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Protein separation with aqueous–aqueous polymer systems by two types of counter-current chromatographs

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ABSTRACT

Two different types of counter-current chromatographs, the cross-axis coil planet centrifuge (*X*-axis CPC) and horizontal flow-through coil planet centrifuge (horizontal CPC), were evaluated for protein separation with an aqueous–aqueous two-phase polymer system. The sample solution, containing 10–200 mg each of cytochrome *c*, myoglobin, ovalbumin and hemoglobin in 2 ml of each phase was eluted with the lower phase.

In both instruments, the effects of the flow-rate, revolution speed, and parameter β (helical diameters of the multilayer coil) on the protein separation were investigated. The best results were obtained from the *X*-axis CPC operated at 750 rpm and a flow-rate of 2.0 ml/min using a multilayer coil with a small helical diameter (β = 0.25–0.60). Four protein samples were well resolved in less than 5 h.

INTRODUCTION

Counter-current chromatography (CCC) is a liquid–liquid partition chromatography utilizing no solid support matrix [1–3]. The stationary phase is retained in the column by the aid of a gravity or centrifugal force. The system eliminates all complications arising from the solid support. Consequently, the method is very useful for separation and purification of biopolymers such as proteins and nucleic acids, using aqueous–aqueous polymer phase systems [4,5]. Polymer phase systems have been introduced by Albertsson [6] for the partition of a variety of macromolecules and cell particles. However, high viscosity and low interfacial tension of polymer phase systems tend to delay phase separation and the operation of the conventional counter-current distribution apparatus becomes tedious and requires a long separation time. Recently some coil planet centrifuge (CPC) apparatus have been modified for performing CCC with polymer phase systems [7,8].

In this paper, the performance of two different CPC models, *i.e.*, the cross-axis coil planet centrifuge (X-axis CPC) and the horizontal coil planet centrifuge (horizontal CPC), were evaluated in the separation of four sample proteins including cytochrome

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c, myoglobin, ovalbumin and hemoglobin with a two-phase polymer system composed of polyethylene glycol (PEG) 1000 and potassium phosphate. The partition coefficient (K) of these proteins were optimized by choosing a suitable pH of the potassium phosphate composition. The separations were performed by varying the chromatographic conditions such as the revolution speed, flow-rate and column dimensions.

EXPERIMENTAL

Apparatus

CCC separations were performed with two different CPC types, the improved cross-axis CPC (X-axis CPC) and the most recent model of the horizontal flow-through CPC, each producing a specific mode of planetary motion [8,9]. These two CCC centrifuges share a common feature in that the system permits continuous elution of the mobile phase through the rotating column without the use of the conventional rotary seal device which is a potential source of leakage and contamination [10].

The design of the X-axis CPC has been reported earlier [8]. The apparatus holds a pair of horizontal rotary shafts symmetrically, one on each side of the rotary frame, at a distance of 7.6 cm from the central axis of the centrifuge. A spool-shaped column holder is mounted on each rotary shaft at a lateral location 15 cm away from the midpoint. As reported earlier, this displacement of the column holder on the holder shaft is essential for providing stable retention of the polymer stationary phase against a high flow-rate of the mobile phase.

Each multilayer coil was prepared from 2.6 mm I.D. PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ, USA) by winding it onto a 7.6 or 3.8 cm diameter holder forming multiple layers of left-handed coils between a pair of flanges spaced 5 cm apart. The larger columns consists of 6 layers of the coil with a 140-ml capacity and the smaller column of 8 layers of the coil with about a 125-ml capacity. In both columns all coiled layers were connected in series by bridging neighboring layers with narrow transfer tubes (0.85 mm I.D., PTFE) across the width of the column. Leak-free connections were made at each joint by using a short sheath of an intermediate-sized PTFE tube (1.6 mm I.D.) as an adaptor. A pair of columns mounted on the rotary frame was connected in series to make up a total capacity of about 280 ml in the larger column and 250 ml in the smaller column. Both inflow and outflow tubes exit together at the center of the top plate of the centrifuge case where they are tightly supported with silicone-rubber-padded clamps.

