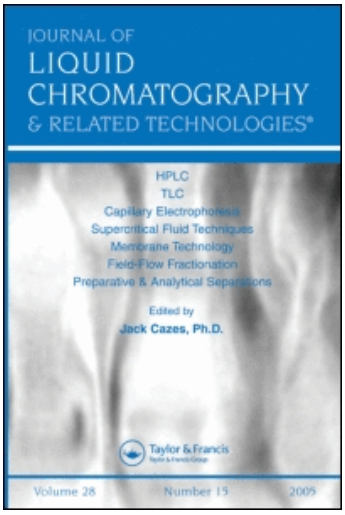

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Y. Shibusawa[a]; M. Yamaguchi[a]; Y. Ito[b]

[a] Department of Analytical Chemistry, School of Pharmacy Tokyo University of Pharmacy and Life Science, Hachioji Tokyo, Japan [b] Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institute, Bethesda, MD, USA

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POLYETHYLENE GLYCOL-POTASSIUM PHOSPHATE AQUEOUS TWO-PHASE SYSTEMS FOR COUNTERCURRENT CHROMATOGRAPHY OF PROTEINS

Yoichi Shibusawa,[1,*] Miwa Yamaguchi,[1] Yoichiro Ito[2]

[1] Department of Analytical Chemistry
School of Pharmacy
Tokyo University of Pharmacy and Life Science
1432-1 Horinouchi, Hachioji
Tokyo 192-03 Japan

[2] Laboratory of Biophysical Chemistry
National Heart, Lung, and Blood Institute
National Institutes of Health
Bethesda, MD 20892, USA

ABSTRACT

Aqueous-aqueous polymer phase systems composed of polyethylene glycol 1000-potassium phosphate were used for the countercurrent chromatographic separation of proteins using the type XL cross-axis coil planet centrifuge. It was found that the peak resolution of proteins is highly dependant on the pH of the solvent system. The best separation of cytochrome c, myoglobin and human serum albumin was achieved with 16.0% PEG 1000-12.5% potassium phosphate buffer at pH 9.2. The resolution and separation time were improved by pH-peak-focusing countercurrent chromatography.

INTRODUCTION

Polymer phase systems were introduced by Albertsson[1] for the partition of a variety of macromolecules and cell particles. However, their high viscosity and low interfacial tension tend to delay phase separation and their use with the countercurrent distribution apparatus becomes tedious and time-consuming.

Countercurrent chromatography (CCC) established by Ito and his collaborators is essentially a form of liquid-liquid partition chromatography in which the stationary phase is retained in the column by the aid of gravity or centrifugal force field.[2-7] Recently, several CCC models such as the type J horizontal flow-through coil planet centrifuge (CPC) and type XL, XLL, XLLL and L cross-axis CPCs have been designed for performing CCC with highly viscous polar solvent systems.[8-14] The cross-axis CPC apparatus with the column holders laterally shifted along the rotary shaft allows retention of the stationary phase of polar solvent systems, such as PEG 1000-KPi (potassium phosphate) buffer by use of the strong centrifugal force field acting across the diameter of the separation column. Consequently the apparatus can retain the stationary phase over 50% of the total column capacity even under a high flow rate of 2.0 mL/min.

The use of aqueous-aqueous polymer phase system and a suitable cross-axis CPC allows the separation and purification of a variety of proteins with minimum denaturation thus preserving their enzymatic activity.[14-21]

In this paper the compositions of PEG 1000-KPi buffer two-phase systems were optimized for CCC separation of proteins by measuring parameters such as the settling time, the two phase volume ratio and the partition coefficients of proteins. The separation of three stable proteins including cytochrome c, myoglobin, and human serum albumin was demonstrated using 16.0% (w/w) PEG 1000-12.5% (w/w) KPi by the conventional CCC technique. The pH-peak-focusing CCC technique[22,23] was also applied for the separation of cytochrome c, myoglobin and ovalbumin.

