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Retention Studies and Protein Separation by Potential Barrier Chromatography

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Abstract

Potential barrier chromatograpy (PBC) exploits the fact that the depth of the adsorption well of the interaction potential between adsorbate and adsorbent can become moderately deep when it is controlled by opposing van der Waals attractive forces and repulsive double layer forces. The moderately deep potential well gives rise to repeated cycles of adsorption and desorption, thereby allowing isocratic elution of proteins to occur. Separation of proteins is achieved because the depth of the adsorption well, which affects the retention time, is very sensitive to the size, charge, and hydrophobicity of the adsorbate molecules. Here, a mixture of six model proteins has been separated using PBC on a commercially available strong anion-exchange column. The selection of the appropriate mobile phase conditions of pH and ionic strength led to fast separations using a two-step isocratic elution procedure. The effects of temperature and various organic additives on the resolution of protein separations have also been investigated.

INTRODUCTION

Most chromatographic methods employed for protein separations are based on the differences of only a single physicochemical parameter of the proteins, such as electric charge (as in ion-exchange chromatography [IEC]), molecular size (size exclusion chromatography [SEC]), hydrophobicity (hydrophobic interaction chromatography [HIC] or reverse phase chromatography [RPC]), or ligand specificity (bioaffinity chroma-

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tography). In contrast, potential barrier chromatography (PBC), which was first proposed by Ruckenstein and Prieve (*1*) in 1976, is sensitive to several factors such as molecular size, electric charge, and hydrophobicity. A distinguishing feature of PBC is that protein retention is determined by opposing van der Waals attractive forces and repulsive electrical double layer forces. This differs from previous methods which are based on a unique attractive force (i.e., attractive electrical double layer forces in the case of IEC and attractive van der Waals (hydrophobic) interactions in the case of HIC and RPC), which usually results in permanent adsorption of the solute (protein) onto the adsorbent. Separation is then achieved by varying the mobile phase conditions (e.g., as in gradient elution), thus attenuating the bindinig attractive force, thereby allowing elution of the various components. However, in PBC repeated cycles of adsorption and desorption take place when the opposing attractive and repulsive forces allow the adsorption energy well to be raised sufficiently for desorption to occur more easily. As a result, an isocratic elution procedure can sometimes be employed. The depth of the adsorption well can be raised or lowered by altering the mobile phase conditions such as pH and ionic strength (thereby affecting the repulsive double layer forces) or by adding a small amount of a suitable organic solvent to the mobile phase (thus attenuating the attractive van der Waals forces). Since the depth of the adsorption well in the interaction potential is extremely sensitive to slight differences in molecular size, electric charge, and hydrophobicity of the adsorbates, proteins that differ slightly in their physicochemical properties will require different lengths of time for an adsorption–desorption cycle. This results in different residence times in the column and consequently separation of these proteins can occur. It is essential that the appropriate mobile phase conditions be chosen to ensure that the adsorption wells become moderately deep so that desorption occurs easily, thereby allowing isocratic elution of the proteins.

However, the properties of the various proteins can be very different. As a result, changes in ionic strength, pH, and organic additive cannot always sufficiently raise the adsorption wells of all the proteins involved. Consequently, isocratic elutions can be employed only when the proteins involved are sufficiently similar. Of course, when the adsorption wells are too close to one another, a separation is also not possible.

Theoretical computations for spherical particles have predicted (*2*) distinct separations based on slight differences in their physicochemical properties such as molecular size, surface potential, or Hamaker constant. Subsequent experiments in this laboratory have demonstrated that this

chromatographic technique can be utilized to successfully separate different protein mixtures (*3–7*) by using a high performance liquid chromatographic (HPLC) system.

Though PBC utilizes conventional IEC columns and buffers for these experiments, the main difference lies in the mode of operation of the columns. Whereas in IEC attractive electrical double layer interactions are essential to the process, in PBC repulsive electrical double layer interactions are always maintained by selecting the mobile phase pH such that both the adsorbent and proteins are similarly charged. For example, when using an anion-exchange column (which is positively charged), the mobile phase pH is adjusted such that it is below the isoelectric points of the proteins used (thus imparting a net positive charge to the proteins), thereby resulting in repulsive double layer interactions.

Care must be taken to distinguish PBC from mixed-mode or multimodal chromatographies, which have found recent applications in protein (*8–12*) and nucleic acid separations (*13–18*). In mixed-mode chromatography, simultaneous retention mechanisms (usually electrostatic and hydrophobic interactions) contribute to chromatographic separation, while in multimodal chromatography, dissimilar stationary phase ligands utilize multiple independent retention mechanisms (again electrostatic and hydrophobic interactions) to effect separations. Both methods employ attractive electrical double layer interactions in conjunction with attractive van der Waals interactions to improve the selectivity of protein separations. Thus, depending on mobile phase conditions, proteins are adsorbed in the IEC mode at low salt concentrations because of electrostatic attraction, or in the HIC mode at very high salt concentrations because of the salting out effect. However, in PBC the electrical double layer interactions are always repulsive. Moreover, since opposing interactions are simultaneously involved at all times, isocratic elution conditions are suitable for PBC. Other distinctive features of PBC in comparison with mixed-mode and multimodal chromatography are discussed later.

Here, the retention times of six different proteins were measured using a strong anion-exchange column, operating at various mobile phase conditions of pH and ionic strength. The retention times of these proteins were expressed in terms of their capacity factors k, which is the ratio of the times a solute (protein) spends in the adsorbed state ($t_{R'}$) and in the mobile phase (t_0):

$$k = \frac{t_{R'}}{t_0} = \frac{t_R - t_0}{t_0} \tag{1}$$

where t_R is the total retention time of the solute:

$$t_R = t_{R'} + t_0 \tag{2}$$

The effect of different organic solvents on the retention of three of the proteins at various mobile phase values of pH and ionic strength was examined in detail. The influence of column temperature on the capacity factors of the six proteins for a given set of mobile phase conditions was examined next. Finally, a mixture of these six proteins was injected into the column in an attempt to separate the proteins in the PBC mode. The mobile phase pH, ionic strength, and organic content were varied to improve the resolution of the separation. The column temperature was chosen so as to minimize enzyme denaturation.

First a brief discussion of the theory underlying PBC is presented, followed by the experimental results and discussion.

THEORY

As mentioned before, the retention of the protein molecules depends on the interaction potential between the proteins and the adsorbent. In potential barrier chromatography (PBC), this interaction potential is a result of two long-range interactions (the attractive van der Waals and repulsive double layer interactions) and various short-range interactions (primarily the Born, hydration, and steric repulsions).

The van der Waals interaction (*19*) arises due to three effects: 1) the dipole-dipole (or Keesom) interaction between polar molecules; 2) the dipole-induced dipole (or Debye) interaction between polar and nonpolar molecules; and 3) the (most important) dispersion (or London) interactions between neutral molecules. Although the van der Waals interaction potential between individual atoms decays rapidly with the inverse sixth power of the distance between their centers (*20–22*), the London–van der Waals interactions have a long-range effect in a macroscopic or macromolecular system due to the contributions of the numerous intermolecular interactions. The London interactions are, to a large extent, additive (*20, 23*). Hamaker (*20*) computed the van der Waals interaction potential, ϕ_{vdW}, between a sphere (an idealized adsorbate) and a semi-infinite flat plate (an idealized adsorbent) by summing the interactions between all pairs of atoms and obtained

$$\phi_{vdW} = \frac{A_{132}}{6}\left[\ln\left(\frac{H+2}{H}\right) - \frac{2}{H}\frac{H+1}{H+2}\right] \tag{3}$$

where A_{132} is the Hamaker constant for materials 1 and 2 immersed in a medium 3, which is given by

$$A_{132} = A_{12} + A_{33} - A_{13} - A_{23} \tag{4}$$

and

$$H = h/a_p \tag{5}$$

A_{ij} represents the Hamaker constant for the interaction between materials i and j, h is the minimum distance of separation between adsorbate and adsorbent, and a_p is the radius of the sphere.

Srinivasan and Ruckenstein (*24*) discussed in detail the importance of the Hamaker constant A_{132} in controlling the van der Waals interactions. The effect of the mobile phase properties on the Hamaker constant has been analyzed qualitatively, and their conclusions are summarized here. Consider, for example, the van der Waals interaction between a protein (subscript 1) and adsorbent (subscript 2) in an aqueous medium (subscript 3). Obviously, strong interactions between the water molecules (A_{33}) or between the protein and adsorbent (A_{12}) result in strong van der Waals interaction forces. Also, weak interactions between the protein and water molecules (A_{13}) or between the adsorbent and water molecules (A_{23}) can further enhance the van der Waals interactions between the protein and adsorbent. If the direct protein–adsorbent interactions (A_{12}) are negligible, then the interactions between the water molecules (A_{33}) clearly dominate the overall van der Waals interactions. Due to the extensive hydrogen bonding present in water, the value of the Hamaker constant A_{33} is quite high. Addition of small electrolyte ions (e.g., ammonium sulfate, potassium chloride) increases the structural order of water, thereby increasing the hydrogen bonding in the solution. Therefore we can expect the Hamaker constant A_{33} to increase, and this results in stronger van der Waals interactions between the protein and adsorbent. However, large anions (such as guanidinium chloride) or hydrocarbon-substituted salts (such as tetraethylammonium chloride) can disrupt the three-dimensional hydrogen bonding of water molecules, thus leading to reduced van der Waals interactions. This can also be achieved by addition of miscible organic solvents (such as ethylene glycol, acetonitrile, alcohols) to the aqueous mobile phase. Since the cohesive forces of these solvents are lower than those of water, the interactions between the molecules of the medium (A_{33}) are attenuated. Furthermore, these solvents can strengthen the various dipole–dipole and dispersion interactions between the

medium and protein and between the medium and adsorbent, and this is reflected in an increase in the Hamaker constants A_{13} and A_{23}. As is evident from Eq. (4), attenuating A_{33} and/or increasing A_{13} and A_{23} leads to an overall reduction in the van der Waals interactions between protein and adsorbent. One can note that the Hamaker constant A_{132}, which includes the effects of the structure of water, of the interactions between water and protein, water and adsorbent, etc., is a measure of the "hydrophobic interactions" between protein and adsorbent.

The electrical double layer interaction is a consequence of the interaction between the electrically charged surfaces of the adsorbates and adsorbent. Proteins (adsorbates) and ion-exchange adsorbents become electrically charged via surface group ionization in the presence of a polar medium such as water. Each of these electrically charged surfaces attracts counterions (ions of charge opposite to that of the surface) from the medium; these counterions form a diffuse layer around the surfaces due to the opposing forces of electrostatic attraction (toward the charged surface) and diffusion (down the concentration gradient). The charged surface and its associated diffuse layer of counterions together constitute the electrical double layer.

When two surfaces bearing similarly charged electrical double layers approach each other, the resulting double layer repulsion can be predicted. The electrical double layer interaction potential ϕ_{DL} between a sphere and a semi-infinite flat plate is given by the approximate expression (*3, 25, 26*)

$$\phi_{DL} = 16\varepsilon\left(\frac{kT}{ze}\right)^2 a_p \tanh\frac{ze\psi_p}{4kT} \tanh\frac{ze\psi_a}{4kT} \exp(-\kappa h) \tag{6}$$

where κ, the reciprocal Debye length, is defined as

$$\kappa = \left(\frac{8\pi I e^2}{\varepsilon kT}\right)^{1/2} \tag{7}$$

and I, the ionic strength of the medium, is given by

$$I = \tfrac{1}{2}\sum_i c_i z_i^2 \tag{8}$$

ψ_p is the surface potential of the adsorbate (protein), ψ_a is the surface potential of the adsorbent, k is the Boltzmann constant, T is the absolute temperature, ε is the dielectric constant of the medium, z_i is the valence of the electrolyte ion, and c_i is the concentration of the electrolyte of species i.

Here the important parameters are the ionic strength of the mobile phase and the surface charges of the proteins and adsorbent. The ionic strength of the medium is varied by addition of electrolyte salts while the surface charges of the proteins can be controlled by altering the pH and ionic strength of the medium. Most adsorbents employed in ion-exchange chromatography have strongly acidic or basic surface groups and hence their surface charge seldom varies appreciably with pH. However, the net charge of the proteins varies as a function of pH of the mobile phase. Thus, at a mobile phase pH far removed from the pI of the protein, the protein has a high surface charge and consequently experiences a strong double layer interaction with the adsorbent. On the other hand, at a mobile phase pH very close to the pI of the protein, there is a very low net surface charge on the protein, thereby giving rise to weak double layer interactions. Furthermore, the net charge affects the configuration of some proteins (*24, 27, 28*), their shape being more globular near the pI and more elongated farther from the pI. While the equations given above are derived for globular shapes, the qualitative conclusions remain valid for other configurations as long as the net charge is uniformly distributed over the protein surface. (The section "Effect of pH and Ionic Strength" contains comments on nonuniformly distributed charges.)

