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Review

Centrifugal precipitation chromatography☆

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ABSTRACT

Centrifugal precipitation chromatography separates analytes according their solubility in ammonium sulfate (AS) solution and other precipitants. The separation column is made from a pair of long spiral channels partitioned with a semipermeable membrane. In a typical separation, concentrated ammonium sulfate is eluted through one channel while water is eluted through the other channel in the opposite direction. This countercurrent process forms an exponential AS concentration gradient through the water channel. Consequently, protein samples injected into the water channel is subjected to a steadily increasing AS concentration and at the critical AS concentration they are precipitated and deposited in the channel bed by the centrifugal force. Then the chromatographic separation is started by gradually reducing the AS concentration in the AS channel which lowers the AS gradient concentration in the water channel. This results in dissolution of deposited proteins which are again precipitated at an advanced critical point as they move through the channel. Consequently, proteins repeat precipitation and dissolution through a long channel and finally eluted out from the column in the order of their solubility in the AS solution. The present method has been successfully applied to a number of analytes including human serum proteins, recombinant ketosteroid isomerase, carotenoid cleavage enzymes, plasmid DNA, polysaccharide, polymerized pigments, PEG-protein conjugates, etc. The method is capable to single out the target species of proteins by affinity ligand or immunoaffinity separation.

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Contents

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1. Introduction

Centrifugal precipitation chromatography is a recently developed technique mainly for separation of proteins based on their solubility in ammonium sulfate (AS) solution and other suitable precipitants. The method has been successfully applied to various kinds of biological samples including serum proteins [1–6], IgM [2,3] and IgG [3], recombinant ketosteroid isomerase [2–5], protein-PEG (polyethylene glycol) conjugate [3,7] polysaccharide [3,8,9], polymerized pigments [10] plasmid DNA RNA and protein [11,12], etc. In this review paper, this new method is introduced by describing the principle, instrumentation, a series of basic studies and three different types of applications including the standard separation, ligand-affinity separation, and immunoaffinity separation of proteins.

2. Principle

Conventionally, salting-out of proteins with AS has been manually performed using multiple test tubes by repeating centrifugation. As is well known, protein is well preserved in AS solution and it has been reported that 80% of protein purification procedure uses at least once this AS salting out technique [13]. However, this conventional AS fractionation is not efficient where one protein species will be spread into 70% of the total fraction. In order to improve the AS salting out technique, centrifugal precipitation chromatography has been introduced. It separates proteins by repeating precipitation and dissolution in a long tubular channel resulting in chromatographic fractionation of proteins [1–5]. The mechanism of centrifugal precipitation chromatography is illustrated in Fig. 1. A long separation channel is divided into two by a sheet of semipermeable membrane (dialysis membrane) and subjected to a strong centrifugal force field as indicated by an arrow. A highly concentrated (C) AS solution is introduced from the upper channel from the right at a higher flow rate (V) and water from the lower channel in the opposite direction from the left at a lower flow rate (v). This countercurrent results in mass transfer of AS from the upper AS channel to the lower water channel through the membrane forming an exponential AS concentration gradient (c) in the lower channel [14]. It is very important to note that this AS concentration gradient is fixed to the channel and stably maintained as long as this countercurrent process is continued. Consequently, protein molecules in the sample solution introduced into the water channel are exposed to a gradually increasing AS concentration and at the critical point they are precipitated and deposited onto the channel bed under the centrifugal force field. In this stage, the proteins are separated along the channel in some extent similar to the conventional salting-out method. After all the proteins in the sample solution were deposited in the channel, the chromatographic elution of proteins is started by gradually reducing the AS concentration in the AS channel. This results in a gradual decline of AS concentration gradient through the channel and once deposited proteins are dissolved and carried out by the mobile phase to the next critical concentration spot to be precipitated again. This repetitive precipitation and dissolution process continues along the channel and all proteins are finally eluted out from the column according to their solubility in AS solution.

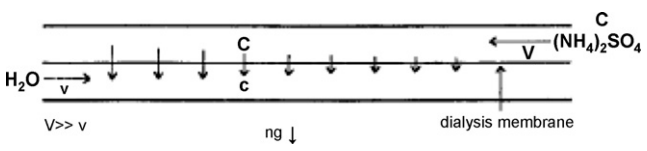

Fig. 1. Principle of centrifugal precipitation chromatography. Two identical channels were partitioned with a semipermeable membrane. Concentrated ammonium sulfate solution is introduced from the upper channel from the right at a flow rate *V* while water is eluted through the lower channel in the opposite direction at a lower flow rate *v*. This results in formation of an exponential AS concentration gradient through the water channel. ng: the centrifugal force that is *n* times the unit gravity (g).

3. Instrumentation

Fig. 2 illustrates the design of the centrifugal precipitation chromatograph. Separation disk was fabricated from a pair of high-density polyethylene disks by making mutually mirror-imaged spiral grooves as shown in Fig. 2a top so that when the dialysis membrane (Fig. 2a bottom) is sandwiched between the disks it forms a pair of spiral channels partitioned with the dialysis membrane as illustrated in Fig. 1. In order to achieve perfect seal, a pair of O-rings was installed at the outer and inner edges of the spiral groove in the lower channel. These disks were tightly pressed by a number of screws to form the separation disk assembly as shown in Fig. 2b which is then mounted on the rotary-seal-free flow-through centrifuge. Fig. 2c shows the flow diagram of the present system. Each terminal of the spiral channel is connected to a flow tube which exits from the top of the centrifuge (see Fig. 3). These flow tubes are not twisted during column rotation so that continuous elution can be carried out without the use of conventional rotary seals [15]. AS solution is introduced into the upper spiral channel from the outer terminal and eluted out through the inner terminal into the waste container (Fig. 2 top, right). Water and sample solution is introduced from the inner terminal of the lower water channel while the effluent is monitored with a UV detector and collected into the test tube with a collector (Fig. 2 c, top left). In this elution mode the AS concentration gradient formed along the spiral channel is kept stable by the aid of the centrifugal force field.

Fig. 3a shows a diagram of the cross-sectional view through the central axis of the seal-free flow-through centrifuge equipped with the spiral disk assembly. The motor drives the gear box through a pair of toothed pulleys and a timing belt. The box contains a set of four identical miter gears, one of which (lower stationary gear) is tightly fixed at the bottom of the centrifuge. Consequently, the rotation of the box at ω causes a rotation of the horizontal gears at the same angular velocity. The rotation of the horizontal gear is further conveyed to the upper miter gear which then rotates at the doubled speed relative to the gear box. Consequently, the spiral disk assembly connected to the upper gear rotates at angular velocity 2ω as indicated in Fig. 2c. This particular rotation/revolution ratio of $2\omega/\omega$ permits the flow tubes to rotate without twisting [15]. Thus the separation can be performed without a risk of leakage at the conventional rotary seal device. Four flow tubes from the spiral disk assembly are bundled together and passed through the hollow central shaft downward and through the hollow shaft of the horizontal miter gear, then through the tube support upward and finally exit the centrifuge at the center of the upper plate of the centrifuge where they are tightly fixed by a pair of clamps as shown in the diagram. The spiral disk assembly can be rotated at a maximum revolution speed of 2000 rpm, while the flow tubes maintain their integrity for many runs. The photograph of the first model of centrifugal precipitation chromatograph shown in Fig. 3b was a gift from the late Dr. Edward F. Chou of the Pharma-Tech Research Corporation in Baltimore, Maryland, USA. The separation column was designed in my laboratory and fabricated at the NIH Machine Shop.

