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Evaluation of the performance of protein separation in figure-8 centrifugal counter-current chromatography

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Abstract

The performance of protein separation using the figure-8 column configuration in centrifugal counter-current chromatography was investigated under various flow rates and revolution speeds. The separation was performed with a two-phase solvent system composed of polyethylene glycol 1000/potassium phosphate each at 12.5% (w/w) in water and with lysozyme and myoglobin as test samples. In order to improve tracing of the elution curve, a hollow fiber membrane dialyzer was inserted at the inlet of the UV detector. The results showed that the retention of stationary phase (Sf) and resolution (Rs) increased with decreased flow rate and increased revolution speed. The highest Rs of approximately 1 was obtained at a flow rate of 0.01 mL/min under a revolution speed of 1200 rpm with a 3.4 ml capacity column.

Keywords

Centrifugal counter-current chromatography; figure-8 column; protein separation; polymer phase system; lysozyme; myoglobin; hollow fiber membrane dialyzer

1. Introduction

Hydrostatic counter-current chromatography (CCC) using a coiled column mounted around the periphery of the centrifuge bowl was developed for improving the performance of analytical CCC separation. The hydrodynamic CCC system, which has been widely applied for the separation and purification of biological samples, fails to perform an efficient analytical separation due to a diminished Archimedean screw effect in small diameter tubing resulting from a strong cohesive force between the inner and outer walls of the tubing which lowers the stationary phase retention in the column [1–6]. In contrast, hydrostatic CCC provides a stable centrifugal force field which facilitates counter-current movement of the two phases through a narrow-bore coiled column which provides an analytical hydrostatic CCC system that can produce a highly efficient separation as reported earlier [7–10].

In our previous studies, a series of novel column designs have been introduced to further improve the retention of the stationary phase and peak resolution under various flow rates and revolution speeds, including triangular coiled [11], zigzag [12, 13], saw tooth [14] and

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figure-8 columns [15]. When the lower phase was mobile, S_f for low viscosity aqueous/organic systems increased from roughly 35% to 45% and R_s increased from roughly 1.3 to 1.7 as the columns were changed from helically coiled to zigzag to saw tooth to figure-8 configurations [16]. Recently, we found that when the coiled column is mounted at different angles against the radially acting centrifugal force, the separation performance was greatly altered. In general, as the column angle against the centrifugal force was decreased, the retention of the stationary phase increased while peak resolution decreased and the retention of the stationary phase became maximum with the column mounted parallel to the centrifugal force [17]. Based on these findings, the performance of the figure-8 column was investigated in a centrifugal hydrostatic CCC for the separation of dipeptide mixtures and DNP-amino acid mixtures by changing the angle between the column axis and centrifugal force. The results indicated that the highest R_s was obtained when the figure-8 column was parallel to the radially acting centrifugal force field, [18].

In the present study, this figure-8 centrifugal CCC was applied for protein separation with a polymer two-phase solvent system, because no satisfactory result has been reported in protein separation using analytical hydrostatic CCC.

2. Experimental

2.1. Apparatus

The present study uses a rotary-seal-free centrifuge fabricated by Pharma-Tech Research Corporation, Baltimore, Maryland, USA. It has an aluminum rotary plate measuring about 34 cm in diameter which holds a separation column. Each column unit is made by hooking a 0.46 mm ID FEP (Fluorinated ethylene propylene) (Zeus Industrial Products, Orangeburg, SC, USA) tubing onto a pair of upstanding screws on the rotary plate making plural layers of figure-8 loops. A total of 18 column units are serially connected with transfer tubing to form a figure-8 separation column with a total capacity of 3.4 ml. Each figure-8 column is mounted around the periphery of the rotary platform arranged equally spaced with the acting centrifugal force field [18]. Each terminal of the column is connected to a PTFE flow tube (0.46 mm I.D., Zues Industrial Products) with a set of tubing connectors (Upchurch Scientific, Palm Spring, CA, USA). The pair of flow tubes is put together and passed through the center of the central shaft downward and the hollow horizontal shaft of a miter gear, then led upward into the vertical hollow tube support, and finally exits the centrifuge from the center of the upper plate where it is tightly held with a pair of clamps [19]. The total space in the feed and return tubing (dead volume) is approximately 0.5 ml.

A metering pump (Shimadzu LC-10ADVP, Columbia, MD, USA) was used for pumping the solvents. In order to improve the tracing, a hollow fiber membrane dialyzer (20 cm, spectrum laboratories, Inc., Rancho Dominguez, CA) was inserted online at the inlet of the detector. The outer dialyzer chamber was flushed with water at a flow rate of 0.01 ml/min. The effluent through the hollow fiber membrane was continuously monitored with a UV detector (LKB Instruments, Stockholm, Sweden).

2.2. Reagents

PEG (polyethylene glycol) 1000, dibasic potassium phosphate, lysozyme (chicken egg), and myoglobin (horse skeletal muscle) were obtained from Sigma Chemicals, St. Louis, MO, USA.

2.3. Partition Coefficient Measurement

The partition coefficients (K_U) of each sample in the two-phase solvent system were determined using the conventional test tube method with a UV spectrophotometer (Genesis

10 UV, Thermo Spectronic, Rochester, NY, USA) at 280 nm. The absorbance of the upper phase was recorded as A_U and that of the lower phase as A_L . Then the K_U value was calculated according to the following equation: $K_U = A_U/A_L$ [20].

2.4. Two-phase Solvent Systems and Sample Solutions

In the present study, a typical polymer two-phase solvent system composed of 12.5% (w/w) PEG1000 and 12.5% (w/w) dibasic potassium phosphate in water (PEG-DPP) was used to separate a set of test samples. The solvent mixture was thoroughly equilibrated in a separatory funnel by vigorous shaking and degassing several times, and the two phases separated shortly before use [21]. The sample mixture was dissolved in the upper phase at a suitable concentration and filtered before application to the column.

