concentration or enrichment of these two proteins was achieved from a large-volume sample load. This technique can potentially be employed in the recovery and enrichment of proteins from large-volume aqueous solutions.

Keywords: Counter-current chromatography; Reverse micelle; Protein separation; Protein enrichment; pH gradient; Ionic strength gradient

1. Introduction

High-speed counter-current chromatography (HSCCC) [1–4] has been applied to preparative separations of various compounds, such as natural products [3], heavy metals [5,6], enantiomers [7,8] and peptides [10,11]. In order to carry out separations in HSCCC, it is usually necessary to search for a two-phase solvent system that provides suitable partition coefficients (*K*) for target compounds. The most commonly used solvent systems are aqueous/organic two-phase systems. Volume ratio adjustment of the components is employed to fine-tune the polarity for both the stationary and mobile phases to optimize the *K* values for the separation. Modifications of the solvent systems for other selectivities include, for example, chelating agents for metal ion separations [5,6] and chiral selectors for enantiomer separations [7,8].

Since most protein molecules are not soluble in organic solvents, special solvent systems are required in CCC separations. Two batch-type liquid–liquid extraction techniques have been developed for protein isolation. The first type involves two-phase aqueous systems that have long been employed in protein extractions. This solvent system is usually prepared by mixing water-soluble polymers, for example poly(ethylene glycol) and dextran, with a phosphate buffer solution. Two aqueous phases are formed after settlement; the upper layer is poly(ethylene glycol)-rich while the bottom layer is rich in dextran [9]. Proteins are distributed between the two aqueous phases according to their differing partition coefficients. Two-phase aqueous systems are being employed in CCC for protein separations [10–12].

The second batch-type technique for liquid–liquid extractions involves applications of reverse micelles. Reverse micelles, formed by surfactants in a non-polar organic solvent mixed with water, provide water-cores in the organic phase that carry water-soluble compounds. Reverse micelles have been applied to large-scale separations of proteins and other biomolecules [13–19]. Anionic surfactant bis(2-ethylhexyl) sulfosuccinate (AOT) molecules form reverse micelle aggregates in isooctane or *n*-hexane. There can be forward and backward extractions: forward extraction (proteins from aqueous solutions to micellar solutions) is achieved by adjusting experimental conditions to let the partition favor the transfer of proteins into the organic micellar phase; and vice versa for backward extraction (proteins from micellar solutions back to aqueous solutions). The major parameter for the partition manipulation is the electrostatic interaction between the protein and charged heads of the ionic surfactant [13]. When the pH of the aqueous solution

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is lower than the isoelectric point (p*I*) of the protein, protein molecules carry positive charge and tend to enter the micelles composed of anionic surfactant through mixing. While another buffer solution with a pH usually higher than the protein's p*I* is brought into contact with the protein micellar phase, it will cause the protein molecules to transfer back to the aqueous solution. The surfactant concentration [20], ionic strength [13,14], charge distribution [21], and types of salt ions [22] can help control both the forward and backward extractions. However, the backward extraction rate of protein from reverse micelle is usually extremely slow [23–25] and the recovery may be diminished by the formation of irreversible protein–surfactant complexes [21,26,27]. It is challenging to find optimum conditions for protein mixture separations. Previous research has dealt with purification and enrichment of single protein compounds. Protein separation was also studied using reverse micellar liquid membranes. Under manipulation of pH and KCl concentration, selected protein molecules were able to be transported across a very thin liquid membrane from one aqueous phase to another aqueous phase [28].

We employed micellar solutions, for the first time, in protein separation using HSCCC. Both pH and KCl concentration gradients were applied in the separations. In addition to efficiency of separation, enrichment of protein was also examined at the same time. This study demonstrated the feasibility of performing protein separation and enrichment in one chromatographic run in HSCCC.

2. Experimental

2.1. Reagents and preparation of sample solutions and solvent systems

HPLC-grade *n*-hexane, acetonitrile and tris(hydroxymethyl)–aminomethane (Tris) were purchased from Tedia (Fairfield, OH, USA). AOT, cytochrome *c* (horse heart, p*I* = 9.6, MW = 12,200), myoglobin (equine skeletal muscle, p*I* = 7.0, MW = 16,900), and lysozyme (chicken egg, p*I* = 11, MW = 14,300) were all purchased from Sigma (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was obtained from Alfa Aesar (Ward Hill, MA, USA), while dipotassium phosphate (K_2HPO_4) and potassium chloride were from Showa (Tokyo, Japan). Water was purified in a Milli-Q apparatus (Millipore, Bedford, MA, USA).

