High-performance metal chelate interaction chromatography of proteins with silica-bound ethylenediamine-N,N'-diacetic acid

MARIA D. BACOLOD and ZIAD EL RASSI*

Department of Chemistry, Oklahoma State University, Stillwater, OK 74078-0447 (U.S.A.)

ABSTRACT

Wide-pore microparticulate silica gel having surface bound ethylenediamine-N,N'-diacetic acid (EDDA) was developed for the separation of proteins by metal chelate interaction chromatography (MIC). The separation of proteins including glycoproteins was carried out by gradient elution using increasing salt concentration. Different selectivities were obtained when changing the nature of the chelated metal on the surface of the stationary phase, manipulating eluent pH and varying the nature and concentration of the salts in the eluent. The retention–pH dependency of iron-free and holo transferrins suggested that MIC could be used for studying metal–protein complexes provided that the metal binding site in the protein molecule is also involved in the protein interactions with the chelated metal on the surface of the stationary phase. The eluting strength of sodium salts with "hard" metal–EDDA columns increased in the order $Cl^- < CH_3COO^- \leqslant HCOO^- < H_2PO_4^-$, whereas with "soft" metal–EDDA columns it increased in the order $CH_3COO^- < H_2PO_4^- < Cl^-$. Compared to another type of metal interaction column such as silica-bound iminodiacetic acid (IDA), the EDDA column exhibited different selectivity and retentivity toward proteins under otherwise identical elution conditions. Metal–EDDA stationary phases can be viewed as complementary to metal–IDA ones.

INTRODUCTION

High-performance metal chelate interaction chromatography (MIC) is a useful technique for the purification and determination of proteins[1–6]. It offers a selectivity different from that of other biopolymer high-performance liquid chromatography (HPLC) techniques such as ion-exchange and hydrophobic interaction chromatography. Indeed, MIC has been the technique of choice for the isolation of interferons[7,8]. Recently, it has been shown that MIC is a suitable HPLC technique for the separation and purification of human erythrocyte glycophorins[3]; traditional approaches by size-exclusion[9] and ion-exchange chromatography[10] were ineffective in purifying the different forms of the membrane proteins. In addition, MIC has been found to be

more effective than hydroxyapatite chromatography for the separation of site specific variants of subtilisin[6].

In metal chelate interaction chromatography, a metal ion is immobilized to the stationary phase via chelating functions bound to the surface. Retention and separation is achieved by the interaction of solute molecules with the chelated metal. Side chain groups of amino acid residues on the surface of the protein molecule, such as histidine, cysteine and to a lesser extent tryptophan, are thought to penetrate the outer coordination sphere of the chelated metal and form coordinate bonds.

Selectivity and retention in MIC can be conveniently modulated and adjusted (i) by changing the type of the metal on the stationary phase[11-13], (ii) by varying the eluent pH[5,12], (iii) by the nature and concentration of the salt[3,11,14,15], and (iv) by the nature and concentration of the competing agent in the eluent[11,16]. Thus far, little attempt has been made to manipulate retention and selectivity by the nature of the chelating functions on the surface of the stationary phase[12,17,18]. Indeed, with agarose or rigid microparticulate stationary phases, iminodiacetic acid (IDA) chelating functions have been the most widely used. This indicates that the potential of MIC has not been fully explored so that the technique will have a common place in a wider range of applications.

This report addresses the need for investigating the potential of new chelating functions in MIC of proteins. In this regard, large-pore silica-bound ethylenediamine-N,N'-diacetic acid (EDDA) was developed in our laboratory. The chromatographic behavior of standard proteins and glycosylated proteins was evaluated with metal–EDDA chelates over a wide range of elution conditions including eluent pH, salts and competing agents. A comparison with IDA stationary phases is also presented.

EXPERIMENTAL

Instrumentation

The chromatograph was assembled from an ISCO (Lincoln, NE, U.S.A.) Model 2350 solvent delivery pump and a Model 2360 gradient programmer with a variable-wavelength detector Model V[4]. A Rheodyne (Cotati, CA, U.S.A.) Model 7010 sampling valve with a 100-μl sample loop was used for injection. Chromatograms were recorded with a Shimadzu (Columbia, MD, U.S.A.) Model C-R5A integrator.