4. Basic studies using test protein samples

A series of the basic studies has been carried out to determine the optimal experimental conditions for separation of proteins as described below:

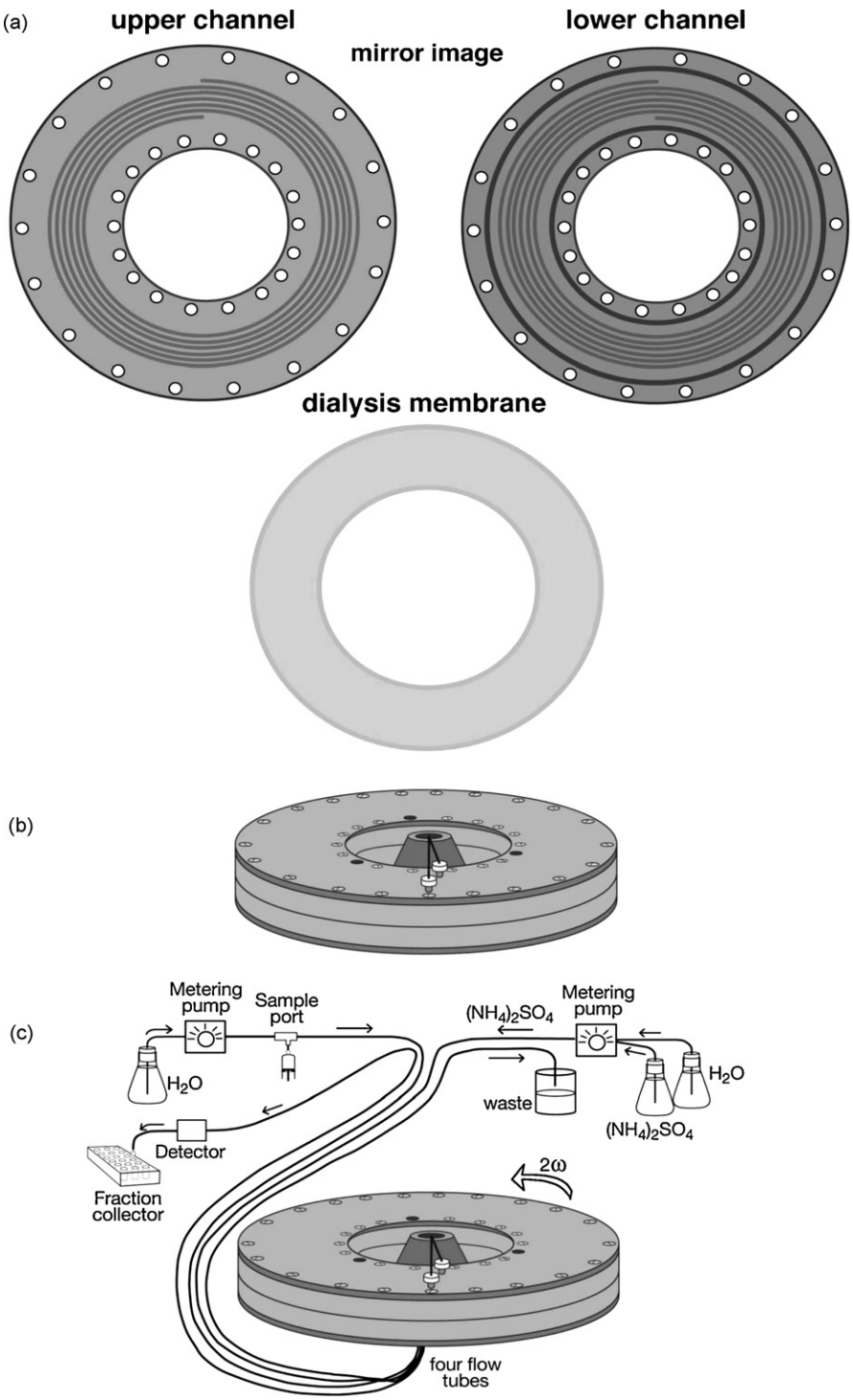

Fig. 2. Column assembly and elution diagram. (a) A pair of disks with mutually mirror imaged spiral grooves and a Teflon seal, (b) separation disk assembly, (c) elution scheme.

4.1. Studies on mass transfer rate of AS and osmosis through the dialysis membrane

As mentioned earlier, the AS solution introduced into the upper channel forms an exponential AS concentration gradient in the lower water channel (Fig. 1). While AS is transferred from the upper channel to the lower channel through the membrane, water in the lower channel also moves through the membrane in the opposite direction to the upper channel which has higher osmotic pressure. This osmosis effect causes a considerable change in the water output through the lower channel since the water input in the lower channel is much less than that of AS solution through the upper channel or $v << V$ as shown in the figure. In order to study the AS transfer rate and the above osmosis effect, the following experiments were conducted:

The 90% saturated AS solution was eluted from the upper channel at a constant flow rate of 1 ml/min, while the water was introduced from the lower channel at various flow rates ranging from 1 to 0.1 ml/min into the column which was either kept stationary or rotated at 1000 and 2000 rpm. At each applied flow rate, the

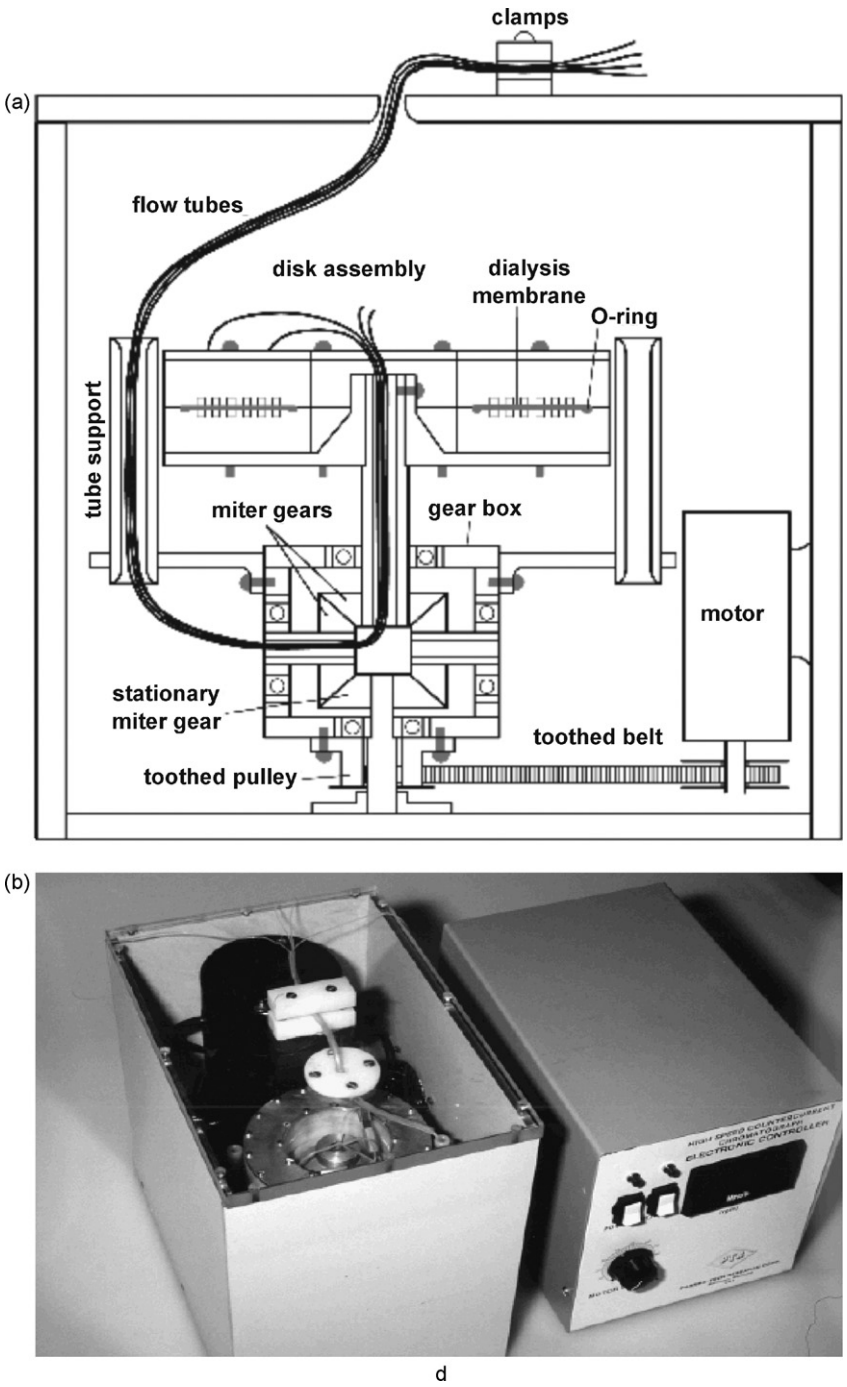

Fig. 3. Centrifugal precipitation chromatograph. (a) Schematic diagram of the design of the apparatus shown by the cross-section through the central axis of the apparatus, (b) photograph of the apparatus.

