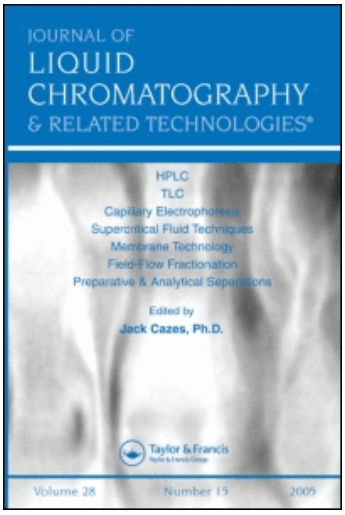

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CENTRIFUGAL PRECIPITATION CHROMATOGRAPHY: NOVEL FRACTIONATION METHOD FOR BIOPOLYMERS, BASED ON THEIR SOLUBILITY

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ABSTRACT

A recently developed chromatographic system called centrifugal precipitation chromatography is reviewed. The system internally generates a concentration gradient of precipitant, such as ammonium sulfate for protein separation, through a long separation channel under a centrifugal force field. Chromatographic elution is performed by gradually lowering the concentration of the precipitant in the gradient, so that the samples are subjected to a repetitive process of dissolution and precipitation along the channel, until they are finally eluted from the column according to their solubility. The separation column consists of a pair of disks equipped with mutually mirror-imaged spiral grooves. A dialysis membrane is sandwiched between these disks to form two identical channels partitioned by the membrane. The disk

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assembly is mounted on a seal-less continuous flow centrifuge. When a concentrated ammonium sulfate solution is eluted through one channel and water through the other channel in an opposite direction, an exponential gradient of ammonium sulfate is formed through the channel. A series of basic studies was conducted to measure mass transfer of ammonium sulfate and osmosis through the membrane, and the effects of various parameters on separation were investigated using a set of standard protein samples. Using an optimized set of conditions, various protein samples were separated, including serum proteins, monoclonal antibodies, recombinant enzyme, and PEG-protein conjugates. The method is extended to the separation of other biopolymers such as polysaccharides and polycatechin using a suitable organic solvent as a precipitant.

Key Words: Centrifugal precipitation chromatography; Solubility; Purification; Biopolymers; Protein; Recombinant enzyme; Monoclonal antibody; Polysaccharide; Dextran

INTRODUCTION

Chromatographic separation is traditionally based on continuous solute partitioning between two phases, one mobile and the other stationary, in the column. However, similar separation can be achieved according to the solubility of solutes in a single solvent that contains a gradient precipitant, provided that the advancing rate of the gradient is made substantially lower than the solvent flow. For example, when the concentration gradient of a precipitant is made through a sephadex column from the top downward, macromolecules, such as protein molecules, can travel through the column at a rate much higher than the gradient. In this way, solutes of different solubilities are subjected to repetitive precipitation and dissolution along the column. Small differences in these solubilities than result in their separation. In the past, this "precipitation and dissolution chromatography" was reported by several workers for separations of proteins[1–3] and synthetic polymers[4] using a separation column filled with a solid support.

Centrifugal precipitation chromatography[5–7] described in this article uses an open channel free of solid support, where a gradient of precipitant is introduced through a semipermeable membrane, while the precipitated sample molecules are retained by centrifugal force. The method eliminates potential problems caused by the solid support used in the conventional chromatographic method such as sample loss and denaturation, carryover of fine precipitate, and clogging.

The present article fully reviews this new chromatographic method including its principle, instrumentation, basic studies on various operational parameters together with various applications for the separation of biopolymers including proteins, polysaccharides, and polycatechins.

PRINCIPLE

The centrifugal separation of solutes according to their solubility can be performed simply by subjecting a mixture of solute precipitates to a gradient elution in a rotating column, in such a way that the precipitant concentration in the mobile phase is gradually reduced. The separation achieved in this way, however, fails to yield high chromatographic resolution, because the sample, once dissolved at the critical precipitant concentration in the gradient, is quickly eluted from the column without the repetitive precipitation and dissolution process that magnifies small differences in solubility, and is essential for high peak resolution. In order to achieve an ideal chromatographic separation, it is necessary to establish a gradient of the precipitant through the column in such a way that: (1) the precipitant gradient should travel through the column at a rate substantially lower than that of the mobile phase and; (2) the traveling rate of the gradient through the column or the precipitant concentration in the gradient should be freely adjustable at a given flow rate of the mobile phase. These two requirements are fulfilled by the separation column equipped with a pair of channels divided by a semipermeable membrane as described below:

Figure 1 illustrates the principle of the method. A long separation channel is divided by a dialysis membrane. A concentrated solution of precipitant (such as ammonium sulfate for protein separation) is eluted through the upper channel and water through the lower channel in the opposite direction, at a much lower flow rate. This forms an exponential concentration gradient of the precipitant through the lower channel as described elsewhere.[5,6,8] This gradient is stable as long as the above countercurrent process of the two liquids is steadily maintained, while the mobile phase is continuously flowing through the channel. Under a centrifugal force field, the sample mixture introduced into the lower channel is subjected to a gradually increasing precipitant concentration and precipitated according to its solubility in the precipitant solution. After all components are precipitated, chromatographic elution is initiated by gradually lowering the precipitant concentration in the upper channel. This results in a proportional decrease in precipitant concentration in the gradient formed in the lower channel, so components are dissolved and again precipitated at a slightly advanced location. Consequently, the sample is subjected to repeat precipitation and dissolution along the channel and finally eluted out in the order of the solubility of its components in the precipitant solution.

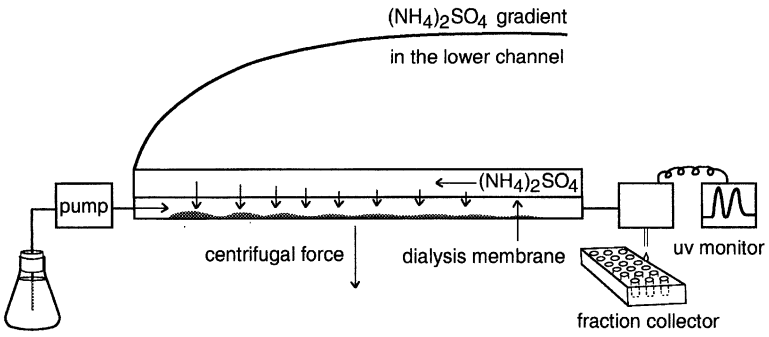

Figure 1. Principle of centrifugal precipitation chromatography. A pair of channels is partitioned with a dialysis membrane. Countercurrent of concentrated ammonium sulfate solution and water through the channel produces an exponential gradient of ammonium sulfate in the lower channel. Proteins introduced into the lower channel are precipitated and deposited at the bottom of the channel by centrifugal force. The chromatographic elution is initiated by gradually reducing the ammonium sulfate concentration in the upper channel that causes proportional reduction of ammonium sulfate concentration in the gradient in the lower channel. This in turn results in repetitive dissolution and precipitation of the proteins along the channel. Consequently, proteins are eluted from the channel according to their solubility in ammonium sulfate solution.

INSTRUMENTATION

The design of the separation disk is shown in Fig. 2a. A pair of plastic disks (each 13 cm diameter and 1.5 cm thick) is equipped with mutually mirror-imaged spiral grooves (1.5 mm wide, 2 mm deep and ca 2 m long) so that a proper alignment forms a single spiral channel. A dialysis membrane (regenerated cellulose, MWCO 6000–8000, Spectrum, San Diego, CA) is sandwiched between these disks to form a desired column structure illustrated in Fig. 1. These two disks are tightly bolted together and mounted on a seal-less continuous flow centrifuge (Fig. 2b).

