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HIGH-PERFORMANCE IMMOBILIZED-METAL AFFINITY CHROMATOGRAPHY OF PROTEINS ON IMINODIACETIC ACID SILICA-BASED BONDED PHASES

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SUMMARY

High-performance separations of proteins, based on immobilized-metal affinity chromatography (HPIMAC), are described. The stationary phase consisted of iminodiacetic acid (IDA) chelate groups, bonded to small particle, wide pore silica gel by means of a polyether hydrophilic leash. After loading the column with metal, retention of proteins was achieved by protein–metal complexation at high concentrations of sodium sulfate. Elution was accomplished by addition of competitive complexing ligands, such as ammonia at constant pH, and/or by a decreasing pH gradient of a specially designed buffer system to maintain buffer capacity constant throughout the gradient. Selective separations, based on differences in the number of histidine residues present on the surface of the proteins, are described. The application of HPIMAC in the separation and purification of structurally similar proteins is presented. The potential application of IDA columns in three chromatographic modes (HPIMA, hydrophobic interaction, and cation exchange) is also described.

INTRODUCTION

In 1975 Porath *et al.*[1] showed that selective metal complexation could be applied to the chromatographic purification of crude protein mixtures by immobilizing metals on agarose gels which contained chelating groups (immobilized metal affinity chromatography, IMAC) (see ref. 2 for the justification of the general term IMAC). Separation was shown to be based on the presence of different kinds and numbers of potential electron donating groups on the surface of the proteins (*e.g.* imidazole, thiol, indole)[3]. In the last few years, IMAC with chelated agarose gels has become a useful separation procedure[2–4]. IMAC follows logically from the extensive research on ligand-exchange chromatography of amino acids[5] and peptides[6]. Indeed, the use of selective metal complexation has proven to be a powerful chiral separation method[7].

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In this paper, results are described on the separation of proteins by high-performance immobilized metal affinity chromatography (HPIMAC) on synthesized hydrophilic iminodiacetic acid (IDA) bonded phases. Initial results have been given in an earlier presentation[8]. Previously, IDA has been bonded to silica by various approaches[9–13], as well as a high-performance liquid chromatographic (HPLC) polymeric matrix[14]. In this work, the ligand IDA plus a hydrophilic polyether leash (see Fig. 1) were synthesized in solution and then bonded to the support, as in previous studies with other ligands[15,16].

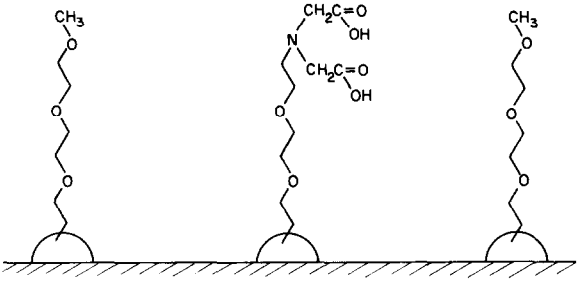

Fig. 1. Structure of diluted iminodiacetic acid (IDA) bonded phase. Diluent is a methyl polyether leash (C_1-ether).

Using gradient elution with decreasing sodium sulfate and increasing ammonia concentrations at constant pH or decreasing pH, rapid, high-performance separations of proteins have been achieved under mild conditions with Cu(II) or Zn(II). Special care has been taken in the selection of the buffer mixture to maintain the buffer capacity constant over the pH range of the gradient. The buffer solution (ALPHIL) was prepared with equivalent amounts of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pK_a 7.5), 2(N-morpholino)ethanesulfonic acid (MES) (pK_a 6.1), and acetic acid (pK_a 4.7). Finally, the same bonded phase, in the absence of a transition metal, was found to resolve mixtures of proteins by either cation exchange or hydrophobic interaction, depending on mobile phase conditions. These stationary phases therefore offer the possibility of high-performance protein separation by several different modes, depending on mobile phase conditions.

EXPERIMENTAL

Equipment

The liquid chromatograph consisted of a Model 420 microprocesor controller, two Model 110A single-piston reciprocating pumps, a dynamically stirred gradient mixing chamber, a Model 210 sample injection valve, and a Model 160 UV detector (all from Beckman Instruments, San Ramon, CA, U.S.A.). The gradient delay volume of the chromatographic system was measured to be 3.1 ml. The chromatograms were recorded on a Model 500 recorder (Linear Instruments, Reno, NV, U.S.A.). The chromatographic data were processed with a Model 2600 chromatography software package (Nelson Analytical, Cupertino, CA, U.S.A.) in conjunction with an

XT personal computer (IBM, Boca Raton, FL, U.S.A.). A pneumatic pump, Model DSTV 122 (Haskel, Burbank, CA, U.S.A.), was used to pack the columns.

Materials

Organic reagents (reagent grade) were obtained from Aldrich (Milwaukee, WI, U.S.A.). Reagent grade sodium pellets were purchased from MCB (Norwood, OH, U.S.A.). Organic solvents, sodium sulfate, sodium bicarbonate, sodium hydroxide and potassium hydroxide were from J. T. Baker (Phillipsburg, NJ, U.S.A.). Ammonium acetate, ammonium sulfate, as well as various high-quality protein standards were obtained from Sigma (St. Louis, MO, U.S.A.). Di-μ-chloro-dichloro-bis(ethylene) diplatinum(II) was obtained from Strem Chemicals (Newburyport, MA, U.S.A.). Triethoxysilane was purchased from Petrarch Systems (Bristol, PA, U.S.A.). Silica gel for bonded phases (Vydac, 5.5 μm particle diameter, 300 Å nominal pore size, S_{BET} 62 and 73 m^2/g) was a gift of the Vydac Separations Group (Hesperia, CA, U.S.A.). The column blanks were obtained from Extrude Hone Co. (Irwin, PA, U.S.A.) and the column end fittings were purchased from Valco (Houston, TX, U.S.A.).

Synthesis

Fig. 2 shows a scheme for the synthesis of the silane that was bonded to the silica gel. Diethylene glycol was converted to the allyl ether by the Williamson method[17]. Allyl alcohol was transformed into the respective amine by substitution of the tosyl derivative. The amine was then alkylated with *tert.*-butylbromoacetic ester. The hydrosilylation was adapted from a procedure described in the literature[18]. The diluent ({3-[2-(2-methoxy)ethoxy]propyl}triethoxy silane: C_1-ether) was synthesized as described previously[16].