When the ionic strength of the medium is increased, the electrolyte ions screen the surface charges of the proteins and the adsorbent, and thus diminish their influence on the counterions extending into the bulk solution. The double layer becomes compressed and the proteins can approach the adsorbent to much shorter distances before experiencing significant double layer repulsions. This is quite obvious when Eqs. (6) and (7) are examined, where the double layer interaction potential is an exponential function of the square root of the ionic strength. Furthermore, the counterions in the mobile phase may bind to the oppositely charged surfaces of the proteins and adsorbent, thus decreasing their net surface charge and thereby lowering the double layer repulsion.

At very short distances between protein and adsorbent, several short-range interactions develop, of which the Born, hydration, and steric repulsions are most important.

Steric repulsion (*29–31*) occurs when macromolecules attached to the adsorbent interact with the approaching proteins, and therefore is a function of the chain length of the adsorbed or bound molecules on the adsorbent. Hydration repulsion (*32*) arises from the difficulty in eliminating water molecules that are strongly held by the charged or polar groups of the surfaces. Born repulsion is due to the resistance of the electron clouds of two atoms to overlap each other, and the corresponding repulsive

potential ϕ_B between a sphere and a semi-infinite flat plate is given by (*1*)

$$\phi_B = \frac{A(\sigma/a_p)^6}{7560}\left[\frac{8+H}{(2+H)^7}+\frac{6-H}{H^7}\right] \tag{9}$$

where σ is the atomic collision diameter. If this collision diameter is regarded as an experimentally determined parameter, then the Born repulsion ϕ_B can be used to effectively describe all short-range repulsive interactions.

The sum of these individual contributions gives the total interaction potential:

$$\phi = \phi_{DL} + \phi_{vdW} + \phi_B \tag{10}$$

What would the profile of this total interaction potential look like? One possible profile is shown by Curve D of Fig. 1, with a potential barrier to adsorption at intermediate distances, a moderately deep adsorption well at short distances, and a very steep repulsive "wall" at very short distances. Of course, the presence of a sufficiently high potential barrier can impede adsorption. It is likely that the total interaction profiles shown in Fig. 2 (which do not possess a potential barrier), or total interaction potentials which exhibit low potential barriers, are encountered in most experimental situations in PBC. Of course, the opposing van der Waals attractive forces and double layer repulsive forces control the depth of the adsorption well in the interaction profile. If the double layer repulsive forces dominate or the van der Waals attractive forces are sufficiently weak, then an adsorption well may not be present in the interaction profile (Curves A and B). Such a situtation arises when the mobile phase pH is far removed from the pI of the protein and at very low ionic strengths. The repulsive double layer interactions can be attenuated by bringing the mobile phase pH closer to the pI of the protein (thereby reducing the surface charge) and/or by raising the ionic strength (thereby compressing the double layer). This results in the formation of an adsorption well as seen in Curves C, D, and E. At sufficiently high ionic strengths or at a mobile phase pH very close to the pI of the protein, the electrical double layer becomes very compressed and the attractive van der Waals interactions dominate. In such cases the adsorption well might be too deep for desorption to occur (Curves D and E). Therefore appropriate choices of mobile phase conditions will generate interaction potential profiles (such as Curve C) with a moderately deep adsorption well. This will allow the pro-

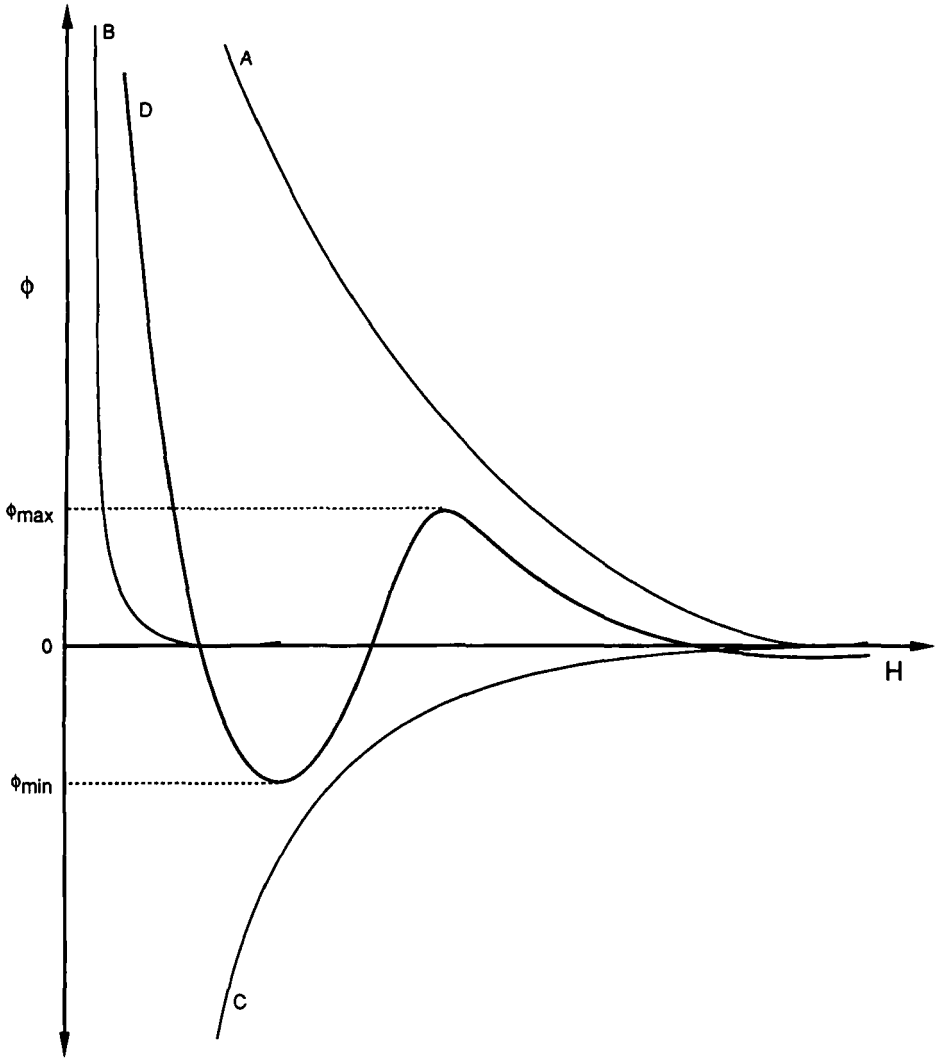

FIG. 1. A representation of the various theoretical interaction potential profiles present in PBC. Curves A and B represent the repulsive double layer and short-range interactions, respectively, while Curve C represents the attractive van der Waals interaction. The total interaction potential is the sum of these combined interactions and is given by Curve D which shows a potential barrier.

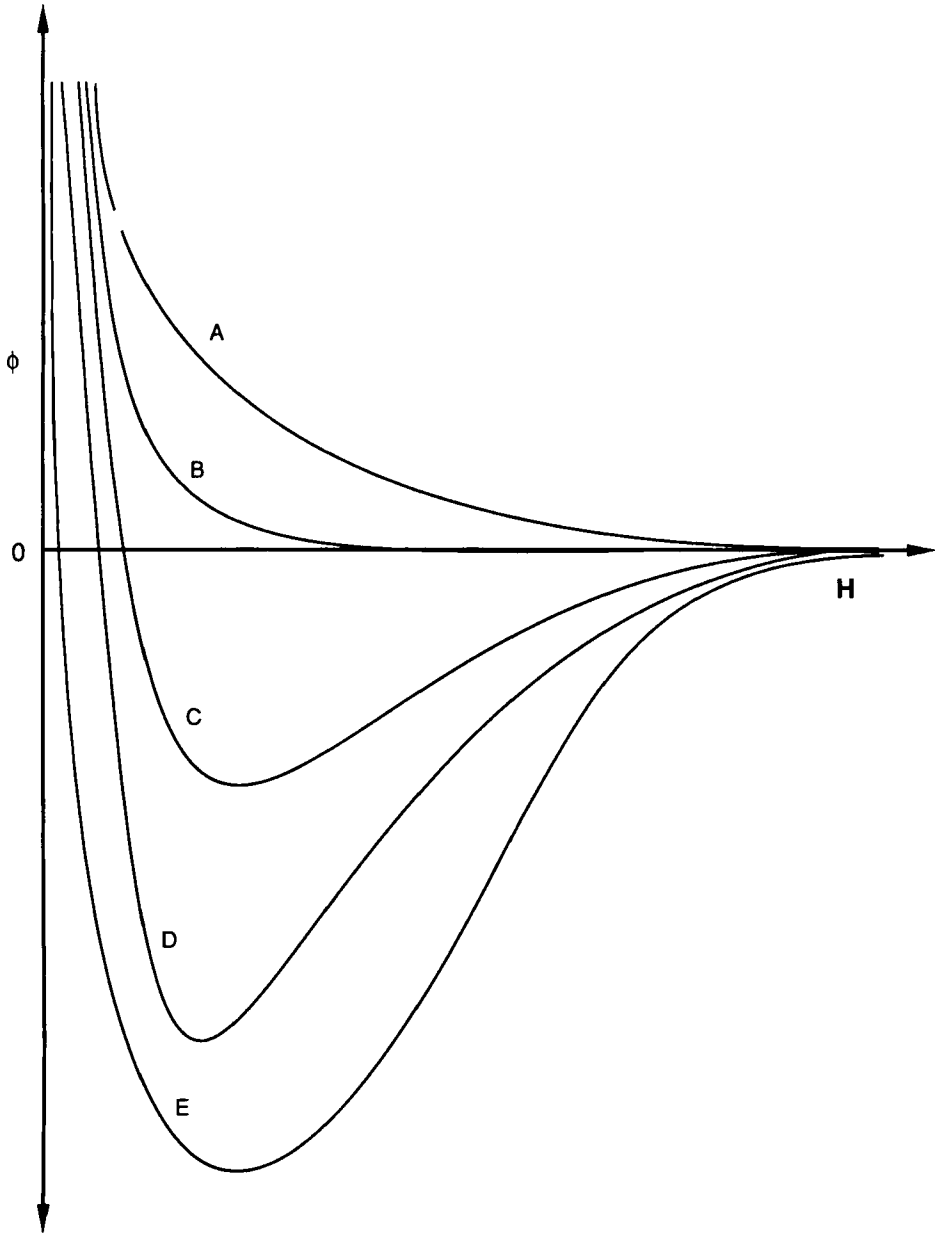

FIG. 2. Some of the total interaction profiles likely to be encountered in experimental PBC. Curves A and B represent cases of excessive double layer repulsion, whereas Curves D and E represent very strong van der Waals attraction. A moderately deep adsorption well is generated, as shown by Curve C, when the double layer and van der Waals forces oppose each other.

tein to undergo repeated cycles of adsorption and desorption, thereby affecting its residence time in the column. Since the depth of the adsorption well is very sensitive to small differences in size, electric charge, and hydrophobicity of the proteins, their residence times will be different. Thus separation of proteins under isocratic elution conditions is possible. Though the term "potential barrier" might be a misnomer (since a potential barrier is not visible in Fig. 2), we still continue to use it for traditional reasons.