This centrifuge is equipped with a set of miter gears, which produces 2 : 1 rotation ratio between the separation disk assembly and the rotary frame. This allows two pairs of flow tubes to rotate without twisting each other, so that the system permits continuous elution without a conventional rotary seal device, which often produces various complications such as leakage and cross-contamination.[9,10] Figure 2c schematically illustrates the elution system of centrifugal precipitation chromatography, and Fig. 2d shows a photograph of the instrument, which is currently available from Pharma-Tech Research Corporation, Baltimore, MD, USA.

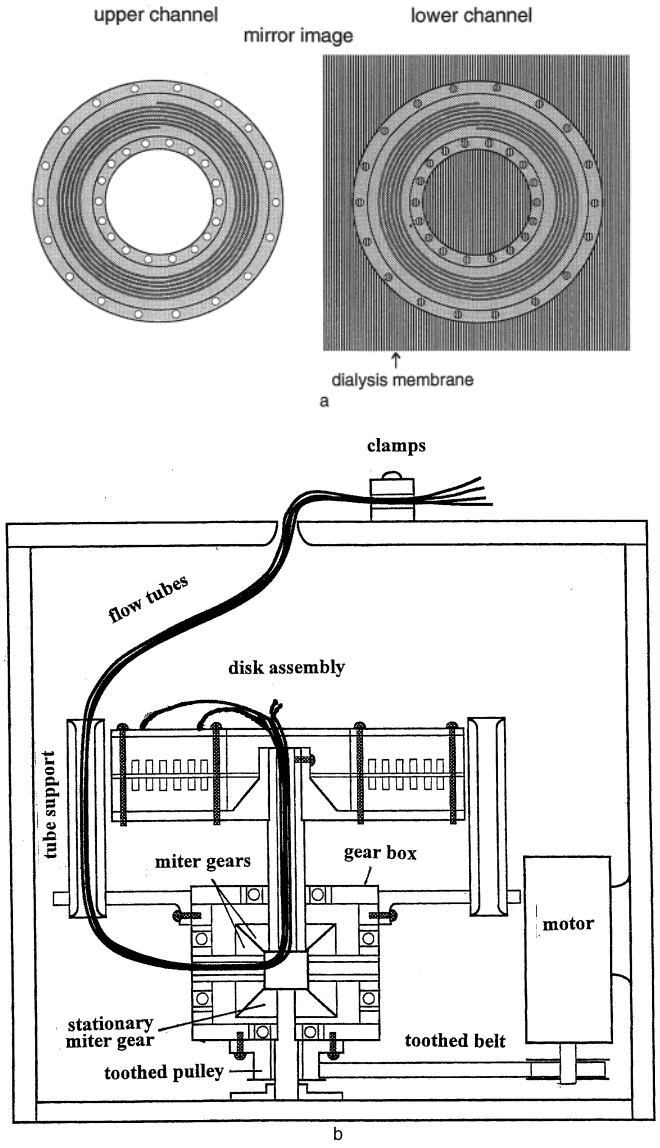

Figure 2. Design of the centrifugal precipitation chromatograph. (a) a pair of separation disks and dialysis membrane; (b) cross-sectional view of the seal-less continuous flow centrifuge equipped with the column assembly for centrifugal precipitation chromatography; (c) schematic diagram of the elution system of centrifugal precipitation chromatography; and (d) photograph of the apparatus.

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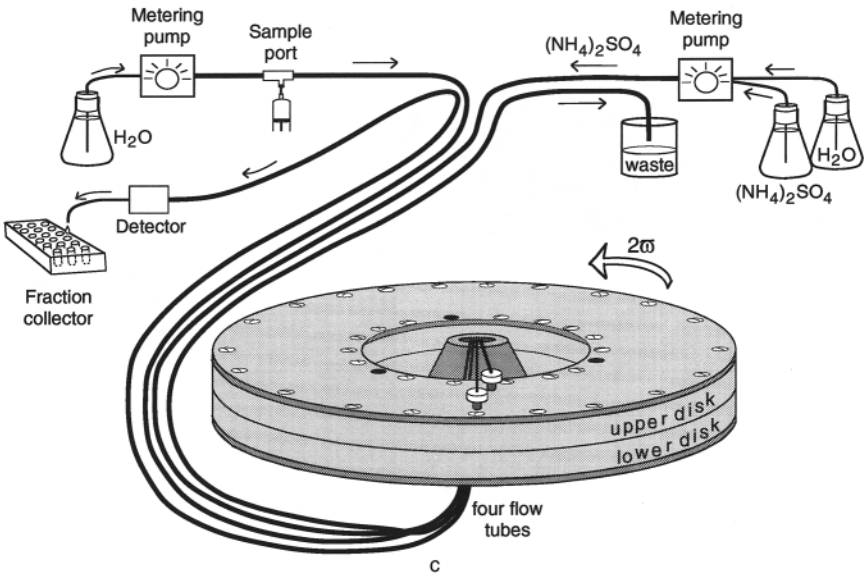

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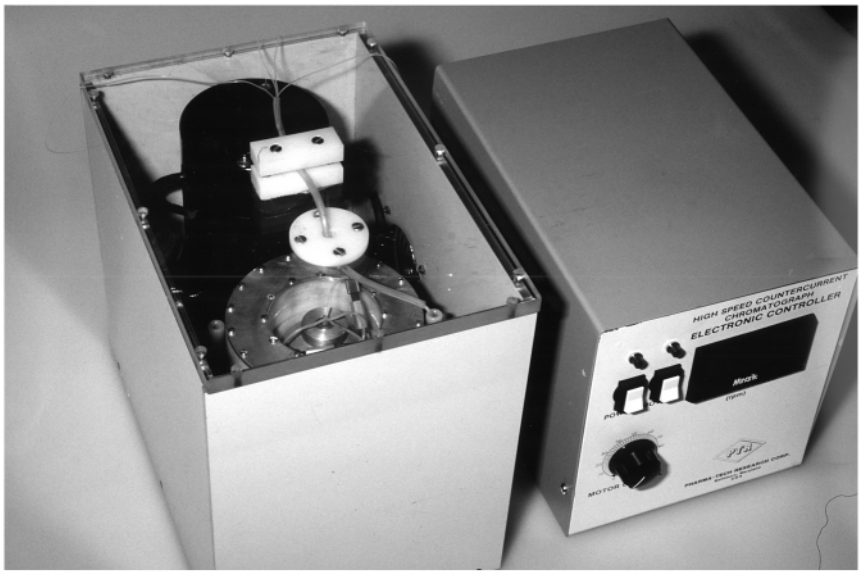

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Figure 2. Continued.

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BASIC STUDIES ON EXPERIMENTAL CONDITIONS FOR PROTEIN SEPARATION

A series of basic studies was conducted to determine the optimum experimental conditions for protein separation in the following two steps: the first step is to measure the mass transfer rates of ammonium sulfate (precipitant) and water through the membrane without protein samples, and the second step is to optimize the separation condition using standard protein samples.

