IMMOBILIZED-METAL AFFINITY CHROMATOGRAPHY (IMAC): A REVIEW

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Contents

Abstract

This article reviews the development of immobilized-metal affinity chromatography (IMAC) and describes its most important applications. We provide an overview on the use of IMAC in protein fractionation and proteomics, in protein immobilization and detection, and on some special applications such as purification of immunoglobulins and the Chelex method. The most relevant application—purification of histidine-tagged recombinant proteins—will be reviewed

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in greater detail with focus of state-of-the-art materials, methods, and protocols, and the limitations of IMAC and recent advances to improve the technology and the methods will be described.

1. OVERVIEW ON IMAC LIGANDS AND IMMOBILIZED IONS

The concept of immobilized–metal affinity chromatography (IMAC) has first been formulated and its feasibility shown by Porath *et al.* (1975). It was based on the known affinity of transition metal ions such as Zn^{2+}, Cu^{2+}, Ni^{2+}, and Co^{2+} to histidine and cysteine in aqueous solutions (Hearon, 1948) and extended to the idea to use metal ions ''strongly fixed'' to a support to fractionate protein solutions. As the chelating ligand used to fix the metal to agarose Porath used iminodiacetic acid (IDA) which is still in use today in many commercial IMAC resins. Already in 1975, Porath speculated that the affinity of immobilized metals to histidine-containing proteins might not be the only application for IMAC—and he was right after all.

In the years following Porath's publications, the new IMAC technology was successfully evaluated by purification of a variety of different proteins and peptides summarized in a first IMAC review by Sulkowski (1985). What started as a crude fractionation of serum soon became what today is the most widely used affinity chromatography technique (Biocompare, 2006; Derewenda, 2004), if not chromatography technique in general. This development was accelerated by the fast maturation of recombinant techniques and modern molecular biology in the late 1970s and by the invention of an improved chelating ligand, nitrilotriacetic acid (NTA) in the 1980s (Hochuli *et al.*, 1987). In the meantime, the purification of recombinant proteins genetically modified on the DNA template level in order to generate oligohistidine extended (His-tagged) polypeptides using NTA-based supports (Hochuli *et al.*, 1988) represents the most important application of IMAC. The principal mechanism of the interaction of a His–tagged protein to an immobilized metal ion is presented in Fig. 27.1.

A possible model is that of an interaction of a metal ion with histidine residues n and n_{+2} of a His tag. This is confirmed by the fact that IMAC ligands can bind to His tags consisting of consecutive histidine residues as well as to alternating tags. As at least consecutive His tags are usually unstructured and thus flexible, other interaction patterns such as $n{:}n_{+1}$, $n{:}n_{+3}$, $n{:}n_{+4}$, and so on could be imagined as well, even within a single molecule (Jacob Piehler, personal communication).

The NTA ligand coordinates the Ni^{2+} with four valencies (tetradentate, coordination number 4) highlighted spherically in Fig. 27.1B, and two valencies are available for interaction with imidazole rings of histidine residues.
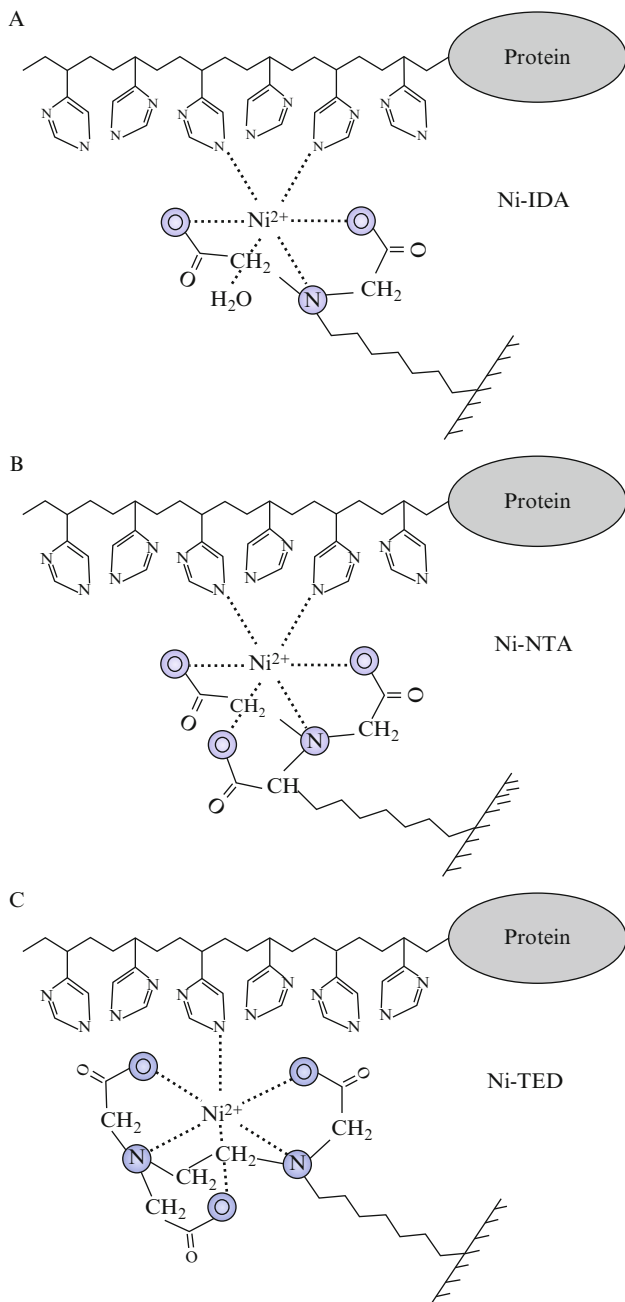
Figure 27.1 Model of the interaction between residues in the His tag and the metal ion in tri– (IDA), tetra– (NTA), and pentadentate IMAC ligands (TED).

