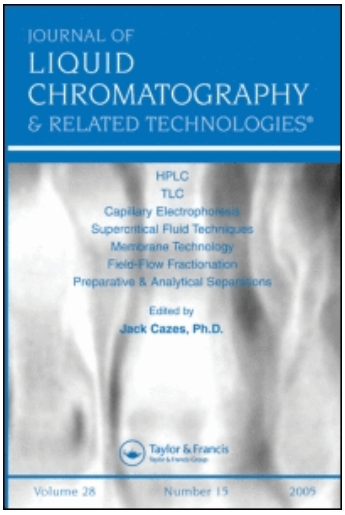

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CONTINUOUS FLOW METHOD FOR MONITORING PROTEIN PRECIPITATION BY AMMONIUM SULFATE

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CONTINUOUS FLOW METHOD FOR MONITORING PROTEIN PRECIPITATION BY AMMONIUM SULFATE

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ABSTRACT

I have proposed "Centrifugal Precipitation Chromatography"[1] as a new method for the precipitation of proteins using a linear gradient of ammonium sulfate (AS). However data on the solubility of proteins under these specific conditions are not available. Therefore, the following method is proposed to obtain these data.

It uses an HPLC pump to form a linear concentration gradient between a protein solution and a concentrated ammonium sulfate (AS) solution (90% saturation) which is continuously monitored with a uv detector at 280 nm. At a low flow rate of 0.1 mL/min the protein is exposed to a gradually increasing AS concentration until it reaches the critical point where precipitation takes place. The resulting light scattering increases the absorbance reading to form a distinct peak on the uv gradient curve where the valley and summit of the peak correspond to the starting and ending points of the protein precipitation, respectively. The method has been tested with a set of stable proteins at various pHs and ionic strengths of potassium phosphate buffer.

Ten protein samples tested may be divided into two groups: The first group represented by albumin precipitates at 60-70% AS saturation and decreases in solubility at an acidic pH. The second group includes globulins which precipitate at 35-40% AS saturation and tend to increase their solubility at an acidic pH.

The present method may be improved by the use of a nephelometric detection system with a small capacity flow cell.

INTRODUCTION

For many years ammonium sulfate (AS) precipitation has been commonly used for fractionation and concentration of proteins mainly due to its preservative effects.[2] The conventional method uses stepwise precipitation or extraction by changing the AS concentration in the centrifuge tubes. However, this manual operation is tedious and inefficient.

Recently we developed a chromatographic fractionation method of proteins using an AS concentration gradient formed through a long separation channel under a centrifugal force field.[1] This method named "centrifugal precipitation chromatography" produces more efficient fractionation of proteins simply by programming the gradient pump to provide a desired AS concentration gradient. In order to facilitate the use of this chromatographic method, it is desirable to develop a method to predict the retention time of proteins which is comparable to those in liquid chromatography.

This paper describes a simple method for predicting the retention time and peak width of protein samples in centrifugal precipitation chromatography.

PRINCIPLE

The present method uses an HPLC gradient pump equipped with two reservoirs containing solvents A and B. A desirable gradient of AS concentration is produced by mixing these solvents by programming the pump. Figure 1 (top) schematically illustrates the program for a linear gradient between solvents A and B. If solvent A is a protein solution and solvent B is water, a decreasing linear gradient of the protein concentration is obtained within a programmed time. The experiment is performed using a concentrated AS solution (90% saturation) as solvent B while monitoring the effluent with a uv monitor at 280 nm. As shown in Figure 1 (bottom), the elution curve starts to decline linearly along the uv absorbance curve until the AS concentration in the gradient reaches the critical level where protein precipitation begins. Protein precipitation in the mixture causes light scattering which increases the absorbance reading. Consequently, at this critical point the uv tracing starts to