The stationary phase was prepared by dissolving AOT in *n*-hexane to make a 0.1 M solution. The mobile phases were composed of Tris–HCl and K_2HPO_4 with different concentrations of KCl. Mobile phase A (0.05 M Tris–HCl, pH 7.35 with 0.1 or 0.2 M KCl) was either used as the solvent for equilibration or for sample preparation. Sample solutions were prepared by dilution of a mixture of myoglobin, cytochrome *c*, and lysozyme, 10 mg/mL each in the mobile phase A. Mobile phase B (0.05 M K_2HPO_4, pH 12.0 with 0.1 or 0.5 M KCl) provided ending conditions of the gradient. Since the gradient elution used two different solutions, the equilibration was conducted by running the starting mobile phase through the separation coil filled with the stationary phase during centrifugation.

2.2. Instrumentation and procedures

The chromatograph used was a Model CCC-1000 (Pharma-Tech Research, MD, USA) HSCCC, mounted in a temperature-controlled oven set at 20 °C. It contained three spool-shape column holders; only one holder was coiled with a 19-m long × 1.6-mm I.D. PTFE (polytetrafluoroethylene) tubing, with a total volume of 38 mL. The CCC gradient elution was achieved using a LabAlliance Series III (State College, PA, USA) dual solvent pumping system. The column was first filled with the stationary phase while the rotation speed of CCC was set at 800 rpm. Buffer A was then pumped in at 1 mL/min until the hydrodynamic equilibrium was reached. Protein sample was injected through a six-port injection valve with a coil of 5 or 38 mL and a gradient elution followed at a flow rate of 1 mL/min in the head-to-tail mode. The effluent was monitored by a Bio-Rad Model 1801 UV detector (Hercules, CA, USA) at 280 nm and 5 mL fractions collected manually.

Each collected fraction was analyzed by a HPLC system equipped with the LabAlliance Series III pumping system, a PLRP-S column (150 mm × 4.6 mm, 300 Å, 15 μm) purchased from Polymer Laboratories (Amherst, MA, USA), and the Bio-Rad Model 1801 UV detector. The HPLC analysis was performed with a linear gradient at a flow rate of 1 mL/min from 0 to 100% mobile phase D in 20 min (mobile phase C: 20% ACN and 0.1% TFA in water; mobile phase D: 80% ACN and 0.1% TFA in water). On-line signals for both CCC and HPLC were acquired using a SISC version 3.1 Chromatography data station (Taipei, Taiwan). Protein standards were run under the same conditions to create a calibration curve and the protein concentrations in the fractions were quantitatively determined by peak area using the SISC data station.

3. Results and discussion

3.1. Separation using pH gradient

A pH gradient elution was examined in the first experiment. At the injection of sample, a mobile phase of lower pH was delivered in order to provide adequate protein partitioning into the micelles. The pH of the mobile phase was increased from 7.35 to 12.0. The mobile phase was made with a 0.1 M KCl solution—the minimum ionic strength to ensure adequate phase separation according to the literature [13]. Potassium ions were used in this study as they have a faster settling time for phase separation in water–AOT–isooctane systems according to published reports [29].

The chromatogram and the concentrations for the three proteins in the collected fractions are illustrated in Fig. 1. The saturated signals in Fig. 1a occurred due to the turbidity caused by stationary phase depletion. At first, the effluent was very clear and no organic phase was observed at the column outlet in ~50 min after the sample injection. Destabilization of the microemulsion system might occur after the protein molecules were partitioned between the aqueous and the reverse micelle phases. Nevertheless, the collected fractions were analyzed using HPLC. The CCC elution began with pH 7.35 at which

