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ION-EXCHANGE CHROMATOGRAPHY OF PROTEINS

THE EFFECT OF NEUTRAL POLYMERS IN THE MOBILE PHASE

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SUMMARY

   The addition of neutral water-soluble polymers such as polyethylene glycol to the eluent of standard ion-exchange chromatography systems affects the separations of proteins in a useful and predictable manner. The relative effects of various polymers are predictable on the basis of their hydrophobicities. Protein retention times are increased, especially for larger and/or more hydrophobic proteins, as predicted by the same order as protein precipitation by neutral polymers. Therefore, this "polymer enhanced chromatography" technique can be particularly useful for the separation of proteins or other biopolymers with the same isoelectric points but different sizes. Examples would include the separation of monomers from dimers and higher order aggregates, or of intact proteins from fragments. Examples of applications with model proteins and the separation of bovine somatotropin monomer and dimer are presented.

INTRODUCTION

   In designing a chromatographic separation system for complex molecules such as proteins, the traditional approach has been to choose a single factor on which to base the separation and then minimize the other possible effects. For a separation on the basis of size, for example, measures are taken to avoid ionic and hydrophobic interactions between the solutes and the solid phase. When one step is insufficient, a series of separations based upon size, charge or hydrophobicity is carried out. In order to have high selectivity in a single step, multiple control factors must be combined. Affinity chromatography provides this combination as a natural biochemical interaction. Such selective control of separations is also being explored in the new multimodal approaches to chromatography. This paper presents a novel approach to multimodal chromatography that allows for control of a separation on the basis of charge, size, and possibly hydrophobicity, in a single step. Standard ion-exchange

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columns and chromatography equipment are used with the addition of neutral polymers to the eluent.

Neutral water-soluble polymers such as polyethylene glycol (PEG) and dextran have been used for decades in biochemical separations. They have been used for the "salting out" or precipitation of macromolecules[1-6], crystallization of proteins from polyethylene glycol solutions[7,8], and in two-phase aqueous extraction systems[9]. Their use is not only safe for maintaining biological activity, but can actually provide added stability[3,5,7]. In "polymer enhanced precipitation," neutral polymers were used to improve the yield and selectivity of protein purification with ionic polymers[10,11]. The technique presented here is related to these in the prediction of the selectivity available and has been termed "polymer enhanced chromatography."

EXPERIMENTAL

The following items were purchased from Sigma (St. Louis, MO, U.S.A.): bovine transferrin, bovine hemoglobin, horse heart myoglobin, bovine pancreas alpha chymotrypsinogen A, ascorbic acid, citric acid, and the polyethylene glycol fractions of various molecular weights. Dextran (15 000–20 000) was purchased from Polysciences (Warrington, PA, U.S.A.), and the polyvinylpyrrolidone (PVP, 40 000) from Kodak (Rochester, NY, U.S.A.).

All separations were carried out at ambient temperature. For the work with model proteins a Spectra-Physics (Houston, TX, U.S.A.) 8100 System with pump and autosampler and integrator was used with a Spectoflow 757 absorbance detector from Kratos Analytical (Ramsey, NJ, U.S.A.) operating at 280 nm. For the study of effects of different polymers on the four model proteins, the column was a TSK-Gel DEAE-5PW column 7.5 cm × 7.5 mm I.D. purchased from P.J. Cobert Assoc. (St. Louis, MO, U.S.A.). The column used for the separation of myoglobin and hemoglobin with added PEG was a SynChropak SAX 25 cm × 4.6 mm I.D. from Synchrom (Lafayette, IN, U.S.A.). In both cases the columns were run isocratically at 1 ml/min with 50 mM sodium phosphate buffer with or without added polymer, pH 7.5. To insure that the addition of polymers to the eluents did not alter the pH, the appropriate weight-to-volume amount of polymer was dissolved in the buffer, then the pH was adjusted and the solution filtered. Protein solutions were approximately 1 mg/ml in eluent buffer (not containing polymer).

