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IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY (IMAC) CHEMISTRY AND BIOSEPARATION APPLICATIONS

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ABSTRACT

This review discusses the principles of immobilized metal ion affinity chromatography (IMAC) and its applications to protein separations. IMAC functions by binding the accessible electron-donating pendant groups of a protein - such as histidine, cysteine, and tryptophan - to a metal ion which is held by a chelating group covalently attached on a stationary support. A common chelating group is iminodiacetate. The ions commonly used are of borderline or soft metals, such as Cu^{2+}, Ni^{2+}, Co^{2+}, and Zn^{2+}. Protein retention in IMAC depends on the number and type of pendant groups which can interact with the metal. The interaction is affected by a variety of independent variables such as pH, temperature, solvent type, salt type, salt concentration, nature of immobilized metal and chelate, ligand density, and protein size. Proteins are usually eluted by a decreasing pH gradient or by an increasing gradient of a competitive agent, such as imidazole, in a buffer. There are still several unresolved issues in IMAC. The exact structures of protein-immobilized metal complexes need to be known so that

retention behavior of proteins can be fully understood and sorbent structures can be optimized. Engineering parameters, such as adsorption/desorption rate constants, sorbent capacities, and intraparticle diffusivities, need to be developed for most protein systems. Engineering analysis and quantitative understanding are also needed so that IMAC can be used efficiently for large scale protein separations.

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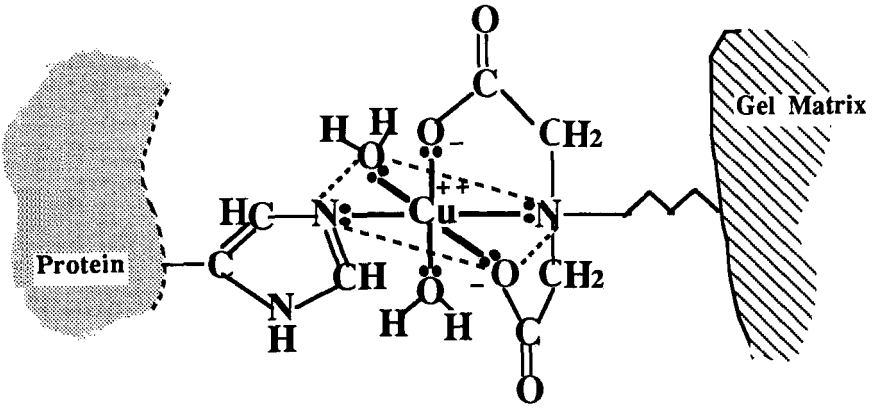
I. INTRODUCTION

The beginnings of immobilized metal ion affinity chromatography (IMAC) can be traced back to 1961 when Helfferich introduced "ligand exchange chromatography" of small molecules.[1] The fundamentals and applications of this technique have been extensively reviewed by Davankov and Semechkin.[2] The chelating resin, Chelex 100 marketed by Biorad, contains iminodiacetate ligands, which were used as a cation exchanger of small metal ions. The use of iminodiacetate as an affinity ligand for macromolecules was not developed until 1975 by Porath *et al.*[3] Porath coined the term "immobilized metal ion affinity chromatography" to encompass all modes of metal chelation interaction chromatography including ligand exchange.[4,5] Porath observed that a protein molecule can bind to metal ions leading to a purification scheme based on metal ion affinity interactions. The use of a chelating agent to immobilize the metal ion on a solid support reduces the degree of freedom of protein-metal interactions. This restriction can reduce protein denaturation and maintain activity while providing substantial purification and separation. The exact structure of a protein-immobilized metal complex is still unknown. A schematic view based on the studies of metal complexes in solution is shown in Figure 1.

An IMAC column can be loaded with a given metal ion (*i.e.* Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}) by perfusing the column with a metal ion solution until equilibrium is reached between the metal chelated to the stationary phase and the metal ion in solution. The solid support (usually of silica or polymer based material) is covalently linked to a ligand such as iminodiacetic acid (IDA). Once the excess metal solution is rinsed and the column equilibrated with the appropriate buffer, a mixture containing biologically active products of interest is passed through the



IDA Metal-Protein Complex

Figure 1. Schematic of the histidine residue of a protein bound to an immobilized copper ion. The metal is chelated to iminodiacetic acid (IDA) coupled to a solid matrix support.[5]

column. All compounds in the mixture with no complementary binding to the ligand will pass unretarded through the column. The products showing affinity for the immobilized metal-ligand complex will be retained.[6]

In sorption of proteins by metal affinity, the exposed electron-donating amino acid residues, such as the imidazole group of histidine, the thiol group of cysteine, and the indoyl group of tryptophan, will contribute to the binding of the immobilized metal. The binding of ligands to biopolymers involves a complex set of van der Waal's, hydrophobic, and electrostatic forces, as well as metal chelation. Proteins can be released from their bound complexes by any mode which reduces the affinity constant between the immobilized metal ion and the protein such as

changing the salt concentration, changing pH, or displacing by a competitive agent which is similar in structure as the amino acid residues involved in binding. Imidazole and histidine are two competitive agents commonly used in IMAC to release bound proteins. A stepwise or gradient elution scheme in one of these desorption methods can be used to selectively recover and purify proteins.

There are many advantages of IMAC over typical methods in affinity chromatography[5]. Different metal ions can be immobilized on the chelator and can be easily removed for regeneration by a stronger chelating agent. The chelating gel can be made stable so that it experiences minimal loss of its metal binding properties. Specific separation methods can be employed by proper choice of the metal, and different sorption characteristics between protein and ligand can be achieved with different metals immobilized to the chelator. In most cases, proteins still possess their biological activity after elution from an IMAC column.[5] A solution can also be made sterile when passed through a metal free IMAC column by removal of the metal ions from the solution. This step prevents the risks of bacterial contamination by removing the metal nutrients needed by the bacteria.

The use of IMAC as a potential purification process can be found in many reviews. Andersson, Davankov *et al.*, El-Rassi and Horvath, Fatiadi, Porath, and Sulkowski have provided background information and applications of IMAC in their excellent reviews.[5,7-12] A history of IMAC is summarized in a table in Appendix A.

A majority of IMAC work has been published by Porath and his associates. Porath's work has focused on the IMAC fundamentals such as the use of agarose or dextran crosslinked polymeric matrices as the support; the choice of the metals used for immobilization such as Cu^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+} and Zn^{2+}; [5,13] the

development of the chelating agents; [5]and the IMAC adsorption-desorption chemistry.[14] Porath's work on the application of IMAC to protein separations includes the purification of serum proteins; [4,15,16] the characterization of metalloproteins and preparation of their free form; [17] the separation of phosphoproteins; [17] the high performance of immobilized metal ion affinity chromatography (HP-IMAC); [5] and the use of IMAC as a probe into the topography of histidine residues on a protein molecule.[18]

II. CHEMICAL PRINCIPLES OF IMAC

II.1 Chromatographic Supports as Matrices for IMAC

There are a variety of supports used for affinity chromatography. The matrix of the support can have a considerable effect on the stability of the complex formed between an immobilized metal and a protein. The general properties required for a chromatographic support are uniform particle size and shape for good column packing, sufficient surface area for high capacity, and mechanical stability.[19] Supports with a small particle size have a high flow resistance and may become clogged. A large particle size reduces the flow resistance but column efficiency is also reduced. Irregular shaped particles may result in nonuniform packing and lead to band broadening. Resistance to attacks by microorganisms is also an important feature.

The first matrix used in IMAC was prepared by covalently binding iminodiacetate ligands to oxirane-activated agarose.[3] Alternative methods of IDA-agarose preparation were later developed by Hemdan and Porath. [20] Agarose is a linear, water soluble polysaccharide composed of alternating 1,3 linked ß-D-galactose and 1,4 linked 3,6 anhydro-α-L galactose units. Aqueous agarose

solutions can gel spontaneously below 50 °C to form beads, pellets or clusters. The polysaccharide chains in agarose form a double helix and aggregate via hydrogen bridges and hydrophobic interactions into fibers of ordered structures. Agarose matrices are hydrophilic, mechanically and chemically stable, and relatively inert. The common trade names for agarose matrices are Sepharose (Pharmacia) and Bio-Gel A (Bio-Rad).

The coupling of IMA gels to silica supports for HPLC practice was first demonstrated by Small *et al.* and Vijayalakshmi, and soon followed by Horvath's and Karger's groups.[21-24] El-Rassi and Horvath were able to bind IDA chelates to hydrophilically coated large pore silica and to separate various proteins with these high performance supports.

High performance matrices of organic polymers have also been studied. In 1986, Kato *et al.* developed a new support for HP-IMAC by immobilizing IDA onto TSK-gel G500 PW, a hydrophilic coated resin of large pore size (1000 Å) and small particle size (10 μm).[25] This new stationary phase for IMAC is commercially available under the trade name of TSK gel chelate 5PW from Toyo Soda (Tokyo, Japan) and TosoHaas (Philadelphia, USA).

The high performance materials based upon synthetic polymers and silicas have obvious advantages over the agarose and dextran supports. They have better mechanical stability, a more rigid form or structure, and smaller volume changes during use. Some drawbacks of the high performance materials are silanol formation at high and low pH with silica-based supports and weaker binding between the immobilized metal and IDA in the TSK resin. Belew *et al.* noticed leakage of the immobilized metal ions from the TSK-gel chelate 5PW when the column is eluted with buffers containing glycine or primary amines at neutral pH.[26]

They were able to prevent metal leakage by lowering the imidazole concentration to 50 mM in phosphate buffer between pH 6.5-8.0 and by avoiding buffers containing primary amines.

II.2. Chelating Ligands in IMAC

In aqueous environments, metal ions are solvated by surrounding water molecules. The metal ion serves as a Lewis acid and water as a Lewis base. However, when water is replaced by a stronger base, a coordination complex is formed. A base such as ammonia, with its single donor atom from nitrogen, can form a monodentate ligand, resulting in a metal complex. Two or more atoms of the same molecule forming a ligand with a metal ion results in a metal chelate.

