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Note

Use of high-performance hydrophobic interaction chromatography for the determination of salting-out conditions of proteins

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The selective precipitation or salting-out of a desired protein by the addition of high concentrations of salt (usually ammonium sulfate) is a classical fractionation technique used in the early stages of many protein purification schemes[1]. Although generally a low resolving technique it still serves the purpose of rapidly concentrating, clarifying and purifying crude protein solutions for subsequent purification steps. A major prerequisite for the use of this technique is knowing the concentration of salt at which the protein of interest starts to precipitate and the concentration of salt at which this precipitation is effectively complete. Hence, some type of physical, chemical or biological assay is required to monitor the protein of interest. Unfortunately, in some cases the presence of high salt concentrations can interfere with these assays[2]. Hence a desalting procedure must be introduced in order to proceed with the assay. Furthermore, these assays may also be complex and time-consuming and may not be sufficiently specific in crude preparations to give quantitative or accurate data. In this paper we report that high-performance hydrophobic interaction chromatography (HPHIC) can serve as a simple yet powerful tool for rapidly determining the optimum salting-out conditions for a given protein. HPHIC is uniquely useful because of its high resolving ability and its insensitivity to the presence of high concentration of salt in the sample. Hence, no sample preparation is necessary other than to simply increase the salt concentration in some samples to promote protein binding to the hydrophobic matrix.

MATERIALS AND METHODS

Chemicals and reagents

Horse cytochrome *c* (Type VI), whale myoglobin, ribonuclease A (Type III-A), egg white lysozyme (grade I), bovine carbonic anhydrase, ovalbumin (grade III) and bovine α-chymotrypsinogen A were obtained from Sigma (St. Louis, MO, U.S.A.). Crude ascites fluid was obtained from Charles River (Wilmington, MA, U.S.A.). Ammonium sulfate, biochemical grade, was obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.).

Chromatography

High-performance liquid chromatography was conducted on either a Varian

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Model 5000 chromatograph, equipped with a Varian CDS 401 data system, or a Beckman Model 344 system consisting of a Model 421A gradient controller, Model 110A pumps and a Model 164 UV–VIS variable-wavelength detector. Chromatography was conducted on analytical columns (250 × 4.6 mm or 50 × 4.6 mm), packed with 5-μm BakerBond wide-pore HI-propyl, obtained from J. T. Baker. All experiments were carried out at a flow-rate of 1.0 ml/min at room temperature. Column effluents were monitored at 280 nm.

Procedure for studying precipitating effects of ammonium sulfate on proteins

Experiments with protein standards involved the use of a stock solution containing all seven proteins, each at a concentration of 1.5 mg/ml, dissolved in 25 m*M* potassium phosphate, 1.0 *M* ammonium sulfate, pH 7.0. This stock solution was then divided into aliquots to which were added the appropriate amounts of solid ammonium sulfate[1], to obtain the desired salt saturation level at 0°C. Solutions were allowed to stand at 0°C for 15 min and then centrifuged in a Brinkman Model 5414 Eppendorf microfuge for 5 min at 3°C (these conditions were used for all centrifugation steps called for in this paper). Samples of the resulting supernatants were then injected directly onto the HPHIC column.

Experiments with ascites fluid used a 1.0-ml sample which was diluted ten-fold with 25 m*M* potassium phosphate, pH 7.0 and clarified by centrifugation. A 150-μl volume of the resulting supernatant was then injected onto an HPHIC column. To the remaining supernatant the appropriate amount of solid ammonium sulfate was added to bring the saturation level of this salt, at 0°C, to 15%. After standing 15 min at 0°C the ascites solution was centrifuged and a 150-μl sample of the supernatant was injected onto the HPHIC column. To the remaining supernatant was added the appropriate amount of solid ammonium sulfate to increase the level of saturation of this salt to the next level. Again this solution was incubated, centrifuged and sampled for HPHIC as indicated above. This process was repeated until a final ammonium sulfate saturation level of 45% had been reached.

RESULTS AND DISCUSSION

Fig. 1 shows the resulting collection of chromatograms for a series of solutions containing the same mixture of commercially available proteins, but different concentrations of ammonium sulfate (expressed in terms of saturation at 0°C). The results clearly show the extent to which these proteins are progressively precipitated as the concentration of ammonium sulfate is increased. It is interesting to note that although many of these proteins precipitate in the reverse order relative to their elution from an HPHIC column (due to the similarity in the forces hypothesized to be involved in both hydrophobic interaction chromatography and the salting-out process[3,4]), this does not always hold. This is illustrated by the difference in the salting-out behavior and the HPHIC retention times of lysozyme and carbonic anhydrase.