For work on the separation of bovine somatotropin monomer and dimer, a fast-protein liquid chromatography (FPLC) pump system, manual injector, and MonoQ HR 5 cm × 5 mm I.D. column, all from Pharmacia (Piscataway, NJ, U.S.A.) were used with an HM Holochrome detector from Gilson (Middleton, WI, U.S.A.) operating at 280 nm. The eluent was 20 mM Tris, pH 10 with a 30-min gradient from 0.10 to 0.25 M sodium chloride. The PEG 3350 level was held constant during the gradient. Somatotropin monomer only, dimer only, and monomer/dimer mixture were provided by S.B. Storrs of the Monsanto Agricultural Company, Animal Sciences Division.

RESULTS

The molecular weights and isoelectric points of the model proteins are given in

TABLE I

MOLECULAR WEIGHTS AND ISOELECTRIC POINTS OF THE FOUR MODEL PROTEINS

Protein	Molecular weight	pI
Myoglobin	16 900	6.8
Chymotrypsinogen A	25 000	9.0
Hemoglobin	64 500	7.0
Transferrin	76 000–80 000	5.2

Table I. These four model proteins and five types of water-soluble polymers were tested. The results are presented as the effect of the polymer type on a given protein in Figs. 1–4. The graphs represent retention time in minutes *versus* the percentage (w/v) of polymer in the eluent. These results are presented in Table II as capacity factors for each model protein under all the conditions tested. Myoglobin, the smallest of the proteins tested, is relatively unaffected by the polymers when shown on the same scale as the other proteins (see Fig. 1). However data in Table II does show that as the concentration of polymer increased, the capacity factor also increased. For transferrin, the largest of the proteins tested, some data points are not included because no peak was detected by 30 min at the higher polymer concentrations. For each polymer, the increases in retention time and capacity factor were greater for the more hydrophobic polymers (as discussed below) and increased as the concentration of the polymer increased.

To illustrate how a polymer affected each protein, Figs. 5 and 6 show the data for PEG 1450 and PEG 3350. Again the graphs represent retention time in minutes *versus* the percentage (w/v) of polymer in the eluent.
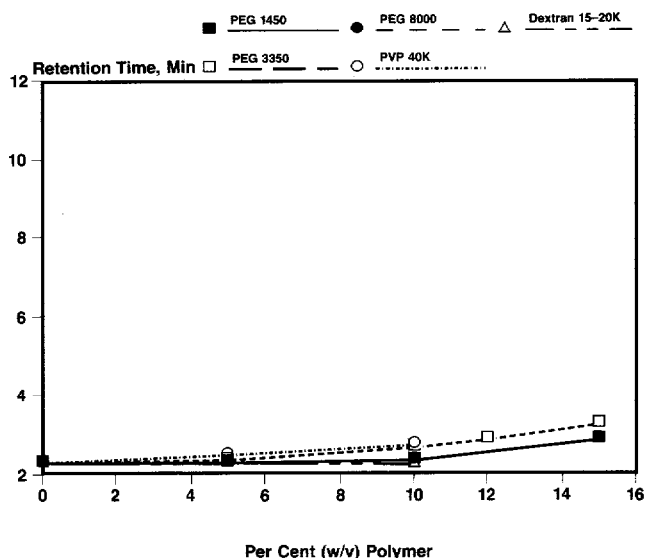

Fig. 1. Effect of type and concentration of neutral polymers in the eluent of an anion-exchange high-performance liquid chromatography (HPLC) system on the retention time of myoglobin.

