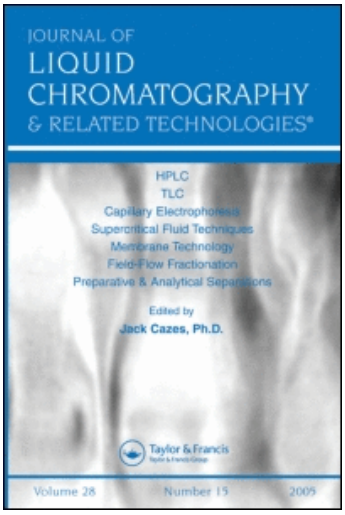

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SEPARATION OF PROTEINS USING AQUEOUS TWO-PHASE SYSTEMS IN ECCENTRIC MULTI-LAYER COIL PLANET CENTRIFUGE

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ABSTRACT

The performance of the eccentric coil planet centrifuges for separating proteins has been tested using polyethylene glycol (PEG)/potassium phosphate/water two-phase systems. The dependency of the separation efficiency on the flow rate of mobile phase and the rotational speed of centrifuge has been studied in the present investigation. The resolution of peaks is decreased with the increase of flow rate of mobile phase and is increased up to a maximum with the increase of rotational speed. The results of this study demonstrate that the eccentric coil planet centrifuge is useful for separation of proteins with aqueous two-phase systems.

INTRODUCTION

Aqueous two-phase systems have been discovered since 1896 and have been studied in detail by P. Å. Albertsson[1,2]. Such systems appeared suitable for separating biological materials with the main advantages of high biocompatibility, good resolution and activity yield, and easy of scale-up.

Some fundamental studies of biomolecule partitioning in aqueous two-phase systems were published somewhere[3,4]. The attempts to use the aqueous two-phase systems in conjunction with high-speed countercurrent chromatography have been made for separating proteins[5]. Several kinds of efficient coil planet centrifuges invented and developed by Dr. Y. Ito have been commercially available[6]. The present study concentrates on investigation of the performance of eccentric coil planet centrifuge for protein separation using PEG/potassium phosphate/water two-phase systems[7]. A series of experiments was conducted to determine phase stability, back pressure, stationary phase retention and separation efficiency by varying the rotational speed and the mobile phase flow rate.

EXPERIMENTAL

Apparatus

Two models of eccentric multi-layer coil planet centrifuge, CCC-1000 and CCC-800, made by Pharma-Tech Research Corp., Baltimore, MD, U.S.A. are used in present studies. The rotary frame of CCC-1000 holds three column holders placed 120° from each other at a distance of 76 mm from the central axis of the centrifuge. Each holder symmetrically supports eight identical column units at a distance of 35 mm from the holder axis. Thus, the ratio of the planet radius to the orbital radius, β, is 0.46 within the range of suitable functioning. Each column unit is made of 2.6 mm I.D. polytetrafluoroethylene (PTFE) tubing by winding it onto a metal rod with a diameter of 5 mm forming two coiled layers. There are 40 helical turns for each rod and the total number of turns in the whole column in CCC-1000 is 960. The total capacity of the entire column measured 110 ml. The rotational speed of CCC-1000 ranges from 0 to 1000 rpm ($85 \times g$). The structure of model CCC-800 is similar to that of CCC-1000. Three coil holders are held on the rotary frame at a distance of 102 mm from the central axis of the centrifuge. Ten identical column units were mounted symmetrically around each coil holder at a distance of 45 mm from the

TABLE 1. Phase Compositions of Systems Used in the Present Studies

System	Phase Compositions (% w/w)					
	Total System		Upper Phase		Lower Phase	
	PEG	Phosphate	PEG	Phosphate	PEG	Phosphate
pH 7.0	10.1	10.9	22.3	5.7	1.0	15.7
pH 5.5	13.7	12.3	16.6	11.8	2.2	19.3

holder axis. Thus, the β is 0.44. There are 50 helical turns made of 2.6 mm I.D. PTFE tubing with a 8 mm helical diameter in each column unit. The total number of helical turns in the whole column in model CCC-800 is 1500 and the capacity of the column is 430 ml. The revolution speed of CCC-800 ranges from 0 to 800 rpm ($73 \times g$).