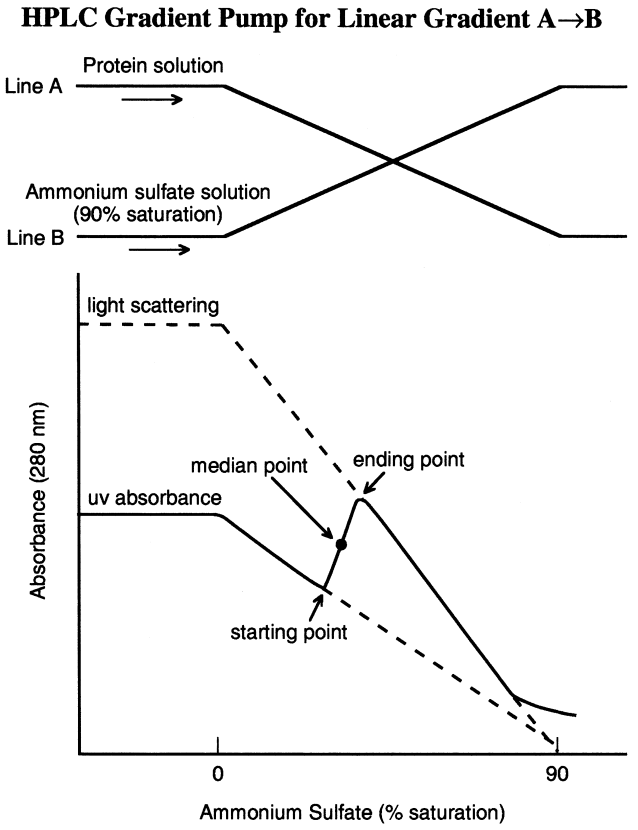

Figure 1. Principle of the present method.

move upward until it reaches the maximum value on the light scattering curve before declining as shown in Figure 1C. In this tracing the valley in the curve corresponds to the starting point and the summit corresponds to the ending point for protein precipitation.

EXPERIMENTAL

Apparatus

A gradient pump (Series 200, Perkin Elmer, Norwalk, CT, U.S.A.) was used to provide a linear gradient between AS and protein solutions. A UV

monitor (Uvicord S, LKB Instruments, Stockholm, Sweden) and a strip-chart recorder (REC 102, Pharmacia, Stockholm, Sweden) were used for recording the elution curve.

Reagents

Ammonium sulfate (AS), monobasic and dibasic potassium phosphates were all of reagent grade (Mallinckrodt Baker, Paris, KY, USA). Water of a chromatographic grade (Fisher Scientific, Fair Lawn, NJ, USA) was used for preparing the protein and AS solutions. Protein samples including human albumin, human alpha-, beta- and gamma-globulins, human fibrinogen, bovine gamma-globulin, bovine hemoglobin, lysozyme (chicken egg white), ovalbumin (chicken egg white) and cytochrome c (horse heart) were obtained from Sigma, St. Louis, MO, USA. Alpha-chymotrypsin (bovine pancreas) was obtained from Calbiochem-Novabiochem, La Jolla, CA, USA.

Procedure

In each experiment, the protein solution (typically 5 mg/mL) was fed through the channel A and 90% saturated AS solution through the channel B to form a linear gradient at a combined flow rate of 0.1 mL/min. The effluent was continuously monitored with a uv monitor (Uvicord S) at 280nm to record the elution curve (chart speed: 1cm/20min). For all studies the concentration gradient was programmed as follows:

Time (min)	Flow Rate (mL/min)	A (Protein) (%)	B (AS) (%)	Curve
3	0.1	100	0	
60	0.1	0	100	1 (linear)
5	0.1	100	0	

After the analysis is completed, the system is ready for the next run. The experiment may be terminated earlier if the valley and summit of the tracing are completed.

The effect of protein concentration on the precipitation curve was investigated using lysozyme dissolved in a wide range of concentrations from 0.1 mg/mL to 10 mg/mL.

RESULTS AND DISCUSSION

Figure 2 shows a typical precipitation tracing for human albumin in plain water obtained by the present method. The uv tracing follows a linear gradient

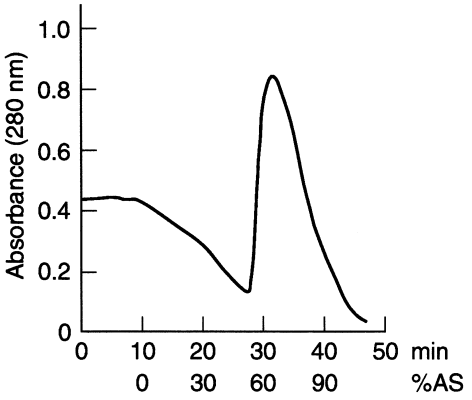

Figure 2. Elution curve of human albumin obtained by the present method. Experimental conditions: Line A: human albumin 5 mg/1mL in water ; Line B: AS 90% saturation; gradient: linear 100 - 0 %; flow rate: 0.1 mL/min; detection: 280 nm.

until it reaches 27 min (AS 56%) where the curve begins to rise indicating the starting point of precipitation. The elution curve then reaches the summit in 32 min (AS 66%) to indicate the ending point of precipitation. A series of experiments was performed to measure a set of stable proteins by varying the pH and ionic concentration of potassium phosphate, the results of which are summarized in Table 1.

