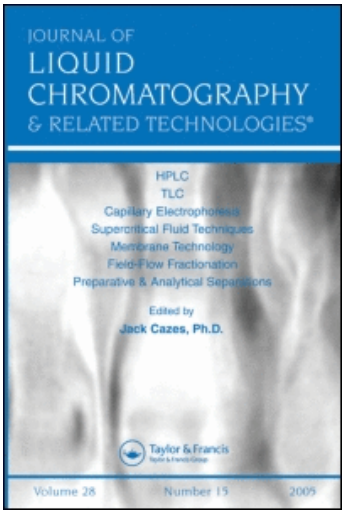

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LIGAND EXCHANGE CHROMATOGRAPHY OF FREE AMINO ACIDS AND PROTEINS ON POROUS MICROPARTICULATE ZIRCONIUM OXIDE

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ABSTRACT

The Lewis acid sites present on the underlying zirconium oxide particles are responsible for the unusual elution sequence for amino acids on copper(II) loaded, phosphated zirconium oxide supports reported in an earlier study. To more thoroughly examine the effect of these strong Lewis acid sites, we have studied ligand exchange chromatography on copper(II) loaded zirconium oxide particles. It is shown here that carboxylate functional groups on amino acid solutes strongly interact with surface Lewis acid sites. Addition of competing hard Lewis bases to the eluent attenuates these specific interactions. The result is a chromatographic system with high selectivity which is also suitable for ligand exchange chromatography of proteins.

INTRODUCTION

Inorganic supports for ligand exchange chromatography have a number of distinct advantages over conventional resin based materials. In general, they have much greater mechanical stability toward flow induced pressure and osmotic pressure changes. Compared to other inorganic supports such as silica, zirconium oxide based supports are chemically stable in strongly alkaline buffers [1,2].

In a previous study, we investigated a porous microparticulate zirconium oxide support which was chemically treated to form a surface layer of zirconium phosphate for use in ligand exchange chromatography [3]. After the resulting material was loaded with copper(II), it was used as a support for the separation of amino acids. The support showed high selectivity between amino acids. In addition, its selectivity was significantly different from that of conventional resin [4,5] and silica [6-9] based supports.

Specifically, basic amino acids are strongly retained on all types of exchangers. Neutral amino acids are less retained than basic amino acids. The main difference in selectivity between the exchangers lies in the elution behavior of the acidic amino acids. On silica and resin based exchangers, acidic amino acids are very weakly retained and always elute before neutral amino acids. On phosphated zirconium oxide, acidic amino acids are generally more retained than neutral amino acids. This behavior is inconsistent with the Doury-Berthod model of ligand exchange chromatography [4,5]. This strongly suggests that an additional retention mechanism is present.

Other studies have shown that Lewis base solutes on native zirconium oxide are retained in large part by ligand exchange at exposed Lewis acid sites [10-16]. A variety of benzoic acid derivatives was used to probe the specific interactions between Lewis base (carboxylate) functional groups and the surface Lewis acid sites [13,14]. These interactions are thermodynamically stronger than other solute-stationary phase interactions, but they can be attenuated by the addition of competing Lewis bases to the

eluent. By careful control of the eluent Lewis base concentration and pH, elution of carboxylic acid solutes could be controlled.

These surface Lewis acid sites may be responsible for the poor efficiencies we observed in the separation of amino acids on phosphated zirconia [3]. Studies of the temperature dependence of the plate height showed that the efficiency increased dramatically at higher temperature. We believe that slow chemical kinetics of ligand exchange are the main problem. This is supported by the fact that tridentate amino acids, which are expected to have slower dissociation kinetics, have much higher reduced plate heights than bidentate amino acids [13,14]. Operating at elevated temperatures improved efficiency by labilizing the ligand exchange kinetics.