Columns

Zorbax PSM 300, a spherical silica, having mean particle and pore diameters of 7.5 μm and 300 Å, respectively, was obtained from DuPont (Wilmington, DE, U.S.A.). The EDDA and IDA stationary phases were made by first reacting Zorbax silica gel with γ-glycidoxypropyltrimethoxysilane in aqueous solution, pH 6.0, for 2 h at 95°C[19]. The products thus obtained were allowed to react with EDDA or IDA using a well established procedure[11]. The surface coverage with EDDA or IDA functions was found to be approximately 2.0–2.2 μmol/m^2, which correspond to 96–105 μmol/ml of packed silica gel, as calculated from the nitrogen content measured by elemental analysis at Galbraith Labs. (Knoxville, TN, U.S.A.). The approximate structures of both stationary phases in metal chelate forms are shown in Fig. 1. The stationary phases thus obtained were packed from an aqueous sucrose–sodium chlo-
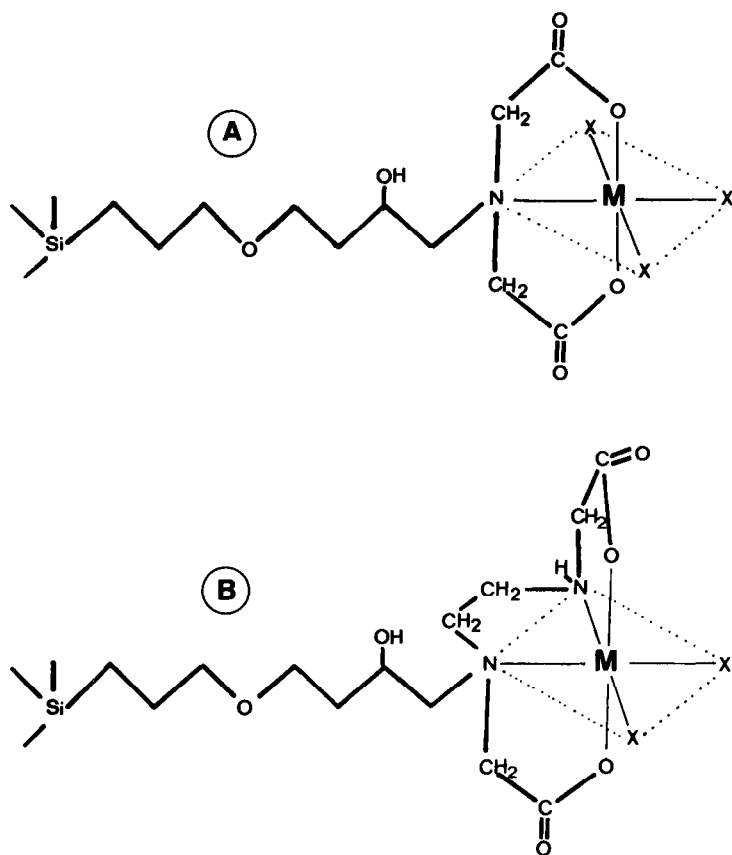
Fig. 1. Schematic illustration of the surface bound metal chelates. (A) Tridentate IDA and (B) tetradentate EDDA. M = Metal; X = protein molecule, salt ion, water molecule or any competing agent.

ride slurry containing 50% sucrose (w/v) at 8000 p.s.i. with 1.0 M sodium chloride solution and using a Shandon column packer instrument (Keystone Scientific, Bellefonte, PA, U.S.A.). All columns were made of 100 × 4.6 mm I.D. No. 316 stainless steel tubes (Alltech Assoc., Deerfield, IL, U.S.A.).

