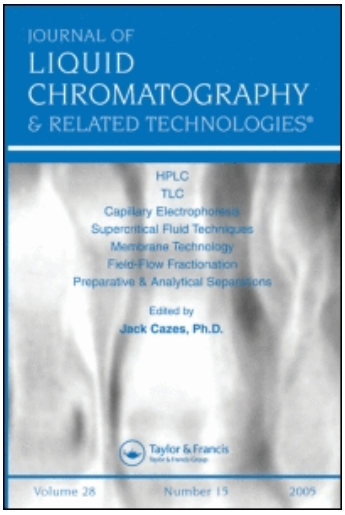

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THE EFFECT OF MOBILE PHASE ON PROTEIN RETENTION AND RECOVERY USING CARBOXYMETHYL DEXTRAN-COATED ZIRCONIA STATIONARY PHASES

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ABSTRACT

We have examined the effect of mobile phase conditions on the elution and recovery of proteins from a carboxymethyl dextran coated zirconia stationary phase. Mobile phases containing the Lewis bases phosphate and fluoride were examined as a function of concentration. We found that proteins only eluted from the stationary phase when some minimum concentration of Lewis base was present in the mobile phase. The effect of ionic strength was also examined. We found that a fairly high ionic strength is needed to elute the proteins with good recovery. The concentrations of the Lewis bases and the ionic strength needed to elute the proteins depended on the identity of the protein and the Lewis base. The pH of the mobile phase also plays a role; when the pH of the system is below the isoelectric point of a protein, it is difficult to elute that protein.

INTRODUCTION

The use of zirconia as a stationary phase support for HPLC has been of great interest in our laboratory.[1] The advantages of zirconia include excellent chemical stability (stable in solution from pH 1 to 14), excellent thermal stability (up to at least 100 °C),[2] and excellent mechanical stability. One particular area of research is the use of polymer coated zirconia materials for bioseparations. The advantages of zirconia can be coupled with a polymer coating that imparts useful surface chemistry to the material. Polymer gels are widely used for biochemical separations due to their excellent biocompatability. However, these gels are not mechanically strong and cannot be used at high flow rates and/or in organic solvents. This problem can be overcome by coating a useful polymer on a rigid support material, such as silica or zirconia. Many examples of polymer-coated silicas have been reported.[3,4,5] However, silica is inherently unstable at high pHs. Bonded phase and coated silicas also have problems with irreversible binding of proteins due to silanol-protein amine interactions, coulombic interactions, or hydrophobic interactions.[6] Zirconia is stable under these conditions, but is problematic in other ways. The surface of zirconia contains several types of sites, the most important chromatographically being the strong, hard Lewis acid sites.[7] These sites arise from the unsatisfied coordination of the surface zirconium atoms. The hard Lewis acid sites will adsorb any hard Lewis bases present in the mobile phase, including, but not limited to, phosphate, fluoride, hydroxide and carboxylic acids.[8,9] As proteins have many carboxylate groups, adsorption of a protein onto a zirconia surface is likely. Thus, we hoped that the polymer coating would shield the Lewis acid sites from the proteins. We have successfully used poly(ethyleneimine) coated zirconia to separate proteins[10] and nucleic acids[11] via ion exchange chromatography. Poly(butadiene) coated zirconia used in the reverse phase mode, has proven to be only marginally successful in the separation of proteins, due to the multiplicity of interactions encountered.[12] The polymer coating imparts the desired hydrophobic character to the phase; however, the zirconia surface also has Lewis acid sites that will adsorb proteins and other Lewis bases. In order to block these surface sites, a strong Lewis base (phosphate) is included in the mobile phase. This in turn creates a negatively charged surface which will retain proteins by coulombic interactions. Counteracting these interactions requires a high concentration of a non-interacting salt to screen the ion exchange sites. Thus, a complicated mobile phase is needed in order to counteract all of these interactions with the proteins. In this paper we examine a hydrophilic polymer (dextran) for use in the chromatography of proteins.

Several reports have examined the coating of dextran on silica for use in protein chromatography.[13,14,15,16,17] These materials include both size exclusion and affinity phases. We have recently reported the synthesis of carboxymethylated dextran (CMD) coated zirconia.[18] The dextran coating is

highly hydrophilic and easily derivatized, owing to its many pendant hydroxyl groups. These alcohol groups can be derivatized with a variety of ligands to create a useful chromatographic phase. We are currently working on an affinity phase based on the CMD-zirconia that has Cibicron Blue groups attached to the CMD polymer.

Before further derivatizing the coated dextran, the behavior of proteins on the CMD-zirconia was explored. In particular, we were interested in identifying any non-specific interactions that might subsequently interfere in the later use of the CMD-zirconia material as a support for affinity chromatography. We had hoped that the carboxymethyl-dextran coating would shield the Lewis acid sites from the proteins. However, previous studies using different probes have shown that this is not the case and the Lewis acid sites are still available to the solutes.[18] Blackwell[19] has shown that proteins can be eluted with fluoride ion (a hard Lewis base) in the mobile phase. We believe that the Lewis acid/base and ion exchange interactions are the dominant interactions on the CMD coated zirconia, and we will investigate these retention mechanisms by measuring the effects of Lewis base concentration, the mobile phase ionic strength, and pH on protein retention and recovery.

