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Immobilized metal-ion affinity systems for recovery and structure–function studies of proteins at molecular, supramolecular, and cellular levels*

Rajasekar R. Prasanna[1] and Mookambeswaran A. Vijayalakshmi[1,2,‡]

[1]Centre for Bioseparation Technology (CBST), VIT University, Vellore 632 014, Tamil Nadu, India; [2]LIMTech.S, Centre de Recherches de Royallieu, B.P. 20 529, 60205, Compiegne Cedex, France

Abstract: Immobilized metal-ion affinity (IMA) adsorption is a collective term that is used to include all kinds of adsorptions where the metal ion serves as the characteristic and most essential part of adsorption center. Of all the IMA techniques, immobilized metal-affinity chromatography (IMAC) has been gaining popularity as the choice of purification technique for proteins. IMAC represents a separation technique that is primarily useful for proteins with natural surface exposed-histidine residues and for recombinant proteins with engineered histidine tag. This review also gives insight into other nonchromatographic applications of IMA adsorption such as immobilized metal-ion affinity gel electrophoresis (IMAGE), immobilized metal-ion affinity capillary electrophoresis (IMACE), and immobilized metal-ion affinity partitioning (IMAP).

Keywords: immobilized metal-affinity chromatography; immobilized metal-ion affinity capillary electrophoresis; immobilized metal-ion affinity gel electrophoresis; immobilized metal-ion affinity partitioning; metal affinity; proteins.

INTRODUCTION

Immobilized metal-affinity chromatography (IMAC), introduced by Porath et al. in 1975 [1], exploits the noncovalent, specific interaction between proteins, nucleic acids, and other biomolecules with immobilized metal ions and the surrounding solute molecules. IMAC uses covalently bound chelating compounds on solid chromatographic supports to entrap metal ions. These metal ions serve as affinity ligands for various proteins, making use of coordinative binding of some amino acid residues exposed on their surface. IMAC is used in cases where rapid purification and substantial purity of the product are necessary. Despite the pseudobiospecificity, the specific binding of protein could be fine-tuned to get excellent selectivity of binding with metal ion. The benefits of IMAC are ligand stability, high protein loading, mild elution conditions, simple regeneration, and low cost [2]. Everson and Parker [3] adapted immobilization of chelating compounds for the separation of metalloproteins. IMAC became popular through the research work of Porath [4–6] and Sulkowski [7–9].

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‡Corresponding author: E-mail: indviji@yahoo.com

IMMOBILIZED METAL-ION AFFINITY (IMA)

IMA adsorption is a collective term that is used to include all kinds of adsorptions where a metal ion, with affinity for analytes in the sample to be fractionated, is fixed to an insoluble matrix. The metal ion is immobilized to a solid support matrix via coordinate bonding with chelating agent. The metal ion serves as the characteristic and most essential part of the adsorption center [4]. Protein adsorption is based on coordinate bond formation between the immobilized metal ion and the electron donor group from the protein surface.

Most commonly used are the transition-metal ions Cu(II), Ni(II), Zn(II), Co(II), Fe(III), which are electron-pair acceptors and can be considered as Lewis acids. Electron-donor atoms (N, S, O) present in the chelating compounds that are immobilized to the chromatographic support are capable of coordinating metal ions and forming metal chelates, which can be bidentate, tridentate, etc., depending on the number of occupied coordination bonds. The remaining metal coordination sites are normally occupied by water molecules and can be exchanged with suitable electron-donor groups from the protein. In addition to the amino terminus, side chains of certain amino acids bind to the chelated metal, due to their electron-donor atoms. Although many residues, such as Glu, Asp (–ve interaction) Tyr, Cys, His, Arg, Lys, and Met (+ve interaction), can interact with the protein, retention in IMAC is based primarily on the availability of histidyl residues under certain pH and ionic conditions. Free cysteines that could also contribute to binding to chelated metal ions are rarely available in the appropriate, reduced state. However, aromatic side chains of Trp, Phe, and Tyr appear to contribute to retention, if they are in the vicinity of accessible histidine residues [8]. Furthermore, the systematic investigation by E. Sulkowski using model homologous series of proteins showed that histidine, a member of Porath's triad (histidine, tryptophan, and cystine) is the preeminent electron donor in the IMAC of proteins.

Adsorption of a protein to the IMAC support is performed at a pH at which imidazole nitrogen in histidyl residues are in the deprotonated state, normally in neutral or slightly basic medium. Usually, buffers with added salt concentration (0.5–1.0 M NaCl) are used to reduce nonspecific electrostatic interactions, while the buffer itself should not coordinatively bind to the chelated metal ion. Elution of the target protein is achieved by protonation, ligand exchange, or extraction of the metal ion by a stronger chelator, such as ethylenediaminetetraacetic acid (EDTA). Protonation by lowering the pH is widely used for elution of the target protein. However, for proteins sensitive to low pH, ligand exchange (e.g., with imidazole) at nearly neutral pH is more favorable. In this case, the IMAC columns must be equilibrated with buffer containing a low concentration of imidazole prior to chromatographic separation to avoid the pH drop caused by the imidazole proton pump effect [9]. Application of a strong chelating agent, such as EDTA, results in elution of the bound proteins, along with the metal, as the metal is ripped off from the column by the strong chelator EDTA. The column must be cleaned and recharged with the metal ion prior to the next separation (Fig. 1).