EXPERIMENTAL

Apparatus

The CCC separations of proteins were performed with two different models of the cross-axis CPC, types XL and XLLL[11]. The type XL CPC was fabricated at the NIH machine shop while the type XLLL was constructed by Ohtake Works, Tokyo,

Japan. The type XL apparatus holds a pair of horizontal rotary shafts symmetrically, one on each side of the rotary frame, at a distance of 10 cm from the central axis of the centrifuge. A pair of spool-shaped column holders is mounted on each rotary shaft at a lateral location 10 cm away from the midpoint.

Each multilayer coil was prepared from 2.6 mm ID polytetrafluoroethylene (PTFE) tubing (Zeus Industrial Products, Raritan, NJ, USA) by winding it onto a 10 cm diameter holder hub forming multiple layers of left handed coils between a pair of flanges spaced 5 cm apart. Each column consists of 4 layers of the coil with a 170 mL capacity. A pair of columns was connected in series on the rotary frame to make up a total capacity of 340 mL. Both inflow and outflow tubes exit together at the center of the top plate of the centrifuge where they are tightly supported with silicone-rubber-padded clamps. The speed of the apparatus is regulated up to 500 rpm with a speed control unit (Bodine Electric, Chicago, IL, USA).

The XLLL CPC is a modified model of the XL CPC. It holds a pair of horizontal rotary shafts symmetrically at a distance of 7.5 cm from the central axis of the apparatus. A spool-shaped column holder is mounted on each rotary shaft at a lateral location 22.5 cm from the midpoint. As reported earlier,[12] this displacement of the column holder along the holder shaft is essential for providing stable retention of the polymer phase against a high flow of the mobile phase. Each multilayer coil was prepared from 1.6 mm ID PTFE tubing by winding it onto a 5.0 cm diameter holder hub making multiple layers of left-handed coils between a pair of flagged spaced 10 cm apart. Each column consists of 4 coiled layers with a 55 mL capacity. A pair of columns was serially connected to provide the total capacity of 110 mL. The speed of the apparatus is regulated at 250 rpm with a speed control unit.

Reagents

Polyethylene glycol (PEG) 1000 (average molecular mass 950-1050), cytochrome c, myoglobin, bovine hemoglobin, human hemoglobin, human serum albumin, lysozyme, α- and γ-globulin were all purchased from Sigma (St Louis, MO, USA). Anhydrous monobasic and dibasic potassium phosphates were obtained from J. T. Baker (Phillipsburg, NJ, USA) and Kanto Chemicals (Tokyo, Japan). Other chemicals were all of reagent grade.

Preparation of PEG-Potassium Phosphate Aqueous Two-Phase Solvent Systems

Two-phase solvent systems composed of 12.5, 16.0 and 25.0% (w/w) PEG 1000 and 12.5% (w/w) potassium phosphate solution were prepared by dissolving 250, 320 and 500 g of PEG 1000, respectively, with 250 g of potassium phosphate in

water to bring the total weight of 2000 g. The desired pH of the solvent system was obtained by changing the ratio of monobasic to dibasic potassium phosphates. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

Measurement of Partition Coefficients (K) of Proteins

The partition coefficient of each protein sample was determined spectrophotometrically by a standard test tube procedure. About 1.5 mL of each phase was delivered into a test tube and about 1 mg of the protein 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 1.0 mL) of each phase was diluted with 2.0 mL of distilled water and the absorbance was measured at 280 nm using a Zeiss PM 6 spectro-photometer (Hanover, MD, USA). The partition coefficient (K) was obtained by dividing the absorbance of the upper phase by that of the lower phase.

Measurement of Settling Time

Using the above equilibrated two-phase solvent system, the settling time was measured as follows: A 2 mL volume of each phase was delivered into a 10-mL capacity graduated glass cylinder which was then sealed with a glass stopper.

The contents was gently mixed by inverting the cylinder 5 times and the cylinder was immediately placed on a flat table to measure the time required for the mixture to settle into two clear layers. The experiment was repeated several times to obtain the mean value.

