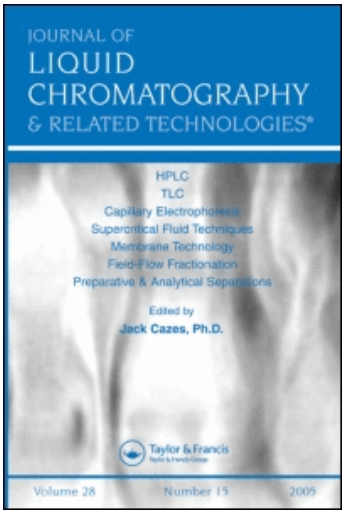

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PURIFICATION OF URIDINE PHOSPHORYLASE FROM CRUDE EXTRACTS OF *ESCHERICHIA COLI* EMPLOYING HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY WITH AN AQUEOUS TWO-PHASE SOLVENT SYSTEM

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ABSTRACT

This is a preliminary report on a novel liquid-liquid partitioning chromatography method for the purification of recombinant protein, uridine phosphorylase (UrdPase). This system utilizes an aqueous two-phase solvent system, consisting of polyethylene glycol and phosphate buffer, with a newly developed high-speed countercurrent centrifuge (X-axis, XLL type). The system is capable of purifying crude recombinant UrdPase in an efficient manner.

INTRODUCTION

Recombinant DNA technology has advanced rapidly over the past decade. It is now possible to express a large number of important mammalian genes in bacteria or yeast. The overexpression of these genes allows a specific protein to be produced in large amounts; sometimes the protein of interest accumulates up to 30-40% of the total cellular proteins(1). Once a protein is overproduced in an expression system, it is essential to select a cost-effective procedure to recover the protein. It is estimated that the cost of isolating and purifying a protein can be 80% of the total production cost. Protein purification is playing a pivotal role in the evolution of biotechnology.

There have been many sophisticated chromatographic methods developed in recent years primarily for the analytical applications, such as capillary electrophoresis, reverse phase HPLC and free flow isoelectric focusing. However, the development of a cost-effective general method of preparative purification, that differs from ion-exchange chromatography is still a challenge.

Aqueous two-phase solvent systems, typically created by mixing solutions of polyethylene glycol and dextran or polyethylene glycol and specific salts of phosphate and sulfate, have long been utilized in the large scale extraction of proteins and other macromolecules(2,3,4,5). However, recovery and purity from a single stage extraction may not be ideal. Multi-stage countercurrent distribution utilizing an aqueous two-phase system has also been successfully employed in the fractionation of proteins from human serum with a centrifugal countercurrent distributor (C-CCD)(6). However, because of its limited number of transfers (60 total) and capacity, the C-CCD method has not been widely employed in the laboratory. Nevertheless, a large body of information pertaining to countercurrent distribution of proteins in various aqueous two phase systems is available in the literature (2,3) and may be used as a guide in initial selection of solvent systems for high speed countercurrent chromatography.

In recent years, high speed countercurrent chromatography (HSCCC) based on the principle of liquid-liquid partition has emerged as a useful method in the isolation of bioactive components from crude extracts of natural products(7,8,9) and in the purification of synthetic polypeptides(10). In general, a non-miscible two-phase solvent system is required for performing HSCCC. These two-phase solvent systems are formed by mixing binary, ternary and even quaternary organic solvents with water. The ability to create a particular two-phase solvent system tailored to isolate a specific component in a crude mixture represents the most critical feature of HSCCC. However, most two-phase solvent systems containing organic solvents are not suitable for the purification of proteins.

Early attempts to employ J-type coaxial HSCCC with an aqueous two-phase solvent system failed. This was due primarily to the fact that the small differences in density and low interfacial tension between the two aqueous phases require a much longer settling time than could be provided by the ordinary J type HSCCC. Consequently, the system design could not retain an adequate amount of stationary phase in the coiled column; thus, resolution has been poor.

