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PROTEIN SEPARATION BY IMPROVED CROSS-AXIS COIL PLANET CENTRIFUGE WITH ECCENTRIC COIL ASSEMBLIES

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ABSTRACT

The new prototype of the cross-axis coil planet centrifuge (X-axis CPC) fabricated in our laboratory provides various improvements over the original unit such as ambient temperature control, good visibility of the rotary frame and substantial reduction of the torque by a round transparent case, direct motor shaft coupling to the rotary frame to stabilize the system, and ease of belt tension adjustment using idler pulleys. The

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capability of the system was demonstrated in the separation of stable proteins with a polymer phase system using a pair of eccentric coil assembly separation columns. Cytochrome C, myoglobin and trypsinogen were well resolved and eluted in 5.5 h at a partition efficiency of 200 theoretical plates. The method provides a gentle environment for proteins without causing their deactivation or loss.

INTRODUCTION

Countercurrent chromatography (CCC) is a continuous liquid-liquid partition method which does not require a solid support. The stationary phase is retained in the column by the aid of gravity or, in more modern versions, centrifugal force. All of the complications arising from the use of solid supports (deactivation, sample loss, etc.) are thereby avoided. Among various CCC systems developed in the past, high-speed CCC has proven most useful since it provides the advantages of high peak resolution and short separation times while using a durable and stable instrument.[1] However, when applied to aqueous-aqueous polymer phase systems that are characterized by low interfacial tension and high viscosity, the usual form of the apparatus does not properly retain the stationary phase.

The cross-axis coil planet centrifuge (X-axis CPC) was introduced to solve the above problem by providing a unique mode of planetary motion in which the column holder rotates about its horizontal axis while revolving around the vertical axis of the centrifuge. This motion satisfactorily retains the stationary phase when used with polymer phase systems required for protein separations.[2]

Previous studies on the separation of stable proteins by the X-axis CPC[3] have demonstrated that the ratio between the revolution radius (X) and the lateral deviation (L) of the coiled column is an important parameter determining both retention of stationary phase and peak resolution. Our recent studies further indicated that an improved version of the X-axis CPC which provides two positions for the column was especially useful in the separation of proteins.[4]

The present paper describes the performance of our new prototype X-axis CPC which provides various improvements over the original unit. The capability of the apparatus was demonstrated in separation of stable proteins with a polymer phase system using a pair of eccentric coil assemblies mounted in an off-center position.