The binding of a metal ion to a ligand is much stronger in a metal chelate than in a metal complex. The greater stability of a metal chelate over a metal complex arises from the loss in free energy produced by ring formation from the polydentate ligand. This thermodynamic stability is called the chelate effect. The lower free energy content (ΔG) of the chelate over the similar metal complex formed from the same monodentate ligands arises from changes in both the enthalpy (ΔH) and the entropy (ΔS), for $\Delta G = \Delta H - T\Delta S$. For small ring formation of five or six members, the predominant contributor to the lower free energy content is the increase in entropy due to formation of the metal chelate from the corresponding metal complex. For macrocyclic ring formation the major driving force is the decrease in enthalpy of the metal chelate over the complex, with minor contribution from the increase in entropy. Some of the forces that contribute to the enthalpy change are the orbital overlap, the ligand field effects, static and electrostatic repulsions between the ligands in the complex, and coulombic interactions arising from ring formation in the chelate. Entropic contributions to the

chelate energy are the number, arrangement, and size of the chelate ring in combination with the number and size of the solvent molecules released by the ligands and the coordinated metal ions with ring formation.

The commonly used chelating ligand for the immobilization of metal ions in IMAC is iminodiacetic acid. Porath and Olin also synthesized another chelator, tris (carboxymethyl) ethylene diamine (TED) immobilized onto an agarose based stationary phase.[16] Ramadan and Porath published a series of papers describing the synthesis and type of agarose bound by mono-, di-, tri- and hexahydroxamate chelating ligands which can immobilize Fe^{3+}.[15,27] Some of the IMAC chelators are shown in Figure 2.

Hochuli *et al.* have developed a nitrilotriacetic acid (NTA) adsorbent (Fig 2) as a new metal chelate for Ni^{2+}-IMAC.[28] When Cu^{2+} is chelated with tridentate IDA, Cu^{2+} has only one coordination site free for biopolymer interactions but Ni^{2+} has three. However, Ni^{2+} is not bound tightly enough by IDA and leaks from the matrix. TED binds Ni^{2+} strongly but has only one coordination site left for ligand exchange. A quadridentate chelating adsorbent comprised of nitrilotriacetic acid (NTA) bound to the matrix can occupy four positions of the metal coordination sphere of Ni^{2+}. The remaining two ligand positions in the octahedral coordination sphere are available for selective protein interactions. This new chelating agent was found to be very selective for neighboring histidine residues on peptides and proteins.

The nature and structure of the immobilized chelate to the solid support have been discussed by McCurley and Seitz.[29] They characterized various batches of immobilized TED using atomic absorption spectroscopy, anodic stripping voltammetry and electron paramagnetic resonance. Their interest in TED was not

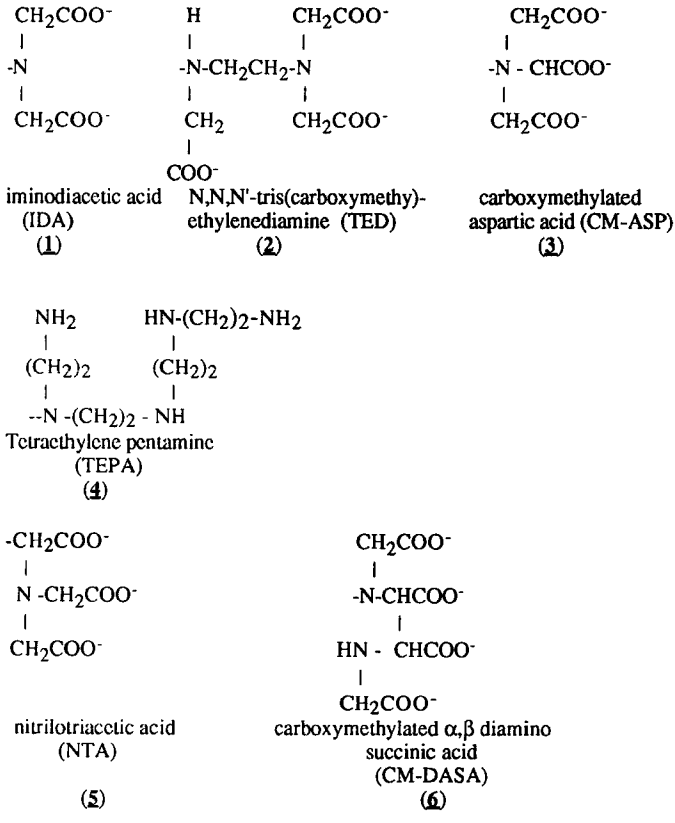


Figure 2. Chelating Ligands in IMAC.[5,28]

only the use of the immobilized chelator as a stationary phase in IMAC but also as a ligand selective indicator in optical sensing.

McCurley and Seitz compared various batches of immobilized TED from Pierce Chemicals and a sample provided by L. Andersson's laboratory in Uppsala. They observed that copper binding capacities were inconsistent and lower than the theoretically calculated values. Results from atomic absorption and anodic stripping

voltammetry indicated that the immobilized TED did not bind Cu^{2+} and that more than one TED was involved in binding each metal ion. Electron paramagnetic resonance spectra were used to compare the immobilized TED to other ligands in solution such as ethylenediaminetetraacetic acid (EDTA), iminodiacetic acid (IDA), N-(2-hydroxyethyl)ethylenediaminetriacetic acid (HEDT), ethylenediamine-N,N'-diacetic acid (EDDA) and nitrilotriacetic acid (NTA). The results showed that the spectra for TED from Pierce had characteristics similiar to EDDA. The complexation constant of the immobilized TED from Pierce determined by competition experiments was similiar to the value for Cu^{2+}-EDDA in solution. A mechanism was postulated in the formation of the immobilized EDDA.

Although there are successful ligands used in IMAC (Figure 2), there can be an expansion in the development of new ligands for IMAC applications. Hancock and Martell state that there is a need for a rational approach toward ligand design for selective complexation of metal ions from solution.[30] An understanding of the principles of selectivity would be invaluable in understanding the metal ion selectivity displayed by biological cation transport systems. For instance, an expansion of IMAC to include metal ions other than transition metals such as Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}, could bring more specificity into applications for IMAC.

A problem with the use of IMAC for commercial purification processes of proteins is the potential toxicity from the metal ions leaching off with the product. This problem can be solved by passing the eluted product through a column of chelating resin, thereby removing the leached metal ions from the product. However, the disposal of the metal ions during regeneration of IMA columns pose some environmental problems for large scale purification. There has been an increasing concern for the potential toxic effects of metal ions as trace contaminants

in the products and byproducts of today's technologies. The typical IMAC metals, Cu^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+}, have potential toxicity at low concentrations, whereas the metals such as Ca^{2+} and Mg^{2+} are environmentally more tolerable at higher concentrations. Other new chelates or metal immobilizers for alkali and alkaline earth metals in IMAC are diacids and crown ethers.[31,32] Diacids have high binding and selectivity for calcium over other alkali, alkaline earth and transition metals because of the steric geometry and special bonding of these chelates for calcium. The immobilization by crown ethers perfused with K^+ ions offers an endless wealth of research in chelate development for IMAC separations of nucleic acids and proteins.[33] Recently, Porath's group has used calcium affinity chromatography by immobilizing Ca^{2+} to a carboxymethylated aspartic acid ligand attached to Sepharose to separate and purify an amyloid glycoprotein.[34] The development of chelating ligands for the less toxic metals is in progress and will help establish IMAC as a commercially attractive operation.

II.3. Coordination Chemistry

The two major questions in coordination chemistry are, "What type of ligand is preferred?" and "What geometric structure is formed in the binding of a particular metal ion to a ligand?" The Principles of Hard and Soft Acids and Bases (HSAB) developed by Pearson can be applied to address these questions.[35] The HSAB approach assumes that in the bonding of two atoms together, one atom plays the role of a Lewis acid and the other a Lewis base. The bond strengths associated with the acid or the base arise from the intrinsic hardness and softness parameters. The HSAB concept states that hard acids prefer hard bases and that soft acids prefer soft bases and that the preferred bonding gives a stronger bond than the non-preferred bonding.

The softness parameter arises from the deformability of the electronic orbital or polarizability of a species. If the electrons are easily moved, the species is soft; if firmly held, the species is hard. Other characteristics that classify hard and soft acids and bases are electronegativity (base), electropositivity (acid), oxidation state, size, bond types (covalent or ionic), availability of donor electrons, and a willingness to accept electrons.[36] A soft base has loosely held valence electrons, has a high polarizability, and is easily oxidized. A hard acid is of small size, has high positive charge density, and does not contain unshared electrons in its valence shell. Hard bases and soft acids are the converses of these descriptions. Some examples of soft acids are monovalent cations of Ag^+, Au^+, Hg^+ and Cu^+; hard acids are represented by K^+, Na^+, Ca^{2+}, Mg^{2+}, and Fe^{3+}; Co^{2+}, Zn^{2+}, Cu^{2+} and Ni^{2+} represent the family of borderline acids. Hard bases include ammonia, amines, sulfates, carbonates, phosphates, and perchlorates; soft bases include sulfur-containing groups and cyanide ion.

When chelated, different metals vary in their reactivity towards proteins. The reactivity of amino acid ligands to different metals can be predicted by HSAB. HSAB assumes there are three main types of ligand donor atoms, of which oxygen (*e.g.*, carboxylate), aliphatic nitrogen (*e.g.*, asparagine and glutamine), and phosphorous (*e.g.*, phosphorylated amino acids) are classified as hard bases; aromatic nitrogen (*e.g.*, histidine and tryptophan) are classified as borderline; and sulfur (*e.g.*, cysteine) is classified as a soft base. The divalent borderline metals, Cu^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+}, coordinate preferentially with aromatic nitrogen atoms (borderline bases) and sulfur atoms (soft bases).

