Journal of Chromatography, 359 (1986) 241–253
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 873

METAL CHELATE-INTERACTION CHROMATOGRAPHY OF PROTEINS WITH IMINODIACETIC ACID-BONDED STATIONARY PHASES ON SILICA SUPPORT

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SUMMARY

An iminodiacetic acid (IDA)-bonded stationary phase on a wide-pore microparticulate silica support was used for the chromatography of amino acids and proteins at pH 5.0 and 6.0. Without chelated metal the retention behavior of the stationary phase parallelled that of other silica-bound cation exchangers used in high-performance liquid chromatography of proteins. In metal chelate-interaction chromatography (MCIC) with IDA, chelated by Cu(II), Zn(II), Ni(II), Fe(II), or Fe(III), amino acids were most strongly retained on Cu(II)–IDA, whereas all metal chelates separated the proteins under investigation but with different selectivity. The effect of salt concentration in the eluent on protein retention was investigated and the pertinent electrostatic and hydrophobic interaction parameters were evaluated. The proteins were separated by MCIC with increasing salt gradient and, using the same column, by hydrophobic-interaction chromatography with decreasing salt gradient. In MCIC the addition of methanol to the mobile phase had disparate effect on protein retention, whereas addition of histidine or glycine, which acted as competing ligands, reduced the retention.

INTRODUCTION

In 1961, Helfferich[1] introduced the concept of using stationary phases with fixed metal chelates and termed the technique "ligand-exchange chromatography". The first comprehensive review of this chromatographic modality by Davankov and Semechkin[2], in 1977, had already revealed the great popularity of the technique as measured by the number of publications. Since then, various silica-based stationary phases bonded with metal chelates have been developed for use in high-performance liquid chromatography (HPLC)[3–7] and found use in the separation of small molecules.

Our interest is in the adaptation of the technique to HPLC of biopolymers. This approach commenced with the seminal work by Porath and his collaborators[8], who have used agarose-bound metal chelates for the separation of proteins and nucleic acids over the past ten years[9,10]. The technique, which was called metal chelate affinity chromatography, has been employed for the separation of biopolymers in several laboratories[11–21] and reviewed briefly by Lönnerdal and Keen[22]. Recently, Fanou-Ayi and Vijayalakshmi[23] and Karger *et al.*[24] have demonstrated the use of

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silica-based stationary phases appropriately bonded with metal chelates for the separation of proteins by HPLC.

The goal of our study is to gain information on the properties of such silica-bound iminodiacetic acid (IDA) stationary phases in free and various metal-chelated forms in HPLC of proteins. In the latter case, the fundamental physico-chemical phenomenon underlying the retention process is the interaction between the protein and the fixed metal chelates of the stationary phase. Thus, this branch of biopolymer chromatography is appropriately called metal chelate-interaction chromatography (MCIC). This term conforms with those used in HPLC for other types of interactive biopolymer chromatography, namely hydrophobic-interaction chromatography (HIC) and electrostatic-interaction chromatography (EIC). For the sake of consistency, the latter expression is preferred to the conventional term ion-exchange chromatography, where the interaction between the biopolyelectrolyte and the fixed charges on the stationary phase is responsible for retention.

In view of the potentially broad range of elution conditions in MCIC, the scope of the present study is rather narrow. Nevertheless, the results shed light on the interaction of amino acids with various metal–IDA chelates, on the effect of salt concentration, pH, and certain additives in the eluent on the retention of proteins having a wide range of isoelectric points. Although the physico-chemical basis of retention, which appears to be much more complex than that in EIC, is yet to be elucidated, the data demonstrate that MCIC is a versatile and convenient chromatographic technique which offers unique selectivities for carrying out a particular separation. Therefore, MCIC may be viewed as an auspicious alternative to the other interactive chromatographic techniques for biopolymer separation by HPLC.

EXPERIMENTAL

Materials

Cytochrome *c* from horse heart (CYT, MW = 12 200), α-chymotrypsinogen A (CHY, MW = 25 200) and ribonuclease A (RNase, MW = 13 700) both from bovine pancreas, lysozyme from chicken egg white (LYS, MW = 14 000), β-lactoglobulin A from bovine milk (LAC A, MW = 35 000), ovalbumin (OVA, MW = 44 000), human serum albumin (HSA, MW = 65 000), and all the amino acids were supplied by Sigma (St. Louis, MO, U.S.A.). Reagent-grade sodium hydroxide, sodium dihydrogen phosphate, iron(III) chloride, iron(II) sulfate, nickel sulfate, copper(II) nitrate, zinc chloride and phosphoric acid, as well as methanol and acetonitrile (HPLC grade) were obtained from Fisher (Pittsburgh, PA, U.S.A.). Distilled water was prepared with a Bransted unit. Vydac silica gel (300 Å mean pore diameter, 5 μm mean particle diameter) was purchased from the Separation Group (Hesperia, CA, U.S.A.). IDA and 3-glycidoxypropyltrimethoxysilane were from Aldrich (Milwaukee, WI, U.S.A.). Reagent-grade disodium salt of ethylenediaminetetraacetic acid (EDTA) was purchased from Fisher.