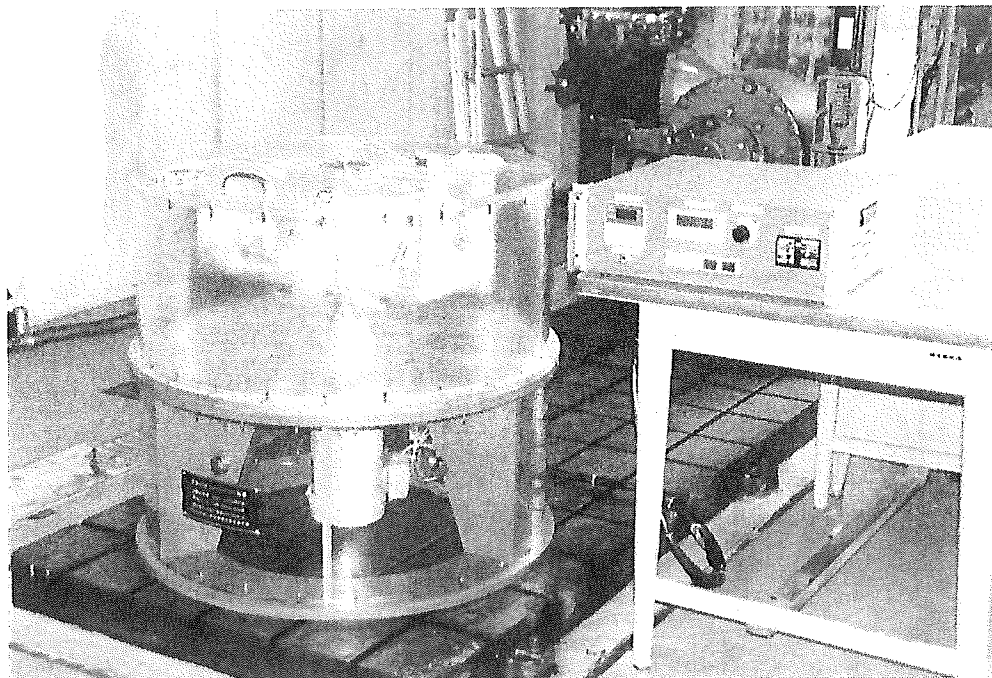

Figure 1. Photograph of the apparatus.

EXPERIMENTAL

Apparatus

The X-axis CPC employed in the present studies was constructed at the machine shop of Nihon University, Chiba, Japan. The basic feature of the apparatus was previously described in detail[4] and a brief description is given here. The apparatus produces a synchronous planetary motion of column holder which rotates about its horizontal axis and simultaneously revolves around the vertical axis of the apparatus at the same angular velocity. The column holder was mounted at an off-center position (X = 10 cm and L = 15 cm) which provides efficient mixing of the two solvent phases and stable retention of the stationary phase in the column.

Figs. 1-3 show the design of the present apparatus. The following improvements were made over the original unit:

Visibility of the rotary frame, the torque and windage were improved by a round transparent centrifuge case (Figs. 1 and 2); a cooling fan is installed on