water output rate from the right end of the channel was measured and the mass transfer rate of AS was determined from the ratio in density between the collected water and the input AS solution or $C_{W\text{-outlet}}/C_{AS\text{-input}}$. Fig. 4 shows the results of the above studies. The upper diagram shows the relationship between the water input rate and the AS saturation rate in water output. All curves show a similar exponential increase of AS saturation rate as the water input rate is decreased reaching over 95% saturation at 1000–2000 rpm. However, in the stationary channel the AS saturation rate becomes less especially in the inverted channel in which the water channel becomes the upper channel. This low saturation rate in the inverted stationary channel may be explained as follows: In this channel orientation, AS transferred from the lower channel to the upper water channel tends to stay above the membrane by the effect of gravity, which interferes with the AS transfer process. In the normal orientation of the channel, AS transferred from the upper channel to the lower water channel is quickly removed by the gravity to accelerate the mass transfer process. In the high centrifugal force field, the AS transferred into the water channel is quickly removed toward one side of the channel regardless of the orientation of the channel. The lower diagram in Fig. 4 shows a relationship between the water input rate and water output rate. All curves form exponential pattern somewhat similar to inverted curves of the upper diagram. The water output is sharply decreased as the water input is decreased reaching 0.25 at 0.1 ml/min. Further experiment using even lower water input revealed that the water output reaches

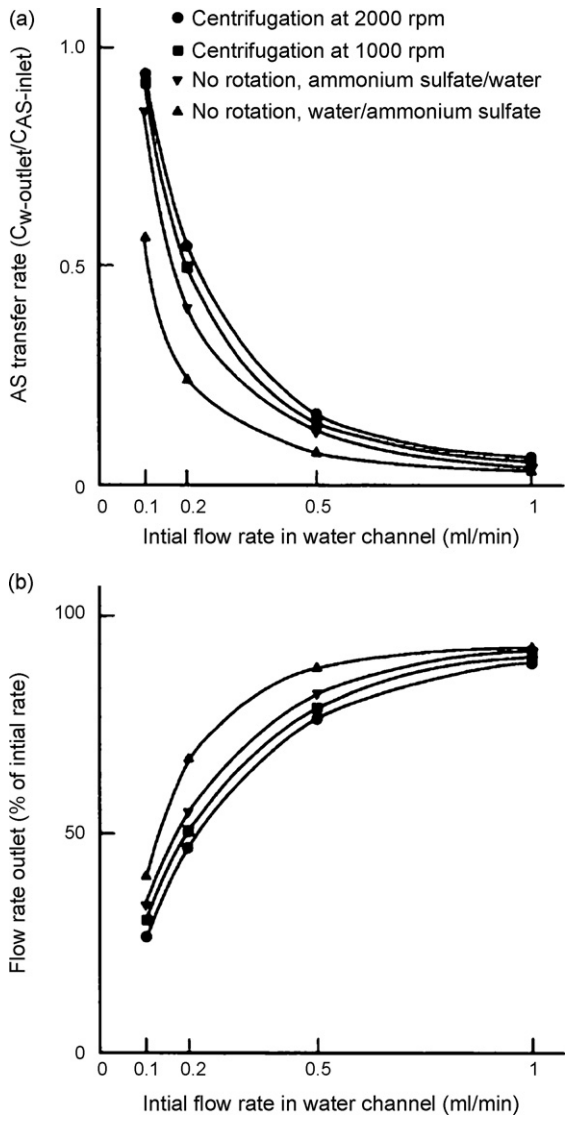

Fig. 4. Ammonium sulfate transfer and osmosis through the semipermeable membrane at various experimental conditions.

0 ml/min when the water input rate becomes at 0.05 ml/min. The results of these studies indicate that the optimal water input rate may be about 0.06 ml/min which would give a high AS saturation rate and concentrated target proteins in the fraction.

4.2. Studies on effects of various other parameters on protein separation

A series of experiments was conducted to determine the optimal conditions using human serum albumin (HSA) and human serum globulin (HSG) as test samples. In each experiment both upper AS and lower water channel were initially filled with 90% saturated AS solution and rotated at a given rpm. This was followed by injection of the sample solution containing HSA and HSG each 5 ml in a buffer solution into the column through a sample port. Then, the separation was started by eluting the AS gradient (with some priming time shown by a flat line) at 1 ml/min while the sample channel is eluted with water at 0.06 ml/min. The effluent from the outlet of the column was continuously monitored with a uv detector at 280 nm and recorded on a strip-chart recorder. The results are summarized in Fig. 5.

Fig. 5a shows the effect of the applied length of AS concentration gradient ranging from 200 min to 1600 min. The peak resolution of the test proteins was sharply improved with the increased length of AS concentration gradient reaching the complete peak resolution at 1600 min. The shoulder of the first peak (HSA) at the bottom chromatogram is considered to be the dimer of HSA formed at a high concentration of AS solution. Fig. 5b shows the effect of inclination of AS concentration gradient on the peak resolution. The results clearly show that the shallower the AS gradient, the higher the peak resolution. The effect of the centrifugal force field is also tested and given in Fig. 5c. The revolution speeds of over 1500 rpm gave similar results while at 1000 rpm showed elevated initial base line and less efficient peak resolution. It was found that pH of the solvent gives significant effects on the separation of the two test samples as shown in Fig. 5d. As the pH is decreased, the position of HSA is shifted toward right and at pH 4.5 it joined to the HSG peak as shown the bottom diagram. Effect of sample size was also tested using lysozyme as the test sample. The result showed that the sample size ranging from 0.1 mg to 10 mg (maximum amount tested) eluted almost at the same point in the AS concentration gradient (chromatogram not shown).

5. Determination of precipitation point of a variety of proteins in AS solution

Before the application of centrifugal precipitation chromatography, it is desirable to determine the precipitation point of various proteins in the AS concentration gradient using a simple elution system [16] since such data is not available in the literature.

Fig. 6a shows the principle of this elution system. The upper diagram shows the elution modes of two pump lines of the gradient maker. Using gradient pumps A and B, protein solution is eluted with pump A at the decreasing linear concentration while the 90% AS solution is eluted with pump B at a increasing linear gradient as shown in the top diagram. When the effluent is continuously monitored with a uv detector, one can obtain a chromatogram of a solid line as shown in the lower diagram where the UV absorbance or scattering in ordinate is plotted against the AS concentration in abscissa. After some flat priming time the uv absorbance curve starts linear decline according to the linear downward gradient elution until the AS concentration reaches the critical solubility point when the protein starts to form precipitation scattering the uv ray that results in the rise of elution curve (starting point). With further increasing AS concentration the protein molecules in the solution are all precipitated and the elution curve starts to fall (ending point). From this elution curve one can calculate the precipitation points including starting, median, and ending point of each protein [16]. Some results are summarized in Fig. 6b, where median precipitation points of 12 different kinds of proteins are plotted against pH. As clearly shown in this figure, with one exception of cytochrome c, precipitation points of all proteins are divided into two ranges, 50–60% AS saturation and 30–40% AS saturation, the latter of which mostly consists of serum globulins. These results suggest that the present system may require a ligand or immuno-affinity separation to single out the target proteins which have a precipitation point at 50–60% AS saturation range.