The geometry of metal complexes is related to the coordination number of the metal. The structure of a coordination compound consists of a core called a

complex made of a metal ion attached to a group of tightly held ligands and to other ions which are loosely associated with the complex ion to preserve electrical neutrality. The bond formation of transition metal complexes to their ligands involves the s,p and d orbitals of the metal and the bonding orbitals of the ligands. Molecular orbital theory has been used to describe the qualitative bond formation and energy levels for metal ligand complexes.[37]

II.4. Amino Acid-Metal Interactions in Free Solution and in Immobilized Form

Amino acids form stable, five-membered chelating rings with many metal ions in solution.[38,39] The common structure of amino acids, $NH_2CHRCOO^-$, consists of the α amino and carboxyl groups, both of which participate in the formation of the chelate. Some amino acids have effective donor atoms in the side chain R, and these may enter into the complex formation and compete with the α amino and carboxyl groups. Amino acids have two donor groups: N, and COO^-. They form complexes either as a metal carboxylate salt or an amine complex or both, in which case, a five membered ring is formed. The borderline ions, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} prefer to form chelate rings, whereas the harder metal ions Mg^{2+} and Ca^{2+} form salts with the carboxylate anions. If the R side group of the amino acid is positively charged (*e.g.*, arginine and glutamine), the complex is less stable because of repulsive effects with the positively charged metal. If the side group is negatively charged (*e.g.*, glutamate and aspartate), the extra charge produces dimerization to give a strong stable complex with the metal.

Histidine and cysteine(ine) have reactive side groups and form strong complexing agents. Histidine can complex in four different ways, forming five, six or seven membered rings.[36] Histidine may combine with a metal ion through the

five-membered ring involving the carboxylate and α-amino groups, the six-membered ring involving the α-amino and imidazole groups, the seven-membered ring involving the carboxylate and imidazole groups, or form a structure in which all three donor groups of histidine are combined with the metal ion. The structure formed is a function of pH.[36] Cysteine has a soft sulfur atom in its side group and combines with soft acids more strongly than any other amino acid. A redox (reduction-oxidation) reaction with cupric ions easily oxidizes cysteine to cystine:

$$4\,RS^- + 2Cu^{2+} = 2\,RSCu + RSSR$$

The studies of amino acids adsorption on IMAC columns are few, and the only qualitative work on the retention of amino acids has been investigated by Hemdan and Porath and El-Rassi and Horvath.[20,23] The retention of neutral amino acids on Ni^{2+}-IDA Sephadex G-25 columns loaded at neutral pH shows L-histidine, L-tryptophan, and L-cysteine are retained most strongly while L-glutamate and L-aspartate are not retained at all. L-arginine and L-lysine display also relatively high retentions on this column packing. Increases in ionic strength and sodium chloride concentration enhance the retention of amino acids. The retention of amino acids may be rationalized in terms of chelation, electrostatic attraction or repulsion, hydrophobic effects and π-bonding.

The retention behavior of α-amino acids was compared on naked IDA and metal loaded IDA attached to silica based gels. El Rassi and Horvath found that Cu^{2+}-IDA-silica retains all the α-amino acids and that retention is greater at pH 6.0 than at pH 5.0.[23] They compared the retention times of α-amino acids on the Cu^{2+}-IDA columns and the stability constants of complexes obtained by amino acids with Cu^{2+} in free solution. The α-amino acids, histidine and cysteine, which form the strongest complexes with Cu^{2+} in free solution, are more strongly retained

by Cu^{2+}-IDA columns. Other metal ions such as Fe^{3+}, Fe^{2+}, Ni^{2+} and Zn^{2+} were also tested. Their work showed that histidine was retained more strongly than any other amino acid studied, and this result is in agreement with that of free solution studies.

II.5. Protein-Metal Interactions in Free Solution and in Immobilized Form

There are several factors to consider in protein-metal ion interactions in free solution.[36] The side chain groups such as carboxyl, imidazole and sulfhydryl are more important in the binding of the metal ion than are the terminal amino or carboxyl groups. The charges on the ligand groups influence the affinity for binding with metal ions. The binding coefficient for metal ions decreases with decreasing pH, because protons begin to compete with the metal for binding to the ligands, i.e. $-NH_2$, $-S^-$, and $-COO^-$. The positive charge on the protonated amino group repels the positively charged metal ion, and protonation of sulfide and carboxylate ions negates the attractive force toward the metal ion.

The tertiary structure of a native protein in solution hinders the close-approach of many functional ligands on the protein to a metal ion. Proteins may have many ligand groups but it may have only one or two metal ions attached to it. Occasionally, a metal ion may be held by only one group. In certain proteins, some ligand groups called "active centers" are found in arrays, which sterically favor the chelation to a metal ion. In other proteins, the ligand donors may not be so well arranged and may hold the metal ion between two different coordinate groups. Any group that can donate electrons to a metal ion can also do so to a proton, so competition between metal ions and protons are very important. Consequently, the protein binding to an immobilized metal is dependent upon pH and ionic strength.[35]

Peptide binding to IMAC columns was also studied qualitatively by Hemdan and Porath using nickel loaded columns at varying salt concentrations.[20] Peptide retention was found to increase with increasing pH. The chromatographic behavior of oligopeptides immobilized on IDA-Ni^{2+} was approximated as the sum of the individual contributions of the constituent amino acids.

Smith *et al.* also did work on immobilized iminodiacetic acid-metal-peptide complexes.[40,41] They described the chemistry as a series of metal ion-ligand substitution reactions. They also compared the chemistry of immobilized IDA-metal complexes to solution studies to understand the stereochemistry, structure, and mechanism of the metal-IDA complexes. The coordination of the immobilized metal ion and the ligand is a combination of thermodynamic and kinetic effects with restricted accessibility due to the presence of the support matrix. The peptide or protein may have more limited accessibility due to its conformation and its size. The space and geometry around the matrix bound metal also limit accessibility to the exposed ligands of the polypeptide or protein.

Porath and Belew have suggested that adsorption is a kinetic, two step process involving an S_N1 mechanism.[4] The role of salts may also play a role in the weakening of the coordinated bonding of water to the metal ion. As the chelated metal becomes coordinatively unsaturated by loss of the water ligand, the metal cation binds a ligand atom from the protein, leading to the formation of a binary complex. In solution, metal ions are coordinated with water molecules as ligands; complexation requires that the water ligands be stripped away so that the new ligand and metal ion can come together and bind. The entropic gain accompanying the expulsion of strongly organized water from the coordination sphere of the immobilized metal into the bulk phase promotes adsorption. The binding strength

of the metal to the amino acid ligand depends on the orbital overlap of the metal with the ligand. The interactions between the immobilized metal ion and protein to form coordinative covalent bonds have been characterized by Porath and Belew as being of three types: charge controlled ionic adsorption (ion pairing); coordinate bond formation (coordination of the metal ion and the electron rich ligand); and covalent bond formation (coupling of two sulfyhydryl groups with concomitant reduction of the metal ion-bond formation by metal catalyzed oxidation-reduction).[4]

III. ADSORPTION-DESORPTION MECHANISMS OF PEPTIDES AND PROTEINS IN IMAC

Protein partitioning in affinity chromatography can be quantitatively described by a sum of the various contributions, which are similar to those in liquid affinity extraction of proteins.[42]

$$\ln K = \ln K_{electrostatic} + \ln K_{hydrophobic} + \ln K_{conformation} + \ln K_{ligand} \qquad [1]$$

where the subscripts denote electrostatic, hydrophillic/hydrophobic surface interactions, and conformational changes that occur with protein and ligand interactions.

Articles concerning the binding of proteins with matrix immobilized metal ligands are scarce. The binding of proteins to metal ions in IMAC is believed to result from the ability of electron rich ligands to displace weakly bonded ligands such as water in metal complexes. Porath *et al.* assumed that retention behavior in IMAC is governed by the surface density of exposed histidine, tryptophan and cysteine centers.[5] Metal-ion protein interactions as well as electrostatic, hydrophobic and charge-transfer effects may be involved in the formation of

specific metal ion-protein coordination complexes. The binding of proteins results from the ability of these electron rich amino acid ligands to displace weakly coordinated water in the metal complexes. The metal can donate or accept electrons from these amino acid ligands. When a protein is exposed to a chelated metal immobilized to a stationary matrix, the protein has the ability to form coordinated bonds and multipoint attachments. This binding is selective at high salt concentrations, since the high ionic strength negates the possibility of any electrostatic interactions.[43]

Adsorption-desorption modes can be performed either by protonation, changing the ionic strength, ligand exchange, or chelate annihilation. Displacement by protonation is a common procedure for elution. Protonation of the electron donor group of the protein surface will reverse its coordination to the chelating gel and result in protein displacement.

Adsorption takes place at high salt concentration when copper, zinc, cobalt or nickel ions (borderline Lewis acid metals) are used. The adsorption capacity and selectivity increases as the salt concentration in the equilibrating buffer increases. Decreasing the salt concentration can result in desorption for some proteins.[44,45] The effect of electrolytes on retention is due to the affinity of the metal ion for its solvated water molecules. The weakening of the forces between the metal ion and water induced by salt facilitates the adsorption processes. Since adsorption becomes increasingly selective with increasing ionic strength, it should be possible to achieve partial and selective elution of proteins by decreasing the salt concentration in the eluent.

However, several proteins can be adsorbed and eluted by immobilizing a hard Lewis acid metal such as Fe^{3+} to an IDA column and by increasing the NaCl

concentration from 0 to 1 M.[45] This type of system represents a departure from traditional IMAC by exploiting electrostatic interaction (*i.e.*, cation exchange) and has been shown to be effective in the chromatography of glycoproteins and phosphoproteins.[45-47]

III.1. pH Effect

The adsorption-desorption step is at the heart of of every affinity chromatographic process. Adsorption of the protein to the IMAC gel is performed at a pH at which the electron donor groups on the protein surface is partially unprotonated. It is common to induce protein adsorption on the chelating gel at a weakly alkaline pH. In order to reduce nonspecific electrostatic interactions, a salt is added to the pH buffer. Phosphate and acetate buffers are commonly used. The "reaction" may be described as:

$$S\text{-}L\text{-}Me^{n+} + XP \underset{\text{high pH (~7-8)}}{\overset{\text{low pH (~4-6)}}{\rightleftharpoons}} S\text{-}L\text{-}Me^{n+}\text{-}XP$$

where S denotes the support, L the chelating ligand, Me^{n+} the immobilized metal ion, and X the reactive side group of the protein P.[3]

The pH plays a complex role in the retention and elution processes because it affects the nucleophillic behavior of the buffer components, the electron donor-acceptor properties of the solutes, and stability of the metal. The selectivity for histidine or cysteine is favored at the pH range 6-8 where the adsorption of proteins

occur. At an alkaline pH, coordination with amino groups will take place, making adsorption more effective but less selective.[48]

Sulkowski studied the retention of bovine serum albumin (BSA) on IDA-Ni^{2+}-Sepharose in 20 mM sodium phosphate, 1.0 M sodium chloride at varying pH.[48] The retention of BSA was the highest at pH 7.0, while the retention at pH 6.0 was negligible, resulting in early elution. A progressive decrease from pH 7.0 to pH 6.0 showed an abrupt transition at pH 6.5, This behavior of BSA on IDA-Ni^{2+}-Sepharose indicates the involvement of histidine that has a $pK_a \sim 6.5$.