A Milton Roy MiniPump was used to pump the solutions, an LKB UV detector (2238 UVICORD S II) to monitor the absorbance and an LKB 2210 recorder to record the chromatogram.

Reagents

PEG 3,400, dibasic and monobasic potassium phosphate (K_2HPO_4 and KH_2PO_4) and hydrochloric acid were purchased from Aldrich Chemical Company, Inc., Milwaukee, WI, U.S.A. Bovine serum albumin (BSA) and lysozyme were obtained from Sigma Chemical Company, St. Louis, MO, U.S.A.

Procedure

The phase compositions and pH values of the PEG 3,400/potassium phosphate/water two-phase systems employed in the present studies are listed in Table 1. The system of pH 7.0 was prepared by dissolving 101 g of PEG 3,400, 70.35 g of K_2HPO_4 and 38.65 g of KH_2PO_4 in 790 g of distilled

TABLE 2. The Partition Coefficients of BSA and Lysozyme in the Systems

	K	
	pH 7.0	pH 5.5
BSA	0.01	0.02
Lysozyme	0.36	3.68

water. The system of pH 5.5 was prepared by dissolving 137 g of PEG 3,400, 79.38 g of K_2HPO_4 and 43.62 g of KH_2PO_4 in 740 g of distilled water, then adjusting the pH to 5.5 by adding 2 M hydrochloric acid. The systems were thoroughly equilibrated at room temperature and the upper and lower phases were then separated. The sample solution consisted of 10 mg each of BSA and lysozyme dissolved in 1 ml of the mobile lower phase.

The experiment was initiated by filling the entire column with the stationary upper phase. Then, the centrifuge was rotated at a desired speed while the mobile lower phase was pumped into the column at a desired flow rate. The sample solution was injected through a loop injection system assembled from Rheodyne sample injection valve with a loop volume of 0.25 ml for CCC-1000 and 0.50 ml for CCC-800. The injection was delayed for an hour or so after beginning mobile phase flow to allow a steady base line to be achieved before early sample constituents emerge. The effluent from the outlet of column was continuously monitored by an LKB detector at 280 nm and the chromatogram was recorded by an LKB recorder. A coiled 1.6 mm I.D. PTFE tubing with a length of 0.5 to 1 m was inserted between the CCC and monitor and immersed in an ice bath to lower the noise level of chromatograms. After the two peaks were eluted, the centrifuge was stopped; and by pumping the mobile phase continuously into the column, the column contents were collected into a graduated cylinder to measure the volume of the stationary phase retained in the column.

The partition coefficients of BSA and lysozyme in the systems are listed in Table 2. 10 mg of BSA or lysozyme was added into a 15 ml

TABLE 3. The Resolution of Peaks and the Retention of Stationary Phase Using System of pH 7.0 in Model CCC-1000

Rotation (rpm)	Flow Rate (ml/hr)	Back Pressure (psi)	Retention (%)	Resolution
200	45	10	25	0.22
600	45	30	35	0.83
800	45	45	37	0.86
1000	45	50	37	0.88
1000	22.5	50	38	0.87
1000	45	50	37	0.88
1000	90	50	26	0.37

TABLE 4. The Resolution of Peaks and the Retention of Stationary Phase Using System of pH 7.0 in Model CCC-800

Rotation (rpm)	Flow Rate (ml/hr)	Back Pressure (psi)	Retention (%)	Resolution
200	115	32	33.8	0.51
400	115	55	32.9	0.86
500	115	70	30.5	0.87
600	115	85	27.7	0.82
800	115	125	27.2	0.64
600	42	85	37.0	1.30
600	80	85	30.4	0.92
600	115	85	27.7	0.82
600	152	85	24.3	0.71

polypropylene centrifuge tube containing 5 ml each of upper and lower phases. The phases were thoroughly equilibrated with the sample at room temperature. The protein concentrations in the upper and lower phases were determined by measuring UV absorbance at 280 nm using a Shimadzu UV-Vis Spectrophotometer. The partition coefficients (K) were obtained by dividing the absorbance of the upper phase by that of the lower phase.

