Nonaqueous Chromatography of Biopolymers by Means of Their Poly(ethylene glycol)-Mediated Solubilization in Alcohols

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SYNOPSIS

Freeze-drying of aqueous solutions of biopolymers and excess poly(ethylene glycol)s leads to formation of hydrophobic complexes. Complexation with PEG increases solubility of low molecular weight DNA, RNA, and several proteins in neat ethanol and methanol. Solubilized nucleic acids can be fractionated, and proteins separated by nonaqueous reversed-phase HPLC. © 1995 John Wiley & Sons, Inc.

INTRODUCTION

Poly(ethylene glycol) (PEG) is widely used as a salting-out agent for proteins[1] and for fractionation of nucleic acids.[2] It has been shown that the salting-out effectiveness of PEG is due to the large unfavorable energy of its interaction with macromolecules.[1] The PEG molecule in an aqueous solution tends to achieve its minimum free energy by exothermic binding water molecules to the ether groups, and to assume the most random conformation (high entropy).[3] When the PEG is above a certain molecular weight, it will tend to fold on itself, thus creating a hydration shell where several bound water molecules will be shared within the folds.[3,4] This hydration shell formation creates an excluded volume from which other molecules are repelled. On the other hand, while interaction of PEG with charged groups of biopolymer is thermodynamically unfavorable,[5] exposed relatively hydrophobic side groups (such as Phe, Trp, Ile, Leu, Val, etc., amino acid side groups of proteins and nucleic acid bases) will interact with inherently hydrophobic PEG, thus helping to break up the water structure associated with PEG. Hydrogen bond formation between electron-deficient protons of a biopolymer and the electron-donating ether groups of PEG can increase stability of the forming complex.[6] Upon drying, these complexes, which will be essentially hydrophobic in the presence of excess PEG, may be soluble in an organic solvent. This complexation would represent an unusual way to modify biopolymers[1] without having PEG covalently attached, and has been proven to increase solubility of chymotrypsin in organic solvents.[7]

In this work, we address the broader question of whether a biopolymer that is complexed to PEG is soluble in neat organic solvents. If so, this may imply a novel strategy towards nonaqueous chromatographic processes involving biopolymers. Low molecular weight alcohols, namely methanol and ethanol, were chosen as representative examples of organic solvents that are widely used in downstreaming processes and yet are good dissolving media for PEG.[8] Solubility of most nucleic acids and proteins in low molecular weight alcohols is somewhat limited.[9]

EXPERIMENTAL

Materials

Bovine pancreatic Zn^{2+}-insulin, hen egg-white lysozyme, bovine pancreatic ribonuclease A (type III-A), bovine pancreatic trypsin (essentially salt free), bovine pancreatic α-chymotrypsin (type II, essentially salt free), bacterial protease (type VIII), human ferrous hemoglobin, and transfer RNA from

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Bakers yeast (type X-SA) were obtained from Sigma. *Torula utilis* RNA and herring low molecular weight DNA were obtained from Fluka. Poly(ethylene glycol) was obtained from Serva or Aldrich (molecular weights of 40,000 or 20,000, respectively). Nucleic acid ladders and other molecular weight standards were obtained from either New England Biolabs or Life Technologies, Inc. Absolute ethanol and methanol were dried with molecular sieves (3 Å, Aldrich). All other chemicals used were obtained from commercial suppliers and were of analytical grade or purer.

Procedures

Proteins were lyophilized for 2 days prior to use from 5 mg/mL solutions or suspensions in deionized water adjusted to the pH that coincided with the pI of the protein; nucleic acids were lyophilized in the same fashion from pH 7.0. Protein concentrations were determined using the bicinchoninic acid (BCA) assay.[10] RNA and DNA were assayed by measuring the absorbance of their aqueous solutions at 260 nm. PEG–protein or PEG–nucleic acid complexes were prepared by lyophilization for 2 days of a mixture of known amounts of PEG and a biopolymer in water adjusted to the pI of protein or to pH 7.0 in case of a nucleic acid.