■ PEG 1450   ● PEG 8000   △ Dextran 15–20K

□ PEG 3350   ○ PVP 40K

Retention Time, Min
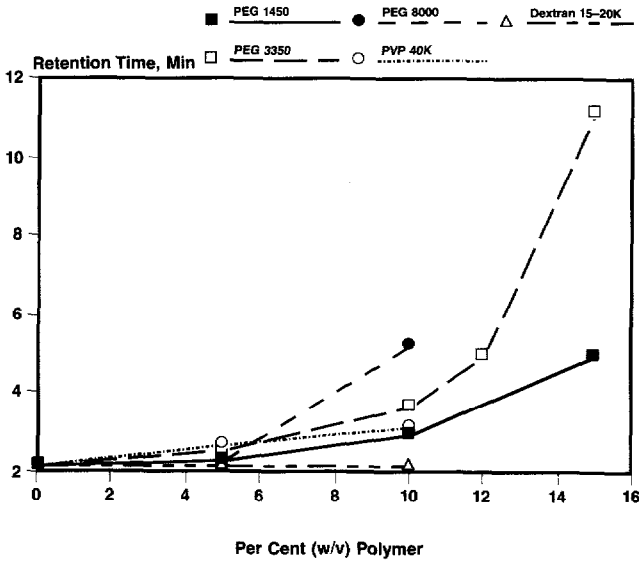
Per Cent (w/v) Polymer

Fig. 2. Effect of type and concentration of neutral polymers in the eluent of an anion-exchange HPLC system on the retention time of hemoglobin.

Under the same conditions as those used for the model proteins, citric acid and ascorbic acid were tested as representative small molecules. They were retained in the absence of polymer with a capacity factor of 0.8. When various concentrations and molecular weights of neutral water-soluble polymers were included in the eluent, the retention times of these small acids were unaffected.

■ PEG 1450   ● PEG 8000   △ Dextran 15–20K

□ PEG 3350   ○ PVP 40K

Retention Time, Min
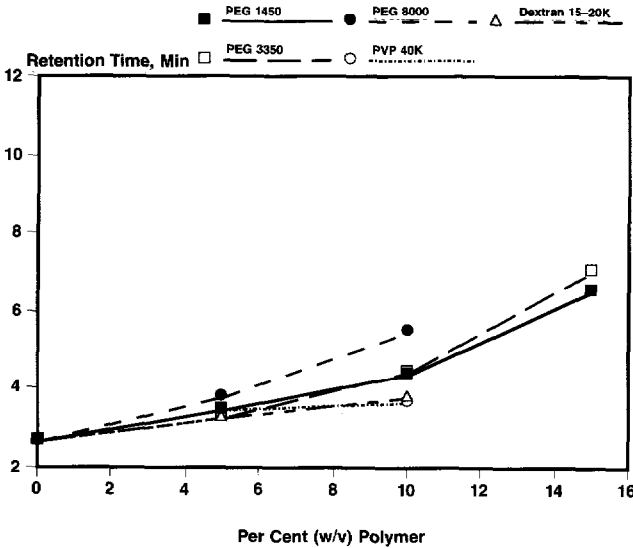
Per Cent (w/v) Polymer

Fig. 3. Effect of type and concentration of neutral polymers in the eluent of an anion-exchange HPLC system on the retention time of chymotrypsinogen A.
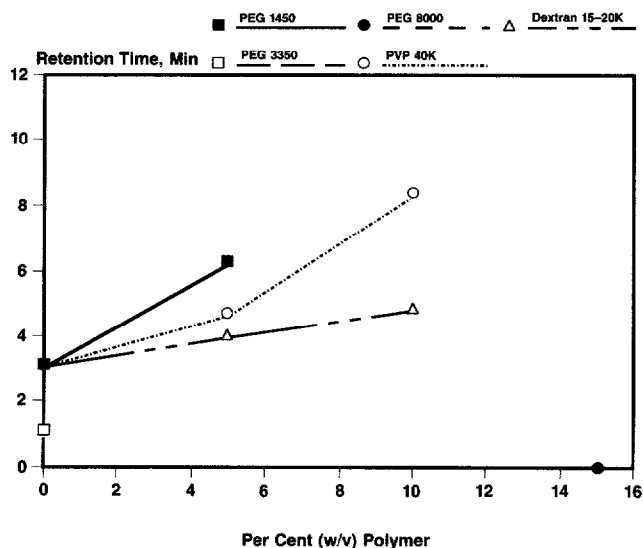
Fig. 4. Effect of type and concentration of neutral polymers in the eluent of an anion-exchange HPLC system on the retention time of transferrin.