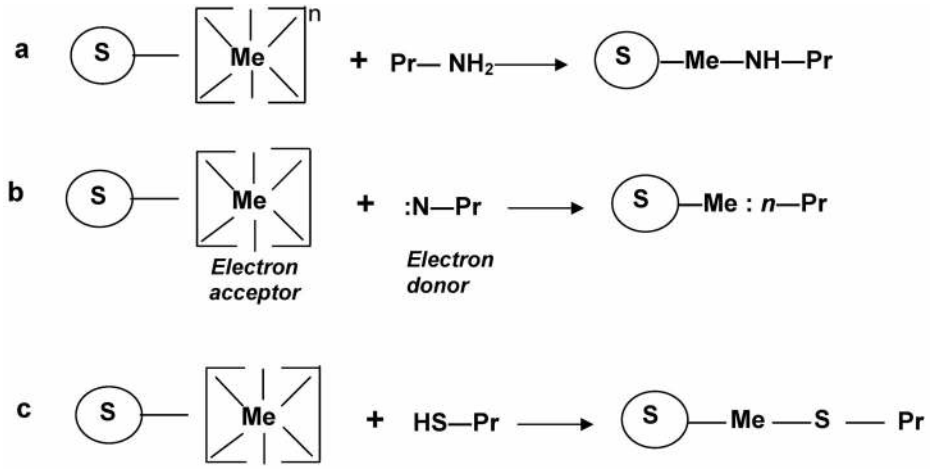

Fig. 1 Interaction mechanisms between an immobilized metal ion and a protein. (a) electrostatic and charge-induced interaction; (b) coordination or electron donor–acceptor interaction; (c) covalent bond formation. S: support; Me: metal; Pr: protein (reprinted with permission from ref. [2], copyright © 1989 Elsevier).

RULES OF INTERACTION BETWEEN HISTIDINE RESIDUES AND IMINODIACETIC ACID (IDA)-Me(II) COLUMNS

1. The absence of histidine residue on protein surface correlates with the lack of retention of that protein on any IDA-Me(II) column (e.g., duck lysozyme).
2. The presence of even a single accessible histidine on a protein surface available for coordination results in that protein binding to IDA-Cu(II) column (e.g., horse heart cytochrome *c*).
3. The presence of two histidine residues available for coordination results in stronger retention on an IDA-Cu(II) column than a single histidine residue.
4. Two histidine residues need to be displayed on the surface to be retained on an IDA-Ni(II) column.
5. The presence of multiple histidine residues results in an increased affinity of binding to all IDA-Me(II) columns.
6. Histidine clusters having -His-(X)*n*-His- in α-helix are required for retention of protein on IDA-Co(II) and IDA-Zn(II) columns (e.g., sperm whale myoglobin).

The above-mentioned results are represented in Table 1.

Table 1 Surface topography of histidine residues and IDA-Me(II) recognition.

Metal ion	Cu(II)	Ni(II)	Zn(II)	Co(II)
His-	+	–	–	–
His-(X)*n*-His-	++	+	–	–
-His-(X)*n*-His- *n* (2,3); α-Helix	+++	+	+	+
-His-X-His, β sheet	+++	+	+	+

The affinity of a protein for a metal chelate depends strongly on the metal ion involved in coordination. In the case of the IDA chelator, the affinities of many retained proteins and their respective retention times are in the following order: Cu(II) > Ni(II) > Zn(II) ≥ Co(II). In contrast to these most com-

monly used metal ions, which have a preference for extra-nitrogen-containing amino acids, metal ions, such as Al(III), Ca(II), Fe(III), Yb(III), prefer oxygen-rich groups of aspartic and glutamic acid as well as phosphate groups of phosphorylated amino acids [10,11]. It may be noted that use of chelated metal ions displaying the highest protein retention does not necessarily translate into the best protein separation, since very high retention could also lead to increased adsorption of impurities [12].

THERMODYNAMIC AND HYDRODYNAMIC PRINCIPLES IN DESIGNING IMA ADSORBENTS

Selection and design of adsorbent matrix is the most crucial component for successful chromatographic separation. The critical factors influencing good separation are binding capacity, binding strength, and selectivity–resolution. These issues are determined by two important parameters:

- Thermodynamic: ligand, ligand strength, binding strength, microenvironment
- Hydrodynamic: pore size, particle size, solute size, flow velocity, mobile-phase physical properties

Thermodynamic design determines the selectivity of an adsorbent, and it can be improved by using new target-specific biomimetic robust affinity ligands. High mass transfer rate and low pressure drop are achieved by optimizing the hydrodynamic design, which determines the productivity and throughput.

Selection of matrix

Classical stationary phases are based on soft-gel matrices, such as cellulose, agarose, and cross-linked dextran. While polysaccharides are biologically compatible and easily activated, they exhibit low mechanical strength and a large pressure drop, which limits their use in large-scale applications. On the other hand, inorganic adsorbents, such as silica, have excellent mechanical properties but exhibit irreversible nonspecific adsorption of proteins and lack stability at alkaline pH conditions.

The support matrix should consist of a molecular network that is permeable to proteins. It should be strongly hydrophilic and chemically inert. For good chromatographic performance, the matrix should be available in the form of small, uniform, rigid, spherical beads. Agarose and sephadex, the first matrices used for IMAC, are useful for many applications but are too compressible for high-performance IMAC. To overcome this limitation, cross-linked agarose was introduced (e.g., sepharose, novarose). Other alternatives include TSK-gel chelate 5PW particles, which consist of a hydrophobic resin core covered by a hydrophilic layer [13], IDA coupled to hydrophilized silica [14,15], membranes consisting of a hydrophylic copolymer, carrying metal chelating groups, the so-called immobilized metal-affinity-membrane adsorbers [IMA-MAs], represent an interesting alternative to conventional matrix, especially in terms of speed and simple scale-up [16]. Affinity membranes operate in convective mode, which can significantly reduce diffusion limitations commonly encountered in column chromatography. As a result, higher throughput and faster processing times are possible in membrane adsorptive affinity systems. Another example of novel IMAC stationary phase is the monolithic supports. Convective Interaction Media (CIM®) disks have the potential to be ideal support systems for IMAC, as they are based on convective flow resulting in flow-unaffected resolution and dynamic binding capacity [17,18]. In addition, due to a very high porosity, pressure drop is negligible [19] and high throughput can be achieved. Monoliths synthesized using three different basic materials have been used for metal-affinity columns so far. Supermacroporous monolithic polyacrylamide cryogel was used for separation of various bacterial cell types [20]. Silica monoliths bearing chelating moieties have been used for separation of very small molecules in order to investigate selectivity toward different metal ions [21]. There are also reports of metal chelate methacrylate-based monoliths.