The solubility of biopolymers or PEG–biopolymer complexes in alcohols was measured by placing them into a screw-cup vial followed by addition of the solvent. The resultant 10 mg/mL (per weight of biopolymer) suspension was shaken at 30°C for 2 days and then centrifuged at 30,000 × *g* and 30°C for 0.5 h. Undissolved residue was removed, the supernatant was evaporated to dryness under vacuum, the resultant solid was redissolved in phosphate buffer, the sample was briefly sonicated, and the biopolymer concentration determined as described above.

Lysozyme activity in 66 m*M* phosphate buffer (pH 6.2) was measured spectrophotometrically on the basis of the lysis of the dried cells of *Micrococcus lysodeikticus*, as described by Shugar.[11]

Electrophoresis of nucleic acids was performed by applying various ladders for sizing on 1–5 w/v% SeaKem™ GTG agarose gels in 10 mm Tris-HCl or Tris-borate and 1 m*M* EDTA buffers (pH 7.5–8.2) at 100 V on a EC 600 apparatus (E-C Apparatus Co., St. Petersburg, FL). Visualization was achieved after ethidium bromide staining.

HPLC was run on a Millipore Waters system composed of a 490E Multiwavelength Detector, A 510 HPLC Pump, a 740 Data Module, a 680 Gradient Controller, and a μBondapak™ C_{18} 3.9 × 300 mm column (particle size 10 μm, pore size 125 Å). A solution of a PEG–biopolymer complex (100 μL) in ethanol was loaded onto the column and then eluted with alcohol using a pump speed of 0.3 mL/min.

RESULTS AND DISCUSSION

The effect of the increase of the DNA solubility in neat alcohols upon complexation with PEG is visualized by absorbance spectra shown in Figure 1. It can be seen that the solubility of herring DNA complexed with PEG (molecular weight of 40,000) in ethanol is more than 20 times higher than that of the original DNA (compare absorbances at 260 nm).

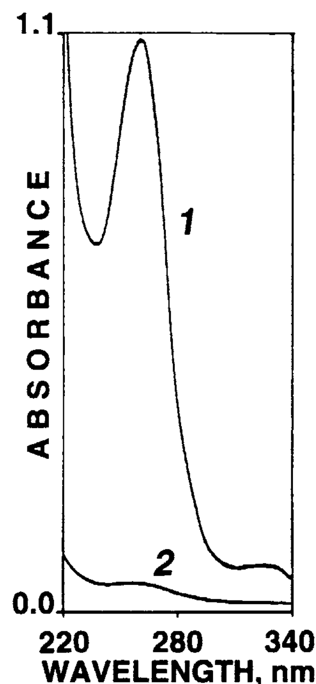

Figure 1 Absorbance spectra of ethanolic solutions of herring DNA/PEG (1 : 10 w/w) complex (1) and original DNA (2). Path length 0.2 cm, blank-anhydrous ethanol. DNA/PEG complex was prepared by lyophilization for 2 days of 5 mg/mL DNA and 50 mg/mL PEG (M_w 40,000) solution in deionized water adjusted to pH 7.0. Herring DNA was lyophilized for 2 days from its 5 mg/mL solution in deionized water (pH 7.0), prior to use. The DNA/PEG complex and DNA were then suspended in anhydrous ethanol at concentrations equal to 100 and 10 mg/mL, respectively, and shaken at 30°C for 2 days; samples were then centrifuged (30,000 × *g*, 0.5 h) to remove undissolved residue.

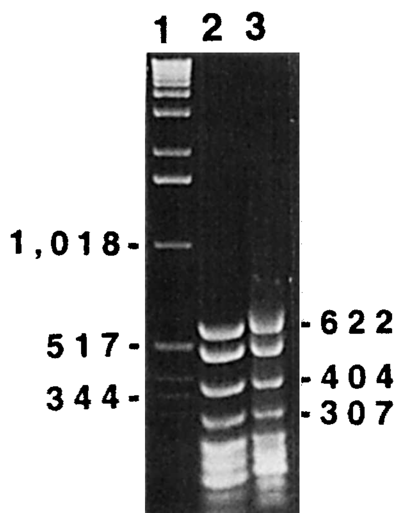

Figure 2 Gel electrophoretic pattern of 1 kb DNA ladder (1), original pBR-*Msp* I DNA digest (2), and the same DNA digest complexed (1 : 10 w/w) with PEG (M_w 40,000) dissolved in dry ethanol by shaking at 30°C for 2 days, and redissolved in Tris-borate EDTA buffer (pH 8.2) following removal of ethanol under vacuum. Staining by ethidium bromide. Numbers indicate the amount of base pairs.