Another major source of poor efficiency may be the kinetics of ion exchange between the copper complexes and the exchanger. The strength of the interaction between the complex and the exchanger will have a significant effect on the separation efficiency. As the strength of this interaction increases, the rate of exchange may well decrease. A decrease in rate can dramatically increase reduced plate heights.

Unmodified zirconium oxide supports have a number of inherent advantages over phosphated supports. First, the support is easier to prepare. Second, in contrast to phosphated zirconium oxide, zirconium oxide particles are stable at high pH [17-19]. However, it is not known whether interactions between amino acids or their copper complexes with a zirconium oxide support would be sufficiently efficient to be useful for chromatography. Previous investigators have shown that amino acids interact very strongly with zirconium hydroxide [20] and titanium oxide [21]. The net effect of these interactions on separation efficiency depends upon the relative strength and kinetics of these processes relative to those between amino acids and the copper complexes.

In light of the surface properties of the underlying zirconium oxide particle [10-16], it is likely that pretreatment with phosphoric acid did not fully mask the strong Lewis acid sites [3,10-16]. These sites are known to strongly interact with

carboxylates and may account for the increased retention of acidic amino acids, especially dicarboxylate amino acids [10-16]. To ascertain whether these sites are responsible for the anomalous behavior of acidic amino acids on phosphated zirconium oxide, the chromatographic behavior of copper(II) loaded zirconium oxide was examined in terms of its ligand exchange characteristics.

EXPERIMENTAL

Chemicals

All amino acids were reagent grade or better and were obtained from commercial sources. 4-Morpholinoethanesulfonic acid (MES) was obtained from Sigma (St.Louis, MO). Copper(II) chloride dihydrate and ammonium hydroxide were obtained from Mallinckrodt (Paris, KY). Sodium hydroxide was obtained as a 50% solution from Curtin Matheson Scientific (Houston, TX). Hydrochloric acid, sodium chloride and HPLC grade tetrahydrofuran, isopropanol and acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ). Acetic acid was obtained from EM Science (Cherry Hill, NJ). Disodium ethylenediaminetetraacetic acid was obtained from J.T. Baker (Phillipsburg, NJ). All chemicals were reagent grade or better.

Water used in these studies was prepared by passing house deionized water through a Barnsted NanoPure water system with an additional organic-free cartridge and a 0.2 μm final filter. The water was subsequently boiled for five minutes then cooled to room temperature immediately prior to use to remove dissolved carbon dioxide.

Chromatographic Supports

The porous zirconium oxide particles were provided by the Ceramic Technology Center of the 3M Company and were described earlier [2,10,12,22]. The particles used in this investigation had a nominal diameter of 5.3 μm ± 1.3 μm, an average pore diameter of 308 Å by mercury porosimetry and an average B.E.T. surface area of

32.5 square meters per gram. The particles were initially pretreated in order to remove as many of the manufacturing impurities as possible [10-12].

Chromatographic columns were prepared in 50 mm by 4.6 mm column blanks using 1/4" Parker end fittings. Titanium screens with 2 μm mesh were used instead of frits to minimize any potential extraneous metal ion contamination from the frits. Columns were packed by the upward slurry technique using isopropanol as the solvent. Packing pressure was 4500 psi (300 atm.). Following the packing procedure, all columns were flushed with freshly boiled water to displace all of the packing solvent prior to introduction of buffers.

Chromatographic Systems

Chromatographic studies were carried out on two systems. The first system consisted of a Hewlett Packard (Avondale, PA) 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) 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) with a Rheodyne (Cotati, CA) 7120 injector valve. The detector was a Perkin Elmer (Norwalk, CT) LC-15 fixed wavelength detector with a 230 nm filter. For high pH eluents, the piston seals were replaced with unfilled Teflon piston seals obtained from Beckman Instruments(San Ramon, CA). Both systems were outfitted with a 50 x 4.6 mm column filled with 10-20 μm zirconia particles. This guard column was placed before the injection valve to scavenge any metal ion contaminants in the buffer.