The horizontal CPC used in the present experiments is a modified version of the high-speed CCC centrifuge described previously [9]. The apparatus holds a set of three column holders symmetrically on the rotary frame at a distance of 10 cm from the central axis of the centrifuge. Each column holder is equipped with a planetary gear which interlocks to an identical stationary sun gear mounted around the central axis of the centrifuge. This gear arrangement produces a desired synchronous planetary motion of the holder, *i.e.*, rotation about its own axis and revolution around the centrifuge axis in the same direction at the same rate. Each column holder can be removed from the rotary frame by loosening a pair of screws on each bearing block.

The column holder was modified to form a composite coil assembly similar to

that in the eccentric CPC previously reported [11]. Each coil assembly consisted of a series of eight coil units which were prepared by winding a single piece of 1.6 mm I.D. PTFE tubing onto 12 cm long, 0.6 cm O.D. stainless-steel pipes forming double layers of coils. Each coil unit consists of about 75 helical turns with about a 9-ml capacity. Eight coil units were arranged around each holder in parallel to and at a distance of 3 cm from the holder axis. Three coil assemblies on the rotary frame were serially connected with flow tubes (0.85 mm I.D., PTFE) to make up the total capacity of about 220 ml. Each interconnection tube runs across the width of the rotary frame along the rotary tube support which was actively counter-rotated to prevent the flow tubes from twisting [9,12]. The inflow and outflow tubes (0.85 mm I.D., PTFE) were each tightly secured onto the side wall of the centrifuge using a pair of silicone-rubber-padded clamps.

Revolution speed of these centrifuges are regulated up to 1000 rpm with a speed control unit (Bodine Electric, Chicago, IL, USA). Dimensions of each column, including the number of layers, total capacity, range of parameter β (helical diameters of the multilayer coil), etc. are summarized in Table I.

Reagents

Polyethylene glycol (PEG) 1000 (mol.wt. = 1000), cytochrome *c* (horse heart), myoglobin (horse heart), ovalbumin (chicken egg) and hemoglobin (bovine) were purchased from Sigma (St. Louis, MO, USA). Anhydrous monobasic and dibasic potassium phosphates were obtained from J. T. Baker (Phillipsburg, NJ, USA). All these chemicals were of reagent grades.

TABLE I

TYPE OF APPARATUS AND DIMENSIONS OF THE SEPARATION COLUMNS USED IN THE PRESENT STUDY

Apparatus (planetary motion)	Coil holder		Type of column	Column			
	Diameter (cm)	Width (cm)		I.D. (mm)	Total length (m)	Total turns	Total capacity (ml)
X-axis CPC (Type XLL)	7.6	5.1	Coaxial multilayer 6 layers all left handed β^a = 0.50–1.00	2.6	53	156	280
	3.8	5.1	Coaxial multilayer 8 layers all left handed β = 0.25–0.60	2.6	47	208	250
Horizontal CPC (Type J)	0.6[b]	12.0	Eccentric double-layer 8 coil/holder right and left handed β = 0.3	1.6	109	2300	220

[a] $\beta = r/R$ where r is the distance from the holder axis to the coil and R, the distance from the holder axis to the central axis of the centrifuge.

[b] Diameter of stainless-steel pipe supporting the coil.

Preparation of polymer two-phase solvent systems and sample solution

The aqueous–aqueous polymer phase systems were prepared by dissolving 150 g of PEG 1000 and 150 g of anhydrous potassium phosphate in 900 g of distilled water. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature, allowing the mixture to completely separate into two layers before use. The sample solution was prepared by dissolving 10–200 mg each of cytochrome *c*, myoglobin, ovalbumin and bovine hemoglobin in 4 ml of the two-phase system consisting of about equal volumes of each phase.

Measurement of partition coefficient (K) of protein samples

The partition coefficient of each protein sample was determined spectrophotometrically by a simple test tube procedure. About 2 ml of each phase was delivered in a test tube and about 1 mg of the sample was added. The contents were thoroughly mixed and allowed to settle at room temperature. After the clear two layers were formed, an aliquot (usually 1 ml) of each phase was pipetted and diluted with 2 ml of distilled water to determine the absorbance at 280 nm using a Zeiss PM6 spectrophotometer. The partition coefficient ($K = C_L/C_U$) was obtained by dividing the absorbance value of the lower phase by that of the upper phase.