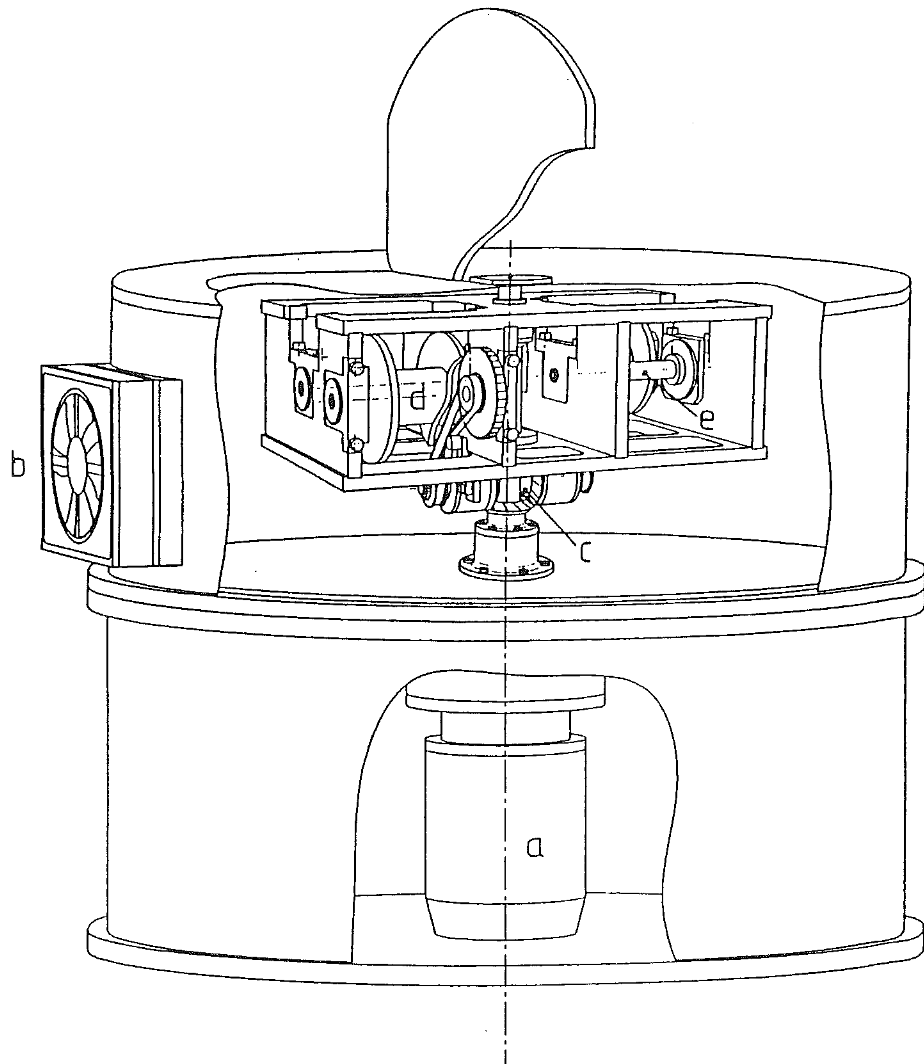

Figure 2. Schematic drawing of the side view of the apparatus. a: motor; b: cooling fan; c: spiral miter gear; d: column holder (off-center position); e: tube holder shaft (central position).

the centrifuge wall to maintain the steady ambient temperature (Fig. 2); the rotary frame is directly driven by the motor mounted at the bottom to improve the stability of the machine (Figs. 1 and 2); and tension of the toothed belts on the rotary frame is precisely adjusted by idler pulleys in both column positions (Fig. 3). These features substantially improved the performance of the apparatus.

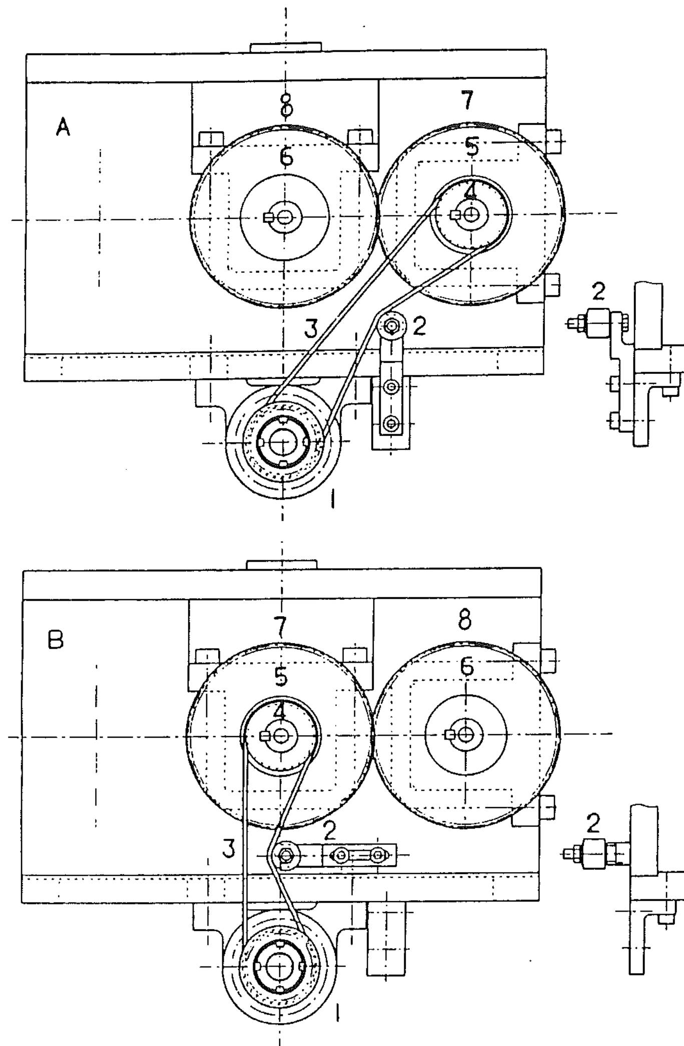

Figure 3. Driving mechanism of column holder and tube holder shaft and adjustment of the tension of the toothed belt by an idler pulley for off-center (A) and central (B) column positions. A set of miter gears at the bottom of the centrifuge axis (not shown) drives the coaxially mounted toothed pulley[1] on the rotary frame. This motion is conveyed through a toothed belt[3] to the identical pulley[4] on the holder. The gear engagement[5,6] between the holder[7] and the tube holder shaft[8] counterrotates the latter to unwind the flow tubes. The tension of the toothed belt is conveniently adjusted by an idler pulley[2] in both positions.