Copper(II) Loading

The packed columns were loaded with copper(II) by flushing the base treated support with 1mM copper(II) chloride in a solution

containing 50% acetonitrile which was 1M in ammonia. Using a flow rate of 0.5 mL/min, the support sorbed 3.9 μmol/m^2. The column was then flushed with the eluent until the baseline at 230 nm was constant.

Following the loading of the copper(II) ion, the column could be stripped by flushing the column with 50 mL of 0.5M sodium chloride in 20mM MES buffer at pH 5.0. This was followed by flushing with 100 mL of 25mM disodium EDTA in 0.1M sodium chloride at pH 5.0. Fifty mL of a 0.1M sodium hydroxide solution was then flushed through the column followed by a rinse with 30 mL freshly boiled water. At the end of each step, the baseline absorbance at 230 nm was constant.

RESULTS AND DISCUSSION

Effect of Ammonia Concentration on Retention

The retention properties of a series of amino acids were examined under two sets of conditions. Table 1 gives the capacity factors for a series of amino acids in 1M ammonia and in 0.5M ammonia/0.5M ammonium chloride. Data obtained with zirconium phosphate are included for comparison. For most amino acids, the capacity factors are greater on the zirconium oxide phase than on zirconium phosphate. The exceptions include: isoleucine, lysine, proline, serine, arginine and histidine. With the exception of isoleucine, these amino acids generally have very high capacity factors on both phases, indicating a strong interaction between either the amino acid complex or the free amino acid with the stationary phase.

The acidic amino acids were much more strongly retained on zirconium oxide than on the phosphated material. Asparagine, glutamine, tyrosine and tryptophan were also more strongly retained on the oxide phase. These amino acids all have Lewis base side chains. This suggests that retention is due in part to interactions of these groups with Lewis acid sites on the particle surface. If

TABLE 1

Retention of Amino Acids on Copper(II) Loaded Phases[a]

capacity factors

	Phosphated Zirconia[3]	Zirconia	
amino acid	1M NH_3	1M NH_3	0.5M NH_3 0.5M NH_4Cl
phenylalanine	0.98	1.13	0.57
tryptophan	1.25	2.13	0.58
methionine	1.30	1.60	0.97
leucine	1.37	1.63	0.60
tyrosine	1.53	2.89	n/m
valine	1.64	1.82	0.59
isoleucine	1.71	1.40	0.47
glutamic acid	1.88	5.78	7.69
aspartic acid	2.22	5.80	11.95
glutamine	2.68	3.59	2.26
threonine	2.82	3.30	3.50
alanine	3.16	5.15	n/m
asparagine	3.50	5.34	4.30
glycine	4.83	4.97	2.24
serine	7.04	6.90	7.40
lysine	7.73	6.59	2.61
proline	10.83	2.11	0.42
histidine	33.09	27.11	1.25
arginine	eno	13.28	2.78
cysteine	eno	eno	eno

a. All eluents contained 0.25mM copper(II) chloride in 50% acetonitrile. Flow rate was 0.5 mL/min at 45°C. n/m = not measured eno = elution not observed

the formation constants for the Lewis base side chains with zirconium are larger than those for ligation to copper, the interaction with the surface zirconium sites will be the dominant retention factor. This complex retention mechanism might lead to a significant decrease in the separation efficiency since the

zirconia support would act more heterogeneously than the zirconium phosphate phase.

The effect of this heterogeneity was studied by examining the reduced plate heights of the amino acids. For all amino acids, the plate heights were significantly greater on zirconium oxide than on zirconium phosphate (e.g. 18 to 283 for leucine and 51 to 316 for glycine for the phosphate and oxide phases, respectively). The increases in reduced plate height were much larger for tridentate amino acids (e.g. aspartic acid increased from 106 to 773 while glutamine rose from 11 to 713).

When 0.5M ammonia/0.5M ammonium chloride buffer was used in place of 1M ammonia, a number of interesting changes were observed. In most cases, the capacity factors decreased due to the higher ionic strength. However, retention of aspartic and glutamic acids, serine and threonine increased. Ionic strength should only have a minor effect on the strong interactions of amino acids with Lewis acid sites.