This ratio has turned out to be most effective for purification of His-tagged proteins. Another tetradentate ligand is carboxymethyl aspartate (CM–Asp; Chaga *et al.*, 1999), commercially available as cobalt-charged Talon resin. In contrast to tetradentate ligands, IDA coordinates a divalent ion with three valencies (tridentate, coordination number 3, Fig. 27.1A) leaving three valencies free for imidazole ring interaction while it is unclear whether the third is sterically able to participate in the interaction. The coordination number seems to play an important role regarding the quality of the purified protein fraction. While protein recovery is usually similar between IDA- and NTA-based chromatography (Fig. 27.2D), a higher leaching of metal ions from IDA ligands compared to NTA is observed in general (Hochuli, 1989) and even increased under reducing conditions (Fig. 27.2C). Although the metal content in the elution fractions (E in Fig. 27.2C) is higher but still within the same order of magnitude, significantly more Ni^{2+} ions leach from the IDA resin in the equilibration and wash steps (W in Fig. 27.2C). Besides considerable metal leaching, purification of His-tagged proteins using an IDA matrix frequently results in lower purity compared to NTA-based purification (Fig. 27.2A and D).
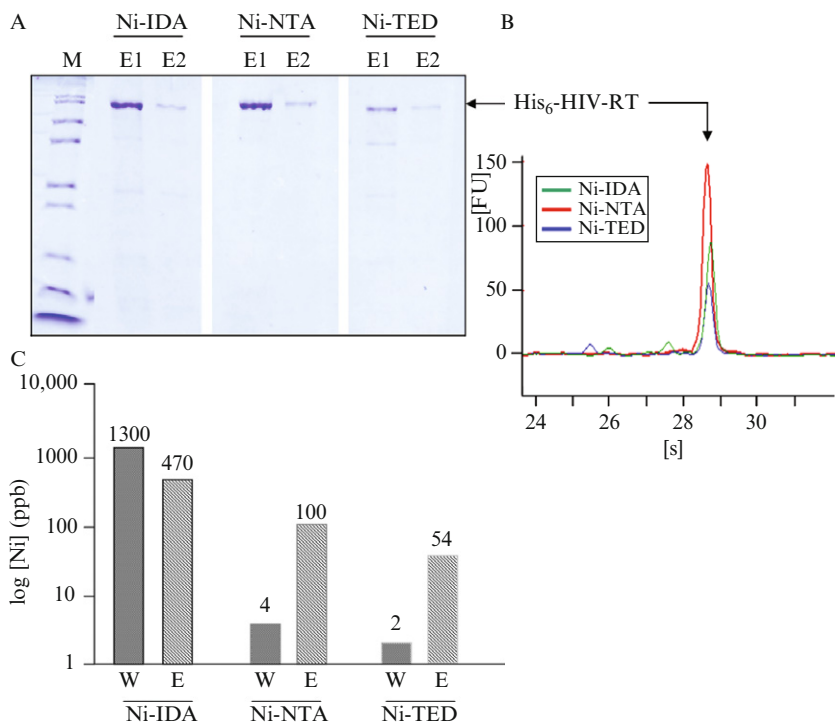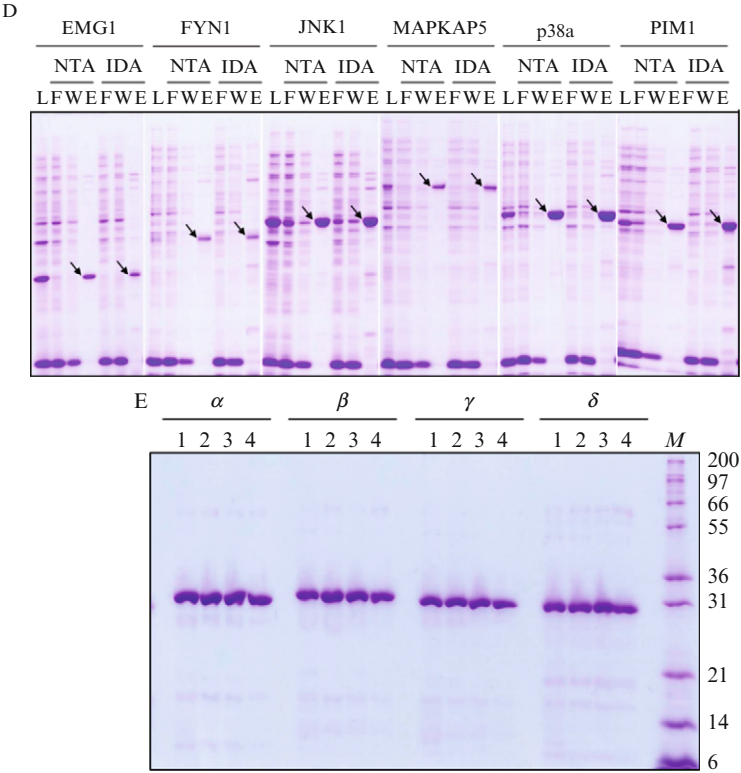

Figure 27.2   (Continued)

Figure 27.2 Purification of His-tagged proteins with NTA, IDA, and TED. (A) H$_6$-HIV-RT was expressed in *E. coli* BL21(DE3) and purified via Ni-IMAC in the presence of 1 m*M* DTT under standard conditions (IDA, NTA; see Section 2.10 for standard conditions) or according to the manufacturer's recommendations (TED). Corresponding aliquots of the IMAC elution fractions (E1, E2) were analyzed by SDS–PAGE and Coomassie staining. (B) Bioanalyzer 2100 lab-on-a-chip analysis of pooled elution fractions. The peaks from the electropherograms corresponding to H$_6$-HIV-RT were overlayed. Peak areas directly correlate to the protein amount in the respective pool fraction. (C) Determination of the nickel content in wash (W) and pooled elution fractions (E) of the H$_6$-HIV-RT purifications described in (A) and (B). Nickel was measured by ICP-MS (intercoupled plasma mass spectrometry) at Wessling Laboratories, Bochum, Germany, and values are provided in μg/l (parts per billion, ppb). (D) QIAgene constructs carrying optimized human genes were used for expression of the indicated proteins in *E. coli* BL21(DE3) LB cultures. Cleared lysates were divided for purification of His-tagged proteins via Ni-NTA (NTA) and Ni-IDA (IDA), respectively. Fractions were analyzed by SDS–PAGE and Coomassie staining as follows: L, cleared lysate; F, IMAC flow-through fraction; W, wash fraction; E, peak elution fraction. (E) Fragments of H$_6$-tagged proteins named α, β, γ, and δ expressed in *E. coli* were purified under standard conditions using NTA and Cm-Asp tetradentate ligands loaded with Ni^{2+} or Co^{2+} as follows: 1, Ni-NTA; 2, Co-NTA; 3, Co-CmAsp; 4, Ni-CmAsp. Aliquots from the peak elution fractions (2 μl each) were analyzed by SDS–PAGE and Coomassie staining.

The reason for the lower purity may be that leaching of metal from the tridentate ligand generates charged groups which could act as a cation exchanger and bind positively charged groups on the surface of proteins. The lowest metal leaching is obtained if a pentadentate ligand is used (Fig. 27.2C) which coordinates the ions extremely tightly, and such resins may represent a valid alternative if low metal ion leaching into the protein preparation is very important. However, in this case only one coordination site remains for His tag binding and recovery of His–tagged protein is usually considerably lower than with IDA or NTA (Fig. 27.2B).

The choice of the metal ion immobilized on the IMAC ligand depends on the application. While trivalent cations such as Al^{3+}, Ga^{3+}, and Fe^{3+} (Andersson and Porath, 1986; Muszynska *et al.*, 1986; Posewitz and Tempst, 1999) or tetravalent Zr^{4+} (Zhou *et al.*, 2006) are preferred for capture of phosphoproteins and phosphopeptides, divalent Cu^{2+}, Ni^{2+}, Zn^{2+}, and Co^{2+} ions are used for purification of His–tagged proteins. Combinations of a tetradentate ligand that ensures strong immobilization, and a metal ion that leaves two coordination sites free for interaction with biopolymers (Ni^{2+}, Co^{2+}) has gained most acceptance and leads to similar recovery and purity of eluted protein. As a typical result using such combinations, Fig. 27.2E shows the purification of several protein fragments derived from different genes that have been expressed and purified as His–tagged proteins by Ni^{2+} and Co^{2+} immobilized on NTA and Cm–Asp tetradentate ligands.

2. IMAC APPLICATIONS

Initially developed for purification of native proteins with an intrinsic affinity to metal ions (Porath *et al.*, 1975), IMAC has turned out to be a technology with a very broad portfolio of applications. On the chromatographic purification side, the range of proteins was expanded from the primary metalloproteins to antibodies, phosphorylated proteins, and recombinant His–tagged proteins. IMAC is being used in proteomics approaches where fractions of the cellular protein pool are enriched and analyzed differentially (phosphoproteome, metalloproteome) by mass spectrometrical techniques; here, IMAC formats can be traditionally bead based or the ligand can be used on functionalized surfaces such as SELDI (surface-enhanced laser desorption/ionization) chips. Other chip-based applications include surface plasmon resonance (SPR) and allow the immobilization of His–tagged proteins for quantitative functional and kinetic investigations. In addition, the IMAC principle has been used as an inhibitor depletion step prior to PCR amplification of nucleic acids from complex samples such as blood in a technology called Chelex (Walsh *et al.*, 1991). The most relevant of the

numerous IMAC applications will be discussed briefly in the following sections. The main application of IMAC—the purification of His-tagged proteins—will then be discussed in detail. This section will include problems and limitations of IMAC, solutions and recent advances in this field.

