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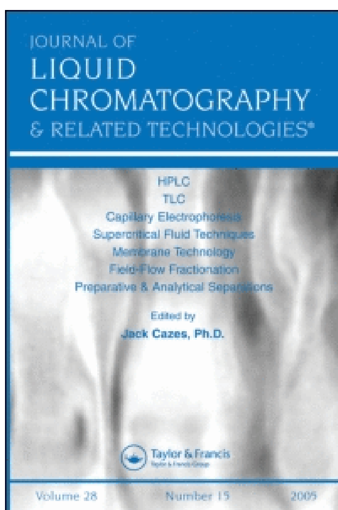

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CENTRIFUGAL PRECIPITATION CHROMATOGRAPHY APPLIED TO FRACTIONATION OF PROTEINS WITH AMMONIUM SULFATE

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ABSTRACT

A novel chromatographic system introduced here internally generates a concentration gradient of ammonium sulfate (AS) along a long channel to fractionate proteins according to their solubility in AS solution. The separation column consists of a pair of discs with mutually mirror-imaged spiral channels that are separated by a semipermeable membrane. The disc assembly is mounted on a seal-less continuous flow centrifuge. A concentrated AS solution is introduced into the upper channel while a water solution is passed through the lower channel in the opposite direction in a rotating column. A mixture of proteins injected into the water channel moves along an AS gradient of increasing concentration that has been established in the water solution. Each protein species precipitates at a different AS concentration along the gradient. By decreasing the concentration of the AS solution its concentration in the water is decreased, causing the protein to redissolve and to reprecipitate further down the channel. The eluate is continuously monitored and collected using a fraction collector.

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The method has been demonstrated on separation of serum proteins and applied to purification of a recombinant ketosteroid isomerase from a crude *E. coli* lysate by adding an affinity ligand to the sample solution. A possible mechanism involved in this affinity separation is discussed. The method may be applied to other biopolymers such as DNA and RNA.

INTRODUCTION

Ammonium sulfate (AS) precipitation is one of the most commonly used procedures in protein purification. Although the conventional method requires tedious manual operation, the statistic studies show that about 80% of the current protein purification protocols include at least one step of precipitation with AS.[1]

A novel chromatographic system introduced below can internally generate a concentration gradient of AS through a long separation channel. Under a centrifugal force acting across the channel, proteins introduced into the channel are eluted through the gradient at different rates and fractionated according to their solubility in the AS solution. The effluent is continuously monitored through a uv detector as in liquid chromatography. The collected fractions are free from impurities of small molecules as well as non-charged biopolymers such as polysaccharides while charged biopolymers such as DNAs and RNAs may also be separated according to their solubility. The capability of the method is demonstrated on the separation of serum proteins and one step purification of recombinant ketosteroid isomerase from a crude *E. coli* lysate by adding an affinity ligand to the sample solution.

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PRINCIPLE OF THE METHOD

Consider a pair of channels partitioned by a dialysis membrane as shown in Figure 1. A concentrated AS solution is introduced into the upper channel from the right terminus at a high rate while water is fed into the lower channel from the left terminus at a lower rate. Under this situation AS transfer takes place from the upper channel to the lower channel through the dialysis membrane at every portion of the column. As this process continues, the two liquids soon establish a steady state equilibrium where an AS concentration gradient is formed in each channel along the entire length. Since the flow rate of AS solution through the upper channel (V) is much greater than that of water through the lower channel (v), the AS concentration (C) in the upper channel is not significantly altered.

Let us assume that the amount of the salt transferred through the membrane is proportional to the difference in AS concentration between the two channels at a given moment. Then,

$$dc = k\,(C - c)\,dt \qquad (1)$$

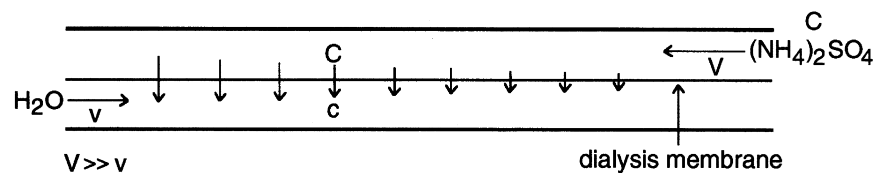

Figure 1. Principle of the present method. Two channels partitioned with a dialysis membrane. Ammonium sulfate (AS) solution and water countercurrent through these channels to produce an exponential concentration gradient of AS in the water channel.

where C and c are the AS concentrations in the upper and lower channels, respectively, and k, a constant which is determined by various factors including permeability of the membrane, area of the membrane per liquid volume, temperature, etc. Solving Eq 1 gives

$$c = C\,(1 - e^{-kt}) \qquad (2)$$

which indicates that an exponential gradient of the AS concentration is formed through the lower channel.