Materials

The following materials were purchased from Sigma Chemical (St. Louis, MO, U.S.A.): cytochrome c from horse heart; lysozyme from chicken egg white; iron-free (*ca.* 90% substantially iron-free) and holo (*ca.* 98% iron saturated) transferrins from human; lactoferrin from bovine colostrum; β-casein from bovine milk; and EDDA. IDA was obtained from W. R. Grace (Nashua, NH, U.S.A.). Reagent grade sodium hydroxide, ethylenediaminetetraacetic acid (EDTA) disodium salt, acetic acid, ferric chloride, zinc chloride, cupric chloride, cobalt chloride, nickelous nitrate, phosphoric acid, sodium acetate, sodium formate, methanol and acetonitrile (both HPLC grade) were obtained from Fisher (Pittsburgh, PA, U.S.A.). γ-Glycidoxypropyltrimethoxysilane was obtained from Aldrich (Millwaukee, WI, U.S.A.).

Procedures

Freshly packed columns with IDA or EDDA siliceous stationary phases were first conditioned with water and then loaded with the appropriate metal by injecting 10 ml of 50 mM metal salt solution using a sampling valve equipped with a 10-ml sample loop. This amount was enough to saturate the column with the desired metal since we have found that the retention of standard proteins did not change for concentrations above 50 mM. After loading the column with a given metal, the excess unchelated metal was subsequently removed from the column by washing it with an ample amount of water followed by the equilibrating mobile phase in order to ensure reproducible results during the ensuing chromatographic separation. The column was unloaded from the metal by washing it with 20 ml of 50 mM EDTA disodium salt. After regeneration with water, the column regained its naked form (without chelated metal) and was ready for reloading with a different metal.

RESULTS AND DISCUSSION

Columns packed with silica-bound EDDA, chelated with Cu(II), Co(II), Ni(II), Zn(II) or Fe(III) metal ions, were evaluated in protein HPLC over a wide range of elution conditions. These metal–EDDA stationary phases were also compared to metal–IDA stationary phases. Phosphorylated and dephosphorylated β-caseins, lactoferrin, iron-free and holo transferrins, cytochrome c and lysozyme were used as model solutes. These proteins, which are well characterized in many aspects, form an attractive set of solutes for elucidation of protein–metal chelate stationary phase associations. Horse heart cytochrome c and egg white lysozyme, each having one surface exposed histidine residue, may serve to evaluate the extent to which proteins that have the same histidine content but are slightly different in molecular weights (MW) and pI values (cytochrome c: MW 12 200, pI 10.6; lysozyme: MW 14 000, pI 11.0) will interact with different metal chelate sorbents. Phosphorylated and dephosphorylated β-caseins may be useful to ascertain the involvement of phosphate groups in metal chelate interaction chromatography. Human serum transferrins and bovine lactoferrin, which are iron-binding proteins[20] (two ferric ions per molecule of protein) of similar molecular weights (about 80 000 for lactoferrin and 75 000 for transferrin), yet differing in their isoelectric points (about 6.0 for human transferrin and 10.0 for bovine lactoferrin), may be regarded as model proteins to assess the implication of the net charge of protein in MIC.

As expected, metal–EDDA columns exhibited weaker interactions with the proteins investigated than their counterparts metal–IDA columns under otherwise identical elution conditions. As illustrated in Fig. 1 metal–IDA columns provide more coordination sites for interaction with the protein molecule, than do metal–EDDA columns. On the other hand, as a result of one fewer donor atom in the IDA molecule, metal–IDA complexes are less stable than metal–EDDA chelates; *cf.* Table I, which compiles the logarithmic stability constants of both metal complexes as measured in free solution[21]. Indeed, certain metal–IDA stationary phases such as Zn(II)–IDA[5] and Cu(II)–IDA[2] are unstable under most elution conditions and change in retention time from run to run is common. In order to circumvent this problem, Figueroa *et al.*[2] added a small amount of salt of the chelated metal to the mobile phase.

TABLE I

LOGARITHMIC STABILITY CONSTANTS OF METAL–EDDA AND METAL–IDA COMPLEX-ES IN FREE SOLUTIONS

Data taken from ref. 21.