III.2. Salt Effects

The characteristics of coordinated or covalent bond-based adsorption of proteins at high pH (*i.e.*, 7 or 8) and high salt make IMAC different from ion exchange chromatography. IMAC resembles hydrophobic interaction chromatography (HIC) in some aspects because protein adsorption with the "borderline" metals increases with the inclusion of salt (*i.e.*, NaCl) in the medium. El-Rassi and Horvath investigated the retention of proteins on naked and metal loaded IDA-silica columns by isocratic elution with 25 mM phosphate buffer at varying ammonium sulfate concentrations.[23] The data were fitted by an expression used to analyze retention data in electrostatic and hydrophobic interaction chromatography of biopolymers:

$$\log k' = A + B \log m + C m \qquad [2]$$

where k' is the retention factor, m is the salt molality, and A is a parameter characteristic of the system. B and C are the electrostatic and the hydrophobic

parameters, respectively. The plot and fit of the data to equation [2] give some insight into the interplay between hydrophobic and electrostatic factors in IMAC. El-Rassi and Horvth attempted to use some theoretical basis and a generic model to explain the retention mechanism in IMAC. However, equation [2] does not involve an explicit parameter for coordinative metal complexation with the protein. Additional evidence that hydrophobic interactions contribute to the retention process in IMAC has been shown by Hubert and Porath.[49] They showed that the presence of ethylene glycol, which weakens hydrophobic and other water structure dependent interactions, decreases retention of various nucleotides to the IMAC matrix.

Retention will occur if electron donating amino acid is brought into close contact with the metal attached to the stationary phase. To achieve this contact, the protein is forced out of the mobile phase by the use of kosmotropic (water structuring) salts, which do not themselves complex with the specific metals. Many investigators have observed that the protein retention on naked IDA columns at high kosmotropic salt concentrations seems to indicate the role of non-specific interactions, particularly hydrophobic interactions.[11,23] Salts may be arranged in a lyotropic or Hofmeister series according to their abilities to precipitate proteins from aqueous solutions.[50,51]

phosphates < sulfates < acetates, fluorides < chlorides < bromides,perchlorates, nitrates < thiocyanates

<---------- kosmotropic ---------- neutral ---------- chaotropic ---------->

sodium < magnesium < calcium, barium < manganese, nickel

The effect of kosmotropic salts in the retention of proteins to IMAC columns and protein stability is of a hydrophobic nature. Kosmotropic salts

increase the rate of refolding of denatured proteins and decrease the susceptibility of protein denaturation.[52] These salts do not directly interact with the protein's charged groups and are preferentially excluded from the surface of the protein. Rather, these salts play a more important role by changing the structure of water or solvent molecules. The addition of kosmotropic salts to the solvent enhances hydrophobic interactions of the protein. The exposure of non-polar groups to the aqueous medium of a protein is accompanied by the formation of an ordered structure of water molecules around the non-polar groups and leads to an unfavorable decrease in entropy of the system. The addition of the kosmotropic salt could change the water structure even further. These salts can cause vicinal water molecules to form a firm hydration sphere and can immobilized more water around the exposed protein's non-polar groups. The cavities formed by the ordered structures in the solvent medium can be related to the change in the free energy of the system. The free energy, ΔG_c required for this cavity formation is related by the surface tension, σ and the surface area A', of the cavity:[53]

$$\Delta G_c = (\gamma^o + \sigma m) A' \qquad [3]$$

where γ^o is the surface tension in pure water and m is the salt molality. Kosmotropic salts increase the surface tension of water, increasing the value of ΔG_c and making it unfavorable for the nonpolar amino acid group to stay in isolated cavities. Interaction of two nonpolar groups will decrease ΔG_c by reduction of the overall surface area or the isolated cavities in equation [3]. It is more unfavorable for the nonpolar groups to stay in the salt solution because of the lower entropy state of the system. The entropy changes act as the main driving force for hydrophobic interactions and cause the hydrated protein to form a second phase.

The hydrophobic interactions not only contribute to the stability of the protein but promote adsorption to the surface of a chromatographic matrix.

Kosmotropic salt-promoted adsorption occurs when the protein is forced out of the solvent and onto the matrix. In the case of IMAC, proteins are thus enriched close to the hydrophobic polymeric or silica matrix covered with immobilized metal ligands when the liquid phase is loaded with very hydrophilic salts. The attached ligands, if sufficiently flexible, are able to penetrate the clefts and crevices of the protein molecule. As a consequence, attractive short range forces come into play. Adsorption takes place when the electron donating amino acid groups of the protein penetrate the outer coordination sphere of the metal, enabling charge transfer and metal bonding forces to take effect.

III.3 Competitive Agents

Another common method of achieving selective desorption is to incorporate certain solutes in the eluent which have a higher affinity for the adsorption sites on the proteins than does the immobilized metal or by using solutes that compete with the proteins for adsorption sites. The use of a selective gradient containing imidazole or histidine at low concentrations (50 mM) and neutral pH is an effective method for displacing the bound protein.

The last resort in the elution of immobilized proteins is to destroy the immobilized chelated metal complex by low pH or by adding histidine or a strong chelating agent such as EDTA. Releasing the chelated metal does elute the protein but destroys the efficacy of the column substrate, and may denature the protein in the eluant.

IV. RETENTION BEHAVIOR OF PROTEINS IN IMAC

The retention behavior of proteins on IDA-silica columns was investigated using gradient elution with increasing sodium chloride concentration in 25 mM

phosphate buffer at pH 5.0 and 6.0 with and without bound metals.[23] Without metal, the IDA-loaded stationary phase is expected to be a strong cation exchanger because the pK_a value of one of the carboxylic groups in the covalently bound IDA is 2.65. A protein mixture consisting of five model proteins, ovalbumin (pI 4.7), β-lactoglobulin A (pI 5.1), cytochrome c (pI 10.6), chymotrypsinogen A (pI 9.5), and lysozyme (pI 11.0), was chromatographed at pH 5.0 and 6.0. The retention compares favorably with the same protein mixture on a silica-based cation exchanger with sulfonic groups. With bound IDA or the strong cation exchanger, the proteins are retained in order of increasing isoelectric point at pH 6.0, whereas at pH 5.0 the retention order of chymotrypsinogen A and cytochrome c are reversed. Retention behavior of proteins is dramatically altered upon chelation of metals on the stationary phase and by changing the pH of the eluent.

The physico-chemical aspects of the retention process are not well understood in IMAC, but there is strong evidence that the binding of proteins by the immobilized metal ion arises with the side chains of histidine, cysteine and tryptophan via electron donor atoms which can coordinate with the immobilized chelated metal. The interaction between an amino acid residue in a protein and an immobilized metal can only take place if this amino acid residue is accessible to the metal chelate functional groups of the stationary phase. This subject has been discussed by Sulkowski, who examined the retention behavior of several proteins varying in histidine, tryptophan and cysteine content.[11,18,54] The observed retention of a series of homologous proteins increased as the number of surface exposed histidine residues increased. The number of histidine centers in a protein can be used as a first approximation to estimate the retention of proteins columns. The lack of histidine on a protein surface usually correlates to the lack of retention,

while one or more surface-accessible histidine centers usually lead to an increased retention (see Table 2 in Appendix B). Sulkowski has also identified which metals to use when separating a particular protein with IMAC. Roughly, a protein needs at least two histidine centers to be retained on a Ni^{2+}-IDA column whereas only one is necessary when Cu^{2+} is used as the chelated metal.

Hemdan *et al.* showed that IMAC can be used as a probe into the topography of histidine residues on a protein molecule.[18] An evaluation of model proteins (thioredoxin, ubiquitin, calmodulin, lysozyme, cytochrome c and myoglobin) interacting with immobilized transition metal matrices provides information on how the surface localization, multiplicity, and density of protein's histidine centers affect retention.

Not all histidine centers are the same in a protein. Studies involving the affinity of model proteins containing a single histidine residue show that accesibility to the IMAC adsorbent is an important criterion for retention. The order of elution on a Cu^{2+} - IDA Sepharose column is found to be the following for a series of proteins carrying a single histidine group: thioredoxin *(E. coli)*, cytochrome c (horse), calmodulin (bovine), ubiquitin (bovine) and lysozyme (chicken). Elution of the stronger bound proteins such as lysozyme requires a decreasing stepwise pH gradient from 7.0 to 6.0 and 5.0. The stronger affinity of lysozyme to the IMAC column is attibuted to the presence of His-15 and the additional tryptophan positioned at 62 or 123. The other proteins have accessible histidine residues that contributed to their binding. The order of retention among these proteins is attributed to the differences in the pK's of the histidine groups. The potential for binding by histidine groups appears to increase as the pK decreases as the following elution order illustrates: cytochrome c (His-33, pK

6.5), calmodulin (His-107, pK 6.1) and lysozyme (His-15, pK 5.2). This pattern is consistent with the concept that histidine groups coordinate stronger with IDA-Me^{n+} in the unprotonated state. One histidine group properly positioned in the protein molecule appears to be sufficient for retention in IMAC.

There is also a correlation between the multiplicity of a protein's histidine groups and retention to an IMAC column. The presence of multiple histidine groups can lead to stronger retention, as the three variants of cytochrome c display. Tuna (no external histidines); horse (one external histidine), and yeast (two external histidines) applied to an IDA-Cu^{2+} Sepharose column in 0.1 M sodium acetate with 1.0 M sodium chloride at pH 7.0 elute from the column in the order of tuna cytochrome c followed by horse cytochrome c, followed by yeast cytochrome c. Decreasing pH gradient to pH 5.0 is needed to displace the horse and yeast cytochrome c's. Tuna cytochrome c contains no free cysteines, and its tryptophans (Trp-59 and Trp-33) and one of its two histidines (His-18) is buried in its hydrophobic core. The other histidine is not accessible to the immobilized metal matrix as a result of the crowding of neighboring hydrophobic groups. The stronger retention of the yeast cytochrome c can be anticipated from the protein's higher number of solvent accessible histidine groups.