The conformation of DNA in ethanol- and methanol-buffer solvent systems has been studied rather extensively, mainly by the circular dichroism method.[12] It was shown that at high alcohol concentrations DNA behaves in an anomalous fashion and tends to assume a compact tertiary structure.[12] Similar trends were observed in solutions containing sufficient concentrations of PEG.[13] Thus, it is possible that in the DNA–PEG complexes dissolved in alcohol the DNA undergoes transition to its unnatural conformation. However, from the bioseparation standpoint, it is important to recover a biopolymer from the solvent in such a way that it would be processible in aqueous solution, rather than to retain its original conformation in the organic solvent.

Significantly, removal of the alcohol from DNA–PEG solution under vacuum followed by redissolution of DNA in aqueous medium resulted in DNA that was virtually identical to the original DNA, as judged by electrophoretic data (Fig. 2). Similarly, solubilities of low molecular weight RNA from *T. utilis* and transfer RNA from Bakers yeast increased 8–22 times upon complexation with a 10-fold excess of PEG; removal of the solvent and subsequent redissolution of the PEG–RNA in 10 m*M* Tris-HCl buffer (pH 8.2) resulted in RNAs electrophoretically identical to the original ones.

The feasibility of HPLC of nucleic acids in neat alcoholic solutions is illustrated by Figures 3 and 4. A 10 : 1 (w/w) complex of PEG (M_w 40,000) and nucleic acid dissolved in dry ethanol was loaded onto a column which had been preequilibrated with ethanol followed by elution, again with dry ethanol. Separation of different fractions of herring DNA (Fig. 3) and *T. utilis* RNA (Fig. 4) was verified by gel electrophoresis. Solutions were chromatographed separately to determine the corresponding retention times. For analysis, fractions (0.5 mL) were then collected around peaks. Ethanol was evaporated and the residues were dissolved in aqueous buffers and electrophoresized as described in the Experimental section.

It can be seen that complexation with PEG is an effective method for the fractionation of nucleic acids in entirely nonaqueous conditions by reversed phase chromatography. The elution order (smaller fractions come out first) suggests that the smaller nucleic acid molecules contain fewer binding sites for PEG and are, therefore, more hydrophilic than larger fractions onto which more PEG molecules are attached. The retention times of the double-stranded DNA and the single-stranded RNA appear to be almost equal, even though there is a 10-fold difference in molecular weights (compare Figs. 3 and 4). This suggests that the presence of the excess PEG bound to biopolymer can substantially level off the indi-

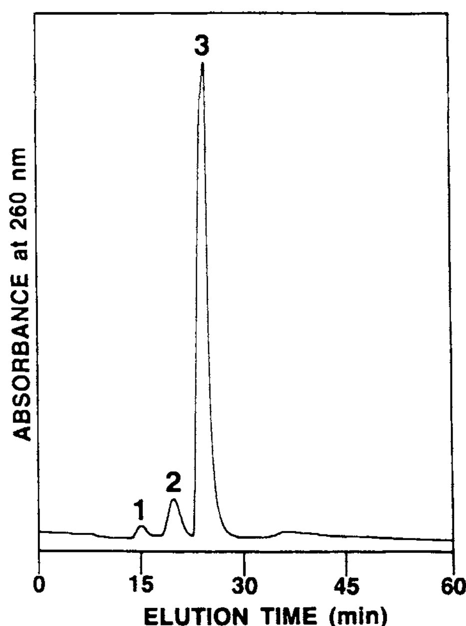

Figure 3 Chromatogram of herring DNA/PEG (1 : 10 w/w) complex dissolved in ethanol. The numbers above the peaks of the profile indicate the main fragment sizes as follows: 1, < 30 bp; 2, 30–70 bp; 3, 70–120 bp. For the details of preparation of the DNA/PEG complex solution that was loaded onto the column, see legend to Figure 1.