The use of an ammonia/ammonium chloride buffer did not significantly improve efficiency relative to that observed in 1M ammonia eluent. Many amino acids showed slightly lower plate height, but others, such as serine, threonine and glutamic acid, showed significantly higher reduced plate heights due to the decrease in the concentration of competing ligand. The increase in ammonium concentration shifted the ligand exchange equilibrium to favor the formation of amino acid/metal ion complexes, which in the case of Lewis acid site complexes, increased the plate heights.

Effect of Displacing Ligand

Table 2 summarizes the results obtained when the concentration of the ammonia/ammonium chloride buffer was varied while maintaining constant total ionic strength. The amino acids do not elute in the absence of the displacing ligand. Addition of 50mM buffer to the eluent caused elution of all amino acids except tyrosine. The plate heights, though, were quite unsatisfactory. The use of higher displacing ligand concentrations significantly decreased the

TABLE 2

Effect of Ammonia/Ammonium Chloride Concentration at Constant Ionic Strength

Eluent[b]	capacity factor[a] Phenylalanine	Alanine	Tryptophan	Tyrosine
0.50M NaCl	eno	eno	eno	eno
0.49M NaCl 0.05M NH_3 0.05M NH_4Cl	6.85 (464)	9.35 (216)	9.84 (758)	eno (n/a)
0.30M NaCl 0.20M NH_3 0.20M NH_4Cl	1.98 (311)	1.94 (198)	2.54 (769)	eno (n/a)
0.50M NH_3 0.50M NH_4Cl	0.70 (127)	0.63 (70)	1.12 (446)	1.53 (235)

a. values in parenthesis are experimentally determined reduced plate heights.
b. All eluents contain 0.25mM $CuCl_2$ at pH 9.2. Flow was 0.5 mL/min at 45°C. eno = elution not observed.

capacity factors for all amino acids, although tyrosine still did not elute.

Use of 0.5M buffer lowered the capacity factors and caused tyrosine to elute. At the same time, the plate heights decreased. We believe that the high displacing ligand concentration caused elution by shifting the ligand exchange equilibrium towards formation of the ammonia complexes (see reaction 1).

$$Cu^{+2}AA + n\ NH_3 \quad - \quad Cu(NH_3)_n^{+2} + AA \qquad 1.$$

The improved efficiency is a result of blocking some of the surface Lewis acid sites. However, even the highest efficiencies shown in Table 2 are still quite poor.

TABLE 3

Effect of Acetate Buffer Concentration at Constant Ionic Strength

Eluent[b]	capacity factor[a] Alanine	Phenylalanine	Tyrosine	Tryptophan
0.50M NaCl	eno	eno	eno	eno
0.49M NaCl .01M HOAc .01M NaOAc	eno (n/a)	eno (n/a)	eno (n/a)	eno (n/a)
0.40M NaCl .10M HOAc .10M NaOAc	0.18 (21)	0.32 (61)	0.34 (96)	0.55 (117)
0.5M HOAc .50M NaOAc	n/a (n/a)	0.16 (n/a)	0.18 (13)	0.26 (43)

a. values in parenthesis are experimentally determined reduced plate heights.
b. All eluents contain 0.25mM $CuCl_2$ at pH 4.8. Flow was 0.5 mL/min at 45°C. eno = elution not observed. n/a = not assessed

Arikawa found a different elution sequence of amino acids when acetate anion was used as the competing ligand [23]. The Lewis basicity of acetate is much higher than ammonia [3,12-14] and consequently, the amino acid elution sequence is quite different. Table 3 summarizes the results obtained when acetate was used in place of ammonia. Small amounts of acetate did not bring about elution of any amino acids, but 0.2M acetate buffer at pH 4.8 caused all amino acids to elute very rapidly. Higher concentrations of acetate decreased the capacity factors without changing the selectivity.