MATERIALS

Zirconia colloid (1000 Å in nitric acid, nominal pH 2, 20% solids by weight, Lot IV-40) was obtained from Nyacol Corp. (Ashland, MA). The dextran (9300 MW), the piperazine-N,N'-bis-[2-ethanesulfonic acid] (PIPES), 2-[N-morpholiono]ethanesulfonic acid (MES), iodoacetic acid and all proteins were obtained from Sigma Chemical Co. (St. Louis, MO).

The relevant physical characteristics of the proteins are listed in Table 1. Dibasic potassium phosphate (reagent grade), sodium fluoride (reagent grade), sodium sulfate (reagent grade) and sodium chloride (reagent grade) were obtained from Mallinckrodt (Paris, Kentucky). Urea (ACS grade) and the 50% (w/w) solution of sodium hydroxide were obtained from Fisher Chemical (Fair Lawn, NJ). Boron trifluoride etherate was obtained from Aldrich (Milwaukee, WI). Sephadex G-10 SEC material was obtained from Pharmacia (Piscataway, NJ).

All water was deionized and then passed through Barnstead ion exchange and Organic Free cartridges followed by a 0.45 μm filter. All water was also boiled for 15 minutes to remove dissolved carbon dioxide prior to use.

Table 1

Protein Solute Sources and Physical Characteristics

Protein	Source	Mw[a]	pI[b]	Symbol
Cytochrome c	horse heart	11,700	9.4	■
Ovalbumin	chicken egg	45,000	4.9	X
Albumin	bovine serum	66,300	4.7	▼
Peroxidase	horseradish	34,000	9	▲
Laccase	pyricularia oryzae	62,000	3.2	⬢
Lysozyme	chicken egg white	14,310	11.0	◆
Transferrin	human	78,000	5.9	$
Hexokinase	bakers yeast	104,000	5	#

[a] molecular weight
[b] isoelectric point

METHODS

Preparation of Zirconia Particles

Zirconia particles for chromatography were prepared from colloidal zirconia by the polymerization induced colloidal aggregation (PICA) method (batch Coac-15).[20] The colloid had been centrifuged to remove fines and then resuspended in a 1% nitric acid solution. Particles were sintered at 750 °C for 6 hours, followed by treatment at 900 °C for 3 hours in a muffle furnace.[20] The particles were then pre-treated in a series of chemical steps. The particles were placed in a glass Erlenmeyer flask and freshly boiled water was added to just cover the surface of the particles. The slurry was then sonicated under vacuum for 10 minutes to remove air from the pores of the particles. This slurry was transferred to a plastic bottle. The water was decanted. Freshly made 0.5 M sodium hydroxide (made from 50% by weight solution) was then added so that the final volume was five volumes the volume of the particles. The slurry was placed on a shaker bath for 24 hours. The particles were allowed to settle and the supernatant was decanted. The particles were then rinsed with water. Freshly made 0.5 M nitric acid was then added in the same proportion as the sodium hydroxide. The slurry was then placed on a shaker bath for 24 hours. The supernatant was then decanted and the particles were rinsed twice with water. They were then dried under vacuum at 110 °C overnight.

The surface area and pore volume of the particles were measured from the nitrogen adsorption isotherm using a Micromeritics ASAP 2000 porosimeter. Pore diameters were estimated using the BJH equation, which assumes

cylindrical pores.[21] Surface areas were estimated using the BET method from the nitrogen sorptometry data.[22] The physical characteristics of these particles are the same as in Reference 18.

Preparation of Carboxymethyl-dextran Coated Zirconia

Carboxymethylated dextran was prepared and coated onto zirconia as described previously.[18] A 0.1 g sample of the CMD was dissolved in 50 mL of 100 mM PIPES, pH 6.5 to make a 5 g/L solution of CMD. To 40 mL of this solution, 4 g of zirconia particles were added. This suspension was sonicated under vacuum for five minutes and then capped. The bottle was then placed on a shaker bath for 2 days, with periodic manual shaking.

After the allotted time, the particles were allowed to settle and the supernatant was decanted. Ethanol (40 mL) was then added and the slurry was shaken for ten minutes. The particles were allowed to settle for thirty minutes and the ethanol was decanted. This procedure was repeated for 50:50 ethanol:chloroform (v:v) and chloroform. The particles were then allowed to air dry at room temperature.

The coated particles were placed in a 30 mL septum flask and 10 mL of chloroform were added. The flask was capped and sonicated for 5 minutes. 1,4-butanediol diglycidyl ether (BUDGE) (17 µL) was added via syringe and the flask was sealed while flushing with nitrogen. A boron trifluoride etherate solution in chloroform was prepared using 0.5 mL of boron trifluoride etherate and 7 mL of chloroform. 0.5 mL of this solution was then added to the flask containing the coated particles. The particle suspension was swirled and allowed to sit for 30-40 minutes. After this time, the solution was removed and the particles were rinsed with ethanol and allowed to dry at room temperature.