2.1. Detection and immobilization

In efforts to make use of the specificity and high affinity of His-tagged proteins to immobilized metal ions, IMAC ligands have been employed for applications such as protein:protein interactions where proteins need to be stably immobilized on surfaces. Two applications—ELISA as a diagnostic tool and chip-based technologies for functional investigations—shall be briefly described.

Ni-NTA ligands attached to surfaces of microtiter plates are used to immobilize His-tagged antigens in its soluble and structurally intact form for serological studies. The directed immobilization via the His tag can be an advantage to standard ELISA where proteins are randomly adsorbed to plastic surfaces which destroys the protein structure and hides part of the protein surface and possible antibody-binding sites. In contrast, IMAC-based ELISA allows the screening of conformation-dependent monoclonal antibodies (Padan *et al.*, 1998) and immunosorbent assays with increased sensitivity (Jin *et al.*, 2004).

Immobilization of His-tagged proteins on chip surfaces for interaction studies with other molecules, for example by SPR, is a widely used protein characterization method. The factor ''stability'' is important to reduce ''bleeding'' of the immobilized molecule, and therefore, due to its favorable binding features described above the NTA ligand is frequently used for immobilization applications (Knecht *et al.*, 2009; Nieba *et al.*, 1997). However, interaction of biotinylated proteins to supports coated with streptavidin is still considerably stronger. A significant improvement in the stability of functional immobilization of His-tagged proteins on glass–type surfaces even at low concentration was achieved by the concept of multivalent chelator heads where a single ligand molecule carries three NTA moieties (tris-NTA; Lata and Piehler, 2005; Zhaohua *et al.*, 2006). This development represents a valid alternative to streptavidin/biotin-based protein immobilization and allows the use of His-tagged proteins without the need for biotin-labeling of proteins following purification. We have synthesized the tris-NTA ligand and coupled it to magnetic agarose beads in order to test whether this approach may be transferred to purification of His-tagged proteins and allow even more specific single-step recovery from complex samples. Initial data (Fig. 27.3) indicate that this may indeed be the case; AKT1 kinase separated from *Spodoptera frugiperda*-derived cell-free lysate reactions could be purified with both bead types and purity was slightly
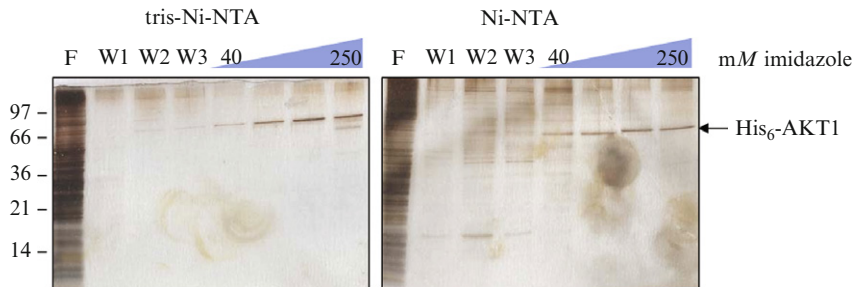
Figure 27.3 Purification of His$_6$–tagged AKT1 kinase using tris–Ni–NTA and Ni–NTA magnetic beads. Human C-terminally His$_6$–tagged AKT1 was expressed cell-free in an insect cell-based lysate (EasyXpress Insect II system) in a 100 μl reaction volume using a pIX4.0 vector construct; purification was performed using magnetic agarose beads functionalized with tris–Ni–NTA (left panel) or Ni–NTA (right panel) under standard conditions (see Section 2.10) in the presence of 0.05% (v/v) Tween-20 using a magnet-equipped tube holder. Aliquots of purification fractions as follows were analyzed by SDS–PAGE and silver staining: F, unbound protein; W1, 2, wash fractions 1 and 2 (10 mM imidazole); W3, wash fraction 3 (20 mM imidazole); 40 → 250, elution-fractions (40, 60, 100, and 250 mM imidazole, respectively). Identity of the purified His$_6$–tagged AKT1 protein was verified by anti-His Western blot analysis (data not shown).

higher using tris–Ni–NTA beads. Whether these findings are of general relevance and whether tris–NTA-based chromatography can be economic in larger scales remains to be evaluated.

IMAC ligands have also been used successfully as reporter in immuno-blot type of applications replacing an antibody. His-tagged proteins transferred to nitrocellulose membrane (Western or dot blot) can be detected by probing with Ni–NTA conjugated to alkaline phosphatase or horseraddish peroxidase reporter enzymes (Lv *et al.*, 2003) in a chromogenic or chemi-luminescence reaction or to quantum dots for fluorescent detection (Kim *et al.*, 2008). This represents an attractive fast and economic alternative to antibody-based detection reactions in cases where the high specificity of an antibody is not required. The specificity of NTA conjugate-based detection has been increased by generation of tris–NTA conjugates (Lata *et al.*, 2006; Reichel *et al.*, 2007).

2.2. Purification of protein fractions

IMAC had originally been developed as a group separation method for metallo- and histidine-containing proteins (Porath *et al.*, 1975). Today, these features are made use of in proteome-wide studies where the reduction of the complexity of the system (the proteome) is indispensable for sensitive analyses of low-abundance proteins. Consequently, preseparation methods such as liquid, reverse-phase, ion-exchange, and affinity

chromatography—such as IMAC—have gradually been used in proteomics to enrich proteins that may otherwise be lost in detection (Loo, 2003; Stasyk and Huber, 2004). The application of IMAC in proteomics has recently been reviewed (Sun *et al.*, 2005) and is focused on the enrichment of phosphoproteins and phosphopeptides and on metal-binding proteins. In the enrichment step, a complex sample such as a cell lysate or blood is passed over the IMAC matrix, washed and the fraction of interest eluted by variation of pH or with high concentrations of imidazole. This fraction is then analyzed by mass spectrometry (MS) or fractionated further by two-dimensional gel electrophoresis followed by MS or by additional liquid chromatography coupled to MS (LC–MS).

Whereas Fe^{3+}, Al^{3+}, and Ga^{3+} are the preferred ions for phospho-protein research and are usually immobilized to IDA, the ions useful for IMAC-based analysis of the metalloproteome are the elements copper, nickel, zinc, and iron which are essential for life. The metalloproteome is defined as a set of proteins that have metal-binding ability and several aspects of this proteomics discipline have been reviewed recently (Shi and Chance, 2008; Sun *et al.*, 2005). Proteins with metal-binding affinity can be enriched by either making use of their ability to bind to certain immobilized Me^{2+} ions (e.g., to Me^{2+}-NTA) or by making use of their bound Me^{2+} ion by catching as a Me^{2+}-protein on an uncharged IMAC ligand (e.g., NTA).

Also, chip-based proteome profiling IMAC methods have been reported (Slentz *et al.*, 2003) and are in use as a tool in clinical screening applications for phospho group- and histidine-containing proteins and peptides (SELDI–IMAC).

IMAC can also be used to bind and separate at least mono- and dinucleotides based on a complex phenomenon accounted for by differential interplay of affinities of the potential binding sites (oxygen in the phosphate group, nitrogen and oxygen on the bases, hydroxyl groups on the ribose) to the immobilized metal (Hubert and Porath, 1980, 1981).

