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Fluoride-modified zirconium oxide as a biocompatible stationary phase for high-performance liquid chromatography

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ABSTRACT

Previously we found that porous microparticulate zirconium oxide strongly adsorbs fluoride from aqueous solutions. The adsorption is due in large part to a Lewis acid–base interaction between coordination sites on the zirconium oxide surface and fluoride. The resulting complex is very stable relative to many other species and therefore fluoride is hard to displace. The chromatographic properties of small molecules and proteins on zirconium oxide particles when fluoride is present in the mobile phase is investigated in this work. In the presence of fluoride, zirconium oxide is a very biocompatible adsorbent, the selectivities of which are analogous to those of calcium hydroxyapatite. The effect of fluoride is very reproducible even after the adsorbed fluoride is stripped by strong base and regenerated in fluoride buffer.

INTRODUCTION

Properly fabricated porous metal oxides have a number of physical characteristics which make them nearly ideal supports for high-performance liquid chromatography (HPLC). They are mechanically stable and can be processed into highly porous microparticulates which have a high proportion of mesopores. In addition, these particles display great resistance to chemical attack. Zirconium oxide, for example, is stable over the pH range 1 to 14 [1]. Such chemical resistance is advantageous when corrosive eluents are used or when the column must be sterilized.

However, a major drawback to the use of these materials as stationary phases is their chemical heterogeneity. The surface of metal oxides are covered with many different types of functional sites [2,3]. Among these are Brönsted acid sites, Brönsted base sites, Lewis acid sites and Lewis base sites. The surface of zirconium oxide has all but the Lewis base sites [3–5] as shown schematically in Fig. 1. These sites will interact differently with different solutes and can lead to badly broadened peaks or irreversible adsorption of strongly interacting solutes.

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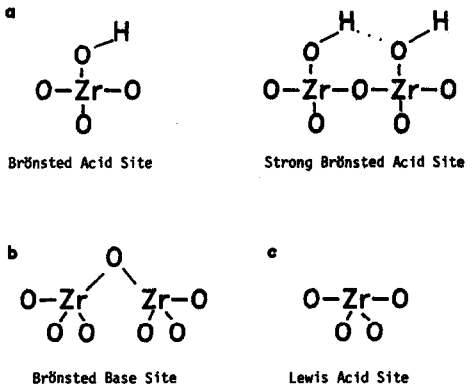

Fig. 1. Surface sites on zirconium oxide. (a) Brönsted acid sites; (b) Brönsted base sites; (c) Lewis acid sites.

The Lewis acid sites are the most troublesome of the various moieties present on the surface of zirconia. These sites arise from the surface discontinuity in bonding between metal and oxygen atoms. In bulk monoclinic zirconium oxide, the zirconium atoms are heptacoordinate. Therefore surrounding each zirconium (4+) ion are seven oxygen atoms with bond lengths of approximately 2.1 Å. The bonding valence is shared nearly equally among these bonds and the result is a very mechanically robust structure. The surface metal ions have satisfied their bonding valence to structural oxygen atoms but their coordinative bonding to other oxygen atoms is interrupted. This results in the existence of a number of accessible coordination sites on the particle surface.

Depending on the pH and other conditions, such sites are ordinarily occupied by hydroxide ions, water molecules or whichever Lewis bases may be present in solution. When a stronger Lewis base is introduced, it displaces the weaker Lewis bases and forms a stronger coordination complex at the surface. Chromatographically, this could lead to irreversible binding of a strong Lewis base unless an even stronger Lewis base is present in solution and acts to displace the bound solute [2]. This chemistry can be used to impart high selectivity to separations based on this elution strategy.

Fluoride interacts strongly with these hard surface Lewis acid sites since it is a very hard Lewis base [6–8]. The physical aspects of adsorption of fluoride onto these sites was investigated earlier [9]. Fluoride appears to be a reasonable candidate for modulating the strength of the Lewis acid site interactions. The effect of fluoride on the chromatographic properties of zirconium oxide, particularly towards protein separations, is the subject of this investigation.

EXPERIMENTAL

Cyclohexylaminopropanesulfonic acid (CAPS), N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid (TAPS), 2-[N-morpholino]ethanesulfonic acid (MES), 3-[N-morpholino]propane sulfonic acid (MOPS), uracil and all proteins were obtained from Sigma (St. Louis, MO, USA). Phenylacetic acid, guanidine hydrochlo-

ride, benzamide and benzenesulfonic acid were obtained from Kodak (Rochester, NY, USA). Imidazole, benzylamine, lithium chloride, sodium thiocyanate, sodium acetate and benzyl mercaptan were from Aldrich (Milwaukee, WI, USA). Phenol, hydrochloric acid, acetic acid and sodium fluoride were from Mallinckrodt (St. Louis, MO, USA), 9-benzenephosphonic acid was from Pfaltz and Bauer (Waterbury, CT, USA) and benzyl alcohol was from J.T. Baker (Phillipsburg, NJ, USA). Sodium sulfate, potassium chloride, ammonium chloride and sodium chloride were obtained from EM Science (Gibbstown, NJ, USA) and 50% sodium hydroxide solution was obtained from CMS Scientific (Houston, TX, USA). Particle pretreatment and solution fluoride measurements were as described in detail earlier [9]. All chemicals were reagent grade or better.

The porous zirconium oxide spherules were provided by the Ceramic Technology Center of the 3M Company and were described earlier [1,9–14]. Particles used in this investigation had a nominal diameter of 5.3 μm $\pm$ 1.3 μm, an average pore diameter of 308 Å by mercury porosimetry and an average BET surface area of 30.5 m^2/g. Particles were suspended in isopropanol and packed at 4500 p.s.i. into 50 × 4.6 mm I.D. columns by the upward slurry method [15]. Titanium screens (2 μm) were used, instead of frits, to minimize protein losses in the inlet [16] and to minimize contamination of the column by solubilized metal ions. Whenever the buffer was changed, the column was regenerated by flushing with approximately 50 ml of 0.1 *M* sodium hydroxide solution followed by 50 ml of freshly boiled deionized water. This treatment is essential since it removes all "irreversibly" bound solutes from the zirconia and reproducibly prepares the surface for equilibration in the next buffer.