CIM® disks are methacrylate-based monolithic columns, mainly intended for very fast analyses, in-process control, and laboratory purification. CIM disks perform exceptionally well with large biomolecules. They exhibit low back-pressure, even at very high flow rates (Fig. 2).

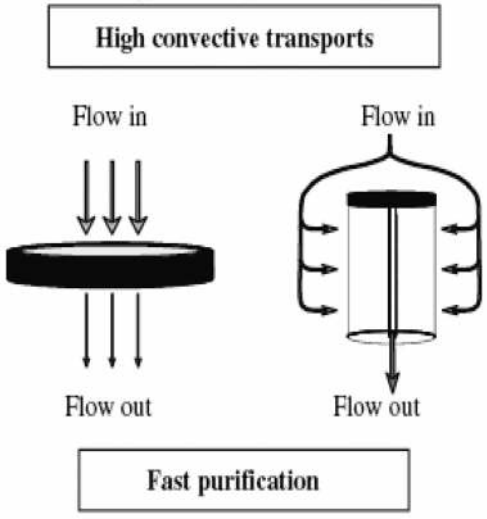

Fig. 2 Axial flow and radial flow in CIM disk and CIM tube monolith.

Metal chelators

The majority of the chelating groups used in IMAC are multidentate chelating compounds providing the strength of the complex formed by the protein, metal ion, and chelating group. These chelating substances are immobilized on the matrix surface via spacer arm (linkage groups), which can vary in length and composition. The final structure formed after the metal ion is chelated must allow some free coordination sites for the adsorption or binding of proteins. The difference in the number of free coordination sites explains why some chelating agents display differences in selectivity toward a target protein. IDA is the standard, most commonly used chelating agent for immobilization of metal ions in IMAC supports [22]. Many other chelators employed in IMAC have been designed with each having their own advantages and limitations. Other extensively used chelators are nitrilotriacetic acid (NTA) developed by Hochuli et al. [23], carboxymethylated aspartic acid (CM-ASP) [12,24] tris(carboxymethyl)-ethylenediamine (TED) [25] and tris(2-aminoethyl) amine (TREN) [16] (Fig. 3).

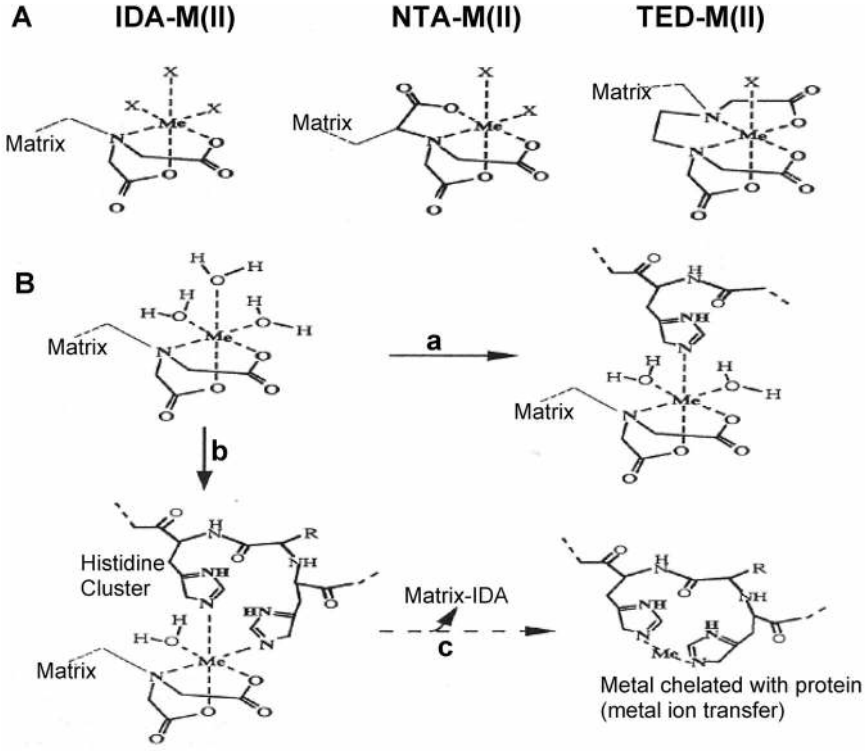

Fig. 3 Structure of adsorption centers in IMA. (A) Structure of the three chelating agents IDA, NTA, and TED. (B) Adsorption of proteins in solution: (a) adsorption of protein with a single accessible surface histidine with IDA-Me(II); (b) adsorption of protein having histidine cluster; (c) phenomenon of metal-ion transfer.

Mobile phase: pH, buffer, and ionic strength

Chelating gels are ion exchangers. Charging the gel with metal ions alters their adsorption properties, although at low ionic strength they still function as ion exchangers, but with altered charge characteristics [4,26,27]. An increase in salt (NaCl) concentration decreases the nonspecific protein adsorption due to ion exchange. To promote protein adsorption to metal and to suppress ionic interactions, the binding buffer should contain high concentration of salts. Sodium chloride (at 0.5–1 M) is used in the IMAC binding buffers to suppress the ionic interaction between sample and matrix, and also between proteins [4]. By increasing the ionic strength of the buffers, the forces between metal ion and water can be reduced. Adsorption of protein on IMAC is carried out at a given pH at which the electron-donor groups on the protein surface are partially unprotonated. The role of pH is complex in the adsorption and elution of proteins as it influences a number of properties such as the nucleophilic behavior of the buffer components, the electron-donor/acceptor properties of the solutes and the metal stability. The pH range between 6 and 8 favors the specific interaction with the histidine residues on the surface of protein. At more alkaline pH conditions, coordinations with amino functional groups are favored, thus decreasing the selectivity [24].