2-[2-(2-Propenoxy)ethoxy]ethanol; {diethylene glycol monoallyl ether} (1). In a 1.0-l three-neck flask, fitted with a mechanical stirrer and thermometer, were placed

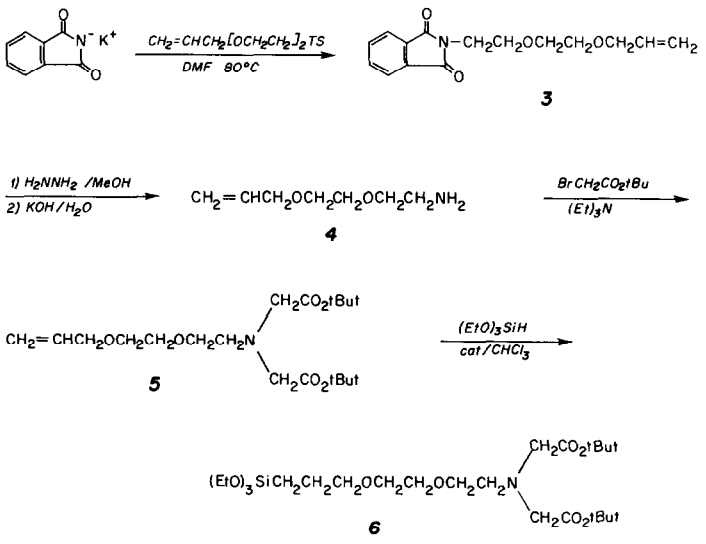

Fig. 2. Synthetic scheme for IDA–silane ligand to be bonded to silica gel.

1.26 moles (134.2 g) of diethylene glycol and 200 ml of dried dioxane. At room temperature, 0.6 moles (13.8 g) of sodium was slowly added (bubbles of hydrogen appear). The mixture was then stirred at reflux temperature for 6 h. After it had cooled to room temperature, 0.6 moles (72.6 g) of allyl bromide was added dropwise, and then the mixture was refluxed for 6 h. The sodium bromide crystals were filtered from the solution, and the filtrate was concentrated. Thin-layer chromatography of the crude material with ethyl acetate showed three spots: R_F 0.2, 0.5 and 0.8. The three spots corresponded to diethylene glycol, diethylene glycol monoallyl ether, and ethylene glycol diallyl ether, respectively. The crude material was then poured in water and exhaustively extracted with ethyl acetate. The ethyl acetate was removed from the organic layer, and the product was distilled (b.p.$_{3.5}$ 86.5–88.5°C, 60%). ^{1}H NMR (C^2HCl_3) δ 3.53 (br s, 9 H, one of these exchangeable with ^2H_2O), 3.90 (d, $=C-CH_2-$), 5.00–5.40 (m, $CH_2=$), 5.50–6.20 (m, $=CH-$); ^{13}C NMR (C^2HCl_3) δ 40.6, 48.6, 49.5, 51.3, and 51.9 ($-CH_2-O-$), 96.2 ($=CH_2$), and 119.0 ($=CH-$).

2-[2-(2-Propenoxy)ethoxy]ethyl 4-methylbenzenesulfonate; {diethylene glycol monoallyl ether-p-toluenesulfonate} (2). In a 250-ml three-neck flask, fitted with a stirrer and thermometer, were placed 0.1 mole (19.1 g) of *p*-toluenesulfonyl chloride and 0.6 moles (45 ml) of pyridine. While maintaining the temperature at −20°C, 0.1 moles (14.6 g) of diethylene glycol monoallyl ether were added. The reaction mixture was left at −10°C for 1 h and then slowly warmed to room temperature. The reaction mixture was again cooled to −10°C, and 40 ml of water were added, followed by 50.0 ml (*ca.* 0.6 moles) of hydrochloric acid (during the last two steps the temperature was maintained below 0°C). The reaction mixture was extracted with three 100-ml portions of ethyl acetate; the combined organic layers were then washed with 5% sodium bicarbonate solution and dried over anhydrous sodium sulfate. The product was concentrated under vacuum to yield 22.8 g (88.0%): R_F (15% ethyl acetate in toluene), 0.40; ^{1}H NMR (C^2HCl_3) δ 2.40 (s, $PhCH_3$), 3.50 (m, 3-CH_2–O), 4.00 (m, 2-CH_2–O), 5.00–5.40 (m, $CH_2=$), 5.50–6.20 (m, $=CH$), and 7.52 (q, –Ph–).

N-[2-(2-(2-Propenoxy)ethoxy)ethyl]-1H-isoindole-1,3(2H)-dione; {N-[2-(2-(2-Propenoxy)ethoxy)ethyl]phthalimide} (3). The conditions employed for this preparation were a modification of those used by Sheehan and Bolhofer[19]. Potassium phthalimide (13.7 g, 0.074 moles) was added to a solution of 22.2 g (0.074 moles) of Compound 2 in 50 ml of dimethylformamide. The mixture was heated at 120°C for 2 h. After it had cooled, 100 ml of chloroform were added, and the mixture was poured into 100 ml of water. The aqueous phase was separated and extracted with two 50-ml portions of chloroform. The combined chloroform extract was washed with 100 ml of 0.2 *M* sodium hydroxide (to remove unreacted phthalimide), followed by 50 ml of water. After drying with anhydrous sodium sulfate and concentrating under vacuum, the residue was chromatographed on 200 g of silica gel, eluting with toluene to give 7.0 g (35% yield) of product: R_F (50% ethyl acetate in toluene), 0.68; ^{1}H NMR (C^2HCl_3) δ 3.30–3.80 (m, 10 H), 4.70–5.20 (m, 2 H), 5.40–6.00 (m, 1 H), 7.60 (d, 4 H).

N-[2-(2-(2-Propenoxy)ethoxy)ethyl]amine (4). This compound was prepared according to the method of Mosher[20] by refluxing 2.9 g (10 mmol) of Compound 3 for 2 h with 50 ml of methanol and 0.7 ml (12 mmoles) of 85% hydrazine hydrate. The reaction mixture was concentrated under vacuum, and the residue was suspended in diethyl ether and shaken at 5°C with 10 ml of 40% potassium hydroxide solution.

The extraction was repeated four times and the extracts dried with potassium carbonate. After concentration under vacuum and distillation, 0.05 g (34% yield) of the product was obtained: b.p. 40°C (1.0 mmHg); R_F (20% *n*-propylamine in ethyl acetate), 0.48; ^{1}H NMR δ 1.62 (s, 2H), 2.85 (t, 2 H), 3.40–3.70 (m, 6 H), 4.02 (d, 2 H), 5.00–5.50 (m, 2 H), 5.60–6.30 (m, 1 H).

