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Purification of alcohol dehydrogenase from bovine liver crude extract by dye–ligand affinity counter-current chromatography

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Abstract

Alcohol dehydrogenase (ADH) was extracted from a crude bovine liver homogenate by dye–ligand affinity counter-current chromatography (CCC) using a cross-axis coil planet centrifuge (*x*-axis CPC). The purification was performed using two types of polymer phase systems composed of 4.4% polyethylene glycol (PEG) 8000–7.0% dextran T500–0.1 M potassium phosphate buffers and 16% PEG 1000–12.5% potassium phosphate buffers, both containing a procion red dye as an affinity ligand at various pH values. The best purification was achieved using the PEG 1000–potassium phosphate system at pH 7.3 containing 0.05% procion red as a ligand. The upper PEG-rich phase containing procion red was used as the stationary phase and a crude bovine liver homogenate was eluted with the potassium phosphate-rich lower phase at 0.5 ml/min. After elution of bovine liver proteins in the homogenate, ADH still retained in the stationary phase was collected from the column by eluting with the PEG 1000-rich upper phase. Collected fractions were analyzed by ADH enzymatic activity and by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) to detect contaminant proteins in the ADH fractions. The ADH was purified directly from crude bovine liver extract within 6 h with minimum loss of its enzymatic activity.
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1. Introduction

From the late 1960s to the early 1970s, dye–ligand affinity chromatography was widely used for the separation and purification of wide variety of proteins. Bohme et al. [1] prepared a column packing material from Sephadex G 200 bonded with cibacron blue F3G-A dye for the purification of the yeast phosphofructokinase [1,2]. Several dyes, such as cibacron, procion, remazol, reactor, and drimarene, were evaluated for their use as affinity ligands for separation of biomolecules and their analogs. The polyaromatic sulfonated dyes including cibacron blue and procion red served as a group specific affinity ligands for a surprisingly broad array of functionally distinct classes of proteins. In order to achieve the dye–ligand affinity chromatography of biomolecules, the reactive dye should be coupled to the functional groups, such as hydroxyl and amino groups, on the surface of the matrices. Cibacron and procion dyes containing mono- or dichlorosubstituted triazine rings are coupled to several types of support material, including cross-linked agarose, beaded agarose, and polyhydroxy methacrylate. However, coupling the dye to the support material requires time-consuming tedious works such as washing out the unbound dyes which adsorbed onto the surface of the matrices [3]. Also, most of these matrices have a high tendency of non-specific adsorption of proteins, resulting in the low recovery of the target proteins from the column.

Counter-current chromatography (CCC) is a generic name for various continuous liquid–liquid partition methods which eliminate the use of solid support matrices [4–6]. Among all existing CCC systems, high-speed CCC is considered the most advanced form in terms of partition efficiency and separation times. CCC utilizes a pair of immiscible solvent phases which have been preequilibrated in a separatory funnel in which one phase is used as the stationary phase and the other as the mobile phase. A type J coil planet centrifuge (CPC) has been successfully used for the separation of plasmid DNA using polyethylene glycol (PEG)–potassium phos-

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phate two-phase system [7]. A spiral disk assembly provides satisfactory retention of the stationary phase for polar solvent systems including aqueous polymer two phase systems [8]. The cross-axis (*x*-axis) coil planet centrifuge is one of the CCC systems that provides reliable retention of the stationary phase of viscous aqueous polymer phase systems used for protein separations. The recovery of the proteins from the separation column is always near 100% because of minimum adsorption loss in the column. If one of the dyes, such as cibacron and procion, is unilaterally partitioned to either phase of the aqueous polymer two phase systems, dye–ligand affinity CCC becomes feasible for separations of protein samples.

In this study, we attempted the dye–ligand affinity CCC separation of alcohol dehydrogenase (ADH) from a crude bovine liver extract using the aqueous–aqueous polymer two-phase systems containing a procion red dye.