Studies on Mass Transfer Rates and Osmosis Through the Membrane

The mass transfer rate was measured by introducing 95% ammonium sulfate solution through one channel at a fixed flow rate of 1 mL/min, while water was introduced through the other channel in the opposite direction at various flow rates ranging from 0.1 to 1 mL/min. Then the water output rate and its ammonium sulfate concentration were measured. The results, shown in Fig. 3a, indicate that the centrifugal force produces significant effects on mass transfer of ammonium sulfate, and at 2000 rpm and a 0.1 mL/min flow rate the ammonium sulfate concentration in the water output reaches near the maximum level of 95%.[5,6]

The osmosis rate through the membrane was also greatest in the column rotating at 2000 rpm at a low flow rate of 0.1 mL/min,[5,6] as shown in Fig. 3b. At a lower flow rate of 0.05 mL/min, no water was collected from the outlet of the water channel. This osmotic effect is beneficial, since it concentrates a small amount of protein in the sample solution to yield quantitative sample recovery as described later.

The results of the above studies imply that during the centrifugation the liquid rotates around each channel as shown in Fig. 3c. This circular motion not only accelerates the mass transfer rate of ammonium sulfate through the membrane, but also provides a beneficial effect by gently mixing the precipitated protein suspensions to prevent them from packing against the wall in the water channel.

The studies on mass transfer and osmosis were also performed using ethanol as a precipitant for polysaccharide fractionation.[11] The data obtained was quite similar, except that the transfer rate of ethanol through the membrane is substantially greater than that of ammonium sulfate.

Optimization of Various Parameters for Protein Separation

In order to determine the optimal experimental condition for protein separation, a series of experiments was performed using human serum albumin

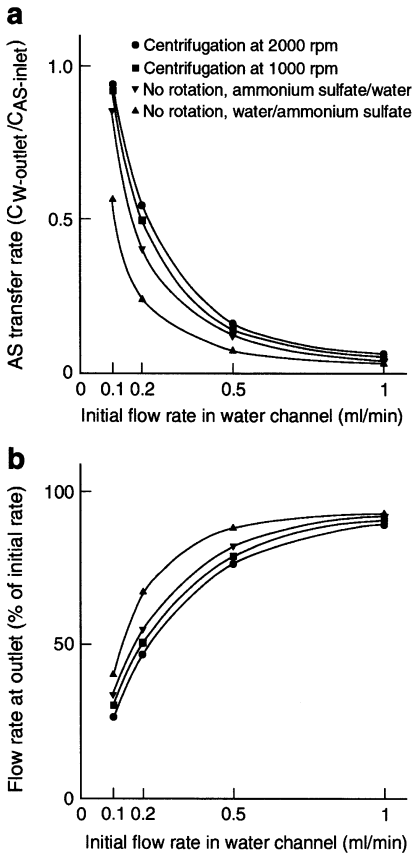

Figure 3. Ammonium sulfate transfer and osmosis rates through the dialysis membrane. (a) Ammonium sulfate transfer rates through the membrane (MWCO 12,000–14,000, 40 μm thick) measured at various water input rates. In the stationary column, unit gravity plays an important role in the transfer. This effect is further enhanced by centrifugal force. At an input flow rate of 0.1 mL/min through the water channel, the ammonium sulfate concentration at the output ($C_{\text{W-output}}$) becomes close to the input concentration of ammonium sulfate ($C_{\text{AS-input}}$). (b) Osmosis rate through the membrane measured at various water input rates. The osmosis curve shows an inverted shape of the ammonium sulfate transfer curve in (a). At an input water flow of 0.05 mL/min the water is completely absorbed into the ammonium sulfate channel, resulting in no water output from the water channel. Experimental conditions: Ammonium sulfate channel 95% saturated solution at 1 mL/min; water channel: water at various flow rate of 1–0.1 mL/min; sample: not used; revolution: 0 (stationary), 1000 and 2000 rpm. (c) Schematic diagram of the cross-sectional view of the upper and lower channels showing circular motion of liquid in both channels induced by centrifugal force.

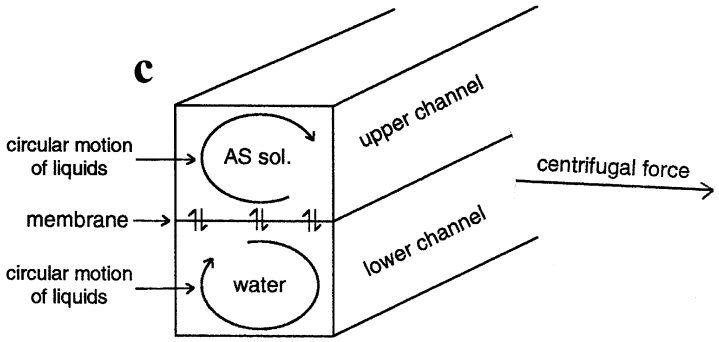

Figure 3. Continued.

and gamma-globulin as test samples to investigate the effects of various parameters such as programmed elution time, ammonium sulfate gradient inclination, revolution speed, pH, and sample size.[6] The results are illustrated in Figs. 4a–e.

The effect of elution time is shown in Fig. 4a where the peak resolution between albumin and globulin increases with the increased elution time. At 1600 min the precipitation point for each protein reaches the reference value of about 65% for albumin and 35% for globulin as obtained by a simple continuous flow method reported elsewhere.[12] This elution time may be shortened by increasing the area of the membrane relative to the capacity of the channel and/or manipulating the gradient of ammonium sulfate concentration as described below.

Figure 4b illustrates the effect of gradient inclination of ammonium sulfate on the peak resolution of proteins. The chromatograms obtained from four different gradient inclinations clearly show that a shallower gradient improves the peak resolution. This suggests that the application of a shallow gradient around the precipitation point of the target protein, if known, will produce an efficient resolution in a short elution time.

The effect of the centrifugal force applied to the column is shown in Fig. 4c. The data indicate that revolution speeds ranging from 2500 to 1500 rpm yield similar chromatograms. At 1000 rpm ($60 \times g$), however, the peaks shifted toward the left. The elevated baseline indicates that small precipitate particles were washed out from the column. Based on these data, 2000 rpm was chosen for the preliminary application.

The effect of pH is shown in Fig. 4d. As expected from the reference data,[12] the albumin peak shifts toward the right at an acid pH, and at pH 4.3 the two peaks are only partially resolved (Table 1). The sample size and recovery rate