6. Applications of centrifugal precipitation chromatography

6.1. Standard separation

Fig. 7 shows examples of the standard separation of various protein samples by the present system. All separations were performed as follows: Both channels were first filled with 95% or 75% saturated

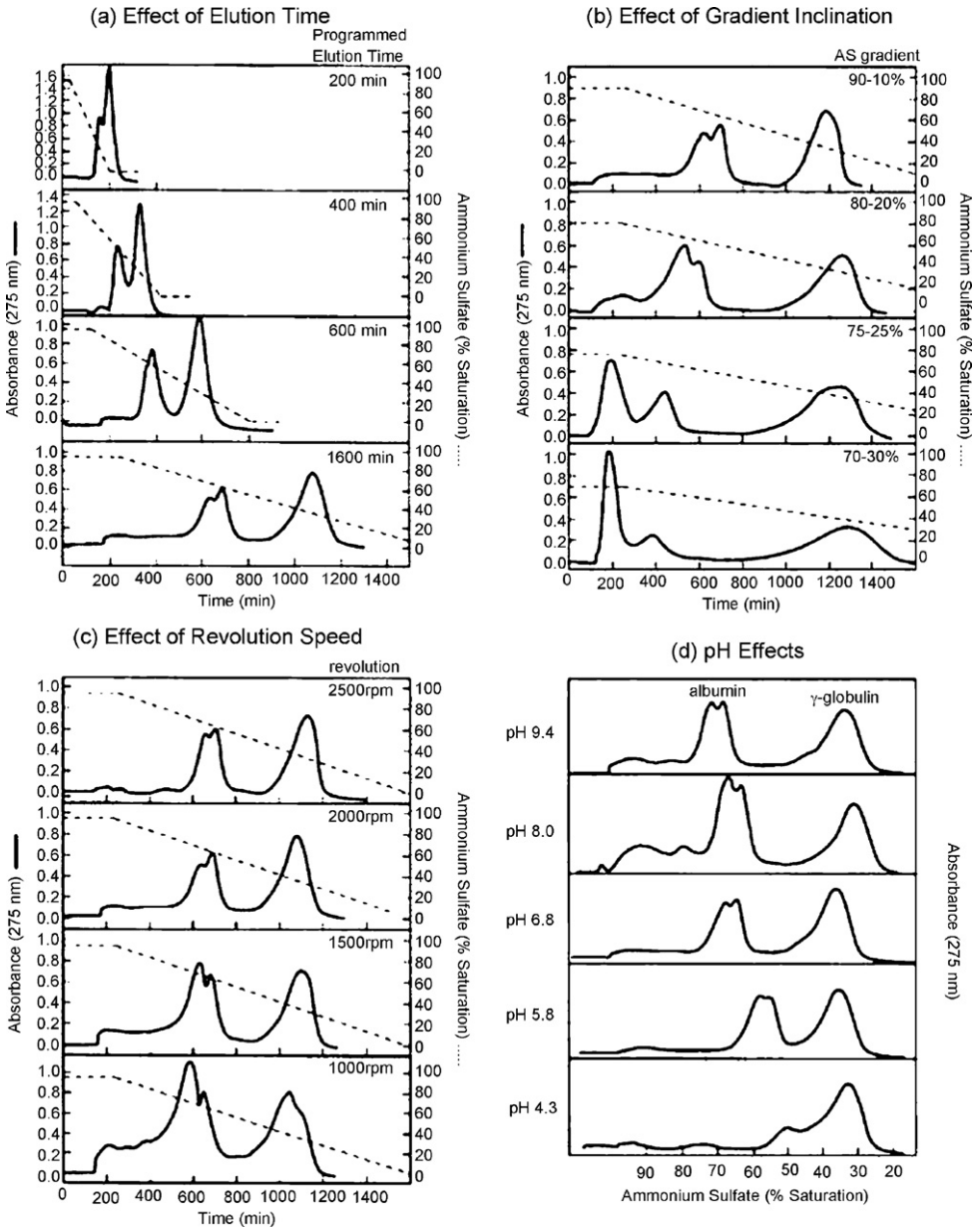

Fig. 5. Optimization of key parameters of centrifugal precipitation chromatography. (a) elution time; (b) inclination of AS concentration gradient; (c) revolution speed; (d) pH. Apparatus: rotary-seal-free flow-through centrifuge; separation disk: a pair of high-density polyethylene disks (13.2 cm in diameter and 1.5 cm in thickness); Separation channel: each 1.5 mm wide and 2 mm deep partitioned with a semipermeable membrane (MWCO 6000–8000, 100 μm thick) each with 5 ml in capacity; flow rate: AS channel: 1 ml/min (Shimadzu SCL10A LC-10AD gradient pump) and water channel: 0.06 ml/min (Harvard Syringe pump, Model 9805320); (c) revolution speed: 2000 rpm unless otherwise indicated; sample: a mixture of HSA and human γ-globulin, each 4 mg.

AS solution followed by sample injection through the sample port. Then, the AS channel was eluted with linear concentration gradient of AS solution (after a priming time of 240 min) at a flow rate of 1 ml/min while the sample channel was eluted with suitable buffer solution at a low flow rate of 0.06 ml/mi under 2000 rpm of column rotation. The effluent from the outlet of the column was continuously monitored with Uvicord s at 280 nm and collected into test tubes for further analysis.

Fig. 7a shows separation of human serum proteins by the standard method. Serum (5 μl) from a healthy human subject was diluted to 1 ml with a buffered saline and injected into the column. As expected from the results of the basic studies, major serum proteins of HSA and HSG were well resolved as shown by the SDS-PAGE analysis. The HSA peak was partially resolved into two peaks, the latter of which is considered to be the dimer on the basis of SDS-PAGE analysis (lanes 2 and 3). Fig. 7b shows purification of monoclonal antibody against human mast cells from hybridoma culture supernatant. The chromatogram shows two major peaks, IgM (target protein) and IgG. A minor peak eluted earlier is thought to be calf albumin which had been added to the hybridoma culture medium. The second IgM peak showed high fluorescent activity about 10 times that of the original serum. This enhanced activity of the IgM fraction may be due to the removal of masking molecules attached on the monoclonal antibody.

The third example is a separation of PEG (polyethylene glycol) -protein conjugates by centrifugal precipitation chromatography. When protein molecule is conjugated with PEG, it increases hydrophobicity and prolongs the circulation time in the blood stream in the patient. It also gives further beneficial effect by increasing the solubility and reducing antigenicity. The PEG-protein conjugates, however, are difficult to separate by the conventional chromatographic methods according to the number of PEG