Conventional CCC Separation of Proteins

For each separation, the coiled column was first completely filled with the stationary upper phase. This was followed by injection of the protein mixture through the sample port. Then, the lower phase was eluted through the coil at 1.0 mL/min while the apparatus was rotated at 500 rpm.

The effluent was continuously monitored with an ISCO UA-5 absorbance monitor (Instrumentation Specialties, Lincoln, NE, USA) at 280 nm and fractionated into test tubes (3 mL/tube) with a CYGNET fraction collector (ISCO).

pH-Peak-Focusing CCC of Proteins

The pH-peak-focusing CCC method used the XLLL CPC. The coil was first completely filled with the stationary upper phase of the solvent system composed of 16.0% (w/w) PEG 1000 and 12.5 (w/w) KPi (pH 9.2). This was followed by injection of 4 mL of the protein mixture through the sample port. Then the lower phase of the above solvent system adjusted at a lower pH was pumped into the column at 1.0 mL/min while the apparatus was rotated at 250 rpm. The effluent was monitored and fractions collected in a similar fashion. The pH of each fraction was manually determined by a model HM-16S pH meter (TOA, Tokyo, Japan).

Analysis

An aliquot of each fraction was diluted with distilled water and the absorbance was determined at 280 nm with a Shimadzu UV-1200 spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

RESULTS AND DISCUSSION

Volume Ratio and Settling Time of Polymer Phase Systems

Table 1 lists the composition of eleven aqueous polymer two-phase solvent systems together with their settling times and phase volume ratios. These polymer phase systems were composed of 12.5% (w/w) (No. 1-3), 16% (w/w) (No. 4-7) and 25% (w/w) (No. 8-11) of PEG 1000, each with 12.5%(w/w) potassium phosphate at different pHs ranging from 6.8 to 9.2. In all these solvent systems, the upper phase is rich in PEG and the lower phase is rich in potassium phosphate.

In all groups, the settling time of these aqueous polymer phase systems increases with the relative concentration of monobasic potassium phosphate. On the other hand, an increase in the PEG concentration results in its decrease. In each group the phase volume ratio increases with the concentration of the monobasic potassium phosphate in the solvent system. The systems 4-7, containing 16.0% (w/w) PEG 1000 yield a desirable phase volume ratio near 1 so that either upper or lower phase can be chosen as the mobile phase without excessive waste of the solvent system. These results indicate that the solvent system composed of 16.0% (w/w) PEG 1000-12.5% (w/w) dibasic potassium phosphate may be most suitable, since the volume ratio of the upper to the lower phases is 0.82 with the relatively short settling time of 96 sec.

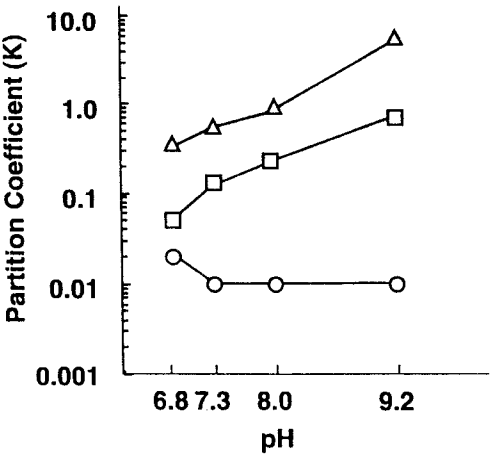

Figure 1. Partition coefficient of cytochrome c (○), myoglobin (□) and human serum albumin (△) at various pH's of the solvent systems.

Table 1

Settling Time and Phase Volume Ratio of Several Aqueous Polymer Phase Systems

No.	Concentration (w/w) % PEG-1000	KH_2PO_4	K_2HPO_4	pH	Settling Time (sec)	Volume Ratio (UP/LP)
1	12.5	4.2	8.3	7.3	213	0.72
2	12.5	2.1	10.4	8.0	180	0.67
3	12.5	0	12.5	9.2	152	0.62
4	16.0	6.25	6.25	6.8	125	1.22
5	16.0	4.2	8.3	7.3	114	1.00
6	16.0	2.1	10.4	8.0	112	0.88
7	16.0	0	12.5	9.2	96	0.82
8	25.0	6.25	6.25	6.8	86	1.73
9	25.0	4.2	8.3	7.3	73	1.61
10	25.0	2.1	10.4	8.0	65	1.40
11	25.0	0	12.5	9.2	57	1.31