CCC separation of proteins

For each separation, the coil was first completely filled with the stationary upper phase. This was followed by injection of the sample solution through the sample port. Then, the mobile phase was eluted through the coil at 0.5–4.0 ml/min while the apparatus was rotated at the desired rpm. The effluent from the outlet of the column was continuously monitored with an LKB Uvicord S (LKB Instruments, Uppsala, Sweden) at 280 nm and fractionated into test tubes (3 ml/tube) with an LKB Ultrorac fraction collector (LKB Instruments).

Analysis of fractions

An aliquot of each fraction was diluted with distilled water and the absorbance was determined at 280 nm with a Zeiss PM6 spectrophotometer.

RESULTS AND DISCUSSION

CCC is a liquid–liquid partition method which is based on difference in the partition coefficient of solutes. For achieving efficient separations of proteins, it is essential to optimize the partition coefficient of each component by selecting the proper composition of the polymer phase system used for separation. Fig. 1 shows the partition coefficient values ($K = C_L/C_U$) of four proteins plotted in a logarithmic scale against the ratio of monobasic and dibasic potassium phosphates in the polymer phase system. The partition coefficients of these proteins generally rise as the relative amount of monobasic potassium phosphate increases, apparently due to the pH shift toward the isoelectric points of the applied proteins. When the concentration of the monobasic to dibasic potassium phosphates exceeds a 1:1 ratio, however, the solvent mixture forms a single phase. An evenly scattered ideal distribution of the four partition coefficient values is observed in the solvent system composed of 12.5% (w/w) dibasic potassium phosphate and 12.5% (w/w) PEG 1000.

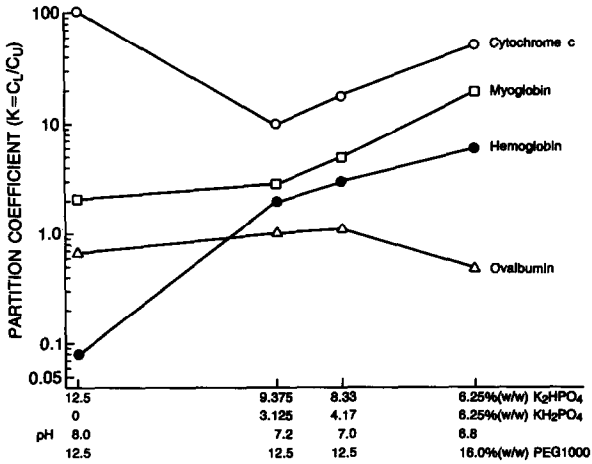

Fig. 1. Partition coefficients (K) of four protein samples in various polymer phase systems composed of PEG 1000 and potassium phosphate. K is the solute concentration in the lower phase divided by that in the upper phase.

Stationary phase retention

In the horizontal CPC, the effects of the revolution speed and flow-rate on the retention of the PEG-rich upper phase were investigated, using the lower phase as the mobile phase. As shown in Fig. 2A, the optimum condition for the retention of the stationary phase is 800 rpm. An increased revolution speed at 1000 rpm resulted in reduction of the retention volume probably due to emulsification caused by excessive mixing of the two phases. On the other hand, a reduced revolution speed down to 600 rpm gave lower retention apparently due to a lack of the centrifugal force field.

In Fig. 2B, the percentage retention for the stationary phase is plotted against the flow-rate at the optimum revolution speed of 800 rpm. It indicates that the retention of the stationary phase rapidly decreases with increased flow-rate. As the flow-

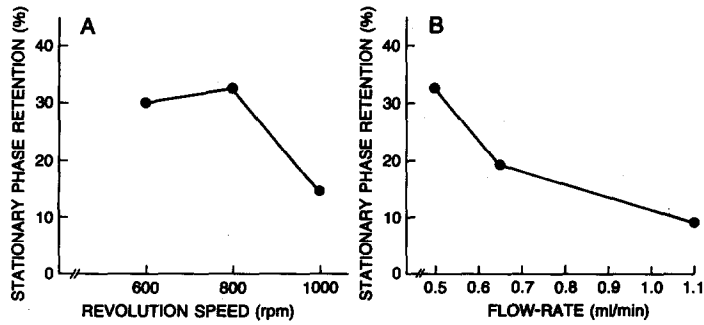

Fig. 2. Effects of revolution speed (A) and flow-rate (B) on the stationary phase retention in the horizontal CPC. (A) 1 ml/min flow-rate; (B) 800 rpm. See Table II for details.