Separation efficiency dramatically improved in acetate buffer. It is not clear whether this improvement resulted from the difference in pH or in the nature of the displacing ligand. At low pH, the amine portion of the amino acid is protonated. This may

TABLE 4

Effect of Sulfate Buffer Concentration at Constant Ionic Strength

Eluent[b]	capacity factor[a] Tyrosine	Phenylalanine	Alanine	Tryptophan
0.50M NaCl	eno	eno	eno	eno
0.42M NaCl 0.01M Na_2SO_4 0.05M MES	1.33 (198)	1.42 (292)	1.53 (292)	1.98 (505)
0.15M NaCl 0.10M Na_2SO_4 0.05M MES	1.82 (356)	1.84 (n/a)	2.13 (n/a)	2.71 (764)

a. values in parenthesis are experimentally determined reduced plate heights.
b. All eluents contain 0.25mM $CuCl_2$ at pH 4.8. Flow was 0.5 mL/min at 45°C. eno = elution not observed.

induce additional retention through ion exchange with the few ionized surface hydroxyls at the zirconium oxide surface, whereas at higher pH the amine group is neutral. This protonation also inhibits the formation of coordinative links with the copper ion since protonated amines are very poor Lewis bases. Thus, coordination should take place via the carboxyl group or side chain Lewis base groups. Clearly, the operating efficiencies with acetate are much more acceptable than with ammonia buffer.

Sulfate binds somewhat more strongly to copper ion than does acetate (log $K_1(SO_2^{=}) = 2.26$ and log $K_1(CH_3COO^-) = 1.88$) [24] and is expected to bring about elution of the amino acids more readily under otherwise similar conditions due to their greater complexing ability towards copper(II). Table 4 shows that a low concentration of sulfate eluted the amino acids, but the selectivity is different than that observed with either acetate or ammonia.

TABLE 5

Effect of Various Salts on Amino Acid Retention

Eluent[b]	capacity factor[a] Alanine	Phenylalanine	Tyrosine	Tryptophan
0.50M NaCl	eno	eno	eno	eno
0.50M NH_4Cl	eno	eno	eno	eno
0.50M NaOAc	1.61 (341)	1.68 (369)	1.95 (385)	2.34 (728)
0.16M Na_2SO_4	eno	eno	eno	22.21 (169)
0.50M NaF	n/a	4.45 (449)	4.96 (481)	4.73 (571)

a. values in parenthesis are experimentally determined reduced plate heights.
b. All eluents contain 0.25mM $CuCl_2$ and 20 mM MES at pH 5.5. Flow was 0.5 mL/min at 22°C. eno = elution not observed.

The exact relationship between ammonia, acetate and sulfate ligation is obscured by other differences in elution conditions. Ammonia was used at high pH while acetate and sulfate were used at low pH. Sulfate required addition of a buffering salt to maintain the pH. While this buffer salt (MES) is a Good's buffer and was chosen to minimize metal ion interactions, its effect on the overall equilibria is not known. However, Good's buffers have been shown to be weak competing Lewis bases towards carboxylic acids [13,14].

The data of Tables 2-4 clearly show that the displacing ligand is critical, thus a series of ligands were tested under nearly identical conditions. The results of this comparison are shown in Table 5. Despite the fact that fluoride forms much weaker complexes with copper(II) (log $K_1[F^- - Cu^{+2}]$ = -0.7) compared to acetate and sulfate, it is evidently a strong displacer. We believe that this

results because fluoride binds very strongly to surface zirconium Lewis acid sites (log $K_1[F^- \text{-} Zr^{+4}]$ = 8.8) [24]. This strongly supports our view that on the copper(II) loaded zirconium oxide, amino acids are held by two chemically distinct processes: ligand exchange at surface copper(II) sites and ligand exchange at surface zirconium(IV) Lewis acid sites. The eluent must have good displacing ability towards both types of sites in order to elute the amino acids. Phosphate masks these interactions as do sulfate and acetate [2,10-19].