To illustrate applications of these concepts, the separation of myoglobin and hemoglobin on an ion-exchange column was tested. The chromatograms in Fig. 7 illustrate how the separation was affected by the presence of PEG in the mobile phase. Under the conditions chosen, the hemoglobin appeared as a shoulder on the un-retained myoglobin peak when no polymer was present in the eluent. With PEG

TABLE II

EFFECT OF THE TYPE AND CONCENTRATION OF POLYMER ON THE CAPACITY FAC-TORS OF MODEL PROTEINS

Conditions: TSK-Gel DEAE-5PW column, 50 mM sodium phosphate buffer, pH 7.5, isocratic at 1 ml/min with constant level of polymer in the eluent.

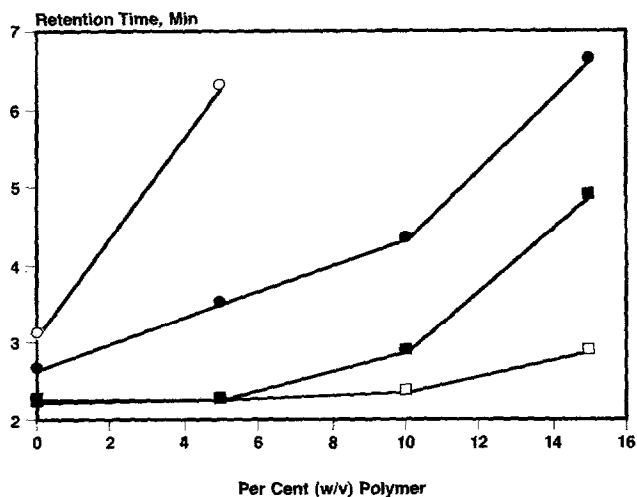|	Myoglobin	Chymotrypsinogen	Hemoglobin	Transferrin
No polymer	0.01	0.20	0.00	0.39
Dextran 15-20K, 10%	0.01	0.70	0.03	1.19
PVP 40 K, 5%	0.12	0.53	0.19	1.08
10%	0.22	0.65	0.42	2.75
PEG 1450, 5%	0.02	0.57	0.02	1.79
10%	0.07	0.94	0.30	[a]
15%	0.30	1.95	1.18	
PEG 3350, 5%	0.08	0.47	0.14	[a]
10%	0.20	0.98	0.62	
12%	0.26		1.21	
15%	0.45	2.15	3.96	
PEG 8000, 5%	0.01	0.70	0.03	[a]
10%	0.08	1.43	1.32	

[a] Peak not detected.

Fig. 5. Effect of PEG 1450 in the eluent of an anion-exchange HPLC system on the retention times of four model proteins. ■ = Hemoglobin; ● = chymotrypsinogen A; □ = myoglobin; ○ = transferrin.

added, however, the retention time of both proteins, especially the hemoglobin, were increased to the point of complete resolution. The results are summarized in Table III as retention times, resolution, and pressure. Both the molecular weight and the (w/v) concentration of the PEG had an effect on the separation. The system pressure was also seen to increase with the addition of PEG to the eluent. Depending upon the equipment used, increased pressure is a potential disadvantage to this technique and did cause limitations to the concentrations of polymer that could be tested in this work. Fortunately, resolution enhancement was observed at low polymer concentrations where the pressure increase was not a concern.
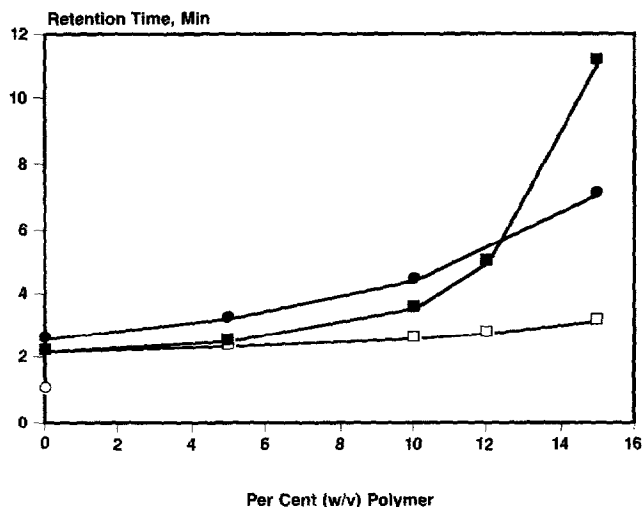