Preparation of the Column

The columns used in the present studies were a pair of eccentric coil assemblies described earlier.[4] Each assembly was prepared by winding a single

Table 1

Partition Coefficients of Proteins in Aqueous Two-Phase Solvent Systems

pH:	9.2	7.2	9.4	7.3	7.0	6.6
PEG 1000 (g/100g)	12.5	12.5	16.0	16.0	16.0	16.0
K_2HPO_4 (g/100g)	12.5	9.4	12.5	9.5	8.3	6.3
KH_2PO_4 (g/100g)	-----	3.4	-----	3.1	4.2	6.3
Protein						
BSA (68,000)	1.95	0.58	10.8	0.82	0.48	0.24
Ovalbumin (45,000)	1.26	0.96	3.16	1.36	1.21	0.91
Cytochrome C (12,000)	0.02	0.08	0.04	0.01	1.36	2.10
Hemoglobin (67,000)	25.0	1.16	33.7	2.10	0.97	0.36
Myoglobin (17,000)	0.59	0.30	0.71	0.13	0.09	0.09
γ-globulins	112	14.9	4.13	17.0	52.0	26.0
Trypsinogen (24,000)	1.79	1.02	5.41	1.46	1.32	1.24
Trypsin Inhibitor (20,100)	9.45	10.5	20.3	19.8	17.5	16.3
α-Chymotrypsinogen A (25,635)	6.00	5.01	29.1	11.8	9.50	6.48
apo-Transferrin	0.17	0.04	0.12	0.06	0.05	0.03
Carbonic Anhydrase (29,000)	4.46	2.53	9.88	5.00	3.67	1.83
Lactalbumin (14,200)	4.06	1.59	5.94	3.50	2.12	1.33

Partition coefficients were calculated from the absorbance of the upper phase divided by that of lower phase.

piece of 1 mm ID PTFE (polytetrafluoroethylene) tubing (Flon Kogyo Co., Tokyo, Japan) onto 7.6 cm long, 5 mm OD nylon pipes forming 20 units of serially connected left handed coils, which were then arranged around the holder with their axes parallel to the holder axis. A pair of identical coil assemblies was connected in series to obtain a total capacity of 28 mL.

Reagents

Polyethylene glycol (PEG) 1000 (M.W. 1000), cytochrome C (horse heart), myoglobin (horse skeletal muscle), ovalbumin (chicken egg), hemoglobin (human), trypsinogen (bovine pancreas), apo-transferrin (bovine), carbonic anhydrase (bovine erythrocytes), trypsin inhibitor (soybean), lactalbumin, α-chymotrypsinogen A (bovine pancreas), γ-globulins (human) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Another ovalbumin (chicken egg), monobasic and dibasic potassium phosphates and bovine serum albumin (BSA) were purchased from Wako Pure Chemicals, Osaka, Japan. All other reagents were of reagent grade.

Preparation of Two-Phase Solvent Systems and Sample Solutions

The compositions of PEG 1000 - potassium phosphate solvent systems are listed in Table 1. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated after two clear phases formed. Samples were prepared by dissolving each protein mixture in 0.4 mL of each phase of the two-phase solvent systems used for separation.

Measurement of the Partition Coefficients of Protein Samples

The partition coefficient of each protein was measured spectrophotometrically using a simple test tube procedure. Two milliliters of each phase of the equilibrated two-phase solvent system was delivered into 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 two clear layers formed, a 1 mL aliquot of each phase was diluted with 2 mL of distilled water and the absorbance was measured at 280 nm using a spectrophotometer (Model UV-160, Shimadzu Corporation, Kyoto, Japan). The partition coefficient (K) was obtained by dividing the absorbance value of the upper phase by that of the lower phase.