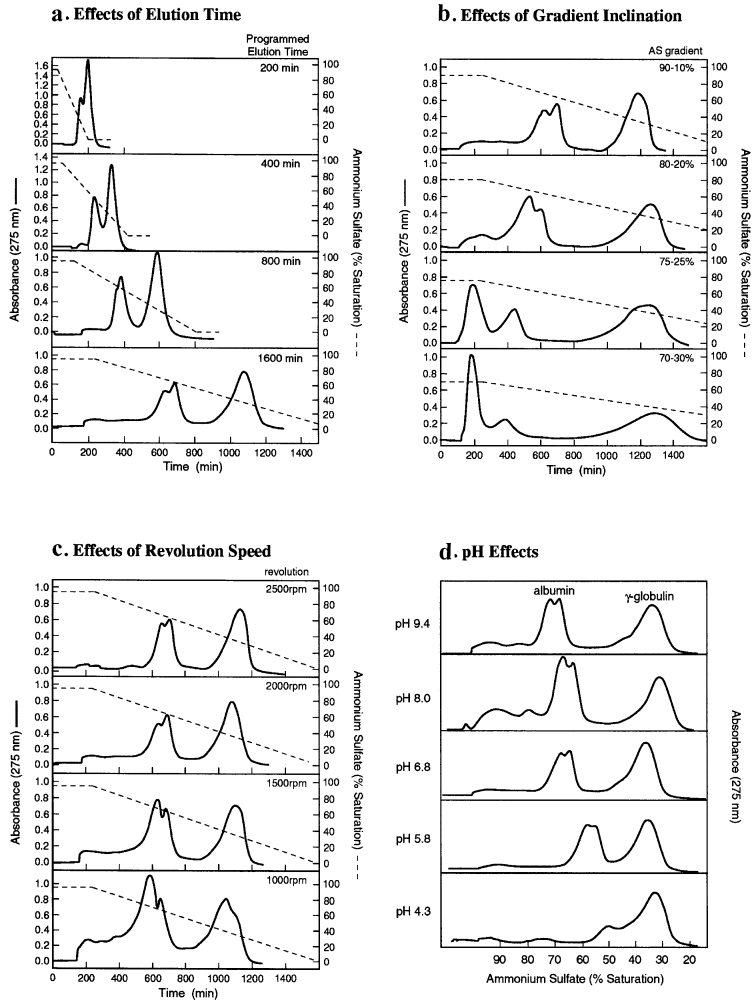

Figure 4. Optimization of key parameters of centrifugal precipitation chromatography. (a) elution time; (b) ammonium sulfate gradient inclination; (c) revolution speed; (d) pH; (e) sample size. Experimental conditions: Apparatus: seal-less continuous flow centrifuge; separation column: a pair of high-density 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 dialysis membrane (MWCO 6000–8000, 100 μm thick) each with a total capacity of 5 mL; flow rate: ammonium sulfate channel at 1 mL/min (Shimadzu SCL-10A and LC-10AD gradient pump), and water channel, 0.06 mL/min (Harvard Syringe pump, Model 980532); Revolution: 2000 rpm unless otherwise indicated in the diagram. Sample: a mixture of human serum albumin and gamma-globulin, each 4 mg (a–d) and lysozyme 0.2–10 mg (e).

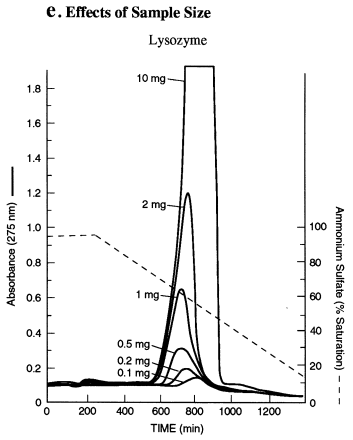

Figure 4. Continued.

were tested using lysozyme as a test sample, since it is available at high purity. Figure 4e illustrates a composite chromatogram obtained from the sample sizes ranging from 10 mg to 0.1 mg in 1 mL solution.[6] It shows little change in precipitation point with a proportional decrease in peak height, indicating quantitative sample recovery (Table 2). This result implies an important advantage of the present system, since the conventional method requires a protein concentration of at least 1 mg/mL for quantitative recovery.[13] This high sample recovery rate of the method is produced mainly by the osmotic effect described earlier.

APPLICATIONS

The present method has been applied to the separation of various protein samples[5–7] including serum proteins, monoclonal antibodies, transcription factors, recombinant enzyme, and PEG-protein conjugate, and more recently the application was extended to the fractionation of polysaccharides (dextran,[11] chondroitin sulfate, and hyaluronic acid[16]) and polycatechins.[17]

Protein Separation

Serum Proteins

The separation of 50 μL of normal human serum was performed under standard experimental conditions, i.e., a linear concentration gradient of

Table 1. Starting/Ending Points (% AS Saturation) of Protein Precipitation in Water and Potassium Phosphate Buffers (KP) at Various pH Values

	100 mM KP Buffer			
	pH 4.3	pH 6.6	pH 9.3	Water
Human albumin	40.2/50.4	56.1/70.5	57.3/73.5	55.5/66.0
Human γ-globulin	27.0/42.6	27.9/41.1	23.7/37.5	36.0/48.9

ammonium sulfate from 75% to 0% saturation applied over 1600 min at 0.06 mL/min at 2000 rpm. The albumin and globulin peaks were well resolved[6] (Fig. 5).

Monoclonal Antibodies

The capability of the method was demonstrated in the separation of a monoclonal antibody against human mast cells obtained from hybridoma culture supernatant.[6] Under the above standard experimental conditions, three peaks were produced as shown in Fig. 6a. The first peak represents albumin of calf serum added to the culture medium, the second peak contained IgM which showed an enhanced antibody activity, and the third peak contained IgG from the calf serum which showed no antibody activity. Figure 6b shows fluorescent antibody activity on the separated fractions. The activity observed in fraction 35 far exceeds that from the original culture supernatant, even after 10 times

Table 2. Median Precipitation Point and Recovery of Lysozyme in Centrifugal Precipitation Chromatography

Sample Size	Precipitation Point (Reference*)	Sample Recovery	
		Peak Height	Peak Area
2 g	58% (55%)	2.00	1.97
1 g	60% (60%)	1.00**	1.00**
0.5 g	59% (64%)	0.42	0.49
0.2 g	58% (74%)	0.19	0.20
0.1 g	56% (–)	0.10	0.10

*Value obtained from continuous flow monitoring method.[12]
**Value obtained from 1 g sample size is set at 1.00.

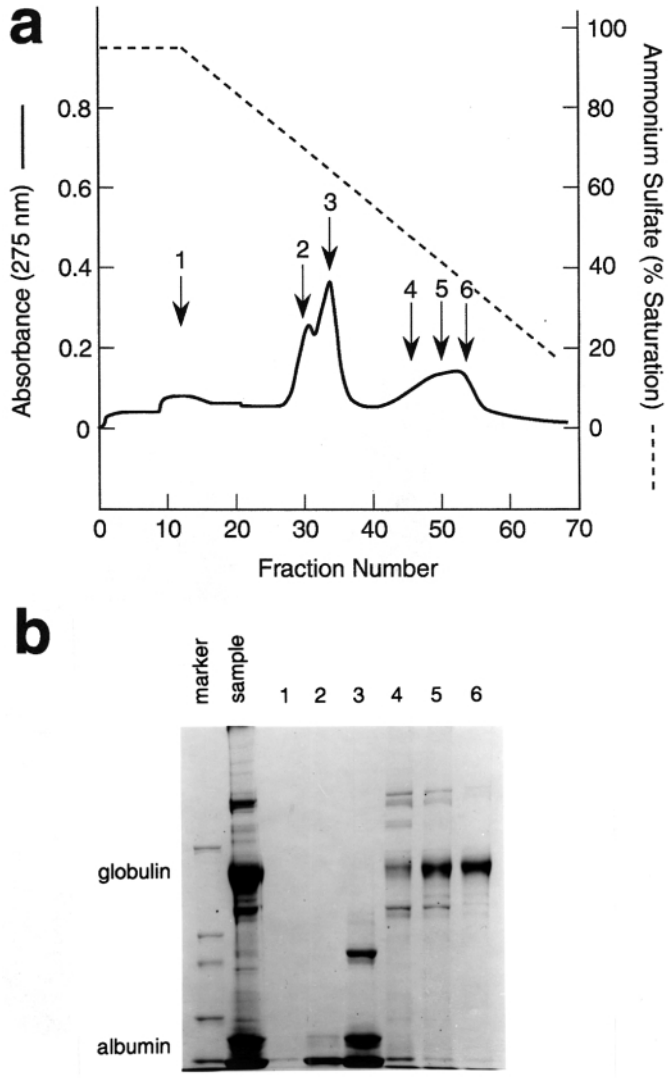

Figure 5. Separation of normal human serum. (a) precipitation chromatogram: (b) SDS-PAGE analysis. Experimental conditions. (a) sample: normal human serum pooled, 0.05 mL in 1 mL buffer solution; ammonium sulfate (AS) channel: AS gradient 95–0% for 1600 min including 15% of the priming time as indicated in the diagram; water channel: 50 mM potassium phosphate (pH 7). Other conditions are described in the Fig. 4 caption. (b) SDS-PAGE analysis was performed using precast *tris*-glycine 6% gel (Novex) at 110 V for 110 min followed by Coomassie brilliant blue staining.