Chromatography

Carboxymethyl-dextran-coated zirconia was packed in 5.0 cm x 0.46 cm stainless steel columns by the stirred upwards slurry technique in HPLC grade isopropanol.[23] The Sephadex G- 10 column was packed by gravity filtration in a 5.0 cm x 0.46 cm stainless steel column. All columns used 0.45 µm screens (Chromtech, Apple Valley, MN) in the end fittings instead of the thicker frits to reduce protein adsorption.[24] Experiments were run on a HP 1090L Liquid Chromatograph with diode array detection and a Chemstation data handling system. Protein recovery was calculated by Equation 1.

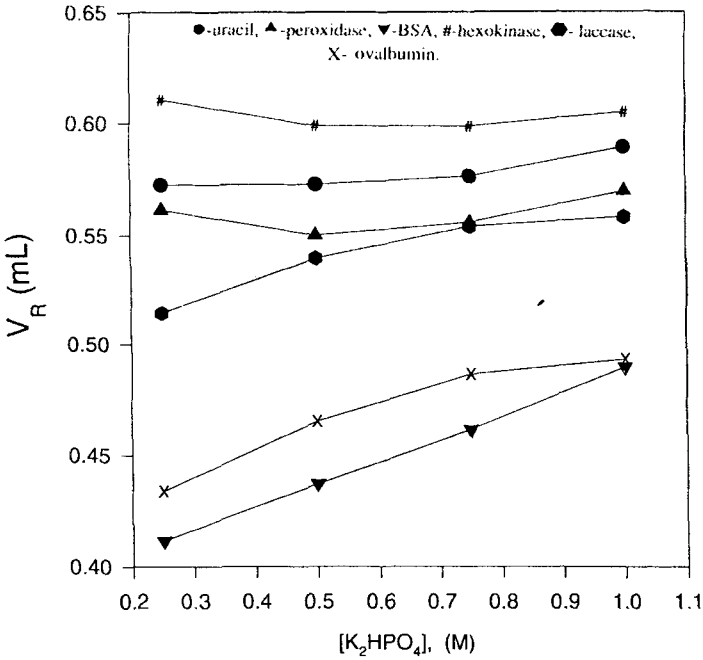

Figure 1. Effect of phosphate concentration on protein elution volume. The mobile phase was potassium phosphate at various concentrations, pH 7. Conditions: The flow rate was 0.5 mL/min. Proteins were 1% by weight in the mobile phase buffer. Detection was at 280nm.

$$\% \text{ Recovery} = \frac{\text{Area}_{\text{CMD-ZrO}_2}}{\text{Area}_{\text{Seph}}} \text{X}(100\%) \qquad (1)$$

where $\text{Area}_{\text{CMD-ZrO}_2}$ is the area count of the peak (as measured by the data system) from the CMD-ZrO_2 column and $\text{Area}_{\text{Seph}}$ is the area count from a Sephadex G-10 column which, we assume, will have no interactions with the solutes used. Area counts are obtained from experiments using protein injections from the same solution and are performed under the same conditions. The area counts used for the calculation are averages of two or more measurements. All other parameters are listed in the figure captions. Retention volumes were calculated from the retention times, as measured by the integrator or integration program (i.e. the peak maximum), multiplied by the flow rate. All retention volumes are averages of two or more separate experiments.

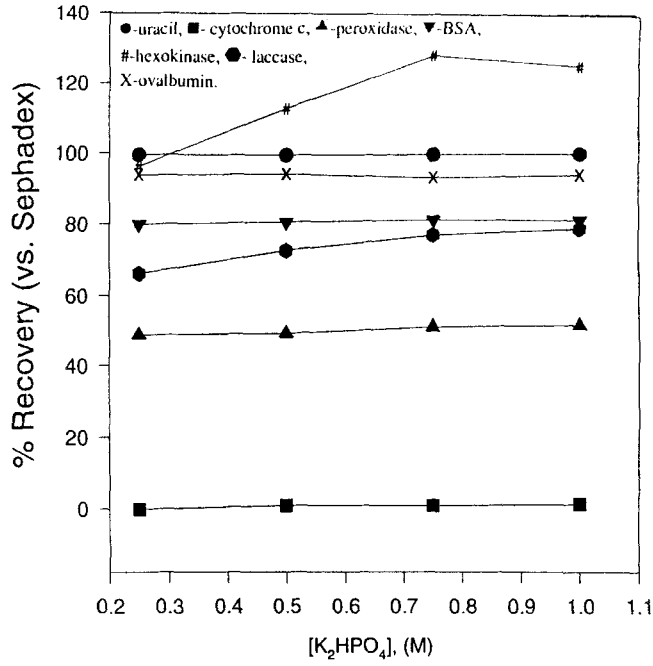

Figure 2. Effect of phosphate concentration on protein recovery. Protein recovery is expressed as the percentage of the area measured compared to that on a column of identical size packed with Sephadex G-10 under the same conditions. All other conditions the same as in Figure 1.