1,1-Dimethylethyl N-[2-(1,1-dimethylethyloxy)-2-oxoethyl]-N-{2-[2-(2-propenoxy)ethoxy]ethyl}glycinate (5). In a 100-ml three-neck flask, fitted with mechanical stirrer and thermometer, were placed 0.9 g (6.2 mmol) of Compound 4, 10 mg of *p*-dimethylaminopyridine, and 100 ml of dried dioxane. The reaction mixture was warmed to 70°C, and while stirring it at this temperature, 7.0 g (36 mmol) of *tert.*-butylbromoacetate and 2.0 g (20 mmol) of triethylamine were simultaneously added. The mixture was left overnight at 80°C and then filtered, and the crystals were washed with dichloromethane. The combined filtrates were concentrated under vacuum. The residue was chromatographed on 20 g of silica gel with ethyl acetate in toluene as mobile phase. The fractions containing the product were concentrated under vacuum to yield 1.5 g (63%) of product: R_F (50% ethyl acetate in toluene), 0.66; IR (film) 3080, 2980, 2940, 2870, 1740 b, 1648, 1480, 1455, 1440, 1395, 1370, 1285, 1250, 1220, 1150 b, 1040, 990, 930, 850, 770, 754 cm^{-1}; ^{1}H NMR (C^2HCl_3) δ 1.41 (s, 18 H), 2.80 (t, 2 H), 3.54 (d, 10 H), 3.98 (d, 2 H), 5.00–5.40 (m, 2 H), 5.55–6.40 (m, 1 H).

1,1-Dimethylethyl N-[2-(1,1-dimethylethyloxy)-2-oxoethyl]-N-{2-[2-(3-(triethoxysilyl)propoxy)ethoxy]ethyl}glycinate (6). The procedure was an adaptation of the hydrosilylation reaction described previously[18]. In a 100-ml three-neck flask, equipped with a magnetic stirring bar, condenser, and thermometer, and sparged with nitrogen, 1.5 g (3.9 mmol) of the olefin 6 was dissolved in 5.0 ml of chloroform, 10 mg of di-μ-chloro-dichlorobis(ethylene) diplatinum(II) catalyst was added, and the reaction mixture was warmed to 55°C for 0.5 h. A solution of triethoxysilane, 0.7 g (4.3 mmol) in 2.0 ml of chloroform, was added slowly to the reaction mixture, maintaining the temperature at 55°C. The solution was refluxed overnight under nitrogen, cooled, and then concentrated under vacuum. Flash chromatography over dry silica gel with 10% ethyl acetate in toluene gave 1.0 g (49% yield) of product: R_F (50% ethyl acetate in toluene) 0.57; IR (film) 3020, 2980, 2940, 2900, 1725, 1450, 1392, 1370, 1295, 1215, 1150, 1100, 1080, 970, 935, 915, 882, 858, 760 b, 670 cm^{-1}; ^{1}H NMR (C^2HCl_3) δ 0.62 (t, 2 H), 1.22 (t, 9 H), 1.45 (s, 18 H), 2.98 (t, 2 H), 3.57 (m, 12 H), 3.88 (q, 6 H).

Bonding and phase characterization

The trialkoxysilanes were bonded to the silica by methods similar to those published previously[16]. The chelate ligand 6 and diluent (C_1-ether) were simultaneously bonded in an appropriate ratio (co-bonding). The deprotection of the carboxylic groups was accomplished according to a previously published procedure[15,21]. For the isobutylene analyses, a sample of bonded silica gel was hydrolyzed in a twenty-fold excess (relative to the amount of IDA on the surface) of trifluoroacetic acid and rotated gently for 2 h at room temperature. After centrifugation, a sample of the supernatant was injected into a gas chromatographic column, packed with Porapak Q as stationary phase. Isobutylene was quantitated by comparing the peak area with standards of similar concentrations.

The surface concentrations of the chelate and diluent were determined as be-

fore[15]. Elemental analysis for measurement of surface coverage was performed by Multichem Lab. (Lowell, MA, U.S.A.). The precision of percent carbon data for a given phase was *ca.* 3% relative standard deviation (R.S.D.). A correction was made for the amount of carbon found in the blank Vydac silica gel. In the calculation of surface coverage, an average reaction of two ethoxy groups per silane molecule was assumed.

Metal loading

The procedure for the determination of the amount of metal loaded on a column was similar to that previously described[15]. The entire system was first washed free of contaminants with 10 m*M* phosphoric acid, 80% *n*-propanol–20% 10 m*M* phosphoric acid and then Milli Q water and subsequently equilibrated with a metal-free buffer solution. (The above column washing procedure was generally used, even when metal loading was not being determined.) The chromatographic system, including the precolumn, but not the analytical column, was next equilibrated with the copper-containing mobile phase. The analytical column was then connected for determination of metal loading by frontal chromatography. The solubility of the metal hydroxide was taken into account in the selection of the loading and elution conditions of HPIMAC.

Chromatographic procedures

The phases were packed into 10 × 0.46 cm I.D. stainless-steel columns, following standard slurry procedures. A precolumn (6.0 × 0.46 cm I.D.), packed with a bonded phase of the diluent alone, was placed between the pump and injector. Mobile phases were prepared by adding the correct weight of salt to a volumetric flask containing a previously prepared ALPHIL solution. The ALPHIL solution consisted of an equimolar mixture of acetic acid, MES, and HEPES. It was prepared by adding the correct weight of acid to a volumetric flask containing Milli Q grade water. The pH was adjusted to the appropriate value with either a concentrated solution of sodium hydroxide or ammonium hydroxide in the ALPHIL mixture, and a small amount of buffered ALPHIL solution was added to the mark. The mobile phases were degassed for 5 min under reduced pressure with rapid stirring. During the HPIMAC experiments, small concentrations of metal were added to the mobile phases to reduce metal bleeding from the column. The temperature was maintained at 25°C. Acetone was utilized as an unretained marker for the hydrophilic columns. The column dead volume was 1.1 ml.