Metal	Logarithmic stability constant	
	EDDA	IDA
Zn(II)	11.22	7.24
Ni(II)	13.65	8.13
Co(II)	11.25	6.94
Cu(II)	16.20	10.57
Fe(III)	–	10.72

Particularly noticeable is the strong binding of most proteins to the Cu(II)–IDA column[11,12]. The elution of proteins from the Cu(II)–IDA column necessitated the use of either a linear gradient by increasing both salt and glycine (a bidentate compet-ing agent) concentrations in the eluent or a gradient by decreasing pH and increasing imidazole (a monodentate competing agent) concentration, which often lead to leach-ing out of the metal and contamination of the separated proteins. It has been demon-strated that the contamination of the protein with metal ions removed by the compet-ing agent from the stationary phase may be avoided by loading the first two-thirds of the column with the metal only[22]. Another alternative may be the use of a naked IDA post column. However, in both cases a metalloprotein may lose its metal to the naked IDA and consequently its biological activity may be reduced as it has been found for holocarboxypeptidase A[23]. In contrast, with the Cu(II)–EDDA column the proteins under investigation were readily eluted and separated with a gradient of increasing sodium chloride concentration in the eluent. On the other hand, the proteins studied could be eluted from both Co(II)–IDA and Co(II)–EDDA columns using the same salt gradient. The retention data obtained on both stationary phases are shown in

TABLE II

COMPARISON OF α-VALUES OF PROTEINS MEASURED WITH Co(II)–IDA AND Co(II)–EDDA COLUMNS

Columns, 100 × 4.6 mm I.D. each; flow-rate 1.0 ml/min; temperature, 25°C. Linear gradient in 15 min from 0 to 1.0 M sodium chloride in 10 mM acetate buffer, pH 5.5, followed by 5 min isocratic elution with 1.0 M sodium chloride in 10 mM acetate buffer.

Pair of proteins	Selectivity, α	
	Co(II)–EDDA	Co(II)–IDA
Iron-free transferrin–holo transferrin	8.79	1.00
Cytochrome c–iron-free transferrin	1.23	1.06[a]
Lysozyme–cytochrome c	1.15	1.12
Lactoferrin–lysozyme	1.32	1.45

[a] Reversal in elution order.

Table II in terms of selectivity. As can be seen in Table II, the Co(II)-EDDA column exhibited higher selectivity than the Co(II)–IDA column and in particular toward holo and iron-free transferrins. However, the Co(II)–IDA stationary phase yielded higher retention for the proteins than did Co(II)–EDDA. As a result, peaks were broader on the former columns than on the latter.

As stated above, the elution of the different proteins from the various metal–EDDA columns was carried out using a linear salt gradient by increasing the sodium chloride concentration in the eluent. Different selectivities were obtained when going from one metal chelate column to another under otherwise identical elution conditions. Fig. 2, which depicts the separation of five proteins on the Co(II)–EDDA column, demonstrates the high selectivity and high efficiency that can be obtained with such columns.