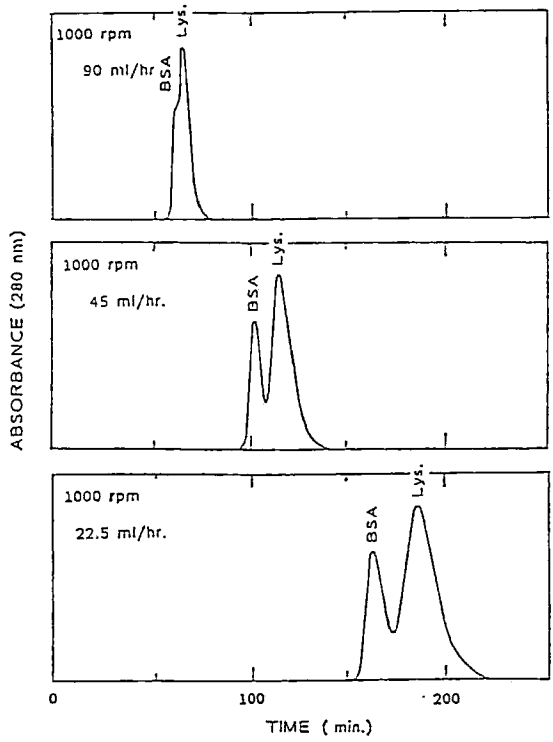

Fig. 1 Separation of BSA and Lysozyme with Different Flow Rate by CCC-1000 Using System of pH 7.0

RESULTS AND DISCUSSION

Performence of two solvent systems composed of PEG 3,400/potassium phosphate/water were tested in two models of eccentric coil planet centrifuge with a wide range of rotational speeds and flow rates. The phase stability is remarkable and the noise level is very low when the PEG-rich upper phase was used as the stationary phase and the effluent was cooled just prior to entering the monitor cell. The experimental conditions and the results obtained by CCC-1000 and CCC-800 using the solvent system of pH 7.0 are summarized in Tables 3 and 4. Figs. 1, 2, 3 and 4 show the chromatograms of BSA and lysozyme obtained at various flow rates of

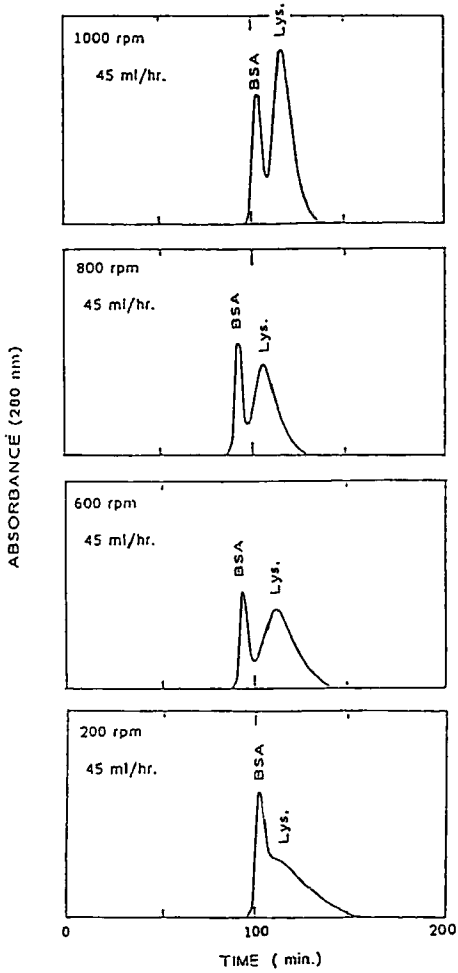

Fig. 2 Separation of BSA and Lysozyme with Different Rotational Speed by CCC-1000 Using System of pH 7.0

