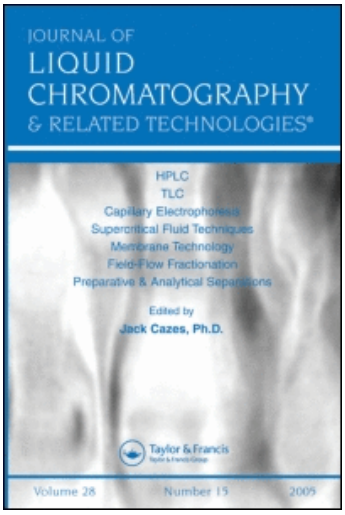

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II. CCC APPLICATIONS

ISOLATION AND PURIFICATION OF 3-OXO-Δ^5-STEROID ISOMERASE FROM CRUDE *E. COLI* LYSATE BY COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

KSI (3-oxo-Δ^5-steroid isomerase) was purified from crude *E. coli* lysate by countercurrent chromatography using a polymer phase system composed of polyethylene glycol 3350 and potassium phosphate (pH 7), each at 12.5%(w/w) in distilled water. Using the cross-axis coil planet centrifuge, a preparative scale separation was performed on 25mL *E. coli* lysate containing ca 50 mg of KSI. About 40 mg of KSI was recovered at 98% purity. A small scale separation was performed on *ca.* 3mg of ^{15}N-labeled KSI using the toroidal coil centrifuge. The present method eliminates sample loss and denaturation caused by the solid support and yields pure proteins in both preparative and analytical separations.

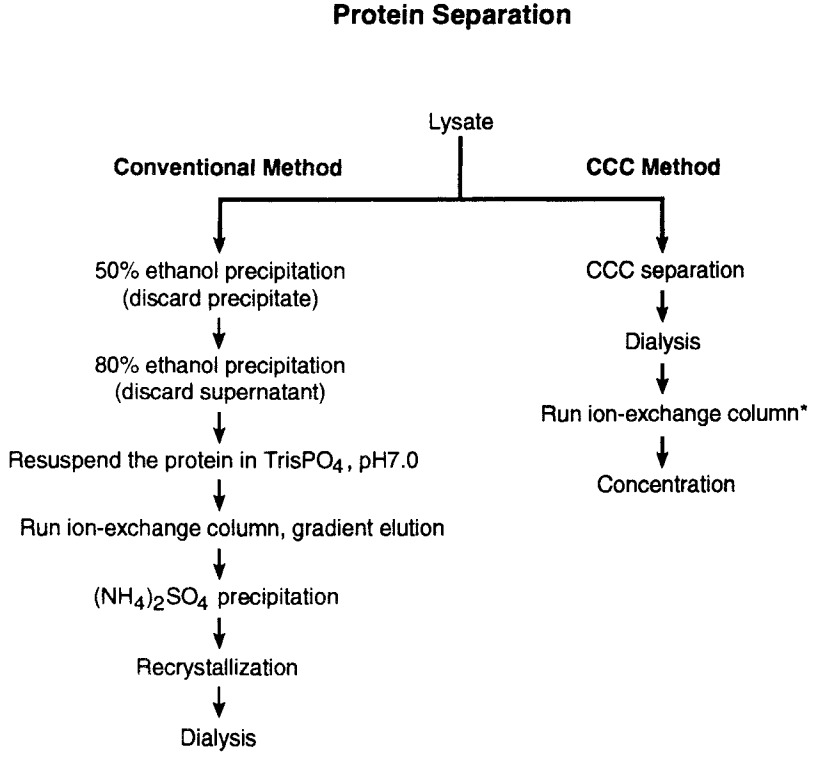

Figure 1. Flow diagram of purification steps of KSI from crude *E. coli* lysate by the conventional (left) and CCC (right) methods. * short column for eliminating PEG.