Fig. 3 illustrates the separation of phospho- and dephosphorylated β-caseins on the Fe(III)–EDDA column using a linear gradient by increasing the sodium chloride concentration in the eluent. Whereas dephosphorylated casein eluted from the Fe(III)–EDDA column with practically little or no retention, phosphorylated casein
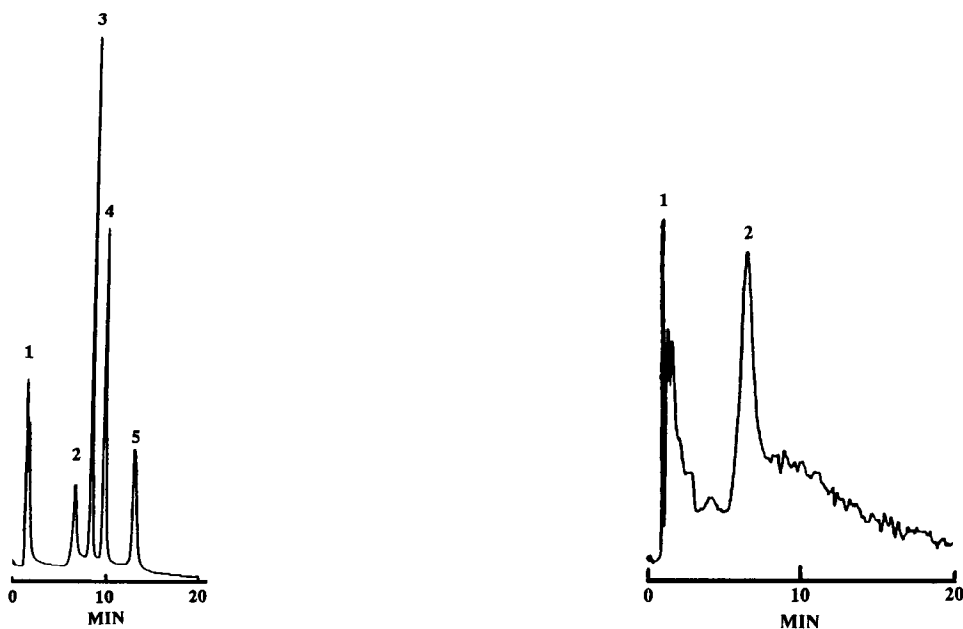

Fig. 2. Chromatogram of standard proteins obtained on Co(II)–EDDA column. Column, 100 × 4.6 mm I.D.; flow-rate, 1.0 ml/min; temperature, 25°C. Linear gradient in 15 min from 0.02 to 1.0 M sodium chloride in 10 mM sodium acetate buffer, pH 5.5, followed by 5 min isocratic elution with 1.0 M sodium chloride in 10 mM sodium acetate. Proteins: 1 = holo transferrin; 2 = iron-free transferrin; 3 = cytochrome c; 4 = lysozyme; 5 = lactoferrin. UV detection at 280 nm.

Fig. 3. Chromatogram of phosphorylated and dephosphorylated β-caseins. Column, Fe(III)–EDDA; flow-rate, 1.0 ml/min; temperature, 25°C. Linear gradient in 15 min from 0 to 1.0 M sodium chloride in 10 mM sodium acetate, pH 5.0, followed by 5 min isocratic elution with 1.0 M sodium chloride in 10 mM sodium acetate. Proteins: 1 = dephosphorylated β-casein; 2 = β-casein. UV detection at 280 nm.
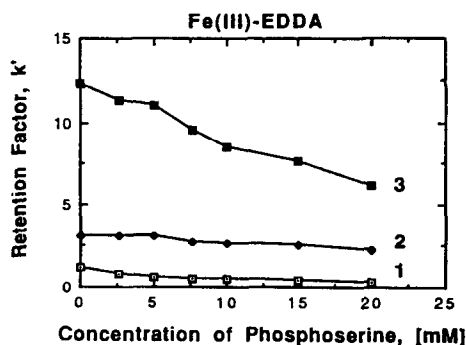
Fig. 4. Plots of retention factor of proteins against phosphoserine concentration in the eluent. Column, 100 × 4.6 mm I.D.; flow-rate, 1 ml/min; temperature, 25°C. Isocratic elution with 10 mM acetate containing 0.15 M sodium chloride at different phosphoserine concentrations, pH 5.0. Proteins: 1 = iron-free transferrin; 2 = cytochrome c; 3 = lysozyme.

was retained by the metal chelate column. This is not unexpected since it is well known that phosphate forms a complex with ferric ions.

In another set of experiments phosphoserine, a phosphorylated amino acid, was added to the eluent and the retention of cytochrome c, lysozyme and iron-free transferrin on the Fe(III)–EDDA column was measured using isocratic elution. As can be seen in Fig. 4, a few millimoles of this amino acid were a useful adjunct for modulating protein retention. Indeed, when adding 20 mM phosphoserine to the eluent, the retention factors of iron-free transferrin, lysozyme and cytochrome c decreased by a factor of 0.2, 0.5 and 0.7, respectively, from their values obtained in the absence of phosphoserine. This is another indication of the predominance of metal interaction with Fe(III)–EDDA stationary phase.