Several authors (*8–18, 33–46*) suggested that mixed retention mechanisms could be exploited for improved selectivity in chromatographic separations. Hofstee and coworkers (*35, 36*) suggested that both electrostatic and hydrophobic interactions between protein and chromatographic stationary phases may be responsible for such mechanisms. However, in these cases the electrical double layer (electrostatic) interactions are invariably attractive in nature, and therein lies the difference with PBC. In mixed-mode or multimodal chromatography, low ionic strengths promote binding of the proteins due to attractive double layer interactions. As the ionic strength is increased, the attractive double layer interactions are attenuated, thereby allowing elution to occur. At still higher ionic strengths, attractive van der Waals interactions dominate because of the salting out effect and the proteins are once again retained on the column. Figure 3 shows a few typical curves for different proteins (*10, 11, 47*) during mixed-mode or multimodal chromatography. Separation of these proteins would be difficult in the conventional pure IEC or HIC modes since their capacity factors (not shown in Fig. 3) are close. Even though selectivity is greatly improved during mixed-mode/multimodal chromatography, a few problems still persist. For a mixture of proteins as depicted in Fig. 3, a long and shallow gradient would be required to effect a separation. Furthermore, many proteins are endowed with a large number of nonpolar residues on their chromatographically accessible surfaces (*35, 41, 48–52*), thus imparting a considerable degree of hydrophobicity. Such proteins (typically Curves D and E in Fig. 3) would not elute easily. However, in the PBC mode, repulsive double layer interactions could be utilized to elute these proteins expeditiously while operating at low ionic strengths (broken lines of Curves B, C, D, and E in Fig. 3). Furthermore, it is possible to use an isocratic elution procedure (for example, at an ionic strength of I_{PBC}) to separate all components of the protein mixture in a relatively shorter time.

Even slight differences in the molecular size, charge, or hydrophobicity of the proteins can give rise to significantly different interaction profiles, which in turn affect the retention time of a particular protein in the column. Thus, by properly tuning the mobile phase conditions such as pH

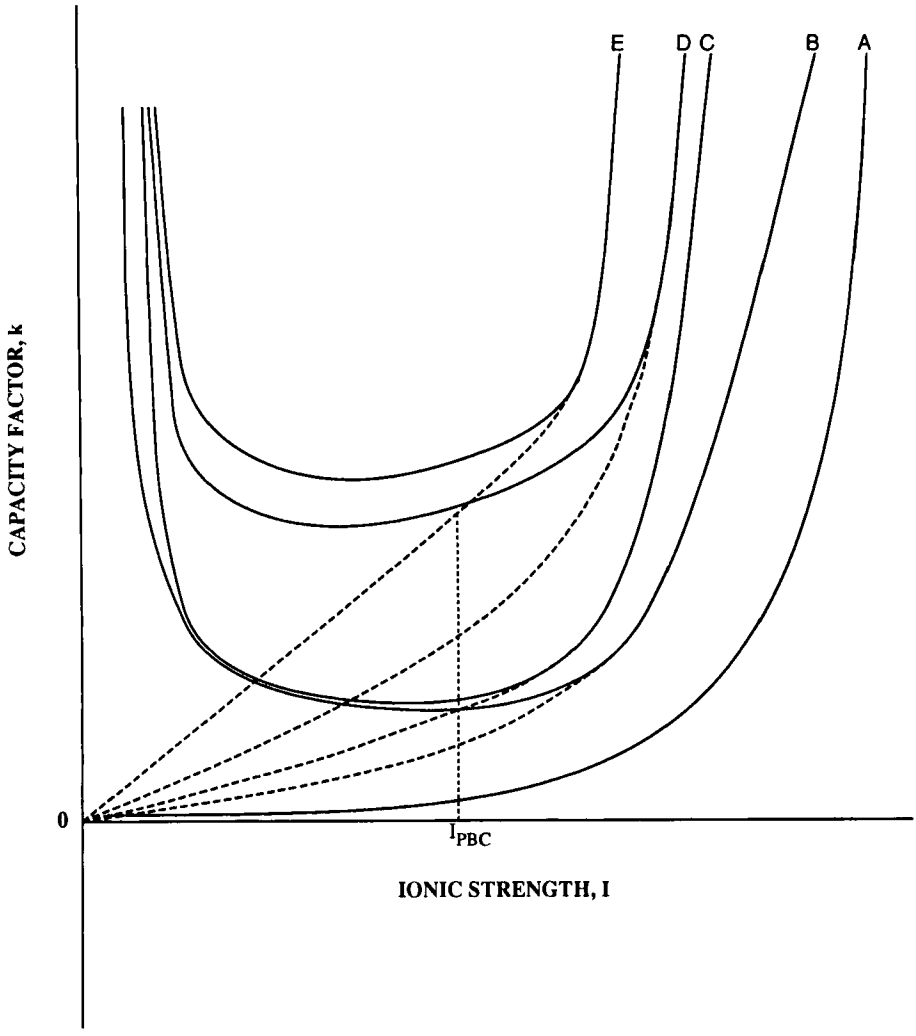

FIG. 3. Capacity factors of different proteins as a function of ionic strength during mixed-mode or multimodal chromatography. Each curve represents the behavior of a single protein as a function of ionic strength. The broken lines of Curves B, C, D, and E illustrate the PBC mode of operation. For isocratic elution of all proteins in PBC mode, I_{PBC} would be the optimum ionic strength.

and ionic strength, different proteins will have different retention times, depending on their respective interaction potentials, and consequently a mixture of proteins can be separated using an isocratic elution procedure. Let us, however, emphasize again that because the properties of the proteins can be very different, it is difficult to raise sufficiently the adsorption wells of all the proteins of a complex mixture. As a result, isocratic elution is possible only for mixtures whose components are sufficiently similar, yet different enough to elute at different times.

EXPERIMENT

The retention studies and protein separations were carried out by using a Pharmacia strong anion exchanger (Mono Q HR 5/5) and an ISCO HPLC precision pump (Model 2350) fitted with a Valco injection valve. The column effluent was monitored at 280 nm using an ISCO variable wavelength UV detector (Model V[4]). All mobile phase titrations were performed by using a digital pH meter (Orion Research 601A) fitted with a combination pH electrode (Orion 91-04). All organic solvents purchased (Aldrich Chemical Co.) were of HPLC grade; other reagents and chemicals used were analytical research grade or of comparable quality. All proteins (highest purity grade) were purchased from Sigma Chemical Co. The isoelectric points of the proteins are given below (*53–55*) (Table 1). The column (5 mm i.d. × 5 cm length) has been described by the manufacturer as a strong anion exchanger based on a beaded hydrophilic resin with the charged group $-CH_2-N^+(CH_3)_3$ on the gel. Various cationic or zwitterionic buffers were chosen for different pH intervals, as shown in Table 2 (*56*), such that the buffer pK_a was within 0.5 pH units of the desired eluent pH.

TABLE 1
Isoelectric Points of Proteins Used

Protein	Source	Isoelectric point	Molecular weight
Ovalbumin	Chicken egg white	4.6	43,500
Bovine serum albumin	Bovine serum	4.9, 5.2	69,000
β-Glucosidase	Almonds	7.3	135,180
Transferrin	Human blood	5.9	76,000
β-Lactoglobulin	Bovine milk	5.13, 5.3	35,000
Superoxide dismutase	Bovine erythrocytes	7, 9	73,000

TABLE 2
Recommended Buffers for Various pH intervals

pH Interval	Buffer	Concentration	pK_a
3.6–4.0	Lactic acid	50 m*M*	3.86
4.0–4.5	Citric acid	50 m*M*	4.76
4.5–5.0	*N*-Methylpiperazine	20 m*M*	4.75
5.0–6.0	Piperazine	20 m*M*	5.68
5.5–6.0	L-Histidine	20 m*M*	6.15
5.8–6.4	bis-Tris	20 m*M*	6.50
6.4–7.3	bis-Tris propane	20 m*M*	6.80

The mobile phases were prepared fresh daily using demineralized distilled water, filtered using a 0.22-μm filter, and sonicated for 20 min prior to use. Since the HPLC pump (ISCO Model 2350) was constructed with stainless steel, all halide salts were scrupulously avoided in preparation of the mobile phases to prevent corrosion problems. Previous investigators in our laboratory have examined the effect of various ions on protein retention during PBC (*4, 24*); based on these results, ammonium sulfate [$(NH_4)_2SO_4$] was chosen as the electrolyte. Protein samples were also freshly prepared by dissolving the protein in the mobile phase to obtain a 1 mg/mL protein solution. The column was first equilibrated with the mobile phase for 30 min at a flow rate of 0.5 mL/min. The column temperature was maintained at 4°C throughout unless specified otherwise. The six proteins [ovalbumin (OVA), bovine serum albumin (BSA), β-glucosidase (β-GLU), transferrin (TRF), β-lactoglobulin (β-LAC), and superoxide dismutase (SOD)] were injected individually in 20 μL pulses. These injections were repeated until reproducible results were obtained.

RESULTS AND DISCUSSION

Effect of pH and Ionic Strength

The variation of the capacity factors with pH for the six proteins OVA, BSA, β-LAC, TRF, β-GLU, and SOD (injected as pure components) at different mobile phase ionic strengths are shown in Figs. 4 through 8. At low or zero ionic strengths, one expects all the proteins to elute near the void volume when the mobile phase pH is below their pI's because the repulsive double layer interactions dominate. This is indeed the case for

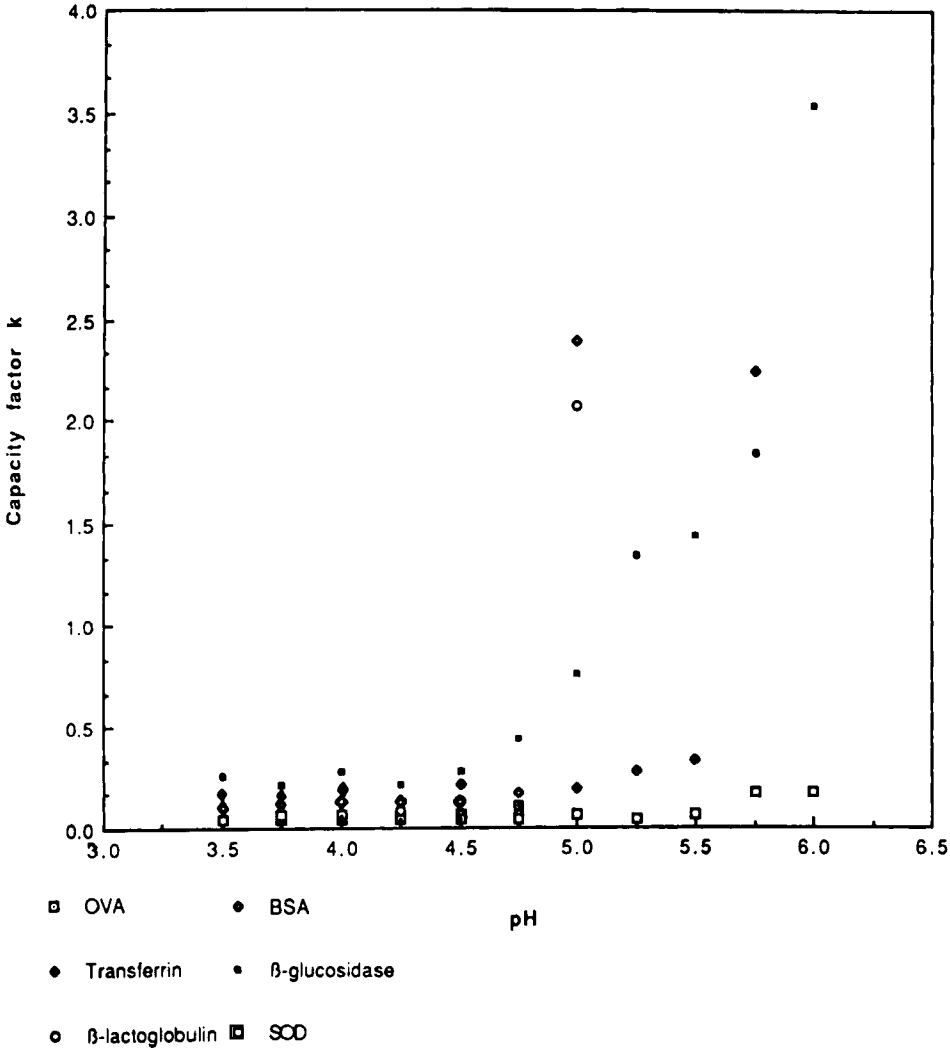

FIG. 4. Capacity factors for six proteins plotted as a function of mobile phase pH. No additional electrolyte added. Note that some of the proteins are permanently retained at their respective pI's and hence do not appear on the plot. Column: Pharmacia Mono Q (5 mm i.d. × 50 mm). Column temperature: 4°C. Mobile phase flow rate: 0.5 mL/min. Sample volume per injection: 20 µL. Individual protein concentrations: 1 mg/mL. Detector sensitivity: 0.5 AUFS.