A cross-axis synchronous planet centrifuge has been designed by Ito(11,12) where the L/R ratio is equal to 2. That ratio permits the continuous partitioning of an aqueous two-phase solvent system and makes it possible to retain an adequate amount of the polymeric phase as stationary phase inside the coiled column. The parameters for the separation of purified proteins (cyctochrome C, myoglobin, ovalbumin and hemoglobin) using this device were published previously(13). In this communication, we report the application of HSCCC to the purification of a recombinant protein, UrdPase, from crude extracts of Escherichia coli.

MATERIALS AND METHODS

Details of the design of the cross-axis HSCCC apparatus have been reported earlier(11,12). The apparatus includes a pair of

multilayer coiled columns made by wrapping 2.6 mm i.d. PTFE tubing onto holders that are mounted on two rotary shafts. Each rotary shaft is held 7.6 cm (R) from the central axis and each column is mounted on the shaft 15 cm (L) from the center of rotation. Two multilayer coil columns with a total volume of 300 mL are connected together.

Experimentally, the two coiled columns were filled with the lower phase and the sample was injected through the sample port. Centrifugation was initiated (750 rpm) and the mobile phase (upper phase solvent) was pumped through the coiled columns at a flow rate of 0.5 mL/min. For most experiments, the effluent from the column outlet was monitored by a LKB Uvicord S at 278 nm and was collected in 3.0 mL fractions. The separations were conducted at room temperature.

E. coli S0744 containing the plasmid pVMK27 (the plasmid containing the udp gene) was grown overnight in a 16 L fermenter as described previously(14,15,16). After centrifugation, the cells were suspended in a minimal volume of 20 mM Tris-HCl (pH 8.0) containing 5 mM $MgCl_2$, lysozyme, DNase and RNase (all 20 μg/mL) and disrupted using a French Pressure Cell (Aminco) at 10,000 psi. The total crude extract was centrifuged at 47,000 x g and the pellet was discarded. The clarified supernatant, containing UrdPase, was used as the starting material. A later experiment indicated that removal of cell debris was unnecessary; and the crude cell extract could be used directly.

Fractions collected from HSCCC were analyzed by SDS-PAGE using 12% acrylamide gels according to the methods of Laemmli (17). Aliquots of 50 μL were mixed with 25 μL of 20% trichloroacetic acid (TCA) for 1 h at 4°C. The samples were centrifuged; the pellets were washed with 5% TCA and were centrifuged. The pellets were then washed 2x with 1 mL of cold acetone. After the last centrifugation, the acetone was aspirated off and the pellets allowed to air dry. The molecular weight standards for electrophoresis were obtained from Pharmacia (Piscataway, NJ).

The aqueous two-phase solvent system was prepared by dissolving 384 g of PEG-1000 (Sigma Chemical Co., St. Louis, MO), 150 g of K_2HPO_4 and 150 g of KH_2PO_4 into 1716 g of distilled water. The solvent mixture was equilibrated in a separatory funnel at room temperature before use. For partition coefficient measurements, individual protein samples (10 μL/1.0 mg) were dissolved into a 4.0 mL aqueous two-phase solvent system in a test tube. The mixture was thoroughly mixed and allowed to settle at room temperature. After two clear layers had formed, an aliquot (1.0 mL) of each phase was pipetted and diluted with 2.0 mL of distilled water to determine the absorbance at 280 nm using a Zeiss PM6 spectrophotometer. The partition coefficient ($K = C_U/C_L$) was obtained by dividing the absorbance value of the upper phase by that of the lower phase.

The chromatographic fractions containing purified UrdPase were pooled together and dialyzed against 20 mM potassium phosphate buffer at pH 7.4 and then applied to a Q Sepharose column (anion exchange) to remove the PEG. The bound UrdPase was washed with several column volume of buffer and eluted with buffer containing 0.5 M NaCl. The UrdPase collected was dialyzed against buffer, concentrated and stored at ca. 4°C. The UrdPase activity was measured by using a Gilford 260 spectrophotometer according to the method outlined by Krenitsky et al. (18).