Chromatographic studies were carried out on two systems. The first system consisted of a Hewlett-Packard (Avondale, PA, USA) Model 1090M liquid chromatograph with a DR5 ternary solvent delivery system and a diode array detector. The optional expanded pH range kit as well as ultrahigh molecular weight polyethylene piston seals (UPC-10) obtained from Bal Seal Engineering (Santa Ana, CA, USA) were installed. Data were processed using a Hewlett-Packard 9000/series 300 computer outfitted with ChemStation software.

The second system consisted of an Altex 110A isocratic pump (Fullerton, CA, USA) with a Rheodyne (Cotati, CA, USA) 7120 injector valve. The detector was a Perkin-Elmer (Norwalk, CT, USA) LC-15 fixed wavelength detector with a 254-nm filter. For high pH eluents, the piston seals were replaced with unfilled PTFE piston seals. Both systems were outfitted with a 50 × 4.6 mm I.D. column filled with 10–20-μm zirconia particles. This guard column was placed before the injection valve to scavenge any contaminants in the buffer.

Protein recovery assays

Lysozyme and myoglobin solutions (*ca.* 10 mg/ml) were made up in 100 m*M* sodium chloride–20 m*M* MES pH 5.5 buffer. Injections of 10 μl were made onto a 50 × 4.6 mm I.D. column packed with 5-μm particles. A linear gradient of 0 to 0.75 *M* Na_2SO_4 in 100 m*M* sodium fluoride–20 m*M* MES pH 5.5 was run in 30 min at 35°C. The column effluent was collected and stored at 4°C for protein analysis later the same day. Each protein was run five times and the effluents saved for analysis. The column was then removed and the analysis repeated five times per protein as a control for protein recovery. Two separate bicinchoninic acid (BCA) protein assay protocols

[17,18] were used to quantitate the protein recovery in each case. The enhanced assay and the microreagent assay were performed using the effluent from a water blank injection as the diluent blank.

RESULTS AND DISCUSSION

Ion exchange is the most common mode of separating proteins and large biomolecules. Retention is reasonably predictable and a great deal of work on the process and its control by such factors as ionic strength, salt composition, pH and organic modifier [19–21] has been done. Recent developments in polymer science have produced polymeric ion-exchange resins with predictable and reproducible properties [22] and advances have been made in producing more mechanically stable materials for use in large scale separations. However, these materials suffer from "selectivity monotony". In anion-exchange chromatography, for example, differences in selectivity between ion-exchange materials are minimal and are mainly due to small secondary effects such as differences in the accessibility of the protein to the exchanger's pore structure and interactions with the exchanger's structural material.

This "selectivity monotony" has, in part, led to a renaissance in the use of materials such as calcium hydroxyapatite. Hydroxyapatite has long been used as a protein adsorbent but its use in preparative-scale HPLC has been limited [23,24]. The underlying mechanism of separation is multimodal [25–28]. Cation exchange takes place at the ionized hydroxyl groups of the phosphate groups on the surface of the particle. Both anion exchange and ligand exchange occur at the calcium (and magnesium) ions sites on the particle. This combination of separation mechanisms confers on hydroxyapatite a selectivity which is unique among all ion-exchange protein separation media. Unfortunately, this unique support is both physically and chemically unstable.

Although zirconium oxide is chemically very different from calcium hydroxyapatite, they share a number of chromatographic similarities. On hydroxyapatite, cation exchange takes place at the ionized hydroxyl groups of chemisorbed phosphate; the number of these sites is pH dependent. Similarly, ionized hydroxyl groups bound to the surface of the zirconium oxide particles serve as sites for cation exchange [2]. The isoelectric point of zirconium oxide is approximately 6.7 [2,29,30] but a wide range of individual pK_a values exist for the various types of Brönsted acid and base sites. This gives zirconium oxide a pH dependent cation-exchange capacity analogous to hydroxyapatite.

More importantly, both phases have Lewis acid sites which are responsible for both ligand and anion exchange. On hydroxyapatite, calcium ions form very labile coordination complexes with oxygen containing moieties and other hard Lewis bases. The calcium ions are also the sites for anion exchange for non-coordinating anionic species. Zirconium oxide also has Lewis acid sites [4,31,32] in the form of the surface zirconium ions. This Lewis acid site has a net 2+ charge [4] and one expects it to show anion-exchange properties for non-coordinating anions.

Ligand-exchange chemistry is slightly different for zirconium as compared to calcium, however, since zirconium (4+) ion has an especially high charge to radius ratio and therefore forms very strong coordination complexes with hard Lewis bases [8,33–35]. It coordinates with the same types of oxygen containing ligands and strong

Lewis bases as does calcium but the lability of such complexes is lower. The result is slower adsorption and desorption kinetics. Earlier investigations [9] have shown that the adsorption kinetics are still relatively fast. However, as long as the adsorption and desorption kinetics are fast on the chromatographic time scale, kinetics will not significantly broaden the elution profiles. Some broadening may be observed due to the heterogeneity of surface binding sites, but this is a relatively minor problem when the overall properties of this material are considered.

In order to determine which functional groups are responsible for the strong retention of proteins by either the ion-exchange or coordination mechanisms, a number of small solutes were studied. Table I summarizes the results of this study. The behavior of guanidine is typical for a cationic solute which has no other interaction with the surface other than electrostatic interactions. At low pH, guanidine is retained in both fluoride and chloride-containing buffers. This indicates that there are cation-exchange sites on the zirconia surface independent of the Lewis acid sites. Since fluoride coordinates at the Lewis acid sites and effectively competes with hydroxide at

TABLE I

SMALL SOLUTE CAPACITY FACTORS (k') *VS.* pH

All eluents contained 20 m*M* eluting salt and 20 m*M* buffer (MES, pH 6.1; TAPS, pH 8.4; or CAPS, pH 10.4). Flow-rate was 0.50 ml/min at 35°C. Diode array detection at 230 and 254 nm was used. Typically 5 μg solute were injected. Values in parentheses are reduced plate heights.