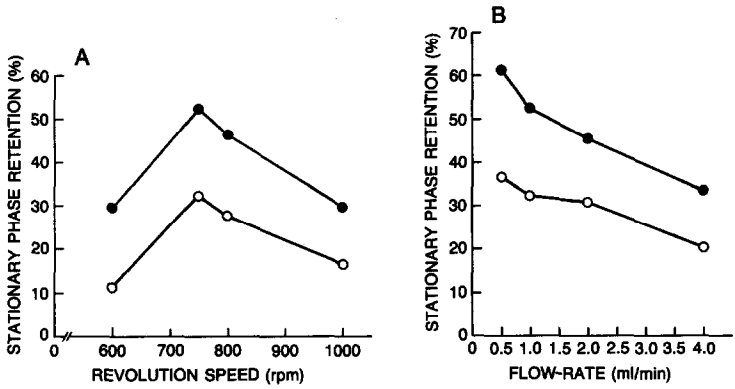

Fig. 3. Effects of revolution speed (A) and flow-rate (B) on the stationary phase retention in the X-axis CPC. (○) larger column (β = 0.50–1.00); (●) smaller column (β = 0.25–0.60); (A) 1 ml/min flow-rate; (B) 750 rpm. See Table III for details.

rate is increased from 0.5 to 1.1 ml/min, the stationary phase retention becomes about 1/4. The optimum operational conditions for the horizontal CPC are found at 800 rpm at a 0.5 ml/min flow-rate with the present aqueous–aqueous polymer phase system.

It has been reported that the present X-axis CPC has a unique capability of retaining a large amount of stationary phase for viscous, low interfacial tension solvent systems [8]. Fig. 3A and B similarly illustrate the effects of the revolution speed (A) and flow-rate (B) on the retention of the PEG-rich upper phase of a 12.5% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate system in the present X-axis CPC. In each diagram, open circles indicate the retention data obtained from the larger column (β = 0.50–1.00) and solid circles, those obtained from the smaller column (β = 0.25–0.60).

The effects of revolution speed on the stationary phase retention was studied by eluting the lower phase at a flow-rate of 1 ml/min. As shown in Fig. 3A, the retention sharply rises with the increased revolution speed from 600 to 750 rpm to reach the maximum level. Further increase of the revolution speed up to 1000 rpm results in a decline of the retention in a linear fashion. The two retention curves display similar shapes, while the retention in the smaller column always exceeds that in the larger column, as expected from the previous data with the organic–aqueous solvent systems [8].

Fig. 3B shows the effects of the flow-rate of the mobile phase on the retention of the stationary phase at a revolution speed of 750 rpm. In both columns, the retention decreases with an increased flow-rate from 0.5 to 4.0 ml/min. The retention level in the smaller column is much greater than that in the larger column. Even at a high flow-rate of 2.0 ml/min, the smaller column holds the stationary phase near 50% of the total column capacity, promising an efficient peak resolution of protein samples in a short elution time.

Separation of proteins with two different CPCs

In order to study effects of the revolution speed and flow-rate on the protein

TABLE II

PEAK RESOLUTION OF THREE PROTEINS OBTAINED BY HORIZONTAL CPC

cy = Cytochrome *c*; myo = myoglobin; ov = ovalbumin. Column capacity: 220 ml; $\beta = 0.3$.

Revolution speed (rpm)	Flow-rate (ml/min)	Peak resolution		Stationary phase retention (%)
		cy/myo	myo/ov	
600	0.5	1.63	0.43	29.8
800	0.5	2.22	0.43	32.6
1000	0.5	1.71	0.29	14.1
800	0.5	2.22	0.43	32.6
800	0.7	1.43	0.60	19.3
800	1.1	0.73	–	9.0

separation, a series of experimental runs was performed with a two-phase solvent system composed of 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate using the lower phase as the mobile phase. From the obtained chromatogram, partition efficiency was computed and expressed in terms of peak resolution, R_s, according to the conventional formula

$$R_s = 2(R_1 - R_2)/(W_1 + W_2) \quad (1)$$

where R_1 and R_2 are retention time or volume of two adjacent peaks, and W_1 and W_2, the peak width of the same peaks expressed in the same unit as R_1 and R_2. The results are summarized in Tables II and III, where R_s values between the first (cytochrome *c*) and second (myoglobin) peaks and between the second and third (ovalbumin) peaks are listed together with the percentage retention of the stationary phase obtained under various combinations of revolution speeds and flow-rates.