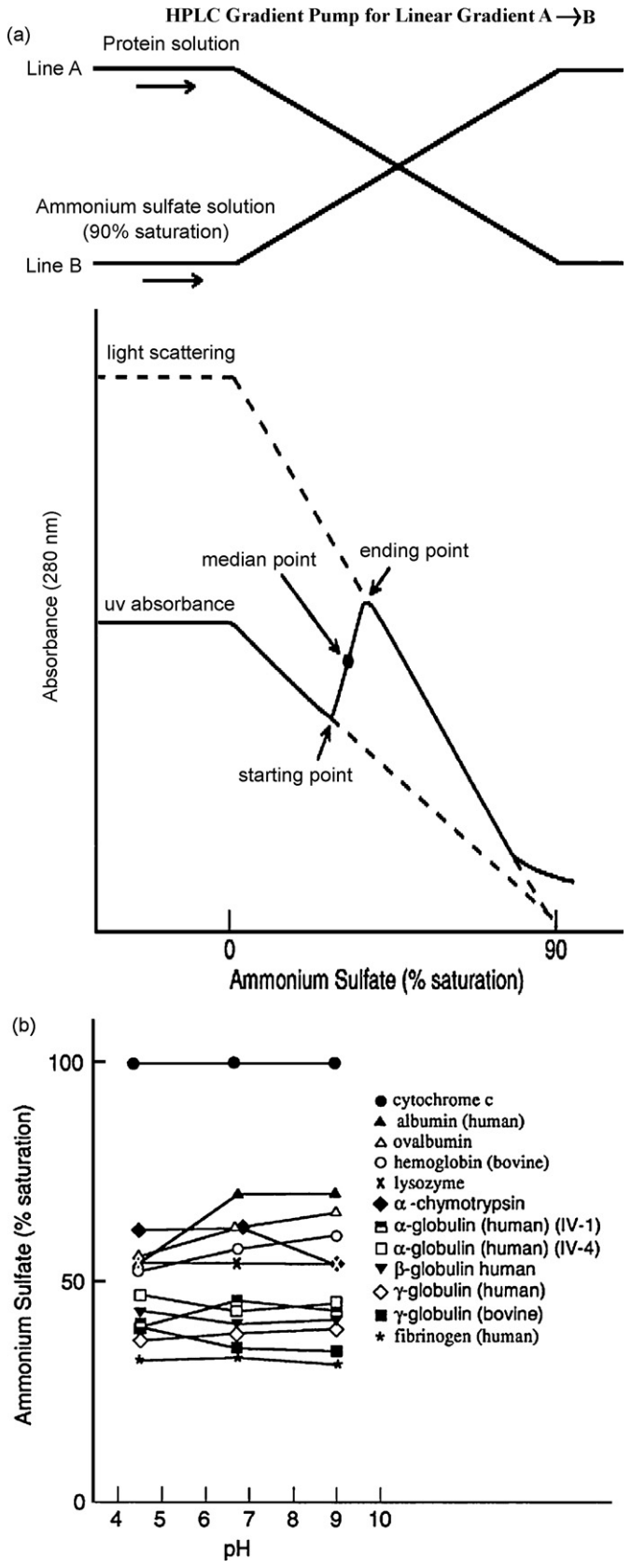

Fig. 6. Simple determination method of precipitation points for various protein samples. (a) Experimental scheme; (b) median precipitation point for various protein samples at acidic, neutral and basic conditions. Except for cytochrome c, all the proteins tested are divided into two groups, one with the precipitation point above 50% AS saturation and the other below 40% AS saturation. The latter group mainly consists of globulins.

molecules per protein molecule. However, It was found that the present method was able to resolve each species as demonstrated by the separation of PEG-lysozyme conjugates using the standard separation method [7]. Fig. 7c shows the results of the experiment. In the chromatogram on the upper diagram, the first peak is free lysozyme; the second peak, PEG-lysozyme; the third peak, $(PEG)_2$-lysozyme; and the last peak is composed of heavier PEG-lysozyme conjugates as shown in the PAGE analysis in the lower diagram.

The fourth and the last example of the standard separation is shown in Fig. 7d where rabbit reticulocyte lysate was resolved into three major peaks: the first peak is rabbit hemoglobin and the third peak RNA with denatured proteins. The second peak eluted around 50% AS saturation point contains the target protein (transcription factors) with many other proteins. This result indicates that it is difficult to single out one target protein by the standard separation unless the method is modified to more sensitive separation method utilizing an affinity between enzyme and substrate (or analog) or antigen and antibody as described later.

In addition to the above examples, the present method has been successfully applied to many other analytes including IgG by AS gradient elution [3–5], dextran by ethanol gradient elution [9], fragments of polysaccharide by ethanol gradient elution [8], plasmid DNA, RNA and protein by the gradient elution of cationic surfactant [11,12], and most recently carotenoid cleavage enzymes from tea leaves by ethanol gradient elution [17].

6.2. *Ligand-affinity centrifugal precipitation chromatography applied to separation of recombinant ketosteroid isomerase*

The example of ligand-affinity separation described here is based on the specific affinity between enzyme and its substrate (or analog). Fig. 8 shows the separation of recombinant ketosteroid isomerase (rKSI) from *E. coli* lysate. The left chromatogram (Fig. 8a) was obtained by the standard technique where the target protein was eluted at about 50% AS saturation point with many other proteins as shown by SDS-PAGE analysis in the lower diagram. In order to improve the separation of rKSI, ligand-affinity separation was performed. The ligand used was estradiol which was conjugated to PEG5000. Ligand-affinity centrifugal precipitation chromatography was performed by adding the ligand-PEG5000 (25 mg) to the sample solution under otherwise identical experimental conditions. The result is shown in Fig. 8c where the protein peak eluted around AS 50% saturation points was remarkably reduced, and instead a new small peak appeared at around 25% AS saturation point followed by a huge peak which was retained in the column until the column was washed with water. In the SDS-PAGE analysis shown in Fig. 8a, the small peak was found to be the target compound rKSI, forming dimers and tetramers due to its high concentration in the fraction. The huge peak retained in the column was found to be ligand-PEG conjugates free of protein (without the stained band in SDS PAGE) which may be reused for the next run. It was found that the rKSI fraction thus obtained also contained a small amount of high molecular weight proteins. These contaminants, however, may be removed by a simple pretreatment of the sample solution by adding AS at 40% followed by centrifugation. The studies were continued to examine whether this method can be applied to a minute amount of rKSI contained in the mutant *E. coli* strain. The result clearly indicated that the method successfully isolated a small amount of rKSI detected by SDS PAGE analysis (data is now shown). This method may also be applied to immunoaffinity separation by using the PEG-antibody or PEG-antigen conjugates.

Here it may be worthwhile to discuss about the unique mechanism that may be involved in this ligand-affinity separation. When the ligand-PEG 5000 conjugate was added to a concentrated AS solution in a test tube, it was found that the conjugate did not