While metal-binding solutes may affect the properties of IMA adsorption sites, other solutes are mostly inert [4]. These solutes include nonionic surfactants, urea, ethylene glycol, and dimethyl sulfoxide (DMSO). These substances are often used to solubilize membrane proteins and other hydrophobic proteins.

PROTEIN PURIFICATION USING IMAC

Native surface histidine (without using affinity tag)

Numerous proteins contain native histidine residues in their amino acid sequence. Histidine residues in proteins are relatively less, only about 2 % of the amino acid content of proteins. Moreover, histidine is mildly hydrophobic, and only a few of them are located on the protein surface [12], thus only a low number of naturally occurring proteins would present some metal affinity and hence be potentially suitable for purification by IMAC. Many proteins having histidine residues in their confirmation have been purified by IMAC without any addition of histidine tags. For use in IMAC, protein-surface histidine residues must also be accessible to the metal ions and their bulky chelating compounds. However, the microenvironment of the binding residue, cooperation between neighboring amino acid side groups, and local conformations play important roles in protein retention. In this way, IMAC can serve as a sensitive tool for revealing histidine topography on the surface of protein. Some interesting examples of using IMAC for purification of proteins with exposed, native surface histidine residues include human serum proteins [1,28], interferon [7], lactoferrin and myoglobin [29], tissue plasminogen activator [30], antibodies [31,32], and yeast alcohol dehydrogenase [33]. Purification of IgG from human serum has been carried out using metal chelate methacrylate monolith (CIM disk, unpublished) (Fig. 4). Anguenot et al. [34] have described the purification of tomato sucrose synthase isoforms by Fe(III)-IMAC. A positive correlation is found between the number of accessible histidines and the strength of binding [6]. Interesting IMAC behavior is exhibited by natural cytochrome *c* from different species, which differ in their histidine content [6]. Similarly, evolutionary variants of the lysozymes show varied affinities for IMAC adsorbents due to differences in the surface topography of histidines [35]. IMAC was employed for the purification of three iso-carboxypeptidases, referred to as carboxypeptideases I, II, and III [36]. IMAC not only helped in separation of the three carboxipeptidases (Fig. 5), it was also found that the results were highly reproducible with high activity yields; sometimes yields exceeding 100 % were achieved (Table 2). IMAC has been used for recovery of both natural and recombinant proteins from plants such as tobacco and potato. Boulis et al. used IMAC to isolate various forms of sporamin, the storage protein present in sweet potatoes, corresponding to different compartment targeting within the plant cell. These different sporamin constructs were expressed in transgenic tobacco plants and were purified to >95 % purity in a single step. The data was suggestive of the differences in the protein expressed in the different cellular compartments (Fig. 6) [37].