2. Experimental

2.1. Apparatus

The cross-axis coil planet centrifuge has a unique feature among the CPC systems available in that it provides reliable retention of the stationary phase for viscous polymer phase systems. The detailed design of the *x*-axis CPC was described elsewhere [9,10]. The present apparatus holds a pair of column holders in the lateral position at 10 cm from the center of the rotary shaft horizontally mounted on the rotary frame at 10 cm from the central axis of the apparatus as shown in Fig. 1. A spool-shaped column holder is mounted on each rotary shaft at an off-center position 10 cm from its mid-point. The large multilayer coil separation column was prepared from a 2.6 mm i.d. polytetrafluoroethylene (PTFE) tube (Zeus Industrial Products, Raritan, NJ, USA) by winding it onto a 5.0 cm diameter holder hub, forming three layers of left-handed coils between a pair of flanges spaced 5 cm apart. A pair of columns mounted on the rotary frame was connected in series with a flow tube (PTFE, 0.85 mm i.d.) resulting in the total capacity of 165 ml. The speed of the apparatus is regulated at 500 rpm with a speed control unit (Bodine Electric, Chicago, IL, USA).

2.2. Reagents

Alcohol dehydrogenase from yeast was obtained from Oriental Yeast (Osaka, Japan) for the determination of the partition coefficient values. Dextran T500 (weight-average molecular mass, $M_r = 500{,}000$); polyethylene glycol (PEG

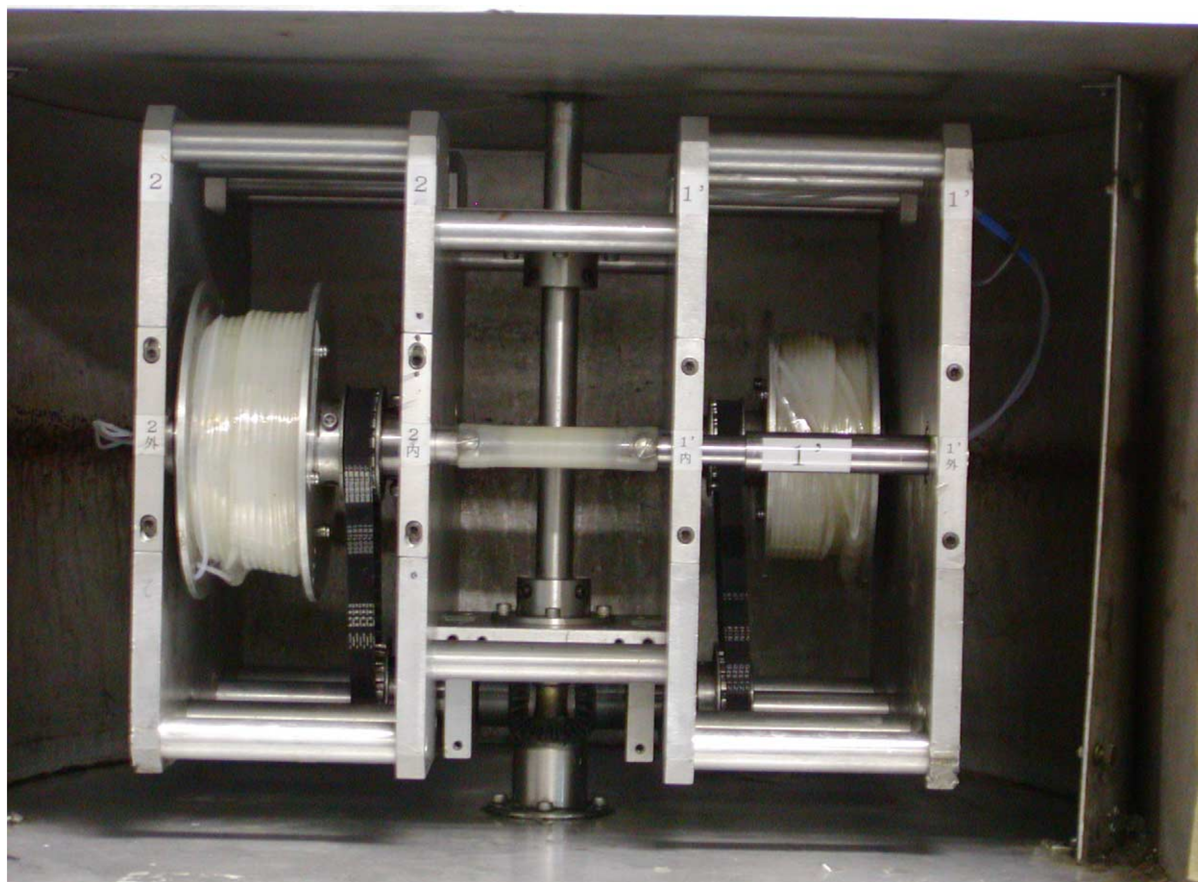

Fig. 1. Type-XL cross-axis coil planet centrifuge equipped with a pair of multilayer coils connected in series.