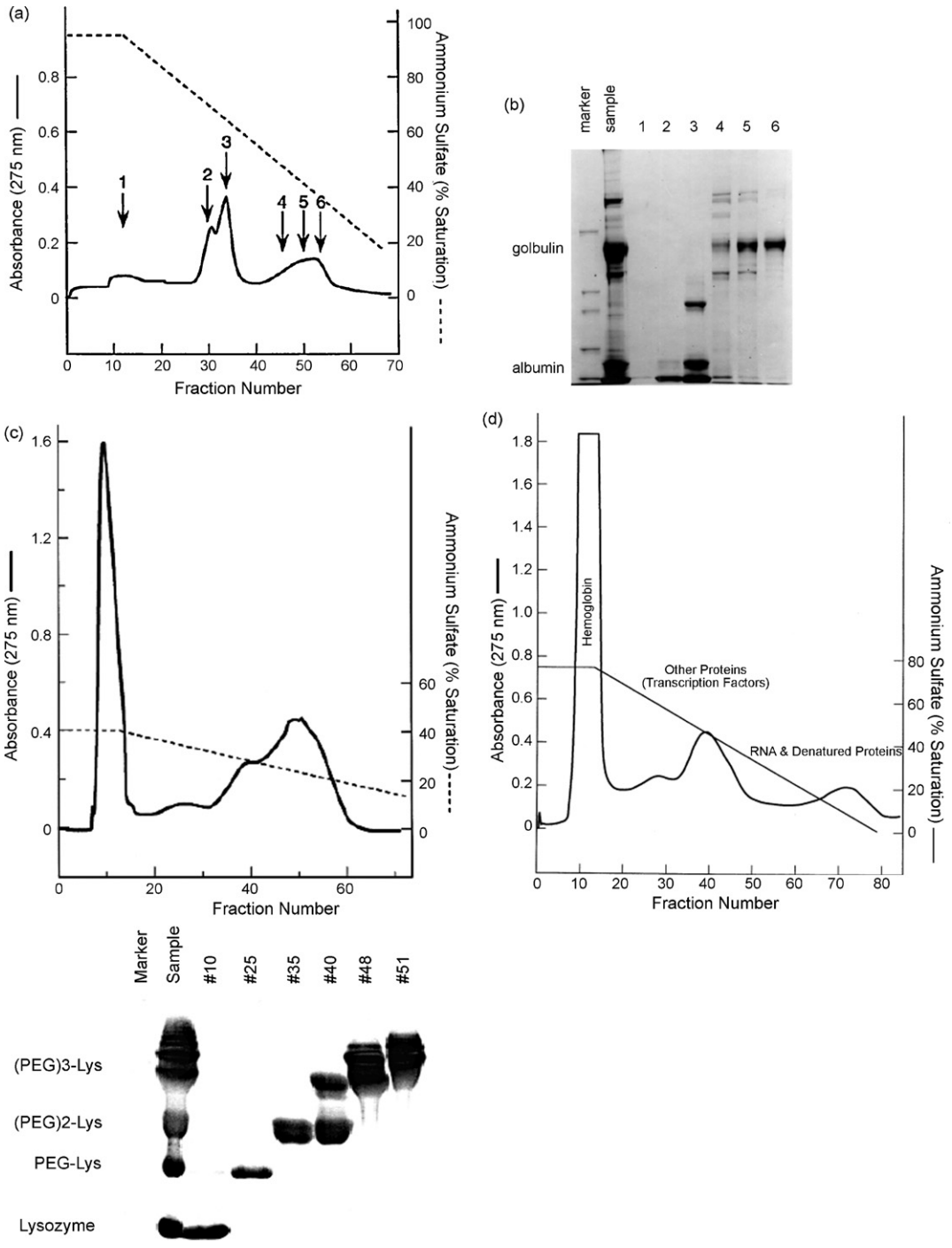

Fig. 7. (a) Separation of normal human serum by centrifugal precipitation chromatography. (a) Precipitation chromatogram, (b) SDS-PAGE analysis. Experimental conditions: upper diagram: sample normal human serum pooled, 0.05 ml mixed in 1 ml buffered solution; AS channel:linear AS concentration gradient 95–0% for 1600 min including 15% of the priming time as indicated in the diagram: water channel:50 mM potassium phosphate (pH 7). Other experimental conditions were described in Fig. 5 caption. Lower diagram: SDS-PAGE analysis was performed using precast tris-glycine 6% gel (Novex) at 110 V for110 min followed by Coomassie brilliant blue staining. (b) Purification of monoclonal antibody from hybridoma culture supernatant. Monoclonal antibody against human mast cells was harvested from the second peak (IgM), which showed high fluorescent activity over10 times that of the original serum. The sample solution was prepared by adding AS to 45 ml of hybridoma culture supernatant (provided by Prof. Tadashi Okada of Aichi Medical University, Aichi, Japan) to bring 60% saturation followed by centrifugation at 15,000 × *g* for 15 min. The precipitates were suspended in 2 ml of 50 mM potassium phosphate buffer solution (pH 7) and introduced into the sample channel. The AS channel was eluted with a linear AS concentration gradient 75–0% at 1 ml/min for 1600 min, including the priming time of 240 min as indicated in the chromatogram while the sample channel was eluted with 50 mM potassium phosphate buffer (pH 7) at 0.06 ml/min under 2000 rpm. (c) Separation of PEG-lysozyme conjugates by centrifugal precipitation chromatography. Upper diagram: Chromatogram obtained by a linear AS concentration gradient from 40% to 0%. Lower diagram: SDS-PAGE analysis of fractions. Experimental conditions, apparatus: rotary-seal-free flow-through centrifuge; separation disk: a pair of high-density polyethylene disks (13.2 cm diameter and 1.5 cm thick); separation channel: each 1.5 mm wide and 2 mm deep partitioned with a semipermeable membrane (MWCO 6000–8000, 100 μm thick) each with 5 ml in capacity; flow rate: AS channel at 1 ml/min (Shimadzu SCL10A LC-10AD gradient pumps) and water channel 0.06 ml/min (Harvard Syringe pump, Model 9805320); revolution speed: 2000 rpm. (d) Separation of reticulocyte lysate containing transcription factors (Max and α-pal). The target transcription factors were eluted in the second peak with many other proteins. Experimental conditions: apparatus: rotary-seal-free flow-through centrifuge; separation disk: a pair of high-density polyethylene disks (13.2 cm diameter and 1.5 cm thick); separation channel: each 1.5 mm wide and 2 mm deep partitioned with a semipermeable membrane (MWCO 6000–8000, 100 μm thick) each with 5 ml in capacity; sample: 1 ml of rabbit reticulocyte lysate; AS channel: AS linear gradient 75–0% at the programmed elution time of 1600 min including 15% of the priming time; revolution: 2000 rpm.

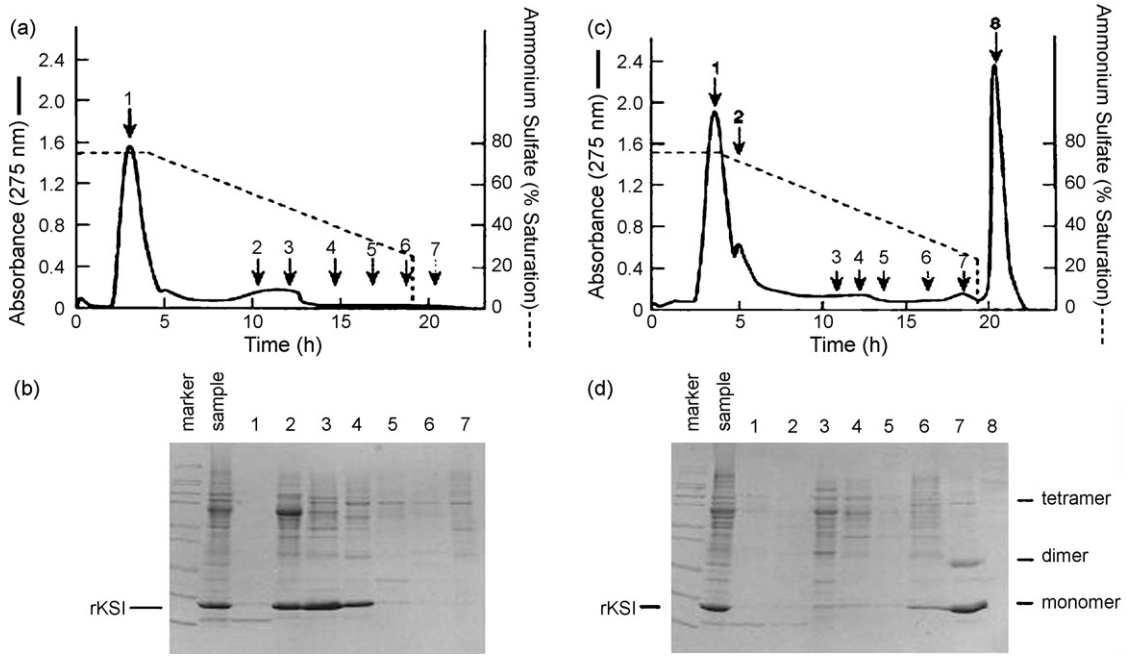

Fig. 8. Purification of recombinant enzyme (rKSI) from a crude *E. coli* lysate using an affinity ligand. (a) and (b): control separation without ligand, (c) and (d): separation with an affinity ligand (estradiol-PEG5000 conjugate) in the sample solution. Experimental conditions: apparatus: rotary-seal-free flow-through centrifuge; separation disk: a pair of high-density polyethylene disks (13.2 cm diameter and 1.5 cm thick); separation channel: each 1.5 mm wide and 2 mm deep partitioned with a semipermeable membrane (MWCO 6000–8000, 100 μm thick) each with 5 ml in capacity; Sample: 0.25 ml of *E. coli* lysate containing recombinant ketosteroid isomerase (rKSI); AS channel: linear AS gradient 75–25% for 900 min including 240 min priming time. In (c) 25 mg of ligand was added to the sample solution. SDS-PAGE: tris-glycine precast 16% gel at 200 V for 55 min followed by Coomassie brilliant blue staining.

show a sign of precipitation and instead formed two liquid phases! Similar two-phase systems composed of PEG-rich upper phase and the inorganic salt-rich lower phase introduced by Albertsson have been efficiently used for partitioning macromolecules such as proteins and nucleic acids according to their partition coefficients [18]. Then, it is most likely that in this separation the ligand-PEG conjugate and high concentration AS solution formed two phases in the column where rKSI with a strong affinity to the ligand (or a high K value) remained in the channel as long as the formation of the two phases was maintained while other proteins with lower K values were eventually eluted out from the column. If this is the case, this method is considered to be the combination of two separation methods, i.e., centrifugal precipitation chromatography and countercurrent chromatography with a polymer phase system. This hypothesis will be validated in the future.