The effect of eluent pH on MIC retention was investigated with various metal–EDDA columns using gradient elution with linearly increasing sodium chloride concentration in the eluent. The results are depicted in Fig. 5 by plots of adjusted retention volumes *versus* eluent pH. As expected, this effect varies from one metal chelate column to another for a given set of proteins[11]. With the exception of the Ni(II)–EDDA column, which did not exhibit an affinity toward holo and iron-free transferrins in the pH range studied, the retention of these two proteins decreased with increasing pH on all other metal–EDDA columns and reached zero at pH values which were different from one column to another. Both transferrins were retained to the same extent on Zn(II)–EDDA at pH 5.0 but eluted with no retention at pH 5.5 and above. On the other hand, on the Fe(III)–EDDA column the holo and iron-free transferrins were only separated at pH 6.25, whereas on Co(II)–EDDA they could be separated at pH values ranging from ca. 5.2 to 6.0 (*cf.* Fig. 5). The decrease in affinity of transferrins toward the chelated metals on the surface of the stationary phase with increasing pH may be explained by the increase in net negative charge of the proteins leading to electrostatic repulsion from the sorbent having the same net charge. The equal affinity of holo and iron-free transferrins toward some of the metal chelate columns at low pH (below or equal to 5.0) may be explained by the dissociation of the iron–protein complex at that pH[20] so that the holo transferrin will lose its iron and
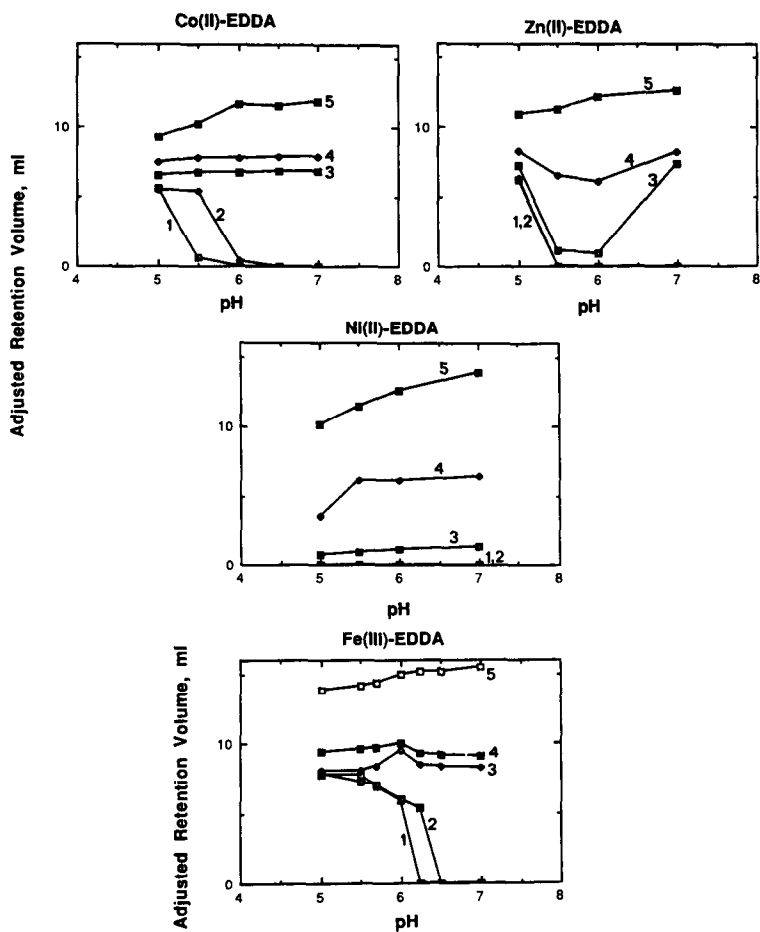
Fig. 5. Plots of adjusted retention volume *versus* pH, measured with different metal–EDDA columns. Column, 100 × 4.6 mm I.D.; flow-rate, 1.0 ml/min; temperature, 25°C. Linear gradient in 15 min from 0 to 1.0 M sodium chloride in 10 mM acetate or phosphate buffer at different pH values, followed by 5 min isocratic elution with 1.0 M sodium chloride in 10 mM buffer. 1 = holo transferrin; 2 = iron-free transferrin; 3 = cytochrome c; 4 = lysozyme; 5 = lactoferrin.