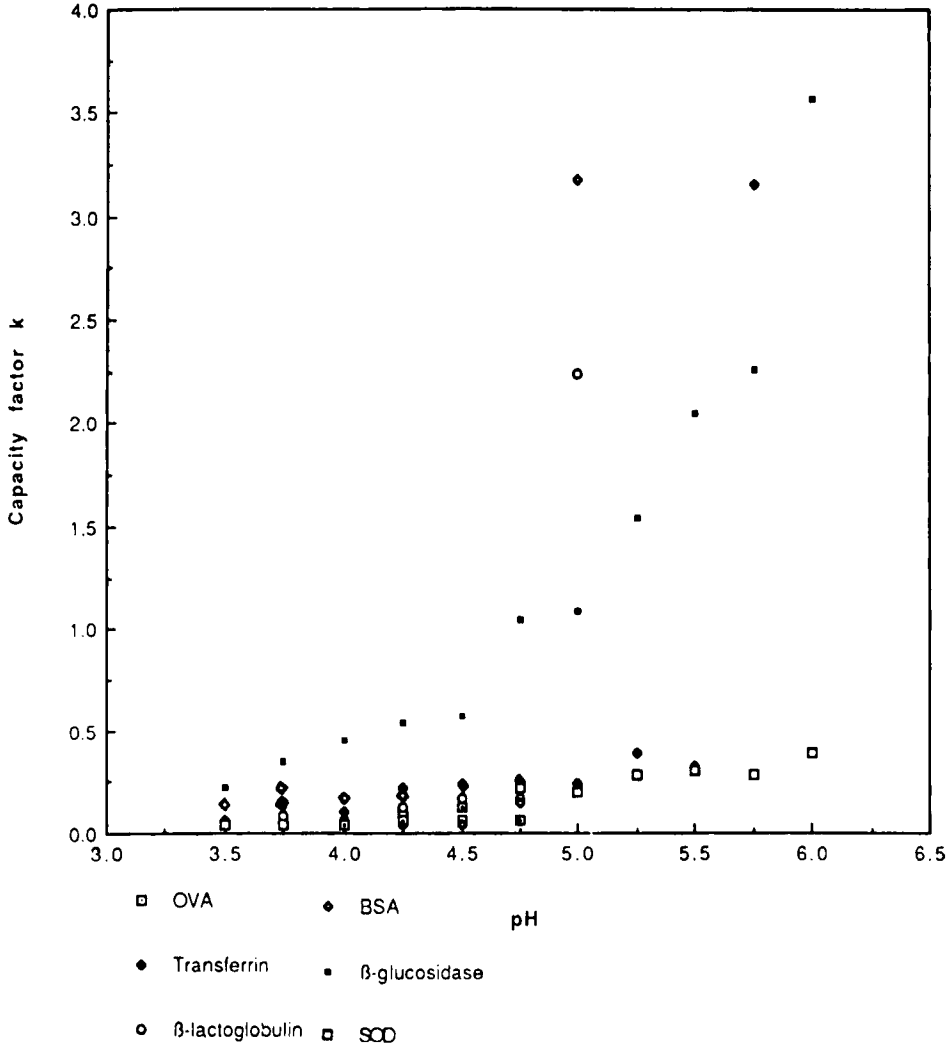

FIG. 5. Capacity factors for six proteins as a function of mobile phase pH. Electrolyte added: 0.005 M $(NH_4)_2SO_4$. All other details as given in Fig. 4.

most proteins, as seen from their very low capacity factors under such conditions (Figs. 4 and 5). However, as the ionic strength is raised, we find that all the proteins are retained for increasingly longer times as seen from their larger capacity factors (Figs. 6–8). It should be noted that at mobile phase pH values close to or above the pI's of the proteins, the corresponding proteins were permanently retained. At a pH of 5.0, OVA (pI = 4.6) is permanently retained on the column, and BSA (pI = 5.0) and β-lactoglobulin (pI = 5.2) give very broad peaks. Furthermore, if we wish to separate the proteins from a mixture, then there should be significant differences in their capacity factors at a given pH. It would seem that this is possible for the six proteins only if we operate in the pH range between 4.25 and 4.75.

In the qualitative discussions, we implied that the net charge is relatively uniformly distributed over the protein surface. In other words, the interactions between protein and adsorbent are repulsive when the net charge on the protein has the same sign as that of the adsorbent. Lesins and Ruckenstein (*57, 58*) have shown that there are conditions under which the charge is not uniformly distributed on the surface of the protein. Even though the net charge on the protein has the same sign as that of the adsorbent, attractive electrostatic interactions can occur. This can be explained by the fact that the protein exposes an oppositely charged patch to the adsorbent. It is easy to identify such conditions by operating at pH values such that the net charge on the protein is of the same sign as that of the adsorbent and then measuring the capacity factor k as the ionic strength is varied. If an oppositely charged patch is responsible for the electrostatic attraction, then increasing the ionic strength will correspondingly decrease the value of the capacity factor. On the other hand, if the interacting patch has the same sign as the net charge of the protein, then increasing the ionic strength will lead to an increase in the capacity factor because the repulsive double layer interactions are attenuated. Since we observe an increase in the capacity factors with increasing ionic strengths, we can assume that no oppositely charged patches exist on the proteins studied.

When a mixture of the six proteins was injected in the column at different mobile phase pH and ionic strengths, the best separation that could be achieved using an isocratic elution procedure was at pH 4.5 and ionic strength 0.01 M $(NH_4)_2SO_4$, as shown in Fig. 9. From the capacity factor data of the individual proteins (Fig. 6), one can anticipate that the peaks of OVA and SOD will overlap in Peak 1, while those of TRF and β-GLU will overlap in Peak 3. This is exactly what was observed, as seen in Fig. 9.

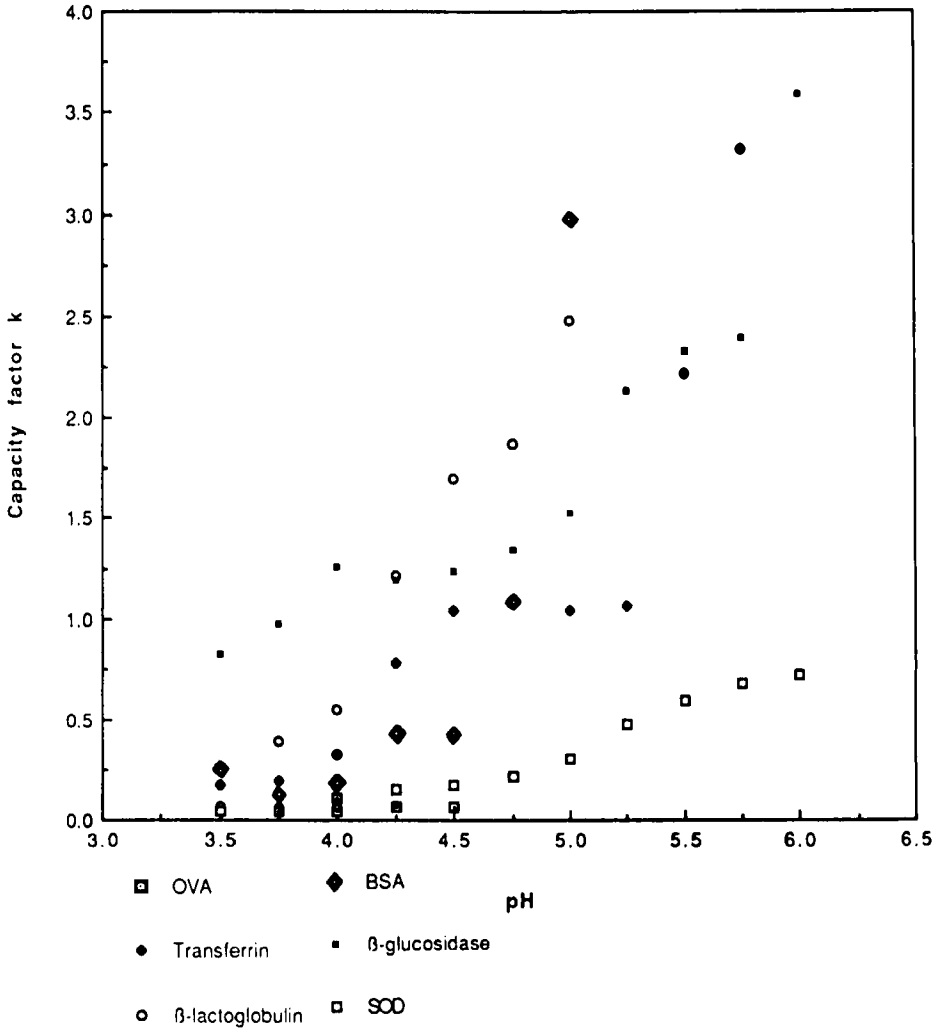

FIG. 6. Capacity factors for six proteins plotted as a function of mobile phase pH. Electrolyte added: 0.01 *M* $(NH_4)_2SO_4$. All other details as given in Fig. 4.

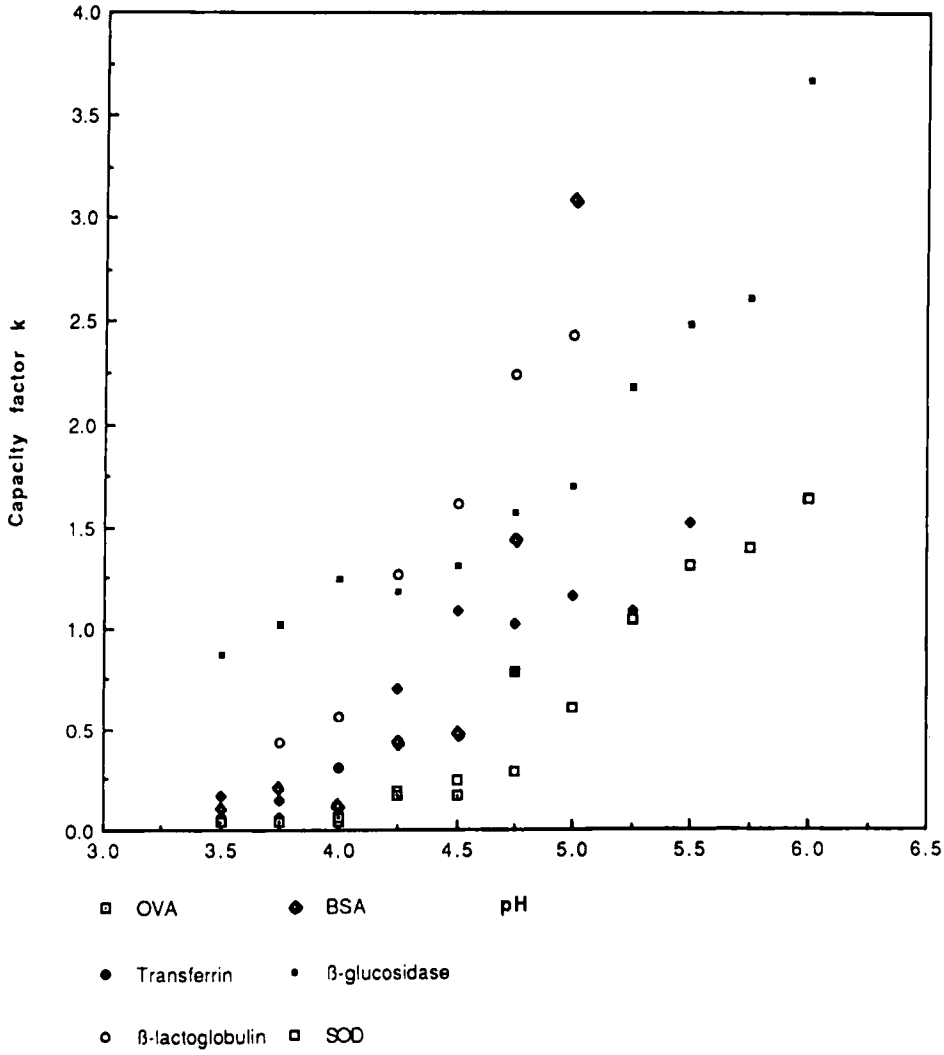

FIG. 7. Capacity factors for six proteins plotted as a function of mobil phase pH. Electrolyte added: 0.05 M $(NH_4)_2SO_4$. All other details as given in Fig. 4.