Retention of proteins on metal chelated columns other than Cu^{2+} chelates requires not only the presence of multiple histidine groups but also the proper location. When horse and yeast cytochromes are applied to a Ni^{2+}-IDA column at 20 mM sodium phosphate with 1.0 M sodium chloride at pH 7.0, horse cytochrome c is not retained and the yeast cytochrome c is eluted isocratically after ten column volumes of eluant. In a comparison study between IDA-Co^{2+} and IDA-Zn^{2+}, yeast cytochrome c is found to have a stronger affinity for IDA-Zn^{2+}

and IDA-Co^{2+} than horse cytochrome c. Another study between dog and sperm whale myoglobins shows slight retention of dog myoglobin on both Co^{2+} and Zn^{2+} columns while sperm whale myoglobin shows significant retention and prolonged elution times. The difference is attributed to the positions of two histidines (his-113 and his-116) found in sperm whale myoglobin but not present in dog myoglobin. The positions of these two histidines and stronger retention are attributed to α-helical localization which shows a favorable distance for strong coordination binding to Co^{2+} -IDA and Zn^{2+}-IDA. The studies with Ni^{2+}-IDA show that a multiplicity of solvent accessible histidines may be required for stronger retention. The surface topography of histidine residues and the recognition by the IDA-metal chelate is listed in Table 2 (Appendix B).

Table 2 is consistent with other results. Smith *et al.* have shown that peptides with the sequences his-trp, his-tyr-NH_2 and his-gly-his have high affinities for Co^{2+}, Ni^{2+} and Cu^{2+} immobilized on IDA-Sephadex.[40] A chelating peptide containing one of these sequences (his-trp) and formed along with genetically engineered proinsulin shows substantial retention on a Ni^{2+}-IDA Sephadex column.[41] Hochuli *et al.* have shown a polyhistidine chelating peptide fused with mouse dihydrofolate reductase is retained on Ni^{2+}-NTA Sepharose.[55] Increasing the number of histidine residues from two to six shows stronger binding to the column. A protein with neighboring histidine residues forms additional bonds with the metal chelate. For example, two additional bonds may form with a metal bound to an NTA adsorbent.

The use of IMAC as a probe to distinguish natural homologous proteins from variants has been extended by Chicz and Regnier to genetically engineered subtilisin.[56] IMAC can be a useful tool to recognize structural changes in

genetically engineered proteins as a result of expression errors. They observed varied retention when specific amino acid substitutions are made through site-directed mutagenesis. These investigators reasoned that neutral and charged amino acids in the environment of a surface histidine accessible to the surface can influence the binding between the histidine center and the immobilized metal matrix and they compared wild type to single and multiple substituted variants of subtilisin.

The location chosen for replacement by a single substituent was Gly 166 because it is essential to the binding of the wild type subtilisin. Chicz and Regnier reasoned that any amino acid substitutions made within a radius of 15 Å should influence the pK_a of histidine 64 and its capacity for binding to a metal. Any changes in retention resulting from the substitution at Gly 166 could only affect his 64 because this is the only electron donating residue with 15 Å of position 166. Substitutions by neutral amino acids at position 166 result in the following order of retention time: Met 166 > Ser 166 > Tyr 166 > Asn 166 > Val 166 at pH 6.2. The influences of electron donating heteroatoms S, O, and N on retention are evident from the sequence.

Substitution at position 166 of charged amino acid at pH 6.2 gives the elution time order: His 166 > Lys 166 > Wild Type > Arg 166 > Asp 166 > Glu 166. Substitutions by negatively charged amino acids decrease the binding by raising the pK_a of the surrounding imidazole groups.

Variants with three to five replacements were examined by substituting phenylalanine for methione at position 50, glutamine for glutamate at position 156 and lysine for glycine at position 166 (FQK variant). At pH 6.2, however, there is a contrast in retention between the wild type and the FQK variants. This

indicates that the active site histidine is pH dependent, sensitive to an acidic environment, and is influenced by its microenvironment. It is probable that the changes in positions 156 and 50 cause the decreased retention at pH 6.2. Substitutions at positions 156 and 166 also appear to affect the histidine 64 microenvironment.

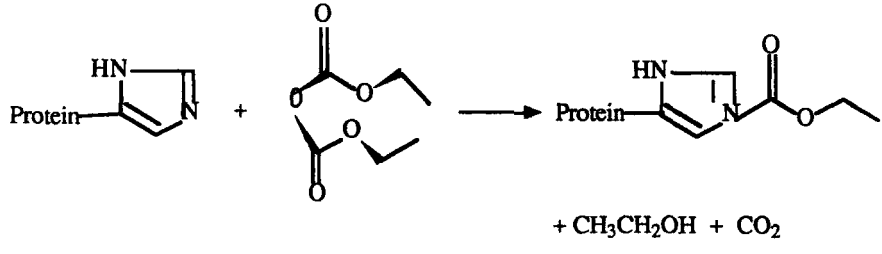
V. APPLICATIONS OF IMAC TO PROTEIN PURIFICATION

Since the pioneering work of Porath, many others have used IMAC in protein separation or purifcation processes (Table 3 in Appendix C).[12,23] IMAC is effective for the purification of genetically engineered proteins. Smith *et al.* developed chelating peptide-immobilized metal ion affinity chromatography (CP-IMAC) based on the assumption that the specific metal chelating peptide will be the one at the terminus of a recombinant peptide chain and will be the preferential binding site for the immobilized metal ion.[41] Recombinant peptides were found to have high affinities for Co^{2+}, Ni^{2+} and Cu^{2+} loaded onto IDA-IMAC columns. A chelating peptide can be cloned to the NH_2 terminus of a fusion protein containing the chelating peptide and the protein of interest. The chelating peptide can later be removed through cleavage techniques. The significance of this work is the demonstration that a preferred binding site can be incorporated into a fusion protein sequence to facilitate purification. Proinsulin is a peptide that has been purified by this technique. Proinsulin contains other histidine residues, but they contribute minor binding to the immobilized metal as a result of their position within the peptide's primary sequence.

Hochuli *et al.* also explored a similiar approach with Ni^{2+}-NTA adsorbent and a fusion protein consisting of mouse dihydrofolate reductase and a

polyhistidine chelating peptide.[55] The polyhistidine peptide was composed of three to six histidine residues attached at the amino or carboxyl terminus. They obtained sufficient purification of the fusion protein on the Ni^{2+}-NTA loaded column by this approach. Their results showed that dihistidine at the carboxyl terminus and hexahistidine at either the carboxyl or amino terminus in were the preferable chelating peptides. Carboxypeptidase A was used to remove the chelating peptide from the protein terminus.

Al-Mashiki and Nakai used columns of Cu^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+} loaded IDA-BGE Sepharose 613/Sephacryl S-300 to separate proteins from blood serum and blood plasma.[57] The packings carrying the different metals were compared in the purification of immunoglobulin and transferrin. Immunochemical analyses of the separated proteins showed no loss in the biological activity. Cu^{2+} loaded columns were the mildest to use and gave the highest IgG activity (>95%). Their paper also presented experimental evidence of the important role of histidine binding to the immobilized metal. They chemically modified the histidine residues of transferrin and IgG with ethoxyformic anhydride or diethyl pyrocarbonate (DEP) and used the method described by Rogers *et al.*[57] The reaction is shown as:



$$+ CH_3CH_2OH + CO_2$$

The DEP treated proteins show diminished binding and elute isocratically with the washing buffer. The control proteins had to be eluted with 0.01 M

imidazole. The decreased binding of the histidine modified proteins with the metal ion illustrates the vital role of histidine in the binding of proteins in IMAC. These results support Porath's early observations that histidine plays a dominant role in the binding between protein and metal. Al-Mashiki *et al.* also applied IMAC to similarly modified histidine groups in the separation of immunoglobulin and lactoferrin from cheese whey.[59] Although histidine was found to be the major binding site to the immobilized metal, a small amount of DEP-Ig was found to adsorb on the column. This suggests the interaction of other amino acids, possibly cysteine, with the immobilized metal .

Zawistowski *et al.* used a Cu^{2+}-IDA Sepharose 6B column for the purification of α–amylases from germinated wheat.[60] A majority of contaminating proteins were removed unretarded with the equilibration buffer. Seven peaks were eluted by varying the glycine concentration in the buffer. Most of the enzymes were eluted with the buffer containing 20 mM glycine. A polyacrylamide gel isoelectric focusing analysis indicates that the protein removed with the 20 mM glycine solution contained mainly the low pI α-amylases, while the early fractions contained high pI α-amylases. The separation of high and low pI α-amylases was accomplished by IMAC with gradient elution by changing the glycine concentration in the buffer.

Allen *et al.* tried to purify bovine fibroblast interferon (BoF-IFN) with zinc chelate affinity chromatography following a partial purification with controlled pore glass (CPG) chromatography.[61] Crude BoF-IFN was purified more than seven fold with CPG chromatography. Additional purification came from ultrafiltration. Further purification by IMAC with Zn^{2+}-IDA agarose loaded column failed, even though consistent and quantitative recoveries of human fibroblast interferon with

Zn^{2+}-loaded IMAC columns have been accomplished.[62] Allen *et al.* did not make further attempts to use other metal ions. Although BoF-IFN contains histidine residues in its primary structure, there are possible reasons for the failure of IMAC for purification. One reason could be the lack of surface and solvent exposed histidine residues for interaction with the immobilized Zn^{2+}. Comparison of human fibroblast interferon and bovine fibroblast interferon shows a different histidine number and distribution of histidine in the structure of each protein.[63] The differences in metal binding between evolutionary species of interferons have been elucidated by Sulkowski *et al.* with IMAC.[54]

Wunderwald *et al.* described a new method for removing active endoproteinases from biological fluids by immobilizing α_2-macroglobulin to Zn^{2+}-bis-carboxymethyl-amino Sepharose.[64] α_2-macroglobulin is known to form complexes with a variety of active endoproteinases . This new method, termed "sandwich affinity chromatography" has been studied and optimized for proteinase binding to the α_2-macroglobulin-Zn^{2+} chelate complex (0.02 M sodium phosphate/0.15 M sodium chloride, pH 6.5). A variety of endoproteinases, trypsin, chymotrypsin, thermolysin, elastase, bromelain, ficin and papain are bound at standard conditions but no binding of exoproteases such as carboxypeptidase A and Y is observed. The effectiveness of α_2-macroglobulin-metal-proteinase complexation is determined by measuring the proteinase activity. In similar studies of "sandwich affinity chromatography", El-Rassi *et al.* immobilized concanavalin A (con A) with Cu^{2+}-IDA on a silica support.[65] Con A is observed to bind strongly to the IMA column via the histidine residues and could only be removed by lowering the pH or adding EDTA at neutral pH. The ConA-Cu^{2+}-IDA columns were used for the purification and separation of various proteins and the removal of various glycoproteins and carbohydrates.