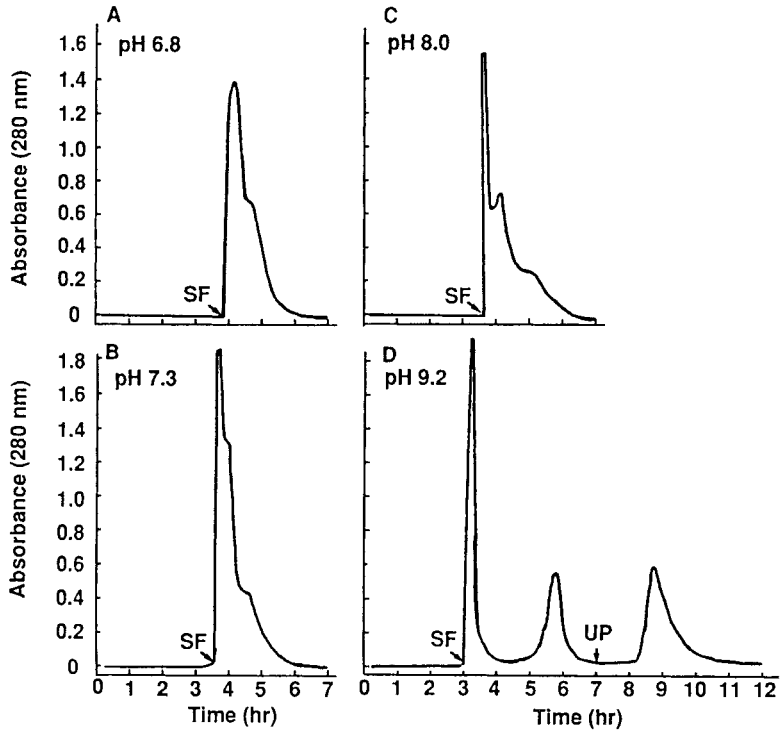

Figure 2. Conventional countercurrent chromatography of cytochrome c, myoglobin and ovalbumin by the XL CPC using three different pH in the solvent systems. A: pH 6.8; B: pH 7.3; C: pH 8.0; and D: pH 9.2. Experimental conditions: apparatus: XL CPC with a pair of multilayer coils of 2.6 mm ID and 340 mL capacity; solvent system: 16% (w/w) PEG 1000-12.5% (w/w) KPi (pH 6.8-9.2), lower phase mobile; sample: cytochrome c (10 mg), myoglobin (20 mg) and human serum albumin (100 mg) in 6 mL of the solvent system (3 mL each phase); flow-rate: 1.0 mL/min (P_IHO elution mode); revolution 500 rpm; stationary phase retention: 42.6% (A), 46.3% (B), 49.3% (C), and 55.9% (D); SF: solvent front; UP: upper phase eluted in the reversed direction.

Partition Coefficients of Proteins

For achieving efficient separation of proteins it is essential to optimize the partition coefficient of each component in the polymer phase systems used for the separation. In this paper, K is defined by the concentration of solute in the upper phase divided by that in the lower phase or $K = C_U/C_L$. Table 2 lists the partition coefficient of nine stable proteins in the aqueous two-phase solvent systems