INTRODUCTION

3-Oxo-Δ^5-steroid isomerase (EC 5.3.3.1, also called Δ^5-3-ketosteroid isomerase, KSI) from *Pseudomonas testosteroni* catalyzes the conversion of a variety of 3-oxo-Δ^5-steroids to their conjugated Δ^4-isomers.[1-3] In order to study the mechanism of this enzyme, it is necessary to purify the enzyme from crude *E. coli* lysate. As shown in Fig. 1 (left column), the method we have been adapting requires many steps where each step may lose some amounts of enzyme.

Countercurrent chromatography (CCC)[4-6] is a form of liquid-liquid partition chromatography in which the stationary phase is retained in the column by gravity or by a centrifugal force, while the mobile phase continuously passes through it. The method has been applied to the separation

of natural products using various organic-aqueous two-phase solvent systems. Recently, this method has been successfully applied to the separation of proteins with aqueous-aqueous polymer phase systems by use of suitable flow-through centrifuge devices such as the cross-axis coil planet centrifuge (CPC)[7] and the toroidal coil centrifuge.[8] The method offers various advantages over conventional chromatographic methods since there is no sample loss or denaturation from the solid support. In addition, it is simple and reproducible.

This paper describes CCC isolation and purification of KSI from crude *E. coli* lysate using a polyethylene glycol(PEG)-potassium phosphate biphasic system.

EXPERIMENTAL

Apparatus

Two CCC centrifuge systems were employed: a type X-1.5L cross-axis CPC[9] for preparative scale separation and a toroidal coil centrifuge[10] for analytical scale separation. The design and principle of these centrifuge systems have been reported in detail.

The cross-axis CPC was fabricated at the NIH machine shop. The rotary frame of the apparatus accommodates a pair of column holders each on a horizontal rotary shaft symmetrically at two locations, either off-center or central position. In the present study, the columns were mounted at the off-center position to promote the mixing the two phases. A set of miter gears and toothed pulleys on the rotary frame causes each column holder to undergo synchronous planetary motion, i.e., one rotation about its own axis for each revolution around the central axis of the centrifuge. This planetary motion not only facilitates the partition process but also permits the flow tubes to rotate without twisting. Thus, the system enables elution of the mobile phase through the rotating column without the use of a rotary seal which would cause various complications such as leakage, heat production and contamination.

Each coiled column was prepared by winding an about 60m long, 2.6mm ID PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ, USA) around the holder hub, making multiple layers of left-handed coils between a pair of flanges spaced 5 cm apart. Two columns were connected in series on the rotary frame to make a total capacity of about 570 mL. The apparatus was rotated at 750 rpm in the present separation.

The toroidal coil centrifuge used in the present studies was a commercial floor model centrifuge (CRU 5000, Damon/IEC Division, Needham Hts, MA, USA). The centrifuge head was designed in our laboratory at NIH and fabricated in our machine shop. The above design allows the flow of the mobile phase through the rotating column without the use of the rotary seal.[11] The column was prepared from a 36m long, 1mm ID PTFE tubing (Zeus Industrial Products) by folding it in two and twisting along its axis to form a number of twisted turns. The twisted tubing was then coiled around a spool-shaped support which snugly fits into the centrifuge bowl. The total capacity of the twisted column was about 36 mL. The column was rotated at 1000 rpm (ca 180 x g).

Reagents

Polyethylene glycol (PEG) 3350 (M.W. 3350), potassium phosphate monobasic (crystals) and potassium phosphate dibasic anhydrous) were purchased from Sigma Chemical Co., St Louis, MO, USA.

Preparation of Cellular Lysate

The cells of *E. coli* NCM533 were transformed by recombinant plasmid pUC18 containing the KSI gene. The harvested cells were spun down at 5000 x g for 20 min and resuspended in 50 mM tris HCl (pH7.5) buffer. The cells were broken by sonicating the suspension at the temperature under 10°C until OD_{600} reading dropped 80-90%. The suspension was centrifuged at 8,000 x g for 20 min. The supernatant was kept frozen as lysate.