RESULTS AND DISCUSSION

UrdPase, which catalyzes reversible phosphorolysis, is employed in the pharmaceutical industry to synthesize a number of important pyrimidine nucleosides. Currently, UrdPase is produced on a large scale by recombinant DNA technology. Many chromatographic procedures including radial-flow (Q Sepharose) ion exchange chromatography(16) have been applied to its purification. In this study the employment of HSCCC with an aqueous two-phase solvent system evidenced a cost-effective procedure for purification of recombinant proteins.

Table 1. Partition Coefficient Studies of Pure UrdPase

Aqueous* Two-Phase Solvent Systems	A	B	C	D	E
pH Values	5.8	6.3	6.8	7.8	9.2
	0.26	0.66	1.57	6.71	0.24

* Aqueous Two-Phase Solvent Systems

A	PEG 1000 (24%)	KH_2PO_4 (8%)	K_2HPO_4 (2%)
B	PEG 1000 (18%)	KH_2PO_4 (10.4)	K_2HPO_4 (4.1%)
C	PEG 1000 (16%)	KH_2PO_4 (6.25%)	K_2HPO_4 (6.25%)
D	PEG 1000 (16%)	KH_2PO_4 (2.5%)	K_2HPO_4 (10.0%)
E	PEG 1000 (16%)	KH_2PO_4 (0%)	K_2HPO_4 (12.5%)

HSCCC:

Apparatus: Cross-Axis (XLL) Centrifuge
Two-Phase Solvent System:
16% PEG (MW. 1000) / 12.5% Potassium Phosphate Buffer at pH = 6.8
Flow Rate: 0.5 ml/min
Rotational Speed: 750 rpm
Fractions: Upper Phase Mobile 3.0 ml/fraction
Sample: 5.8 ml of Recombinant UrdPase (overexpressed) 542 U/ml