In the horizontal CPC (Table II), a fixed flow-rate of 0.5 ml/min produced the highest peak resolution at 800 rpm as shown in the top three rows. The shift of the revolution speed in either direction results in a lower R_s value. This loss of peak resolution may be secondary to the reduced stationary phase volume retained in the column since the percentage retention values appear to bear a somewhat significant correlation with the R_s values. At the optimum revolution speed of 800 rpm, the flow-rate was varied from 0.5 to 1.1 ml/min (bottom three rows in Table II). With one exception, the peak resolution decreases with an increased flow-rate and, again, R_s values between the first and second peaks show similar correlation with the percentage retention of the stationary phase.

These experimental results strongly suggest that, in the horizontal CPC, the retention level of the stationary phase may play a major role in governing the peak resolution of proteins within the applied experimental conditions.

Fig. 4 shows a chromatogram of the four proteins obtained by the horizontal CPC under the optimum operational conditions. The separation was performed at 800 rpm at a flow-rate of 0.65 ml/min using the lower phase as the mobile phase. The four components were eluted in the order of their partition coefficient values within 15 h. After the elution of cytochrome *c* ($K = 103.7$), myoglobin ($K = 2.08$), and

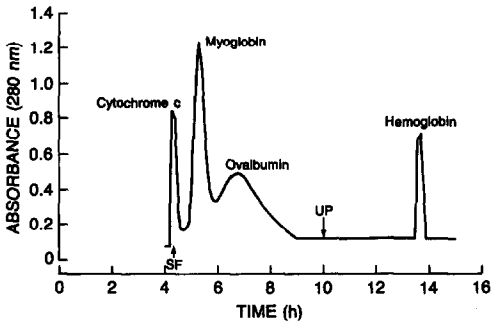

Fig. 4. Chromatogram of proteins obtained by the horizontal CPC. Experimental conditions: column: eccentric dual layer coil assemblies × 3, 1.6 mm I.D. PTFE, 220 ml capacity; sample: 10–200 mg of each protein in 4 ml solvent; solvent system: 12.5% (w/w) PEG 1000–12.5% (w/w) K_2HPO_4 in distilled water; mobile phase: lower phase; flow-rate: 0.65 ml/min; revolution: 800 rpm; SF = solvent front; UP = upper phase eluted in the reversed direction.

ovalbumin ($K = 0.63$), the PEG-rich upper phase was pumped into the column in the reversed direction to facilitate rapid elution of hemoglobin ($K = 0.08$) still remaining in the column. From the obtained chromatogram, the separation efficiency may be computed and expressed in terms of theoretical plate number using the conventional gas chromatographic formula.

$$N = (4R/W)^2 \tag{2}$$

where N denotes the theoretical plate number; R, the retention time or volume of the peak maximum; and W, the peak width expressed in the same unit as R. Using the above equation, the separation efficiency thus obtained for the third peak (ovalbumin) was $n = 50$, while the R_s between the second and the third peaks is 0.60.

The peak resolutions of proteins in the two different columns on the X-axis CPC have been studied under various experimental conditions, the results being summarized in Table III. In the smaller coil ($\beta = 0.25$–0.60), the effects of the revolution speed on the peak resolution were examined at a 1.0 ml/min flow-rate (top four rows). The maximum peak resolution was obtained at 750 rpm. The lower (600 rpm) or higher (1000 rpm) speed results in considerable decrease of peak resolution probably due to the lower stationary phase retention as observed in the horizontal CPC. At a 750 rpm revolution speed (5th–8th rows in Table III), an increase of the flow-rate up to 2 ml/min does not affect the peak resolution, despite a considerable decrease in stationary phase retention. However, further increase of the flow-rate at 4.0 ml/min results in a detrimental loss in the peak resolution probably due to a sharp decline in the retention level.