RESULTS AND DISCUSSION

Bonded phase: selection and characterization

A significant aspect in the design of chemically bonded phases for protein separations by HPIMAC is the selection of the chelate ligand for bonding to the silica gel. As active site, IDA functionality was chosen because of the high complexation constants toward metals such as Cu(II) and its previous successful use in IMAC[2–4]. The strong binding of metals to the IDA chelating groups aids in minimizing bleeding of metal from the column.

IDA was attached to the silica gel by a long hydrophilic leash (see Fig. 1). This

spacer was selected in order that the chelate complex be highly accessible to proteins and that the phase be hydrophilic. Hydrophilic phases are desirable in order to enhance the specific protein–metal interaction relative to the general mode of hydrophobic interaction and to reduce the possibility of protein unfolding upon adsorption[22]. In addition, the active ligands were co-bonded with a hydrophilic ether ligand (a mixture of protected iminodiacetic acid silane 6 and C_1-ether silane was simultaneously bonded to the silica gel) in order to reduce potential bis-complex formation between two adjacent chelate groups[15] and to enhance the hydrophilic environment (Fig. 1).

The characterization of the bonded phase is important for stationary phases incorporating secondary chemical equilibria[23] especially when such phases involve the use of diluents, such as the C_1-ether in Fig. 1. In this work, we used two approaches previously shown to be accurate measures of the concentration of chelate attached to the diluted bonded phase[15]: (1) analysis of the isobutylene released in the deblocking of the carboxylic acid after bonding and (2) analysis of the amount of metal loaded on the column at saturation. It was found that the amount of chelate measured by both methods of quantitation agreed within 2%. Determination of the amount of diluent was then obtained from elemental analysis after subtraction of the amount of chelate ligand on the surface. Two diluted bonded phases, IDA(I) and IDA(II), with chelate surface concentrations of 0.60 and 0.46 μmol/m^2, respectively, and silica surface areas of 73 and 62 m^2/g, respectively, were synthesized for this work. Each phase was found to be stable over a period of at least four months, based on the constancy of metal loading within this time period. Periodically, during this time period, the column was washed free of metal and reloaded. As an example, a typical metal loading on the IDA(I) column was 44 μmoles of Cu(II).

Chromatographic retention: high salt concentration

Coordination between metals [*e.g.*, Cu(II), Zn(II)] and ligands (*e.g.*, imidazole) of intermediate hard acid–base character is a short-range interaction (coordinative complexation), and, in order for an electron-donating side chain of a protein to exchange with a ligand coordinated to a metal, that side group must penetrate the outer coordination sphere of the metal[24]. Thus, for retention to occur, the electron-donating group must first be brought into close contact with the metal attached to the stationary phase. One approach to achieve this contact is by forcing the protein out of the mobile phase via high concentrations of antichaotropic salts which do not themselves complex with the specific metal[25].

It is first to be noted that protein retention *per se* can occur with IDA columns at high antichaotropic salt concentrations, even when no metal is present. Table I shows the total retention volume, V_g, of a series of proteins on the IDA column in the absence of metal when a gradient of decreasing ammonium sulfate concentration at pH 7 was used. As the table shows, the elution order under these conditions is similar to that obtained on a column consisting of the diluent alone (*i.e.* C_1-ether-bonded phase) which was previously shown to operate in the hydrophobic-interaction chromatography (HIC) mode[16]. In general, retention for the same gradient and column dimensions was less on the IDA column, most likely due to the hydrophilicity of the IDA group. Some selectivity differences are observed when both columns are compared; *e.g.*, transferrin and lysozyme are not well resolved on the

TABLE I

HIC RETENTION OF PROTEINS ON IDA-BONDED PHASE, COMPARED WITH ETHER-BONDED PHASE

Protein	V_g *(ml)*	
	*IDA-bonded phase**	*Ether-bonded phase***
Cytochrome *c* (equine)	8.7	9.1
Ribonuclease A (bovine)	10.6	14.3
Ovalbumin (egg)	11.0	15.9
Carbonic anhydrase (bovine)	12.1	16.4
Serum albumin (bovine)	12.2	16.0
Lysozyme (egg)	12.9	17.5
Hemoglobin (human)	14.8	–
Transferrin	15.0	17.1
Myoglobin (equine)	17.5	18.4
Chymotrypsinogen A	18.6	20.1
Papain	19.7	24.8

* Conditions: column, IDA(I) (100 × 4.6 mm I.D.), mobile phases, A = 3.0 *M* ammonium sulfate, 0.05 *M* ALPHIL (pH 7.0); B = 0.05 *M* ALPHIL (pH 7.0). Gradient, linear from A to 97%B in 20 min; flow-rate 1 ml/min, temperature 25°C.

** This phase consisted of diluent alone (C_1-ether) and was previously used for HIC[16].

ether column but are widely separated on the IDA column, with an inversion in elution order. As another example, ribonuclease A and ovalbumin are better resolved on the ether column. However, the efficiency of the IDA metal-free column with a large salt concentration mobile phase was not as high as the hydrophilic ether column. Nevertheless, IDA phases, as electrostatic phases in general[26], can be operated with HIC mobile phases.

The behavior of the IDA column, loaded with divalent metals, was found to be completely different with high antichaotropic salt concentrations, if the salts themselves did not complex with the metal (*e.g.* sodium sulfate). Table II presents the behavior of three standard proteins with different initial concentrations of sodium sulfate in the mobile phase. At 1.2 *M* salt concentrations in mobile phase A, the three

TABLE II

INFLUENCE OF METAL ON PROTEIN RETENTION AT HIGH SODIUM SULFATE CONCENTRATIONS

Protein	V_g *(ml)*	
	*Cu(II)**	*No metal***
Cytochrome *c*	1.1	1.1
Lysozyme	–***	1.8
Myoglobin	–***	1.3

* Conditions: column, IDA(I) (100 × 4.6 mm I.D.) +. 44 μmol of Cu(II); mobile phases, A = 1.2 *M* sodium sulfate, 0.08 *M* HEPES, $2 \cdot 10^{-6}$ *M* Cu(II) (pH 7.5); B = 0.08 *M* HEPES, $2 \cdot 10^{-6}$ *M* Cu(II) (pH 7.5). Gradient, linear from A to B in 20 min, flow-rate 1 ml/min, temperature 25°C.

** No metal present in the column. All other conditions are the same.

*** No elution.

proteins were essentially unretained. When the IDA column was saturated with Cu(II), cytochrome *c* was unretained, but now lysozyme and myoglobin were not eluted. At an initial concentration of 1.5 *M* sodium sulfate, cytochrome *c* was still unretained, but with the addition of the metal the protein was not eluted from the column. Clearly, the addition of metal caused a significant increase in retention for the proteins relative to the HIC condition alone.