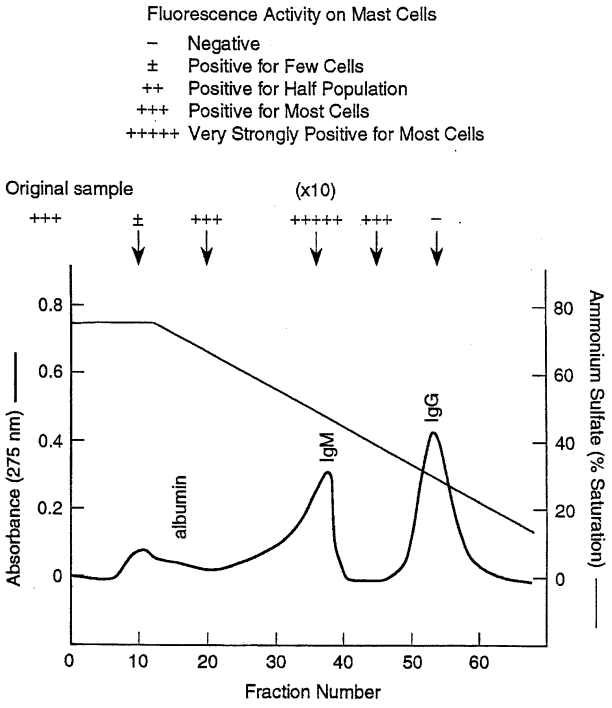

Figure 6. Purification of monoclonal antibody from hybridoma culture supernatant. (a) Chromatogram; (b) fluorescent antibody activity of purified fractions. The monoclonal antibody against human mast cells were harvested from the second peak (IgM), which showed high fluorescent activity over 10 times that of the original serum, probably due to elimination of a masking factor. The sample solution was prepared by adding ammonium sulfate 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 $1500 \times g$ 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 ammonium sulfate channel was eluted with a linear gradient 75–0% at 1 mL/min for 1600 min, including the prime time of 240 min as indicated in the chromatogram, and the sample channel with a 50 mM potassium phosphate buffer (pH 7) at 0.06 mL/min under 2000 rpm.

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dilution. This enhanced activity in the IgM fraction may be due to the removal of a masking compound from the IgM molecule by the present method. IgG monoclonal antibody in ascitic fluid was also separated in the present method[14] (Fig. 7).

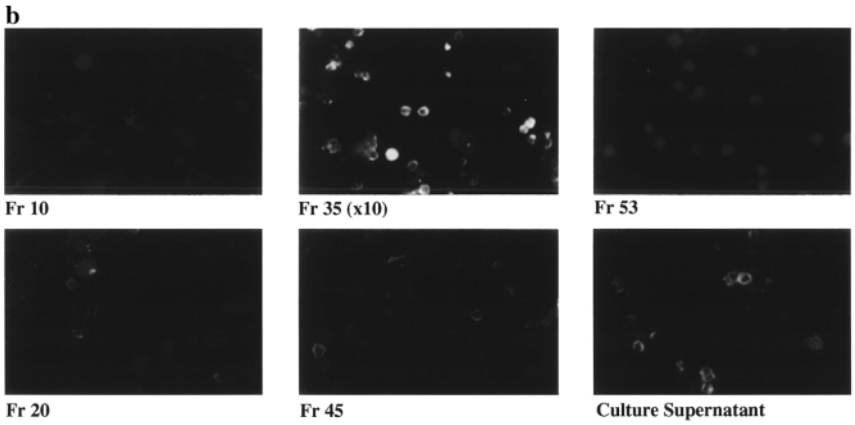

Figure 6. Continued.

Rabbit Reticulocyte Lysate

The method was applied to the separation of rabbit reticulocyte lysate, which contained a large amount of hemoglobin.[6] The run produced four peaks as shown in Fig. 8. The first large peak represents hemoglobin. The second and third peaks were only partially resolved and contained various other native proteins, while the fourth peak contained denatured proteins and RNAs from the reticula. The transcription factors, max and alpha-pal, were detected in the third peak among other proteins. These factors may be isolated by the present method using an affinity ligand, which specifically binds to these target proteins to alter their solubility in ammonium sulfate solution. This possibility is demonstrated in the following experiments.

Affinity Precipitation of Recombinant Ketosteroid Isomerase

In order to demonstrate the feasibility for affinity precipitation, recombinant ketosteroid isomerase (rKSI) was purified directly from a crude *E. coli* lysate.[5,6] In Fig. 9 the left chromatogram (a) was obtained without ligand. It shows a large impurity peak followed by the second peak, which contained the target protein mixed with other proteins (b). In order to isolate the target protein, I used an affinity ligand of estradiol-polyethylene glycol 5000 (Shearwater Polymers, Birmingham, Alabama, USA), which specifically binds to the target protein. The experiment was performed by adding 25 mg of this ligand to the sample solution under otherwise identical conditions. The results are shown in the

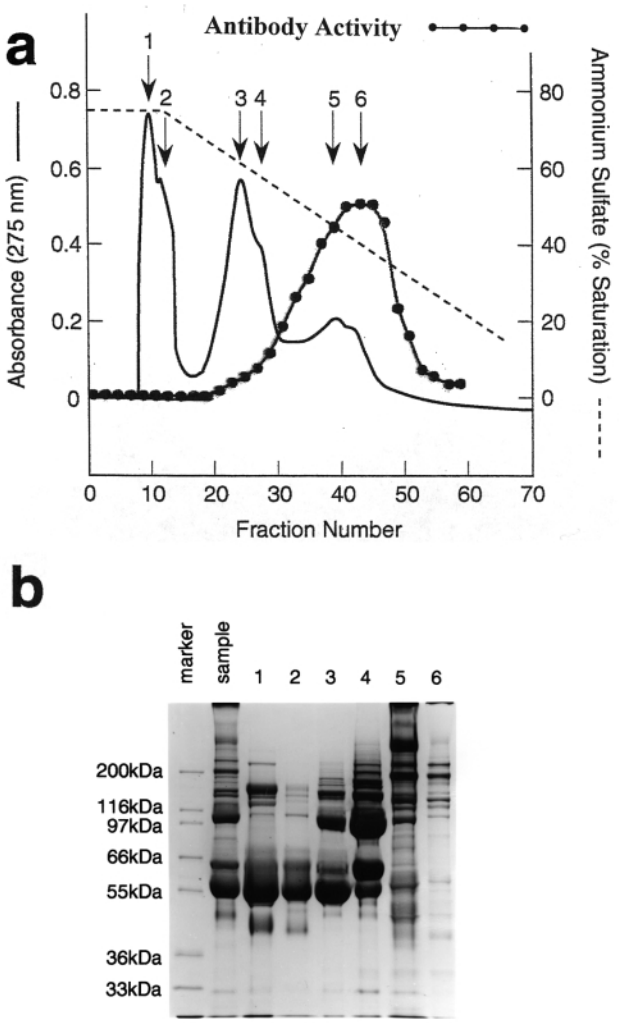

Figure 7. Purification of anti-MFIII monoclonal antibody from ascitic fluid. The separation (a) and PAGE analysis (b) was performed according to the experimental conditions described in the Fig. 5 caption.