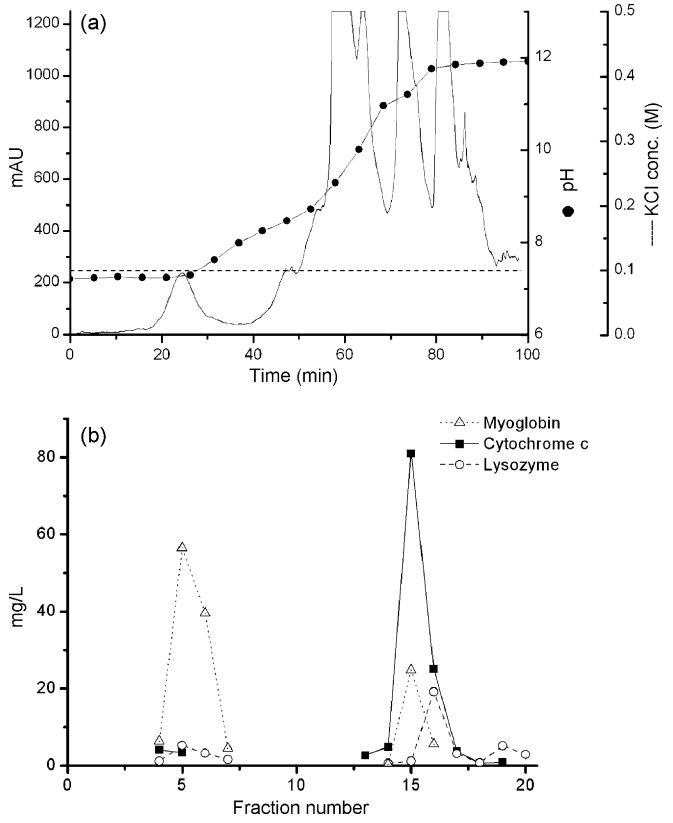

Fig. 1. Separation using pH gradient elution with 0.1 M KCl. Experimental conditions: stationary phase, 100 mM AOT in *n*-hexane; mobile phase A, 0.05 M Tris–HCl, 0.1 M KCl at pH 7.35; mobile phase B, 0.05 M K_2HPO_4, 0.1 M KCl, pH 12; sample concentration, 500 mg/L, respectively, for myoglobin, cytochrome *c*, and lysozyme in 5 mL; flow-rate 1 mL/min; column volume 38 mL; phase retention ratio 42%. (a) Chromatogram obtained using on-line UV detector monitored at 280 nm; pH measured in each 5 mL fraction; (b) protein concentrations of 20 fractions acquired by HPLC analysis.

myoglobin stayed preferably in the mobile phase as a result of electrostatic repulsion against the anionic AOT reverse micelles. Fractions 4–7, shown in Fig. 1b, contained mainly myoglobin as the sample injection solvent front arrived at the column outlet. In addition, a certain amount of cytochrome *c* and lysozyme was eluted out along with myoglobin. As the pH became close to 10, cytochrome *c* molecules started to appear in the mobile phase, and were collected mainly in fractions 14–17. However, considerable amount of myoglobin and lysozyme molecules also emerged with cytochrome *c* in these fractions. Lysozyme recovery was markedly low even when the pH rose up to 12. Furthermore, cytochrome *c* loss was observed in fractions 12–14 along with some organic phase droplets floating on the collected aqueous solutions. Although these three proteins were not well resolved, the major portions of them emerged on the chromatogram in an order, as expected, essentially according to their p*I* values.

A separation was carried out using hexane/buffer solvent system, without adding AOT to hexane. The experiment was performed in the conditions exactly same as the previous run. Only a large peak appeared at the solvent front. The collected fractions of this peak were also analyzed using HPLC. The results (not shown) indicated that all the proteins were collected at the solvent front without being separated. This test ensured that the separation was truly achieved by the presence of the micellar phase in the stationary phase.