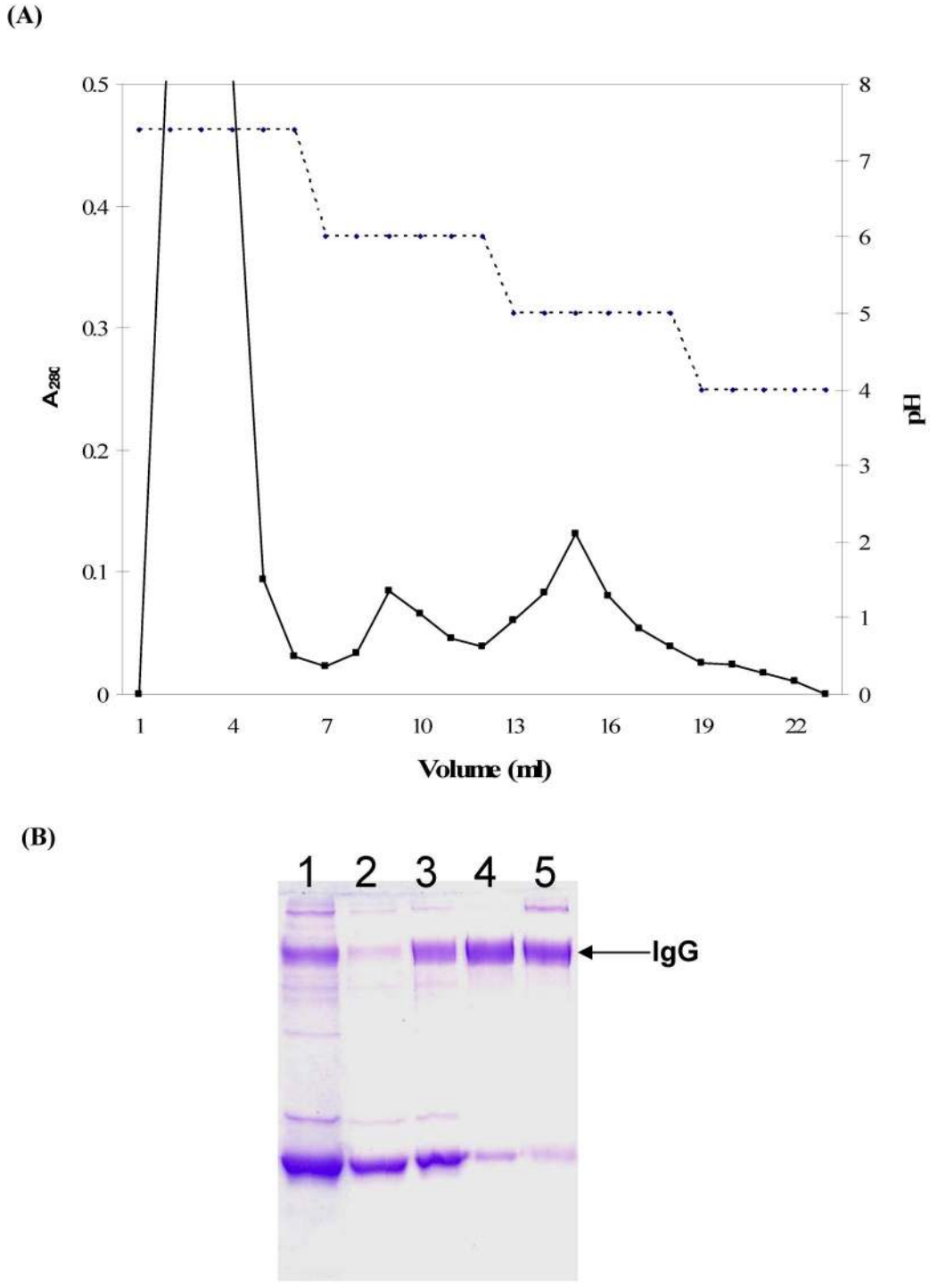

Fig. 4 (A) Chromatogram of total human serum (150 µl) on CIM-IDA-Ni(II) disk (12 × 3 mm) monolithic column. Elution was done using decreasing pH step gradient (pH 6.0–4.0). Buffer composition: 25 mM MMA + 0.5 M NaCl for all pH indicated. Flow rate: 3 ml/min. (B) Fig 6: SDS-PAGE under non-reducing conditions of peak fractions; lanes: 1- load; 2- pH 7.4 wash; 3- pH 6.0; 4- pH 5.0, and 5- pH 4.0.

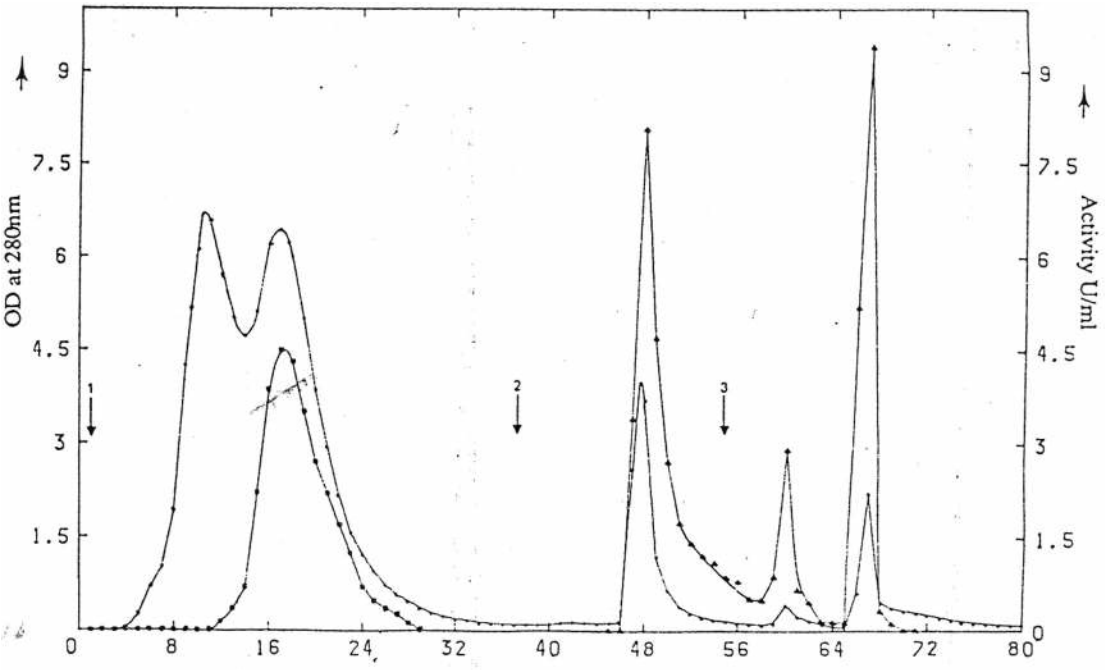

Fig. 5 Chromatography of the three carboxypeptidases and the acid protease by tandem desalting-metal chelate affinity chromatography on sephadex G-50-Cu(II)-IDA-sepharose 6B (reprinted with permission from ref. [36] copyright © 1986 Elsevier).

Table 2 Purification of serine carboxypeptidases isoforms (I, II, and III) by tandem desalting-metal chelate affinity chromatography on sephadex G-50-Cu(II)-IDA-sepharose 6B.

Extract		Protein (mg)	Activity (U)	Specific activity (U/mg)	Purification factor	Activity yield (%)
Crude extract		6930	660	0.095	1	100
0–90 % $(NH_4)_2SO_4$ extract		3132	662	0.211	2.2	100
After tandem	Peak I	173	483	2.79	29	73
desalting affinity	Peak II	20.5	99	4.54	47	15
chromatography	Peak III	80	354	4.42	46	54
Total yield of carboxypeptidase isoforms enzyme activity (I + II + III)						142