Solute	k' (h)					
	pH 6.1		pH 8.4		pH 10.4	
	NaF	NaCl	NaF	NaCl	NaF	NaCl
Guanidine	1.15 (6.6)	0.65 (36.7)	1.29 (11.3)	0.65 (35.3)	0.47 (61.1)	0.60 (86.7)
Imidazole	0.41 (9.4)	−0.01 (33.3)	0.04 (14.5)	0.03 (12.0)	0.02 (31.1)	0.01 (37.9)
Benzylamine	0.58 (7.3)	−0.01 (7.5)	0.79 (6.1)	0.39 (7.6)	0.20 (19.2)	0.18 (17.8)
Uracil	0.01 (7.2)	0.50 (26.7)	0.06 (5.0)	0.52 (44.5)	0.01 (23.2)	0.06 (29.7)
Benzamide	−0.02 (7.6)	0.00 (7.1)	−0.02 (7.8)	−0.01 (7.0)	−0.02 (17.0)	−0.04 (16.3)
Benzyl alcohol	−0.03 (7.6)	−0.02 (7.4)	−0.03 (8.4)	−0.02 (7.5)	−0.03 (17.4)	−0.04 (18.8)
Benzyl mercaptan	−0.01 (13.0)	0.03 (12.4)	−0.01 (12.4)	0.01 (14.1)	0.03 (35.6)	0.09 (158)
Phenol	−0.02 (7.5)	0.11 (8.1)	0.00 (8.3)	0.13 (11.0)	−0.03 (14.0)	−0.02 (14.6)
Phenylacetate	−0.05 (8.1)	0.65 (39.6)	−0.06 (7.3)	0.34 (29.2)	−0.11 (19.3)	−0.11 (27.2)
Phenylsulfonate	−0.08 (10.6)	0.89 (8.3)	−0.10 (11.2)	−0.05 (11.3)	−0.12 (16.9)	−0.13 (25.1)
Phenylphosphonate	−[a]	−	−	−	3.26 (287)	6.78 (154)

[a] Elution not observed.

these low pH values, the cation-exchange capacity is increased. The chloride does not coordinate to those sites so it does not change the cation-exchange capacity. At higher pH, fluoride cannot effectively compete with hydroxide for the Lewis acid sites so the cation-exchange capacity does not increase when fluoride is in the eluent. The remaining cation-exchange capacity at pH 10.4 likely results from the ionized surface hydroxyl groups. The buffer salt has little effect on the number of these sites.

Imidazole acts like guanidine except that in buffers above its pK_a, it is not retained since it is uncharged. Benzylamine also displays this behavior except there is some slight retention at pH 10.4 which we attribute to hydrogen-bonding interactions. This interaction is also observed to a small extent in chloride buffer at pH 8.4 since retention is anomolously high compared to retention in chloride buffer at pH 6.1. Uracil shows retention patterns unlike the other cationic solutes, however. With fluoride in the buffer, little to no retention is observed at any pH. However, if the Lewis acid sites are available (occupied by coordinated water molecules) as in the chloride buffers of low pH, uracil is retained. This indicates that the oxygen atoms of uracil, although individually weak Lewis bases, work cooperatively with the proper geometry to form a complex with the Lewis acid site. This interaction is strong enough to enhance retention in solutions where no stronger Lewis base is present. At high pH, uracil cannot compete with hydroxide and is no longer retained in the chloride buffer.

The neutral solutes show little retention under any of the conditions used here. However, benzyl mercaptan is slightly retained in chloride buffers at pH 10.4 possibly due to hydrogen bonding interactions as seen with the benzylamine at high pH.

In the presence of fluoride, anionic solutes are not retained and are excluded from the pores to some extent. However, in the presence of chloride buffers at low pH, the anionic solutes are free to interact with the Lewis acid sites which bear a 2+ charge. Phenol is weakly retained in the chloride buffers up to pH 10.4. At this pH, the phenolic hydroxyl is ionized and the retention is diminished. This loss of retention may also be caused by the unfavorable competition between the phenoxy anion and hydroxide present in solution. Phenylacetic acid is retained quite strongly at low pH but is decreasingly retained as pH is increased. This indicates a direct competition between the carboxyl group of phenylacetate and solution hydroxide species.

In previous work in this laboratory, Rigney *et al.* [1,14] showed that species such as carboxylic and sulfonic acids will not elute from zirconia based columns except in the presence of acetate or phosphate in the mobile phase. The fact that both phenylacetate and phenylsulfonate elute in the absence of fluoride indicates that the elution buffer species (MES, TAPS and CAPS) are interacting with the surface to an observable extent. At high pH, phenylacetate is excluded to the same extent in chloride buffer as in fluoride buffer.

Phenylsulfonate proves to be a stronger Lewis base than phenylacetate at low pH but is more readily displaced by hydroxide ion. At pH 8.4, phenylsulfonate is less retained than phenylacetate indicating a reversal of strength. This may be due to a hydrogen-bonding contribution to the phenylacetate retention, however. Phenylphosphonate is not displaced by either chloride or fluoride at low pH. At higher pH, hydroxide ion has some difficulty in displacing the phosphonate indicating a very strong complexation constant between zirconium cation and phosphonate. A fair difference in retention is observed at high pH in the presence of fluoride compared to

that of chloride. Fluoride decreases the capacity factor of phenylphosphonate by a factor of two indicating that fluoride does have some displacing power relative to that of the phosphonate anion. Phosphonates and phosphates can be readily displaced by buffers containing higher concentrations of fluoride, which is in agreement with the thermodynamic coordination equilibria involved.

Taken together, the results indicate a surface the dominant features of which are Lewis acid sites and ionizable hydroxyl groups. Other groups such as the Brönsted base groups may be present in small numbers with relatively little effect on the overall retention mechanism for anionic solutes. These Brönsted base sites may be responsible for the hydrogen bond accepting properties observed for strong hydrogen bond donor solutes at high pH. At low pH, they may contribute to the anion-exchange capacity provided by the Lewis acid sites. The properties of the ionizable hydroxyl groups are determined by the solution pH and have a relatively predictable effect on solute retention.