3.2. Separation using pH gradient with higher ionic strength

Since significant phase depletion was observed, we then tried to improve the mobile/stationary phase stability by raising the KCl concentration of the mobile phase to 0.2 M. The results are shown in Fig. 2. The effluent stayed clear in most of the process until the elution time reached to ∼65 min. As can be seen in Fig. 2b, myoglobin remained essentially un-retained and was collected at early stage as before and was still cross-contaminated with some cytochrome *c* and lysozyme. The major portion of cytochrome *c* appeared, however, earlier than before. Apparently, partitioning of cytochrome *c* into the reverse micelle phase declined due to the higher ionic strength. Higher concentration of chloride counter-ions competed with the positive charge of protein binding to AOT, and thus lowered the protein partitioning in the stationary phase [18]. The distribution diagram of the collected proteins was effectively changed especially for cytochrome *c* compared with the previous run using 0.1 M KCl. As can be seen, higher ionic strength helped not only

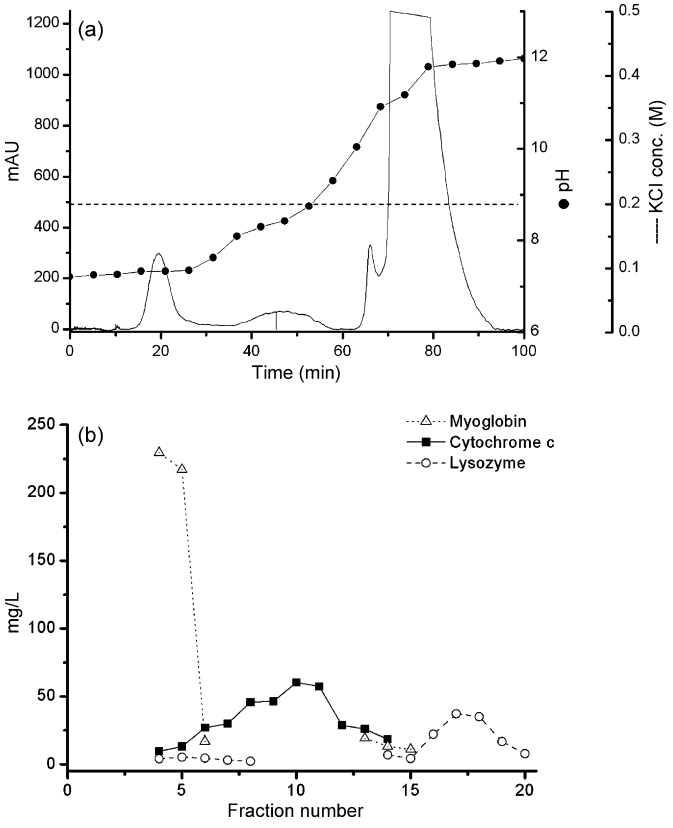

Fig. 2. Separation using pH gradient elution with 0.2 M KCl. Experimental conditions: stationary phase, 100 mM AOT in *n*-hexane; mobile phase A, 0.05 M Tris–HCl, 0.2 M KCl at pH 7.35; mobile phase B, 0.05 M K_2HPO_4, 0.2 M KCl at pH 12; sample concentration, 500 mg/L, respectively, for myoglobin, cytochrome *c*, and lysozyme in 5 mL; flow-rate 1 mL/min; column volume 38 mL; phase retention ratio 50%. (a and b) as in Fig. 1.

to stabilize the stationary/mobile phase equilibrium but also to improve the resolution between cytochrome *c* and lysozyme. Unfortunately, the total amount of lysozyme from fractions 14–20 was still merely ~30% of the injected sample. The off-scale signals after 65 min were due to turbidity. The stationary phase was pushed out with nitrogen gas after the elution was stopped at 100 min. White precipitate observed in the stationary phase was considered responsible for the low recovery of lysozyme and was probably denatured protein.

3.3. Separation using pH and ionic strength gradients simultaneously

Since the higher ionic strength appeared to improve the separation resolution and reduce phase depletion effectively, we planned to further increase the ionic strength. Accordingly, we tried to combine ionic strength with the pH gradients. The mobile phase with 0.2 M KCl concentration was initially pumped into the column to allow adequate partition of the proteins. The pH as well as the KCl concentration was increased during the elution. The chromatogram, shown in Fig. 3a, reveals three well-separated peaks. The gradually increasing KCl did not affect the mobile/stationary phase stability. As a result, the effluent stayed very clear during the elution. The un-retained myoglobin overlapped with cytochrome *c* and very small amount of lysozyme. Then most of cytochrome *c* was collected from fractions 7–13, as shown in Fig. 3b. Except for a small amount of myoglobin appearing in fraction 14, lysozyme was recovered from fractions 15–20 as the KCl concentration varied from 0.4 to 0.5 M. Using the pH and KCl gradients in this experiment significantly enhanced the separation of cytochrome *c* and lysozyme [14]. Although the myoglobin retention in reverse micelle was unfavorable in terms of electrostatic interaction above pH 7, strong hydrophobic interaction with the reverse micelles might have strictly exerted a force on some myoglobin molecules until pH ~ 10.5 and KCl ~ 0.35 M. However, this small myoglobin peak would not affect the collection of most fractions for either cytochrome *c* or lysozyme during the elution. The recovery of lysozyme was successfully enhanced from 30% of previous run to 90% in this experiment due to the high KCl concentration in the later stage of the elution [14].