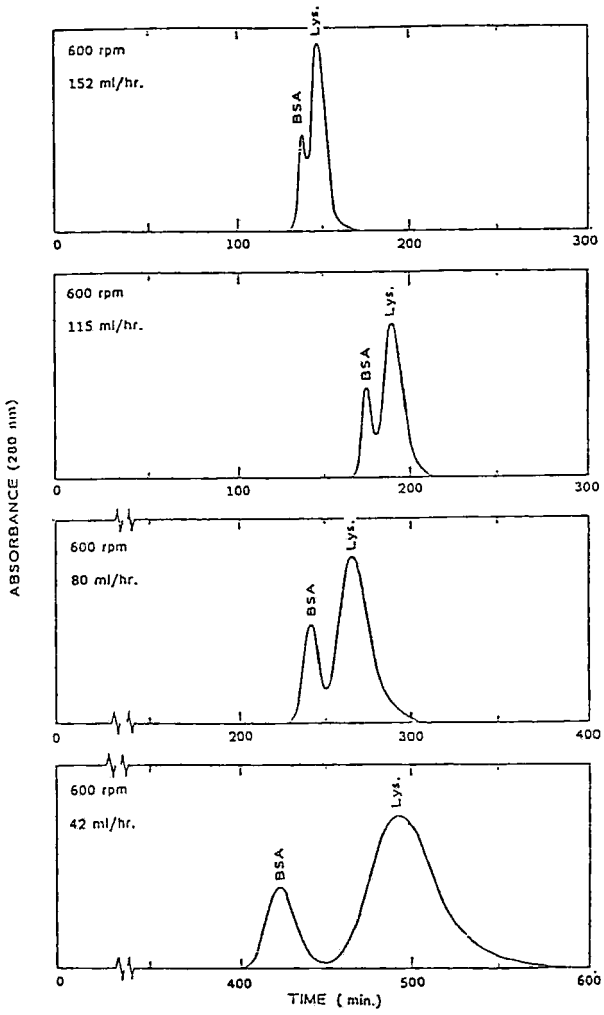

Fig. 3 Separation of BSA and Lysozyme with Different Flow Rate by CCC-800 Using System of pH 7.0

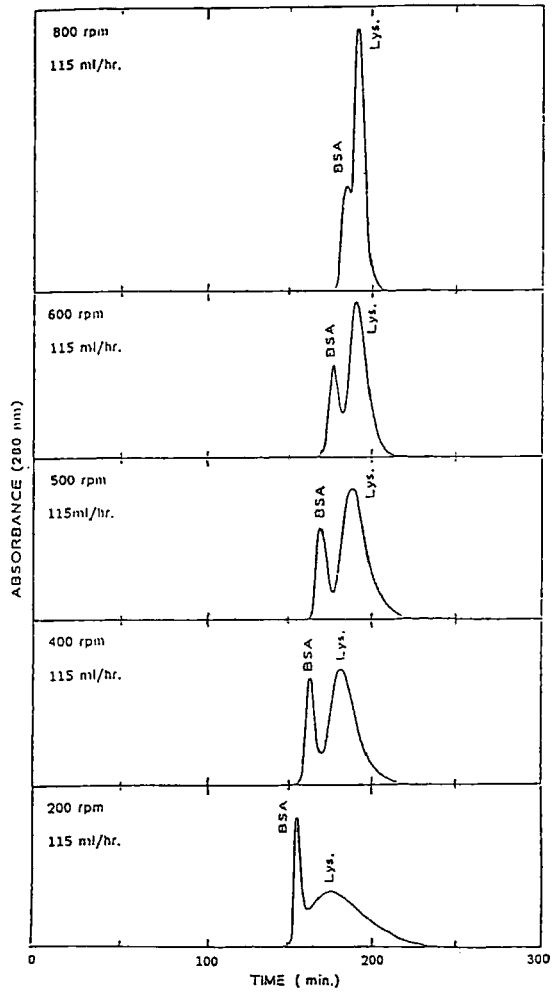

Fig. 4 Separation of BSA and Lysozyme with Different Rotational Speed by CCC-800 Using System of pH 7.0

mobile phase and rotational speeds. In the figures, the zero on the time axis is the moment of sample injection. The chromatograms demonstrate that the peak resolution between BSA and lysozyme varies with both the rotational speed and flow rate. In some conditions, such as at a rotational speed of 600 rpm and a flow rate of 42 ml/hr. in model CCC-800 (Fig. 3, bottom), the two proteins were completely resolved.

