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Note

Horizontal flow-through coil planet centrifuge equipped with a set of multilayer coils around the column holder

Counter-current chromatography of proteins with a polymer-phase system

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During the preceding decade, the horizontal flow-through coil planet centrifuge has been introduced for performing preparative counter-current chromatography (CCC)[1,2]. The apparatus holds a coil assembly around the column holder which undergoes synchronous planetary motion to provide efficient mixing of the two solvent phases in the coil. Because the system permits stable retention of low-interfacial-tension two-phase solvent systems, the apparatus has been extensively used for separations of peptides and other polar compounds with hydrophilic butanol solvent systems[3]. The partition efficiency of the original apparatus, however, is largely limited by its long coil assembly (50 cm), which tends to be deformed under a strong centrifugal force field, thus the maximum revolutional speed which can be applied safely is 400 rpm. Recently, the design of the apparatus has been improved by shortening the column holder shaft to about one-third (18 cm) while the column capacity was almost recovered by doubling the layer of coil in each column unit. Using conventional butanol two-phase solvent systems, the capability of the apparatus has been demonstrated by efficient chromatographic separations of dipeptides and partition of bovine insulin at a high revolutional speed of 800 rpm[4].

The present paper describes a new design of the column assembly which consists of a set of multilayer coils symmetrically arranged around the holder shaft to double the column capacity. The capability of the present apparatus was demonstrated in protein separation with an aqueous–aqueous polymer two-phase system.

EXPERIMENTAL

Apparatus

Fig. 1 shows a photograph of the apparatus. The overall design of the apparatus was described previously[4,5]. The rotary frame of the apparatus holds a column holder and a counterweight holder diametrically at a distance of 10 cm from the

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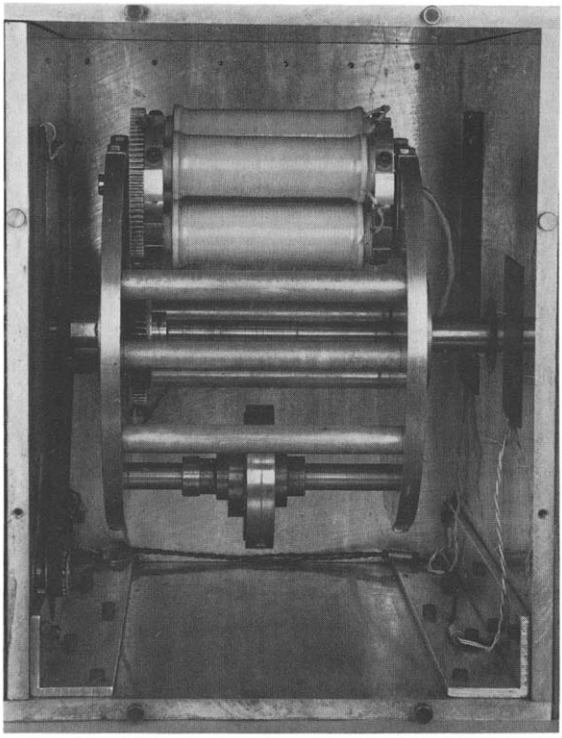

Fig. 1. Photograph of the present apparatus. The apparatus holds a set of multilayer coil separation columns connected in series while the mechanical balance of the centrifuge system is maintained with a counterweight placed in the opposite side of the rotary frame. The desired planetary motion of the holder is effected by coupling the planetary gear on the column holder shaft to an identical stationary sun gear mounted on the central stationary shaft. Temperature of the unit can be regulated up to 50°C with a set of electric heating pads and a temperature control unit.

central axis of the centrifuge. Engagement of the planetary gear, mounted on the holder shaft, to an identical stationary sun gear on the central axis of the apparatus produces a desired planetary motion of the holders, *i.e.*, revolution of the holder around the central axis of the apparatus and simultaneous rotation of the holder about its own axis at the same angular velocity in the same direction.

The column holder is removable from the rotary frame by loosening a pair of screws on each bearing block. The holder accommodates four identical multilayer coils symmetrically around the holder shaft. Each multilayer coil was prepared from an approximately 25 m length of 1.6 mm I.D. polytetrafluoroethylene (PTFE) tubing (Zeus Industrial Products, Raritan, NJ, U.S.A.) by winding it onto a spool-shaped holder hub, measuring 12.5 cm in length and 1.25 cm in diameter, making 6 layers of tightly wound coil. In order to secure the multilayer coil against the holder, the entire column and the flanges were covered with a heat-shrinkable poly(vinyl chloride) tube which was tempered by a heat gun. Four identical multilayer coils were symmetrically mounted around the holder shaft at a distance of 3.5 cm from the holder axis. Using short pieces of PTFE tubing (2 mm O.D.), these four columns were connected in series in such a way that the external terminal of the first column was connected to the

internal terminal of the second column, the external terminal of the second column to the internal terminal of the third, and so forth. The total capacity of the entire column measured about 200 ml. A pair of flow tubes from the column was first led through the center hole of the holder shaft and then passed through the side hole of the short coupling pipe to enter the opening of the central stationary pipe. At the exit of the centrifuge, they are securely clamped with the tube support. As described elsewhere, these flow tubes are free from twisting and serve for continuous elution through the rotating column without the use of the conventional rotary seal device[1].