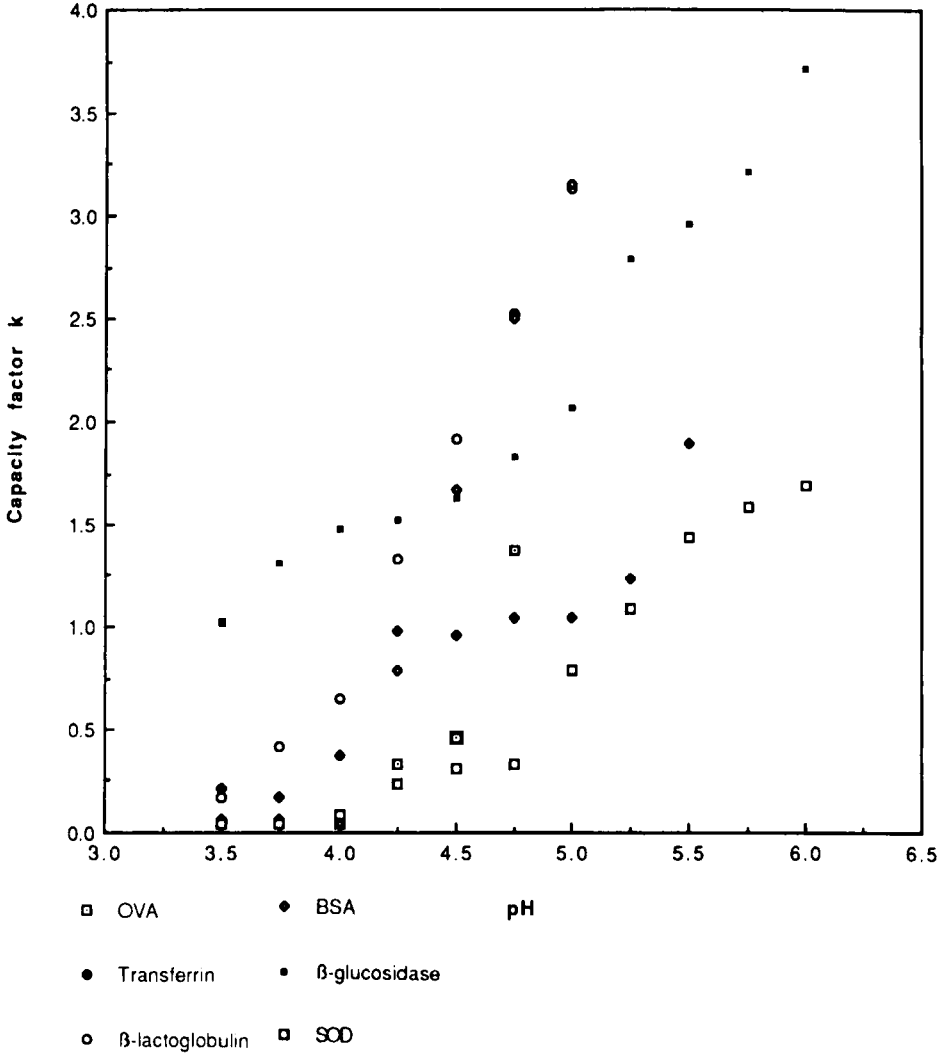

FIG. 8. Capacity factors for six proteins plotted as a function of mobile phase pH. Electrolyte added: 0.10 M $(NH_4)_2SO_4$. All other details as given in Fig. 4.

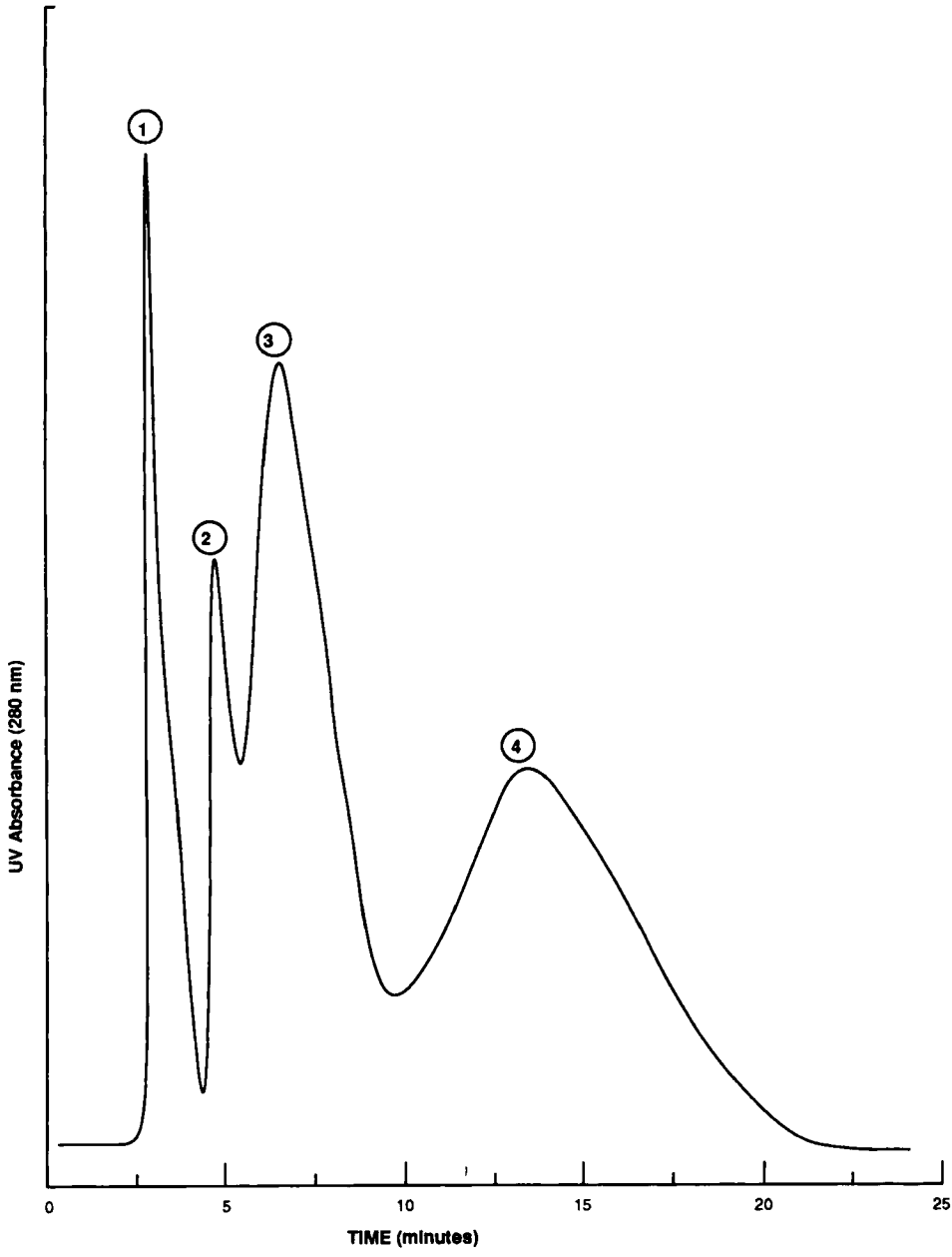

FIG. 9. Chromatogram showing the partial separation of a multicomponent protein mixture (β-GLU, SOD, OVA, β-LAC, TRF, and BSA) using an isocratic elution procedure. Peak 1, OVA and SOD; Peak 2, BSA: Peak 3, TRF and β-GLU; Peak 4, β-LAC. Mobile phase: 20 m*M* *n*-methylpiperazine buffer with 0.01 *M* $(NH_4)_2SO_4$ at pH 4.5. Sample volume of injection: 60 μL. All other details as given in Fig. 4.

Because of the large differences in the pI's of the proteins, it is quite difficult to separate the six proteins individually using a single-step isocratic elution procedure. Instead, a two-step elution procedure was used. First, a mobile phase of pH 6.0 and 0.005 M $(NH_4)_2SO_4$ ionic strength was passed through the column. When a mixture of the six proteins was injected, four of the proteins (OVA, BSA, β-LAC, and TRF) were permanently retained, while the other two (SOD and β-GLU) separated out and eluted from the column (Fig. 10a). The four proteins were retained due to the strong electrostatic attraction between the negatively charged proteins (whose pI is below the mobile phase pH) and the positively charged anion-exchange column. The other two proteins (SOD and β-GLU) are still positively charged (as their pI's are above the mobile phase pH) and hence experience double layer repulsions in the column. However, due to their significantly different interaction potentials, they were retained for different times and consequently separated out.

Next, the mobile phase conditions were changed to a pH of 4.5 and ionic strength of 0.01 M $(NH_4)_2SO_4$, and under these conditions the retained four proteins separated out and eluted as shown in Fig. 10b. Since this mobile phase pH was below the pI's of the proteins, they now experience significant double layer repulsion and hence elute out of the column. Once again, the differences in their interaction potentials result in the separation of these four proteins. It must be emphasized here that though initially four of the proteins are bound to the column in an ion-exchange mode, all proteins separated out in the PBC mode eventually.

Effect of Organic Solvents

The effects of various organic solvents on three selected proteins (β-GLU, BSA, and TRF) were studied for different conditions of mobile phase pH and ionic strengths. The capacity factors of these proteins decreased with the addition of organic solvents as seen in Figs. 11 through 16. One can also see that peak broadening was considerably reduced, especially in the case of β-GLU and BSA.

We have already stated that organic solvents can affect the structures of water and protein and alter the interactions between proteins and water. With the alcohols methanol, ethanol, and 2-propanol, one expects that the modification of the structure of water will be greater for the higher alcohols. This results in a reduction of the Hamaker constant A_{33} for the interaction between the water molecules, thereby lowering the overall Hamaker constant A_{132} and the attractive van der Waals interaction (Eqs.

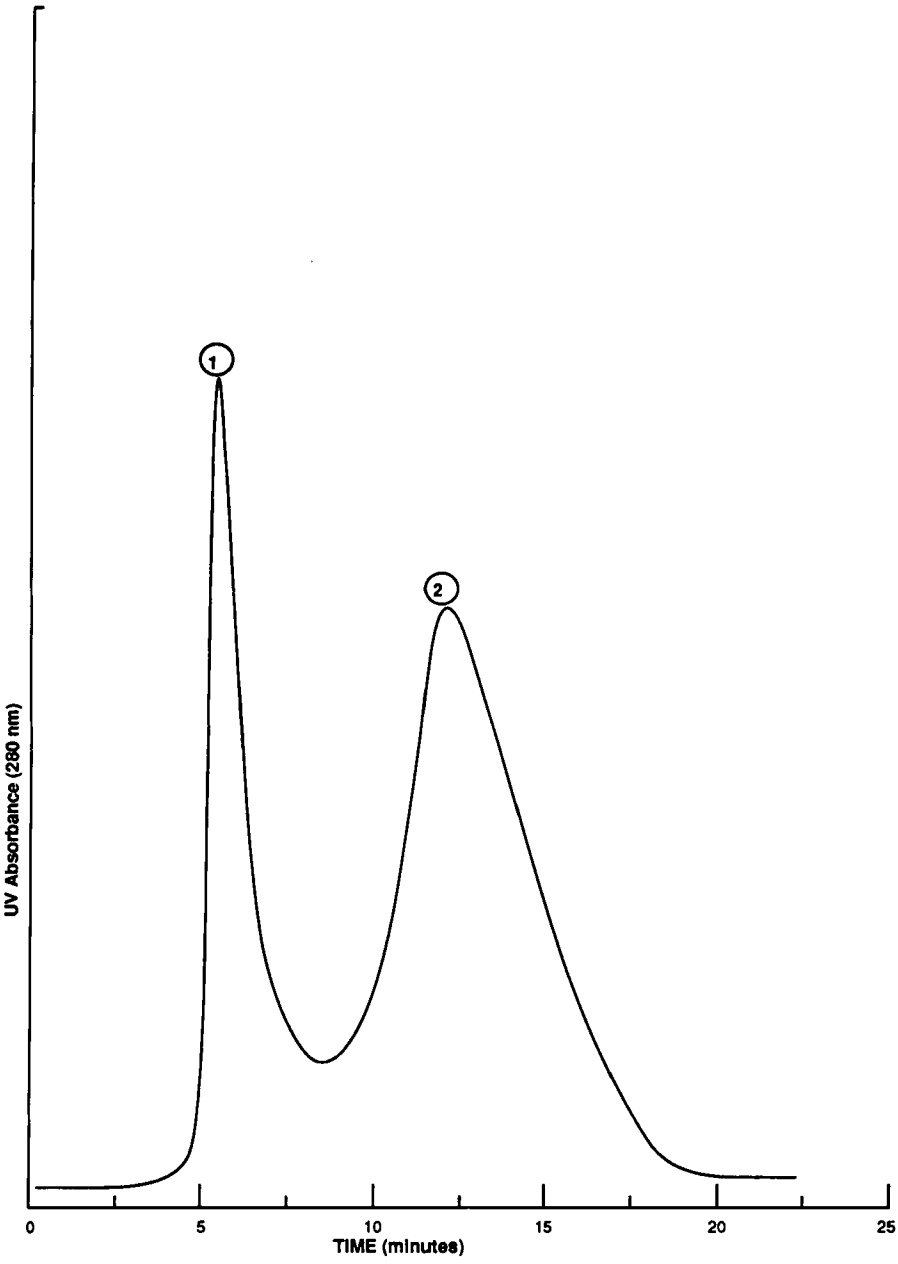

FIG. 10a. Chromatogram for the first step of the two-step elution of a protein mixture. The remaining four proteins are retained on the column. Peak 1, SOD; Peak 2, β-GLU. Mobile phase: 20 m*M* bis-Tris buffer with 0.005 *M* $(NH_4)_2SO_4$ at pH 6.0. All other details as given in Fig. 9.