Fig. 6. Effect of PEG 3350 in the eluent of an anion-exchange HPLC system on the retention times of four model proteins. For key to symbols, see Fig. 5.
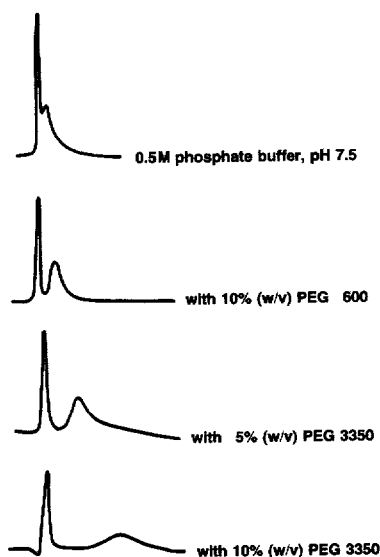
Fig. 7. Separation of myoglobin and hemoglobin on a SynChrom SAX column, 50 mM phosphate buffer, pH 7.5, 1 ml/min. The concentration and molecular weight of PEG added to the eluent were varied.

In another application, the influence of PEG 3350 on the separation of bovine somatotropin monomer and dimer is shown in Fig. 8. Without PEG, neither the monomer nor the dimer was retained under the conditions chosen. The retention times of both somatotropin species, especially the dimer, were increased by an increase in the level of PEG in the eluent. A summary of the results for these separations in included in Table IV.

DISCUSSIONS

The precipitation of proteins by neutral polymers is a complex event. While there exists numerous experimental data and theoretical treatments[1–8,12–14], a com-

TABLE III

SEPARATION OF MYOGLOBIN AND HEMOGLOBIN

Conditions: SynChrom SAX column, 50 mM phosphate buffer, pH 7.5, isocratic, 1 ml/min. The eluent contained PEG as listed in the first column.

Eluent	Retention time (min)		Resolution	Pressure (p.s.i.)
	Myoglobin	Hemoglobin		
0.05 M phosphate buffer, pH 7.5	2.24	2.73	0.25	1100
with 10% (w/v) PEG 600	2.25	3.54	0.56	1500
with 5% (w/v) PEG 3350	2.92	5.10	0.70	1460
with 10% (w/v) PEG 3350	2.91	9.12	1.7	2460

TABLE IV

SEPARATION OF BST MONOMER AND DIMER

Conditions: Pharmacia MonoQ column, 20 mM Tris, pH 10, with a 30 min gradient from 10 mM to 25 mM NaCl. PEG 3350 concentration in eluent was held constant during the separation. na = Data not available.