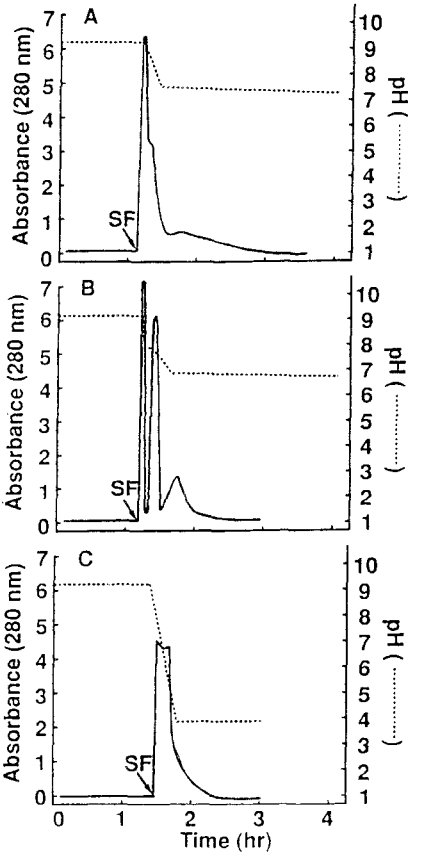

Figure 3. pH-peak-focusing countercurrent chromatography of cytochrome c, myoglobin, and ovalbumin by the XLLL CPC at different mobile phase pH. Experimental conditions: apparatus: XLLL CPC with a pair of multilayer coils of 1.6 mm ID and 110 ml capacity; stationary phase: upper phase of 16.0% (w/w) PEG 1000-12.5% (w/w) KPi (pH 9.2); mobile phase: lower phase of the above solvent system at pH 7.3 (A) and pH 6.9 (B) and that of 16.0% (w/w) PEG 1000-20% (w/w) NaH_2PO_4 at pH 4.0 (C); sample: cytochrome c (10 mg), myoglobin (20 mg) and ovalbumin (100 mg) in 6 mL of the solvent system (3 mL each phase); flow-rate: 1.0 mL/min in the P_iHO elution mode; revolution: 250 rpm; stationary phase retention: 32.6% (A), 25.2% (B) and 14.8% (C); SF: solvent front.

composed of 12.5, 16.0 and 25.0% (w/w) PEG 1000-12.5% (w/w) potassium phosphate buffer at a wide range of pH from 6.8 to 9.2. The system with 12.5% (w/w) PEG and 12.5% (w/w) potassium phosphate at pH 6.8 gives a single phase. The K values of cytochrome c and myoglobin are mostly less than unity while those

of lysozyme, α- and γ-globulins are always larger than 1.0. As the pH of the system is increased from 6.8 to 9.4, the K values of myoglobin, bovine and human hemoglobins, lysozyme, α- and γ-globulins are increased in all two-phase solvent systems examined. The K values of many proteins such as ovalbumin, human hemoglobin, lysozyme and α-globulin become greater as the concentration of PEG increases from 12.5 to 25.0%(w/w) regardless of the solvent pH.

In the present solvent system, the PEG-rich upper phase is much more viscous than the phosphate-rich lower phase. Therefore, it is more advantageous to use less viscous lower phase as the mobile phase.

In order to demonstrate the capability of the cross-axis CPC in protein separation, a mixture of cytochrome c, myoglobin and human serum albumin were subjected to CCC fractionation using the 16.0%(w/w) PEG 1000-12.5%(w/w) KPi at various pH. The use of stable colored protein samples facilitates observation of the solute peaks. The 1/K values of these three proteins are plotted against the pH of the solvent systems (Fig. 1). The 1/K values of cytochrome c increases as the solvent pH increases. On the other hand, those of both myoglobin and human serum albumin decrease with the increased solvent pH. From Fig. 1, a large difference in the 1/K values between cytochrome c and myoglobin is obtained at pH 9.2 indicating the optimum operational condition for separation of these two proteins. In this solvent system, human serum albumin is retained in the column for a long period of time because of its low 1/K value (0.2).

Conventional CCC Separations of Proteins

Fig. 2 shows the CCC separation of cytochrome c, myoglobin and human serum albumin using a polymer phase system composed of 16.0% (w/w) PEG 1000-12.5%(w/w) KPi at several pH values ranging from 6.8 to 9.2. The separation was performed at 500 rpm at a flow-rate of 1.0 ml/min using the lower phase as the mobile phase.

Three components were not resolved at pHs 6.8 (A) and 7.3 (B), because of their low K values. As the pH is increased to 8.0, three components were partially resolved (C). Further increase to 9.2 resulted in their complete separation (D).