The experimental plate heights indicate that the fluoride did not significantly improve the efficiency. Indeed, in some cases the efficiency decreased. This is probably due to the overall heterogeneity of the system and the complexity of the retention process.

Effect of Temperature on Amino Acid Retention

Figure 1 shows the effect of temperature on the capacity factor and the reduced plate height. Alanine was chosen for this study because it has a comparatively low reduced plate height. As in our earlier work [3], the capacity factor increased upon increasing the temperature. The increase here was two to three times greater than on the phosphated phase [3] (slopes: 0.0858/°C and 0.2115/°C for the phosphate and oxide phases, respectively).

On the phosphated phase, the reduced plate heights did not decrease until the temperature exceeded 50°C. The decrease in reduced plate height with temperature had a slope of -0.40/°C. On the zirconium oxide phase, the reduced plate height began to decrease at temperatures greater than 35°C. The efficiency improved quite rapidly and then reached a limiting value at higher temperature. The overall rate of change was -0.81/°C.

Flow Rate Effects

Figure 2 shows the effect of flow rate on the plate height in the form of a van Deemter plot. The increase in reduced plate height was quite dramatic at low flow rates, but appeared to

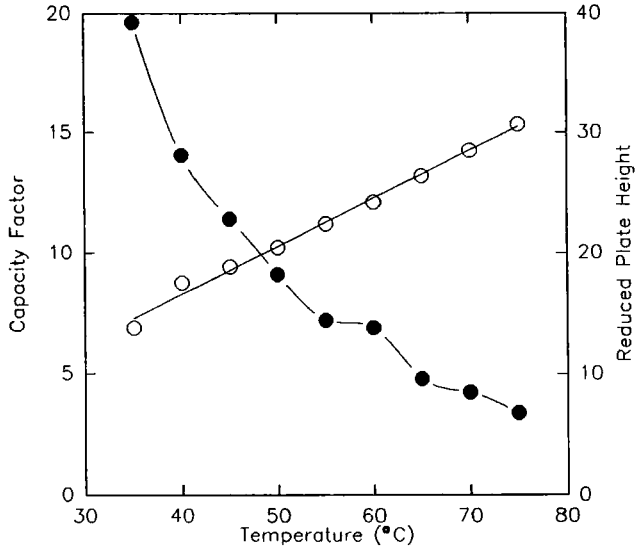

Figure 1. Capacity factor (O) and efficiency (●) for alanine on copper loaded zirconium oxide. Eluent was 1M ammonia in 50% acetonitrile containing 0.25mM copper(II) chloride. Flow rate was 0.5 mL/min with detection at 254 nm.

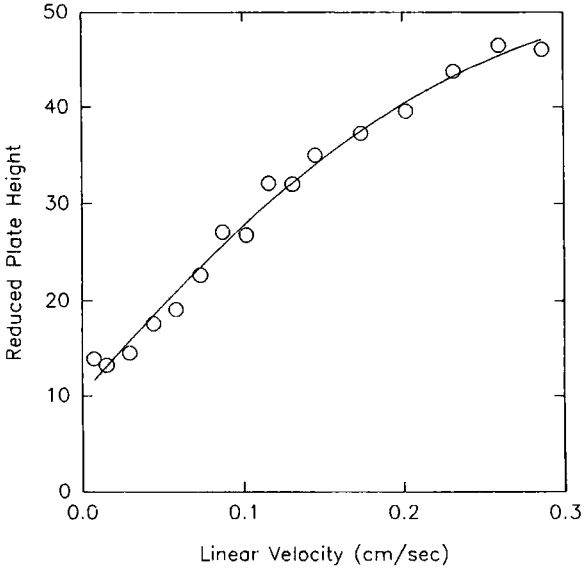

Figure 2. Van Deemter plot for copper loaded zirconium oxide. Test solute was alanine operating at 45°C with detection at 254 nm. Eluent was 1M ammonia in 50% acetonitrile containing 0.25mM copper(II) chloride.

diminish as the linear velocity was increased above normal operating rates. This behavior is comparable to the phosphated system and indicates a slow chemical kinetic process.