The retention of the stationary phase in the column is expressed by the volume percentage of the stationary phase retained in the column. As shown in Figs. 5, 6, 7 and 8, at a constant rotational speed, the retention decreases with an increased flow rate of the mobile phase in both models. When the rotational speed is increased at a constant flow rate, however, the retention decreases in CCC-800 and increases in CCC-1000.

The resolution of two peaks from each other is calculated according to the conventional chromatographic formula

$$R_s = \frac{2\Delta t}{W_1 + W_2}$$

where Δt is the distance between the peaks measured at the peak maximum while W_1 and W_2 are the widths of the peaks expressed in the same unit of time or volume as Δt. The results are shown in Figs. 9, 10, 11 and 12. In both models, the peak resolution is highest at the lowest flow rate of mobile phase tested and sharply reduces with the increased rate at a constant rotational speed. The tests were performed in a wide range of rotational speed from 200 rpm to the maximum of 1000 and 800 rpm for CCC-1000 and CCC-800, respectively. The resolution is poor at lower rotational speed such as 200 rpm in both models. As the rotational speed is increased, the resolution increases monotonously up to the maximum at the highest speed of 1000 rpm in model CCC-1000. In model CCC-800, however, an optimal speed is found around 500 rpm for maximum resolution. The resolution decreases with further increase of the rotational speed.

It seems that the retention of the stationary phase is one of the most important parameters on performance of planet centrifugal