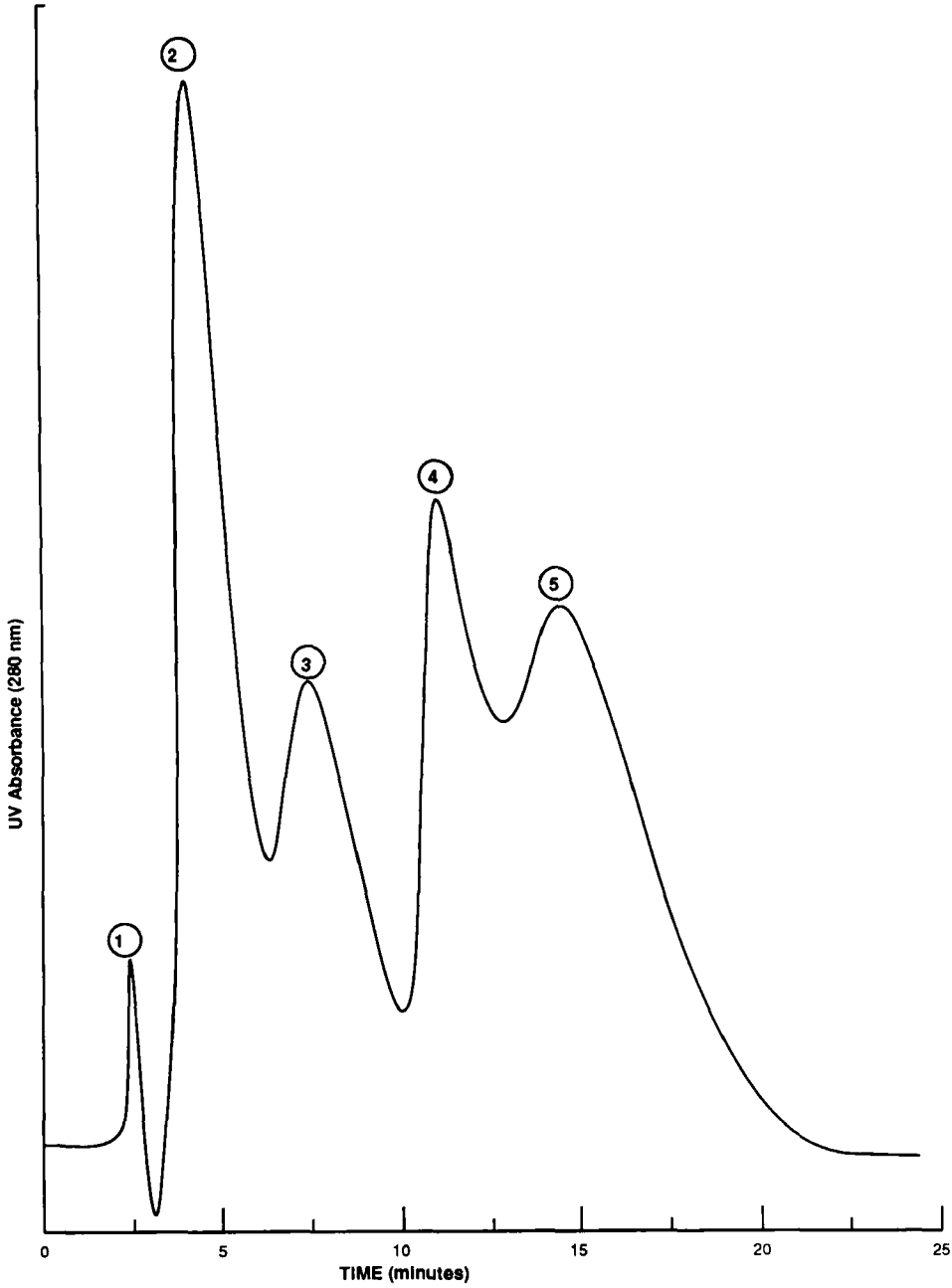

FIG. 10b. Chromatogram for the second step of the two-step elution procedure illustrating the separation of the four proteins retained during the first isocratic step. Peak 1, solvent; Peak 2, OVA; Peak 3, BSA; Peak 4, TRF; Peak 5, β-LAC. Mobile phase: 20 m*M* *n*-methylpiperazine buffer with 0.01 *M* $(NH_4)_2SO_4$ at pH 4.5. All other details as in Fig. 10a.

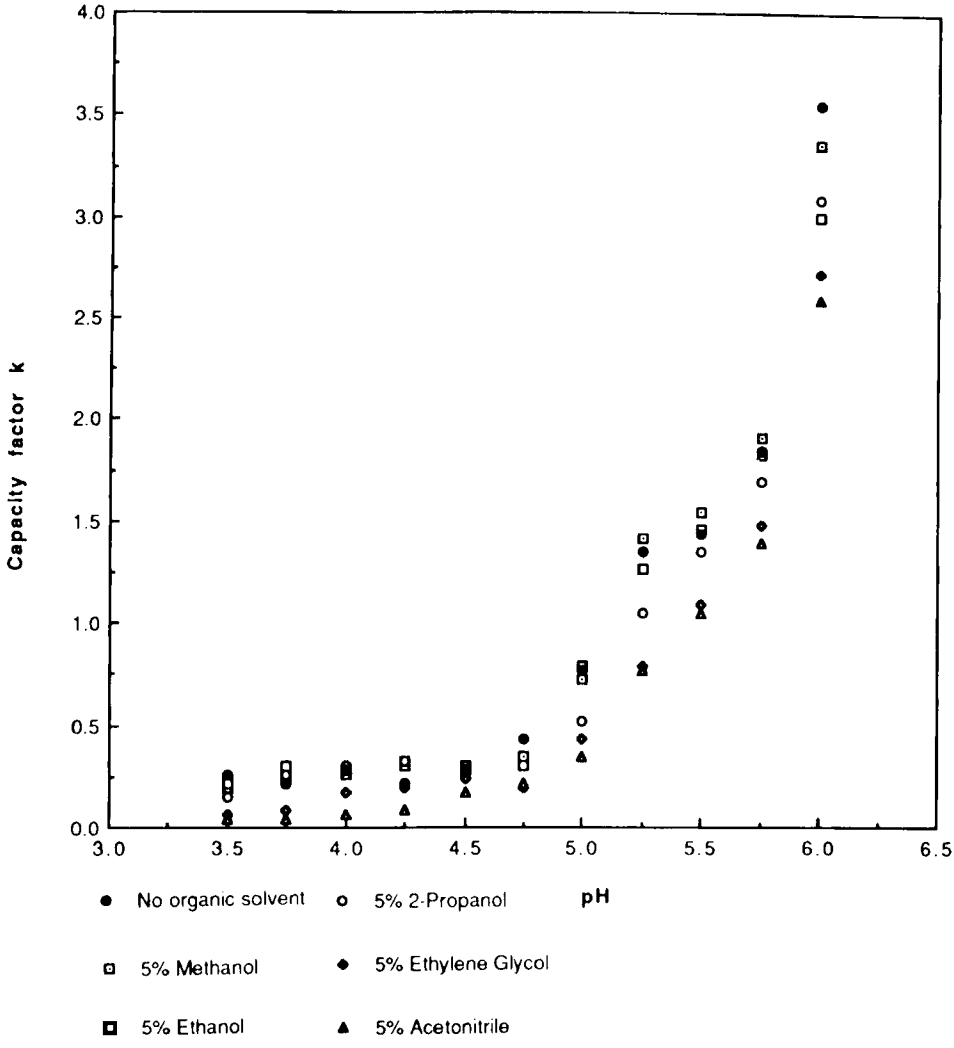

FIG. 11. Capacity factors for β-glucosidase for five diferent organic solvents added to the mobile phase. Electrolyte added: 0.005 *M* $(NH_4)_2SO_4$. Organic content is reported as volume/total volume basis. All other details as in Fig. 4.

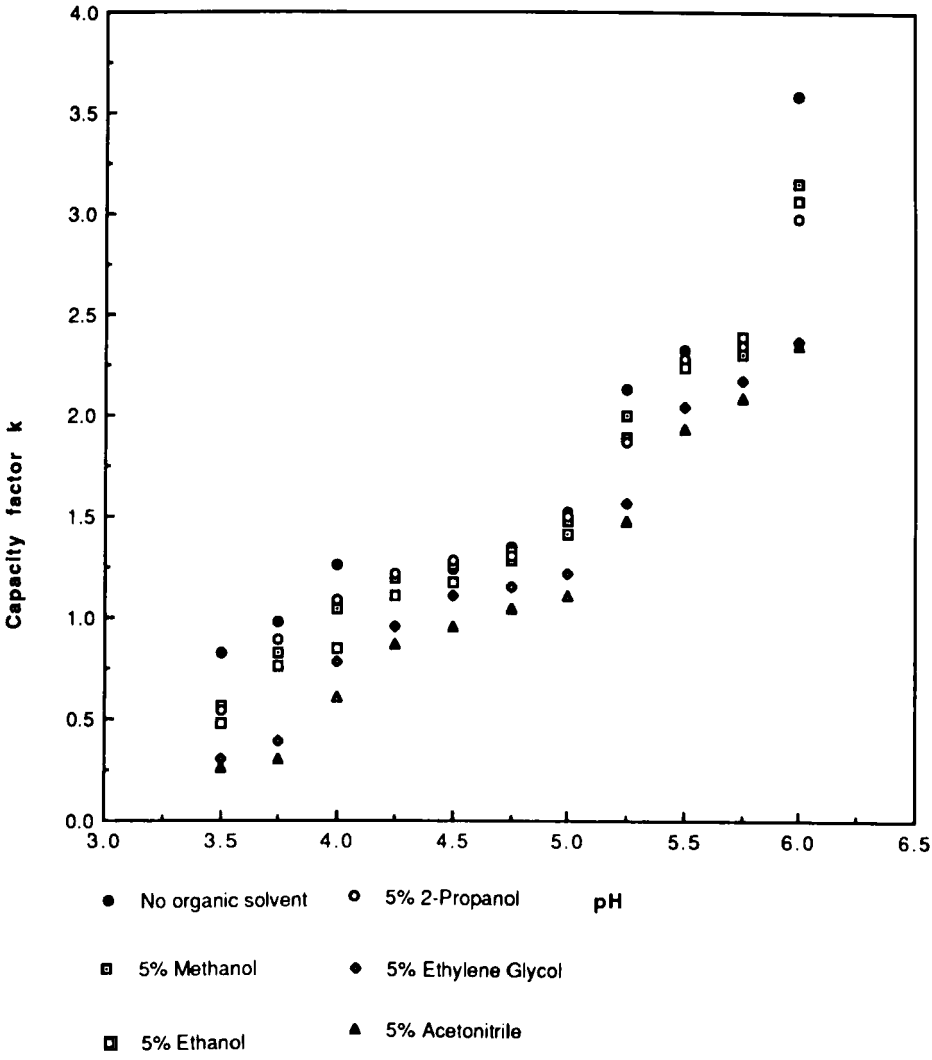

FIG. 12. Capacity factors for β-glucosidase for five different organic solvents added to the mobile phase. Electrolyte added: 0.01 M $(NH_4)_2SO_4$. All other details as in Fig. 11.