Among various proteins tested, some produced problems in measurement: Myoglobin from horse skeletal muscle showed no distinct initiation point nor peak probably due to the heterogeneity of the sample which consisted of monomers, dimers, trimers, and tetramers as observed by PAGE analysis. Cytochrome c from horse heart showed no evidence of precipitation even at 90% AS saturation while trypsinogen from bovine pancreas precipitated at a very low AS concentration below 5%. Insulin was not dissolved well in either water or potassium phosphate buffer solution at suitable concentrations.

As observed from the table, most proteins precipitate in a narrow range within 10% to 12% (ending point - starting point) of AS concentration while some serum proteins such as alpha-globulin give much wider values of around 20% suggesting the heterogeneity of the sample.

Fig. 3 shows a set of AS precipitation curves for various proteins each at pH values of 4.5, 6.7 and 9.0 in 10 mM potassium phosphate buffer solution. Except for cytochrome c, these proteins may be divided into two major groups: the albumin and hemoglobin which have high % AS precipitation of 60-65%; and globulins and fibrinogen which have low precipitation values of 35-45%.

Table 1

Starting/Ending Points* of Protein Precipitation in Water and Potassium Phosphate Buffer (KP) at Various pH Values

	Acidic		Neutral		Basic		Water
KP (mM):	**10**	**100**	**10**	**100**	**10**	**100**	
pH:	**4.1**	**4.3**	**6.7**	**6.6**	**9.0**	**9.3**	
Protein							
Hemoglobin (bovine)	43.5/62.1	6.9/25.2	52.5/63.0	54.6/65.7	43.5/67.8	51.3/57.3	41.7/62.7
Ovalbumin (chicken egg)	50.4/60.6	40.5/48.6	56.4/67.5	55.5/69.6	60.0/73.2	57.6/73.8	54.8/60.0
Albumin (human)	47.7/59.7	40.0/50.4	63.0/75.6	56.1/70.5	63.6/76.5	57.3/73.5	55.5/66.0
α-Chymotrypsin (bovine pancreas)	59.1/71.4	55.1/65.8	56.4/67.5	53.4/63.6	45.0/63.0	45.0/56.4	72.6/56.4
Lysozyme (chicken egg)	51.0/60.0	44.7/52.5	49.8/58.2	47.7/53.7	49.5/58.8	48.6/56.1	52.5/66.0
α-Globulin (human)	36.0/57.3	25.5/49.5	27.6/58.5	31.5/50.7	33.0/57.0	27.6/53.4	33.3/48.0
β-Globulin (human)	34.5/51.6	16.5/45.6	31.8/48.0	30.0/49.5	29.1/53.7	26.4/42.9	36.3/47.7
γ-Globulin (human)	30.0/43.8	27.0/42.6	30.0/48.8	27.0/41.1	33.0/45.9	23.7/37.5	36.0/48.9
γ-Globulin (bovine)	30.9/46.5	21.3/27.6	25.2/42.0	25.5/40.5	24.9/42.6	24.0/41.1	30.0/45.0
Fibrinogen (human)	28.5/35.4	20.7/31.5	29.1/37.5	22.8/34.2	26.7/34.8	17.4/29.1	29.7/37.2

* % AS saturation.