A quite different group-specific separation application of IMAC is represented by the affinity of antibodies to immobilized metal ions. As the molecular basis for this interaction an endogenous metal-binding site on the heavy chain (Hale and Beidler, 1994) and an arrangement of histidine residues on the antibody (Porath and Olin, 1983) have been discussed. Adsorption of immunoglobulins from different sources on IMAC matrices has been reported by many authors (human IgG, Porath and Olin, 1983; humanized murine IgG, Hale and Beidler, 1994; goat IgG, Boden *et al.*, 1995). Antibody purification has been successfully performed using various IMAC formats including gels (Hale and Beidler, 1994; Vancan *et al.*, 2002), methacrylate polymer (Mészárosová *et al.*, 2003), and membraneous hollow fibers (Serpa *et al.*, 2005). The mild elution of the protein with salts, costs, and the robustness of IMAC matrices have been identified as advantageous over traditional protein A or G chromatography (Serpa *et al.*, 2005).

   The Chelex method shall be mentioned here as well in order to complete the list of applications which make use of IMAC ligands. Unlike classical IMAC, however, where an immobilized metal ion is used to purify a (poly)peptide by its affinity for this transition metal, Chelex represents a nucleic acid sample preparation method that depletes metal ion inhibitors of PCR as the downstream application (Walsh *et al.*, 1991). As such, Chelex resins such as Bio-Rad's Chelex 100 are uncharged ligands like IDA coupled to a usually agarose-based matrix. In brief, the procedure works as follows: a blood or tissue sample is incubated with Chelex resin in the presence or absence of proteinase K followed by separation of beads from supernatant containing the nucleic acids. The resulting nucleic acid fraction is not pure but suitable for amplification by PCR because metal ions have been removed from the sample which may otherwise catalyze rupture of the DNA at high temperature during PCR and thus cause PCR inhibition. Chelex is mainly applied as a fast and inexpensive method to prepare small samples obtained from biopsies and puncture aspirations for amplification of small DNA fragments by PCR (García González *et al.*, 2004; Gill *et al.*, 1992).

2.3. Purification of His-tagged proteins

2.3.1. His tags and their effects on protein expression

The most important application of IMAC is purification of recombinant proteins expressed in fusion with an epitope containing six or more histidine residues, the His tag (Fig. 27.1). Due to the relatively high affinity and specificity of the His tag a single IMAC purification step in most cases leads to a degree of purity of the target protein preparation that is sufficient for many applications. The structure of the tag, that is its position, sequence, and length, can influence production of a protein on several levels: expression rate, accessibility for binding to the IMAC ligand, protein three-dimensional structure, protein crystal formation, and—although to a minor extent—solubility and activity. The most common form of a His tag consists of six consecutive histidine residues (H_6) which provides a number of six metal-binding sites high enough to shift the association/dissociation equilibrium more to the association side leading to stable binding in most cases (Table 27.1; Knecht *et al.*, 2009). In Biacore experiments, the dissociation rate of a hexahistidine-tagged protein to Ni-NTA has been determined to approximately 1×10^{-6} to 1.4×10^{-8} M at pH 7.0 to 7.4 (Knecht *et al.*, 2009; Nieba *et al.*, 1997). However, the situation on a planar chip surface is significantly different from a porous agarose bead with respect to flow characteristics, ligand density, and protein concentration. Also, the stability of the interaction of the His-tagged protein to the IMAC ligand is influenced by the grade of accessibility of the tag and by the overall number of chelating residues (histidine, cysteine, aspartate, and glutamate)

Table 27.1 Reported His tag sequences (single letter amino acid sequence code)

His tag		Reference/vector
H H H H H H	H_6	Hochuli *et al.* (1988)
H H H H H H H H	H_8	pQE-TriSystem, pTriEx
H H H H H H H H H H	H_{10}	pQE-TriSystem-5, -6
H H H H H H H H H H H H H H	H_{14}	
H Q H Q H Q H Q H Q H Q	$(HQ)_6$	Pedersen *et al.* (1999)
H N H N H N H N H N H N	$(HN)_6$	US patent 7176298
H G H G H G H G H G H Q	$(H G/Q)_6$	Pedersen *et al.* (1999)
H H Q H H A H H G	$(HHX)_3$	Pedersen *et al.* (1999)
K D H L I H N V H K E H A H A H N K	HAT	Imai *et al.*, 2001 and US patent 7176298
$(H X\star)_{3-6}$	$(HX^a)_n$	US patent 5594115
$H X_1 H R H X_2 H^b$	$(HXH)_2R$	US patent application 2004/0029781

[a] X can be D, E, P, A, G, V, S, L, I, and T.
[b] X_1 can be A, R, N, D, Q, E, I, L, F, P, S, T, W, V; X_2 can be A, R, N, D, C, Q, E, G, I, L, K, M, P, S, T, Y, V.

on the surface of a protein (Bolanos–Garcia and Davies, 2006; Jensen *et al.*, 2004) and is thus individual to a significant extent. In most cases, that is, if the His tag is accessible, its affinity—or better termed avidity here—to Ni-NTA is high enough for column chromatography even under stringent conditions. Table 27.1 lists the tag sequences reported in the literature and some unpublished ones tested recently in our laboratories.

A different situation compared to a "standard" purification of a soluble protein (see Section 2.10) can be encountered when a membrane protein is to be recovered in the presence of detergents because the deterent micelle may cover part of or the complete His tag. In such cases, the use of longer tag sequences or the use of a linker can be helpful to allow binding of the protein to the IMAC resin (Mohanty and Wiener, 2004). A range of variations of the His tag including alternating sequences has been proposed (Table 27.1) and improved binding to IMAC resins postulated but in our and in the hands of others (Knecht *et al.*, 2009) they have no practical advantage over the classical H_n tags. What is found to be of greater importance than the sequence of the His tag itself is its position (N- or C-terminal) and the amino acids at the N-terminus. The nature of the amino acid following the N-terminal methionine has been reported to prevent N-terminal methionine processing and to have a positive effect on the general protein expression rate in *Escherichia coli* (Dalbøge *et al.*, 1990; Hirel *et al.*, 1989). We and others have evaluated these reports and confirmed that

namely, lysine and arginine in position 2 of a protein N-terminus show this effect (Pedersen *et al.*, 1999; Schäfer *et al.*, 2002a; Svensson *et al.*, 2006). The high success rate of using N–terminal His (and Strep II) tags is, however, not only based on the stimulatory effect of the second amino acid on expression but also seems to have stabilizing impact on the mRNA structure in the translation initiation region. We compared bacterial and eukaryotic expression of N– versus C-terminal positioning of the His (and Strep II) tag on several proteins and analyzed expression level and solubility (Fig. 27.4). N-terminal tags improved protein expression in most cases.