After the elution of cytochrome c (K=0.02) and myoglobin (K=0.77), the column was eluted with the upper phase (originally used as the stationary phase) in the opposite direction to collect the human serum albumin (K=5.01) still retained in the column. Table 2 lists K values in a broad range of pH in these solvent systems. The table is useful for selection of solvent systems for separation of these proteins.

Table 2

Partition Coefficient (C_U/C_L) of Proteins In Several Aqueous Polymer Phase Systems

	Partition Coefficient (K = C_U/C_L)										
	12.5% PEG 1000-12.5% KPi			16.0% PEG 1000-12.5% KPi				25.0% PEG 1000-12.5% KPi			
Proteins	**pH: 7.3**	**8.0**	**9.2**	**pH: 6.8**	**7.3**	**8.0**	**9.2**	**pH: 6.8**	**7.3**	**8.0**	**9.2**
Cytochrome C	0.15	0.10	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.02
Myoglobin	0.37	0.36	0.48	0.05	0.13	0.23	0.77	0.09	0.37	0.58	15.8
Human Serum Albumin	0.64	0.71	1.56	0.36	0.55	0.93	5.10	11.1	166	86.0*	106
Ovalbumin	0.86	0.82	1.74	2.13	1.80	2.29	2.94	14.1	47.5	25.6	178
Bovine Hemoglobin	0.38	0.65	12.8	0.17	0.18	0.58	22.0	0.15	4.00*	26.3	51.6
Human Hemoglobin	0.83	2.34	24.0	0.41	0.85	3.64	78.0	2.14*	14.7*	27.3	88.0
Lysozyme	1.20	1.28	1.52	1.70	1.83	2.23	3.61	20.0	59.0	48.7	77.0*
α-Globulin	2.72	3.92	9.00*	3.36*	7.00*	9.60	21.5	13.4*	44.0*	61.0*	92.0
γ-Globulin	3.88	7.06	47.6	6.90	1.22*	14.7*	25.0*	---	---	---	28.0*

Two-phase systems were composed of 12.5-25% of PEG 1000 and 12.5% potassium phosphate (KPi).

* A part of proteins dissolved in the two-phase systems; --- not dissoloved in the two-phase systems.

pH-Peak-Focusing CCC of Proteins

In the past, CCC separations of protein mixture containing cytochrome c, myoglobin, ovalbumin were performed with the cross-axis CPCs using the conventional technique.[13] In these separations, the ovalbumin produced a broad peak requiring a long elution time. A new elution method, called pH-peak-focusing CCC, can produce a sharp elution peak by manipulating the pH of both stationary and the mobile phases[22] to shorten the separation time in analogous to the conventional stepwise or gradient elution. The method was demonstrated in the separation of DNP amino acids using organic and aqueous solvent systems.[23] In the present study, this technique was used for the separation of proteins using the XLLL CPC equipped with a small capacity column for shortening the separation time.

Fig. 3 shows three chromatograms obtained by pH-peak-focusing CCC. In these experiments, the column was first filled with the upper stationary phase of the 16.0% (w/w) PEG 1000-12.5% (w/w) KPi at pH 9.2 followed by elution with the lower phase having different pHs. The sample solution containing about 10 mg cytochrome c, 20 mg of myoglobin and 100 mg of ovalbumin was loaded into a 110 mL capacity column of the XLLL CPC. The separation was performed at 250 rpm at a flow-rate of 1.0 mL/min.

The results indicated that the pH of the mobile phase has an important effect on the separation. At pH 7.3 (Fig. 3A), the first two peaks were only partially resolved and the third ovalbumin peak showed a broad peak. When the pH was lowered to 6.8 (Fig. 3B), all peaks were well resolved and eluted in two hours. Further reducing the pH of the mobile phase to 4.0 (Fig. 3C) resulted in loss of resolution.

The present studies indicate that the partition coefficients of the proteins provide an important guide for the separation of proteins. Conventional CCC requires a long separation time, which is substantially shortened by the pH-peak-focusing CCC technique.

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