Chromatographic elution; ammonia at constant pH

A common approach for the desorption of proteins in IMAC is to add strong complexing agents, such as histidine, glycine, and EDTA to the mobile phase[2]. In the process of protein elution, the metal may be stripped from the chelate by these strong displacers. When we tried this approach with the IDA–Cu(II) column at low concentrations of glycine in solvent B, the baseline shifted dramatically as the gradient progressed. The large change in UV absorbance during gradient elution was due to the bleeding of metal ion from the IDA–Cu(II) column. The loss of significant amounts of metal during chromatography requires that the column be re-equilibrated with high concentrations of metal after each run. Sometimes, this reloading can be problematical, since it is not always possible to load the column with high concentrations of metal at the pH of the starting chromatographic conditions due to precipitation of the metal hydroxide. Even with loading at the same pH, it is possible that many column volumes of mobile phase may be required for return to the starting conditions, thus increasing the time between runs. Our goal was to find conditions that minimized bleeding in order to reduce re-equilibration time and achieve rapid separations and, moreover, to allow the convenient use of Cu(II). Cu(II) is one of the more important divalent transition metals in IMAC, since it has the highest binding constant to IDA of all of these metals. The rate of ligand binding and dissociation with Cu(II) is generally also the highest among the divalent metals[27].

We next explored ligand competitors (displacers) that would not significantly strip metal from the support. Ammonia was chosen as displacer because of the lower metal-binding constants (relative to the previously mentioned displacers) and because of the ability to obtain conveniently reproducible low concentrations via pH control. Table III presents the results for the elution of four proteins when ammonia was added to mobile phase B. When no ammonia was present (first column of Table III), the proteins were not eluted. However, when small amounts of ammonia were added to mobile phase B, the weakly coordinated proteins were eluted. In order to desorb the more strongly interacting myoglobins, higher concentrations of the displacer were necessary.

The importance of ammonium hydroxide as a displacer of proteins can be further seen from the fact that at an equivalent mobile phase surface tension for sodium sulfate (1.5 *M*) and ammonium sulfate (1.9 *M*), the three proteins in Table II at pH 7 on the Cu(II)-loaded column were fully retained with the former salt (Na^+) and unretained with the latter salt (NH_4^+). Thus, hydrophobic interaction is not the controlling factor in the retention process under these conditions with metal. However, some hydrophobic interaction of non-polar groups on the surface of a protein with the stationary phase could occur as a re-enforcement of the binding of the protein to Cu(II) in the stationary phase.

Fig. 3 presents the high-performance separation of three standard proteins on

TABLE III

INFLUENCE OF AMMONIA ON THE DESORPTION PROCESS OF PROTEINS

Conditions: column, IDA(I) (100 × 4.6 mm I.D.) + 44 μmol of Cu(II); mobile phases, A = 1.2 *M* sodium sulfate, 0.05 *M* ALPHIL, $2 \cdot 10^{-6}$ *M* Cu(II) (pH 7); B = 0.05 *M* ALPHIL, $2 \cdot 10^{-6}$ *M* Cu(II) (pH 7), and either 0 *M* (a), 0.1 *M* ammonium hydroxide (b) or 0.1 *M* ammonium hydroxide plus 0.50 *M* ammonium acetate (c). Gradient, linear from A to 97% B in 20 min, flow-rate 1 ml/min, temperature 25°C.

Protein	V_g *(ml)*		
	*0**	$5.5 \cdot 10^{-4}$ *M**	$3.3 \cdot 10^{-3}$ *M**
Lysozyme	—**	12.4	9.4
Ribonuclease A	—**	17.8	10.9
Myoglobin (equine)	—**	—**	25.1
Myoglobin (whale)	—**	—**	33.5

* Free ammonia concentration in solvent B.
** No elution.

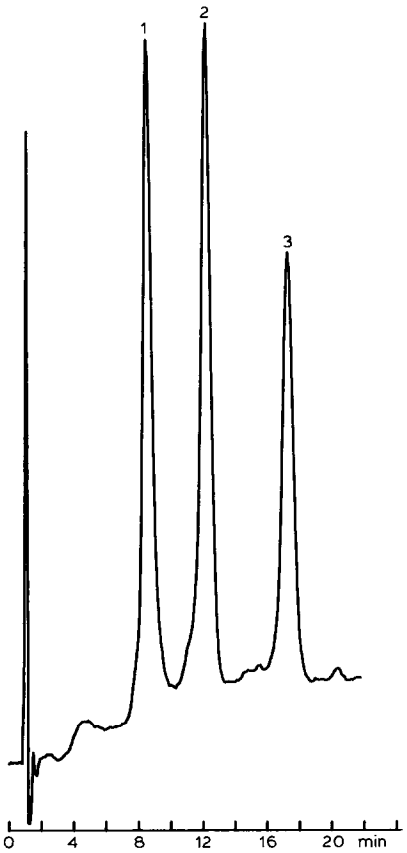

Fig. 3. Separation of a protein mixture on the IDA(II)–Cu(II) column. The protein mixture (1, lysozyme; 2, ribonuclease A; 3, dog myoglobin) was chromatographed at a flow-rate of 1.0 ml/min at 25°C with a 20-min linear gradient from 0 to 97% B; mobile phase A = 1.2 *M* sodium sulfate, 0.05 *M* ALPHIL, $2 \cdot 10^{-6}$ *M* Cu(II) (pH 7.0); mobile phase B = 0.05 *M* ALPHIL, $2 \cdot 10^{-6}$ *M* Cu(II), 0.5 *M* ammonium acetate (pH 7.0); detector wavelength, 280 nm.

IDA(II)–Cu(II) at pH 7 with a 20-min gradient of increasing concentration of ammonia. The elution order is different from that in which metal is not present (*e.g.*, reversal of order for ribonuclease A and lysozyme, *cf.* Table I). High-efficiency separation was observed and little or no bleeding of Cu(II) from the column was found, even with a gradient of 0.5 *M* ammonium acetate and 0.1 *M* ammonium hydroxide. Given the UV sensitivity of Cu(II) at the detection wavelength of 280 nm, any significant bleeding would have been easily observed. The initial rise in the baseline at the beginning of the gradient in Fig. 3 is probably due to the minute bleeding of the metal from the equilibrated system when ammonia reaches the mixer and column. As further evidence of the low level of metal bleeding, it was found that only 5–10 column volumes of mobile phase A were required to return to starting conditions. In order to achieve this result, it was necessary to have a trace amount of Cu(II) ($2 \cdot 10^{-6}$ *M*) in mobile phases A and B. The addition of this trace amount of metal did not appear to affect retention, since the V_g values in Table I for the ether column were not altered when $2 \cdot 10^{-6}$ *M* Cu(II) was added to the mobile phase. Given the high-performance capabilities of these columns and mobile phases, we next explored selectivity.