Instruments

The liquid chromatograph was assembled from a Micromeritics (Norcross, GA, U.S.A.) Model 750 solvent delivery pump with a Model 753 ternary solvent mixer and a Model 740 control module. Samples were injected by a Rheodyne (Berkeley, CA, U.S.A.) Model 7010 sampling valve with 20- or 100-μl sample loop. A

Kratos (Ramsey, NJ, U.S.A.) Model 770R variable-wavelength UV detector was used to monitor the column effluent at 280 nm. Chromatograms were recorded with Model C-R3A integrator (Shimadzu, Columbia, MA, U.S.A.).

Columns

Vydac 300 Å silica gel was first made to react with 3-glycidoxypropyltrimethoxysilane and then with IDA by a method similar to those described in the literature[9,23]. The stationary phase in methanol slurry was packed into 100 × 4.6-mm No. 316 stainless-steel tubes (Handy and Harman, Morristown, PA, U.S.A.) using methanol at 8000 p.s.i.[25].

Procedures

Columns were washed successively with water, aqueous 50 m*M* EDTA, water, methanol, and again water. Thereafter, the stationary phase was loaded with the metal by perfusing the column with 200 ml of 15 m*M* solution of the corresponding metal salt in water. Subsequently, the column was washed with 100 ml of water and the same volume of the eluent buffer as used in the subsequent experiments.

The metal was removed from the stationary phase by perfusing the column with 100 ml of 50 m*M* EDTA solution, water, methanol, and again water before loading it with another metal.

The retention of α-amino acids and proteins on naked IDA, *i.e.* the chelating stationary phase devoid of metal, was determined with 25 m*M* phosphate buffer (pH 5.0 or 6.0), containing 1 m*M* EDTA as the eluent to avoid metal binding by the stationary phase from the eluent in contact with metal parts of the chromatographic system. In experiments with gradient elution on naked IDA, both the starting eluent and the gradient former contained 5 m*M* EDTA. In all cases, the column was maintained at 25°C.

RESULTS AND DISCUSSIONS

Retention of amino acids

The retention behavior of commonly occurring α-amino acids was investigated both on naked IDA and on an IDA stationary phase loaded with a given metal. The results are listed in Tables I and II in terms of retention factor and retention modulus. The modulus, η, is the ratio of the retention factors of a given eluite, measured on the same column with a chelated metal and without metal, respectively. Thus, the modulus is a measure of the retention-modulating effect of the chelated metal, with the retention on naked chelating stationary phase as the reference. The moduli are not listed in Tables I and II when any of the two retention factors is less than 0.1.

Only Cu(II)–IDA retained all of the α-amino acids to a measurable extent under the conditions investigated. The retention values are given in Table I. The retention of all α-amino acids on Cu(II)–IDA was greater at pH 6.0 than at pH 5.0. The moduli are greater than unity, indicating that IDA chelated with Cu(II) has stronger retentive properties than naked IDA, with the exception of arginine and lysine, which have a modulus less than 1 at pH 5.0. The data presented in Table I show no direct correlation between the retention factors of α-amino acids on the Cu(II)–IDA column and the stability constants of the complexes obtained by the amino acids with Cu(II) in free solution[26]. Indeed, such a correlation is not expected,

TABLE I

RETENTION FACTORS, k', RETENTION MODULI, η, OF AMINO ACIDS, AS MEASURED ON A Cu(II)–IDA COLUMN, AND THE LOGARITHMIC STABILITY CONSTANTS, log K_1 AND log K_2, FOR COMPLEXING ONE AND TWO AMINO ACIDS RESPECTIVELY WITH Cu(II) IN FREE SOLUTION AT 25°C AT AN IONIC STRENGTH OF 0.1 μ

Values of stability constants were taken from ref. 26.