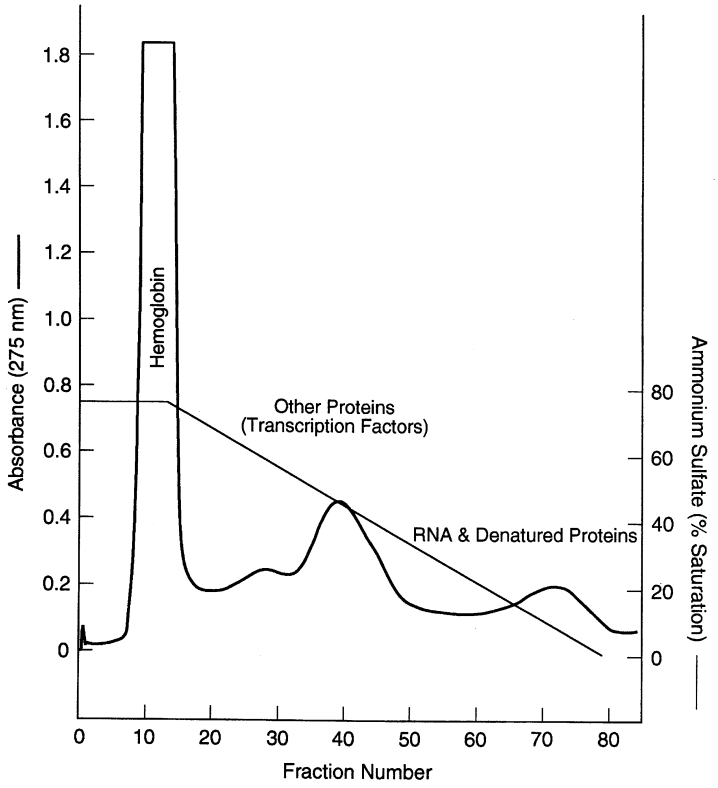

Figure 8. Separation of reticulocyte lysate containing transcription factors. The precipitation chromatogram show three major peaks: the first peak represents hemoglobin; the second peak, a mixture of proteins containing transcription factors (Max and α-Pal); and the third peak, a mixture of denatured proteins and RNA from reticulum. Experimental conditions. sample: 1 mL of rabbit reticulocyte lysate; AS channel: ammonium sulfate gradient 75–0% at the programmed elution time of 1600 min, which contains 15% of the priming time. Other conditions are described in the Fig. 5 caption.

right chromatogram (c). The protein peak became much lower and a small peak appeared followed by a large peak. The SDS PAGE analysis (d) revealed that the target protein was all eluted in the newly formed small peak, which showed monomer, dimer, and tetramer of rKSI, due to its high concentration and high purity. The large peak eluting after the small peak contained no target protein and, therefore, is considered to be an excess of free ligand.

The experiment was continued to demonstrate the ability of the present method to separate a small amount of protein.[6] A mutant *E. coli* strain cultured

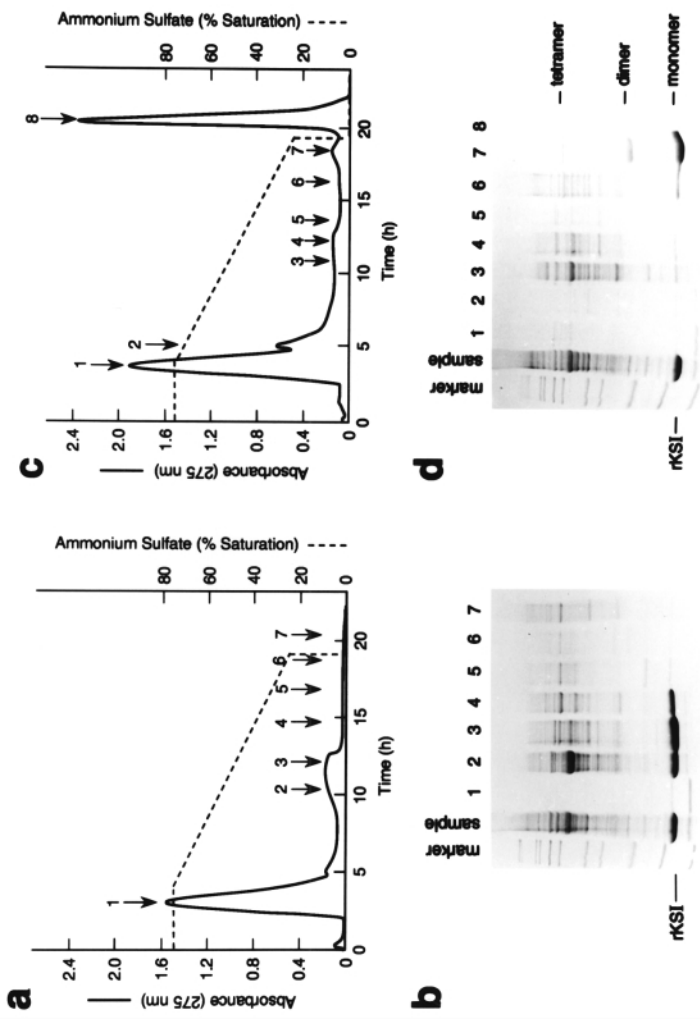

Figure 9. Purification of recombinant enzyme from a crude *E. coli* lysate using an affinity ligand. (a) and (b): Separation without ligand; (c) and (d) separation with an affinity ligand (estradiol-PEG5000) in the sample solution. Sample: 0.25 mL of *E. coli* lysate containing recombinant ketosteroid isomerase (rKSI). AS channel: AS gradient 75–25% for 900 min including 240 min of 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.

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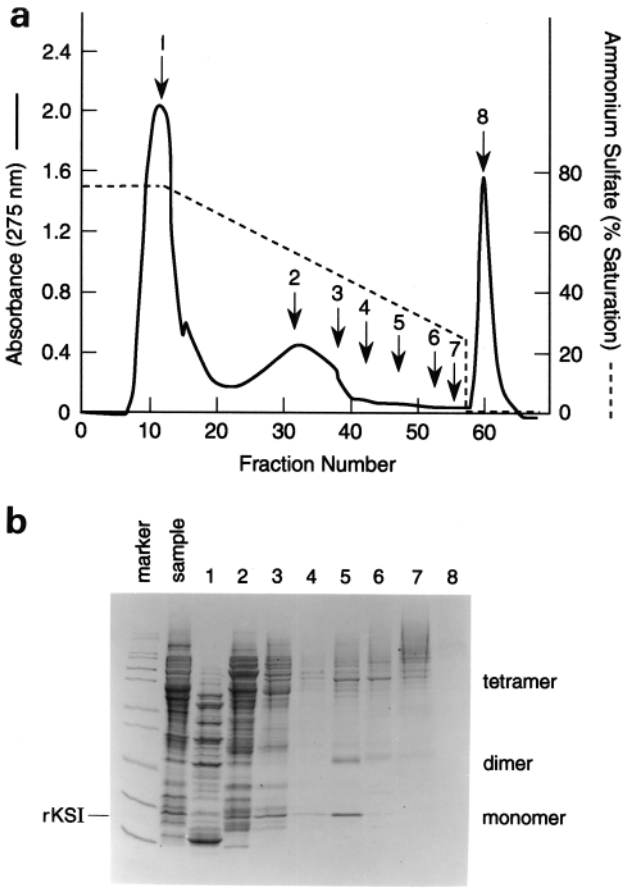