Systematic investigation of the 5′ region of mRNAs showed that hairpin loops forming in the translation initiation area frequently are the reason for low expression as they prevent the ribosome:mRNA binding (Cèbe and Geiser, 2006). Sequence optimization can be performed to destabilize hairpin formation and improve expression, and a similar result was obtained when the proteins were expressed with an N–terminal H_6 tag. The effects described in Fig. 27.4 can be explained with this initiator effect of preventing secondary structures on the mRNA in the translation initiation region. Similar observations were also made by others (Busso *et al.*, 2003; Svensson *et al.*, 2006) and this may be the reason for the attractiveness of N-terminal
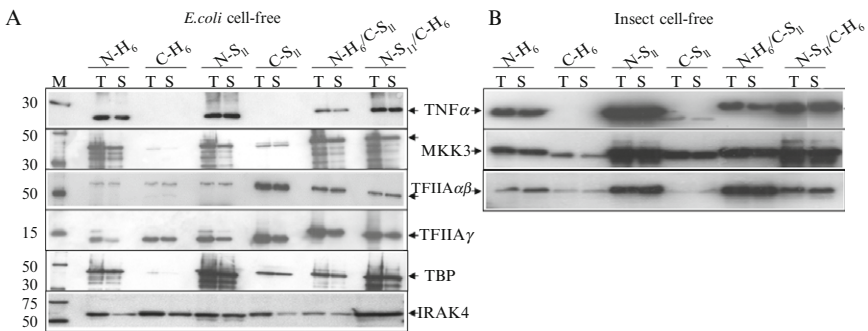

Figure 27.4 Effect of His tag position on protein expression. Proteins were expressed from PCR product templates generated by two-step PCR using the EasyXpress Linear Template Kit in *E. coli*- (A) and insect cell-derived (B) lysates (EasyXpress Protein Synthesis and EasyXpress Insect II Kits, respectively). Initiator (adapter) primers in the Linear Template Kit were designed in order to prevent formation of secondary structure in the translation initiation region on the mRNA and introduce the following tag sequence(s): N-H_6, N-terminal His$_6$ tag; C-H_6, C-terminal His$_6$ tag; N-S_{II}, N-terminal Strep II tag; C-S_{II}, C-terminal Strep II tag; N-H_6/C-S_{II}, N-terminal His$_6$ and C-terminal Strep II tags; N-S_{II}/C-H_6, N-terminal Strep II and C-terminal His$_6$ tags. Corresponding aliquots of total (T) and soluble protein (S, supernatant after centrifugation at $15,000 \times g$ for 10 min) were loaded on a SDS gel. Protein bands were visualized by Western blot analysis using a mixture of Penta anti–His and anti–Strep tag antibodies. Protein sizes are (kDa) TNFα, 21; TBP, 38; TFIIAαβ, 55; TFIIAγ, 12.5; MKK3, 39; IRAK4, 55. M, His-tagged protein size markers (kDa).

His tags. However, in some cases such as the one of IRAK4 the C-terminal His tag has a more pronounced effect on both expression rate and solubility (Fig. 27.4). Recently, expression of an insect toxin in *E. coli* was reported where the similar observation of higher solubility and thermostability of the C-terminally His-tagged form was made (Xu *et al.*, 2008). The authors discussed that the C-terminal tag stabilized the overall protein structure. Other groups found the His tag to contribute slightly negative to solubility when compared to the untagged protein but to improve yield when fused to the C-terminus (Woestenenk *et al.*, 2004). All in all, these data suggest that an evaluation of at least N- and C-terminally tagged variants of a protein will increase the chance to obtain reasonable expression and quality of a recombinant protein. For expression of proteins to be secreted, tags should be placed at the C-terminus to prevent interference with membrane trafficking.

2.4. General considerations of protein purification by IMAC

There are several advantages of IMAC for purification of His-tagged proteins compared to other affinity chromatography principles as the reason for being the most widely used chromatographic technique (Biocompare, 2006; Derewenda, 2004). Besides low costs and the simplicity of use the robustness of IMAC is certainly its most striking feature: (i) the His-tag: ligand interaction works under both native and denaturing conditions such as 8 M urea or 6 M guanidinium hydrochloride (Hochuli *et al.*, 1988) enabling subsequent on-column refolding (Jungbauer *et al.*, 2004) as well as (ii) both oxidizing and reducing conditions, (iii) protein binding withstands a broad spectrum of various chemicals of different types (Table 27.1 summarizes chemical compatibilities for Ni-NTA IMAC and some limitations), (iv) its relatively high affinity and specificity allows high capturing efficiency even in the presence of high protein titers, and (v) the scalability of the purification procedure.

Despite the broad compatibility, IMAC has its limitations. Obviously, the use of chelating agents has to be avoided which can be a disadvantage as EDTA, a potent inhibitor of metalloproteases, can only be applied in low concentrations. Care should also be taken with the use of other potentially chelating groups such as Tris, ammonium salts, and certain amino acids (Table 27.2).

Until recently, the use of strong reducing agents such as DTT in IMAC processing has been regarded as problematic because of reduction of nickel and, as a consequence, suspected increase of nickel concentrations in protein preparations. However, we found that moderate concentrations of DTT are fully compatible with NTA-based purification. This is shown, for example, in Fig. 27.5A for HIV-1 reverse transcriptase (RT) purified with unaffected efficiency in the presence of up to 10 mM DTT. Also, RT activity is not influenced by these conditions and both end-point

Table 27.2 Chemical compatibility of purification of His-tagged protein using agarose-based IMAC (Ni-NTA) resins and its limitations

IMAC chemical compatibility			
Component	Limitation (up to)	Component	Limitation (up to)
Buffers		**Salts**	
Na–phosphate	Recommended, limit not known	NaCl	4 M
Phosphate citrate	Limit not known	MgCl$_2$	4 M
Tris–HCl, HEPES, MOPS	100 mM	CaCl$_2$	5 mM^c
Citrate	60 mM	NaHCO$_3$	Not recommended
Detergents (in 300 mM NaCl)		Ammonium salts	Not recommended
n-Hexadecyl-β-D-maltoside	0.0003% (w/v)	**Protease inhibitors**	
n-Tetradecyl-β-D-maltopyranoside	0.005% (w/v)	EDTA	1 mM^a
n-Tridecyl-β-D-maltopyranoside	0.016% (w/v)	Commonly used protease inhibitors[d]	Compatible in effective concentrations
Brij 35	0.1% (v/v)	Complete cocktail (EDTA-free)	1× concentrated
Digitonin	0.6% (w/v)	**Denaturants**	
Cymal 6	1% (w/v)	Urea	8 M
n-Nonyl-β-D-glucopyranoside (NG)	1% (w/v)	Gu–HCl	6 M
n–Decyl-β-D-maltopyranoside (DM)	2% (w/v)	**Amino acids**	
n-Dodecyl-β-D-maltoside (DDM)	2% (w/v)	Histidine	1–2 mM^b
C12–E9	1% (w/v)	Glycine	Not recommended
n-Octyl-β-D-glucopyranoside (OG)	1.5% (w/v)	Cysteine	Not recommended
Triton X-100, Tween, NP-40	2% (v/v)	Glutamate	Not recommended
Triton X-114	2% (v/v)	Aspartate	Not recommended
Fos-Cholines	0.05% (w/v)	Arginine	500 mM

Dodecyldimethyl–phosphineoxide	0.15% (w/v)	**Organic solvents**	
N,N–Dimethyldodecylamine–N-oxide (LDAO)	0.7% (w/v)	Isopropanol	60% (v/v)[e]
CHAPS	1% (w/v)	Ethanol	20% (v/v)
Laurosyl–sarcosine	1% (w/v)	**Reducing reagents**	
SDS	0.3% (w/v)[a]	β-ME	20 mM
Other		TCEP	20 mM
EGTA	1 mM^{a}	DTT	10 mM
Imidazole	10–20 mM^{b}	DTE	10 mM
Hemoglobin	Not recommended		
Glycerol	50% (v/v)		