Amino acid	*pH 5.0*		*pH 6.0*		*Logarithmic stability constants*	
	k'	η	k'	η	*log* K_1	*log* K_2
Glycine	0.14	–	0.28	–	8.15	14.97*
Alanine	0.14	–	0.34	2.84	8.13	14.92
Valine	0.17	–	0.40	–	8.11	14.90
Leucine	0.24	2.40	0.64	5.34	8.11	14.90
Isoleucine	0.19	1.90	0.48	4.80	8.40	15.40
Methionine	0.38	3.80	1.28	10.70	8.11	14.72
Phenylalanine	0.69	3.45	2.14	8.23	7.86	14.77
Serine	0.17	–	0.43	–	7.89	14.48
Threonine	0.17	–	0.67	–	8.01	14.73
Cysteine	1.48	–	39.0	–	–	–
Aspargine	0.26	–	1.31	–	7.86	14.42
Glutamine	0.19	–	0.62	–	7.75	14.23
Tyrosine	0.26	–	0.95	9.5	7.81	14.74
Tryptophan	1.34	5.6	4.71	19.6	8.29	15.48
Aspartate	0.09	–	0.38	–	8.57	15.35
Glutamate	0.09	–	0.17	–	7.87	14.16
Histidine	34.6	41.7	**	–	10.20	18.10
Lysine	0.42	0.60	3.05	3.76	7.56***	13.90
Arginine	0.71	0.75	4.57	4.27	7.93	14.57

* At 25°C and 0.5 μ.
** Not eluted.
*** At 20°C and 0.1 μ.

as Cu(II) is presented in the IDA column not in free but in chelated form. Nonetheless, α-amino acids such as histidine and cysteine which form the strongest complexes with Cu(II) in free solution, are also most strongly retained by the Cu(II)–IDA column.

Retention data obtained with the naked IDA column and with the same column containing one of the metals in chelated form are shown in Table II. Surprisingly, the retention of the basic amino acids is reduced when Ni(II) and Zn(II) is bound to IDA, with the exception of histidine, which is more strongly retained on all metal chelates listed in Table II than on naked IDA at pH 6.0. The retention of phenylalanine at pH 6.0 is also smaller on all the metal chelate columns than on naked IDA. The greatest enhancement of retention by the metals shown in Table II occurs when Fe(III) or Fe(II) are anchored by the covalently bound chelating functions. It is noted that the stationary phase under consideration is designed for biopolymer chromatography and is therefore not expected to retain amino acids strongly at pH 5.0 and 6.0, even at salt concentrations as low as 25 m*M* in the eluent. Among all amino acids, including those not shown in Table II, histidine exhibits the strongest

TABLE II

RETENTION MODULI OF AMINO ACIDS ON AN IDA COLUMN WITH DIFFERENT METAL CHELATES AND RETENTION FACTORS ON A NAKED IDA COLUMN

Column	*pH*	*Retention modulus*				
		Phenylalanine	*Tryptophan*	*Histidine*	*Lysine*	*Arginine*
Fe(III)–IDA	5.0	1.20	1.50	2.92	1.63	1.56
	6.0	0.82	1.20	4.53	1.90	1.75
Fe(II)–IDA	5.0	1.00	1.29	2.35	1.39	1.41
	6.0	0.81	1.21	3.80	1.60	1.40
Ni(II)–IDA	5.0	1.00	1.21	0.97	0.52	0.45
	6.0	0.73	1.00	5.67	0.60	0.54
Zn(II)–IDA	5.0	1.00	1.12	0.76	0.60	0.54
	6.0	0.65	1.0	1.85	0.63	0.61
		Retention factor				
		Phenylalanine	*Tryptophan*	*Histidine*	*Lysine*	*Arginine*
Naked IDA	5.0	0.20	0.24	0.83	0.73	0.95
	6.0	0.26	0.24	0.67	0.81	1.07

interaction with all the metal chelate, in agreement with the observation[26] that in free solution histidine has the greatest complex formation constants with these metals.

Chromatography of proteins

The retention behavior of proteins on silica-bonded stationary phases bearing various metal–IDA chelates was investigated using gradient elution with increasing sodium chloride concentration in 25 m*M* phosphate buffer at pH 5.0 and 6.0. Similar experiments were carried out on naked IDA but with 5 m*M* EDTA in the eluent. The results are depicted on the chart in Fig. 1.