CCC Separations of Proteins

For each separation, the coil was completely filled with the PEG-rich upper stationary phase and the sample solution (ca. 1 mL) was charged into the column through the sample port. Then, the phosphate-rich lower mobile phase was pumped into the column at 0.2 mL/min, while the column was rotated at 800 rpm in a counterclockwise direction. The effluent from the outlet of the column was collected in test tubes (0.4 mL/tube) using a fraction collector (Model SF-200, Advantec Co., Tokyo, Japan).

Analysis of CCC Fractions

Each fraction was diluted with 2.5 mL of distilled water and the absorbance was measured at 280 nm.

Evaluation of Partition Efficiency

The partition efficiencies of protein separations were computed from the chromatogram and expressed in terms of theoretical plate number (N) and peak resolution (Rs). Both values are based on an assumption that each peak represents the distribution of a single component.

RESULTS AND DISCUSSION

Table 1 shows partition coefficients of various proteins in the PEG 1000 - potassium phosphate systems over a broad range of pH values. This data is useful for predicting the retention time of various protein samples.

Fig. 4A illustrates the separation of cytochrome C (2.5 mg), myoglobin (8 mg) and ovalbumin from Sigma Chemical Company (30 mg) by the X-axis CPC. In previous studies[4] it was found that ovalbumin from this source gave an extremely broad peak compared with those of the other two proteins. In the present experiment, the partition efficiency computed from the myoglobin peak is 101 TP (theoretical plates) and the resolution between cytochrome C and myoglobin is 1.2. The myoglobin and ovalbumin peaks, however, are once again only partially resolved due to the broad ovalbumin peak. The breadth of this peak is apparently due to heterogeneity of the ovalbumin as described in detail in the accompanying paper.[5] Fig. 4B illustrates the separation of cytochrome C, myoglobin and a sample of ovalbumin obtained from the Wako