The ability to use this technique to determine and optimize the salting-out conditions for a given protein from a complex biological mixture is demonstrated for a monoclonal antibody (MAb) (immunoglobulin G, IgG) present in ascites fluid. Data from these experiments are shown in Figs. 2 and 3. Fig. 2 is a collection of

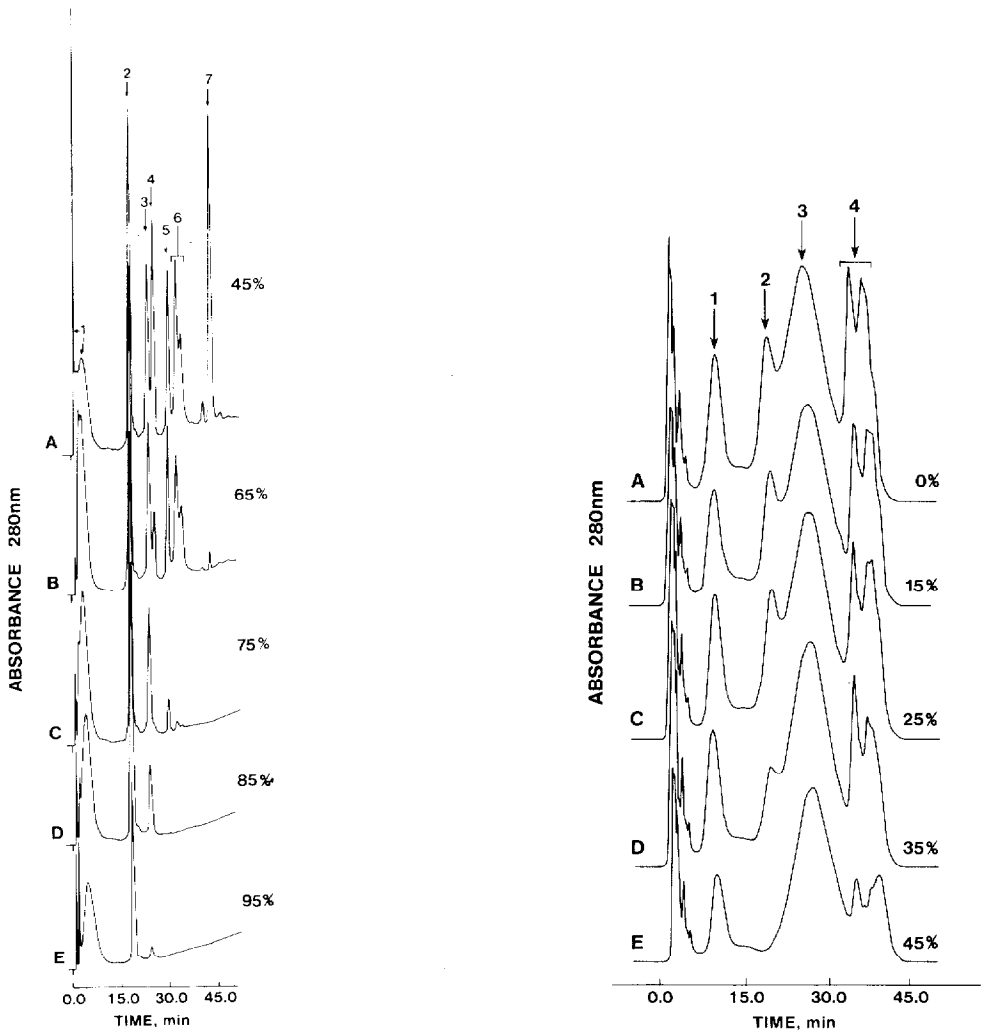

Fig. 1. Monitoring the effect of increasing concentrations of ammonium sulfate on the solubility of several protein standards using HPHIC chromatography. The concentration of ammonium sulfate (expressed in percent saturation at 0°C) in the test sample is indicated next to the resulting HPHIC chromatograms. Analytical conditions used for conducting chromatography are as follows: column: 50 × 4.6 mm, 5-μm HI-propyl; mobile phase: buffer A = 2.0 *M* ammonium sulfate and 25 m*M* potassium phosphate, pH 7.0; buffer B = 25 m*M* potassium phosphate, pH 7.0; linear gradient: 0% to 100% B in 30 min; sample volume injected: 100 μl. Peaks: 1 = cytochrome *c*; 2 = myoglobulin; 3 = ribonuclease A; 4 = lysozyme; 5 = carbonic anhydrase; 6 = ovalbumin; 7 = α-chymotrypsinogen A.