6.3. *Immunoaffinity centrifugal precipitation chromatography*

The basic studies for immunoaffinity separation with the present method were performed using a rabbit polyclonal antibody against human serum albumin (HSA). The mechanism of separation is illustrated in Fig. 9: The separation is initiated by filling the column with 75% saturated AS solution as in the standard separation procedure. This is followed by injection of sample solution through the sample port. The sample solution is prepared by mixing the HSA and suitable amount of rabbit polyclonal antibody to form antigen–antibody complex in the sample tube. Then, the AS channel is eluted with 40–50% AS saturated solution while the sample channel is eluted with buffered solution at a low flow rate. This results in precipitation of the antigen–antibody complex at the channel bed while other proteins with higher precipitation points will be all eluted out from the channel (Fig. 9 (1)). After all those proteins are washed out from the channel, the antigen-releasing reagent such as high-pH glycine solution is added to the sample channel to dissociate antigen–antibody complex to release the target protein which is eluted from the channel (Fig. 9 (2)). The antibody still remained in the channel is washed out with water (Fig. 9 (3)). The mechanism is somewhat similar to the immunoaffinity column chromatography except that the present system retains the antigen–antibody complex in the column by the centrifugal force without using a solid support which might irreversibly adsorb or denature the target proteins.

The actual immunoaffinity centrifugal precipitation chromatographic technique was demonstrated in two steps: the separation of HSA commercial sample by rabbit polyclonal anti-HSA and the similar separation of HSA from the human blood plasma.

6.3.1. *Separation of HSA with rabbit anti-HSA*

The experiment was performed as follows: The sample suspension was prepared by adding 2 mg of rabbit polyclonal anti-HSA (Sigma Chemical Co., St. Louis, MO, USA) to the buffered sample solution containing 1 mg of HSA (Sigma Chemical Co.) to form antigen–antibody complex in a test tube. In each separation both AS and sample (water) channels were first entirely filled with 40% AS saturated solution buffered at pH 4 followed by injection of the above sample suspension. Then, under 2000 rpm of column rotation, the AS channel is eluted with 40% saturated AS solution at a flow rate of 1 ml/min while the sample channel was eluted with buffered solution at a low flow rate of 0.06 ml/min. After 5 h of elution when all unbound HSA and impurities in the sample solution were eluted out, a releasing solution containing 0.5 M glycine in 40% saturated AS solution at pH 10 by ammonium hydroxide (shown by an arrow in Fig. 10a) until the released HSA was eluted out. The antibody still remained in the channel was collected by eluting water through both channels. As shown in the chromatogram (Fig. 10a) a small HSA peak was detected at fraction 12 which formed a single band in SDS-PAGE analysis (Fig. 10b). A huge peak at fraction 17–22 contained almost antigen-free antibody as shown in Fig. 10b, lanes 4–6. A small peak at fraction 7(Fig. 10a) may be the unbound HSA in the sample solution (Fig. 10b, lane 1).

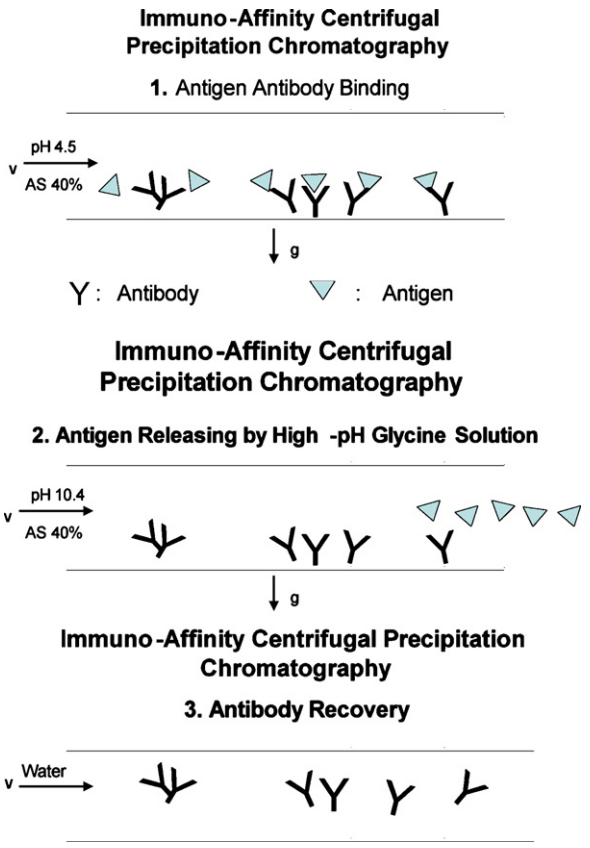

Fig. 9. Mechanism of immunoaffinity centrifugal precipitation chromatography.

6.3.2. Separation of HSA from human plasma with rabbit anti-HSA

The present method was applied to separate HSA from human blood plasma according to the method described above except that the releaser was in 50% AS saturated solution to enhance the releasing action. The sample solution was prepared by adding 2 mg of rabbit polyclonal anti-HSA to 20 μl of human plasma to form antigen–antibody complex in a test tube. The results are shown in Fig. 11a and b. Peaks eluted at fractions 5–10 in the chromatogram (Fig. 11a) shows no band in SDS PAGE analysis (Fig. 11b, lane 5). The target protein was eluted at fraction 13 (Fig. 11a) which showed a single band in SDS PAGE analysis (Fig. 11b). The freed antibody still retained in the channel was quickly eluted out by water at a higher flow rate (Fig. 11b, lane 6).

7. Preparative centrifugal precipitation chromatography

A variety of applications of centrifugal precipitation chromatography described above have been performed with a small spiral channel of 6 ml capacity having a small mass transfer area of semipermeable membrane at ca 30 cm^2 which limits the maximum sample loading capacity to ca 10 mg. In order to increase the sample loading capacity, two new designs for separation column have been proposed. In the first model, a long piece of dialysis tubing of 4 mm in diameter (MWCO 3500 or 8000, Spectrum, San Diego, CA, USA) was inserted into a cavity of convoluted tubing of 5.7 mm average ID and 2.2 m long (Zeus Industrial Products, Orangeburg, NC, USA). The column was then fixed on the rotary plate in a spiral configuration using nylon ties [19] or later embedded into a spiral groove made on a flat plastic rotary disk [20]. In this design, the mass transfer area for AS is increased to ca 280 cm^2 or over 9 times that of the original design. The separation was performed by eluting AS concentration gradient through the dialysis tubing while the protein was introduced through the space between the dialysis tubing and the convoluted tubing. The separation was similarly performed by eluting the AS gradient from the external terminal and the sample solution from the internal terminal of the spiral channel under 800 rpm (average ca 100 × *g*). Using this system, 100 mg of the sample protein mixture (HSA and human γ-globulin, each 50 mg) was successfully resolved in 6 h [20].