Table IV presents results on retention of a variety of proteins when ammonium hydroxide is used as a displacer at pH 7.0 and Cu(II) and Zn(II) as metals. A cursory comparison with the IDA metal-free column (Table I) reveals a significant change in elution order with the addition of metal to the column. Thus, equine myoglobin and hemoglobin were not eluted from the column with Cu(II) (Table IV), but were eluted in reasonable time from the IDA metal-free column with 3 *M* ammonium sulfate

TABLE IV

RETENTION OF PROTEINS IN HPIMAC ON IDA(I)

Protein	V_g *(ml)*	
	*Zn(II)**	*Cu(II)***
Cytochrome *c* (equine)	–	1.1
Ovalbumin (egg)	–	6.1
Trypsin (bovine)	–	7.5
Serum albumin (bovine)	–	8.3
Lysozyme (egg)	3.7	9.4
Ribonuclease A (bovine)	2.5	10.9
α-Chymotrypsinogen A (bovine)	8.8	11.4
Myoglobin (dog)	–	17.7***
α-Lactalbumin (metal-free)	–	18.7
Myoglobin (equine)	11.9	25.1
Myoglobin (whale)	21.9	–§
Hemoglobin (human)	–§	–§

* Mobile phases, A = 1.2 *M* sodium sulfate, 0.05 *M* ALPHIL, $2 \cdot 10^{-4}$ *M* Zn(II) (pH 7.0); B = 0.05 *M* ALPHIL, 0.1 *M* ammonium hydroxide, $2 \cdot 10^{-4}$ *M* Zn(II) (pH 7.0). Gradient, linear from A to 97% B in 20 min, flow-rate 1 ml/min, temperature 25°C.

** Mobile phases, A = 1.2 *M* sodium sulfate, 0.08 *M* HEPES, $2 \cdot 10^{-6}$ *M* Cu(II) (pH 7.0); B = 0.5 *M* ammonium acetate, 0.1 *M* ammonium hydroxide, 0.05 *M* HEPES, $2 \cdot 10^{-6}$ *M* Cu(II) (pH 7.0). Gradient, linear from A to 100% B in 20 min, flow-rate 1 ml/min, temperature 25°C.

*** IDA(II), ALPHIL was used instead of HEPES with Cu(II).

§ No elution.

(Table I). Interestingly, these two proteins were unretained on the IDA metal-free column when the starting mobile phase gradient was 1.2 *M* sodium sulfate.

The retention order on the IDA–Cu(II) column can be interpreted in terms of the number of exposed histidine (His) groups on the surface of a protein in the native state, in agreement with the findings of Sulkowski *et al.*[28]. (At pH 7.0 tryptophan also interacts, but only weakly[4], and exposed cysteine groups, while strongly interacting[4], do not occur in the examples chosen for this work.) As already noted, ribonuclease A was eluted before lysozyme from the IDA metal-free column, whereas the order was inverted with IDA–Cu(II). For ribonuclease A, the greater number of His on the protein surface capable of interacting with bound Cu(II) resulted in the longer retention[4,28].

It is well known that ligands bind more weakly to Zn(II) than Cu(II). In all cases, the proteins were found to be eluted earlier from an IDA–Zn(II) column than from an IDA–Cu(II) column (Table IV), as expected from the relative binding strengths of the two metals. Indeed, whale myoglobin remained on the Cu(II) column but did emerge from the Zn(II) column. Thus, Zn(II) with ammonium hydroxide at constant pH as displacer may find value for proteins that bind strongly to metals.

Ribonuclease A was eluted before lysozyme from the IDA–Zn(II) column (Table IV), as found for the IDA metal-free column (Table I). Evidently, with Zn(II) and the mobile phase conditions in Table IV, the binding of these proteins to the metal was not sufficiently strong to overcome salt-induced hydrophobic interaction with the diluted IDA column. However, as will be shortly seen, proteins that interact more strongly with Zn(II) were able to overcome the hydrophobic interaction process. It may also be noted that $2 \cdot 10^{-4}$ *M* Zn(II) was added to mobile phases A and B in order to minimize bleeding. As with the Cu(II) column, only 5–10 column volumes were necessary for return to the starting conditions.

Consider next the three myoglobin species. The elution order in Table IV for both metal-loaded columns can again be explained in terms of the surface histidine residues available for interaction with the chelate metal bonded phase. Sperm whale myoglobin (three exposed histidine residues)[29] is thus more strongly retained than equine myoglobin (two exposed histidine residues), and dog myoglobin (one exposed histidine) is eluted first. This example illustrates the unique selectivity possible with HPIMAC.

Finally, it is to be noted that hemoglobin is not eluted from either metal-loaded column under these conditions. This protein contains a total of 34 histidine residues[30], and it is likely that a sufficient number are exposed for strong interaction with the metal on the surface. In fact, previous studies have shown that hemoglobin is extremely difficult to elute from agarose/metal chelate columns[31]. Given this result, we explored alternative gradients to elute very strongly bound proteins.

Chromatographic elution: pH gradient with ammonia

A convenient approach for eluting proteins that are strongly bound to the metal-loaded columns is to reduce the pH of the mobile phase. The pK_a of histidine (His) is close to 6, depending on its environment in the protein[32]. As the His group becomes protonated with decreasing pH, the binding of this group to a metal will gradually weaken until full release occurs. With ammonia present in the mobile phase, the exchange of this displacer for the protein attached to the metal will be

facilitated. (This enhancement of ammonium hydroxide as a displacer will be compensated to some extent by the decrease in free ammonia concentration with decreasing pH. Nevertheless, proteins are eluted more rapidly in the presence of ammonia with a pH gradient than in its absence.) Interestingly, even with a pH value close to 5, the binding constant of the metal to IDA will be large[33]. Thus, the binding of the protein to the metal is affected to a far greater extent than the binding of the metal to IDA, as the pH is decreased from 7 to 5. We have explored this approach of pH gradients for the release of strongly bound species.