There are still key questions in IMAC that need to be solved. One issue studied by Belew *et al.*, Hutchens, Yip, Porath, and Wong is the quantification of chromatographic parameters that could be vital in the purifications of proteins on a preparative or large scale.[66-68] Quantitative data are important in designing preparative separations. Belew *et al.* studied the interactions of four model proteins on Chelating Sepharose Fast Flow and TSK-gel chelate 5PW sorbents.[66] From the adsorption isotherms, they determined the affinity constants and capacities of the sorbents for the proteins. They observed that the adsorption capacity of the sorbent decreases as its affinity for the protein increases. This may be due to multipoint attachment of the proteins to the adsorption sites and the subsequent changes in protein conformation upon adsorption.

The capacities of IMA adsorbents for the proteins are usually much smaller than those for small ions. This appears to be a consequence of steric effects between the adsorption sites and the proteins. The results of Hutchens *et al.* also indicate some ligands may be inaccessible due to the size of the proteins.[67] Wong found that at maximum loading, each lysozyme, myoglobin, and chymotrypsinogen molecule covers about 5, 9, and 11 IMA sites respectively.[68]

Result studies show that the determination of equilibrium isotherms by frontal analysis can be significantly complicated by protein aggregation and slow intrinsic adsorption kinetics.[69,70] When protein aggregation is significant, effluent histories show multiple plateaus. The breakthrough times of the multiple plateaus vary with protein concentration and flow rate. The breakthrough curves also show slow adsorption of proteins in IMAC. A nonlinear chromatography model considering both protein aggregation and slow adsorption kinetics was developed to analyze the data. The model simulations predict closely that as protein

concentration increases or flow rate decreases, the aggregation rates become relatively fast compared to the controlling mass transfer rates, the two breakthrough curves tend to merge.[69,70] Comparing the lysozyme data with simulation results, they found that the resistance due to slow adsorption is approximately equivilent to an intraparticle diffusion resistance of lysozyme in 280 μm particles.

VI. SUMMARY

This review provides a summary on the chemical principles, adsorption mechanisms, and protein retention behaviors in IMAC systems. The various parameters such as concentration, pH, salt concentration, flowrate and protein structure and how biochemical adsorption responds to the changes in its environment are important in large scale operations. Detailed engineering analysis as well as fundamental engineering data, such as equilibrium parameters, mass transfer coefficients, and adsorption rate constants, are still to be developed for most protein systems. It is our hope that future studies in this area can provide the necessary information so that large-scale metal ion affinity separations can be widely used.

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APPENDIX A

Table 1. History of Immobilized Metal Ion Affinity Chromatography (IMAC)

Year	Event	Reference
1961	-Helfferich first suggest "ligand exchange chromatography".	Helfferich (1961)[1]
1975	-Porath develops "metal chelate affinity chromatography" as a new approach for protein fractionation.	Porath *et al.* (1975)[3]
1978	-Investigation of charge transfer adsorption in metal chelate chromatography.	Porath (1978)[14]
1981	-First studies of immobilized calcium affinity chromatography.	Borrebaeck *et al.* (1981)[71]
1982	-Binding mechanism of IMAC suggested.	Lonnerdal and Keen (1982)[43]
1983	-High performance metal chelate affinity chromatography using silica.	Small *et al.* (1983)[21]
	-Effects of various parameters, i.e. metal ion loading, salt, detergent, solvents, sample load, pH and elution in IMAC.	Porath and Belew (1983)[4] Porath and Olin (1983)[16]
	-Porath officially terms this process as "Immobilized Metal Ion Affinity Chromatography".	*ibid.*
	-Sandwich Affinity Chromatography: Immobilizing α_2-macroglobulin to Zn^{2+} -IDA-Sepharose to remove active endoproteinases.	Wunderwald *et al.* (1983)[64]
1985	-Sulkowski summarizes a decade of past and present work and future expansion of IMAC.	Sulkowski (1985)[11]
1986	-Isolation of phosphoproteins by immobilized Fe^{3+}-affinity chromatography.	Muszynska , *et al.* (1986)[17]
	-HPLC of metal chelate chromatography using a hydrophilic resin based polymer, TSK gel chelate 5PW.	Kato *et al.* (1986)[25]

Table 1. Continued

1986 (cont)	-Study of protein interactions with immobilized metal ions-Metal ion transfer (MIT)	Muszynska, *et al.* (1986)[72]
	-Metal chelate interaction of proteins with IDA stationary phases on silica supports in HPLC. Quantitative information of pH and salt effects on the retention of model amino acids and proteins.	Corradini *et al.* (1986)[73]; El-Rassi and Horvath (1986)[23]
1987	-New metal chelate adsorbent developed, NTA (nitrilotriacetic acid).	Hochuli *et al.* (1987)[28]
	-Chelating peptide-Immobilized metal ion affinity chromatography (CP-IMAC) developed for the isolation of genetically engineered proteins.	Smith *et al.* (1987)[40] Hochuli *et al.* (1988)[55]
	-Protein recognition studies of immobilized metal ligands.	Hutchens and Porath (1987)[74]
	-Quantitative studies of protein adsorption in IMAC by frontal analysis.	Belew *et al.* (1987)[66]
	-Evaluation of TSK gel chelate 5PW in the HPLC analysis applications of IMAC.	Belew, *et al.* (1987)[26]
	-Semipreparative isolation of carboxypeptidase isoenzymes using metal chelate chromatography.	Krishnan, *et al.* (1987)[75]
	-IMAC reviewed as an analytical method.	Fatiadi (1987)[10]
1988	-Quantitative analysis of protein interaction with immobilized metal ligands.	Hutchens, *et al.* (1988)[67]
	-Chemiluminescence detector is successfully combined with a metal chelate column to determine small amounts of biological constituents.	Hara, *et al.* (1988)[76]
	-Separation of immunoglobulins, transferrin and lactoferrin from agricultural products using IMAC. Experimental evidence that histidine is the strongest and most important amino acid residue in binding to the immobilized metal ligand.	Al-Mashikhi and Nakai (1988)[57]; Al-Mashikhi, *et al.* (1988)[59]

Table 1. Continued

1988 (cont.)	-IMAC used to evaluate metal binding properties of uterine estrogen receptor proteins. Receptor isoforms were separated. Later, IMAC was used to characterized and locate metal ion-specific binding domains of the DNA-binding form of estrogen receptor protein.	Hutchens and Li (1988)[77] Hutchens, *et al.* (1989)[78]
1989	-Purification of human serum amyloid component (SAP) glycoprotein using immobilized calcium affinity chromatography.	Chaga *et al.* (1989)[47]
	-IMAC as a facile probe for the surface topography of histidine residues in model proteins.	Hemdan *et al.* (1989)[18]
	-IMAC rationalized in terms of histidine interactions and metal ion transfer. Detailed work relating protein interactions with immobilized metal ligands in IMAC.	Sulkowski (1989)[12]
	-IMAC as a probe ro distingusih variants of genetically-engineered proteins.	Chicz and Regnier (1989)[56]
	-Elimination of *E. coli* peptide (ECP) contaminants in recombinant human interferon-β1 by Zn^{2+}- IDA chromatography. Selection of the spacer arm is important in separation.	Utsumi *et al.* (1989)[79]
	-Metal affinity precipitation of proteins is introduced.	Van Dam *et al.* (1989)[80]
1990	-Interactions of peptides and protein surface structures with free metal ions and surface-immobilized metal ions.	Hutchens and Yip (1990)[81]
	-First demonstration of direct Fe^{3+} binding to apoferritin using IMAC.	Boyer *et al.* (1990)[82]
	-Liquid-liquid extractions using immobilized metal affinity partitioning is introduced. Mathematical modeling is used to quantitate the results.	Suh and Arnold (1990)[83]

APPENDIX 2

Table 2. Surface Topography of Histidine Residues and IDA-Me^{2+} Recognition.[12] (+) = retention; (-) = no observed retention when metal ion was loaded on resin.

Ligand/Me^{2+}	Cu^{2+}	Ni^{2+}	Zn^{2+}	Co^{2+}
1. -His-	+	–	–	–
2. -His$(X)_n$His-	+	+	–	–
3. -His$(X)_n$His- n(2,3); α-helix	+	+	+	+
4. -HisHis-	+	+	+	+

APPENDIX C

Table 3. Application of IMAC to Protein Purification by Conventional Column Chromatography and High-Performance Liquid Chromatography.[9]

PROTEIN	SOURCE	COLUMN	METAL	OPERATING CONDITIONS	REFERENCE
Albumin	Human serum	IDA Sepharose	Zn, Cu	Stepwise elution with buffer solutions of different pH.	Hansson and Kagedal (1981)[84]
Albumin	Human serum	IDA	Ni	Column equilibrated with 20 mM sodium phosphate/1.0 M NaCl, pH 6.8. Stepwise elution with same buffer except at pH 6.1, 5.7 and 3.8.	Andersson *et al.* (1987)[85]
α-Amylases	Germinated wheat	IDA Chelating Sepharose-6B	Cu	Wheat meal slurried in 0.05 M Tris-HCl/0.15 M NaCl, 10^{-4} M $CaCl_2$, and adjusted with 1 M NaOH to pH 7.5. Columns equilibrated with same buffer solution. Elution of product with sequential buffers containing 20, 50, 100 and 150 mM glycine/buffer.	Zawistowska *et al.* (1988)[86]
Cadmium binding protein	*Escherichia coli* cells	bis-CM amino agarose	Cd	Eluted from 10^{-2} M Tris-HCl (pH 8.6) to 10^{-3} M Tris-HCl (pH 8.6) and with 10^{-3} M Tris-HCl/0.5 M NaCl (pH 8.6) at 4°C.	Khazaeli and Mitra (1981)[87]
Carboxy-peptidase	*Aspegillus niger* cells	IDA Sepharose-6B	Cu	Stepwise elution with 5 mM glycine/HCl followed by 0.1 M M glycine/HCl, pH 3.0.	Krishnan and Vijayalakshmi (1986)[88]
Serine carboxy-peptidase and acid protease	*Aspegillus niger* cells	IDA Sepharose-6B	Cu	Eluted from 50 mM acetate, pH 5.5 to 50 mM acetate/75 mM EDTA, pH 5.5.	Krishanan and Vijayalakshmi (1985)[89]