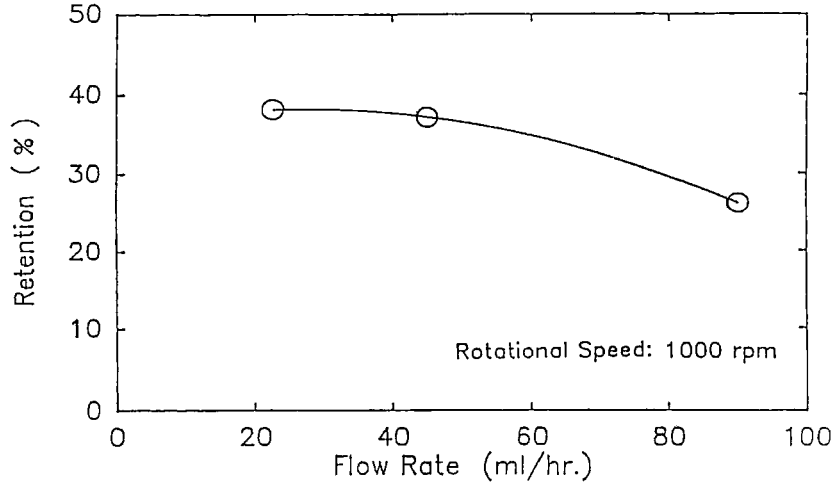

Fig. 5 Stationary Phase Retention vs. Flow Rate in CCC-1000, pH 7.0

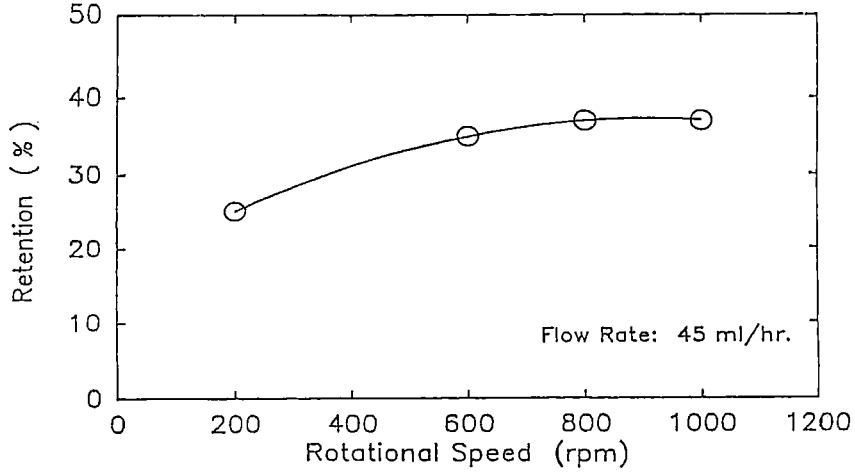

Fig. 6 Stationary Phase Retention vs. Rotational Speed in CCC-1000, pH 7.0

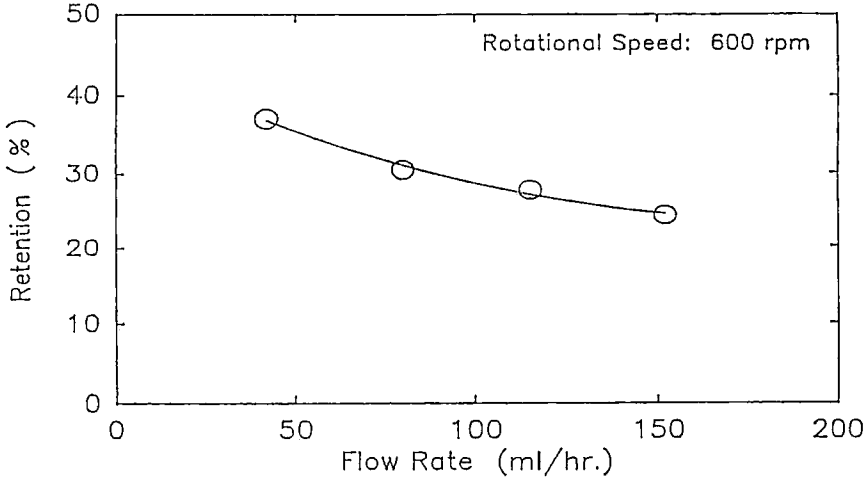

Fig. 7 Stationary Phase Retention vs. Flow Rate in CCC-800, pH 7.0

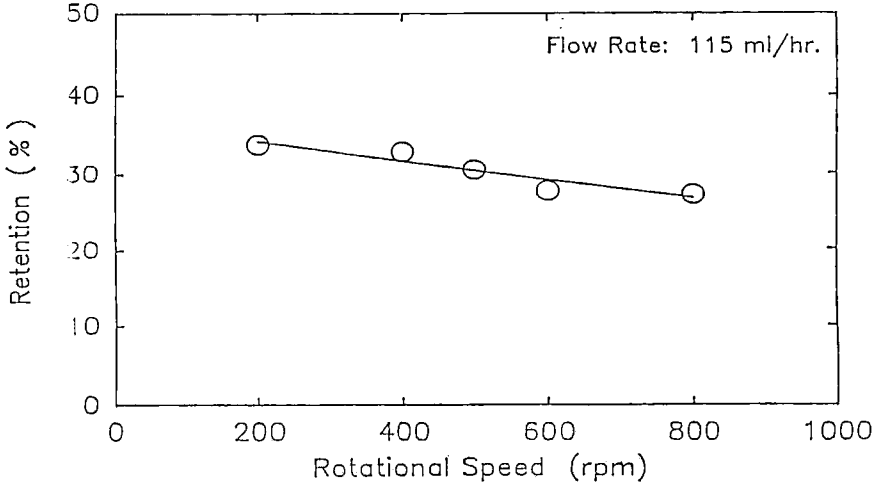

Fig. 8 Stationary Phase Retention vs. Rotational Speed in CCC-800, pH 7.0

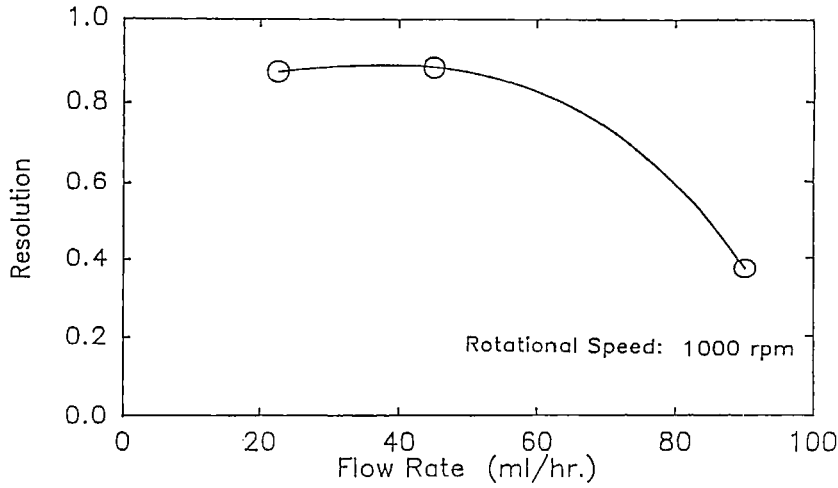

Fig. 9 Resolution vs. Flow Rate in CCC-1000, pH 7.0

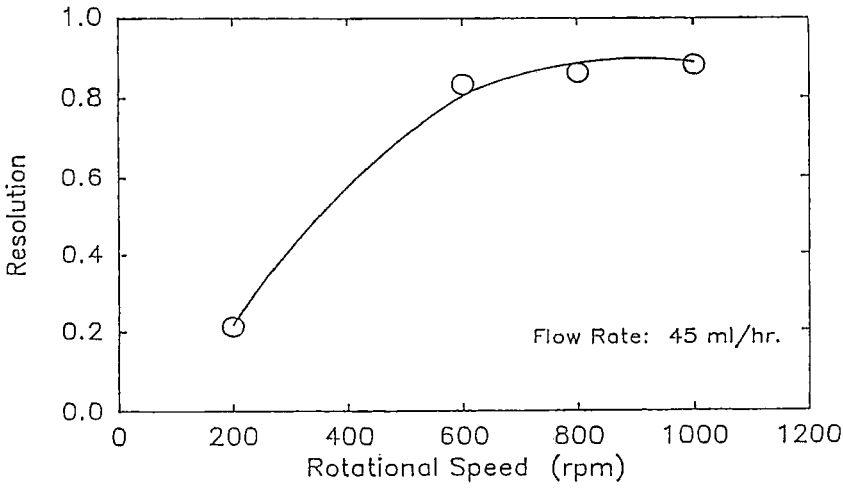

Fig. 10 Resolution vs. Rotational Speed in CCC-1000, pH 7.0

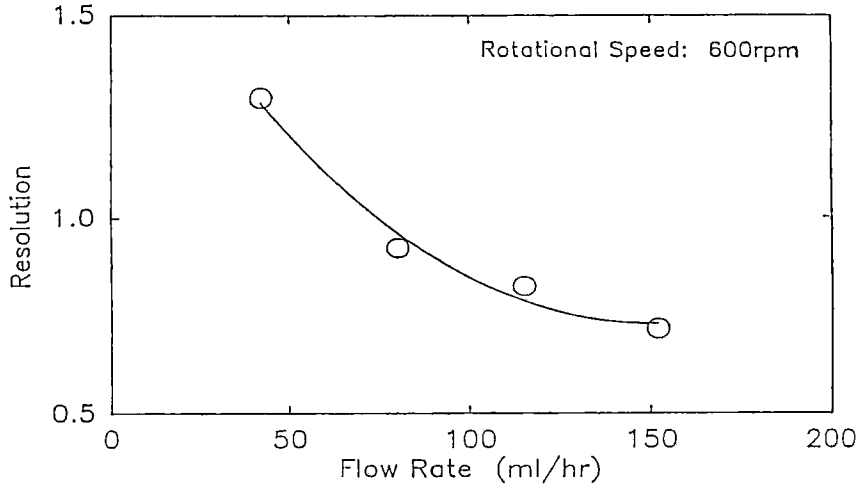

Fig. 11 Resolution vs. Flow Rate in CCC-800, pH 7.0

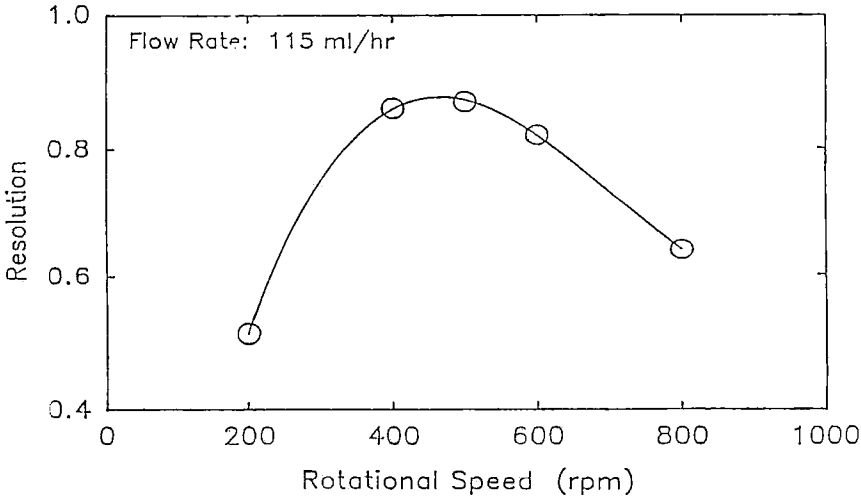

Fig. 12 Resolution vs. Rotational Speed in CCC-1000, pH 7.0

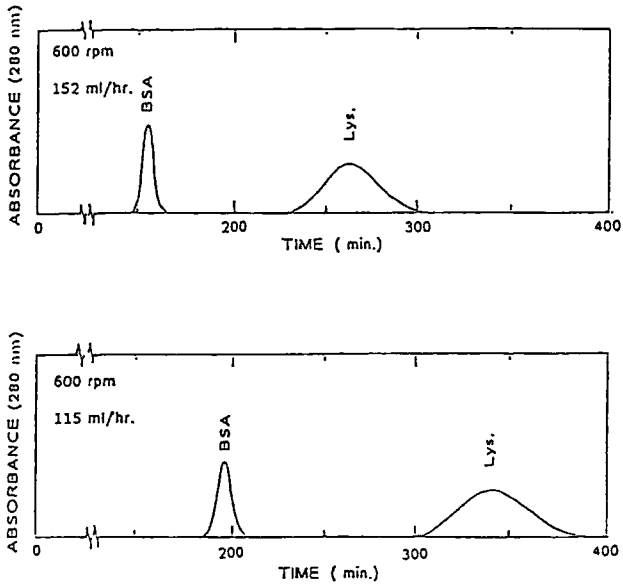

Fig. 13 Separation of BSA and Lysozyme with Different Flow Rate by CCC-800 Using System of pH 5.5

TABLE 5. The Resolution of Peaks and the Retention of Stationary Phase Using System of pH 5.5 in Model CCC-800