[a] Has been used successfully in the indicated concentration but should be avoided whenever possible.
[b] Dissociates His–tagged proteins at high concentrations.
[c] Should be avoided in combination with Na–phosphate.
[d] Include, for example, Aprotinin, Leupeptin, PMSF, and related serine protease inhibitors: Pepstatin, Antipain, Bestatin, E64, Benzamidine.
[e] Compatible with Ni–NTA purification and endotoxin removal according to Franken et al. (2000), but in this concentration is incompatible with reuse of the chromatographic media (data not shown).
This table provides some of the most relevant tested substances and concentrations and may not be complete or represent the maximal concentrations compatible with purification of His–tagged proteins. Abbreviations: β-ME, β-mercaptoethanol; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; DTT/DTE, dithio–threitol/–erythritol; Gu–HCl, guanidinium hydrochloride.
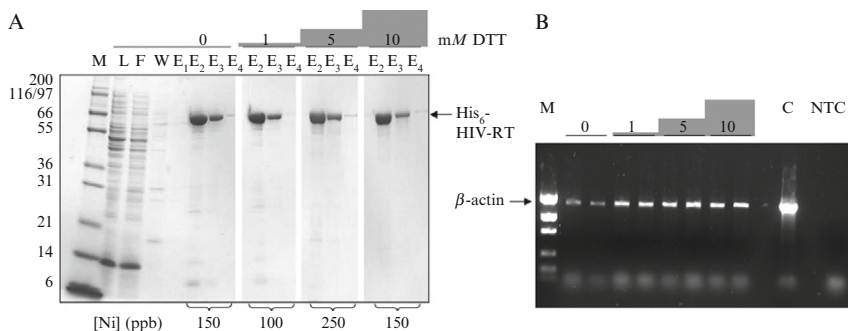
Figure 27.5 IMAC under reducing conditions. (A) His$_6$-tagged HIV–1 reverse transcriptase (RT) was purified under standard conditions in the presence of the indicated DTT concentrations. Aliquots of each chromatographic fraction of the purification without DTT (M, markers; L, lysate; F, flow-through; W, wash; E, elution fraction) and of corresponding volumes of elution fractions 2–4 containing DTT were analyzed by SDS–PAGE and Coomassie staining. Elution fractions were pooled and analyzed for nickel content by ICP-MS at Wessling Laboratories, Bochum, Germany; nickel concentrations are given in μg/l (parts per billion, ppb). (B) Equal amounts of HIV-1 RT purified in the presence of the indicated concentrations of DTT were used in duplicates to reverse transcribe a 1.5 kb β-actin cDNA which was subsequently amplified by PCR and the PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Reducing conditions of at least 1 mM DTT were found to be required for full RT activity (compare weaker amplification with RT purified in the absence of DTT). C, Omniscript positive control; an equal amount of Omniscript RT protein was used; NTC, no template negative control.

(Fig. 27.5B) and quantitative real–time RT-PCR (data not shown) show no inhibitory effect which could origin from high heavy–metal–ion concentrations. Even though nickel ions may become reduced by DTT leading to a color change of the resin bed they do not increasingly leach from the ligand (Fig. 27.5A), and resins processed under reducing conditions can be repeatedly reused and regenerated (data not shown). These findings suggest that despite a color change as a consequence of nickel reduction by DTT the resin is still functional. TCEP, a different, nonthio–based reducing reagent is more and more replacing DTT and β-ME in protein purification by IMAC as it seems to be more selective to reduction of disulfide bonds, is odorless and more stable in aqueous solution. We recommend the use of TCEP with Ni–NTA chromatography in a concentration of 1–5 mM.

2.5. Copurifying proteins on IMAC and what to do about it

IMAC leads to high protein purity upon single-step chromatographic purification in many cases (Figs. 27.2A, D, E and 27.5A; Bornhorst and Falke, 2000; Schmitt *et al.*, 1993). The higher the purity is the closer the amount of IMAC resin applied in the chromatographic step has been

correlated to the amount of recombinant His-tagged protein present in the sample to be processed. The reason is that proteins naturally displaying surface motifs suitable to interact with an immobilized metal ion may bind to the resin although usually with slightly lower affinity than a flexible His tag. Most His-tagged proteins will therefore displace proteins with natural or accidental surface motifs. However, there are some proteins where the local density of chelating amino acids such as histidine is so high that they will bind to immobilized metal ions almost inevitably. In general, mammalian systems have a higher natural abundance than bacterial systems of proteins containing consecutive histidines (Crowe *et al.*, 1994), and a prominent example in human cells is the alpha subunit of transcription factor TFIIA which has seven consecutive and surface-exposed histidine residues and can be purified via IMAC from natural sources under native conditions (DeJong and Roeder, 1993; Ma *et al.*, 1993) and which is observed frequently as a signal band of 55 (the $\alpha\beta$ precursor) or 35 kDa (the α subunit) of TFIIA in Western blots using an anti-His tag antibody. Another example is the human transcription factor YY1 with 11 consecutive histidines (Shi *et al.*, 1991). In *E. coli*, the proteins observed to copurify with His-tagged target proteins can be divided into four groups: (i) proteins with natural metal-binding motifs, (ii) proteins with histidine clusters on their surfaces, (iii) proteins that bind to heterologously expressed His-tagged proteins, for example by a chaperone mechanism, and (iv) proteins with affinity to agarose-based supports (Bolanos-Garcia and Davies, 2006). Whether or not one of the *E. coli* proteins is copurified is not easily predictable. For example, a protein from group (ii) sometimes reported to copurify with Ni–NTA is the 21 kDa SlyD, however, we have never observed this one in our lab upon purification from *E. coli* BL21(DE3), DH5α, *M*15(pREP4), and other strains. This may be explained by the fact that many of these impurities are stress-responsive proteins, suggesting that the cultivation conditions and the bacterial strain have an influence on their abundance and, a consequence, their appearance as a contaminating species in the target protein preparation; it is therefore recommended to induce as little stress as possible during cultivation of *E. coli* cells (e.g., by using shake flasks without baffles). Furthermore, some copurifying proteins seem to have a binding preference for Co over Ni (or other ions) and others vice versa.

Several options to get rid of these copurified proteins or prevent their adsorption early on have been evaluated and some of them shall be discussed in the following section. These options include (i) performing additional purification steps, (ii) adjusting the His-tagged protein to resin ratio, (iii) to use an engineered host strain that does not express certain proteins, (iv) using an alternative support, and (v) tag cleavage followed by reverse chromatography.

Suitable additional purification steps include classical chromatographic techniques such as ion–exchange (IEX) and size exclusion chromatography (SEC) whereas IEX has the higher separation power. However, as SEC not

only serves to separate molecules by size and helps to remove aggregates of high molecular weight but also can be used to desalt the preparation and provide conditions suitable for certain downstream applications, it is frequently used as a standardized procedure by, for example, high-throughput labs such as structural biology consortia (Acton *et al.*, 2005; Gräslund *et al.*, 2008) who perform protein crystallization or NMR spectroscopy. Although IMAC–SEC (as opposed to IMAC–IEX) can be performed as a standardized procedure without having to take into account the protein biochemistry such as pI the separation range of a given SEC column will not be suitable for all separation tasks and a range of SEC columns may have to be held in place. Another issue with the application of IEX and SEC is that in order to make use of the full power provided by these technologies, costly equipment such as an automated chromatography system is required which frequently precludes multiparallel processing leading to low throughput. Affinity purification such as IMAC can usually be done as a bind–wash–elute procedure in a bench top/gravity flow mode. By introduction of a second affinity tag (e.g., StrepII, GST, Flag) into the expression construct a two-step purification leading to high-purity protein preparations is enabled as a bench-top two-step affinity chromatography procedure (Cass *et al.*, 2005; Prinz *et al.*, 2004).