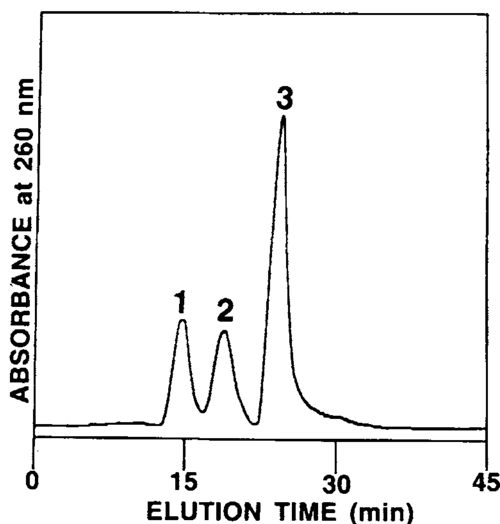

Figure 4 Chromatogram of *T. utilis* RNA/PEG (1 : 10 w/w) complex dissolved in ethanol. The numbers above the peaks of the profile indicate the main fragment sizes as follows: 1, < 10 bases; 2, < 15 bases; 3, 15–30 bases. The RNA/PEG complex was prepared analogously to the procedure described for DNA/PEG complex (see legend to Fig. 1).

viduality of it's molecules. The study of protease chemically modified by PEG demonstrated that the PEG-enzyme aggregates five orders of magnitude larger than single PEG-protease molecules were formed in organic solvents.[14] Thus, the mode of separation would depend on the extent of interactions between PEG–biopolymer complexes, resulting in the formation of aggregates of different sizes and hydrophobicity. The nature of these interactions remains yet to be studied.

Next, we examined PEG-enhanced protein solubility in neat alcohols. Complexation of insulin, lysozyme, trypsin, α-chymotrypsin, human hemoglobin, ribonuclease A, and protease with 5- or 10-fold excess of PEG (molecular weight of either 20,000 or 40,000) resulted in an increase in protein solubility in methanol or ethanol by a factor of 3 to 20, as measured by the BCA assay following evaporation of alcohol and redissolution of protein in aqueous buffer (for procedure, see the Experimental section). The absorption spectrum of a methanolic solution of human hemoglobin complexed with PEG (Fig. 5) reveals four distinctive peaks at 276, 400, 486, and 595 nm, due to absorption by aromatic amino acids and the heme, the Soret band, and the β and α bands, respectively. In this spectrum, the Soret bands, as well as β and α bands, which are due to the π-π^* electronic transitions in heme,[15] are shifted from their original positions at 410, 510, and 540 nm, respectively, compared to the spectrum of hemoglobin in aqueous buffer.[16] These shifts suggest that the electronic environment of the heme is perturbed when water is replaced by an organic solvent of lower polarity. These changes, however, appear to be reversed when methanol is removed under vacuum and the PEG–hemoglobin complex is dissolved in an aqueous solution. Namely, the positions of the bands resulting from the heme absorbance of the PEG–hemoglobin complex redissolved in 10 mM Tris buffer at pH 7.0 were identical to the ones of the original hemoglobin. This indicates that hemoglobin can be recovered unchanged once its complex with PEG is redissolved in aqueous medium.

Example of reversed-phase HPLC of a PEG–protein solution mixture is given in Figure 6. It can be seen that facile protein separation in neat ethanol