RESULTS AND DISCUSSION

Effect of Phosphate and Fluoride Concentration

In order to investigate the Lewis acid/base interactions, the concentrations of two strong, hard Lewis bases (phosphate and fluoride) were varied and the effect on protein retention and recovery was observed. If Lewis acid/base interactions were important, we would expect that the protein retention volume would decrease and protein recovery would increase as the Lewis base concentration in the mobile phase was increased. Figures 1 and 2 show the effect of phosphate concentration on the retention (Figure 1) and recovery (Figure 2) of various proteins. Cytochrome c (see ■ in Figure 2) does not elute at the lowest concentration of phosphate and its recovery is very poor in all cases. We attribute this to the high charge on this protein at pH 7. At this pH, cytochrome c will have a positive charge and will be strongly retained on the

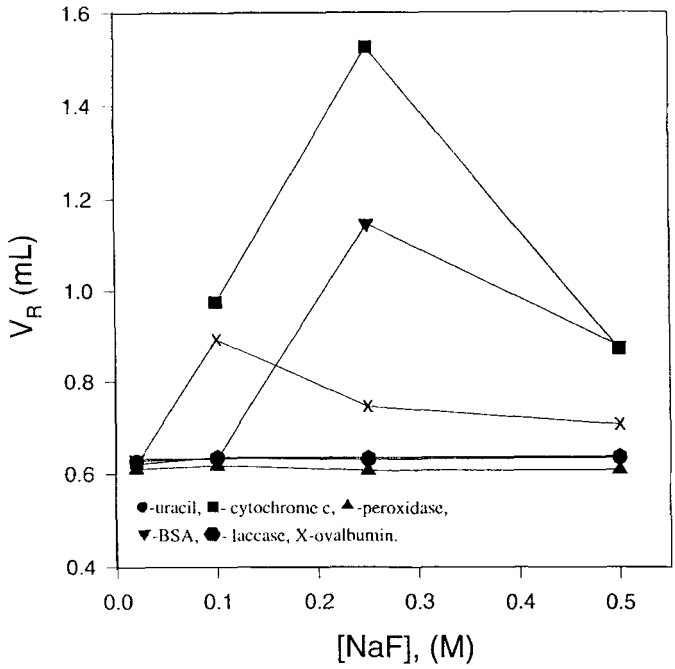

Figure 3. Effect of fluoride concentration on protein elution volume. The mobile phase was 20 mM MES + 250 mM Na_2SO_4 + the fluoride concentration shown at pH 5.5. Other conditions are the same as in Figure 1

negatively charged phosphate adsorbed on the zirconia surface. The small size of the protein also allows it to penetrate the dextran layer and interact strongly with the surface. Peroxidase, which also has a high pI but is larger (about twice the molecular weight), has a higher recovery, although it is still only about 50% recovered. The other proteins show little, if any, change in retention or recovery as the phosphate concentration is changed. Ovalbumin, BSA and laccase all show a slight increase in retention volume as the phosphate concentration is increased. However, they are all eluted before the totally included volume, as measured by uracil. At pH 7, these proteins are negatively charged, as is the zirconia surface. Thus, they are kept from entering the pore space by an ion exclusion mechanism. As the phosphate salt concentration (as well as the ionic strength) is increased, the surface charge is screened from the protein's charge, allowing the protein access to more of the pore space and thus increasing the elution volume. The recoveries of these proteins, however, does not change and are all near 100%.

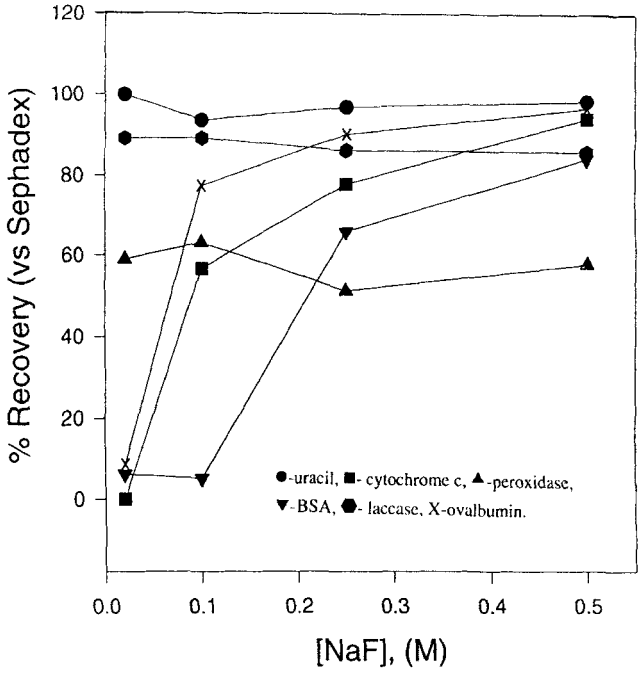

Figure 4. Effect of fluoride concentration on protein recovery. Protein recovery is expressed as the percentage of the area measured compared to that on a column of identical size packed with Sephadex G-10 under the same conditions. All other conditions are the same as in Figure 3.