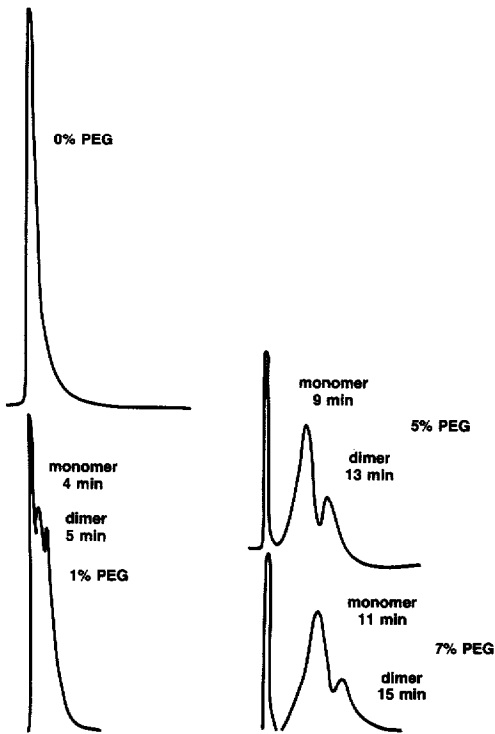| PEG 3350 (%, w/v)	Retention times (min)		Pressure (p.s.i.)
	Monomer	Dimer	
0	3	3	250
1	4	5	na
2.5	6.5	8	300
5	9	13	360
7	11	14.5	420
10	13	38	450

Fig. 8. Separation of bovine somatotropin monomer and dimer on a Pharmacia MonoQ column. The eluent was 20 mM Tris, pH 10, with a 30 min gradient from 10 mM to 25 mM NaCl with 0–7% PEG 3350 which was constant during the gradient.

plete understanding of the phonemenon has not been obtained. It is generally agreed, however, that the observed effects of PEG and other neutral polymers on protein precipitation can be qualitatively explained by the excluded volume theory. According to this theory, water-soluble polymers in aqueous solutions are able to interact with a large number of water molecules, resulting in reduction of the solvating power of water for the protein. The major parameter here is the size (molecular weight) of the polymer: the larger the polymer, the more effective it is as precipitant.

Lee and Lee[7] and Arakawa and Timasheff[14] obtained some evidence for a secondary effect that they termed "preferential interaction" between PEG and the proteins studied. This interaction is proposed to be hydrophobic in nature[14] to explain the opposite effect of PEG on RNase A and β-lactoglobulin. Thus, an important parameter here is the chemical nature (*i.e.*, hydrophobicity) of the protein, which is not considered in the excluded volume theory.

Empirically, the controlling protein parameter is size (volume), with hydrophobicity and charge being secondary. As with salting out, larger proteins precipitate at lower polymer concentrations than smaller ones. The controlling polymer parameter is hydrophobicity as determined by its composition and molecular weight. More hydrophilic polymers are more selective but less efficient than more hydrophobic ones. For the related phenomenon of two-phase aqueous systems, Albertsson has proposed a ranking of the hydrophobicities of water soluble polymers (Fig. 9); in addition, for certain types of polymers such as PEG, hydrophobicity increases as molecular weight increases[9]. The region of aqueous polymer systems is a narrow band within the range from the aqueous salts to the non-polar solvents. This is an unexplored region for applications to chromatographic elution.

In polymer-enhanced precipitation[10,11], a combination of a charged polymer and a neutral polymer is used to selectively precipitate protein(s) of interest. The charged polymer is used to form a charge complex with the appropriate protein(s). The role of the neutral polymer is less straightforward and is probably two-fold. First, it enhances the precipitation of the protein–charged polymer complex, very likely as a result of the excluded volume effect. Secondly the appropriate choice of the neutral polymer appears to enhance the selectivity of the charged polymer, probably through the hydrophobic interactions between the neutral polymer and proteins in solution.
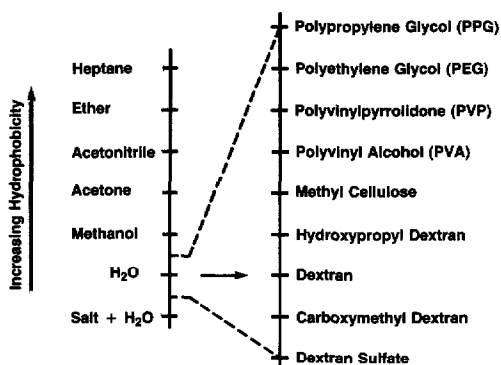

Fig. 9. The hydrophobic ladder as modified from ref. 9. Ether = Diethyl ether.