Table 3. Continued

PROTEIN	SOURCE	COLUMN	METAL	OPERATING CONDITIONS	REFERENCE
Carboxy-peptidase G	-----	IDA epoxysilylated silica	Zn	Column preequilibrated with 10 mM HEPES/ NaOH, pH 8.0; sample is introduced, followed by 200 µL pulse of 0.5 M KCl for removal of contaminants, elution of product with 200 µL pulse of 10 mM EDTA.	Small, *et al.* (1983)[21]
Coagulation factor XII	Human plasma	IDA Sepharose 4B	Zn	Stepwise elution with buffers of different pH, rechromatography of the fraction containing the product with decreasing pH gradient.	Weerasinghe, *et al.* (1981)[90]
Human clotting factor XII	Human plasma	IDA	Zn	Column equilibrated with 0.15 M NaCl /50 mM Tris-HCl, pH 8.0 and was washed with 0.8 M NaCl/0.1 M Na-acetate, pH 4.5. Elution with 0.8 M NaCl /0.1 M Na-acetate, pH 4.5.	Weerasinghe, *et al.* (1985)[91]
Collagenases	Mammalian connective tissue culture	IDA Sepharose	Zn	Stepwise elution with buffers of different pH, containing 0.05% Brij, 0.03% toluene and 1 mM $CaCl_2$.	Cawston and Murphy (1981)[92]
Collagenases	Porcine synovia	IDA Sepharose 6B	Zn	Stepwise elution with buffers of different salt content and pH.	Cawston and Tyler (1979)[93]
Copper binding fragments	Human ceruloplasmin	IDA Sepharose	Zn, Cu	After tryptic digest, the peptide fragments were applied to Zn^{2+}-IDA and eluted with 0.1M Na-phosphate/0.15 M NaCl, at pH 8 and pH 6.2. The nonbinding fragments were applied to a Cu^{2+}-IDA and washed with 0.1 M Na-acetate/0.15 M NaCl, pH 7 and eluted with equilibrated buffer and eluted with 0.1 M Na -acetate/0.15 M NaCl, pH 2.9.	Ortel *et al.* (1983)[94]

Table 3. Continued

PROTEIN	SOURCE	COLUMN	METAL	OPERATING CONDITIONS	REFERENCE
Dolichos biflorus lectin	Horse gram	bis-CM amino Sepharose 4B	Ca	Loading the column at pH 8.2 and elution of protein with 10mM EDTA at the same pH.	Borrebaeck *et al.* (1981)[71]
Epidermal grow factor	Shrew submaxillary gland	TED Sepharose 6B	Ni	Stepwise elution with buffer solutions of different pH (7.4-> 6.0).	Yip *et al.* (1985)[95]
α-Fetoproteins albumin	Crude serum	IDA Sepharose 6B	Ni	pH gradient from 20 mM Na-phosphate/1 M NaCl, pH 6.8 to 20 mM Na-phosphate/1 M NaCl, pH 5.3.	Andersson *et al.* (1987)[96]
Fibrinogen	Human plasma agarose	bis-CM amino Sepharose 4B	Zn	Decreasing pH gradient from 8.0 to 5.5.	Scully and V. Kakkar (1982)[97]
Fibrinogen	Human plasma	bis-CM amino agarose Sepharose 4B	Zn	Equilibrated with 0.8 M NaCl/ 25 mM Na-phosphate and eluted with 0.8 mM NaCl/25 mM Na-phosphate/5 mM Na-acetate, pH 4.0 at 40 mL/hr gradient.	Scully and Kakkar (1981)[98]
Gastrointestinal polypeptides	Porcine colon Sepharose 4B	IDA Zn	Ni, Cd,	Columns preequilibrated with 0.1 phosphate containing 1.0 M NaCl, pH 7.4. After loading, stepwise elution with 0.1 M sodium acetate, pH 4.0.	Conlon and Murphy (1976)[99]
Gastrointestinal polypeptides	Porcine colon Sepharose 4B	IDA	Co, Cu	As above but stepwise elution with 0.5 M EDTA, containing 0.5 M NaCl, pH 7.0.	*ibid.*

Table 3. Continued

PROTEIN	SOURCE	COLUMN	METAL	OPERATING CONDITIONS	REFERENCE
Glutathione reductase (NADPH -specific disulfide reductase)	*Halobacterium halobium*	IDA agarose	Cu, Ni	Supernatant from Sepharose 4B buffered in 1.23 M $(NH_4)_2SO_4$ was loaded in Cu^{2+}-loaded column at the same buffer. Proteins eluted with a linear gradient of 0-100 mM imidazole (400 mLs collected). After passing through a Sepharose 4B column, protein solution is adjusted to pH 6.7 (1.0 M NH_4OH) and loaded to a Ni^{2+} column, equilibrated with buffer /4.0 M NaCl. Protein is eluted by a decreasing pH gradient to 5.2.	Sundquist and Fahey (1988)[100]
Glycogen phos-phorylase	Chicken breast muscle	IDA Chelating Sepharose Fast Flow	Fe	Columns equilibrated with 0.020 M Tris/HCl, pH 7.7; extracts were applied and column was washed with 0.02 M Mes/ NaOH, pH 6.5; and later with 0.02 M sodium phosphate , pH 6.4.	Chaga *et al.* (1989)[47]
Glycophorins	Human erythrocytes	IDA Nucleosil Silica	Cu	Decreasing pH and salt gradient from 7.0 to 5.0 and from 0.7 to 0 M NaCl.	Corradini *et al.* (1988)[73]
Granule proteins	Guinea pig granulocytes	IDA Sepharose 6B	Cu	Decreasing pH gradient from 7.7 to 2.8 with buffers containing 0.5 M NaCl.	Torres *et al.* (1979)[101]
Granulocyte macrophage colony stimulating factor (GM-CSF)	Murine myeloid leukemic cells	IDA Sepharose 6B	Cu	Equilibrated in 50 mM 1 M NaCl, pH 5.0 and eluted with 100 mL equilibration buffer followed by 100 mL equilibration buffer + 50 mM EDTA and 100 mL of 50 mM $NaHCO_3$ containing 0.1% (w/v) SDS.	Hilton *et al.* (1988)[102]

Table 3. Continued

PROTEIN	SOURCE	COLUMN	METAL	OPERATING CONDITIONS	REFERENCE
Immuno-globulins	Blood serum and plasma, Cheese whey	IDA Sepharose 6B	Cu, Zn, Ni, Co	Columns equilibrated with 0.5 M NaCl/0.05 M Tris/acetate, pH 8.2. Column was eluted with 0.05 Tris-acetate/0.5 M NaCl, pH 4.0 and later with 0.01 M imidazole in the same buffer, pH 4.0.	Al-Mashikhi and Nakai (1988)[57] Al-Mashikhi, *et al.* (1988)[59]
Inter-α-trypsin inhibitor	Human serum	IDA Sepharose 6B	Zn	Stepwise elution with phosphate, pH 6.0, containing 0.8 M NaCl, pH 6.0, followed by EDTA/NaCl, pH 7.4.	Salier *et al.* (1980)[103]
Interferons	Human fibroblast	IDA Sepharose 6B	Zn	Decreasing pH gradient from 6.0 to 4.0 with eluents at 1.0 M NaCl.	Edy *et al.* (1977)[104]
Interferons	Human	IDA agarose	Cu	----------	Berg and Heron (1980)[105]
Recombinant mouse Interferon β	*Escherichia coli* cells	IDA Sepharose	Cu	Column equilibrated with 0.1 M Na-acetate/0.5 M NaCl, pH 5.6 and eluted with 0.1 M Na-acetate/ 0.5 M NaCl, pH 4.5.	Matsuda *et al.* (1986)[106]
Interferons	Mammalian	IDA Sepharose 6B	Zn, Co, Ni, Cu	Decreasing pH gradient from 6.0 to 4.0.	Chadha *et al.* (1982)[107]
Interferons	Human cornea cells	IDA Sepharose	Zn	Removal of contaminants with phosphate, pH 7.4 and acetate, pH 6.0, both containing 1 M NaCl, then decreasing from 6.0 to 4.0.	Thacore *et al.* (1982)[108]

Table 3. Continued

PROTEIN	SOURCE	COLUMN	METAL	OPERATING CONDITIONS	REFERENCE
Interferons	Human fibroblast	IDA Sepharose 6B	Zn	Decreasing pH gradient from 6.0 to 4.0.	Heine *et al.* (1981)[109]
Interferons	Human lynphoblastoid	IDA	Cu	Decreasing pH gradient from 7.0 to 2.0	Yonehara *et al.* (1981)[110]
Interferons α-2A (fusion protein)	recombinant *Escherichia coli*	NTA Sepharose	Ni	Column equilibrated with 0.05 M sodium phosphate buffer, pH 8.0. Product was eluted with a pH gradient pH 8 to 5 in the same buffering solution.	Hochuli (1988)[111]
α-Lact-albumin	Bovine milk 5PW	IDA TSK-Chelate	Cu	Column equilibrated Tris/0.5 M NaCl, pH 7. Prepared protein solution was eluted by a gradient increase of the buffer from 0-20 mM imidazole.	Reid and Stancavage (1989)[112]
Lactate dehydro-genase	Chicken breast muscle	IDA Chelating Sepharose FF	Fe	*(Same as glycogen phosphorylase)*	
Lactoferrin	Human milk amino	bis CM Sepharose 4B	Cu	Decreasing pH gradient from 8.2 to 2.8 with buffers containing 0.5 M NaCl.	Lonnerdal *et al.* (1977)[113]
Lactoferrin	Bovine milk	IDA Spherosil XOB030 oxirane activated	Cu	Elution steps with 0.5 M NaCl, 20 mM glycine and 10 mM histidine all in 50 mM Tris-HCl, pH 7.5.	Vijayalakshmi (1983)[22]
Lactoferrin	Cheese Whey	IDA Sepharose	Cu	*(Same as immunoglobulins)*	
Lipoxidase	Soya bean	IDA TSK-Chelate 5PW	Zn	Gradient with increasing glycine concentration from 0 to 0.2 M in 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl.	Kato *et al.* (1986)[25]