With a centrifugal force applied across the channel, the sample solution containing proteins is injected into the lower channel. As the elution proceeds, the proteins are exposed to a gradually increasing AS concentration until the concentration reaches the critical level where precipitation ("salting out") takes place. Once solid particles are formed, they are quickly precipitated at the distal wall of the lower channel by the centrifugal force.

The group of proteins with lowest solubility in the AS solution is the first to be precipitated and does so in an early portion of the lower channel; while others continue to advance through the lower channel until they reach their own critical points. Thus, proteins are precipitated along the distal wall of the lower channel according to their solubility. After all proteins in the sample are precipitated in this manner, the concentration of the AS fed into the upper channel is now gradually decreased.

This results in a gradual change of the gradient curve in the lower channel in such a way that the AS concentration at every point is proportionally decreased as indicated by Eq 2. This dissolves the once precipitated proteins, but they will again be precipitated at a slightly more advanced location of the channel. Thus, the proteins are subjected to a repetitive process of dissolution and precipitation until they are eluted out from the column. The eluates are monitored at a suitable wavelength (280 nm) and fractions collected to isolate the aimed proteins.

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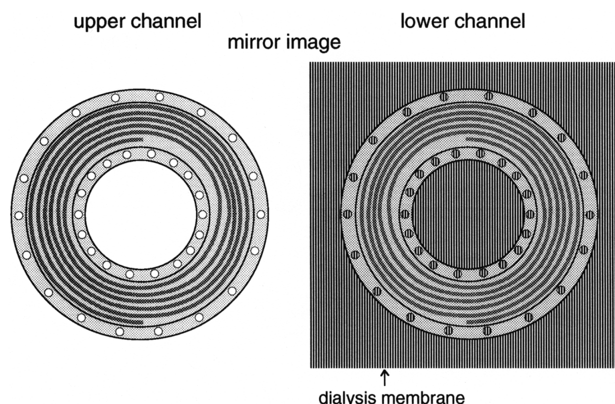

Figure 2. Design of the separation discs. A pair of discs equipped with mutually mirror-imaged spiral grooves. With a proper alignment of these two discs a single spiral channel is formed, and inserting a dialysis membrane between the discs provides a pair of channels (each 5 mL capacity) as indicated in Figure 1. These two discs and a membrane are tightly pressed between a pair of aluminum plates with a number of screws to provide leak-free seal.

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EXPERIMENTAL

Design of the Apparatus

The separation disc assembly was designed in my laboratory and fabricated at the machine shop of the National Institutes of Health, Bethesda, MD, USA. The separation column was made of a pair of flat discs (high-density polyethylene, 13.5 cm in diameter and 1.5 cm in thickness) with a spiral-shaped narrow groove (1.5mm wide, 2mm deep and ca 2 m in length) at the periphery as shown in Figure 2. The spiral groove of the left disc is made in a mirror image to that of the right disc so that with a proper alignment these two spiral grooves can be made to form a single spiral channel. The dialysis membrane sheet (regenerated cellulose, molecular weight cut-off 12,000-14,000, Spectrum, Laguna Hills, CA, USA) is sandwiched between these discs to form two channels (5mL capacity for each channel) referred to in Figure 1. The leakage-free seal is made by tightly pressing the two discs between a pair of aluminum plates with a number of screws. This column assembly is mounted on the seal-less continuous flow centrifuge (Pharma-Tech Research Corporation, Baltimore, MD, USA) that allows continuous elution through multiple flow lines of the rotating column without the use of rotary seals.[2,3]

The entire elution system of the present method is schematically illustrated in Figure 3. A gradient pump (Perkin Elmer model 200 series, Perkin Elmer, Norwalk, CT, USA) was used to elute the AS gradient through the upper channel