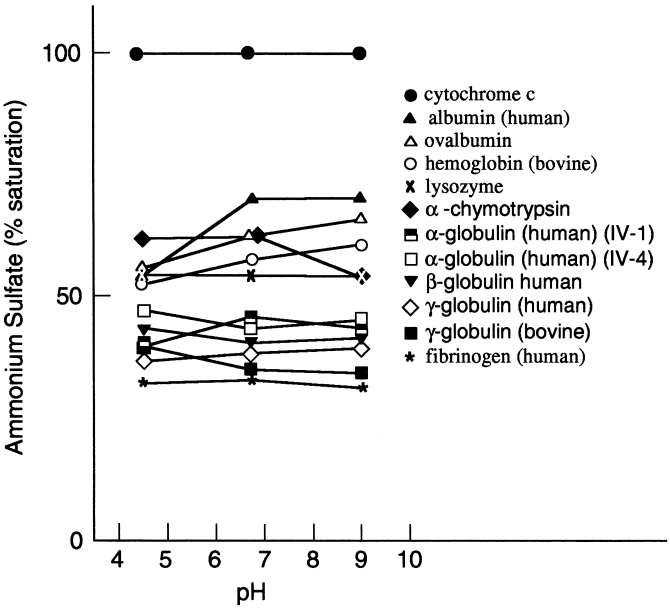

Figure 3. Median precipitation points of stable proteins at various pH in AS solution. Experimental conditions: Line A: the respective protein sample at 5 mg/mL in 10 mM phosphate buffer solution at the indicated pH; Line B: a mixture of saturated AS solution and 100 mM potassium phosphate at the indicated pH at a volume ratio of 9:1.

The first group (albumin and hemoglobin) is pH sensitive and increases solubility at high pH. The second group (globulins) shows an opposite trend that the solubility decreases at high pH. These results indicate that separation of these two groups of proteins which often coexist in the sample solution may be improved by precipitating at a high pH.

The % AS precipitation values of these protein groups bear an important implication for the practical application by centrifugal precipitation chromatography. For example, if the target protein is a monoclonal antibody present in the supernatant of a cell culture medium, the method would effectively separate IgM or IgG from albumin and low molecular weight compounds which had been added to the medium. On the other hand, when the sample is a cell lysate which contains a number of proteins with intermediate precipitation values around 40 - 55%, a meaningful fractionation of the target protein is usually difficult by one step operation. In this case, the result may be improved using an affinity ligand which can specifically bind to the target protein to alter its solubility in AS solution.[1]

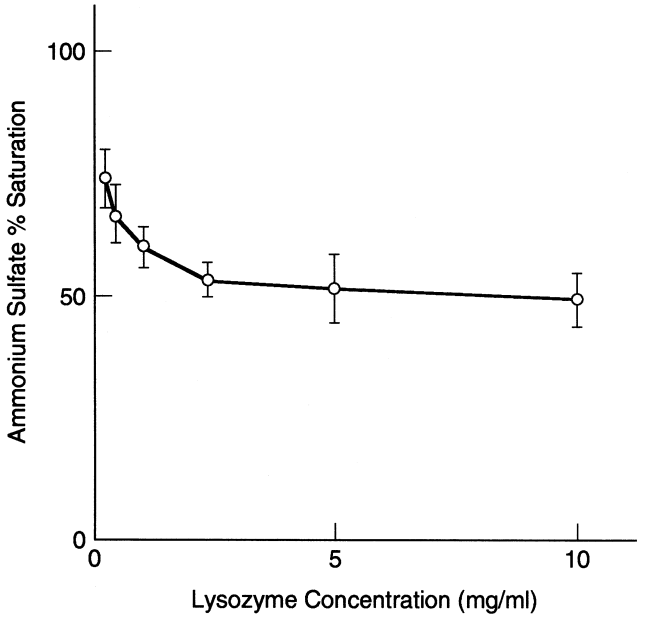

Figure 4. Effects of protein concentration on the precipitation of lysozyme in AS solution. Experimental conditions: Line A: lysozyme 5 mg/mL in water; Line B: 90% saturated AS solution; gradient: linear 100 - 0 %; flow rate: 0.1 mL/min; detection: 280 nm.

The effects of protein concentration on the AS precipitation is illustrated in Figure 4 where the median precipitation value of lysozyme is plotted against its concentration ranging from 0.1 to 10 mg/mL. It indicates that the precipitation value is not significantly affected at the concentration from 2.5 to 10 mg/mL measuring around 53-50% AS saturation. As the protein concentration decreases below the above stable range, however, the precipitation value sharply increases reaching 75% at 0.1 mg/mL.

CONCLUSIONS

The present method provides AS precipitation values of various proteins which are useful for fractionation of target proteins by both the conventional manual method and recently developed centrifugal precipitation chromatography. The amount of the protein sample required for each determination may be greatly reduced by miniaturizing the gradient pump and using a nephelometric detector with a small-capacity flow cell.

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