Protein Separations

Rigney found that proteins could only be eluted from zirconium oxide supports at very high pH [2,22]. The eluted species were very poorly resolved and the efficiency was very poor. Subsequently, Schafer prepared a zirconium phosphate surface from porous zirconium oxide and demonstrated that proteins were retained by cation exchange and could be eluted with reasonable peak shapes at neutral pH [17-19]. Retention was due to cation exchange on the exposed phosphate groups.

The original goal of the present work was to separate proteins by ligand exchange on zirconium oxide supports. However, by analogy to the phosphated zirconium oxide, we felt that in order for this material to work, the strong adsorption sites would have to be masked. Acetate appeared to improve the efficiency of amino acid elution by blocking the zirconium(IV) Lewis acid sites, but earlier experiments by Rigney showed that acetate buffers do not mask the surface sufficiently to allow protein elution [2,22]. Similarly, sulfonate buffers did not help. Here we report on the use of fluoride as a possible displacing agent for the separation of proteins on metal ion loaded columns. The ability to elute proteins without phosphate in the mobile phase was a significant development in the understanding of the adsorption process, since only an interaction between fluoride and surface zirconium ions can account for the elution of proteins under these conditions [11].

Table 6 shows the results obtained when a variety of proteins were separated on a zirconium oxide column which had been treated with a high concentration of fluoride with and without added copper(II). In the absence of copper(II), all proteins were eluted with high recovery, as determined by comparing peak areas with and without a column in place. In most cases, the proteins were not retained to any significant extent due to the use of high salt

TABLE 6

Protein Retention on Zirconium Oxide with Fluoride Buffers[a]

protein	pI	capacity factors without Cu^{+2}	with Cu^{+2}
trypsin inhibitor	4.6	0.13	0.50
β-lactoglobulin	5.1	0.43	eno
lipase	5.2	0.25	3.31
myoglobin	6.8	0.53	8.83
immunoglobulin G	7.6	0.30	eno
α-chymotrypsin	8.8	-0.03	4.45
lysozyme	11.0	0.30	2.04

a. Gradient from 100% A to 100% B in 30 min then to 100% A in 10 min, hold 5 min, change to 100% C in 0.1 min with a 5 min hold followed by a change to 100% A in 0.1 min with a 20 min equilibration period. Flow rate was 0.5 mL/min at 35°C. Detection was at 280 and 410 nm.

A = 0.5M Na_2SO_4 20mM NaF 20mM TAPS pH 8.4;
B = 0.5M $(NH_4)_2SO_4$ 20mM NaF 20mM TAPS pH 8.4;
C = 20mM NaF 20mM TAPS pH 8.4 (without Cu^{+2}) or 1M NH_3 1mM $CuCl_2$ (with Cu^{+2}).

concentrations in the eluents. Lysozyme, myoglobin and β-lactoglobulin were slightly retained.

Addition of a copper loading step to the gradient elution procedure allowed the reproducible loading of copper(II) onto the support prior to each run. When copper was preloaded onto the column, a significant difference in protein retention was observed. In all cases, retention increased. This increase occurred without regard to net protein charge, which implies that ionic interactions are not solely responsible for the increased retention. The increased retention was, therefore, attributed to the ligand exchange interaction between immobilized copper ions and the proteins. This demonstrates that the original goal of ligand exchange separations of proteins on the pH stable support is feasible. It also demonstrates that the poor efficiencies observed for the amino acid separations are not directly relevant to the