Special care was taken in the selection and preparation of the buffer solutions for the pH gradients. First, the buffers were chosen to have a similar and high buffer capacity over the whole pH range of the gradient (7.5–4.7). Secondly, similar concentrations of each buffer were used, and each buffer mixture contained all components over the whole pH range. Thirdly, the buffers were selected to have a low binding capacity toward metals and to be transparent in the UV range of detection. In order to meet the above criteria, the buffer solution was prepared with equivalent amounts of HEPES (pK_a 7.5), MES (pK_a 6.1), and acetic acid (pK_a 4.7)[34].

Fig. 4A(i) shows a pH titration of 0.05 *M* ALPHIL buffer (pH 4.7) with 0.05

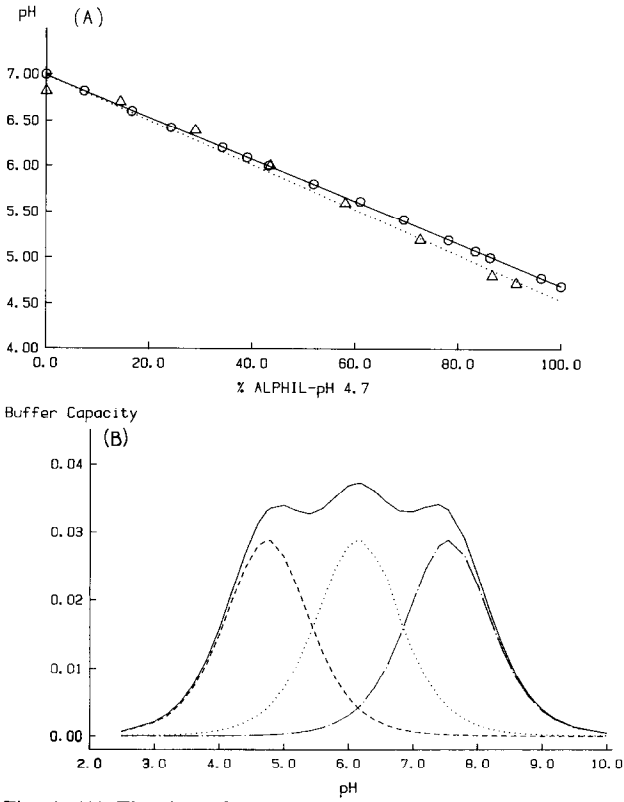

Fig. 4. (A) Titration of 0.05 *M* ALPHIL buffer (pH 7.0) with 0.05 *M* ALPHIL buffer (pH 4.7); i (○—○): manual titration; ii (△...△): pH after elution from column. The delay volume and column dead volume have been subtracted. (B) Buffer capacity of ALPHIL and its components. – – –, Acetic acid; ....., MES; –.–.–, HEPES: ———, ALPHIL.

M ALPHIL buffer (pH 7.0). Fig. 4A(ii) shows a similar titration, but in this case the buffer solutions were pumped through the column, and the pH of the eluate was determined. As can be seen in Fig. 4A, the pH gradient from 7.0 to 4.7 was linear, whether in solution or after passage through the chromatographic column. Fig. 4B shows a plot of buffer capacity for each buffer and their combination (ALPHIL) over a wide pH range, and a constant capacity of 0.035 ± 0.002 for 0.05 *M* ALPHIL is observed in the pH gradient range. The linearity in Fig. 4A is a result of this constant buffer capacity. If the buffer capacity of the mobile phase were not constant during the gradient, the pH would vary widely and sharp changes in pH leading to step gradients could occur.

A pH gradient from 7.0 to 5.5, produced with the ALPHIL buffer and 0.5 *M* ammonium acetate in solvent B was next examined in HPIMAC. In Fig. 5, dog, equine, and whale myoglobin are successfully resolved with a 20-min gradient and

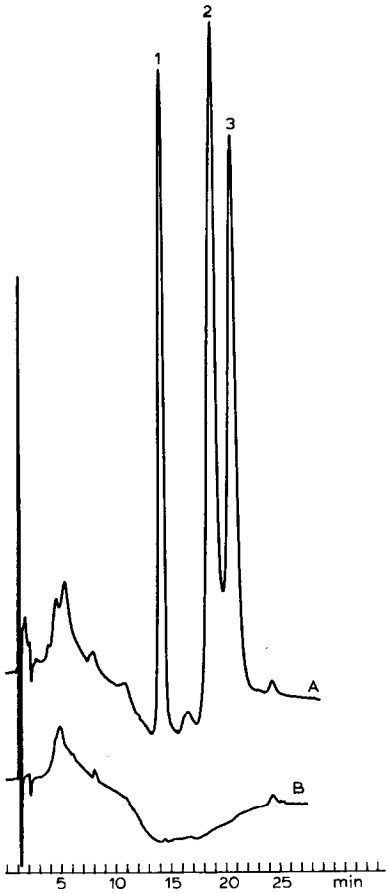

Fig. 5. HPIMAC of a myoglobin mixture on IDA(II)–Cu(II) column. The protein mixture (1, dog myoglobin; 2, equine myoglobin; 3, whale myoglobin) was chromatographed at a flow-rate of 1.0 ml/min at 25°C using a 20-min linear gradient from 0 to 100% B; mobile phase A = 0.08 *M* sodium sulfate, 0.05 *M* ALPHIL (pH 8.0), $2 \cdot 10^{-6}$ *M* Cu(II); mobile phase B = 0.05 *M* ammonium acetate, 0.05 *M* ALPHIL (pH 5.5), $2 \cdot 10^{-6}$ *M* Cu(II). Detector wavelength, 280 nm.

Cu(II) as the loaded metal. For these, as well as other proteins, the elution order is similar to that obtained with ammonia at pH 7.0 in Table IV. As previously, return to starting conditions required only 5–10 column volumes. Separate studies revealed retention reproducibility of 1–2% from run to run. Similar results on reproducibility were obtained at constant pH 7.0 with ammonia.

The peak sharpness in Fig. 5 is especially noteworthy. Shallow pH gradients with gradient times of 1 h also produced relatively sharp, symmetrical peaks. Moreover, hemoglobin, which was not eluted from the column under the conditions in Table IV, was now eluted as a sharp peak within the pH gradient on IDA–Zn(II). Hence, the combined pH and displacer gradient provides a means of eluting proteins with rather strong metal-binding constants.