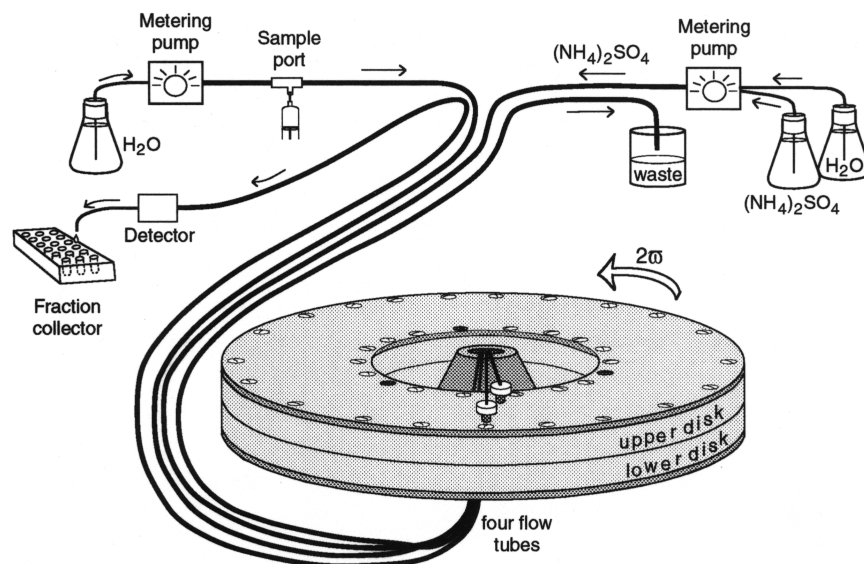

Figure 3. Schematic illustration of the chromatographic elution system of the present method. The two pairs of flow tubes led from the rotating column assembly are passed through the central hollow shaft downward and then supported at the top of the centrifuge. The protein sample introduced into the inlet of the column is continuously eluted with buffer solution by the first metering pump (left) and the effluent from the outlet of the column is continuously monitored by a uv monitor and fractionated into test tubes using a fraction collector. AS solution is fed from the second metering pump (right) which introduces a gradually decreasing AS concentration to elute proteins according to their solubility.

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while a syringe pump (Harvard Apparatus, South Natick, MA, USA) for elution the proteins through the lower channel. The effluent was monitored through a Uvicord S (LKB Instruments, Stockholm, Sweden) and the elution curve was traced with a strip chart recorder (Pharmacia LKB RC102, Stockholm, Sweden).

Materials and Methods

Reagents

Ammonium sulfate, dibasic and monobasic potassium phosphates were all of analytical reagent grade and purchased from Mallinckrodt Baker, Paris, KY, USA). An affinity ligand, β-estradiol-17-methylPEG (polyethylene glycol) - 5,000 was

supplied from Shearwater Polymers, Birmingham, AL, USA (under a CRADA contract). The crude *E. coli* lysate containing recombinant ketosteroid isomerase (2 mg/mL) was prepared as previously described.[4]

Measurement of AS and Water Transfer Rates

A series of experiments was performed to study the rates of AS and water transfer through the membrane without protein samples. In these experiments a 95% saturated AS solution was eluted through the AS channel at a flow rate of 1 mL/min while the flow rate of water through the other channel is varied from 1 mL/min to 0.05 mL/min. The first two experiments were performed without rotation of the column by eluting the water through the upper channel and through the lower channel. In the following two experiments the column was rotated at 1,000 rpm and at 2,000 rpm while the water was eluted through the lower channel.

In each experiment, after the mass transfer equilibrium was established between the channels, the effluent from the outlet of each channel was collected at a given interval to measure both the output flow rate and the % AS saturation. The latter was determined by comparing the 1-mL weight of the effluent to that of AS saturated solution (1.235g).

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Fractionation of Serum Proteins

The performance of the present system was examined by the separation of normal human serum according to the following procedure: Both channels were first completely filled with 95% saturated AS solution. After the column was rotated at 2000 rpm, the buffer solution (50 mM potassium phosphate at pH 7) was eluted through the water channel at 0.06 mL/min using a Harvard syringe pump (a pair of 60 mL capacity plastic syringes were connected with a tee junction at their outlets and driven at 30 μL/min). After 10 min of priming elution, 0.5 mL of sample solution containing 50 μL of normal human serum in 50 mM potassium phosphate buffer was introduced into the channel via a sample loop. Using a Perkin Elmer 200 series pump, the gradient elution was programmed as follows: 75% AS for 4 hrs, then a linear gradient from 75% to 25% for 15 hrs. The effluent from the outlet of the water channel was continuously monitored with a uv monitor at 275 nm and fractionated into test tubes at 20 min intervals.