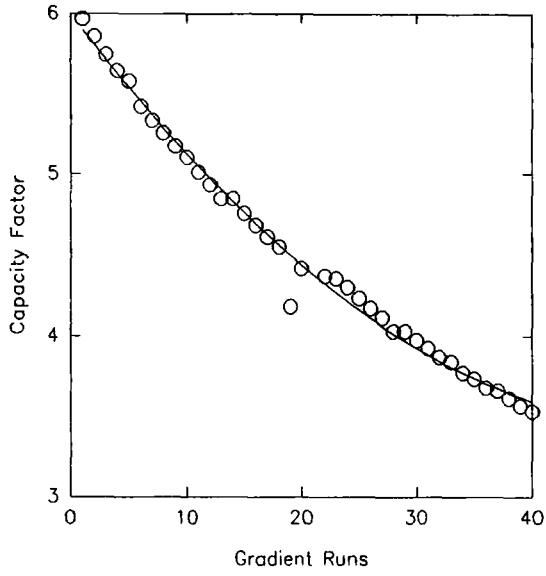

Figure 3. Myoglobin retention stability without copper ion in the mobile phase. Gradient elution was described in Table 4. Buffer C was replaced with 20mM sodium fluoride in 20mM TAPS at pH 8.4.

separation of proteins since the proteins are rather efficiently separated. This difference arises in part because the amino acids chelate copper ions while the proteins presumably have more independent interactions with the copper ions.

The separation of proteins on the copper ion loaded phase depends upon the reproducible loading of the exchanger. As is true for all exchangers, solutes which interact strongly with the metal ions tend to strip the metal ions from the exchanger. If saturation of the exchanger is not maintained, the capacity factors will decrease as copper is stripped. This effect is observed in Figure 3, where the capacity factor for myoglobin is monitored when there was no copper regeneration step in the gradient procedure. The decrease in capacity factor continues until the capacity factor reaches that obtained on a copper ion free support. Regeneration

with copper ion readily restores the capacity factor to its original value.

CONCLUSION

Investigation of the zirconium oxide phase led to some interesting discoveries. The large decrease in separation efficiency compared to that on the phosphated phase showed that the zirconium(IV) Lewis acid sites on the underlying particle are involved in the retention of amino acids on copper(II) loaded zirconium phosphate. Interactions of the solutes with these Lewis acid sites produces a great deal of complexity in the ligand exchange equilibria. This accounts for the poor reduced plate heights observed with buffers which do not contain strong, hard Lewis bases to mask these interactions. This also accounts for the overall selectivity differences between zirconia based phases and silica based phases. Acidic amino acids have an additional carboxylate group which is available for interaction with the surface zirconium ions, whereas neutral and basic amino acids do not have these extra hard Lewis base groups.

The use of strong Lewis base buffers improved the operating efficiency of the zirconium oxide phase. Acetate effectively blocked these sites from entering into the ligand exchange equilibria and dramatically improved the plate heights. Subsequent experiments showed that other Lewis bases such as sulfate and fluoride have a similar effect. However, despite improvements in reduced plate heights, the overall system cannot compete with more highly efficient means of amino acid separation such as reversed phase or conventional ion chromatography.

Proteins can be separated on copper loaded zirconium oxide phases in a rather efficient manner. Proteins have a number of exposed side chains which can interact with metal ions, but do so in a different manner than amino acids. Amino acids form strong complexes via chelation of proximal ligands. Proteins provide the

same types of ligands, but there is generally a larger spatial separation of these groups so the entropic advantages of chelation are not realized to the same extent as for the amino acids. Therefore, the interactions are individually weaker.

The result is a system which can be used for ligand exchange separation of proteins. The zirconium oxide phase is stable to both pH and temperature and is readily reloaded with copper(II) in a reproducible manner. Separations using this phase are efficient and offer unique selectivity due to the lack of hydrophobic interactions with the support observed when using conventional ligand exchange resins. An additional advantage to this system is the stability afforded by the mechanical rigidity of the support. Shrinking and swelling of the support are not possible, therefore bed fissuring and collapse will not occur, as in the case of resin based materials.

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