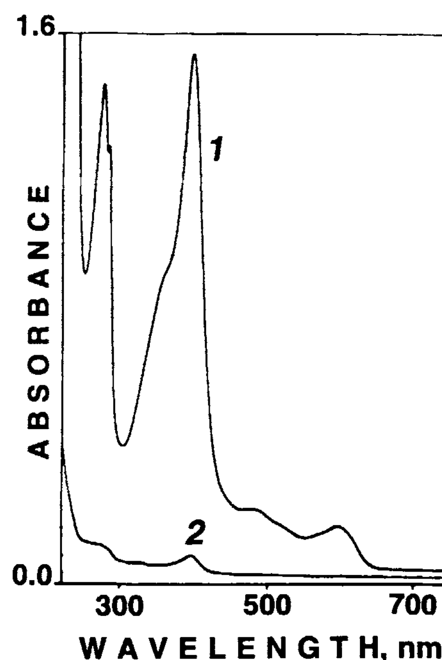

Figure 5 Absorption spectra of methanolic solutions of hemoglobin/PEG (1 : 5 w/w) complex (1) and original hemoglobin (2). Path length 0.1 cm, blank-anhydrous methanol. Hemoglobin/PEG complex was prepared by lyophilization for 2 days of 5 mg/mL human hemoglobin and 25 mg/mL PEG (M_w 20,000) solution in deionized water adjusted to pH 6.8. Hemoglobin was lyophilized for 2 days from its 5 mg/mL solution in deionized water (pH 6.8), prior to use. The hemoglobin/PEG complex and hemoglobin were then suspended in anhydrous methanol at concentrations equal to 100 and 20 mg/mL, respectively, and shaken at 30°C for 2 days; samples were then centrifuged (30,000 × g, 0.5 h) to remove undissolved residue.

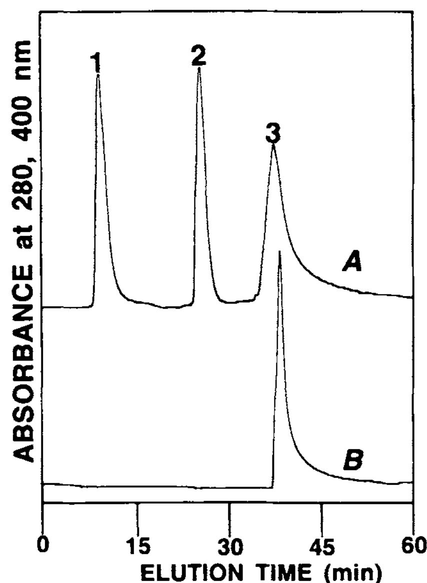

Figure 6 Chromatograms of ethanolic solution of the mixture of PEG-complexed proteins recorded at 280 nm (A) and 400 nm (B). Protein/PEG (1 : 10 w/w) complexes were prepared separately for each protein by complexation with PEG (M_w 40,000) as described in the legend to Figure 5. Prior to lyophilization, pH of protein solution was adjusted to the pI value of the protein. Numbers above the peaks of the profile (chromatogram A) indicate lysozyme (1), protease (2), and human hemoglobin (3).

is achieved. This separation is related to the size of protein molecule: the observed retention times increase in the order lysozyme (M_w 14,400) < protease (M_w 27,800) < hemoglobin (M_w 64,500), suggesting that the larger proteins in which greater number of hydrophobic amino acid residues is exposed to the protein surface complex more PEG molecules and, thus, larger PEG–protein aggregates may be more hydrophobic.

The lysozyme fraction recovered from the ethanol solution and dissolved in 66 mM phosphate buffer (pH 6.2) exhibited enzymatic activity undistinguishable from that of the original lysozyme. Hence, the conditions chosen for solubilization of the enzyme are mild enough for processing. This is a remarkable result, considering the fact that the use of organic solvents in reversed-phase chromatography can be very detrimental to the native protein structure and, notably, the interaction with the column contributes to the irreversible denaturation of the enzyme.[17] Hence, from the example given in Figure 6, it can be concluded that PEG-mediated solubilization of proteins can result in a convenient medium for HPLC. The presence of PEG may protect proteins from irreversible denaturation, so that they can be renatured by simple redissolution in aqueous buffers.

CONCLUSIONS

The phenomenon of PEG-mediated solubilization of proteins and nucleic acids in neat ethanol and methanol is described. The alcoholic solutions of biopolymers can become a convenient medium for nonaqueous HPLC. It is hypothesized that biopolymers can be recovered unchanged upon redissolution of their complexes with PEG in aqueous buffers.

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