Fig. 2. Monitoring the effect of increasing concentrations of ammonium sulfate on the solubility of a monoclonal antibody (MAb) (immunoglobulin G) from mouse ascites fluid. The concentration of ammonium sulfate (expressed in percent saturation at 0°C) in the test sample is indicated next to the resulting HPHIC chromatograms. Analytical conditions used for conducting chromatography are as follows; column: 250 × 4.6 mm, 5-μm HI-propyl; mobile phase: buffer A = 2.0 *M* ammonium sulfate and 25 m*M* potassium phosphate, pH 7.0, buffer B = 25 m*M* potassium phosphate, pH 7.0; linear gradient: 50% to 70% B in 30 min then 70% to 100% B in 5 min; sample volume injected; 150 μl. Peaks 1 = transferrin; 2 = MAb; 3 = albumin; 4 = host (mouse) polyclonal immunoglobulin G.

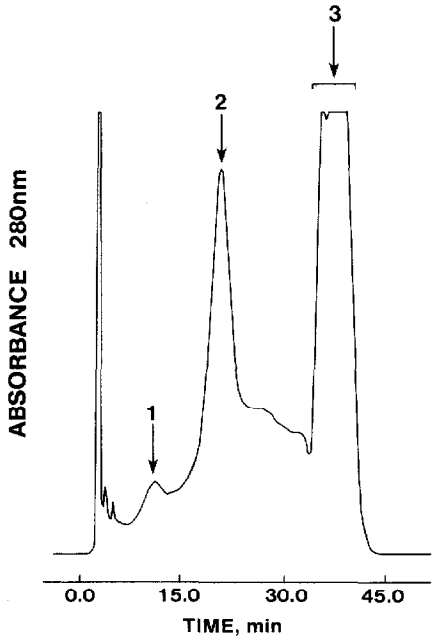

Fig. 3. HPHIC of the material which is precipitated from the mouse ascites fluid of Fig. 2 using an ammonium sulfate cut between 32 to 43% saturation. Analytical conditions used to conduct HPHIC are the same as that indicated in Fig. 2. Peaks: 1 = transferrin; 2 = MAb (IgG); 3 = host (mouse) polyclonal IgG.

HPHIC chromatograms, analogous to that shown in Fig. 1, of an ascites fluid treated with different amounts of ammonium sulfate. Data show that the MAb begins to precipitate at 35% saturation and is completely precipitated at the 45% saturation level. Based upon these experiments a fresh sample was prepared and the material that precipitated from the ascites fluid between the 32% and 43% ammonium sulfate saturation levels (referred to as a salt cut) was collected. This precipitated material was centrifuged and the pellet resuspended in 25 m*M* potassium phosphate, pH 7.0. A sample of this solution was then centrifuged and reinjected onto the HPHIC column. The resulting chromatogram, shown in Fig. 3, indicates that substantial purification of the MAb is achieved with only minor contamination from transferrin and serum albumin. However, a major contamination in this preparation is the host (mouse) polyclonal IgG. Due to the general similarity in the chemical and physical structure of this class of macromolecules it is not surprising that some of the polyclonal IgGs show similar salting-out properties to the monoclonal IgG.

The use of HPHIC has been shown to be capable of determining quickly and simply the necessary conditions for salting-out a given protein without requiring any significant sample preparation. The only condition which generally must be met before injecting the sample onto the column is that the salt concentration be high enough to permit the protein of interest to bind to the hydrophobic media. Under appropriate conditions, however, the concentration of salt in the sample can be below that required to facilitate the binding of the protein of interest to the chromatography

media (as shown in the top two chromatograms in Fig. 2) since normal mixing, which occurs during sample movement down the column, can increase the salt concentration so that eventual binding occurs. Higher salt concentrations in the injected sample than that needed to achieve protein binding and differences in pH between the injected sample and mobile phase have little or no effect on the resulting chromatography (although the pH of the mobile phase can be an important parameter in manipulating the selectivity of hydrophobic interaction chromatography[5,6]). The key requirement for using this procedure to monitor the salting-out process is that the HPHIC columns resolve or partially resolve a peak which corresponds to the protein of interest.

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