Without metal, the iminodiacetic acid-bonded stationary phase is expected to be a relatively strong cation exchanger under the conditions of the experiment, because the pK_a value of one of the carboxylic groups in the covalently bound IDA is 2.65[27]. The protein mixture, consisting of ovalbumin (p*I* 4.7), β-lactoglobulin A (p*I* 5.1), cytochrome *c* (p*I* 10.6), α-chymotrypsinogen A (p*I* 9.5), and lysozyme (p*I* 11.0), was chromatographed on naked IDA. Typical chromatograms obtained at pH 5.0 and 6.0 are illustrated in Fig. 2.

The retention data in Fig. 1 and the chromatograms in Fig. 2 closely parallel the results obtained in our laboratory with the same protein mixture on a silica-based cation exchanger with sulfonic acid groups (unpublished results). On both naked IDA and the strong cation exchanger the proteins are retained in order of increasing isoelectric point at pH 6.0, whereas on a typical weak cation exchangers with fixed carboxylic functions of pK_a 4.0–5.0 the retention order of α-chymotrypsinogen and cytochrome *c* are reversed. Although the retention values at pH 5.0 are different from those at pH 6.0, such behavior is also observed on a typical strong cation exchanger under similar conditions.

However, the retention behavior of proteins is dramatically altered upon che-

METAL	pH	RETENTION VOLUME [ml] 0–5	5–10	10–15	15–20	20–25	25–30	30–35	35–40	>40
Zn^{2+}	6.0	+ x			□	◇	○			
	5.0	+		□ x	◇ ○					
Ni^{2+}	6.0	+ x			□	◇		○		
	5.0	+			x □	◇	○			
Fe^{2+}	6.0	+				x	□		◇ ○	
	5.0	+						□	x○	◇
Fe^{3+}	7.0	+ x				□		◇ ○		
	6.0	+				□	x		◇,○	
	5.0					+	□	○	x,◇	
Cu^{2+}	6.0				□					
	5.0				□					
—	6.0	+ x				□	◇	○		
	5.0	+			□	x	◇ ○			

Fig. 1. Retention volume of proteins on naked IDA and metal chelated IDA columns. Column, 100 × 4.6 mm I.D.; flow-rate, 1 ml/min; temperature, 25°C. Linear gradient in 40 min from 0 to 0.5 M sodium chloride in 25 mM phosphate buffer of pH 5.0, 6.0, or 7.0. The symbols are: (+) ovalbumin, (□) α-chymotrypsinogen A, (×) β-lactoglobulin, (◇) cytochrome c and (○) lysozyme.

lation of metals on the stationary phase and by changing the pH of the eluent, as illustrated in Fig. 1. Most proteins under consideration are retained very strongly by Cu(II)–IDA and only α-chymotrypsinogen is eluted under the conditions used. Quite unexpectedly, the retention of this protein is less on Cu(II)–IDA than on naked IDA. In contradistinction, Zn(II)–IDA and Ni(II)–IDA exhibited a retentive behavior mildly different from that of naked IDA with slight changes in selectivity, but at pH 5.0 the proteins were eluted more rapidly from Zn(II)–IDA than from naked IDA.

Both Fe(III)–IDA and Fe(II)–IDA were found to be much more retentive stationary phases than naked IDA, and the selectivity of the two kinds of iron chelate-interaction columns was vastly different. Ovalbumin, which was not retained on the other columns except on Cu(II)–IDA, was also retained on Fe(III)–IDA at pH 5.0.

The observed retention values depend, of course, not only on the strength of protein–metal chelate interactions but also on the amount of metal bound under operating conditions to the IDA ligates. According to the literature[27,28], the binding constants of IDA in free solution and immobilized form are similar, and the strength

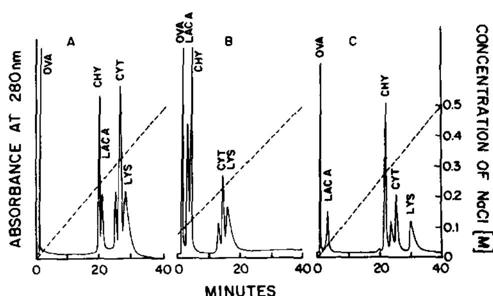

Fig. 2. Chromatograms of proteins on silica-bound "soft" IDA stationary phase. Column, 100 × 4.6 mm I.D.; flow-rate, 1 ml/min; temperature, 25°C; buffer, 25 mM phosphate. Linear gradient in 40 min from 0 to 0.5 M sodium chloride in (A) (pH 5.0) and (C) (pH 6.0) and from 0.08 M to 0.5 M sodium chloride in (B) (pH 5.0).