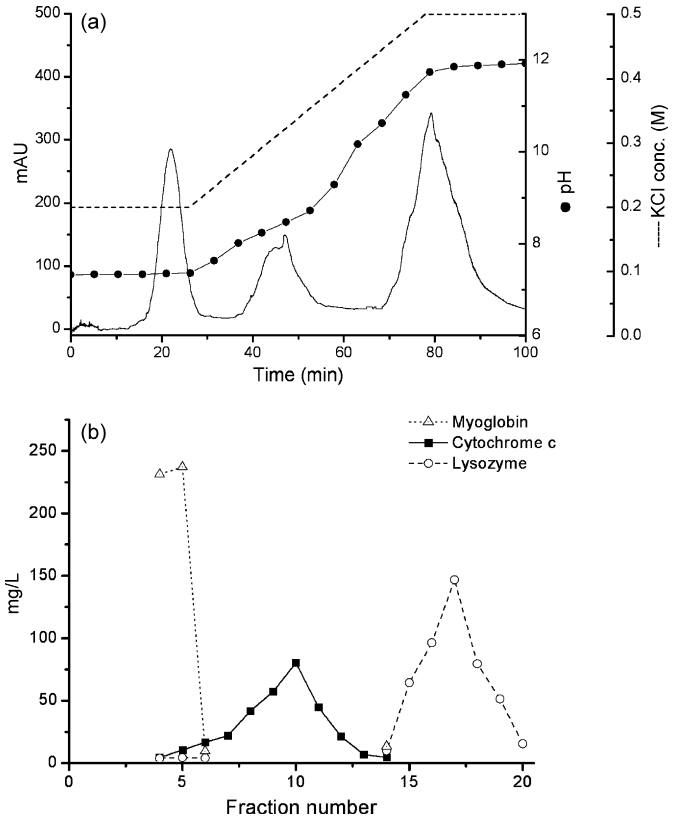

Fig. 3. Separation using pH and KCl concentration gradient elution. Experimental conditions: stationary phase, 100 mM AOT in *n*-hexane; mobile phase A, 0.05 M Tris–HCl, 0.2 M KCl at pH 7.35; mobile phase B: 0.05 M K_2HPO_4, 0.5 M KCl at pH 12; sample concentration, 500 mg/L, respectively, for myoglobin, cytochrome *c*, and lysozyme in 5 mL; flow-rate: 1 mL/min; column volume 38 mL; phase retention ratio 50%. (a) Chromatogram obtained using on-line UV detector monitored at 280 nm; pH measured in each 5 mL fraction; KCl concentration computed using the fractions of mobile phases A and B; (b) as in Fig. 1.

3.4. Protein enrichment and separation using pH and ionic strength gradients

In order to carry out a preparative run for a sample of large volume, we tried a sample injection (300 mg/L of each protein) in a total volume of 38 mL which equalled the total volume of the HSCCC column. The program of the gradient elution was the same as the previous run. Fig. 4a shows the on-line signal of an initial plateau-like region followed by two other separated peaks. The first signal lasted a little more than 40 min, i.e. 40 mL elution volume, comparable to the sample volume. The broad flat peak corresponded to the elution of myoglobin. After the baseline lowered, the pH and KCl concentration gradients were started. Most cytochrome *c* was collected from fractions 14–18. The equilibrium between the stationary and mobile phases stayed undisturbed during the sample injection and the whole elution. The second and the third peaks were quite large and well resolved. As can be seen in Fig. 4b, fractions 7–12 contained mainly myoglobin but still blended with cytochrome *c*. In fractions 19 and 20, some myoglobin appeared again in which cytochrome c and lysozyme were just resolved. The concentrations of cytochrome *c* were 439, 1030, and 361 mg/L in fractions 15–17. The protein concentrations in these fractions increased over the injected samples. In addition, lysozyme was mainly collected in fractions 21–24. The recovery concentrations of lysozyme in fractions 21 and 22 reached over two times that of the sample solution. The stationary phase was removed with nitrogen gas after the separation. Some precipitate (much less than on experiments 1 and 2) at the interface between the upper and lower phases was observed. Fortunately, the precipitate did not create any phase depletion during the elution. Protein recovery in the total fractions was 83% for myoglobin, 90% for cytochrome *c*, and 82% for lysozyme. The recovery for cytochrome *c* reached 79% in fractions 13–18 and that for lysozyme reached 67% in fractions 21–24. Considering that analyte concentration is usually diluted in chromatographic elution, the measured concentrations in peak fractions were very