Rotation (rpm)	Flow Rate (ml/hr)	Back Pressure (psi)	Retention (%)	Resolution
600	115	60	24.8	3.07
600	152	65	19.7	3.04

countercurrent chromatography. The peak resolution is strongly dependent on the retention of the stationary phase. The higher the retention is, the higher resolution is obtained regardless of the applied flow rate or rotational speed except at low rotational speeds such as 200 rpm in CCC-800.

The chromatograms obtained using the solvent system of pH 5.5 in CCC-800 are shown in Fig. 13 and the experimental conditions and R_S values are listed in Table 5. The partition coefficient of lysozyme changes with the pH value, from 0.36 at pH 7.0 to 3.68 at pH 5.5 while that of BSA remains almost unchanged. Consequently, the separation factor of lysozyme over BSA is much higher in the system of pH 5.5 than that in the system of pH 7.0. This higher separation factor results in the base line separation of these two proteins even at the flow rate being as high as 152 ml/hr. The results indicate that the eccentric coil planet centrifuge is capable of performing preparative separation of proteins in aqueous two-phase systems.

CONCLUSIONS

The eccentric multi-layer coil planet centrifuge is successfully used for separating proteins with aqueous two-phase systems. Stable retention of the stationary phase is obtained when the more viscous phase with higher affinity to the tube wall, such as the PEG-rich upper phase of PEG/salt systems, is used as stationary phase. The separation efficiency is generally dependent on the two major parameters, i.e., the flow rate of mobile phase and the rotational speed of the centrifuge, both via retention of stationary phase in the coils. At a constant rotational speed, increasing the flow rate of mobile phase will decrease the retention, so that the resolution of solute peaks will be reduced. At a constant mobile phase flow rate, an increased rotational speed improves the peak resolution to reach the maximum level. Further increase of the rotational speed, however, results in no significant change or decrease of the resolution.

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