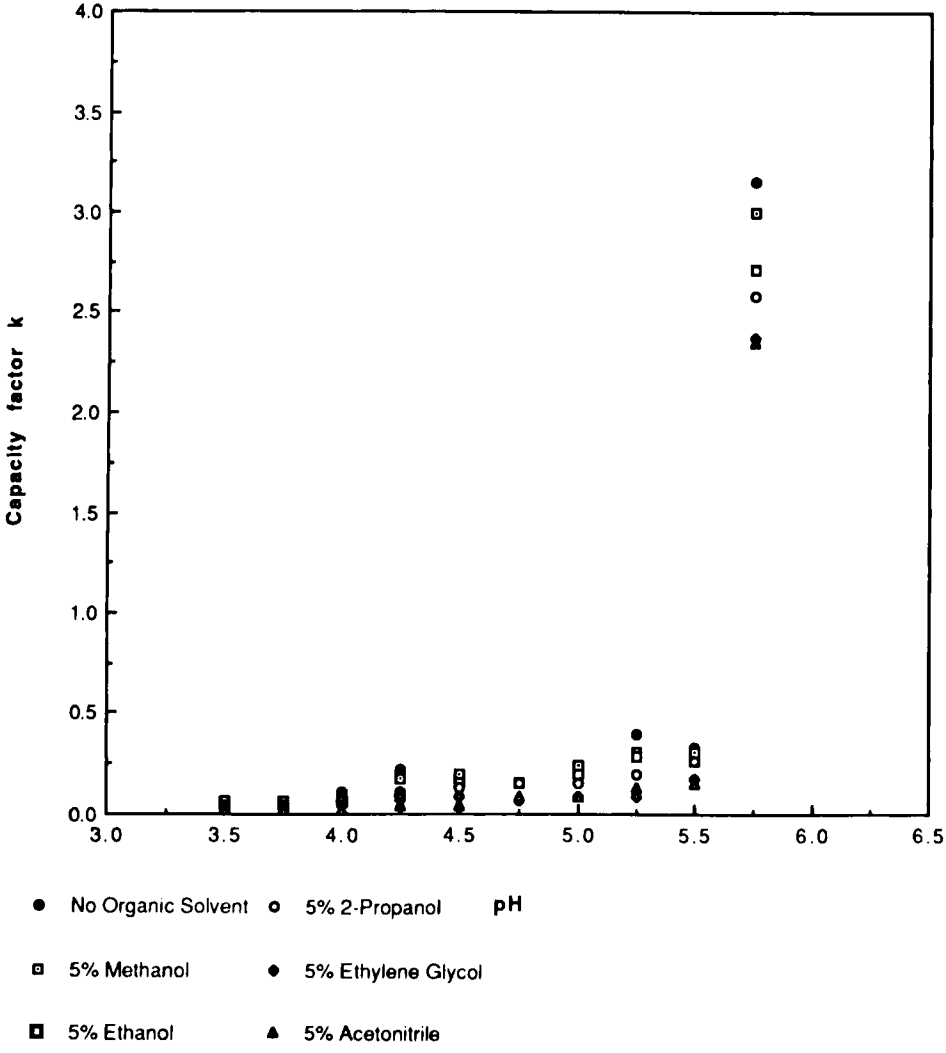

FIG. 13. Capacity factors for transferrin for five different organic solvents added to the mobile phase. Electrolyte added: 0.005 M $(NH_4)_2SO_4$. All other details as in Fig. 11.

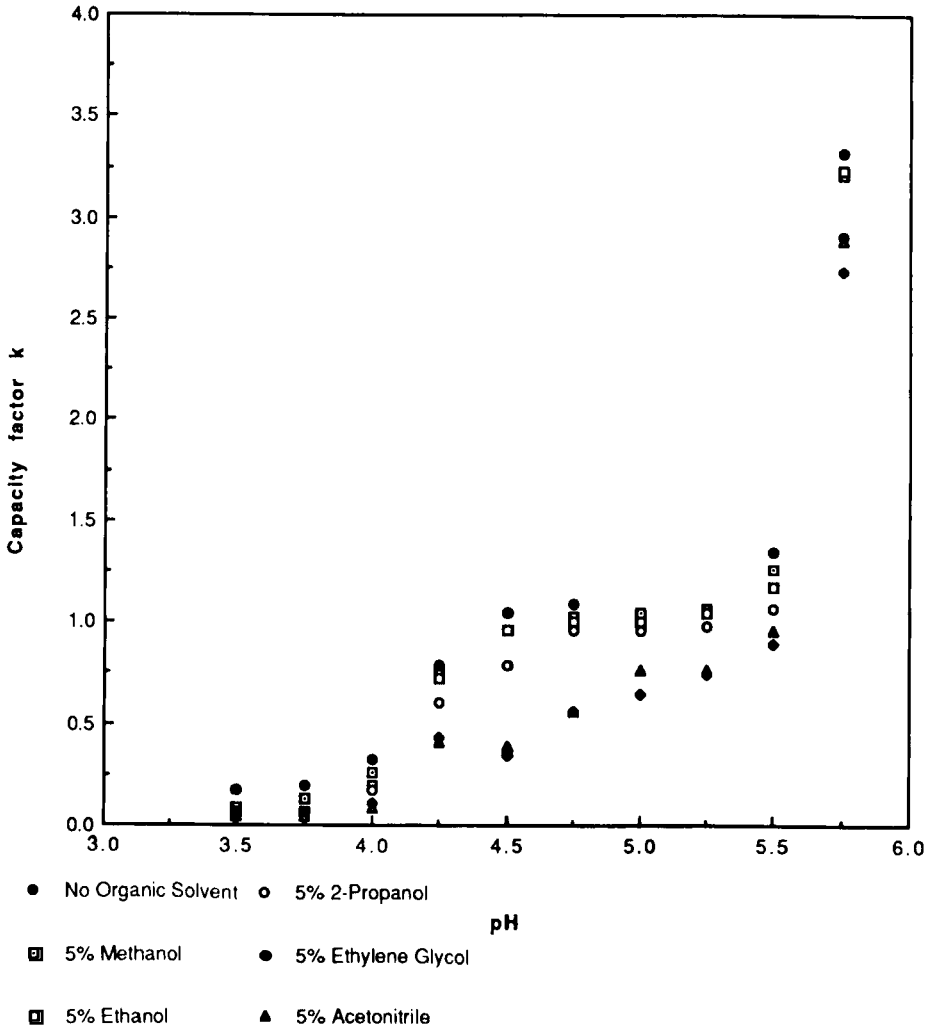

FIG. 14. Capacity factors for transferrin for five different organic solvents added to the mobile phase. Electrolyte added: 0.01 M $(NH_4)_2SO_4$. All other details as in Fig. 11.

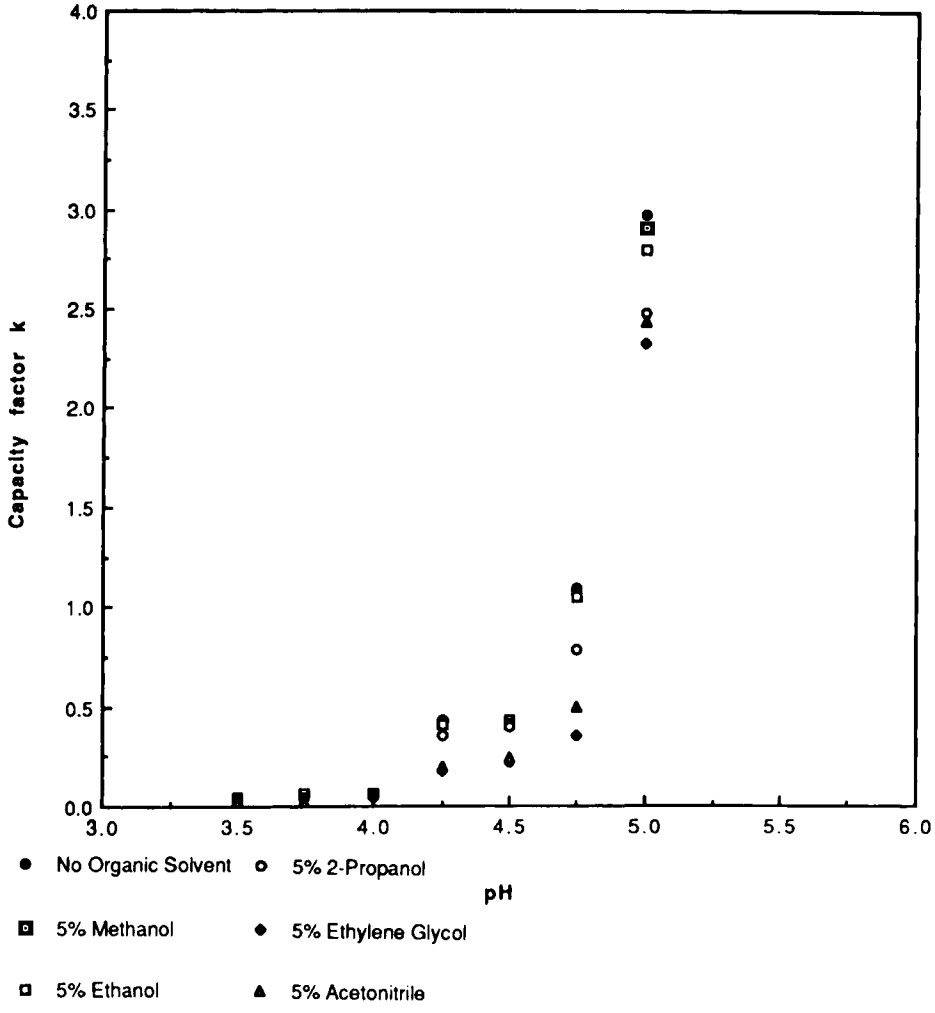

FIG. 15. Capacity factors for bovine serum albumin for five different organic solvents added to the mobile phase. Electrolylte added: 0.01 M $(NH_4)_2SO_4$. All other details as in Fig. 11.

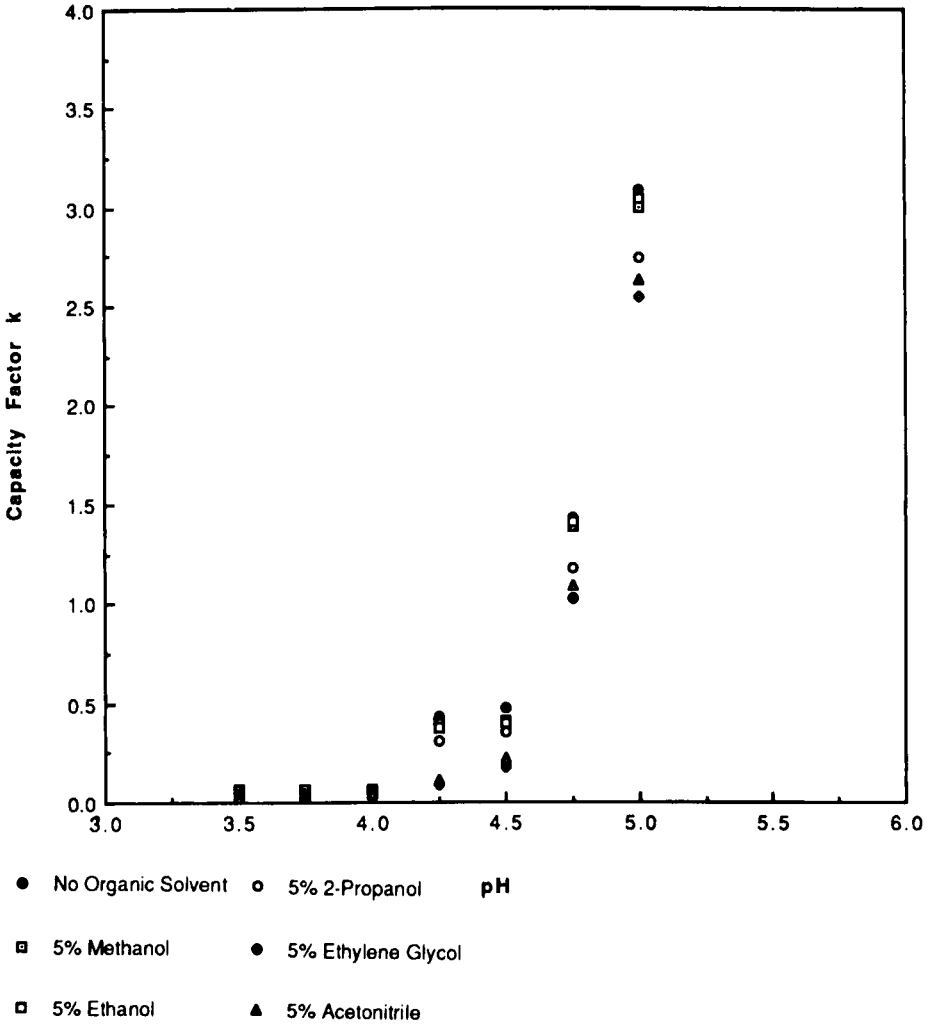

FIG. 16. Capacity factors for bovine serum albumin for five different organic solvents added to the mobile phase. Electrolyte added: 0.05 M $(NH_4)_2SO_4$. All other details as in Fig. 11.