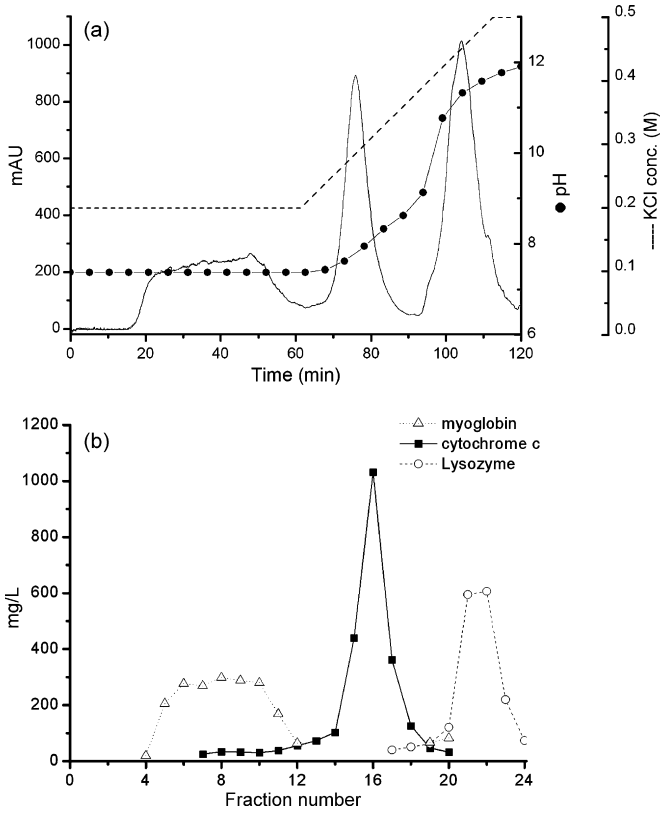

Fig. 4. Protein separation and enrichment using pH and KCl concentration gradient elution. Experimental conditions: stationary phase, 100 mM AOT in *n*-hexane; mobile phase A, 0.05 M Tris–HCl, 0.2 M KCl at pH 7.35; mobile phase B, 0.05 M K_2HPO_4, 0.5 M KCl at pH 12; sample concentration, 300 mg/L, respectively, for myoglobin, cytochrome *c*, and lysozyme in 38 mL; flow-rate 1 mL/min; column volume 38 mL; phase retention ratio 50%. (a and b) as in Fig. 3.

high, over 3-fold for cytochrome *c* and 2-fold for lysozyme. The feasibility of simultaneous separation and enrichment was demonstrated in this experiment. Since myoglobin was non-retained, no enrichment was attained, and a relatively broad signal was observed as expected.

4. Conclusions

Reverse micelle solvent systems, KCl-containing buffer solution/AOT-containing hexane, are hereby applied in protein separation and enrichment. Myoglobin, cytochrome *c*, and lysozyme with relatively close p*I*'s (7.0, 9.6 and 11, respectively) were investigated. Separation efficiency was significantly enhanced by performing pH and ionic strength gradient elution simultaneously. A sample of a relatively large amount (in a volume equal to the column capacity) was examined using the optimized experimental conditions. Although complete resolving of the three compounds was not achieved due to non-specific protein/micelle interactions, cytochrome *c* and lysozyme were recovered with high enrichment. The current technique may provide separation and enrichment simultaneously for proteins from aqueous solutions.

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