Figure 10. Purification of a small amount of enzyme from a crude *E. coli* lysate using an affinity ligand. Cell lysate of a mutant *E. coli* strain containing a small amount of KSI was subjected to centrifugal precipitation chromatography by adding 25 mg of the above ligand in the sample solution. The results show that the minute amount of proteins may be purified from cell lysate by using a suitable ligand in the sample solution.

with nitrogen-depleted media produces a very small amount of rKSI, and, therefore, the cell lysate of this *E. coli* strain was subjected to precipitation chromatography by adding the affinity ligand to the sample solution. The result is shown in Fig. 10. The chromatogram (a) similarly shows a large impurity peak followed by the main protein peak. Although, no measurable protein peak was detected near the free ligand peak, the SDS PAGE analysis (b) showed a distinct

rKSI band in fraction 5 with its dimer and tetramer at their corresponding positions. The result indicates that the present method can purify a small amount of protein from a crude *E. coli* lysate by adding a suitable ligand to the sample solution. As seen in Figs. 9d and 10b (SDS PAGE analyses), the rKSI fractions obtained by the above affinity separation are contaminated by proteins with large molecular mass. However, in practical application these impurities can be eliminated with a simple pretreatment of the crude cell lysate by ammonium sulfate precipitation according to the following protocol: A typical sample volume (50–100 mL) of crude cell lysate is first precipitated by adding 30% ammonium sulfate followed by centrifugation at $1500 \times g$ for 30 min, and the sediment discarded. The target protein present in the supernatant is then precipitated by adding ammonium sulfate to 60% saturation, followed by centrifugation. The precipitates are dissolved in a suitable volume of buffer solution and loaded into the separation column.

PEG-Protein Conjugate[15]

It is well known that a drug remains for a longer period of time in circulating blood if it is conjugated with PEG (polyethylene glycol), thus extending its time effectiveness. When a drug has multiple conjugation sites, it is desirable to determine the optimum number of PEG molecules per each drug molecule to maximize its potency while minimizing any side effect. However, the separation of each conjugate is difficult using a conventional method such as size exclusion chromatography.

Ability of the method to fractionate each species of drug conjugate is demonstrated by the separation of PEG5000-lysozyme conjugates by the present method. As shown in Fig. 11a, centrifugal precipitation chromatography produced multiple peaks, and PAGE analysis of the fractions (Fig. 11b) revealed a good resolution between PEG-free lysozyme (#10), one (#25), two (#35), and higher PEG conjugates (#41–51).

Polysaccharides

Dextran[11]

Crude dextran can be fractionated by centrifugal precipitation chromatography using ethanol as a precipitant. Ethanol is compatible with the regenerated cellulose membrane, and also facilitates sedimentation of the precipitates by its low density (0.79 g/mL). Accurate calibration of the ethanol concentration in the gradient required construction of a new device, which we call "on-line flow

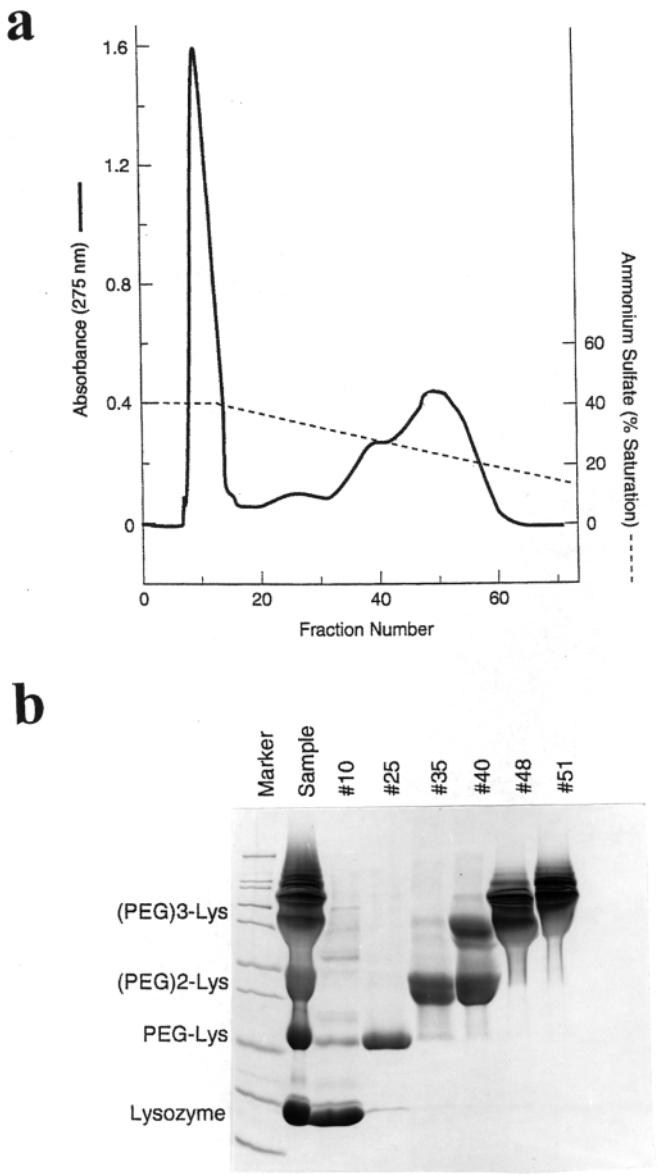

Figure 11. Separation of PEG-lysozyme conjugates by centrifugal precipitation chromatography. (a) Chromatogram obtained by a linear gradient of ammonium sulfate from 40–0%; (b) SDS-PAGE analysis of fractions. Other experimental conditions are described in the Fig. 9 caption.

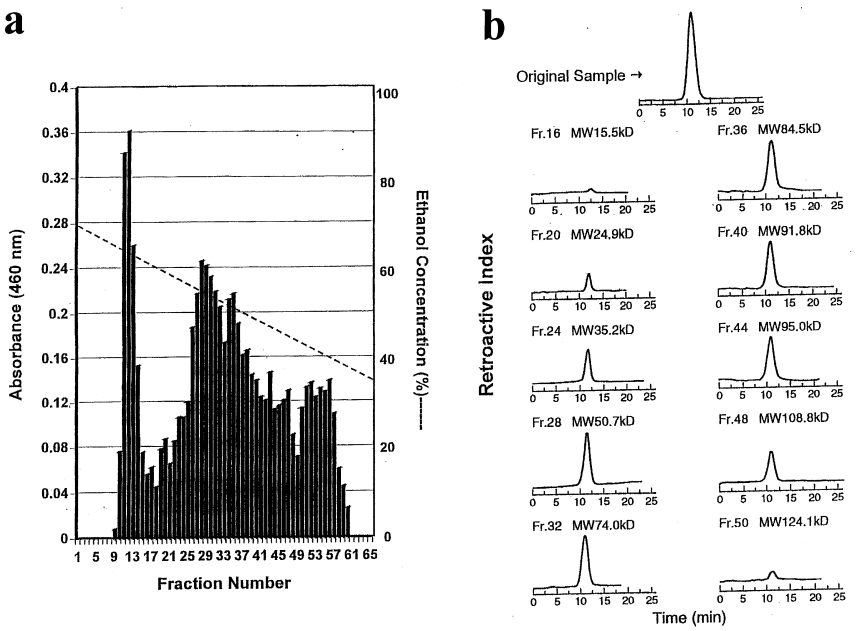

Figure 12. Dextran fractionation by centrifugal precipitation chromatography. Experimental conditions are revolution speed: 2000 rpm; flow rates: 1.0 mL/min (ethanol channel) and 0.12 mL/min (sample channel); sample size: 10 mg dextran crude sample dissolved in 1 mL water; ethanol gradient in ethanol channel: ethanol concentration 70–0% in 400 min; detection: (a) 460 nm by phenol-sulfuric assay, (b) 275 nm by size exclusion chromatography (SEC); SEC analysis (TSK-GEL G5000PWXL column, 30 cm long, 7.8 mm ID, TosoHaas), mobile phase, 50 mM potassium phosphate (pH 6.9) at a flow rate of 0.7 mL/min.