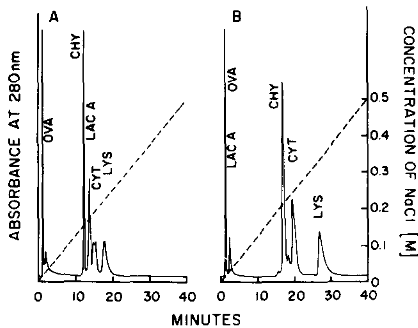

Fig. 3. Separation of proteins by MCIC on Zn(II)–IDA. Column, 100 × 4.6 mm I.D.; flow-rate, 1 ml/min; temperature, 25°C. Linear gradient in 40 min from 0 to 0.5 M sodium chloride in 25 mM phosphate buffer of pH 5.0 in (A) and of pH 6.0 in (B).

of binding follows the order Fe(III) > Cu(II) > Ni(II) > Zn(II) > Fe(II). The similar retention behavior of Zn(II)–IDA and naked IDA could be thus attributed to a low degree of chelation by Zn(II). However, as seen in Fig. 1, the proteins are actually eluted faster from the Zn(II)–IDA than from naked IDA, whereas the most weakly binding Fe(II) greatly enhances the retention. We must agree with those who state that the theoretical basis of the energetics of ligand-exchange chromatography is still insufficient for an interpretation of chromatographic retention in the hermeneutics of a sound physico-chemical model[2]. This seems to be particularly true for the MCIC of proteins. Nevertheless, as illustrated in Figs. 3 and 4, the use of metal chelate interactions allows the efficient separation of proteins. The separations shown were carried out with relatively weakly interacting Zn(II)–IDA and Ni(II)–IDA columns and illustrate the differences in selectivity in comparison to that obtained with naked IDA (cf. Fig. 2). According to our experience, the efficiency of columns containing strongly interacting metal chelates is lower than suggested by the chromatograms in Figs. 3 and 4. This is probably due to the relatively slow kinetics of the interaction between the proteins and the bound metal chelate functions of the stationary phase.

Factors affecting retention

Salt concentration in the eluent. The chromatograms in Fig. 5 illustrate that the

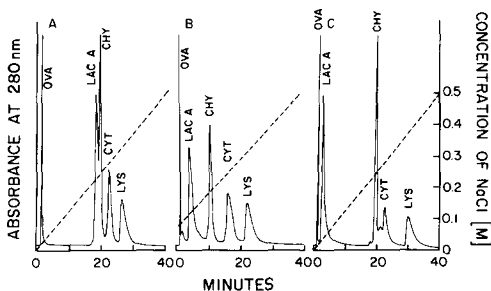

Fig. 4. Separation of proteins by MCIC on Ni(II)–IDA. Column, 100 × 4.6 mm I.D. Other conditions as in Fig. 2.

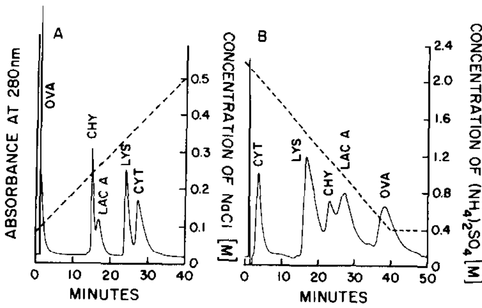

Fig. 5. Separation of proteins by MCIC on Fe(III)–IDA. Column, 100 × 4.6 mm I.D.; flow-rate, 1 ml/min; temperature, 25°C. (A) Linear gradient in 40 min from 0.08 to 0.5 M sodium chloride in 25 mM phosphate (pH 5.0). (B) Linear gradient in 40 min from 2.2 M to 0.4 M ammonium sulfate in 25 mM phosphate (pH 6.0).

separation of proteins on Fe(III)–IDA can be carried out by either increasing or decreasing salt gradients. Thus, this column can be used in two ways: with increasing salt gradient in MCIC and with decreasing salt gradient in HIC, because at low and high salt concentrations the proteins are bound to the stationary phase via metal chelate and hydrophobic interactions, respectively. Due to the nature of these retention mechanisms, the elution order of the proteins is almost palindromical, giving rise to vastly different selectivities.