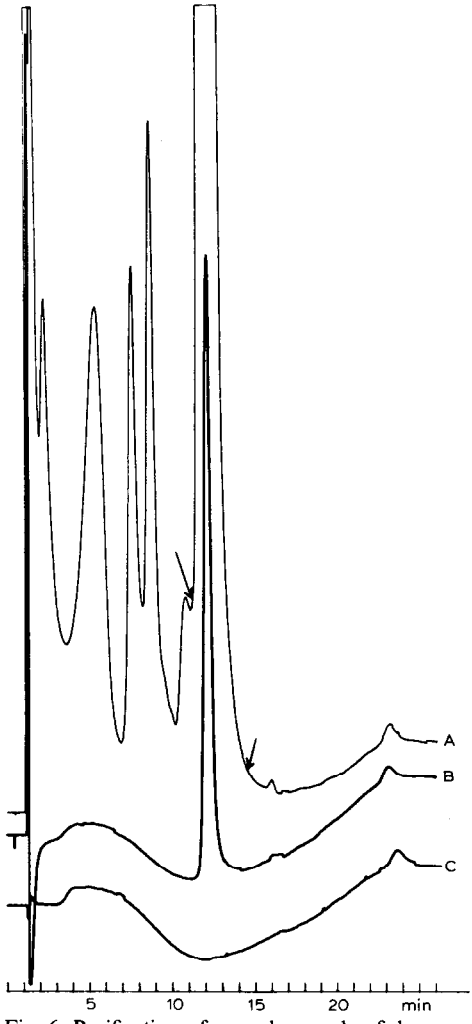

Fig. 6. Purification of a crude sample of dog myoglobin by HPIMAC on IDA(II)–Cu(II). Chromatographic conditions were the same as in Fig. 5. A = Crude sample of dog myoglobin; B = dog myoglobin, collected from A; C = blank gradient after chromatogram A.

As an example of the high performance of this IMAC approach with linear pH gradients, Fig. 6A shows the rapid separation of a crude sample of dog myoglobin. Fig. 6B presents the chromatogram of the sample, collected at the arrows in Fig. 6A. The absence of impurities is readily apparent. Fig. 6C shows a blank gradient after elution of the impure sample, in order to demonstrate the absence of ghost peaks, and suggests a high mass recovery of dog myoglobin. In other studies, we have found, in agreement with others[14], that mass recovery is high. When the experiment in Fig. 6 was conducted with detection at 405 nm, the peak assigned to dog myoglobin increased in size, indicating that the heme was not released from the myoglobin under the chromatographic conditions. In the case of this protein, conformational unfolding often leads to release of the heme group[35], and the above result is thus suggestive of maintenance of the native state. Other workers have demonstrated that the conditions of IMAC are sufficiently mild for maintaining the biological activity of many purified proteins[4,14] HPIMAC thus appears well suited for the purification of active proteins.

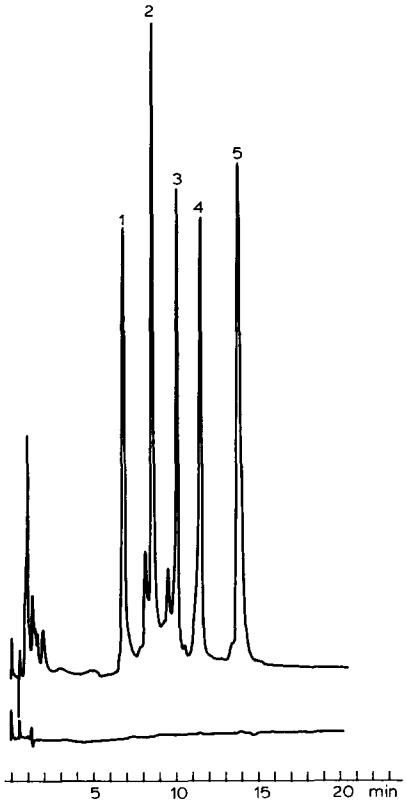

Fig. 7. Cation-exchange chromatography on IDA(II) column. The protein mixture (1, dog myoglobin; 2, whale myoglobin; 3, ribonuclease A; 4, chymotrypsinogen; 5, lysozyme) was chromatographed at a flow-rate of 1.0 ml/min at 25°C, using a 20-min linear gradient from 10 m*M* HEPES to 100% of 0.5 *M* ammonium acetate in 10 m*M* HEPES (pH 7.0).

Multimodal operation

With the choice of mobile phase conditions, the IDA bonded phase without metal can also function in either the cation-exchange or the hydrophobic interaction modes. The successful use of the IDA column in the hydrophobic interaction mode has been previously discussed in connection with Table I. Fig. 7 presents the separation of standard proteins on the IDA column in the cation-exchange mode with an increasing salt concentration gradient at 25°C (pH 7.0). The order of elution corresponds to that expected for cation exchange, *i.e.* increasing retention with increase of the p*I* value of the protein[36]. The results clearly show that highly efficient cation-exchange separations of proteins can be achieved on chelate-bonded-phase columns. When Cu(II), Ni(II) or Zn(II) was loaded on the column, separations with similar low salt concentration gradients (sodium sulfate instead of ammonium acetate and trace amounts of metal in the mobile phase) gave slightly lower retention and the same elution order as in cation-exchange chromatography.

The results of this work demonstrated that the IDA columns can be used in three different modes, depending on mobile phase conditions. It should be emphasized that the selectivity is quite different in the three modes. IDA columns thus represent rather versatile stationary phases for protein separation.

CONCLUSIONS

The use of hydrophilic IDA-bonded phases for HPIMAC has been demonstrated. When the columns are loaded with metals, a distinct selectivity of proteins is obtained compared to that in hydrophobic interaction and cation-exchange chromatography. Chromatographic conditions are described for high-performance protein separations by IMAC. Separations comparable to those obtained with other high-performance chromatographic modes have been observed. Special buffer solutions with constant buffer capacity over a broad pH range have been developed for these separations. Selectivity is based in large part on the number of histidine residues present on the surface of the proteins. The columns appear to be stable in terms of the amount of metal loaded and are reproducible from run to run. Based on this work and that of others[4,14] IMAC allows recovery of proteins with high mass and biological activity. Therefore, it can be concluded that HPIMAC can be used as a selective method or protein separation and purification. In addition, the type of column described here can be utilized in the hydrophobic interaction and cation-exchange modes under different mobile phase conditions when metal is not present.

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