The effect of the fluoride concentration on protein retention and recovery (Figures 3 and 4) is quite different from that of the phosphate mobile phases. The recoveries of several of the proteins (cytochrome c, BSA and ovalbumin) are very low at the lowest fluoride concentration (20 mM). However, the recovery improves remarkably as the concentration of sodium fluoride is increased. In sharp contrast to phosphate systems, even cytochrome c is eluted with close to 100% recovery when the fluoride concentration is increased to above 0.25 M. The retention volumes of three of the proteins change over the concentration range examined. Cytochrome c is very sensitive to the amount of fluoride present, as are ovalbumin and BSA. The retention behavior of ovalbumin and BSA (hydrophobic proteins)[25] is possibly due to the increased contribution of hydrophobic interactions as the ionic strength increases. The behavior of the cytochrome c can be fully rationalized as follows. At 20 mM sodium fluoride, there is not enough fluoride to block the Lewis acid surface sites and the cytochrome c is irreversibly retained. As the fluoride concentration is increased, the Lewis acid sites are blocked by fluoride adsorption, but a

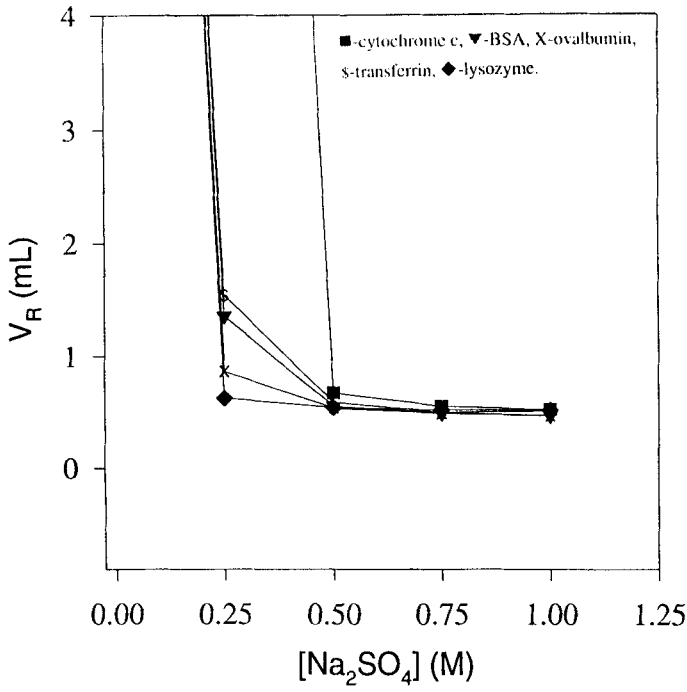

Figure 5. Effect of ionic strength on the retention time of proteins in fluoride containing mobile phases. All mobile phases contain 20mM MES + 100mM NaF + the sodium sulfate at the concentration shown. All other conditions are the same as in Figure 1.

negative charge is established on the surface due to the adsorbed fluoride. This negative charge retains the positively charged cytochrome c. Finally, between 0.25 and 0.50 M fluoride, the ionic strength becomes high enough to screen the surface charge from the cytochrome c, which results in the observed decrease in retention. Thus, it appears that Lewis base concentration is not the sole controlling factor in retention of proteins. Ionic strength is also an important factor.

Effect of Ionic Strength

The effect of the ionic strength of the mobile phase on the retention and recovery of proteins was examined by varying the concentration of sodium sulfate, a non-interacting salt, in the presence of a fixed concentration of the two Lewis bases (phosphate and fluoride). The two Lewis bases were examined

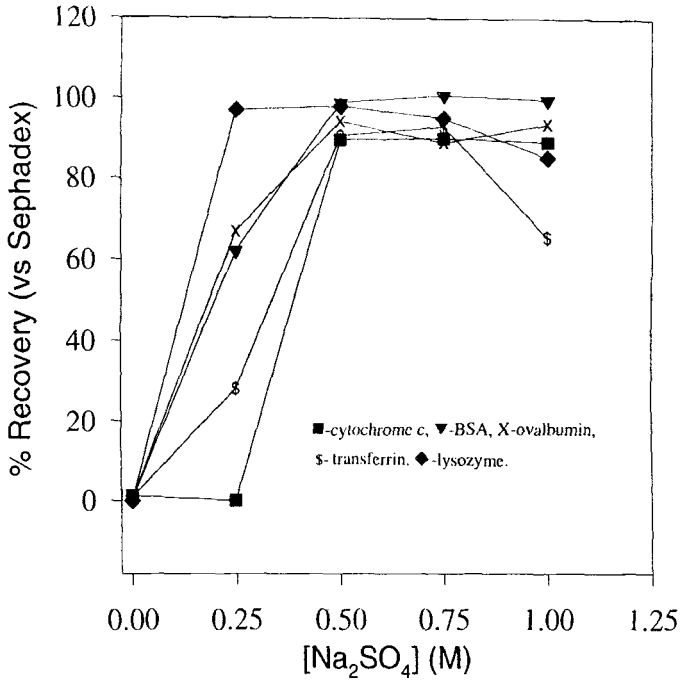

Figure 6. Effect of ionic strength on the recovery of proteins in fluoride containing mobile phases. Protein recovery is expressed as the percentage of the area measured compared to that on a column packed with Sephadex G-10 under the same conditions. All other conditions are as in Figure 1.

separately at a concentration of 100 mM each. It is important to note here, that the two different Lewis base buffers do not have the same ionic strength.