injection method"[16] where the effluent from the outlet of the column is directly introduced into the sample loop of the conventional HPLC elution system. Thus, the effluent can be subjected to the RI detection at desired regular intervals without the risk of loss of ethanol by evaporation.

In Fig. 12a the crude dextran was fractionated at different gradient elution times at 400 min from 70–0% ethanol in water. The elution yielded three peaks (a). The fractions obtained were subjected to high performance size exclusion chromatography (HPSEC) analysis. The results illustrated in Fig. 12b shows that there is a high correlation between ethanol solubility and molecular mass of the dextran, indicating that the present method may be useful for providing molecular weight standards of dextran.

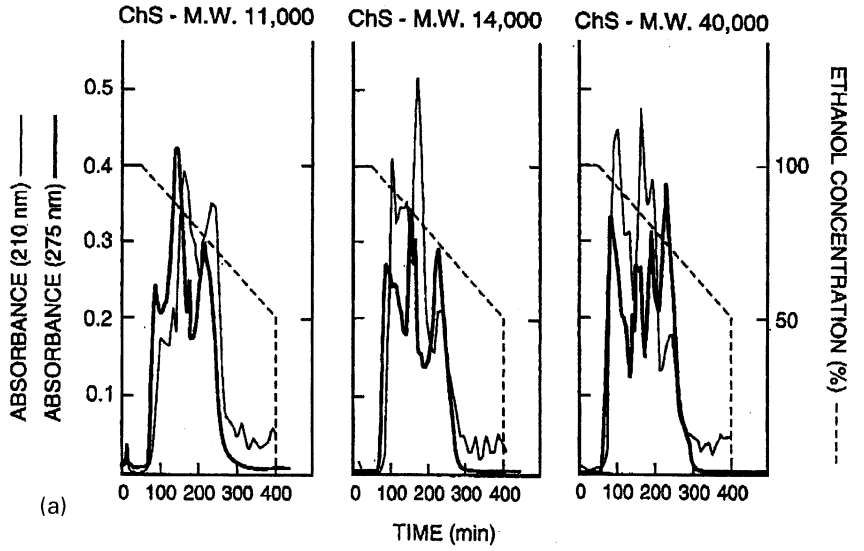

Figure 13. Precipitation chromatograms of fragments of chondroitin sulfate (a) and hyaluronic acids (b). Experimental conditions are apparatus: seal-less continuous flow centrifuge; separation column: a pair of high-density polyethylene disks (13.2 cm diameter and 1.5 cm thick) with separation channels of 1.5 mm wide and 0.5 mm deep in the upper disk and 1.5 mm wide and 2 mm deep in the lower disk partitioned with a membrane (MWCO 6000–8000, 100 μm thick); elution: upper channel with water at 0.06 mL/min and the lower channel with ethanol gradient, 100% for 30 min and linear gradient 100–50 for 6 h at a flow rate of 1.0 mL/min; sample: (a) chondroitin sulfate and (b) hyaluronic acid, each 5 mg dissolved in 1 mL of distilled water; revolution: 2000 rpm; detection: 275 nm.

(continued)

Chondroitin Sulfate and Hyaluronic Acid

Fragments of chondroitin sulfate and hyaluronic acid were each subjected to centrifugal precipitation chromatography using aqueous ethanol as precipitant. Both samples produced several peaks as indicated in Fig. 13. NMR analysis of the first and second peaks (Fig. 13b left) of hyaluronidase fragments with about 20,000 Da showed some evidence of separation, most likely according to the surface hydrophobicity of the molecules.

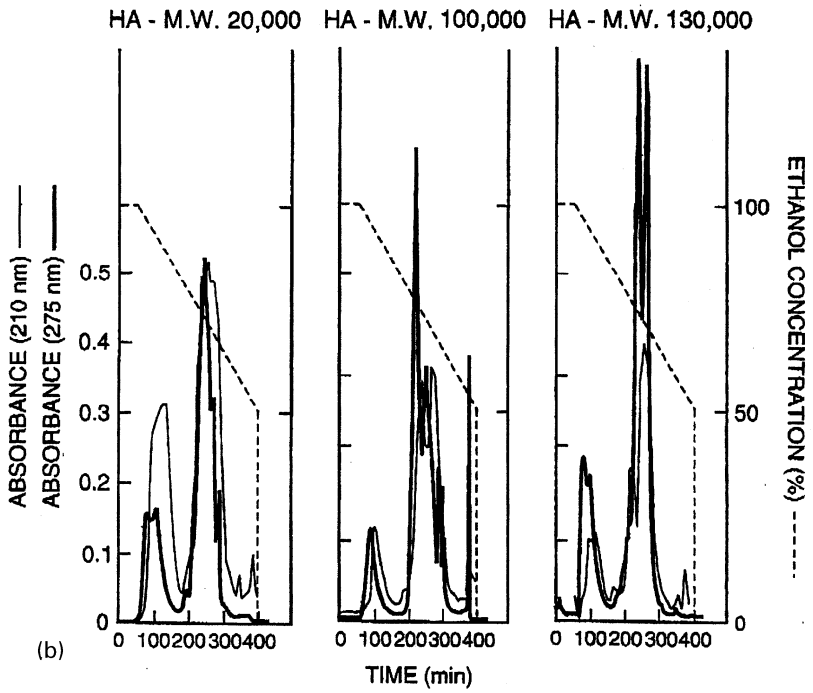

Figure 13. Continued.

Other Biopolymers

In addition to proteins and polysaccharides, the present method was applied to the separation of polymeric pigments extracted from black tea and red wine.[17] Fractionation was performed using a precipitant gradient of hexane or methyl *tert*-butyl ether in ethanol. High performance liquid chromatography analysis of fractions revealed that monomers eluted first, whereas fractionated polymers can be found at the end of the chromatographic run.

Fractionation of nucleic acids using an ethanol gradient is currently underway in our laboratory.

CONCLUSIONS

1. The method uses ammonium sulfate in an open column to give a high recovery of proteins while preserving their biological activities.[18]

2. Due to the osmotic effect, a minute amount of proteins can be quantitatively recovered in a concentrated state.
3. Low molecular weight impurities, as well as non-ionizable macromolecules, are simultaneously eliminated either by dialysis through the membrane or by their rapid elution from the column.
4. The method can separate monoclonal antibodies from a culture medium or ascitic fluid.
5. The method can also be used for affinity separation by adding a suitable ligand to the sample solution.
6. The method can be applied to other polymers including polysaccharides, nucleic acids, polycatechin, etc.

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