1000 (number-average molecular mass, M_n = 950–1050); PEG 8000 (M_n = 6000–7500); mono- and dibasic potassium phosphates for the preparation of aqueous polymer two-phase systems were purchased from Kanto Chemicals (Tokyo, Japan). Other chemicals were all of analytical reagent grade.

2.3. Preparation of bovine liver crude extract

About 200 g of bovine liver was homogenized with 200 ml of ice-cold water in a household mixer for 15 min. The homogenate was centrifuged at 30,000 rpm for 1 h and the supernatant was filtered through a glass filter G3. The filtrate was again filtered through a Millipore filter (pore size: 0.45 μm) (Nihon Millipore Kogyo K.K., Yonezawa, Japan) before loading onto the CPC column. The bovine liver crude extract thus obtained was stored at −20 °C until use.

2.4. Preparation of PEG-potassium phosphate and PEG-dextran aqueous two-phase solvent systems containing dye ligand

The solvent systems used for dye–ligand affinity CCC were prepared by modifying the standard polymer phase systems used for the protein separation [11,12]. The PEG 8000-dextran T500 systems were prepared by dissolving 44 g of PEG 8000, 70 g of dextran T500 and 0.5 or 2.0 g procion red or 2.0 g of blue dextran in 886 g of a 100 mM potassium phosphate buffer solution. The pH of the system was similarly adjusted by choosing the proper ratio between the mono- and dibasic potassium phosphates. This two-phase solvent system consisted of the PEG-rich upper phase and dextran-rich lower phase where both blue dextran and procion red were partitioned in the upper PEG-rich phase.

The PEG-phosphate polymer phase systems were similarly prepared by dissolving 160 g of PEG 1000, 125 g of potassium phosphate, and 0.5 or 2 g of procion red in 715 g of water. Alternatively, 2 g of blue dextran was added to the same solvent systems. The ratio of monobasic to dibasic potassium phosphates determines the pH of the solvent system, affecting the partition coefficients of the ADH. These polymer solutions form two layers, the upper layer is rich in PEG and the lower layer is rich in potassium phosphate where procion red was mostly distributed in the upper phase and blue dextran in the lower phase. The solutions were thoroughly mixed in a separatory funnel and allowed to settle into two clear layers before use.

2.5. Determination of partition coefficient of alcohol dehydrogenase

The partition coefficients of the ADH were determined in each two-phase solvent system composed of PEG 8000/dextran T500/100 mM potassium phosphate buffers and PEG 1000/potassium phosphate buffers with and without procion red or blue dextran as an affinity ligand at various pH values. A standard enzyme solution (5 mg/ml in 100 μl) was added to 3 ml of the polymer two-phase systems (1.5 ml of each phase). After equilibration, aliquots of the upper and lower phases were diluted 10 times with distilled water and ADH enzyme activities in both phases were determined with the method described by Bonnichsen and Brink [13].

2.6. Counter-current chromatography of bovine liver crude extract

In each experiment, the CPC column was first entirely filled with the stationary upper phase containing procion red, followed by injection of the sample solution (a mixture of 3 ml of bovine liver crude extract containing PEG, dextran and/or potassium phosphate to meet the composition of the two-phase solvent system used for the separation) using an EYELA type SV-6000 sample injector (Tokyo Rikakikai, Tokyo, Japan). Then the lower phase was eluted through the column at a flow rate of 0.5 ml/min while the apparatus was rotated at 500 rpm. The effluent from the outlet of the column was monitored using an EYELA UV-9000 absorbance monitor (Tokyo Rikakikai) at 280 nm, and fractionated into test tubes (3 ml) using an LKB 2112 Redirac fraction collector (LKB Instruments, Stockholm, Sweden). Proteins in the CCC fractions were analyzed by 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli [14]. The contaminant proteins in the purified ADH fractions were detected by Coomassie brilliant blue staining.