Figures 5 and 6 show the effect of the ionic strength of the mobile phase on the retention (Figure 5) and recovery (Figure 6) of proteins in fluoride mobile phases. These mobile phases contained 100 mM sodium fluoride and 20 mM MES buffer at pH 5.5 and a variable concentration of sodium sulfate. Figure 5 shows that at sodium sulfate concentrations below 0.25 M, no protein eluted from the CMD-coated zirconia. When the ionic strength was increased by adding 0.250 M sodium sulfate, all proteins except cytochrome c were eluted with some recovery. There is some selectivity in the retention volumes at this sodium sulfate concentration, which disappears when the ionic strength is increased. When the sodium sulfate concentration is 0.5 M or greater, the proteins all elute at about the same volume, and there is very little resolution between them.

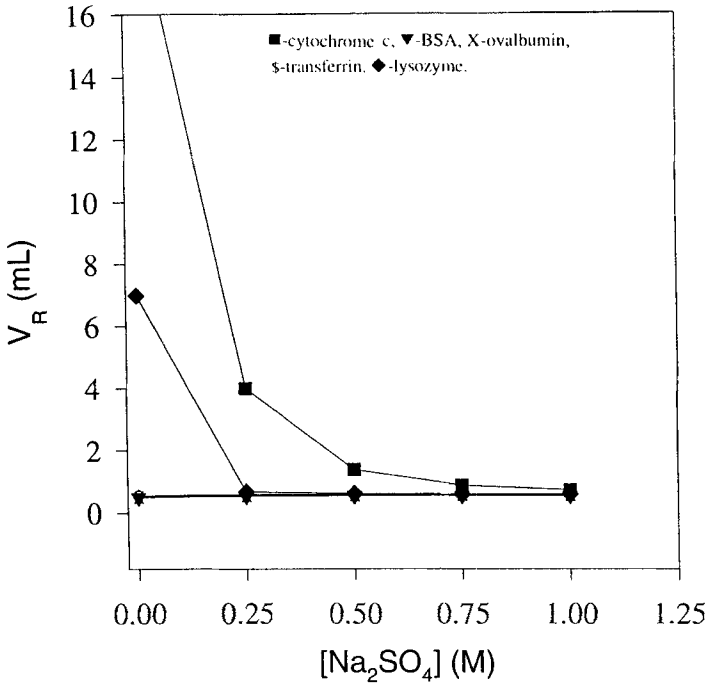

Figure 7. Effect of ionic strength on the retention of proteins in phosphate containing mobile phases. All mobile phases contain 100mM K_2HPO_4 at pH 7 + sodium sulfate at the concentration shown. All other conditions are the same as in Figure 1.

The effect of ionic strength on protein recovery is shown in Figure 6. When the sodium sulfate concentration is 0.5 M or above, the proteins all elute with 80% or better recovery. It appears that a high ionic strength is required to screen the ion exchange sites originating from the surface adsorbed fluoride.

Phosphate containing mobile phases show different behavior than that of the fluoride mobile phases. These experiments were all run with mobile phases containing 100 mM potassium phosphate at pH 7.0. Unlike the fluoride case, all the proteins except cytochrome c do elute when there is no sodium sulfate present in the mobile phase (Figure 7). The trend in protein recovery (Figure 8) is also different from the fluoride case. To be completely recovered, Cytochrome c requires a sodium sulfate concentration of at least 0.75 M to be recovered completely. All other proteins are eluted with some recovery (lysozyme is the lowest at 38%), even in the sulfate free mobile phase. Lysozyme does not behave as expected, since it has a higher pI than cytochrome c, and thus, should have a higher negative charge and be retained more than the

cytochrome c. This may be due to the moderately larger size of lysozyme in comparison to cytochrome c. Lysozyme also has fewer[18] basic residues (positively charged) than does cytochrome c.[23] Since cytochrome c is smaller, the average surface charges (charge per unit surface area) of the two proteins is different, with cytochrome c having a higher average positive surface charge.[25] This will make it more attractive to the negative charges on the surface of the zirconia than the lysozyme.

We should note, here, that this does not appear to be a good size exclusion chromatographic phase for proteins. Under mobile phase conditions which give us good recovery of all the proteins, the elution volume differences between the proteins are very small. However, the fact that most of the proteins elute at approximately the void volume (as measured by uracil), implies that the pore space is accessible to the proteins. This is very important for later studies on derivatized supports. If the proteins can not access the pore space, where most of the ligands are located, the capacity of the column will be very poor, and the phase will not be very useful. It appears that we do not have to worry about this problem for proteins that are as large as the ones tested here.