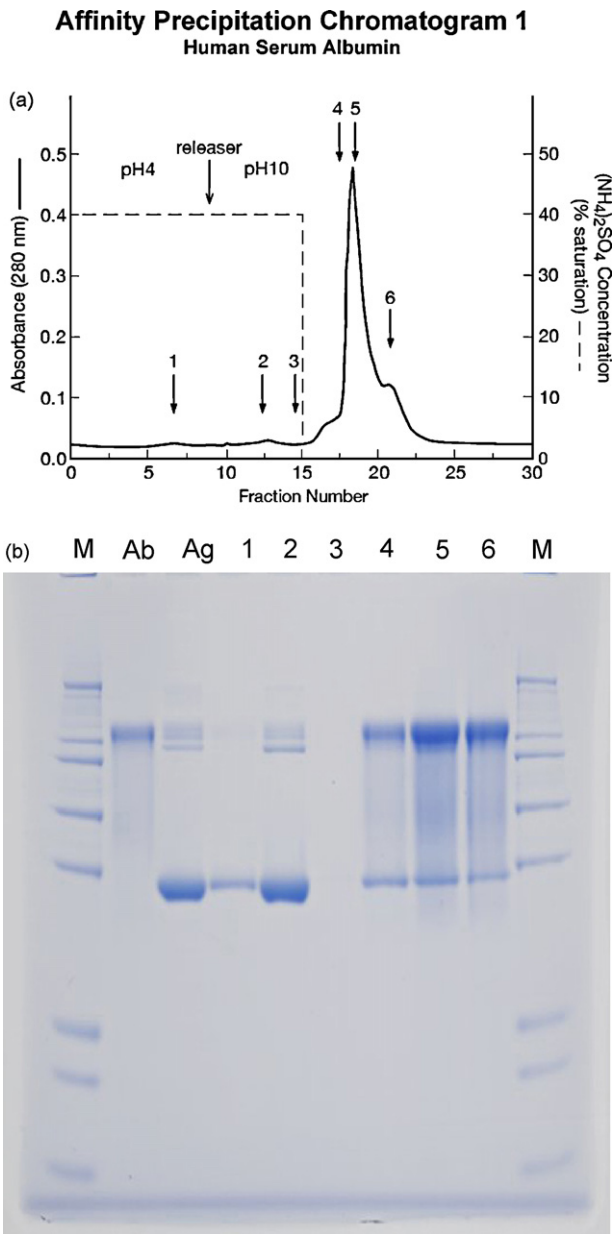

Fig. 10. Separation of HSA by immunoaffinity centrifugal precipitation chromatography. (a) Chromatogram; (b) SDS-PAGE analysis of collected fractions. Experimental conditions: (a) apparatus: rotary-seal-free flow-through centrifuge; separation disk: a pair of high-density polyethylene disks (13.2 cm diameter and 1.5 cm thick); separation channel: each 1.5 mm wide and 2 mm deep partitioned with a semipermeable membrane (MWCO 6000–8000, 100 μm thick) each with 5 ml in capacity; sample: HSA (Sigma Chemical Co., St. Louis, MO, USA) 1 mg mixed with 2 mg of polyclonal rabbit anti-HSA antibody. AS channel: 40% saturated AS solution at pH 4–4.5 at a flow rate of 1 ml/min for ca 5 h followed by elution with the releasing solvent composed of 0.5 M glycine in 40% saturated AS solution at pH 10 adjusted by ammonium hydroxide until the target HSA was eluted; water channel: water at 0.03 ml/min throughout the experiment; revolution: 2000 rpm; fractionation: 40 min/tube; detection: 280 nm. (b) Gel: precasted tris-glycine gel (Invitrogen); M: marker; Ab: antibody; Ag: antigen (HSA).

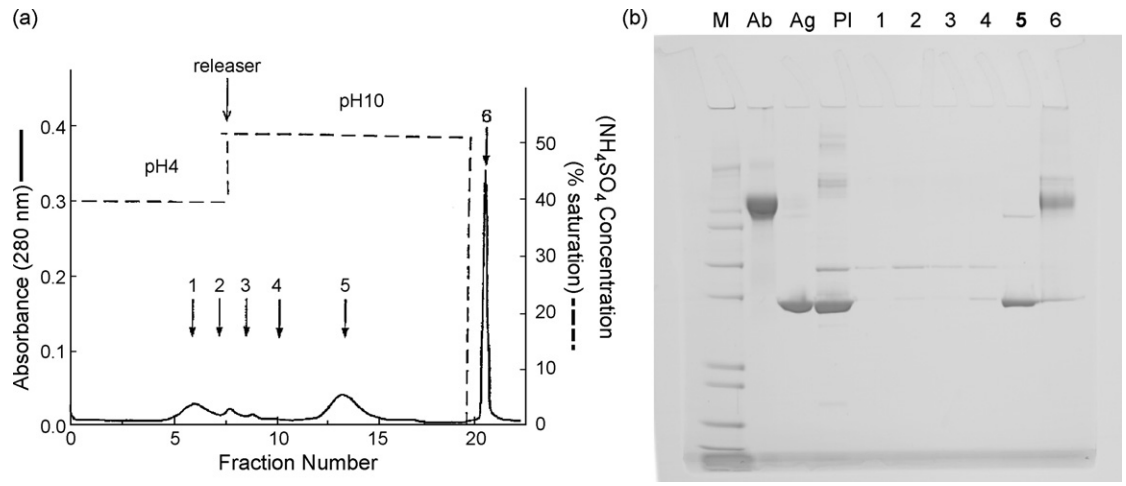

Fig. 11. Isolation of HSA from human blood plasma by immunoaffinity centrifugal precipitation chromatography. (a) Chromatogram and (b) SDS-PAGE analysis of collected fractions. Sample: human blood plasma 20 μl mixed with 2 mg of rabbit polyclonal anti-HSA antibody. Releasing solution: 0.5 M glycine in 50% saturated AS solution at pH 10 adjusted by ammonium hydroxide. After HSA is eluted, antibody was recovered by pumping water at 1 ml/min. The rest of the experimental conditions are described in the Fig. 10 caption. M: marker; Ab: antibody; Ag: antigen; Pl: human blood plasma.

The second model used a commercial product, MidGee Hoop Cross Flow Filter cartilage (Amersham Biosciences, Piscataway, NJ, USA) for the separation channel. It was made of a 60 cm-long polysulfone tube containing two pieces of narrow-bore hollow fiber dialysis tubing (MWCO 10,000) that was originally designed for quick dialysis and/or concentration of macromolecules from dilute sample solution. In the present experiment, the above two cartilages were symmetrically mounted on the rotary plate in a spiral form using a number of metal clamps. Using a single cartilage or two cartilages connected in series, the optimal elution conditions were determined for separation of HSA and human γ-globulin, where a mixture of 50 mg of each sample was successfully resolved in 13 and 25 h, respectively [21].

8. Centrifugal precipitation chromatography vis. conventional separation method

As described above, centrifugal precipitation chromatography separates analytes according to their solubility in suitable precipitating reagents typically ammonium sulfate. The ability of ammonium sulfate to preserve the natural structure of proteins permits one to safely perform a wide range of applications for separation of enzymes and other sensitive analytes without loss of their activity. This unique feature of the method will give an advantage over the conventional methods using a solid matrix such as size exclusion chromatography and ion exchange chromatography which may have problems of irreversible adsorption and denaturation of proteins by the solid support. As in affinity column chromatography, the present method also provides affinity separations which can single out one desired species using PEG-ligand conjugates or antibodies. The present method, however, facilitates the use of various kinds of ligands simply by introducing them into the liquid mobile phase using the same separation column so that it avoids tedious column packing procedure necessary for the conventional methods.

The present method based on the solubility of the analyte in ammonium sulfate solution may be extremely useful as a supplemental separation method for the conventional column chromatography based on other separation parameters.

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