2.7. Measurement of enzyme activity

ADH in the CCC fractions was determined by an enzymatic analysis [13]. When ethanol (the substrate of ADH) is oxidized to acetaldehyde by ADH, NAD^+ (nicotin amide adenine dinucleotide) is reduced to NADH, increasing the absorbance at 340 nm, which quantitatively indicates the enzymatic activity of ADL.

3. Results and discussion

3.1. Partition coefficient of the standard ADH

CCC is an aqueous–aqueous partition method where the separation is based on the difference in partition coefficient of solutes between the two solvent phases. To achieve efficient separation of ADH from bovine liver homogenate, it is essential to optimize the partition coefficient of ADH by selecting a proper pH of the polymer phase systems. In conventional CCC, the two kinds of aqueous–aqueous polymer two-phase systems, i.e., PEG-dextran and PEG-phosphate biphasic systems have been used for the separation of proteins [11,12]. Since both cibacron blue and procion red dyes have been effectively used as ligands for group specific affinity chromatography of biomolecules [3], affinity CCC was

Table 1
Partition coefficient of alcohol dehydrogenase in PEG-dextran systems with and without ligand dyes

Ligands	Concentration (%)	pH		
		6.5	6.8	7.7
Blue dextran	0	0.32	0.41	$<10^{-4}$
	0.2	504	506	91.4
Procion red	0.05	0.86	0.91	$<10^{-4}$
	0.2	2.37	3.55	0.01

Solvent systems: 4.4% PEG 8000–7.0% dextran–100 mM potassium phosphate buffer.

performed by dissolvent these two dyes in the stationary phase of aqueous polymer phase systems.

Table 1 shows the partition coefficients of the standard ADH in the solvent systems composed of 4.4% PEG 8000–7.0% dextran T500 with and without two kinds of dye ligands. In these systems, both blue dextran and procion red are always partitioned in the upper PEG-rich phase. The partition coefficients of ADH in the dye-free two-phase solvent systems are 0.32, 0.41 and smaller than 10^{-4} at pH 6.5, 6.8, and 7.7, respectively, indicating that ADH has a higher affinity to the dextran T500-rich lower phase.

As shown in Table 1 (middle column), the partition coefficients of the enzyme was extremely increased when blue dextran was added to the solvent systems at 0.2% in all pH groups. The addition of procion red to the solvent system results in the increase of the partition coefficients of ADH at pH 6.5 and 6.8. The increase of procion red concentration from 0.05 to 0.2% of the solvent systems resulted in an increased partition coefficient of ADH from 0.86 to 2.37 at pH 6.5 and from 0.91 to 3.55 at pH 6.8. At pH 7.7, the partition coefficients of ADH are very low but they are increased significantly.

Table 2 shows the partition coefficient of ADH in the 16% PEG–12.5% potassium phosphate buffer systems at pH 6.8, 7.3, and 8.0 with and without dyes. In these solvent systems, the blue dextran was mostly partitioned to the phosphate-rich lower phase whereas procion red was mainly distributed to the PEG 1000-rich upper phase. As the pH of the dye-free solvent system was increased to 6.8, 7.3, and 8.0, the partition coefficients of ADH increased to 0.42, 2.34 and greater than 10^4, respectively. When the 0.2% concentration of blue dextran was added to the solvent system, the partition coefficient of ADH slightly increased from that in the dye-free system at pH 6.5. The partition coefficients of ADH in these solvent systems containing 0.2% blue dextran at pH 7.3 and 8.0 decreased compared with those obtained in the dye-free solvent systems, because ADH has a high affinity to blue dextran that partitioned in the potassium phosphate-rich lower phase. On the other hand, procion red was mostly partitioned in the upper phase regardless of the pH, and the partition coefficients of ADH became greater than 10^4 in the solvent systems containing 0.05% procion red. The results clearly indicated that using these dyes as affinity ligands it will be possible to separate ADH from a bovine liver crude extract by CCC.