Affinity Separation of Recombinant Enzyme

The present method was applied to purification of a recombinant ketosteroid isomerase (rKSI) from a crude *E. coli* lysate using an affinity ligand, β-estradiol-17-methylPEG-5,000. The separation was similarly performed according to the serum fractionation described above except that an affinity ligand was added to the

sample solution. After the two channels reach equilibrium, 0.5 mL of crude *E. coli* lysate containing about 1 mg of rKSI and 25 mg of the affinity ligand was injected through the sample loop. The effluent was similarly monitored with a Uvicord S at 275 nm and collected into test tubes at 20 min intervals. The control experiment was performed without adding ligand to the sample solution under otherwise identical conditions.

SDS PAGE Analysis of Fractions

The chromatographic fractions were desalted and concentrated with Centricon 10 (Amicon, Beverly, MA, USA), and analyzed by SDS PAGE using a precasted tris-glycine gels and a colloidal Coomassie brilliant blue staining kit (Norvex, San Diego, CA, USA).

RESULTS AND DISCUSSION

Studies on AS and Water Transfer Rates Through the Membrane

A series of preliminary experiments was performed to investigate whether the two channels are equivalent, the effect of rotation and flow rate through the water channel.

The results of the experiment measuring the AS transfer rate through the membrane are summarized in Figure 4a. As described earlier, the first two experiments were run without column rotation using a fixed flow rate at l mL/min through the AS channel. As expected the AS concentration increased in the output from the water channel as the flow rate is decreased. However, it was found that gravity plays a significant role in this transfer through the membrane. When the water channel is the lower channel, the AS diffusing through the membrane momentarily increases the density of the layer just below it. Gravity then aids mixing of this layer more rapidly, reducing its AS concentration and aiding subsequent diffusion through the membrane. This gravitational effect is enhanced when the centrifugal force is applied across the channel as indicated in the following two experiments where the more dense layer formed by the AS migrating through the membrane is quickly shifted toward the distal wall of the channel again accelerating AS transfer through the membrane.

Figure 4b shows water moving across the membrane resulting in a decreased output from the water channel and an enhanced output from the AS channel. This water transfer is also a function of the difference in AS concentration between the two channels, as is the AS transfer through the membrane. At 2,000 rpm the water output becomes less than one fourth of its input rate.

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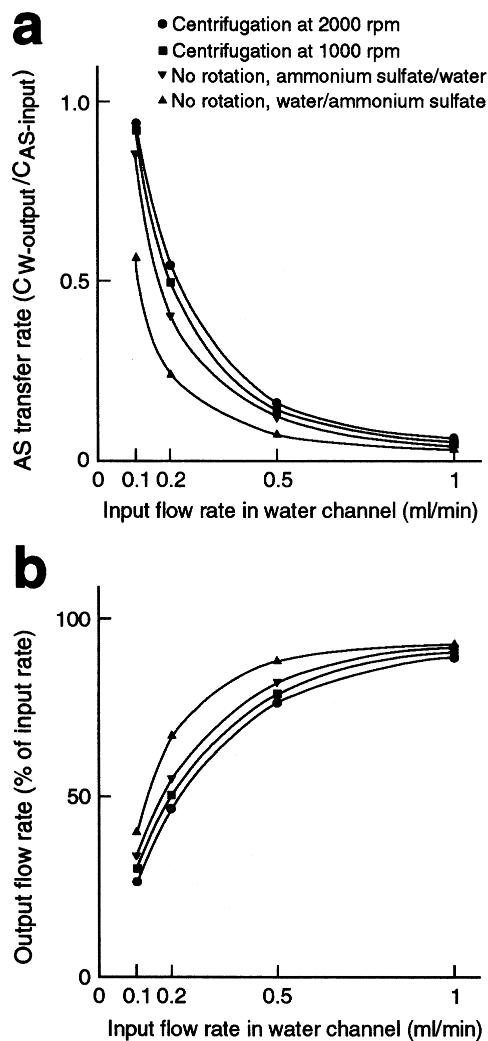

Figure 4. a) Ammonium sulfate (AS) transfer rate through the membrane. In the stationary column the gravity plays an important role in AS transfer. This effect is further enhanced by a centrifugal force. At an input flow rate of 0.1 mL/min through the water channel, the AS concentration at the output ($C_{W\text{-output}}$) becomes close to the input concentration of the AS channel ($C_{AS\text{-input}}$..). b) Change of the flow rate through the water channel. Water transfer across the membrane reduces the output flow rate through the water channel depending on various factors such as the input flow rate of the water channel, channel orientation in the stationary column, and a strength of the applied centrifugal force field. At an input flow rate of 0.05mL/min through the water channel under 2,000 rpm, the water is totally absorbed into the AS channel resulting in no output flow from the water channel.