KSI Activity Assay

The activities of KSI were determined under standard conditions.[10,11] The assay was performed by isomerizing 58 μM 5-androstene-3,17-dione in 1.7% (v/v) methanol in the presence of KSI at 25°C in a 3.0 mL potassium phosphate buffer, pH 7.0. All determinations were made in a 3 mL quartz cuvette at 248 nm, which is the maximum absorption wavelength of the product (M = 16300 $M^{-1}cm^{-1}$), using a Gilford Response or Response II UV spectrophotometer. One unit of enzyme is defined as a sufficient amount of enzyme to catalyze the isomerization of 1 μmol of 5-androstene-3,17-dione per minute under the standard conditions. The specific activity of KSI under this condition is 55,000 units.

Preparation of Two-Phase Solvent System and Sample Solution

For CCC separation of the enzyme, a solvent mixture composed of 12.5% (w/w) PEG 3350 and 12.5% (w/w) potassium phosphate (pH = 7) was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated after two clear layers were formed.

The sample solution was prepared by adding PEG 3350 and potassium phosphate, each at 12.5% (w/w), to the crude KSI lysate solution to simulate the composition of the polymer phase system used for separation. Precipitates which formed in the sample solution were eliminated by centrifugation because they showed no KSI activity.

Partition Coefficient Measurement

Determination of the partition coefficient of KSI in the polymer phase system was performed as follows: To a test tube containing 2 mL each of the upper and lower phases, 0.1 mL of the enzyme solution was added. The contents were thoroughly equilibrated by mixing and, after two clear layers were formed, the enzyme activity was determined for both phases. The partition coefficient (K) was expressed as the ratio of the activity in the upper phase to that in the lower phase. The K value of the KSI in the above solvent system is 4.0.

CCC Separation of KSI

In each separation, the coil was completely filled with the PEG-rich upper phase (stationary phase) followed by injection of the sample solution through the sample port. Then, the phosphate-rich lower phase (mobile phase) was pumped into the column while the column was rotated. The effluent from the outlet of the column was continuously monitored at 280 nm with a UV monitor (Uvicord S, LKB Instruments, Stockholm, Sweden) and collected into test tubes using a fraction collector (Ultrorac, LKB Instruments).

Analysis of CCC Fractions

An aliquot of 20 μL in each fraction was diluted and analyzed by KSI activity assay. The KSI purity was assessed by UV scan and SDS-PAGE gel analysis.