The revolutional speed of the apparatus can be regulated up to 800 rpm with a speed control unit (Bodine Electric, Chicago, IL, U.S.A.). The present apparatus is also equipped with a column-temperature control system which regulates the ambient temperature from room temperature to 50°C with a set of electric heating pads and a temperature control unit (RFL, Boonton, NJ, U.S.A.).

Procedure

The aqueous–aqueous two-phase polymer system was prepared by dissolving 150 g of polyethylene glycol 1000 (Sigma, St. Louis, MO, U.S.A.) and 150 g of anhydrous dibasic potassium phosphate (J. T. Baker, Phillipsburg, NJ, U.S.A.) in 900 ml of distilled water. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated shortly before use. The sample solution consisted of 100 mg of each cytochrome *c* and lysozyme (both from Sigma) dissolved in 3 ml of the above solvent system.

The experiment was initiated by filling the entire column with the stationary upper phase. This was followed by sample injection through the sample port. Then, the apparatus was rotated at 800 rpm while the mobile lower phase was eluted through the column at a flow-rate of 0.5 ml/min or 1.0 ml/min. The effluent from the outlet of the column was continuously monitored with an LKB Uvicord S at 275 nm and then fractionated into test tubes at 3 ml/tube with an LKB fraction collector. After the two peaks were eluted, the apparatus was stopped and the column contents were collected into a graduated cylinder to measure the volume of the stationary phase retained in the column. An aliquot of each fraction was diluted with distilled water and the absorbance was determined with a Zeiss PM6 spectrophotometer at 280 nm and 550 nm (for cytochrome *c*) to draw a chromatogram.

RESULTS AND DISCUSSION

Fig. 2 shows a chromatogram of cytochrome *c* and lysozyme obtained with the aqueous–aqueous polymer-phase system composed of 12.5% (w/w) polyethylene glycol 1000 and 12.5% (w/w) anhydrous dibasic potassium phosphate in distilled water. The fractions containing cytochrome *c* peak were easily identified by their color and quantitated by its absorbance measured at 550 nm as indicated by the broken curve. At a flow-rate of 1.0 ml/min, the two peaks were completely resolved in 5 h. The partition efficiency may be calculated from the chromatogram according to the conventional gas chromatographic formula, *i.e.*, $N = (4t_R/W)^2$, where N denotes the partition efficiency expressed in terms of theoretical plate number; t_R, the retention time of the peak maximum; and W, the peak width expressed in the same unit as t_R. Using the above formula, the partition efficiency computed from the second peak was

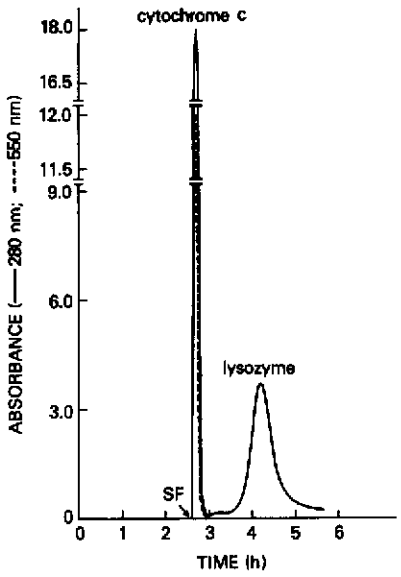

Fig. 2. Separation of cytochrome *c* and lysozyme with the aqueous–aqueous two-phase system composed of 12.5% (w/w) polyethylene glycol 1000 and 12.5% (w/w) anhydrous dibasic potassium phosphate in distilled water. Sample size: each component 100 mg; flow-rate: 1 ml/min; mobile phase: lower phase; revolution: 800 rpm. SF = Solvent front.

about 350 theoretical plates. The retention of the stationary phase was 26% of the total column capacity and the maximum column pressure measured at the outlet of the pump was 170 p.s.i. Application of a lower flow-rate of 0.5 ml/min substantially improved the peak resolution but with a longer elution time of 10 h.

Among various separation methods, the liquid–liquid two-phase partition method with polymer-phase systems is particularly suitable for separations of biopolymers and cell particles, because of its gentle separation procedure with a non-hostile environment provided by the media. However, high viscosity and low interfacial tension between the two phases tend to delay the phase settling, resulting in long separation times. Although various centrifuge devices[6–10] have been introduced to overcome this problem, high cost of these instruments generally prevents universal use of the method.

The present method yields a high partition efficiency in relatively short separation times (1 theoretical plate is produced in less than 30 s), while the apparatus is simple in design and relatively inexpensive. The method may be applied to various other biopolymers and cell particles by choosing the proper phase composition. We believe that the present method will be extremely useful for separation of various biological samples.

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