As mentioned earlier, adjusting the amount of His-tagged protein to be recovered to the binding capacity of the used IMAC resin can help to improve protein purity by preventing copurification of proteins with certain affinity to IMAC resins. However, the amount of His-tagged protein is usually unknown unless a pilot experiment is performed to estimate the content of target protein. While this is an option, the better way would be to exclude the presence of such copurifying proteins by expressing the target protein in an engineered strain where the respective genes have been knocked out. However, to our knowledge results from work with such strains have not yet been reported and the experience with knockout strains in protein production is still low. Also, it does not seem realistic that an *E. coli* strain can be generated which lacks 17 proteins reported to bind to IMAC resins including as important functions as superoxide dismutase and iron-uptake regulation (Bolanos-Garcia and Davies, 2006) and which is still well viable under stress situations such as protein overproduction.

A different approach to improve the purity of proteins recovered from IMAC has been reported that made use of dextran-coating of an agarose matrix, the constituting material of the most widely used chromatographic supports (Sepharoses, Superflow, Agaroses), and which prevented copurification of proteins with affinity to these matrices (Mateo *et al.*, 2001). Dextran-coated beads, however, are not readily commercially available as IMAC resins and this measure only helps to preclude proteins with affinity to agarose and not to the immobilized metal or to the target protein. Silica-based IMAC supports also prevent adsorption of proteins with affinity for

agarose and, in addition, have good pressure stability which makes them suitable for high-resolution HPLC applications but the silica resins frequently suffer from low binding capacity and limited resistance to high pH sanitization procedures. A very recent method that avoids the need to use solid chromatographic supports completely is called affinity precipitation (Hilbrig and Freitag, 2003) and has been applied to IMAC (Matiasson et al., 2007). Here, the IMAC ligand is chemically coupled to a responsive polymer which, after binding to the His-tagged protein, can be aggregated upon change of environmental conditions such as pH or temperature and can thus be precipitated by centrifugation. Protocols for its use are still relatively complicated but as soon as robust and easy to use commercial materials are available this method may have the potential to play an important role in IMAC applications. Using a ligand in solution could overcome steric hindrance of the binding of some His-tagged proteins to an immobilized ligand as well as mass transport limitations of porous chromatographic media. Moreover, it is in line with a trend in industrial-scale chromatography toward single-use disposable materials.

Yet another approach has recently been reported that can be applied for protein separation from lysates after cell-free expression (Kim et al., 2006). An E. coli-derived lysate was preincubated with Ni–NTA magnetic agarose beads to remove proteins with affinity to Ni–NTA prior to template addition and protein expression; the expression capacity of the S30 extract was found to remain unaltered and the Ni–NTA purified His-tagged protein fractions to be of higher purity than without pretreatment.

While the aforementioned strategies to improve the purity of MAC protein preps have proven useful in many cases they are not generally applicable and successful. There is a method, however, that almost meets the criterium of universal applicability regarding improvement of purity, and it has the additional benefit of resulting in a protein native or near-native structure: proteolytic His tag cleavage using a His-tagged protease followed by reverse IMAC (Block et al., 2008). This strategy overcomes the copurification issue by passing the proteolytically processed protein under similar or identical conditions over the same column, and the proteins that bound to the IMAC resin as impurities in the initial purification step will bind to the same resin again while the cleaved, that is untagged, target protein is collected in the flow-through fraction (reverse or subtractive IMAC mode). It can be performed with both exo- and endoproteases that themselves carry an (uncleavable) His-tag (Nilsson et al., 1997; Polayes et al., 2008) but the exoproteolytical approach has the advantage that it is faster and results in a protein with native structure with no vector-derived amino acids (Arnau et al., 2006; Block et al., 2008; Pedersen et al., 1999). This approach is especially suitable for demanding downstream applications such as protein crystallization or biopharmaceutical production. Notably, the method requires only a single chromatography column to achieve an

extremely high degree of purity. An application is shown in Fig. 27.6 where TNFα was crystallized in its IMAC one-step purified form (A, B, C, D) or in its subtractive-IMAC processed form as described above (A, E, F, G). Although already His-tagged TNFα purified on Ni–NTA appeared highly pure upon SDS–PAGE and Coomassie staining (Fig. 27.6A, lane IMAC) analysis on a silver-stained 2D gel showed impurities (Fig. 27.6B). These impurities are removed by reverse mode IMAC resulting in a protein preparation of extremely high purity (Fig. 27.6E). Figure 27.6 also demonstrates the influence the His tag can have on protein crystallization. While both the tagged and the mature native form of TNFα eluted from a SEC column as a trimer (Fig. 27.6C and F), they crystallized under significantly different conditions and resulted in different crystal forms (His$_6$-TNFα: tetragonal, Fig. 27.6D; TNFα: rhombohedral, Fig. 27.6G). Nevertheless, calculated structures both were in accordance with the one deposited in the pdb (data not shown). However, when we attempted to perform the same experimental workflow with His-tagged and native mature IL-1β, we were unable to crystallize the His–tagged cytokine (Block *et al.*, 2008; and data not shown). Our data confirm the observation also made by many others that it is frequently possible to crystallize tagged proteins but suggest that it makes sense to provide a tag cleavage option when designing an expression construct.