Separation of Serum Proteins

Figure 5 shows fractionation of human serum proteins using the present method. In Figure 5a, the application of a linear concentration gradient of AS for 15 hrs produced 2 major peaks. SDS PAGE analysis of these fractions (Figure 5b) shows that serum albumin (fraction 2) and globulins (fractions 4-6 from the second major peak) are well separated from each other although not from other proteins.

Affinity Separation of rKSI From Crude *E. coli* Lysate

The present method was applied to the separation of a recombinant enzyme, ketosteroid isomerase (rKSI), from a crude E. coli lysate under similar experimental conditions. In the control experiment shown in Figures 6a and 6b, after the large peak containing intensely uv-absorbing small molecule of unknown structure, the target protein was eluted with many other proteins (fractions 2 and 3) and showed little enrichment as expected, since most proteins including KSI have their critical precipitation points in AS solution somewhere between 40 % and 60 % saturation. To overcome this problem we constructed an affinity ligand that would affect the precipitation behavior of the target protein. To this end, β-estradiol-17-methyl-PEG-5,000 was added to the sample solution. The resulted chromatogram in Figure 6c shows a substantial change in its elution pattern: The rKSI now eluted in fraction 7 giving a small absorption peak near the 25% AS saturation point followed by a huge peak which represents the free ligand. The SDS PAGE analysis in Figure 6d shows that fraction 7 is highly enriched in target protein where it forms three dark bands representing monomer, dimer and tetramer as indicated. Clearly, this fraction contains far fewer contaminating proteins than the corresponding eluate without the ligand.

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The mechanism of this affinity-ligand separation merits further discussion. It appears that the ligand-protein complex has a lower solubility than the protein alone so that it is precipitated earlier to retain longer in the column. However, estradiol-PEG5,000 forms two phases when AS solution is added and this type of polymer phase system has been used for partitioning proteins.[5] This suggests that the ligand in the sample solution may locally form two phases in the channel with the stationary lighter phase rich in ligand and the mobile heavier phase rich in AS. In this situation, the target protein bound to the ligand will stay longer in the column, since it should have the highest partition coefficient to the stationary phase among all proteins.[6] Whichever explanation is correct, the method should work well for purification of other proteins by adding suitable ligands to the sample solution.

CONCLUSION

The present chromatographic system internally generates a concentration gradient of AS through a long channel under a centrifugal force field. Proteins introduced into the channel travel through the channel at different rates according to