Table 3. Continued

PROTEIN	SOURCE	COLUMN	METAL	OPERATING CONDITIONS	REFERENCE
α_2-Macroglobulin	Human plasma	IDA Sepharose 6B and 4B	Zn	Stepwise elution with phosphate buffer, pH 6.0, followed by sodium cacodylate, pH 5.0.	Kurecki *et al.* (1979)[114]
Maltate dehydrogenase	Oriental yeast			*(Same as lipoxidase)*	
α-D-Mannosidases	Monkey brain	IDA Sepharose 4B	Co	Preequilibration with 20 mM potassium phosphate, pH 7.5; washing with 1.0 M NaCl in the presaturant; elution with 20 mM Tris-acetate, pH 7.5.	Mathur *et al.* (1984)[115]; (1988)[116]
Rabbit bone metalloproteinase	Rabbit calvariae	IDA Sepharose	Zn	Equilibrated with 25 mM sodium borate, pH 8, 0.15 M NaCl, 5 mM $CaCl_2$ and 0.05 % Brij 35. Column eluted with stepwise elution of buffers of different salt content and pH.	Galloway *et al.* (1983)[117]
Monoclonal IgG	Mouse ascites fluid	IDA TSK Chelate 5PW	Zn	Gradient elution with increasing imidazole concentration from 1 to 11 mM in 20 mM phosphate containing 0.5 M NaCl, pH 7.0.	Belew, *et al.* (1987)[66]
Myoglobin	Canine heart Vydac (Silica)	IDA	Cu	Decreasing pH gradient from 8.0 to 5.5.	Figueroa *et al.* (1986)[24]
NADH nitrate reductase	Squash	IDA Sepharose	Zn	Preequilibration with 0.1 M phosphate, pH 7.5, Stepwise elution with 1.0 M phosphate, pH 6.2.	Redinbaugh and Campbell (1983)[118]

Table 3. Continued

PROTEIN	SOURCE	COLUMN	METAL	OPERATING CONDITIONS	REFERENCE
Nonhistone proteins	Rat liver	IDA Sepharose 6B	Cu	Stepwise elution with 20 mM glycine, 10 mM histamine and 10 mM histidine in 10 mM phosphate buffer, pH 7.9, containing 0.5 M NaCl and 10% glycerol.	Kikuchi and Watanabe (1981)[119]
Nucleoside diphos-phosphate	Rat liver	IDA Sepharose	Cu, Zn	Linear histidine gradient from 0 to 15 mM in 0.01 M maleate, pH 6.6.	Ohkubo *et al.* (1980).[120]
Ovatransferrin	Egg white	IDA Sepharose 6B	Cu	Stepwise elution from 0.05 M Tris-acetate/0.5 M NaCl, pH 8.2 to 0.05M acetate-Tris/0.5 M NaCl, pH 4.0	Al-Mashikhi Nakai (1987)[121]
Phosphoproteins	----	IDA	Fe	Increasing pH gradient from 5.7 to7.2 at constant buffering capacity.	Andersson and Porath (1986)[46]
Plasminogen activator	Human uterine tissue	IDA Sepharose 4B	Zn	Imidazole gradient from 0 to 0.05 M in 20 mM Tris-HCl, pH 7.5, containing 1 M NaCl.	Rijken *et al.* (1979)[122]
Plasminogen activator	Melanoma, Bowers tissue culture	IDA TSK gel chelate 5PW	Zn	Increasing glycine gradient from 0 to 0.1 M in 0.05 M Tris-HCl, pH 8.0, containing 0.5 M NaCl and 0.01% Tween 80.	Matsuo *et al.* (1986)[123]
Phosphoty-trosyl-protein phosphatase	Ehrlich ascites tumor cells	IDA agarose	Zn	Stepwise elution with 20 mM and 60 mM histidine and 50 mM EDTA.	Horlein *et al.* (1982)[124]

Table 3. Continued

PROTEIN	SOURCE	COLUMN	METAL	OPERATING CONDITIONS	REFERENCE
Platelet-derived growth factor (PDGF)	Human platelets	IDA Superose 12	Cu	Column equilibrated with 0.5 M NaCl/ 0.02 M sodium acetate, 1mM imidazole, pH 7.4. Samples applied in equilibrating buffer and eluted from column with a linear gradient of imidazole (1 mM/min) and an inverse gradient of NaCl (10 mM/min) at a flow rate of 0.5 mL/min.	Hammacher *et al.* (1988)[125]
Polyphenol oxidase	Jerusalem artichoke tubers	IDA Sepharose 6B	Cu	Columns equilibrated with 50 mM TRIS-HCl, pH 7.5 containing 0.15 M NaCl, followed by elution with the same buffer containing 10 mM histidine or 20 mM glycine.	Zawistowski *et al.* (1988)[60]
α_1-Proteinase inhibitor	Human plasma	IDA Sepharose	Zn	*(Same as α_2-macroglobulin)*	
Proteins	Human serum	TED Sepharose	Fe, Ni	Tandem columns: Fe(III) -IDA ->Ni(II)-IDA or Ni(II)-IDA->Fe(III)-IDA. Stepwise elution with buffers of different pH.	Porath and Olin (1983)[16]
Proteins	Human serum	TED Sepharose	Al, Ga In, Tl	Tandem columns: Al-IDA->Ga-IDA-> In-IDA->Tl-IDA. Stepwise elution with buffers of different pH.	Porath *et al.* (1983)[13]
Proteins	Human serum Sepharose	IDA, TED	Zn, Cd	Elution with ammonium sulfate or histidine at constant ionic strength, pH 8.0.	Andersson (1984)[126]
Proteins	Human serum mate	Hydroxa- Sepharose 6B	Fe(III)	Stepwise with NaCl in 0.05 M sodium acetate, pH 5.5.	Ramadan and Porath (1985b)[15]

Table 3. Continued

PROTEIN	SOURCE	COLUMN	METAL	OPERATING CONDITIONS	REFERENCE
Proteins	Human serum	IDA Sepharose 6B	Cu, Zn	Tandem columns: Zn (II)-IDA->Cu(II)-IDA. Stepwise elution with >0.5 M NaCl in buffers of different pH.	Porath *et al.* (1975)[3]
Prothrombin activator	*Bothrops neuwiedi* snake venom	IDA Sepharose	Zn	Pooled fractions of protein from DEAE Sephacel column were applied to Zn^{2+}-chelate column in 50 mM Tris-HCl/0.4 M NaCl, pH 8.3. The column was washed with starting buffer followed by stepwise elution with 50 mM Mes /0.4 M NaCl, pH 6.0; and later with buffer containing 50 mM EDTA to remove all bound protein.	Govers-Riemslag *et al.* (1987)[127]
α_1-SH glyco-protein	Human plasma	IDA	Zn NaCl.	Stepwise elution with pH 8.0, 6.5 and 4.5 buffers containing 0.8 M	Lebreton (1977)[128]
9.5 Sα1-glycoprotein	Human serum	CM-Asp agarose	Ca	Column loaded with 20 mM boric acid-NaOH /25 mM $CaCl_2$,pH 9. Column equilibrated with 20 mM imidazole-HCl/ 0.25 M $CaCl_2$, pH 7.0. 40 mL diluted serum applied to column and eluted with 0.1 M EDTA-NaOH, pH 9.0	Mantovaara *et al.* (1990)[34]
Superoxide dismutase and catalase	Human erythrocytes	IDA Sepharose 6B	Cu	Stepwise elution, first with 0.05 M phosphate, pH 7.0, then with 0.05 M Tris-HCl, pH 7.0; gradient elution from 0 to 0.2 M histidine in 0.05 M Tris-HCl, pH 6.0. All buffers in 0.5 M NaCl.	Miyata-Asano *et al.* (1986)[129]
Superoxide dismutase	-------	IDA agarose	Cu	Stepwise elution with buffers of constant ionic strength but different pH.	Lonnerdal and Keen (1982)[43]

Table 3. Continued

PROTEIN	SOURCE	COLUMN	METAL	OPERATING CONDITIONS	REFERENCE
Superoxide dismutase	human blood	IDA Sepharose 6B	Cu	Equilibrated from K-phosphate/0.1 M NaCl, pH 6.4 then 400 mL K-phosphate w/o NaCl ; eluted with 10 mM Na-acetate, pH 5.0 and 20 mM Na-citrate buffer, pH 5.0.	Weselake *et al.* (1986)[130]
Superoxide dismutase	human blood	IDA	Zn	same as Weselake *et al.* (1986)	Strange *et al.* (1988)[131]
α_1-Thiol Proteinase inhibitor	Human plasma	IDA Sepharose	Zn	Column equilibrated with 20 mM borate, pH 8.0, containing 10 μM p-APMSF. After sample introduction, proteins eluted with a linear gradient of same buffer containing 0-50 mM L-histidine.	Ohkubo *et. al.* (1988)[132]
Transferrin	Blood Serum	IDA		*(same as immunoglobulins)*	
Trypsin isoinhibitors	Swine colostrum	IDA Sepharose	Zn	Sample loading at pH 8.0; elution with 50 mM EDTA, pH 7.0.	Yoshimoto and Laskowski (1982)[133]
Tyr(P) phosphatases	rabbit kidneys	IDA agarose	Zn	Column washed at 10 mM HEPES 0.3 M NaCl, 0.01% Brij 35, and latter washed with same buffer but with 1.0 M NaCl added. The pH was changed to pH 6.3 with 50 mM MES/1.0 M NaCl and a decreasing pH gradient to 50 mM MES/1 M NaCl, pH 5.5.	Shriner and Brautigan (1987)[134]