become an iron-free protein. Therefore, we believe that MIC will find use in studying protein–metal complexes provided that the metal-binding site in the protein molecule is also involved in the interaction process between the protein and the chelated metal on the surface of the stationary phase. The monotonic increase in the retention of lactoferrin with eluent pH on the various metal–EDDA columns (Fig. 5) may reflect the presence of imidazole groups on the surface of the protein molecule. Indeed, with the high histidine content of such a molecule[20] and the high pI value (net positive charge over a wide range of pH) an interaction of that kind may be favored. In contrast, due to their net negative charge at pH values above 5.5–6.0, acidic transferrins may be hindered from interacting with the chelated metals of the stationary

phases despite their high content of histidine. The plots of retention *vs.* eluent pH for cytochrome *c* and lysozyme on the Zn(II)–EDDA column are U-shaped curves (see Fig. 5). This may be explained by the presence of both carboxyl and imidazole groups in the binding site to the Zn(II)–EDDA column. These same proteins showed little or no change in retention on other columns when varying the eluent pH. It has been advocated that a cluster of groups rather than a single group[24,25] may be involved in the binding of proteins to the chelated metal on the surface of the stationary phase.

To study the effect of the nature and concentration of the salt in the eluent on MIC retention and selectivity, isocratic measurements were carried out with various salts at pH 5.5. The salts studied were sodium chloride, sodium formate, sodium acetate and sodium phosphate, and the columns examined were Co(II)– and Fe(III)–EDDA. Co(II) is a representative of "soft" metal ions, whereas Fe(III) is somewhat on the borderline of "hard" metal ions[26]. In all cases retention decreased with increasing salt concentration in the eluent in the concentration ranging from 0 to 0.5 M. Typical results are shown in Fig. 6 by plots of logarithmic retention factor *versus* the logarithmic salt concentration in the eluent. As shown in Fig. 6 straight lines were obtained for all the proteins under investigation. The intercepts of these lines with the ordinate (y-intercept), which are summarized in Tables III and IV, were used to rank the eluting strength of the different salts. A greater negative intercept reflects a stronger eluting salt. According to this empirical consideration the eluent strength with Fe(III)–EDDA column increased in the order of $Cl^- < CH_3COO^- \leqslant HCOO^- < H_2PO_4^-$ (except for lysozyme), whereas with Co(II)–EDDA column it increased in the order $CH_3COO^- < H_2PO_4^- < Cl^-$. These results are in agreement with the observation that "hard" metals such as Fe(III) coordinate preferably with oxygen containing ions (*e.g.* phosphate, acetate, formate), whereas "soft" metal ions such as cobalt have preference for large donor atoms, *e.g.* chloride ions in this study.

Based on the above results, MIC selectivity can be varied by keeping the eluting strength of the salt roughly the same while changing the nature of the salt in the eluent. As shown in Fig. 6 and Table V, different selectivities were achieved on the Fe(III)–EDDA column by exchanging sodium acetate for sodium formate; both salts have about the same eluting strength.
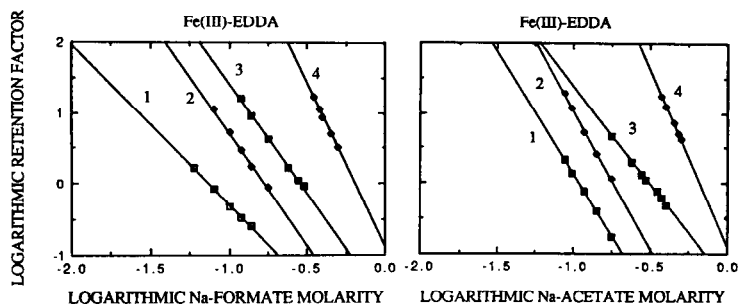