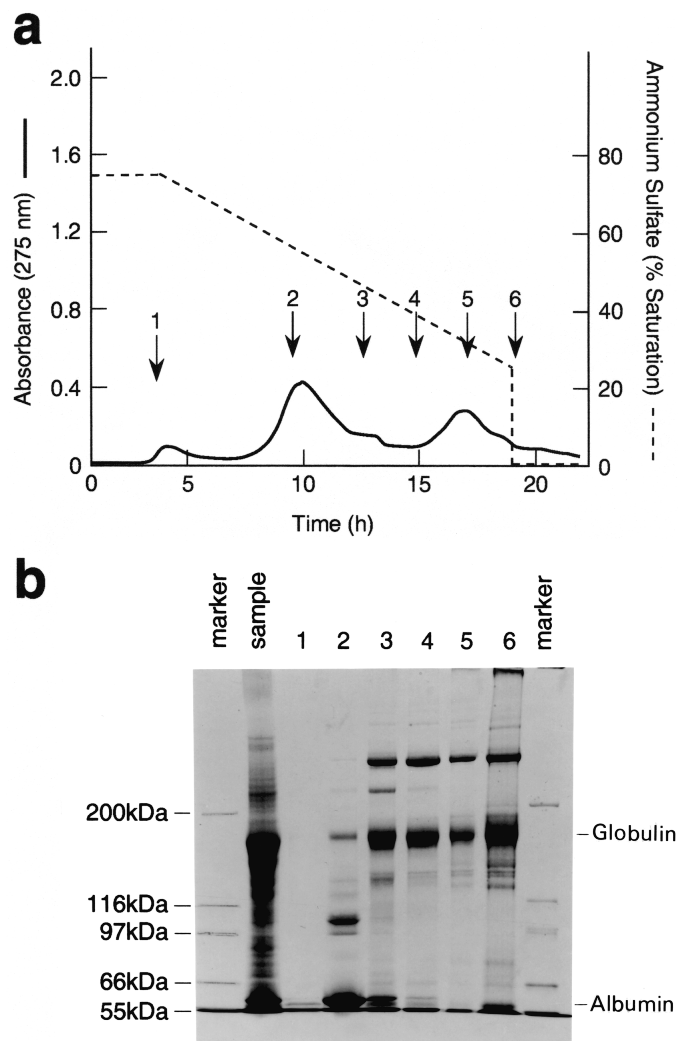

Figure 5. Fractionation of human serum by the present method. (a) chromatogram and (b) SDS PAGE analysis of fractions. The experimental conditions were as follows: apparatus: a sealless continuous flow centrifuge; column: high-density polyethylene discs with spiral channels (upper and lower channels) each 1.5mm wide, 2mm deep and 2 m long with a 5mL capacity; sample: normal human serum 50 μL in 0.5mL water; upper channel: 75% saturated ammonium sulfate (AS) at 1 mL/min for 4 hrs followed by an AS linear gradient from 75% to 25 % for 15 hrs; lower channel: 50mM potassium phosphate (pH 7) at 0.06mL/min; revolution: 2,000 rpm (ca 250xg); detection: 275 nm; recording: 1cm/50min. Analysis of fractions: SDS PAGE using a Novex precasted 6% tris-glycine gel at 110V for 100 min and Coomassie brilliant blue staining.

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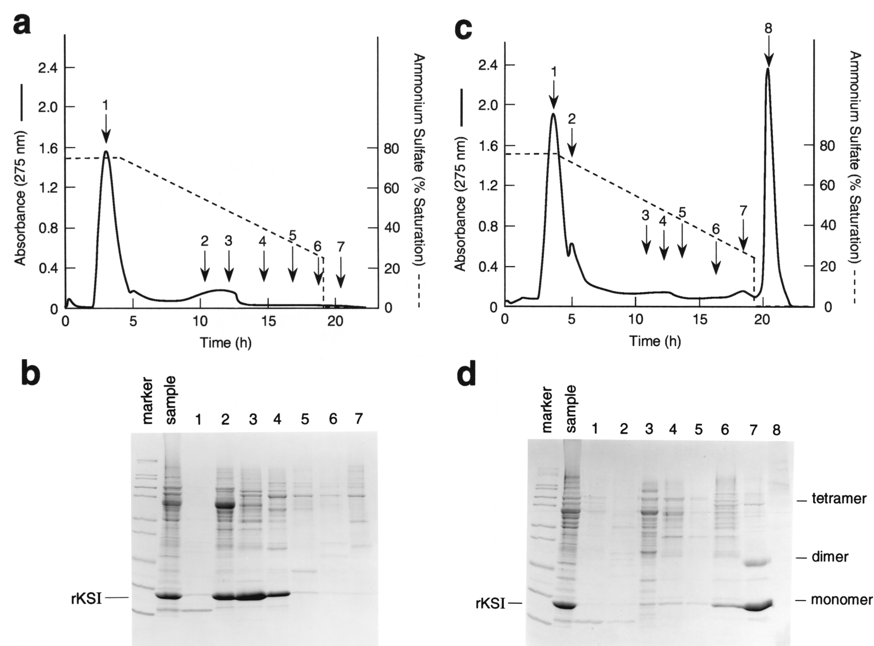

Figure 6. Purification of recombinant ketosteroid isomerase (rKSI) from crude E. coli lysate using an affinity ligand. Sample: (a) 0.5mL of E. coli lysate; (c). 0.5mL of E. coli lysate + 25mg of β-estradiol-17-methylPEG-5,000; SDS PAGE analysis (b and d) was performed using a precasted 16% tris-glycine gel at 200V for 45 min for each set of fractions. Other experimental conditions are described in the Figure 5 caption.

their solubility in AS solution. The effluent is continuously monitored and fractionated as in liquid chromatography. The method was demonstrated on separation of serum proteins and successfully applied to purification of rKSI from a crude *E. coli* lysate using an affinity ligand.

The future applications include the separation of monoclonal antibodies from ascites and culture medium, protein-PEG conjugates, and various natural and recombinant enzymes from cell lysate using suitable ligands in the sample solution. Although the present studies were performed at room temperature, the future experiments should be conducted in a refrigerated cabinet where the temperature is maintained between 0-4°C to preserve bioactivities of unstable proteins. The method may also be applied to other biopolymers including DNA and RNA using a pH gradient.

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