Table 2
Partition coefficient of alcohol dehydrogenase in PEG-potassium phosphate buffer systems with and without ligand dyes

Ligands	Concentration (%)	pH		
		6.8	7.3	8.0
Blue dextran	0	0.42	2.34	$>10^4$
	0.2	0.68	1.57	5.43
Procion red	0.05	$>10^4$	$>10^4$	$>10^4$

Solvent systems: 16% PEG 1000–12.5% potassium phosphate buffer.

Since blue dextran has a limited solubility to the polymer phase systems in addition to its high viscosity, procion red is chosen as an affinity ligand in the present study.

3.2. Purification of ADH from the bovine liver homogenate by CCC

ADH was separated from the bovine liver crude extract by the *x*-axis CPC using the 4.4% PEG 8000–7.0% dextran T500 aqueous polymer two-phase systems at pH 6.5 containing 0.05% procion red as an affinity ligand. The separation was initiated by filling the entire column with the PEG-rich upper stationary phase containing procion red. This was followed by injection of the sample solution containing 3 g of the liver homogenate, 0.15 g of PEG 8000 and 0.26 g of dextran T500 through the sample port. The separation was performed at 500 rpm at a flow-rate of 0.5 ml/min using the dextran-rich lower phase as the mobile phase. The solvent front emerged at a retention volume of 140 ml. Fig. 2 shows the elution profile where the absorbance of proteins at 280 nm (open circles) and the ADH enzymatic activity (closed circles) are plotted against the retention volume. Proteins in the bovine liver extract were mostly eluted from the column in two peaks and one shoulder in the retention volumes from 150 to 240 ml, whereas the ADH enzymatic activity was detected at the retention volumes from 150 to 240 ml. The retention of the stationary PEG 8000-rich upper phase measured after purification was 9.5% of the total column capacity. Because of the poor retention of the stationary phase, the ADH fractions were overlapped with other proteins. We concluded that the PEG 8000-dextran T500 aqueous polymer two-phase system is not suitable for the purification of ADH from the bovine liver crude extract. Because the addition of the procion red ligand to the solvent systems results in a little change of the partition coefficients of the ADH at all pH groups, the dye–ligand CCC is also not feasible with these solvent systems.

The other polymer phase systems, composed of the PEG 1000 and potassium phosphate buffers, were then tested for the purification of ADH from the same crude extract. Because the partition coefficient of ADH was greatly influenced by the addition of procion red to these solvent systems, it is feasible that the group specific affinity CCC

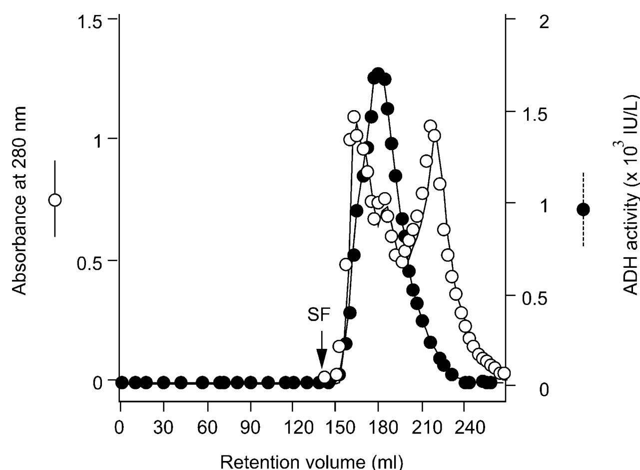

Fig. 2. Dye–ligand counter-current chromatography of alcohol dehydrogenase from crude bovine liver extract. Experimental conditions: apparatus: the XL cross-axis coil planet centrifuge with a pair of multilayer coil columns of 2.6 mm i.d. and 165 ml capacity; solvent system: 4.4% (w/w) PEG 8000–7.0% (w/w) dextran T500–10 mM potassium phosphate–0.05% (w/w) procion red at pH 6.5; stationary phase: PEG 8000-rich upper phase; mobile phase: dextran-rich lower phase; sample: 3 g of bovine liver crude extract, 0.15 g of PEG 8000 and 0.26 g of dextran T500; flow-rate: 0.5 ml/min; revolution: 500 rpm; stationary phase retention: 9.5% of the total column capacity (165 ml); SF: solvent front.