The Lewis acid sites are much more complex, however. An equilibrium exists between all the Lewis bases present in the buffer and the Lewis acid sites. The displacing strength of each Lewis base is consistent with its thermodynamic formation constant; the higher the constant, the stronger the displacing effect. Due to the extensive hydrolysis of zirconium ion in aqueous solution, it is usually studied in extremely acidic solution (1–4 *M* $HClO_4$) to prevent hydrolysis and the formation of binuclear complexes thus little thermodynamic data exist at pH values relevant to this work. Therefore, the relative strengths of interaction must be experimentally determined for each Lewis base of interest.

In the presence of fluoride, cations are retained most strongly. They are held at the anionic ionized hydroxyl groups and/or coordinated fluoride ions. Such species are readily eluted isocratically with very good reduced plate heights (h = 6–10). Solutes containing weak Lewis base groups are either very weakly or not retained and also show very good reduced plate heights.

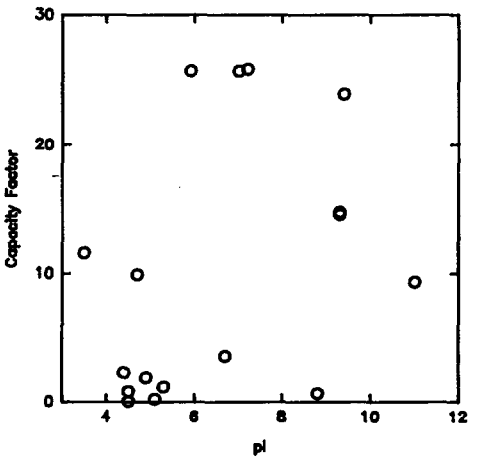

Fig. 2. Correlation of capacity factor with p*I*. Gradient elution from 0 to 0.5 *M* Na_2SO_4 in 30 min was used. Both buffers contained 20 m*M* sodium fluoride and 20 m*M* MES at pH 6.2 at 35°C. Flow-rate was 0.5 ml/min and detection was at 280 nm.

In the absence of fluoride, Lewis base containing ligands are increasingly retained since the Lewis acid sites are no longer blocked by fluoride. The reduced plate heights of these solutes are much higher (h = 15–70) due to the strong interactions with Lewis acid sites. The kinetics of ligand association and dissociation are slower than for ion-exchange interactions so the peaks become correspondingly wider. The cationic solutes do not show an increase in reduced plate height as do Lewis bases when fluoride is not present. While each of these simple interactions is small, a biopolymer containing many such groups can be very strongly retained due to the sum of a large number of such contributions.

For complex biopolymers, zirconium oxide particles display mixed mode retention analogous to calcium hydroxyapatite. Fig. 2 shows the absence of any correlation between isoelectric point of the protein and capacity factor at pH 6.2. It should

TABLE II

PROTEIN RETENTION AS A FUNCTION OF ELUENT pH[a]

Separations performed on a 50 × 4.6 mm I.D. column at 35°C with a flow-rate of 0.5 ml/min. Detection was at 280 nm with approximately 100 μg protein injected.

Protein	pI	Capacity factor at pH:		
		4.8[a]	6.2[b]	8.4[c]
Albumin, human	5.2, 4.6–5.3	25.46	–	–
Alkaline phosphatase	4.4	0.23	2.30	3.95
Cellulase	4.5, 4.2, 3.9	0.05	0.06	3.67
Cholinesterase, acetyl	4.5	–[d]	–	0.00
α-Chymotrypsin	8.8	0.28	0.68	9.92
Creatine phosphokinase	6.6, 6.7, 6.9	–	3.57	–
Cytochrome c	9.4, 9.0	20.23	23.89	25.87
Ferritin	4.1–4.6	–	–	0.00
Fetuin	3.2–3.8	6.34	11.60	0.02
β-Glucuronidase	5.1, 5.9	0.02	0.22	0.08
Hemoglobin	6.9–7.4	21.18	25.77	–
Hexokinase	4.9, 5.3	0.22	1.91	2.80
β-Lactoglobulin	5.3, 5.1	0.19	1.19	0.61
Lysozyme	11.0	8.73	9.35	11.20
Malate dehydrogenase	5.1	0.07	–	–
Myoglobin, equine	6.8, 7.3	25.61	25.63	–
Ovalbumin	4.7	9.27	9.92	–
Peroxidase, horseradish	4.0–8.4	1.66	2.35	0.02
Ribonuclease A	9.3	13.28	14.60	–
Ribonuclease B	9.3	8.71	14.78	–
Transferrin	5.9	23.80	25.65	–
Trypsin inhibitor	4.5	0.47	0.84	0.14

[a] Mobile phase was a 30 min linear gradient from 0 to 0.5 M Na_2SO_4 in 20 mM acetate buffer containing 20 mM sodium fluoride.

[b] Mobile phase was a 30 min linear gradient from 0 to 0.5 M Na_2SO_4 in 20 mM MES buffer containing 20 mM sodium fluoride.

[c] Mobile phase was a 30 min linear gradient from 0 to 0.5 M Na_2SO_4 in 20 mM TAPS buffer containing 20 mM sodium fluoride.

[d] Elution not observed.

be noted that without fluoride in the eluent, the proteins did not elute. When fluoride was present at pH 8.4, most proteins with low p*I* values are weakly retained while proteins with high p*I* values are well retained. This is similar to results found for cation-exchange supports, however, contributions from the Lewis acid sites and the protonated Brönsted base sites produce a more pronounced mixed retention behavior.