2.5. Separation Procedure

In each separation, the column was entirely filled with the stationary phase (upper phase), followed by sample injection, and the column was rotated at 1000 rpm while the mobile phase was pumped into the column at a given flow rate. The effluent from the outlet of the column was continuously monitored with a Uvicord IIS (LKB, Stockholm, Sweden) at 280 nm and the elution curve was traced using a strip-chart recorder (Pharmacia, Stockholm, Sweden). After the desired peaks were eluted, the run was terminated and the contents was pushed out by pressurized air into a graduated cylinder to determine the volume of the stationary phase retained in the column. The stationary phase retention (S_f) was computed by dividing the volume of the retained stationary phase by the column volume.

2.6. Evaluation of Partition Efficiency

The partition efficiency of the separation column was evaluated by computing theoretical plate number (N) for each peak and the peak resolution (R_s) between the peaks using the following conventional equations:

$$N=(4t_r/W)^2 \quad (1)$$

$$R_s=2(t_2 - t_1)/(W_1+W_2) \quad (2)$$

where t_R and W indicate the retention time and the baseline peak width in Eq 1 and those for the specified peaks in Eq 2, respectively.

3. Results and discussion

Our previous studies have shown that the performance of centrifugal CCC was improved by the novel design, figure-8 column [15]. When the column was parallel to the acting centrifugal force field, the best R_s was obtained in both DNP-amino acid and dipeptide separations, each done with a suitable two-phase solvent system. We speculated that the double loop of the figure-8 column provides two separate segments each producing droplet flow of mobile phase to increase the interface area for mass transfer and improve the peak resolution (R_s) [18]. In this study, the protein samples were separated by this figure-8 centrifugal hydrostatic CCC system. K_U values of myoglobin and lysozyme in the solvent system, PEG-DPP, were 0.51 and 1.69, respectively.

3.1. The tracing improvement by hollow fiber membranes

In the traditional counter-current chromatography separation, the effluent was directly monitored with a UV detector. However, when the polymer two-phase solvent system, PEG-DPP, was used for protein separation, the tracing was disturbed by steady carryover of the stationary phase. Fig. 1A shows that the lysozyme and myoglobin were separated by figure-8 centrifugal counter-current chromatography using PEG-DPP. The flow rate was 0.01 mL/min and the revolution speed was 1000 rpm. However, the excessive noise in the output signal made it difficult to judge the separation of the sample peaks. To solve this problem, a hollow fiber membrane dialyzer was inserted in the outlet stream prior to the detector. Fig 1B clearly shows the chromatogram for protein separation by centrifugal CCC with a figure-8 column using the hollow fiber membrane dialyzer.

3.2. Performance of protein separation under various flow rates

Fig. 2 shows the performance of protein separation, lysozyme and myoglobin, using the PEG-DPP solvent system with various flow rates (0.01–0.06 mL/min) by figure-8 centrifugal counter-current chromatography. The revolution speed was 1000 rpm. The results indicate that both peak resolution (R_s) and retention of stationary phase (S_f) increase with decreasing flow rate. When the lowest flow rate of 0.01 mL/min was used for protein separation, S_f was over 35% and R_s was 0.95. This suggests that the higher efficiency of protein separation can be obtained with lower flow rate. The lower flow rate can provide enough time to produce a prolonged droplet flow of the mobile phase, which provides a large interface area to enhance the mass transfer process. This may explain high performance obtained by a lower flow rate of the mobile phase in the figure-8 column [18].

3.3. Performance of protein separation under various revolution speeds

Fig. 3 shows the performance of protein separation, lysozyme and myoglobin, with PEG-DPP solvent system with various revolution speeds using figure-8 centrifugal counter-current chromatography at a flow rate of 0.01 mL/min. In all separations R_s and S_f increases with the applied revolution speeds from 800 rpm to 1200 rpm. When the revolution speed was 1200 rpm, S_f was over 37% and R_s was approximately 1. This indicates that higher efficiency of the protein separation can be attained with a higher revolution speed. The higher revolution speed would produce smaller droplets which increase both the interface area and the retention volume of the stationary phase, so that the mass transfer process was enhanced. Along with a slower flow rate, this may explain improved partition efficiency of figure-8 column at a high revolution speed.

4. Conclusions

Overall results of our present studies indicate that figure-8 centrifugal counter-current chromatography can be used for protein separation where signal tracing was remarkably improved by passing the effluent through a hollow fiber membrane dialyzer. The efficiency of protein separation is enhanced by lower flow rate and/or higher revolution speed.

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Figure 1. Chromatograms for protein separation by centrifugal counter-current chromatography using figure-8 column. Sample: lysozyme (chicken egg), myoglobin (horse skeletal muscle); solvent system: PEG-DPP; flow rate: 0.01 mL/min; rotational speed: 1000 rpm; A) without hollow fiber membrane dialyzer; B) with hollow fiber membrane dialyzer to improve the tracing.

Figure 2.

The performance of the protein separation at various flow rates by centrifugal counter-current chromatography using the figure-8 column. Samples: lysozyme and, myoglobin; solvent system: PEG-DPP; rotational speed: 1000 rpm; capacity: 3.4 mL.

Figure 3.

The performance of the protein separation at various rotational speeds by centrifugal counter-current chromatography using figure-8 column. Samples: lysozyme, myoglobin; solvent system: PEG-DPP; flow rate: 0.01 mL/min; capacity: 3.4 mL.