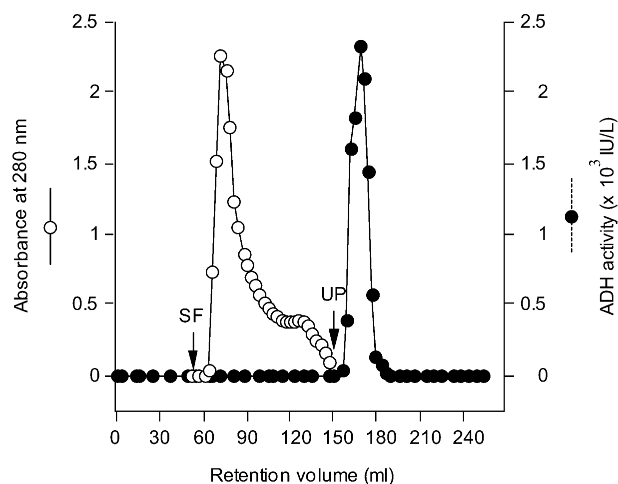

Fig. 3. Dye–ligand counter-current chromatographic purification of alcohol dehydrogenase from crude bovine liver homogenate. Experimental conditions: apparatus: the XL cross-axis coil planet centrifuge with a pair of multilayer coil columns of 2.6 mm i.d. and 165 ml capacity, solvent system: 16% (w/w) PEG 1000–12.5% potassium phosphate–0.05% (w/w) procion red at pH 7.3; stationary phase: PEG 1000-rich upper phase; mobile phase: potassium phosphate-rich lower phase; sample: 3 g of bovine liver crude extract, 0.67 g of PEG 1000 and 0.52 g of potassium phosphate; flow-rate: 0.5 ml/min; revolution: 500 rpm; stationary phase retention: 28.2% of the total column capacity (165 ml) prior to the application of the elution with the upper phase; SF: solvent front; UP: elution with the upper phase.

of ADH can be achieved. The separation was similarly performed at 500 rpm at a flow-rate of 0.5 ml/min using the potassium phosphate buffer system at pH 7.3, where the PEG-rich upper phase containing procion red at 0.05% was used as the stationary phase.

Fig. 3 shows the chromatograms of the bovine liver crude extract obtained by the *x*-axis CPC using the 16% PEG 1000–12.5% potassium phosphate buffer system at pH 7.3, where absorbance of the proteins at 280 nm (open circles) in the fractions and the ADH activity (closed circles) are plotted against the retention time. The solvent front emerged at the retention volume of 54 ml. The majority of protein mass was eluted at the retention time of between 60 and 150 ml. After the elution of the major protein peak, the PEG 1000-rich upper phase was pumped into the column in the reversed direction to facilitate rapid elution of ADH still remaining in the column. The purified enzyme was eluted together with the procion red dye. The fractions were diluted with 10-folds of distilled water and dialyzed with an ultrafiltration membrane YM10 (Milipore, Bedford, MA, USA) to remove the dye and PEG 1000. The upper stationary phase retained in the column was estimated as 28.2% of the total column capacity (165 ml) prior to the application of the reversed elution with the upper phase. Because of the sample solution obtained from a bovine liver extract was very curde, it was clear that the carry over of the PEG 1000-rich upper phase was caused a poor retention of the stationary phase. The enzymatic activity of ADH was detected in the fractions obtain at retention times between 165 and 180 ml, and the separation was completed within 6 h. The chromatographic fractions were analyzed by 12.0% (w/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis with Coomassie brilliant blue staining. These results indicated that the ADH is separated in the fractions eluted at the retention volume between 165 and 180 ml without detectable contaminants of other proteins.

4. Conclusions

The results of the above studies indicate that the purification of the ADH from bovine liver crude extract can be carried out in a one-step operation using the *x*-axis CPC with a PEG 1000-potassium phosphate systems containing 0.05% procion red as an affinity ligand. The results show that, by manipulating several parameters such as buffers, polymer molecular mass, and dye–ligand, they can allude to the fact that the process could be rapid if using new CCC technology. Its ability to preserve the enzymatic activity in the support free separation column is noteworthy, and we expected that the present system may also be effectively used for separations of other types of proteins. This is the first report of the dye–ligand affinity CCC techniques for purification of the target protein from a crude extract.

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