Since the pK_a values of the surface hydroxyls of zirconium oxide are approximately 8.1 a larger proportion of these hydroxyl groups are ionized at pH 8.4 than at pH 6.2. Cationic proteins will be more strongly retained under these conditions and anionic proteins will be excluded from the surface to a greater degree. At pH 4.8, a minute fraction of the surface hydroxyl groups are ionized and the surface charge will be determined primarily by coordinated fluoride ions and protonated Brönsted base sites. A summary of the results is given in Table II. A wide variety of patterns are observed. Some proteins show increased retention, others go through a minimum in retention, etc. Clearly several factors are responsible for retention. Most importantly, a very wide variety of proteins are retained.

To explore which factors are responsible for retention, the role of the cation and the anion in the eluent were explored. Four proteins with distinctly different isoelectric points and molecular weights were evaluated to assess the effect of changes in ionic strength and chemical parameters have on retention. Fig. 3 shows the effect of varying the cation and the ionic strength. Lipase (Fig. 3D) was not sensitive to either

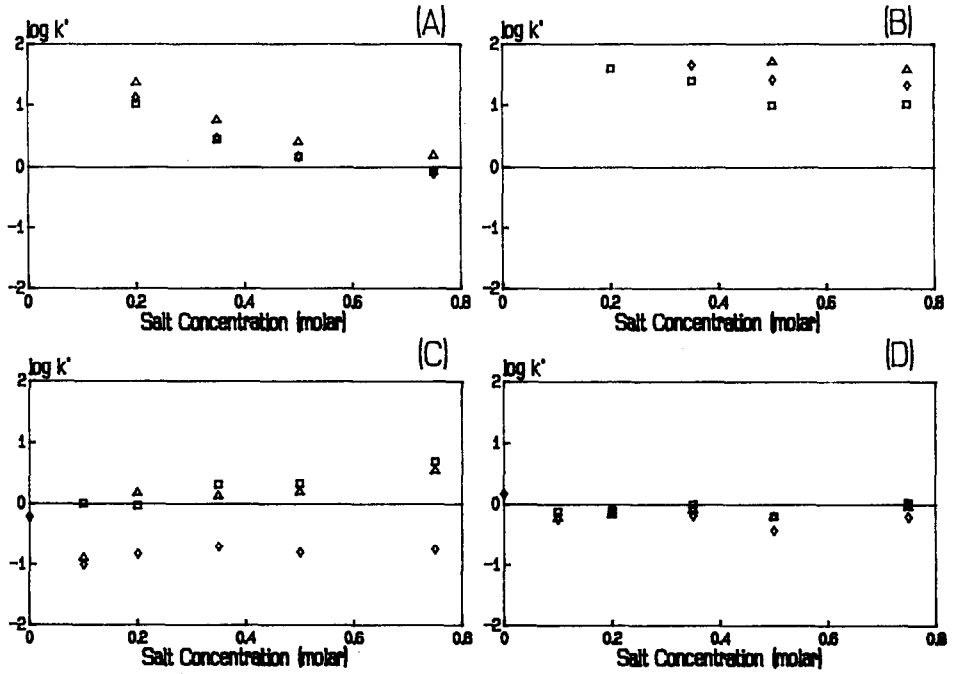

Fig. 3. Protein retention *versus* cation concentration. (A) lysozyme; (B) myoglobin; (C) trypsin inhibitor; (D) lipase. ◇ = NaCl; △ = NH_4Cl; □ = KCl. Isocratic elution at 35°C and 0.5 ml/min flow-rate were used. All buffers contained 20 m*M* NaF and 20 m*M* TAPS at pH 8.4.

the ionic strength or the cation used. Trypsin inhibitor (Fig. 3C) behaved similarly except that sodium salts promoted elution. Lysozyme (Fig. 3A) behaved as expected, based on a retention mechanism dominated by ion exchange, since retention increased at low ionic strength and was independent of the cation used. The ammonium cation appears to be a weaker eluent but since the study was performed at pH 8.4, a fair amount of the ammonium ion is unprotonated, thereby lowering the total ionic strength of the eluent. Myoglobin (Fig. 3B) is difficult to elute under these conditions and a trend is difficult to observe. Again it is important to reiterate the fact that without a small amount of fluoride in the eluent (20 m*M*), none of these or any other proteins are eluted from the zirconia particles at this pH.

Under these conditions, the surface of the particles should have a net negative charge. Coordinated fluoride and ionized hydroxyl groups contribute to this charge. A consequence of this high charge density is the formation of an ionic double layer. If this layer were partly responsible for the mixed mode ion-exchange behavior, there should be a large cation dependence on protein retention. This was not observed and may be ruled out as a contributing retention process.

Fig. 4 shows the effect of variations in the type of anion and its concentration on protein capacity factors. Again, lipase (Fig. 4D) and trypsin inhibitor (Fig. 4C) show little effect for any anion except for fluoride. Fluoride is an extremely potent eluent and therefore leads us to believe that these proteins are primarily retained by