Fig. 10 shows the important properties for the protein and each polymer. For the protein and the neutral polymer, the same factors have the same qualitative influence as in polymer-induced precipitation. The data presented here can also be explained by these control factors. The neutral polymers decrease the solvating power of the eluent. This shifts the equilibrium of the level of solute in the mobile phase *versus* on the solid phase, so the interaction between the protein and the solid phase is increased. Because this technique uses an immobilized ion exchange support instead of a soluble charged polymer, it has been given the related name of "polymer enhanced chromatography."

If the mechanism of polymer enhanced chromatography is indeed related to protein precipitation and the excluded volume theory, the above results should be qualitatively explained in the same way. If the excluded volume theory is applicable to our observation, it predicts selectivity in how polymers affect protein chromatography. Larger and/or more hydrophobic proteins should be more readily effected than smaller and/or more hydrophilic ones. Furthermore, the effectiveness of a polymer is determined by its hydrophobicity. The series of experiments with model proteins and different polymer types verifies these predictions. In general, the protein retention time increased as the concentration of polymer was increased. As predicted, the effect was greater for more hydrophobic polymers. For a given concentration of PEG, the effect increased as the average molecular weight of the PEG increased. Dextran, the least hydrophobic of the polymers tested, had very little effect on the protein retention. PVP is ranked between dextran and PEG on the hydrophobicity scale (Fig. 9). The particular sample of PVP tested was higher in molecular weight than the PEGs but was still less effective in shifting the protein retention.

Myoglobin, the smallest of the model proteins, was least influenced by the added polymers (Fig. 1). However Table II does show that at 10–15% polymer, there was an increase in capacity factor. Hemoglobin is a protein similar in charge and hydrophobicity to myoglobin, but approximately four times larger (see Table I). As predicted by the excluded volume theory, hemoglobin was more strongly influenced by the presence of polymers than the myoglobin. Transferrin, the largest of the proteins tested, was the most strongly affected by the polymers (Fig. 4). In this case, several of the concentrations of polymers used increased and/or broadened the peak to the point of being undetectable. Chymotrypsinogen is between myoglobin and hemoglobin in size (see Table I) yet was more strongly influenced by the presence of
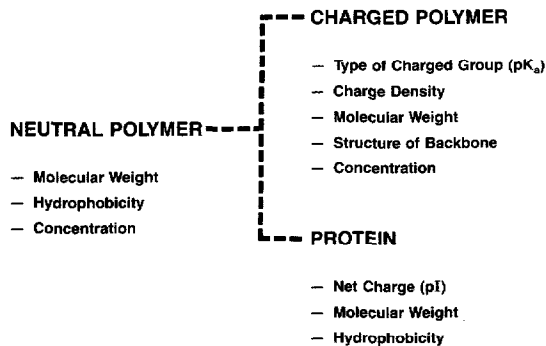
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                                 ┌── CHARGED POLYMER
                                 │
                                 │    — Type of Charged Group (pKₐ)
                                 │    — Charge Density
NEUTRAL POLYMER ──┤    — Molecular Weight
                                 │    — Structure of Backbone
                                 │    — Concentration
— Molecular Weight      │
— Hydrophobicity         │
— Concentration           │
                                 └── PROTEIN

                                      — Net Charge (pI)
                                      — Molecular Weight
                                      — Hydrophobicity
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Fig. 10. Controlling factors in polymer-enchanced precipitation[10,11].

polymers (see Table II and Figs. 5 and 6). It is possible that in addition to size, a protein's hydrophobicity may play an important role in the extent its solubility is affected by the presence of the polymer. Also note that at the pH used (7.5) chymotrypsinogen with a pI of 9.0 has an overall positive charge, although net charge alone is insufficient to explain protein retention in ion-exchange chromatography[15]. Fig. 6 shows a crossover of the lines for hemoglobin and chymotrypsinogen that may represent the point at which size becomes more dominant than hydrophobic and ionic effects. Further exploration of the effects of pH and ionic strength on polymer enhanced chromatography is needed to obtain a better understanding of these interactions.