In order to gain further insight into the effect of salt over a wide concentration range, the retention of the proteins on both naked IDA and Fe(III)–IDA columns was investigated by isocratic elution with 25 mM phosphate (pH 6.0), containing ammonium sulfate at different concentrations. The results are presented in Fig. 6 by plots of the logarithmic retention factor against the logarithm of ammonium sulfate molality in the mobile phase. The experimental data were fitted to a three-parameter equation which has recently been introduced for the analysis of retention data in EIC and HIC of biopolymers[29]. It is given by

$$\log k' = A + B \log m + Cm \tag{1}$$

where m is the salt molality. Among the parameters A, B, and C, the last two are of particular interest. B is the limiting slope at sufficiently low salt concentrations and

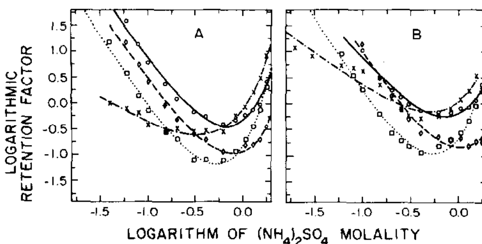

Fig. 6. Plots of the logarithmic retention factor of proteins on naked IDA (A) and on Fe(III)–IDA (B) *versus* logarithm of ammonium sulfate molality. Isocratic elution with 25 mM phosphate buffer (pH 6.0) at different ammonium sulfate concentrations. Symbols as in Fig. 1.

TABLE III

ELECTROSTATIC (*B*) AND HYDROPHOBIC (*C*) INTERACTION PARAMETERS IN EQN. 1 FOR PROTEIN RETENTION

Determined isocratically with 25 m*M* phosphate buffer (pH 6.0) at various concentrations of ammonium sulfate in the eluent. Parameter *A* (eqn. 1) is also listed. Non-linear regression analysis was performed with the Statistical Analytical System (SAS Institute, Cary, NC, U.S.A.). *R* = coefficient of correlation.

Protein	*Naked IDA*				*Fe(III)–IDA*			
	A	*B*	*C*	*R*	*A*	*B*	*C*	*R*
Cytochrome *c*	−2.51	−2.84	1.57	0.9874	−2.11	−2.98	1.27	0.9637
Lysozyme	−2.18	−2.88	1.85	0.9806	−1.54	−2.29	1.34	0.9595
β-Lactoglobulin A	−1.72	−1.21	1.61	0.9819	−1.20	−1.43	1.22	0.8965
α-Chymotrypsinogen A	−3.36	−3.10	2.52	0.9887	−3.10	−3.12	2.41	0.9814
Ovalbumin	–	–	1.76	0.9775	–	–	1.48	0.9988

is termed electrostatic-interaction parameter, because it is a measure of the effect of salt on the magnitude of the electrostatic interaction between the protein and the stationary phase. *C* is the limiting slope at sufficiently high salt concentrations and is called hydrophobic-interaction parameter because it expresses the magnitude of the corresponding salt-mediated hydrophobic interaction.

The results of data analysis according to eqn. 1 are shown in Table III. Because eqn. 1 has been derived for protein retention on ion exchangers, it is not surprising that the retention data obtained on naked IDA show a better fit than those on Fe(III)–IDA, as measured by the coefficient of determination. The worst fit was obtained for β-lactoglobulin A, which exhibited the strongest specific interaction with Fe(III)–IDA. Even so, eqn. 1 seems to be useful for studying the effect of salt concentration on the retention in MCIC, at least under the conditions used here (cf. Fig. 6). This is not entirely unexpected, since metal chelate interactions are in essence also of electrostatic nature but more complex than those encountered in EIC.

Table III shows that the hydrophobic-interaction parameters for the different proteins decrease when Fe(III) is chelated on the stationary phase. For the proteins investigated, the parameter *C* decreases by about 20%, with the exception of α-chymotrypsinogen A, which shows a 5% decrease. This indicates that the presence of the metal has a relatively minor effect on *C*, which is believed to be the product of the hydrophobic contact area between the protein and stationary phase ligates and the surface tension of the salt[30]. The observed reduction in the value of *C* indicates that, upon chelating Fe(III) with the IDA ligates, the stationary phase becomes apparently less hydrophobic.

The electrostatic parameter *B* shows a slight increase for all proteins when IDA is chelated with Fe(III), with the exception of lysozyme. α-Chymotrypsinogen exhibits almost the same retention behavior on both naked IDA and Fe(III)–IDA. The greatest increase in *B* upon chelation of IDA with Fe(III) is observed with β-lactoglobulin A, which appears to have the strongest metal chelate interaction among these proteins. Ovalbumin was not retained to a measurable extent at low salt concentrations. At ammonium sulfate concentrations higher than 0.25 *M* the logarithmic retention factor increased linearly with the salt molality so that the hydrophobic-interaction parameter *C* could be evaluated.