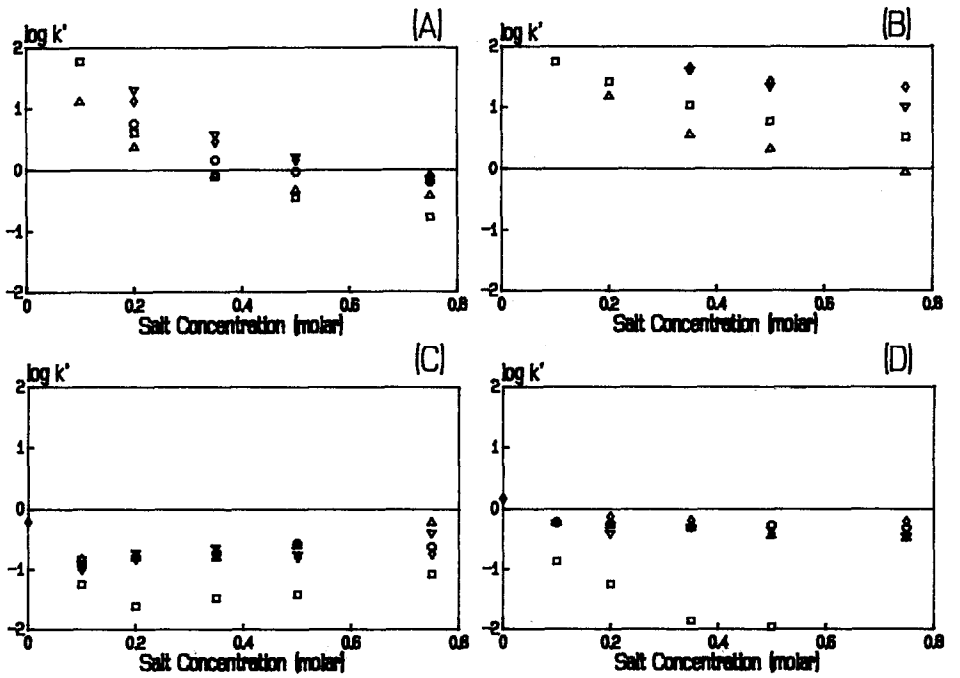

Fig. 4. Protein retention *versus* anion concentration. (A) lysozyme; (B) myoglobin; (C) trypsin inhibitor; (D) lipase. □ = NaF; ◇ = NaCl; △ = Na_2SO_4; ○ = NaSCN; ▽ = sodium acetate. Isocratic elution at 35°C and 0.5 ml/min flow-rate were used. All buffers contained 20 m*M* NaF and 20 m*M* TAPS at pH 8.4.

ligand exchange. None of the other salts are strong enough Lewis bases to displace the functional groups of the protein which are coordinated to the Lewis acid sites on the surface.

Lysozyme (Fig. 4A) shows the same dependence of retention on ionic strength as in the preceeding cation effect study (Fig. 3). Fluoride is an anomolously strong eluent indicating that at least some of the retention of lysozyme is due to ligand exchange. At lower salt concentrations, sulfate proves stronger than fluoride due possibly in part to the higher ionic strength of sulfate over fluoride at the same molarity. Myoglobin (Fig. 4B) is eluted more easily with sulfate or fluoride than with chloride. For all salt concentrations, sulfate proves the stronger eluent. However, at low salt concentration, only fluoride can elute myoglobin. For all proteins, anions other than fluoride or sulfate show little or no difference in eluotropic strength.

A brief study of the displacement mechanism parameters was undertaken for these test proteins. The logarithm of the capacity factors for each protein was plotted against the logarithm of the reciprocal displacing salt concentration for each of the salts tested above. The slope of such a plot is considered by some to be a measure of the displacement stoichiometry for each protein and is named the Z value [36,37]. For each protein, the Z values did not change significantly between eluting salt species. Lysozyme, for example, had Z values from 1.67 to 2.92 and myoglobin had values from 0.76 to 2.12. Lower displacement stoichiometry was observed for trypsin inhibitor and lipase with Z ranging from -1.4 to -0.17 and -0.15 to 0.32, respectively. There was no consistent trend in values for any of the proteins with relation to the eluting salt although the correlation coefficients for the regressions to calculate Z were fairly good in most cases. The relatively low stoichiometric values may be due in part to the inflexibility of the inorganic ion-exchange material. In organic resins and derivatized silicas, the ionic groups of the support have some degree of mobility associated with them because of the flexibility of the support or from the spacer arm between the point charge and the support backbone. Such flexibility allows greater access of the charged groups to the ionic sites on the globular protein. In the case of a rigid inorganic ion exchanger with the ionic sites firmly planted on the surface, such flexibility is not possible and less coulombic interactions are expected on a steric basis. In the case of denatured proteins, both types of ion exchangers should show similar Z values since the steric restraints on the solute are greatly diminished.

The great ability of fluoride to displace Lewis bases from the Lewis acid sites on the surface is a consequence of the strength of the coordination complex formed between fluoride and zirconium. Fluoride has a very large formation constant with tetravalent zirconium ($K_1 = 10^{8.94}$) [38]. Few Lewis bases form stronger complexes with zirconium ion therefore fluoride can displace almost any other Lewis base. Thus fluoride elutes bound proteins by displacing the coordinating groups on the accessible surface of the protein. Sulfate also forms a moderately strong complex with zirconium ion ($K_1 = 10^{3.67}$) [38] and we expect it to displace some Lewis bases. This may help to explain why sulfate elutes myoglobin more strongly than does fluoride.

The use of only fluoride as an eluent allows both displacement of Lewis bases and acts simultaneously as a displacer for ion-exchange mediated retention. Sulfate can displace weak Lewis bases but can also act as a more effective displacing salt than fluoride for ion exchange. This is an important difference which allows the optimization of the selectivity between proteins with similar total retention but different

amounts of ligand-exchange contributions to retention. When a protein has two proximal Lewis bases which both bind to a single Lewis acid site, it will form a less labile complex than the simple addition of their individual effects. An example of this is the comparison between oxalate and acetate ions. Acetate forms a very weak complex with zirconium ion ($K_1 = 10^{0.3}$) but oxalate forms an exceptionally strong complex ($K_1 = 10^{9.80}$) [38]. Sulfate readily displaces acetate ion but is not able to displace oxalate. Fluoride is able to displace both if it is present in sufficient concentration.

The anomolous behavior of sulfate *versus* fluoride for the elution of myoglobin may arise from such an effect. If no proximal Lewis bases are on the protein's surface, then a relatively weak Lewis base should be able to displace the individual bases from the Lewis acid sites. This would leave a relatively large contribution from the ion-exchange interactions. Sulfate will overcome these remaining ionic interactions more efficiently than fluoride. Lipase and trypsin inhibitor are both held by strong ligand-exchange contributions to retention but do not show this effect. This may be due to the presence or proximal Lewis bases on their surface combined with a relatively weak contribution from ion exchange. The ionic strength of the fluoride necessary to overcome the ligand-exchange retention is in excess of that required for the ionic retention. Clearly, the effect of formation constant on eluotropic selectivity requires further study.