The effects of revolution speed on the peak resolution were similarly investigated in the larger column at a flow-rate of 1.0 ml/min (9th–12th rows in Table III). The results show that the R_s values between the first and second peaks (cy/myo) are rather insensitive to the revolution speed, while the best resolution between the second and third peaks (myo/ov) is found at 750 rpm associated with the highest retention level of the stationary phase as observed in the smaller column. At a constant revolution

TABLE III

PEAK RESOLUTION OF THREE PROTEINS OBTAINED BY X-AXIS CPC

cy = Cytochrome *c*; myo = myoglobin; ov = ovalbumin.

Column capacity (ml) β value	Revolution speed (rpm)	Flow-rate (ml/min)	Peak resolution		Stationary phase retention (%)
			cy/myo	myo/ov	
250 ml					
β = 0.25–0.60	600	1.0	1.59	0.56	29.1
	750	1.0	2.10	0.85	52.1
	800	1.0	1.88	0.51	46.4
	1000	1.0	1.75	0.45	29.6
	750	0.5	2.26	0.82	61.4
	750	1.0	2.10	0.85	52.1
	750	2.0	2.28	0.86	45.4
	750	4.0	1.01	–	33.5
280 ml					
β = 0.50–1.00	600	1.0	2.46	0.81	11.3
	750	1.0	2.29	0.90	32.3
	800	1.0	2.53	0.68	27.5
	1000	1.0	2.11	0.53	16.6
	750	0.5	3.35	0.99	36.6
	750	1.0	2.29	0.90	32.3
	750	2.0	1.73	–	30.9
	750	4.0	0.18	–	20.2

speed of 750 rpm, the lowest flow-rate of 0.5 ml/min yields the best peak resolution, while the highest flow-rate at 4.0 ml/min results in a serious loss of peak resolution apparently due to the low stationary phase retention.

Fig. 5 shows a chromatogram of the protein samples by the X-axis CPC using

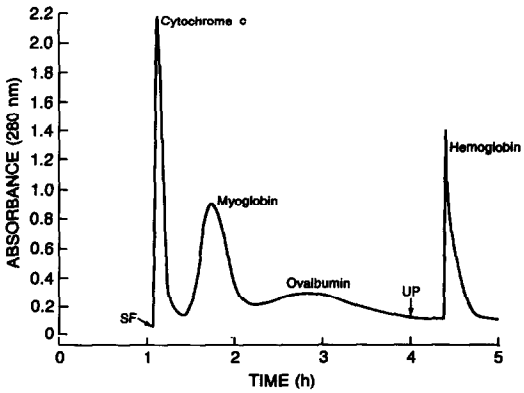

Fig. 5. Chromatogram of proteins obtained by the X-axis CPC. Experimental conditions: column: 2.6 mm I.D. PTFE multilayer coils × 2, β = 0.25–0.60, 250 ml capacity; sample: four proteins 10–200 mg each in 4 ml solvent; solvent system: 12.5% (w/w) PEG 1000–12.5% (w/w) K_2HPO_4 in distilled water; mobile phase: lower phase; flow-rate: 2 ml/min; revolution: 750 rpm; SF = solvent front; UP = upper phase eluted in the reverse direction.

the smaller column (β = 0.25–0.60). The separation was performed at 750 rpm and at a high flow-rate of 2 ml/min. All components were well resolved in less than 5 h. The hemoglobin with a low partition coefficient was collected from the column by applying the reversed elution as described earlier. The partition efficiencies computed from the chromatogram range from 550 TP (theoretical plates) for the first peak to 35 TP for the third peak, while R_s = 0.86 between the second and third peaks.

CONCLUSION

The peak resolution of the proteins is greatly influenced by the revolution speed and the flow-rate of the mobile phase in both horizontal and X-axis CPCs. The horizontal CPC was able to resolve the protein peaks at a low flow-rate of 0.5 ml/min in 15 h. In the X-axis CPC, the smaller column (β = 0.25–0.60) provided stable retention of the stationary phase against a high flow-rate of 2 ml/min, yielding a comparable separation of the proteins within 5 h. The results of the present studies succesfully demonstrate the capability of the two CCC instrument to perform protein separations with an aqueous–aqueous polymer phase system. The present methods will be useful in separation and purification of various biopolymers.

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