Fig. 6. Plots of logarithmic retention factor of proteins *versus* logarithmic salt molarity. Column, 100 × 4.6 mm I.D.; flow-rate, 1.0 ml/min; temperature, 25°C. Isocratic elution with 10 mM acetate buffer, pH 5.5, at different salt concentrations. Proteins: 1 = iron-free transferrin; 2 = cytochrome *c*; 3 = lysozyme; 4 = lactoferrin.

TABLE III

VALUES OF y-INTERCEPT OF PLOTS OF LOGARITHMIC RETENTION FACTOR OF PRO-
TEINS *VERSUS* THE LOGARITHMIC SALT MOLARITY IN THE ELUENT

Column, 100 × 4.6 mm I.D., Co(II)–EDDA; flow-rate, 1.0 ml/min; temperature, 25°C. Isocratic elution
with 10 mM acetate buffer, pH 5.5, at different salt concentrations.

Protein	y-Intercept		
	Sodium acetate	Sodium phosphate	Sodium chloride
Iron-free transferrin	− 3.54	− 3.03	− 4.73
Cytochrome c	− 2.05	− 2.81	− 3.11
Lysozyme	− 1.29	− 2.10	− 2.53
Lactoferrin	− 0.97	− 1.69	− 2.01

TABLE IV

VALUES OF y-INTERCEPT OF PLOTS OF LOGARITHMIC RETENTION FACTOR OF PRO-
TEINS *VERSUS* THE LOGARITHMIC SALT MOLARITY IN THE ELUENT

Column, 100 × 4.6 mm I.D., Fe(III)–EDDA; flow-rate, 1.0 ml/min; temperature, 25°C. Isocratic elution
with 10 mM acetate buffer, pH 5.5, at different salt concentrations.

Protein	y-Intercept			
	Sodium chloride	Sodium formate	Sodium acetate	Sodium phosphate
Iron-free transferrin	− 2.07	− 2.55	− 3.36	− 4.00
Cytochrome c	− 2.04	− 2.44	− 2.84	− 2.78
Lysozyme	− 1.94	− 1.68	− 1.41	− 2.15
Lactoferrin	− 1.06	− 0.88	− 0.89	− 1.51

TABLE V

SELECTIVITY, α, MEASURED WITH TWO SALTS OF COMPARABLE ELUTING STRENGTH

Column, 100 × 4.6 mm I.D., Fe(III)–EDDA; flow-rate, 1.0 ml/min; temperature, 25°C. Isocratic elution
with 0.12 M sodium acetate or formate buffer, pH 5.5.

Pair of proteins	Selectivity, α	
	Sodium acetate	Sodium formate
Cytochrome c–transferrin	6.75	8.85
Lysozyme–cytochrome c	2.66	5.29

The slopes of the plots of log k' *vs.* log salt molarity, which measure the magni-
tude of interaction between the protein and the metal chelate stationary phase[11], were
also calculated. A greater negative slope reflects a stronger interaction. As determined
from these slopes, lactoferrin exhibited the strongest interaction with the Co(II)– and
Fe(III)–EDDA columns, while all other proteins, *i.e.* cytochrome c, lysozyme and

iron-free transferrin, interacted with the metal chelate columns to a lesser extent than lactoferrin and at slightly different magnitude among each other.

In conclusion, EDDA stationary phases in an appropriate metal form are very suitable for the separation and determination of proteins. Such stationary phases can be viewed as complementary to IDA stationary phases, since they afford different selectivity and retentivity toward proteins. In addition, metal–EDDA stationary phases are stable as manifested by the constancy of the retention of proteins under elution conditions used in this study.

ACKNOWLEDGEMENTS

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