Greater insight into the retention mechanism is obtained by plotting the logarithm of the capacity factor versus the logarithm of the fluoride concentration as shown in Fig. 5. A straight line indicates a single retention mechanism predominantly based on displacement by fluoride ion while curvature in such a plot may signify mixed retention mechanism. We hypothesize that the degree of curvature is a rough indication of the degree of contribution from other retention processes. Myoglobin displays a nearly linear relationship and the displacement mechanism is consistent with results based on cation and anion effect studies. As expected, lysozyme, lipase

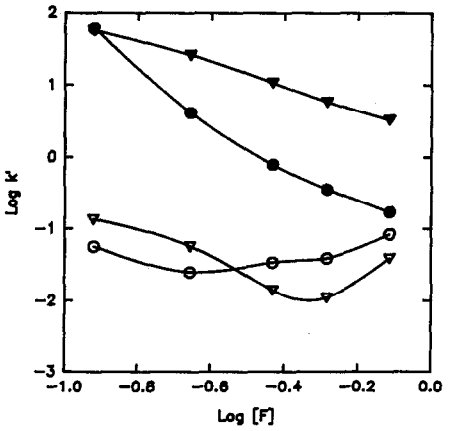

Fig. 5. Retention as a function of Lewis base concentration. △ = lysozyme; ○ = myoglobin; □ = trypsin inhibitor; ▽ = lipase. Isocratic elution at 35°C and 0.5 ml/min flow-rate were used. All buffers contained 20 m*M* TAPS at pH 8.4.

TABLE III

EFFECT OF ANION TYPE ON GRADIENT ELUTION CAPACITY FACTORS OF VARIOUS PROTEINS

Separations performed with a 30-min linear gradient from 0 to 0.5 *M* Na_2SO_4 or 0 to 1.5 *M* sodium chloride in 20 m*M* MES buffer at pH 5.5 containing 0.5 *M* sodium fluoride. Flow-rate was 1 ml/min at 35°C. Protein loadings were typically 10 μg and detection was at 280 nm.

Protein	Capacity factors	
	Sulfate	Chloride
Myoglobin	3.54	3.44
α-Chymotrypsin	2.34	1.39
Bovine albumin	14.18	–[a]
Lysozyme	0.23	0.09
Cytochrome *c*	5.09	3.91
Apotransferrin	16.60	32.00
Hemoglobin	3.89	3.04
Human albumin	17.00	–
Ribonuclease A	3.25	2.95
Ribonuclease B	3.56	0.22
Ovalbumin	10.55	0.40

[a] Elution not observed.

and trypsin inhibitor all exhibit multiple retention mechanisms. Lipase shows a fairly linear relationship between log k' and log $[F^-]$ at low fluoride concentrations. However, at high fluoride concentrations, a sharp change is observed and some other form of retention mechanism may take over. This would be the case if the concentration of fluoride necessary to displace the ligated Lewis bases on the protein was in excess of that required to overcome the electrostatic retention contributions.

When the retention mechanisms of various proteins are different, one can easily adjust the chromatographic selectivity. By changing the eluting salt during an ionic strength gradient, one can drastically change the order of elution. Table III shows the difference in retention between a gradient of sodium sulfate and a gradient at the same ionic strength using sodium chloride. Such a change has a dramatic effect on the retention of transferrin and ovalbumin while proteins like myoglobin and ribonuclease A are hardly affected.

Ligand exchange between the zirconium sites and the Lewis base groups on the proteins is expected to be relatively slower than simple ion exchange. Experimentally, this is observed in the effect of flow-rate on the reduced plate height at different pH values. For slow kinetic processes, the efficiency decreases as the flow-rate is increased. At low pH, the zirconium oxide surface will be saturated with fluoride ions [9]. This will make the support act as a high capacity cation-exchange material with some additional anion-exchange character due to the protonated Brönsted base sites. This surface should be very efficient and have a relatively small "*C* term" in a Van Deemter plot. However, at higher pH, there will be less fluoride masking the Lewis acid sites and the slower ligand-exchange mechanism will dominate the "*C* term" in the Van Deemter equation. Fig. 6 shows this effect for two proteins.

As stated earlier, we hypothesize that lysozyme is retained mainly by an ion-

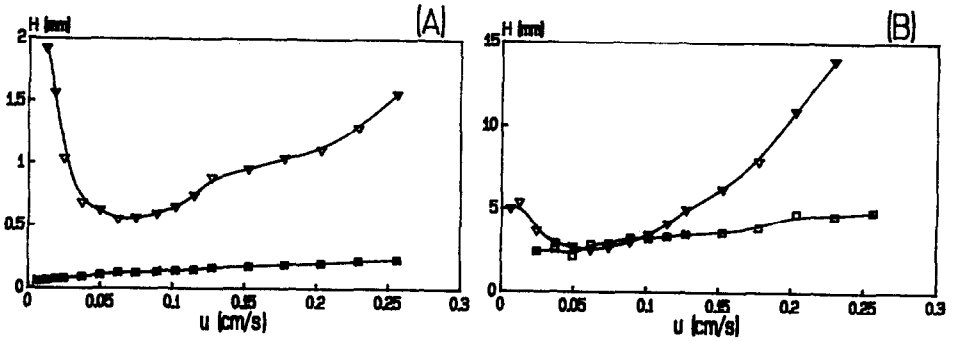

Fig. 6. Van Deemter plots. (A) lysozyme; (B) myoglobin. ▽ = 20 m*M* CAPS, pH 10.4; □ = 20 m*M* MES, pH 6.1. Isocratic elution at 35°C and 1.00 ml/min flow-rate were used. All buffers contained 20 m*M* NaF and 200 m*M* Na_2SO_4. H = plate height, u = linear velocity.

exchange interaction. At low pH, the plate height is quite satisfactory. At higher pH, the weak contribution of ligand exchange to retention becomes overwhelming due to its sluggishness. The efficiency therefore becomes highly flow dependent. We also believe that myoglobin is retained mainly by a ligand-exchange mechanism. At high pH, the efficiency is relatively poor due to the slowness of ligand-exchange kinetics on the relatively scarce Lewis acid sites. When the flow-rate is increased to 1.0 ml/min, the myoglobin separation becomes so inefficient at pH 10.4 that it is almost useless.