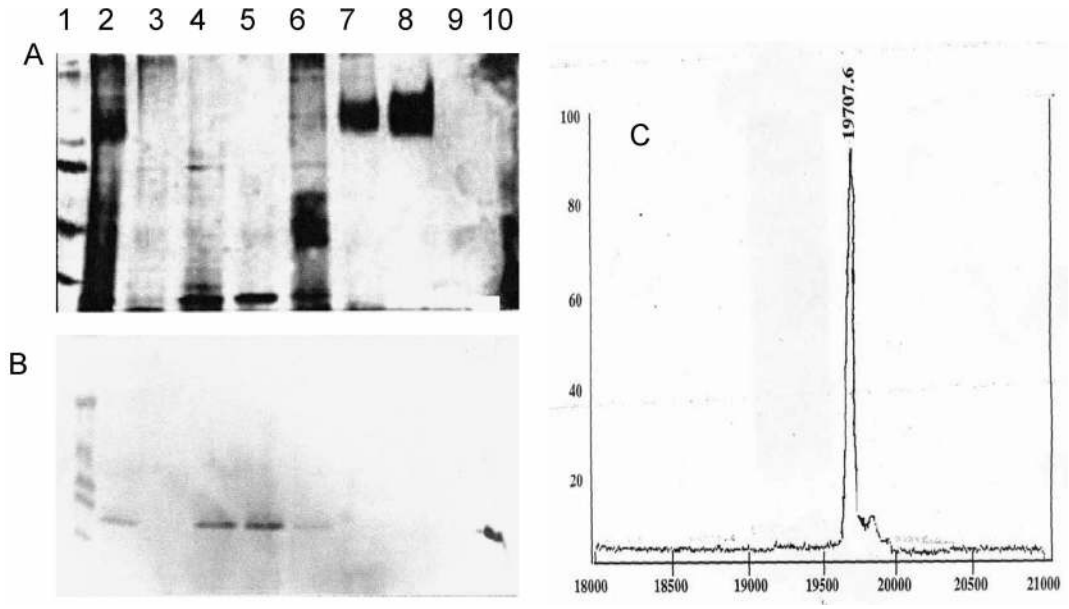

Fig. 6 (A) SDS PAGE and (B) western blot analysis of Δpro Sporamin samples obtained after IMAC. Lanes 1–3 show standard protein marker, injected sample, and nonretained sample, respectively, Lanes 4–9 show 5, 10, 20, 50, 100 mM and EDTA elution fractions, respectively, and lane 10 shows standard sporamin from sweet potato (reprinted with permission from ref. [37], copyright © 2003 Elsevier). (C) Mass calculation of Δpro Sporamin after MS spectra deconvolution.

Metal-affinity tags (histidine tagging)

Application of metal-affinity tags have proven highly advantageous to protein purification. Genetically engineered affinity tags are attached to amino or carboxy terminals of the recombinant proteins to significantly improve the resolution and also to speed up the purification process. The first histidine-rich fusions were made on the basis of the high affinity of certain natural proteins containing histidine residues near the N-terminus. For instance, an octapeptide derived from angiotensine I was fused to the N-terminus of the TEM-β-lactamase and expressed in *E. coli*. One-step purification of the recombinant protein from the resolubilized inclusion body material was achieved on IDA-Zn(II) [38]. In the past, numerous histidine tags were employed, from very short ones, e.g., HisTrp, utilized for isolation of sulfitolized proinsulin [39] to rather long extensions, containing up to eight repeats of the peptide Ala-His-Gly-His-Arg-Pro, attached to various proteins [40]. However, today by far the most widely used histidine tags consist of 6 consecutive histidine residues. After the appearance of Hochuli's papers [23,41], describing a new chelating matrix Ni-NTA and fusions with short peptides, containing 2–6 neighboring histidines, these hexa-histidine tags have become very popular. An ideal affinity tag should enable effective but not too strong a binding, and allow elution of the desired protein under mild, non-destructive conditions. In the case of recombinant proteins expressed in *E. coli*, many host proteins strongly adhere to the IMAC adsorbents, especially when charged with Cu(II) or Ni(II) ions, and are eluted with the target proteins. Histidine tags seem to be compatible with most of the expression systems used today. Thus, His-tagged recombinant proteins can be successfully produced in prokaryotic and eukaryotic organisms, either as intra- or extracellular proteins [42]. Many authors have reported improvements in protein stability, and protein expression levels following insertion of a metal affinity tag [43,44]. Recently, metal-chelate methacrylate monolith has been used for purification of tumor necrosis factor-α (TNF-α) analog LK-801 and green fluorescence protein with 6 histidine tag (GFP-6His)

[45]. IMAC has been used in combination with mass spectrometric analysis in the field of proteomics [46–48].

APPLICATIONS OF IMMOBILIZED METAL-ION AFFINITY BEYOND IMAC

In the last decade, some nonchromatographic techniques have appeared, such as metal-affinity precipitation of proteins with attached histidine-affinity tails through formation of a metal chelate complex, with new Cu(II)-loaded copolymers [49]. Immobilized metal-ion affinity partitioning (IMAP) is another related technique for preparative extraction of proteins based on different content and distribution of histidine residues. Aqueous two-phase systems containing metal-IDA-poly(ethylene glycol) (PEG) in PEG-dextran and PEG-salt systems have been used not only for extracting proteins [50] but also for affinity partitioning of human blood cells such as erythrocytes [51] or lymphocytes [52]. Immobilized metal-ion affinity gel electrophoresis (IMAGE) on, e.g., PEG-IDA-Cu(II) in agarose gels [53], and immobilized metal-ion affinity capillary electrophoresis (IMACE) with soluble polymer-supported ligands [54] are examples of further applications of the same basic principle, although none of these techniques has become as popular as IMAC itself. Other application of metal-affinity-based protein adsorption is in biosensor technique. Zhu et al. [55] immobilized hexahistidine-tagged proteins on nickel-coated glass slides, which resulted in superior-quality protein chip. Metal-affinity-based immobilization of proteins is also applied in studies on the structure and function of proteins present on the surface of cell membranes. Nickel-charged metal-chelating lipid membranes were used by Celia et al. [56] to capture his tagged major histocompatibility complex (MHC) molecules for subsequent investigation of their binding to T-cell receptors.

Metal-chelate probing of active-site histidines

Berna et al. [57] employed IMAC to study the conformational changes of native bovine α-chymotrypsin during its activation process from chymotrypsinogen to its different active subspecies. The involvement of His-40 of chymotrypsin in IDA-Cu(II) recognition was documented. The differential retention of the subspecies onto novarose-IDA-Cu(II) was explained by a modification of the microenvironment of the His-40 during the activation pathway from zymogen to α-chymotrypsin. The active-site His-57, although well exposed on the protein surface (more than His-40), did not seem to engage in the coordination bond formation with IDA-Cu(II), apparently due to the involvement in strong hydrogen bonding and hence the rigidity of this residue. The IMA probe, IDA-M(II), offered insights into the accessibility and the chemical bonding potential of a histidine residue. Mapping of surface-accessible histidine of different serine proteases by Boden et al. [58] was done using IMA interaction. The (IMA) interaction of different serine proteases, namely, porcine and bovine trypsins and BPN' and Carlsberg subtilisins, was studied on sepharose-IDA-Cu(II). Both trypsins were resolved into their different subspecies, whereas the subtilisins appeared as only one species. The use of diethyl pyrocarbonate-modified enzymes demonstrated the contribution of histidine(s) as the sole interacting site(s). The use of different peptidic and chemical inhibitors complexed to the enzymes confirmed the contribution of histidine(s) as the interacting site(s) and further resulted in different chromatographic patterns for the free and complexed serine proteases. It was also possible to use IMAC combined with immunodetection for deducing the structure of the anti-diethylenetriaminepentaacetic acid (DTPA)-indium antibody used for tumor imaging [59].