The results in Fig. 6 and Table III give insight into the interplay of electrostatic and hydrophobic interactions between the proteins and the stationary phases. The corresponding parameters, obtained from retention data measured on naked IDA, are very close to those found from retention values obtained with these proteins under similar conditions on a strong cation exchanger, prepared with a similar silica support. The results also suggest that MCIC can be viewed as a kind of electrostatic-interaction chromatography, the selectivity of the metal chelate column being significantly different from that of the naked ion exchanger. However, it should be noted that at other eluent pH values the picture may be quite different from that presented here, due to the changes in the electrostatic properties of both the proteins and the stationary phase. Furthermore, other stationary phases with IDA ligates, prepared in another way, may exhibit a different retention behavior. Be that as it may, MCIC offers a unique approach to increasing the selectivity of chromatographic systems for the analysis and purification of proteins at the cost of some trial-and-error search for the most appropriate metal. In our experience, however, such experiments can be carried out with great ease, as the removal of the chelated metal by EDTA and the reloading of the stationary phase with another metal proceed rapidly.

Effect of methanol and amino acids. Additives are frequently used in the mobile phase to modulate the selectivity of separation and/or improve the peak shape. Our interest was focused on the effect of an organic solvent, methanol, and histidine, which is known to form strong complexes with various metals and has already been used in MCIC[15].

The effect of methanol on the retention by Fe(III)–IDA was investigated by using isocratic elution with mixtures of 0.15 *M* ammonium sulfate in 25 m*M* phosphate buffer (pH 6.0) and methanol. The results are depicted in Fig. 7 by plotting the retention factor against the methanol concentration in the mobile phase. The results can be discussed in view of the electrostatic-interaction parameters listed in Table III that suggest that β-lactoglobulin A and cytochrome *c* have the strongest specific interaction with Fe(III)–IDA. The effect of increasing methanol concentrations is to enhance the retention by strengthening this interaction in a fashion similar to that frequently observed with complex formation in the presence of organic solvents[3]. In contradistinction, the retention of lysozyme decreases with increasing methanol concentration; apparently, methanol reduces the interaction between this

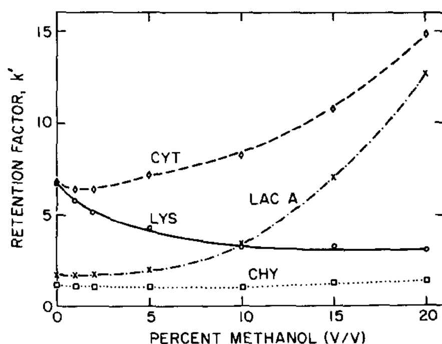

Fig. 7. Plots of the retention factors of proteins on a Fe(III)–IDA column against methanol concentration in 25 m*M* phosphate buffer (pH 6.0), containing 0.15 *M* ammonium sulfate.

protein and the Fe(III)–IDA. The results suggest that methanol affects the interaction between proteins and Fe(III)–IDA in such a way that strong and weak interactions are intensified and attenuated by the organic solvent, respectively. The retention behavior of α-chymotrypsinogen, which has essentially the same retention on naked IDA and Fe(III)–IDA (*cf.* Table IV), is not affected by methanol, in agreement with this observation. Fig. 7 also suggests that the selectivity in MCIC can be modulated by adding to the eluent a few percent of an organic solvent which is not expected to denature the proteins.

The data in Tables I and II show that, among all amino acids, histidine was most strongly retained in MCIC with the different metal chelates investigated. Therefore, it was of interest to examine the effect of the histidine concentration in the eluent on the retention of proteins on the Fe(III)–IDA column. Isocratic elution was used with 0.15 *M* ammonium sulfate in 25 m*M* phosphate buffer (pH 6.0), and histidine was added to the mobile phase. The results are illustrated in Fig. 8 by plots of the logarithmic retention factors against histidine concentration. It is seen that the logarithmic retention factor decreases linearly with increasing histidine concentration. The retention of cytochrome *c* and β-lactoglobulin A, which interact most strongly with the chelated Fe(III), show the greatest decrease by the competitive ligand. The slopes for lysozyme and α-chymotrypsinogen A are about the same but half as great as those for the other proteins. This suggests that other effects, such as binding of histidine to the stationary phase and/or to the proteins, are involved in the attenuation of retention with increasing histidine concentration. In any case, the addition of histidine —and probably that of other amino acids— can greatly alter the selectivity in MCIC and give rise to changes in elution order, particularly when gradient elution is employed. The use of a competing ligand may be advantageous when the interaction between the proteins and the metal chelate stationary phase is very strong, such as that observed with Cu(II)–IDA. Fig. 9 shows the separation of the proteins on Cu(II)–IDA by gradient elution with decreasing pH and increasing concentration of histidine, sodium chloride, and acetonitrile.