Fig. 7 and Table III show enhanced resolution of myoglobin and hemoglobin by the addition of PEG to the eluent of an anion-exchange system. Hemoglobin is an oligomer made up of four polypeptide chains, each of which is similar to the single chain of myglobin. Thus the isoelectric points and the hydrophobicities of these two proteins are very similar, but hemoglobin is approximately four times larger than myoglobin. The presence of PEG in the eluent increased the retention times of the proteins, especially the larger hemoglobin. The effect was greater when the higher molecular weight, and therefore more hydrophobic PEG was used. Peaks were significantly broadened although peak areas were relatively constant, indicating consistant recovery is possible. The broadening is likely due to the heterogeneous nature of the interaction between the protein surface and the solid phase. Using a gradient of the polymer should decrease the peak broadening, but this has not yet been tested.

Fig. 8 shows how the separation of bovine somatotropin (BST) monomer and dimer is affected by the addition of PEG 3350 to the mobile phase of an ion-exchange system. This separation is a real life example which is ideally suited to polymer enhanced chromatography. Because of the need to separate the BST from other contaminants it is necessary to carry out an ion-exchange step at this point of the purification. It is desired to minimize the level of dimer in the final product. The monomer and dimer are obviously very much alike in every way but size. Polymer enhanced chromatography allows the ion-exchange step to also separate monomer from dimer. The retention times of both species are increased, but that of the larger dimer is more affected than the monomer. Just 5% PEG 3350 increased the resolution of the two components from 0.25 to 0.70 without a dramatic increase in pressure. Note that peak broadening is not as significant as in the myoglobin–hemoglobin separation, perhaps because a salt gradient was used in this case.

CONCLUSIONS

The addition of neutral water-soluble polymers to the eluent of ion-exchange chromatography systems selectively increases the retention times of protein solutes. It could be argued that the polymer alters the solid phase in some manner or blocks the pores in the solid support. This does not seem likely because any blocking of charges or pores should act to decrease, rather than increase, retention times. Furthermore, the retention times of small molecules (ascorbic and citric acid) were unaffected by the polymer. It might also be hypothesized that the polymer leads to protein self-association, although this has not been detected in studies of polymer-induced precipitation[2,6].

The observed increased retention times are most probably due to an increased protein–solid phase interaction. That is, the protein is less soluble in eluent containing the polymer and is therefore more likely to interact with the solid phase.

The effect of polymers on the ion-exchange retention of proteins can be predicted qualitatively by the excluded volume theory which is also invoked to explain neutral polymer-induced precipitation of proteins. According to this concept, polymer competes with the protein for water. In the extreme case, the protein becomes dehydrated enough to precipitate. In this application to chromatography, polymer added to the eluent shifts the equilibrium of the protein from solution in the mobile phase to interaction with the solid phase.

For the combination of ion-exchange chromatography with neutral polymers presented here, the greatest power of the technique is when the proteins to be separated have very similar charge properties but different sizes. Two such applications have been presented: the separation of myoglobin and hemoglobin, and the separation of bovine somatotropin monomer and dimer. While these separations are achievable by other chromatographic methods, ion exchange is preferred for preparative separation because of its high loading capacity and its use of aqueous rather than organic eluents. The presence of a neutral polymer in the product is not necessarily a complication since it may improve the product stability or be required in the final formulation.

Based upon the results and hypothesis presented here, neutral polymers would increase the interaction between biopolymers and chromatographic supports in general. Other potential applications of polymer-enhanced chromatography include separating intact molecules from fragments or breakdown products, or separating antibody–antigen complexes from the free components. It is likely that this technique will be applicable to the separations of nucleotides, cellular fragments, etc., in the same manner that polymer-induced precipitation and two-phase aqueous systems have been extended to these areas. Extension of this work may also include hydrophobic interaction chromatography using decreasing gradients of neutral polymers rather than salts.

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