3 and 4). In addition, the alcohols can adsorb onto the surface of the proteins and thus modify the protein-water interactions. It is likely that the hydrophobic moieties of these solvents adsorb onto the exposed hydrophobic regions of the proteins, while the polar part of these adsorbed molecules interact with the water molecules. This increases the value of the Hamaker constant A_{13} which further reduces the overall Hamaker constant A_{132} and thus attenuates the attractive van der Waals interactions between protein and adsorbent. As a result, the retention time is decreased.

The capacity factor data show that the order in which the alcohols affect the van der Waals attraction depends on the mobile phase pH conditions. This is probably because the pH affects the protein configuration (*24, 27, 28*), and hence the exposure of its hydrophobic regions, thereby affecting the adsorption of the alcohol molecules on the protein. The change in ionic strength influences only the double layer interactions, and not the van der Waals interactions. Though electrolyte ions can affect the structural order of water, the ionic strengths employed here are too low for any significant effect on the van der Waals interactions.

One can see that the other two solvents, ethylene glycol and acetonitrile, reduce the capacity factors much more than the alcohols. These solvents probably modify the structure of water to a greater extent and also have stronger interactions with the protein molecules as compared to the alcohols. This considerably decreases the interactions between the water molecules (A_{33}) and increases the protein-water interactions (A_{13}), which results in a significant reduction in the attractive van der Waals interactions between proteins and adsorbent.

The effect of these solvents on protein separations was examined next. Acetonitrile was found to be the most effective in improving the resolution of separation and in reducing the overall elution time. The chromatograms from the two-step elution procedure are shown in Fig. 17. Thus organic solvents can be used to modify the van der Waals interactions between proteins and adsorbent, thereby permitting faster and improved separations. In general, such additives lower the dielectric constant of the mobile phase. This enhances the double layer interactions at constant surface charges, but decreases the dissociation of the weak acidic groups and hence the charge. It is likely that the former enhancement and the latter attenuation cancel one another at low volume fractions of the organic additive.

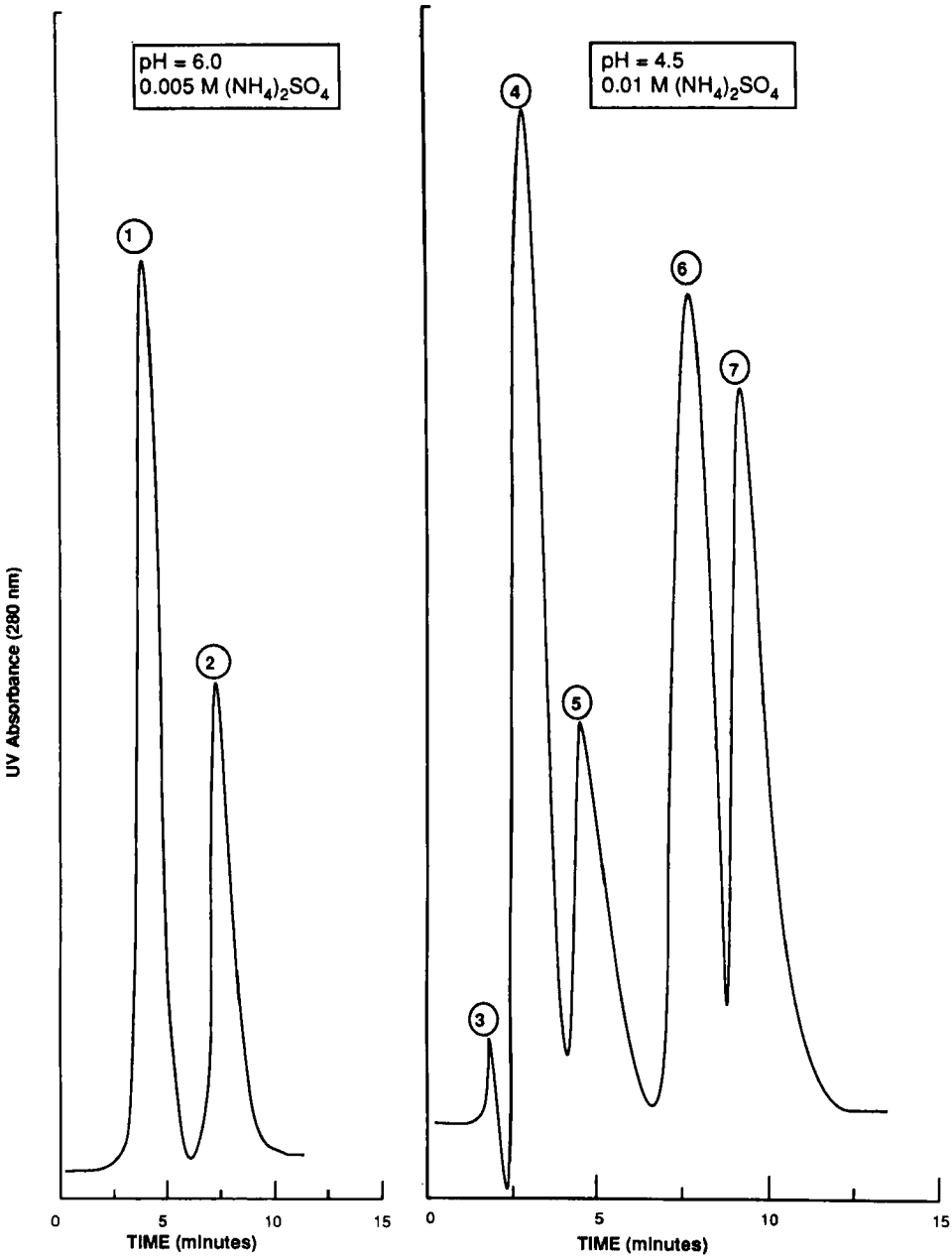

FIG. 17. Chromatogram illustrating the improved resolution in the separation of a multicomponent mixture of proteins during the two-step elution process when 5% volume/total volume acetonitrile is added to the mobile phase. First step: Peak 1, SOD; Peak 2, β-GLU. Second step: Peak 3, solvent; Peak 4, OVA: Peak 5, BSA; Peak 6, TRF; Peak 7, β-LAC. All other details as given in Figs. 10a and 10b.

Effect of Temperature

The capacity factors of the six proteins plotted as a function of column temperature (Fig. 18) show a slight effect with increasing temperature. In the case of the enzymes β-GLU and SOD, two major peaks were observed at higher temperatures. This is probably due to the denaturation of the enzymes, which results in separate elution of the active and denatured forms. Though Eqs. (3), (6), (7), and (9) show a functional relationship between the interaction potentials and temperature, the other physicochemical properties of the system, which also determine the interaction potentials (e.g., dielectric constant, Hamaker constant, etc.), are themselves functions of temperature. Furthermore, altering the temperature causes conformational changes in the protein structure, which cannot be predicted easily. Since it is quite difficult to predict the effect of temperature on the total interaction potential, it is best to operate under temperature conditions which ensure only minimum denaturation of proteins.

CONCLUSION

Retention studies of six model proteins, using a strong anion-exchange column, have shown a definite functional relationship between their capacity factors and changes in mobile phase pH and ionic strength in the potential barrier chromatography (PBC) mode. PBC, which is based on opposing van der Waals attractions and double layer repulsions between proteins and adsorbent, has been successfully utilized to separate a multicomponent protein mixture using a two-step elution procedure. The double layer repulsions are controlled by altering the mobile phase pH and ionic strength, while the attractive van der Waals interactions are modified by adding small amounts of organic solvents to the mobile phase. Among the five organic solvents examined, ethylene glycol and acetonitrile had the greatest effect on attenuating the van der Waals interactions as evidenced by the considerably lower capacity factors of the proteins β-glucosidase, bovine serum albumin, and transferrin. Though the addition of organic solvents does not in itself achieve separation of a protein mixture, their presence can lead to improved resolution of separation and shorter elution times. Finally, an optimum column temperature has to be chosen to avoid enzyme denaturation. Since these separations were achieved using isocratic elution conditions, this allows easier scale-up of PBC, and could prove to be very valuable in preparative chromatography. Furthermore, due to the mild aqueous mobile phase conditions and short contact times with the chromatographic adsorbent, there is very

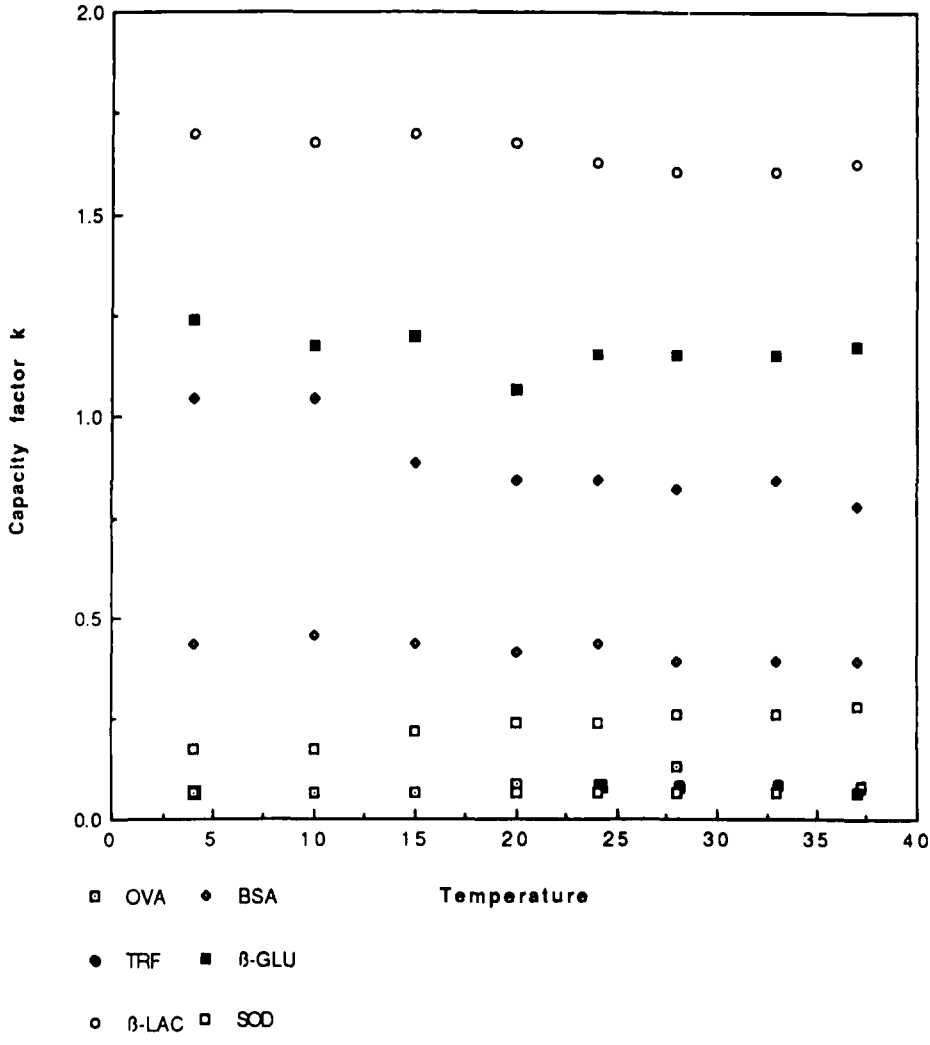

FIG. 18. Capacity factors for six proteins plotted as a function of column temperature (in °C). Mobile phase: 20 m*M* *n*-methylpiperazine buffer with 0.01 *M* $(NH_4)_2SO_4$ at pH 4.5. All other details as given in Fig. 4.

little likelihood of protein denaturation, and this is very useful for enzyme purifications. Also, by carefully choosing appropriate mobile phase conditions of pH, ionic strength, organic content, and temperature, it should be possible to separate natural mixtures of closely related proteins, such as isozymes, using PBC.

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