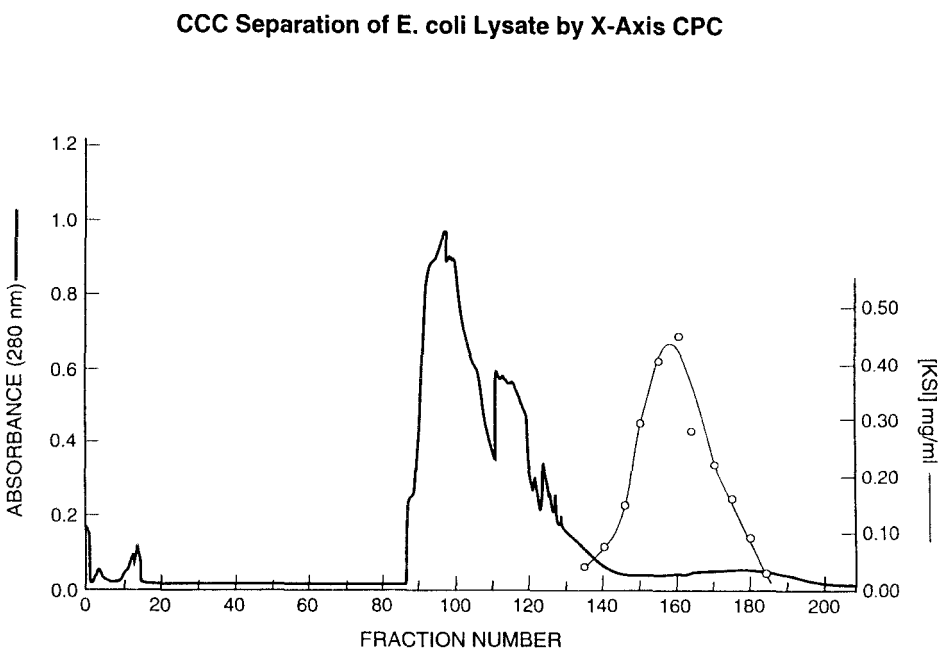

Figure 2. CCC separation of KSI from a crude *E. coli* lysate by the cross-axis coil planet centrifuge. Experimental conditions: Apparatus: cross-axis CPC equipped with a 2.6 mm ID and 570 ml capacity column; Sample: 25 mL of crude *E. coli* lysate containing about 50 mg of KSI; solvent system: 12.5% (w/w) PEG 3350 and 12.5% potassium phosphate (pH=7); mobile phase: lower phase; flow rate: 2.5 mL/min; fractionation: 5 mL/tube; revolution: 750 rpm. Retention of the stationary phase was 20%.

RESULTS AND DISCUSSION

Fig. 2 shows the preparative separation of KSI using the cross-axis CPC. About 25 mL of the crude KSI lysate containing 50 mg of KSI was loaded. In the diagram, the thick line indicates the UV absorbance curve at 280 nm, and the thin line the enzyme concentration determined by the KSI activity assay. As shown in the UV curve, a large amount of impurities was eluted shortly after the solvent front (fractions 86-120) while the enzyme (K = 4) was retained much longer in the column. Because this enzyme does not have strong absorbance at 280 nm, UV detection is difficult. The activity assay revealed that the KSI peak was eluted in fractions 140 to 180 as indicated in the diagram. In this separation, about 40 mg of enzyme was recovered and its purity was over 98% as examined by the UV scan.

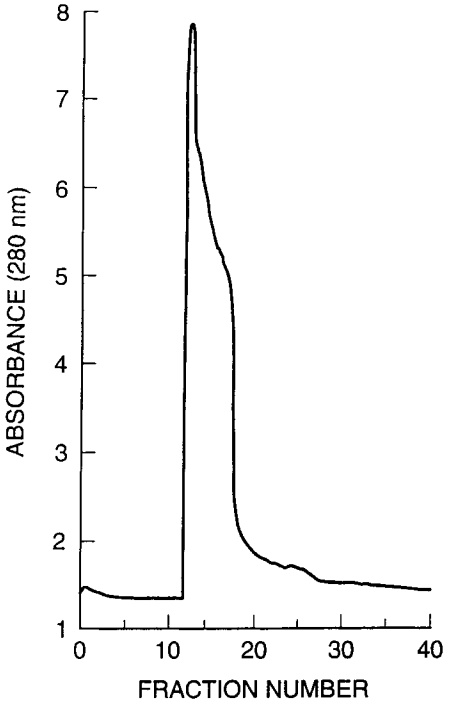

Figure 3. CCC separation of ^{15}N-labeled KSI from crude *E. coli* lysate by the toroidal coil centrifuge. Experimental conditions: Apparatus: toroidal coil centrifuge equipped with a 1.07 mm ID and 36 mL capacity twisted column; Sample: 6 mL crude lysate solution containing about 3 mg ^{15}N-labeled KSI; solvent system: 12.5% (w/w) PEG 3350 and 12.5% (w/w) potassium phosphate (pH=7); mobile phase; lower phase; flow rate: 0.4 mL/min; fractionation; 1.6 mL/tube; revolution: 1000 rpm. Retention of the stationary phase was 22.4 %.

Fig. 3 shows the analytical separation of a second sample of KSI by the toroidal coil centrifuge. Since this sample was an ^{15}N-labeled enzyme for NMR studies, a small-scale separation was required for the preliminary study. It is difficult to purify a small amount of enzyme by the method we have been adapting in our laboratory, which involves multiple steps, as shown in Fig. 1. We took the advantage of CCC because the sample would not be lost by adsorption onto the solid support and only a few steps were required. Using a twisted column with a 36 mL capacity, 1 mL of lysate solution was separated by the toroidal coil centrifuge.