In addition to being essential for elution of proteins from zirconium oxide particles, fluoride serves another important purpose. Unless buffers are scrupulously pure, a number of Lewis base impurities will be present. Carbonate is ubiquitous.

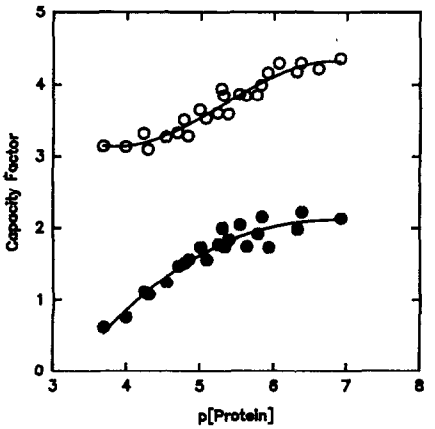

Fig. 7. Loading study on fluoride modified zirconium oxide. ○ = Lysozyme; ● = lipase. Isocratic elution at 35°C and 0.5 ml/min flow-rate were used. Eluent was 0.35 *M* potassium chloride with 20 m*M* NaF and 20 m*M* TAPS at pH 8.4.

TABLE IV

PROTEIN RECOVERY ± S.D. (n = 5)[a]

Protein	Enhanced assay	Micro assay
Lysozyme	103.9 ± 2.5%	101.2 ± 2.2%
Myoglobin	107.7 ± 6.1%	95.7 ± 4.1%

[a] See experimental for details.

When fluoride is not present in the mobile phase to block the Lewis acid sites, over time, carbonate builds up on the particle surface and changes the overall character of the surface. Since carbonate is not monodentate, it has a slow desorption rate and prevents other Lewis bases from reaching the Lewis acid sites. This masks the ligand-exchange mechanism and results in an inorganic cation-exchange material with less selectivity than the original material. Phosphate would act similarly and should be avoided if long term reproducibility between base flushings is desired. Therefore, for reproducible and consistent retention properties, it is essential to control interactions at the Lewis acid sites.

An important characteristic of separation materials, especially multimodal supports, is the effect of solute load on peak shape. Mixed mode materials are often readily overloaded if one type of site is less populous than the other retention site. The result is a complex relationship between capacity factor and solute load. This could lead to serious problems if a preparative separation is desired. Fig. 7 shows that the capacity factors do not change significantly even though the amount of protein injected was varied by over three orders of magnitude although some decrease in capacity factor is expected as sample size is increased [39]. This is consistent with earlier

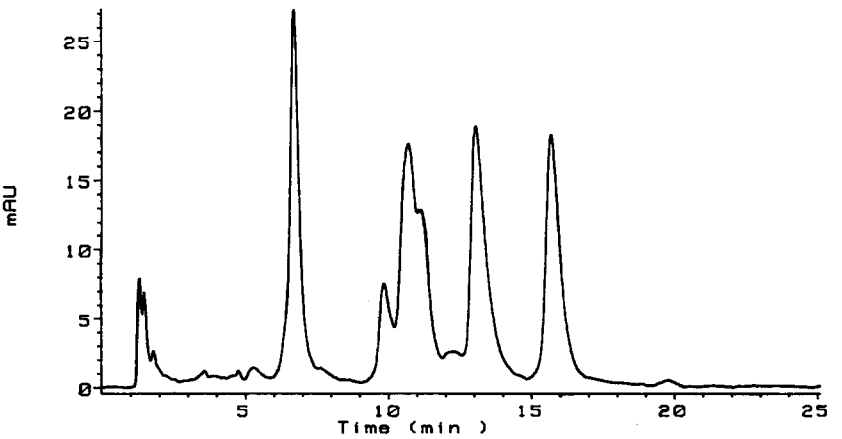

Fig. 8. Protein separation on fluoride modified zirconium oxide. A linear gradient of 0–0.75 M Na_2SO_4 in 100 mM NaF and 20 mM MES at pH 5.5 was used. Flow-rate was 0.5 ml/min at 35°C. Protein loadings were 4.4 μg lysozyme, 15.4 μg α-chymotrypsin, 13.6 μg myoglobin and 15.4 μg cytochrome c.

findings that the Lewis acid site density is quite high relative to the hydroxyl group density [9]. The variation in capacity factors for similar mass loadings for a given protein is an effect due to variations in injection volume. In the loading study, injection volumes varied from 20 to 0.1 μl and for isocratic elution would be expected to have a small effect on the retention volume of proteins.

For preparative separations, protein recovery is an important characteristic. Lysozyme and myoglobin were used as test proteins since their retention mechanisms are quite different. The results of mass recovery studies are summarized in Table IV. Quantitative recovery was accomplished by using two protein assay protocols. This demonstrates that both the ion-exchange mechanism and ligand-exchange mechanisms are free from irreversible binding of proteins under these conditions.

CONCLUSIONS

When zirconium oxide supports are used in fluoride-containing media, a very biocompatible surface is established. The unique overall retention properties of this material are operationally analogous to those of calcium hydroxyapatite. However, the system does not have the chemical and physical weaknesses that limit the use of hydroxyapatite. Fig. 8 shows that very efficient separations can be implemented in any of a number of elution schemes. There are few limitations to what may be present in the eluent compared to hydroxyapatite. However, strongly acidic solutions should be avoided since fluoride forms hydrofluoric acid at low pH. This substance is detrimental to the integrity of the packing material as well as the chromatographic system components.

The substantial base stability allows sterilization of the packing material and stripping of "irreversibly" bound proteins with 0.1 *M* sodium hydroxide solutions. Such treatments strip all solutes including fluoride. Equilibration of the stripped particles with a fluoride solution of the desired ionic strength and pH restores the selectivity and efficiency of the column to its original condition. The sorption/stripping cycle has been repeated at least fifty times on one column with no loss of efficiency or selectivity. This phase is a useful candidate for analytical and preparative separations of biomolecules.

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