Immobilized metal-ion affinity gel electrophoresis

IMAGE is a technique that uses the two concepts, namely, affinity electrophoresis (AE) and IMAC. AE merges the principle of electrophoretic separation and the interaction of biomolecules with a ligand immobilized within the electrophoresis gel support. The principle of IMA in AE was exploited by

Goubran-Botros and Vijayalakshmi in early 1990s [60]. The IMAGE method uses transition-metal chelates, e.g., iminodiacetic acid-Me(II) [IDA-Me(II)], as ligands that are incorporated in electrophoresis gels. In the beginning, the ligand was immobilized onto agarose beads and incorporated in an agarose gel [60]. Later, ligands coupled to soluble polymers [53] and monomeric polymerizable derivatives of the ligands that could be copolymerized into polyacrylamide gel [61].

The dissociation (K_d) constant of the complex (protein + immobilized ligand) is calculated using the following equation [62,63]:

$$1/(R_0 - r) = 1/(R_0 - R_c)\,[1 + (K_d/c)] \quad (1)$$

where R_0 and r are the relative migration distances of protein in the absence and the presence of an affinity ligand, respectively, R_c is the migration distance for the protein ligand complex, and c is the concentration of the affinity ligand.

Baek et al. [64] employed IMAGE to study the affinities of cytochrome *c* from different species for IDA-Cu(II) (Fig. 7), using four different systems (Table 3).

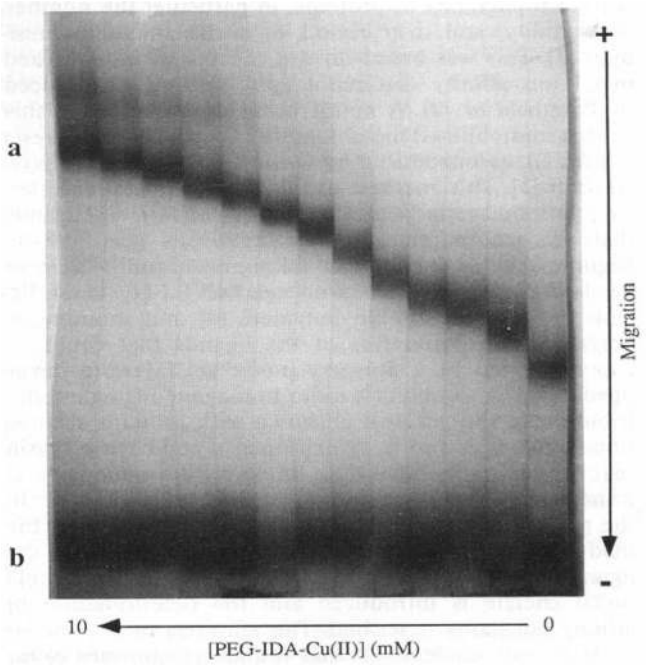

Fig. 7 IMAGE of cytochrome *c* from (a) *Candida krusei* and (b) tuna heart in polyacrylamide/PEG-IDA-Cu(II) system. Ligand concentrations: 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 7.5, 10 mM (reprinted with permission from ref. [64], copyright © 1996, Wiley-VCH).

Table 3 Dissociation constants (in mM) of cytochrome *c* binding to IDA-Cu(II) with four different affinity gel systems.

	Agarose	Polyacrylamide		
Cytochrome *c*	PEG-IDA-Cu(II)	PEG-IDA-Cu(II)	ADA-IDA-Cu(II)	VBIDA-Cu(II)
Candida krusei	8.9 ± 0.35	4.5 ± 0.2	5.5 ± 0.3	ND[a]
Tuna heart	BT[b]	BT	BT	ND

[a]ND, not determined, adsorption isotherm not of Langmuir type.
[b]BT, values below threshold, affinity very low.

It was demonstrated with a model protein RNase A, the possibilities of discriminating the denatured and renatured species in a mixture. Distinct K_d values for denatured and renatured species and the percentage of renaturation could be estimated (Table 4).

Table 4 Follow-up of unfolding and refolding of RNase A using IMAGE.

RNase A	Activity	Number of species	K_d (mM) (average value)
Native RNase A[1]	100	1	22.0
Denatured RNase A[2] pH 8.0, 90 °C, 3 min	46.7	2 (50 %) (50 %)	22.0 10.4
Denatured RNase A[2] pH 8.0, 90 °C, 3 min, 10 μm $CuCl_2$	73.4	1	22.0
Denatured RNase A[2] pH 8.0, 90 °C, 6 min	17.7	2 (20 %) (80 %)	24.0 10.4
Denatured RNase A[2] pH 8.0, 90 °C, 6 min, 10 μm $CuCl_2$	46.6	2 (60 %) (40 %)	22.0 10.4
Denatured RNase A[3] pH 8.0, 90 °C, 60 min	0.0	1	10.7

[1]Denotes a total of 20 experiments.
[2]Denotes a total of 10 experiments at each condition.
[3]Denotes a total of 6 experiments under the specified condition.