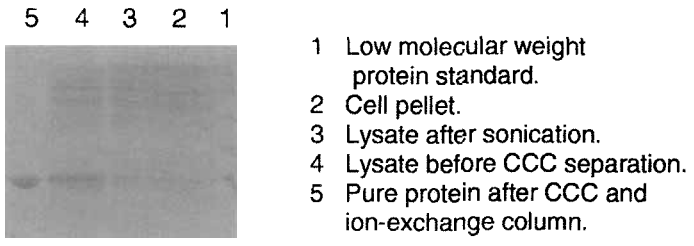

Figure 4. SDS-PAGE analysis of CCC-purified KSI.

As shown in Fig. 3, large amounts of impurities were eluted near the solvent front while the KSI was totally retained in the column. After stopping the centrifugation, the enzyme was recovered from the column contents. Desalting of fractions was performed by dialysis against water. Then, a short ion-exchange column was used to remove the PEG.

The enzyme fractions were combined and concentrated by a Centricon. Fig. 4 shows SDS-PAGE analysis of the KSI fraction which revealed a single band of the pure KSI.

Determination of the molecular weight and the purity of the enzyme was conducted by ES-MS which confirmed that the KSI is pure and, as expected, approximately 20% was labeled (Fig. 5).

CONCLUSIONS

KSI was purified from a crude *E. coli* lysate by CCC using two CCC centrifuge systems, the cross-axis CPC for a preparative-scale separation and the toroidal coil centrifuge for an analytical-scale separation.

Compared with the conventional KSI purification method, the present method offers various advantages: A large amount of crude KSI lysate can be directly loaded into the CCC column without complication, and the analytical-scale CCC purification provides a higher yield because of the fewer steps

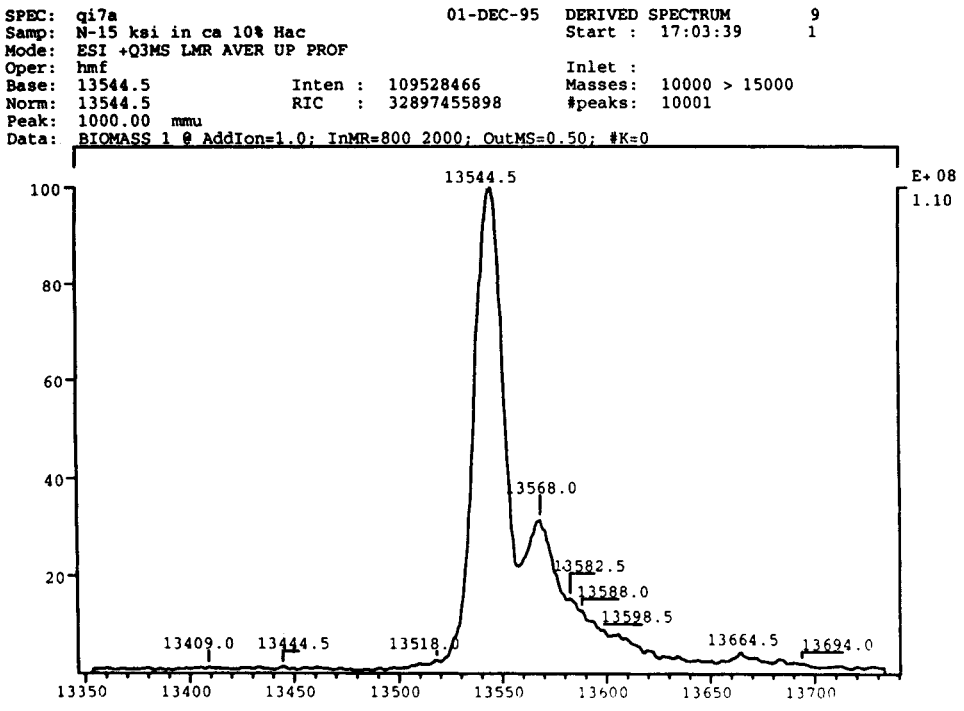

Figure 5. ES/MS analysis of CCC-purified KSI (20% ^{15}N-labeled KSI with molecular weight of 13568.0).

required, no sample loss by the adsorption of solid support, and high concentration of polymer preserves the enzymatic activity even at room temperature. The polymers can be removed from the fraction by dialysis and/or passing through a short ion-exchange column as indicated in Fig. 1.

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