Effect of pH

Since it is apparent that the zirconia surface charge resulting from adsorbed Lewis bases is important, a study of the effect of pH on retention and recovery was undertaken. We chose mobile phase conditions (100 mM K_2HPO_4 + 0.75 M Na_2SO_4) under which the proteins all eluted at pH 7. Figures 9 and 10 show the effect of changing the pH of a phosphate mobile phase on protein retention and recovery. We can see that at pH 3, most proteins do not elute, and those that do (lysozyme) have a higher retention volume than at the other pHs. As the pH is increased, the proteins become less retained. This can be explained as the combination of two effects. The first, is the charge on the protein itself. At pH 3, all of the proteins used as solutes have a positive charge and will be retained (in these cases, irreversibly) by the negatively charged phosphate/zirconia surface. As the pH is raised, some of the proteins (BSA and ovalbumin) will become negatively charged, and will no longer be retained by the zirconia surface. The other mechanism accounts for the behavior of the high pI proteins (cytochrome c, peroxidase and lysozyme). As the pH is lowered, the concentration of hydroxide present in the solution will decrease. This shifts the equilibrium between phosphate and hydroxide for the surface Lewis acid sites in favor of phosphate. This will increase the amount of phosphate adsorbed and change the surface charge.[26] Thus, the proteins that are negatively charged will be affected by the pH due to the increased charge on the surface. Of course, the protonation state of the adsorbed phosphate will also change, decreasing the surface charge. However, it appears from these results, especially the behavior of lysozyme and cytochrome c, that the surface charge increases as the pH is decreased.

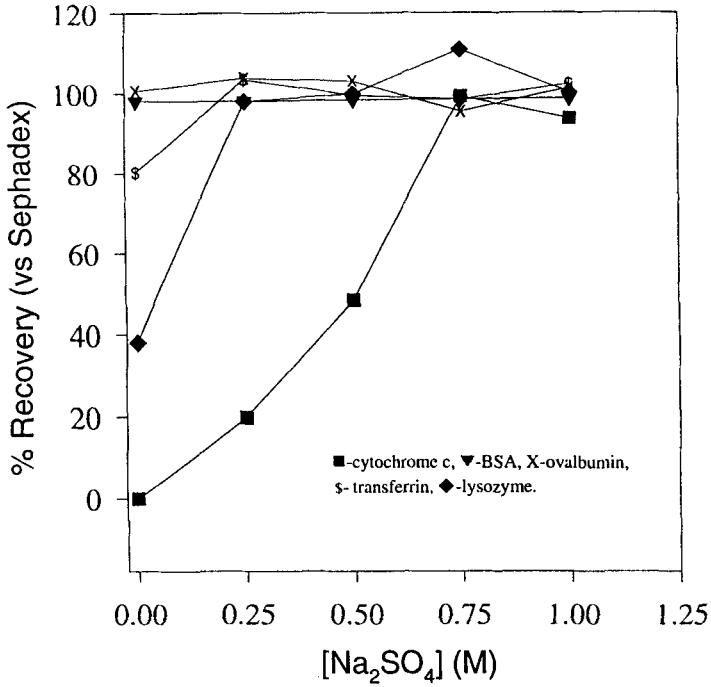

Figure 8. Effect of ionic strength on the recovery of proteins in phosphate containing mobile phases. Protein recovery is expressed as the percentage of the area measured compared to that on a column packed with Sephadex G-10 under the same conditions. All other conditions are as in Figure 5.

Effect of the Type Of Lewis Base

As discussed above, a Lewis base is necessary to effect elution of proteins. As can be seen in Figures 1 and 3, protein retention is not strongly influenced by the concentration of either phosphate or fluoride, once a minimum concentration has been reached. This is most likely due to the fact that both of these anions are strongly adsorbed by zirconia and the equilibrium will strongly favor the adsorbed state. Thus, changing the concentration will only affect those proteins that have a very strong affinity for zirconia. However, when these Lewis bases adsorb to the surface of the zirconia, a negative charge is formed on the surface. At the mobile phase pHs used, the adsorbed Lewis bases will impart a cation exchange character to the zirconia support material. Proteins with a positive charge (i.e. proteins whose pI is higher than the pH of the mobile phase), will be retained by the surface. Increasing the ionic strength of the mobile phase by adding a neutral salt will screen the ion exchange sites and the proteins are eluted from the material.