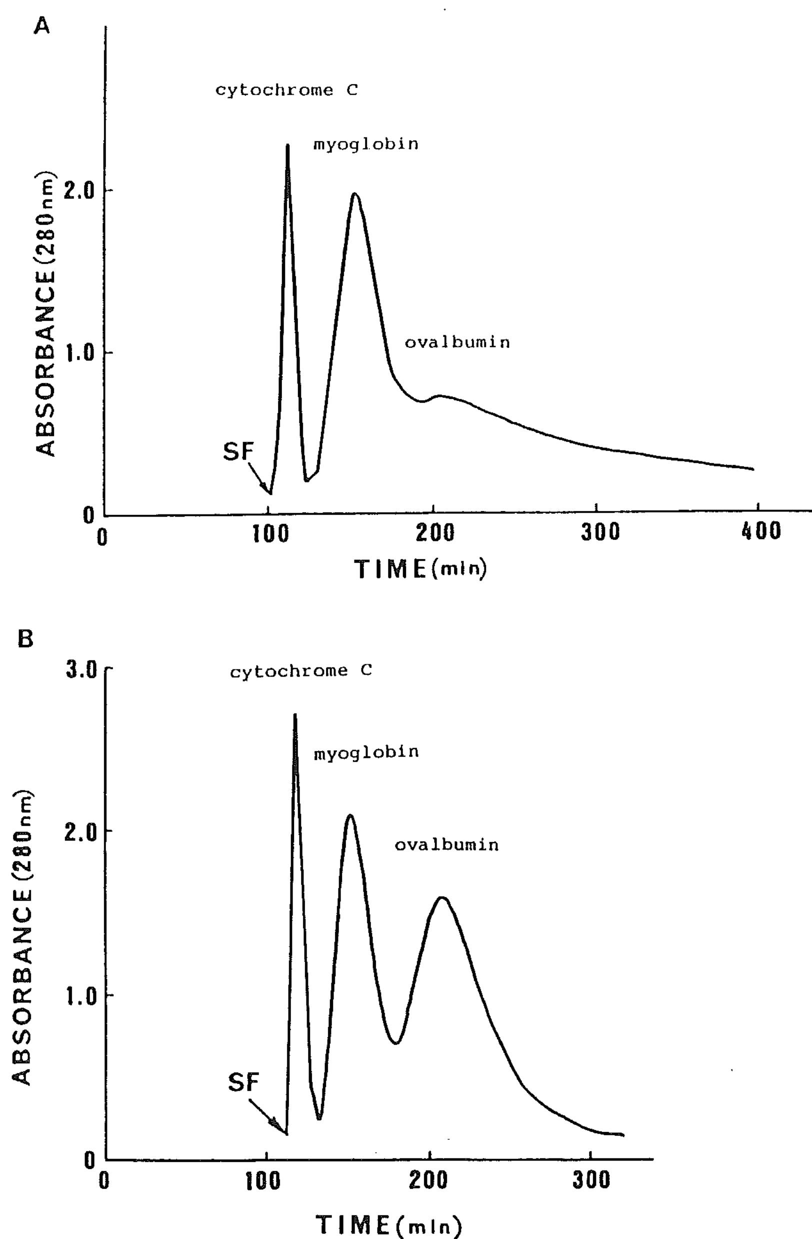

Figure 4. CCC separation of cytochrome C, myoglobin and two different commercial ovalbumin products by X-axis CPC.

Experimental conditions: Apparatus: X-axis CPC equipped with a pair of eccentric coil assemblies, 1 mm ID and 28 mL capacity; Sample: (A) cytochrome C (2.5 mg), myoglobin (8 mg), and ovalbumin (Sigma) (30 mg); (B) cytochrome C (2.5 mg), myoglobin (8 mg) and ovalbumin (Wako) (30 mg); Solvent system: 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate; Mobile phase: lower phase; Flow rate: 0.2 mL/min; Revolution: 800 rpm. SF = solvent front

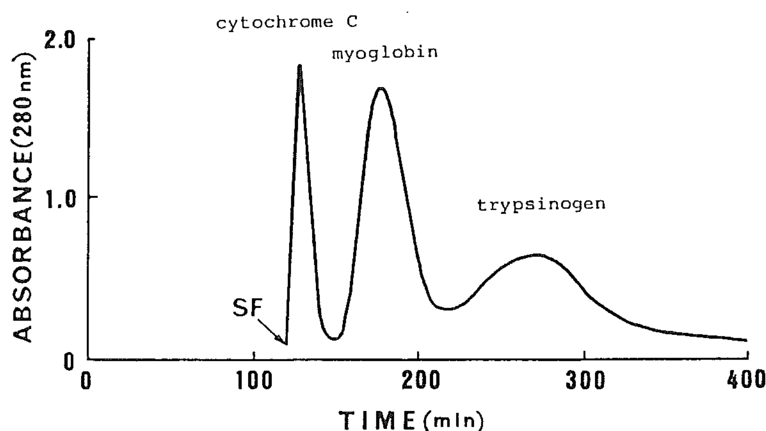

Figure 5. CCC separation of proteins by X-axis CPC.
Experimental conditions: Sample: cytochrome C (2.5 mg), myoglobin (8 mg) and trypsinogen (10 mg). Other experimental conditions are same as those described in Fig. 4 caption. SF = solvent front

Chemical Company under the same experimental conditions. The partition efficiency computed from the myoglobin peak is 183 TP, the resolution between the cytochrome C and myoglobin peaks is 1.1 and that between the myoglobin and ovalbumin peaks, 0.9. In this separation the resolution between myoglobin and ovalbumin peaks is much improved because the ovalbumin (Wako) consists exclusively of monomer in contrast with the ovalbumin from Sigma which contains both dimer and monomer forms.

Fig. 5 shows a separation of cytochrome C, myoglobin and trypsinogen where all components are well resolved. The partition efficiency computed from the myoglobin peak is 200 TP. The peak resolution between the cytochrome C and myoglobin peaks is 1.3 and that between the myoglobin and trypsinogen peaks, 1.1.

The overall results of the present studies indicate that the new prototype of the X-axis CPC yields an efficient CCC separation of 2.5 - 30 mg of proteins in several hours. The method provides a gentle environment for biological samples without loss or deactivation caused by the solid support matrix.

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