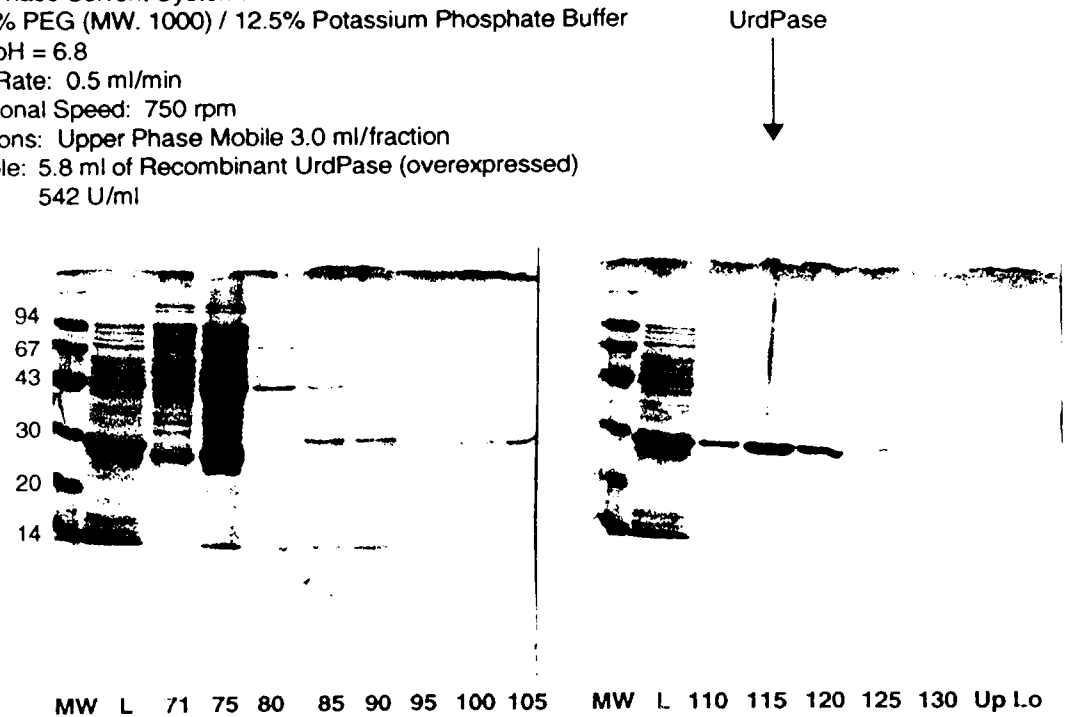

Figure 1. SDS Gel Profile of the Fractions Collected from HSCCC Separation of Recombinant UrdPase

The selection of UrdPase as a model enzyme was based on the considerations that UrdPase is a known, thermally stable, and available in large quantities in both pure and crude forms. Thus, the HSCCC evaluations were greatly simplified, particularly in studying the effects of pH, salt concentration, and polymeric phases.

Selection of an appropriate two-phase solvent system, the key to any successful HSCCC separation, was based on a series of partition coefficient (K) studies employing pure UrdPase. The results shown in Table 1 indicated that the partition coefficient, K (U/L), of UrdPase in an aqueous two-phase solvent system composed of PEG-1000 and potassium phosphate buffer could range from 0.24 to 6.71 depending upon the polymer composition and on the pH of the phosphate buffer. For instance, UrdPase has a K(U/L) value of 1.57 at pH 6.8. A slight shift of pH in either direction resulted in dramatic changes of K values; K (U/L) = 0.66 at pH 6.3 and K (U/L) = 6.71 at pH 7.8. Apparently, pH, surface charge and partition coefficient are closely interrelated.

The partition of crude UrdPase was also measured at pH 6.8. Comparison of the UV absorption values of crude UrdPase partitioned into the upper and lower phase, with those of pure UrdPase suggested that selection of an aqueous two-phase solvent system composed of PEG-1000 (16%) and potassium phosphate (12.5%) would have the advantage of removing impurities effectively.

In Figure 1, the HSCCC elution profile of UrdPase as exemplified by SDS polyacrylamide gel analysis revealed that the major UrdPase peak was well separated from other proteins and eluted at fraction 115. The UrdPase activity (180 U/mL) was also measured further confirming its purity. The preliminary results clearly demonstrated the capability of HSCCC in purification of recombinant proteins.

Several observations call for further studies. For example, carry-over of the mobile phase has made it difficult to predict the retention of the stationary phase using the conventional methods. Some variations were observed in terms of solvent front

and UrdPase peak using the same polymeric solvent system. It appears likely that UrdPase sample concentration, PEG interaction, and pH all have significant effects on the outcome of HSCCC separations.

Currently, we are systematically investigating the effects of sample concentration, loading capacity, pH and PEG molecular weights on the separation. One important issue related to the purification of recombinant protein is how to remove the PEG from the purified UrdPase. Smearing shown in the SDS-PAGE of fraction 115 could be attributed to traces of PEG. One effective procedure to remove PEG is to pass the mixture through a Q or hydrophobic column and recover the UrdPase subsequently.

Aqueous two-phase solvent systems consisting of polyethylene glycol and dextran or polyethylene glycol and specific salts of phosphate or sulfate offer a number of advantages in purification of proteins. For instance, these aqueous polymeric phases are extremely gentle on the enzyme. Also the polymer phase, concentration, and pH of the salt solution can be adjusted to optimize the recovery and/or resolution of a desired protein.

In conclusion, the newly developed Cross-Axis (XLL) high speed countercurrent system is capable of providing adequate retention of an aqueous polymeric phases. Its use in purifying of recombinant proteins was demonstrated using recombinant UrdPase as the target enzyme.

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