Glycine is another amino acid additive that has been used in MCIC[15]. Since the stability constants of metal complexes are smaller for glycine than for histidine, the former is expected to be a less competitive ligand. Table IV shows the retention volumes of the proteins in gradient elution with glycine at pH 5.0 and 6.0 on a

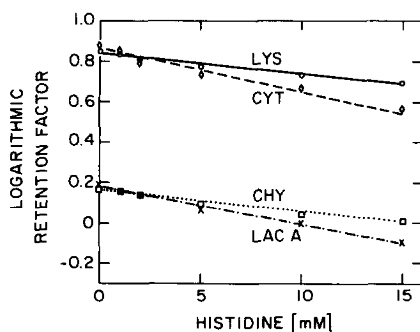

Fig. 8. Plots of the logarithmic retention factors of proteins on a Fe(III)–IDA column against the concentration of histidine in 25 m*M* phosphate buffer (pH 6.0), containing 0.15 *M* ammonium sulfate.

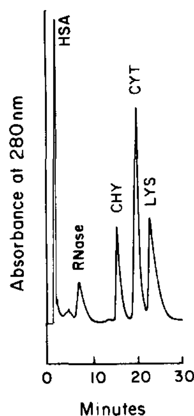

Fig. 9. Separation of proteins by MCIC on Cu(II)–IDA. Column, 100 × 4.6 mm I.D.; flow-rate, 1 ml/min; temperature, 25°C. Linear gradient in 30 min from 0 to 40% (v/v) B in A; eluent A, 25 m*M* Tris–HCl containing 0.1 *M* sodium acetate (pH 8.0); eluent B, 0.1 *M* phosphate buffer (pH 4.5), containing 0.8 *M* sodium chloride, 10% (v/v) acetonitrile and 10 m*M* histidine.

Cu(II)–IDA column. With glycine in the mobile phase, all proteins were eluted, whereas without glycine all proteins except chymotrypsinogen A were very strongly retained by Cu(II)–IDA, as shown in Fig. 1. Another dramatic effect of glycine is that even at very low glycine concentrations ovalbumin was eluted in the void volume.

CONCLUSIONS

Silica-based stationary phases with IDA functions are polytypic, since they can be used for protein separations in the electrostatic, hydrophobic and metal chelate-interaction modalities of chromatography. The different selectivities exhibited by the stationary phase upon chelation of various metals can be exploited in both analytical

TABLE IV

RETENTION VOLUME OF PROTEINS ON Cu(II)-IDA IN GRADIENT ELUTION WITH INCREASING GLYCINE AND SALT CONCENTRATION

Protein	*Retention volume (ml)*	
	*pH 5.0**	*pH 6.0***
Ovalbumin***	–	–
β-Lactoglobulin A	9.0	4.5
α-Chymotrypsinogen A	9.0	8.5
Cytochrome *c*	18	23
Lysozyme	25	38

* 40-min linear gradient from 0.05 *M* sodium chloride and 1.56 m*M* glycine to 0.8 *M* sodium chloride and 25 m*M* glycine in 25 m*M* phosphate.

** 40-min linear gradient from 0.1 *M* sodium chloride and 3.1 m*M* glycine to 0.8 *M* sodium chloride and 25 m*M* glycine in 25 m*M* phosphate.

*** Not retained.

and preparative liquid chromatography. The use of a given column in different ways may afford a convenient way to expand the scope of its application and offer a degree of versatility not available with other stationary phases. In HPLC on columns and instruments with metal parts in contact with the eluent MCIC may have been practiced frequently and inadvertently because various ion exchangers and other types of stationary phases are known to complex metal ions. Elimination of steel frits and the use of chelator precolumns to scavenge metal ions from the eluent and perhaps use of a chelating agent in the eluent are needed to preclude unintentional involvement of MCIC.

ACKNOWLEDGEMENTS

This work was supported by Grants Nos. GM 20993 and CA 21948 from National Institutes of Health, U.S. Department of Health and Human Resources and by a Contract from the National Foundation of Cancer Research.

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