As seen in Table 4, it was possible to follow the denaturation and protection from denaturation by using ionizable copper chloride ($CuCl_2$) salt in micromolar concentration in the solution. Thus, the native species showed a higher average K_d value (22.4 mM) reflecting lower affinity due to only 2 of the 4 histidine residues being exposed on the surface. When total denaturation happened (90 °C, 60 min), there was only one band of average K_d value of 10.7 mM, denoting higher affinity due to all the four histidine residues being exposed. In the partially denatured systems (90 °C, 3 min and 90 °C, 6 min), unprotected enzyme showed the equal or higher percentage of denatured species showing an average K_d value of 10.4 mM, while the enzyme protected by added $CuCl_2$ in the solution clearly revealed a higher percentage of native species with an average K_d value of 22.4 mM. IMAGE technique was also applied to study the change of the surface histidines topography of RNase A when chemically glycosylated on exposed carboxylic groups with glucosamine using carbodiimide as cross-linker, under mild conditions [65]. The Zn(II) binding region in growth hormone was studied using IMAGE by Anisimove et al. [66]. Using IMAGE structural study of natural and chemically induced oligomeric ribonucleases was done to study the relationship between surface histidine topography in oligomeric forms of these ribonucleases and their catalytic properties [67].

Immobilized metal-ion affinity capillary electrophoresis

IMACE is a technique that combines the principles of high-performance capillary electrophoresis (HPCE) with IMAC. IMACE is useful as an analytical tool for the study of protein interaction with immobilized metal-ion chelates, hence it can be used for studying protein surface topography and structure–function relationship. Haupt et al. [54] developed a novel affinity capillary electrophoresis method (IMACE) that employs small ligand bound to a replaceable soluble polymer matrix in the dynamic coating mode rather than employing the affinity ligands to the capillary wall [68,69]. Metal chelate IDA-

Cu(II) coupled to PEG (matrix) was used as a model system. In IMACE, separation is based on the different charge to mass characteristics for the protein ligand complexes compared to the unbound protein, which results in different migration times in HPCE. Using this system, interactions of different model proteins, namely, RNases A and B, cytochromes *c*, chymotrypsin, and kallikrien were investigated. The affinity of a protein analyzed in IMACE is expressed by the change in its migration according to the time spent in the complexed (protein-ligand complex) and free form (unbound protein) during electrophoresis. The dissociation constant (K_d) is calculated using the following formula:

$$t_N = t_{Nmax}\, C/K_d + c \tag{2}$$

To eliminate the influence of electroosmotic flow (EOF), all protein migration times (t) were divided by the migration time of EOF marker (t_{EOF}), yielding normalized migration time (t_N). Differential migration time $t_N = t_N - t_{N0}$ were obtained by subtracting the normalized migration time in the absence of the ligand (t_{N0}) from normalized migration time; c is the concentration of the ligand.

The dissociation constant (K_d) obtained from the equation could be correlated to the qualitative and quantitative aspects of the accessible histidine residues in the protein. This in turn can give information about the structural differences resulting in different functional properties. Structural study and the structure–function relationship of natural and chemical induced oligomeric ribonuclease were also studied using IMACE [66]. Effects of chemical glycosylation with glucosamine of bovine α-chymotrypsin on its catalytic and structural properties, particularly their modification of the affinity for the transition-metal chelate, IDA-Cu(II) was studied by Jiang et al. [70] using IMACE. The general rules for protein interaction with matrix-bound metal chelates established for IMAC are maintained in IMACE, and it also allowed for using similar conditions as in IMAC, especially regarding high salt concentrations, usually employed with IMAC.

Immobilized metal-ion affinity partitioning

Aqueous two-phase partitioning of proteins and cell organelles was introduced and developed by Albertsson [71]. IMAP was designed for the separation of different cell populations in aqueous two-phase systems [72]. IMAP is an extension of IMAC. In IMAP, the partitioning is influenced not only by the relationship between cell surface and physical properties of the phases, but more selectively by the affinity of immobilized metal ion to the histidine residues of proteins exposed on the membrane surface. Aqueous two-phase systems containing metal-IDA-PEG in PEG-dextran and PEG-salt systems have been used not only for extracting proteins but also for affinity partitioning of human blood cells such as erythrocytes or lymphocytes. This system has been successfully used for cell separation with the advantage of a shear-free system [51]. Healthy and pathological cells have been studied by IMAP, and it was possible to differentiate malarial red blood cells (RBCs), cancerous fibroblasts, and lymphoma cells from their healthy counterparts using PEG-IDA-Ni(II) as affinity ligand [73] with excellent cell viability and with little modification of cell surface. The effect of introducing an immobilized metal-ion ligand in the lower phase of the PEG/dextran system was studied on the erythrocytes and lymphocytes partition [74], and it was clearly demonstrated that the partition in IMAP systems is correlated with the affinity between the cell surface and the ligand as the cells were attracted to the ligand-containing phase. The potential of IMAP was evaluated using dextran-PEG + PEG-IDA-M(II) systems to separate mononuclear cells from cord blood, and it was shown that the cell population where the surface antigen did not have any histidine residues (e.g., CD_{34}) does not partition in a selective manner in the IMAP system [52].

CONCLUSIONS

The understanding of molecular mechanisms that govern protein adsorption to metal chelate complexes has increased significantly since the time it was introduced by Porath in 1975, and almost all of

Poraths's and Sulkowski's [1,8] vision many years ago has come true. An outstanding result in IMAC has been the finding that the protein metal-chelate interaction is not only useful for purification purposes but also can be used to probe protein molecular surface at a remarkably high resolution. This article reviews the rapidly increasing use of IMA systems for protein purification as well as for protein characterization. IMAC has become the popular technique for purification of proteins in recent times especially after the emergence of engineered histidine affinity tags. This review also highlights the central role of protein-metal-affinity systems in a wide variety of applications including protein extraction using two-phase IMAP and IMA concept coupled to other nonchromatographic techniques such as IMAGE and IMACE. A very striking feature is the positive cell sorting of human cells. This is based on the trans-membrane proteins modification on the cell surface due to pathological conditions such as cancer, malaria, etc., resulting in the non-accessibility by occlusion or other phenomenon of the putative histidine residue.

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