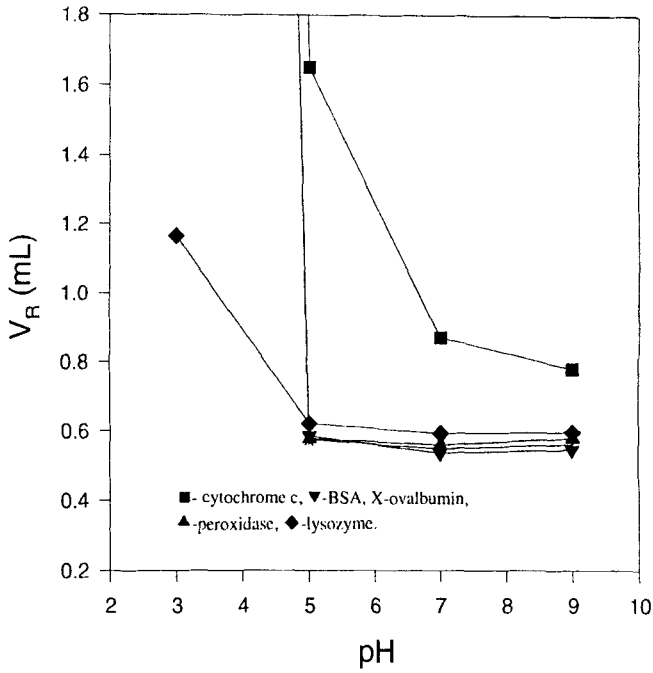

Figure 9. Effect of mobile phase pH on the retention volume of proteins. All mobile phases contain 100 mM K_2HPO_4 + 0.75 M Na_2SO_4 adjusted to the pH reported either by concentrated HCl or NaOH. All other conditions are the same as in Figure 1.

Although the two different mobile phases both contain strong Lewis bases, they do exhibit some differences. When there is no sodium sulfate in the mobile phase, the proteins are not eluted at all by the fluoride mobile phase, yet all proteins, except cytochrome c, are eluted from the phosphate mobile phase. This can be explained by examining the ionic strength of the two mobile phases. When there is no sodium sulfate in the mobile phase, the ionic strength of the 100 mM potassium phosphate mobile phase is 0.6 M, while the ionic strength of the sodium fluoride/MES mobile phase is only 0.24 M. This difference in mobile phase ionic strength is sufficient to explain the differences in the eluting strength of the two mobile phases. Another difference between the two mobile phases is the ionic strength at which cytochrome c is eluted. The phosphate mobile phase requires a higher concentration of sodium sulfate (0.75 M) to fully elute the cytochrome c than the fluoride mobile phase (0.5 M). This is can be explained by examination of the surface charge imparted to the surface by the two mobile phases. Fluoride will only have one negative charge per adsorbed molecule, while phosphate will have between one and two at the pHs used for the two mobile phases. This difference appears in the behavior of

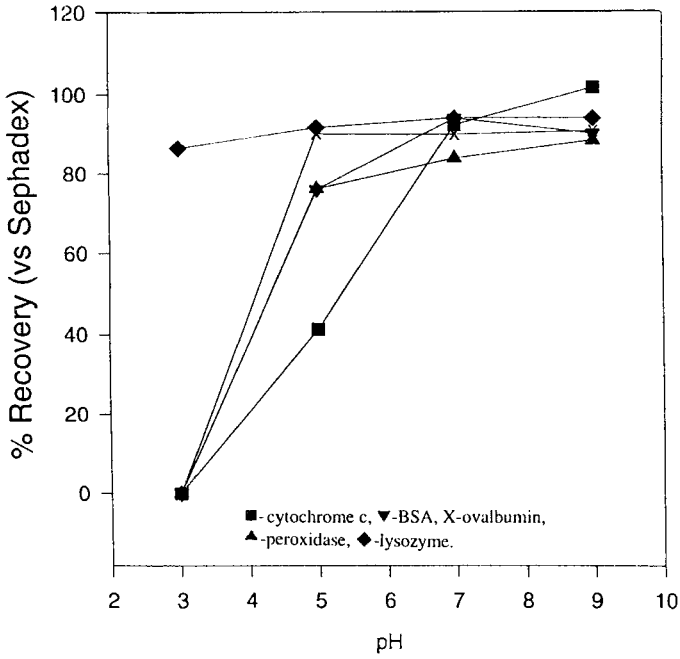

Figure 10. Effect of mobile phase pH on the recovery of proteins. All mobile phases contain 100 mM K_2HPO_4 + 0.75 M Na_2SO_4 adjusted to the pH reported either by addition of concentrated HCl or NaOH. All other conditions are the same as in Figure 1.

the highly charged protein cytochrome c. It will take a higher ionic strength to screen the charge on the phosphate-adsorbed surface than that on the fluoride-adsorbed surface. Thus, the ability of a Lewis base to elute a protein will depend on how highly charged the protein is.

CONCLUSIONS

The elution behavior of a set of highly varied (pI, MW) proteins was examined on CMD-coated zirconia. We found that both Lewis acid/base interactions and coulombic (ion exchange) interactions are taking place. The Lewis acid/base interactions are eliminated by adding a hard Lewis base (e.g. phosphate or fluoride) to the mobile phase. The ion exchange effects can be minimized by increasing the ionic strength of the mobile phase. We also found that both phosphate and fluoride buffers work well in eluting proteins. However, there are differences between the two mobile phase buffers in the surface charge they produce on zirconia.

Although, this does not appear to be a suitable phase for size exclusion chromatography, the proteins can access all the pores in the material, which is important for affinity chromatography.

We have also shown that, by choosing the correct mobile phase conditions, non-specific interactions can be eliminated and the CMD-zirconia phase can make a suitable support for affinity ligands.

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