2.6. IMAC for industrial-scale protein production

IMAC for production of proteins in industrial scale, for example for use as biopharmaceutical, has not been used until quite recently due to worries regarding the potential immunogenicity of a His tag sequence and because of allergenic effects of nickel leaching from an IMAC matrix. However, nickel concentrations typically observed in protein preparations obtained from tetradentate IMAC resins are low and content in expected daily doses of a biopharmaceutical will be far below the typical daily intake of nickel and the permanent nickel body burden (Block *et al.*, 2008). Removal of artificial sequences from recombinant proteins by the use of protease has been discussed above as the way to go for use in humans but proteins carrying a His tag have been successfully used for vaccination (Kaslow and Shiloach, 1994; Stowers *et al.*, 2001) or are presently commercialized as drugs (unpublished). IMAC is a chromatography method that can simply be scaled linearly from milliliter to liter bed volumes (Block *et al.*, 2008; Hochuli *et al.*, 1988; Kaslow and Shiloach, 1994; Schäfer *et al.*, 2000) and Ni–NTA Superflow columns in dimensions up to 50 l are in use for biopharmaceutical production processes (F. Schäfer, personal communication). Compatibility of IMAC matrices with a wide range of chemicals such as chaotropics, salts, organic solvents, and detergents (Table 27.2) facilitates adaption to the specific needs of the production of the individual protein.
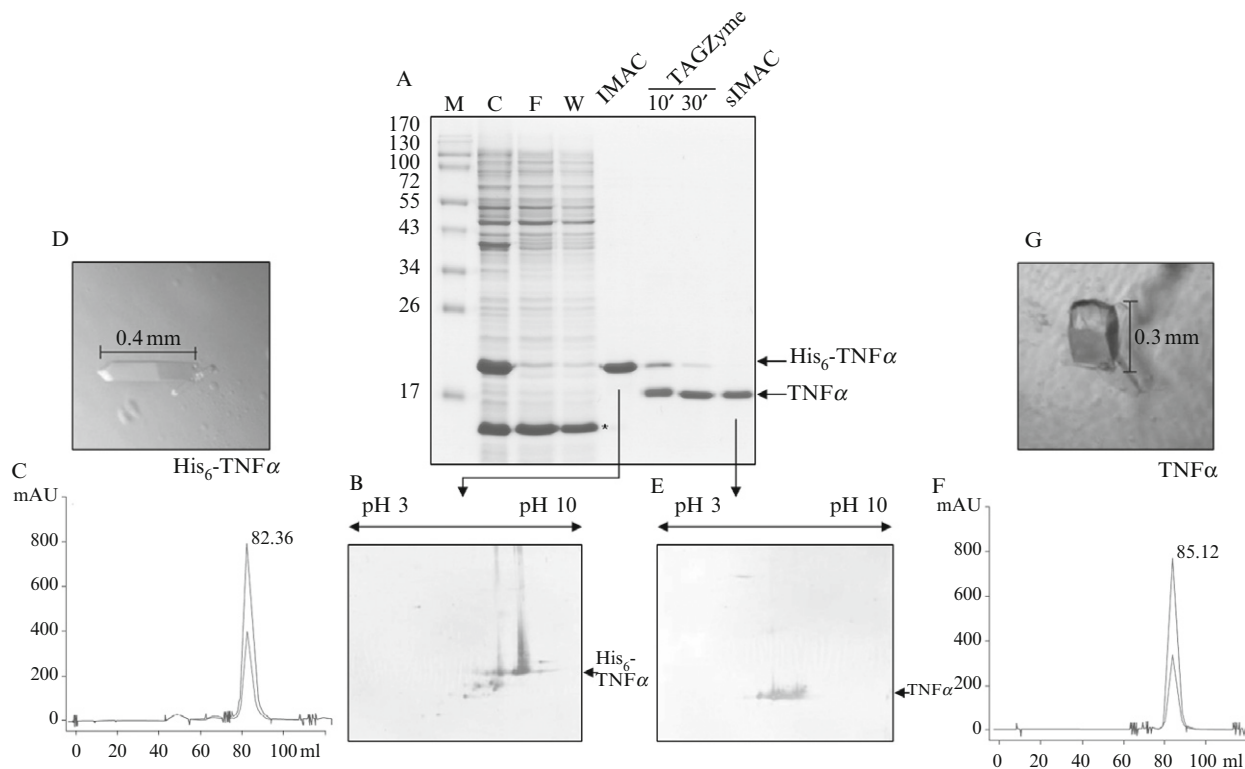
Figure 27.6 Removal of copurifying proteins by His tag cleavage and reverse IMAC. (A) His$_6$-TNFα expressed in *E. coli* was purified via Ni-NTA Superflow and processed using the TAGZyme exoproteolytic system as described (Schäfer *et al.*, 2002a). (B, E) 2D gel electrophoresis and silver staining of His$_6$-TNFα and TNFα, respectively, was performed as described (Block *et al.*, 2008). The subband pattern in the first dimension between pI 6.7 and 5.8 for both His$_6$-TNFα and TNFα is in accordance with the report for TNFα produced in yeast (Eck *et al.*, 1988). (C, F) Analytical size exclusion chromatography (SEC) on HR 10/30 Superdex 200 was run with 1× TAGZyme buffer (Schäfer *et al.*, 2002a). (D, G) His$_6$-TNFα tetragonal crystal (D) formed in 2.7 *M* MgSO$_4$, MES, pH 5.5 and diffracted to 2.5 Å (homelab X-ray source, FR591 Nonius Bruker); TNFα rhombohedral crystal (G) formed in 1.8 *M* NH$_4$SO$_3$, 200 m*M* Tris–HCl, pH 7.8 and diffracted to 2.0 Å (ESRF synchrotron). The size of typical crystals (mm) is indicated by the bar in (D) and (G).

For example, bacterial endotoxins (lipopolysaccharides) can be eliminated from the protein product during chromatography by including a wash step using a detergent (Triton X-114, Block *et al.*, 2008) or an organic solvent (60% isopropanol; Franken *et al.*, 2000). The suitability of IMAC for industrial production purposes has been demonstrated and it can be expected that IMAC-based processes become increasingly used in the future due to its robustness and relatively low requirements for individual optimization.

2.7. High-throughput automation of IMAC

Due to its robustness, universal applicability, and widespread use, IMAC is also an ideal tool for multiparallel screening for protein expression and solubility. This is mostly done in the convenient 96-well format using agarose- or magnetic bead-based IMAC resins in SBS footprint filter or microplates on 96-well magnets or plate centrifuges (Braun *et al.*, 2002; Büssow *et al.*, 2000). As the complete expression and the simple bind–wash–elute IMAC purification workflow is easily miniaturizable in microplate formats it was also suitable for hands-on free automation using liquid handling laboratory robots (see Lesley, 2001, for an overview). The automated steps covered the workflow to various extents, ranging from protein purification from manually generated *E. coli* lysates (Lanio *et al.*, 2000), *E. coli* or insect cell lysis, lysate clarification, and protein purification (Garzia *et al.*, 2003; Schäfer *et al.*, 2002b; Scheich *et al.*, 2003), to automation of complete workflows from construct cloning to protein analysis (Acton *et al.*, 2005; Hunt, 2005; Koehn and Hunt, 2009). Recently, we added another series of protocols and consumables for purification of His-tagged proteins from *E. coli* or eukaryotic cells or cell-free lysates to the list of options: a conceptually new lab automation instrument allows isolation of microgram to milligram amounts of proteins from a variable number of samples (with a random-access sample feeding option) using ready-to-go prefilled cartridges that provide enzymes, buffers, and Ni-NTA magnetic beads for lysis and purification. Figure 27.7 describes an expression and purification screening using a set of 24 optimized-gene constructs for production of human proteins. Between 1.4 and 35 μg of highly pure protein was obtained under native conditions. Proteins that could not be purified under native conditions were obtained upon purification under denaturing conditions, and Western blot analyses using an anti–His antibody showed the absence of cross-contaminations between wells (data not shown). Protein resulting from such high-throughput purification experiments can be used for functional assays (e.g., interaction studies), characterization of protein random mutagenesis, solubility analyses, and clone screening.

The next step following an expression screening is frequently a scale-up with a limited number of proteins or clones for production of milligram amounts
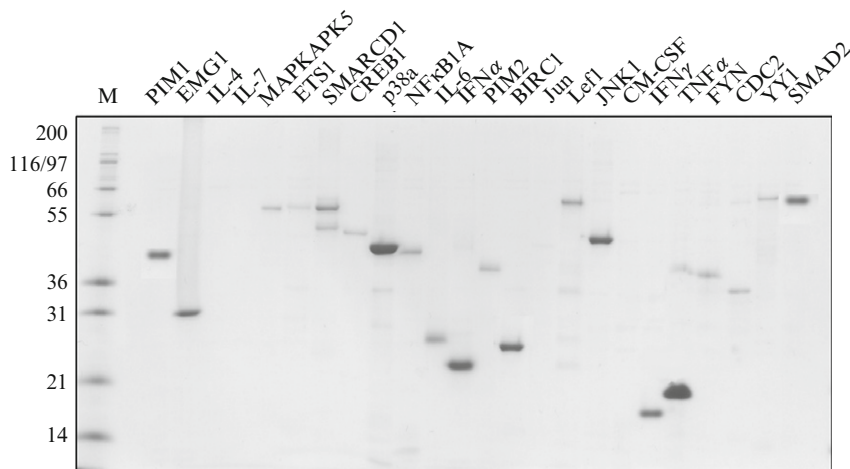
Figure 27.7 High–throughput protein purification screening on a new automation platform. *E. coli* BL21 (DE3) cells were transformed with QIAgene constructs carrying optimized human genes for expression of the indicated proteins in 1 ml LB cultures in a 96-deep well block. Cells were harvested by centrifugation and the pellet block placed onto the sample input drawer of the QIAsymphony SP instrument. Cells were resuspended and lysed and His$_6$-tagged proteins purified from the crude lysates using Ni-NTA magnetic agarose beads and buffer solutions provided in the QIAsymphony cartridge setup. A 5 μl of each elution fraction was analyzed by SDS–PAGE and Coomassie staining. Expected protein sizes (kDa) for the individual proteins were as follows: PIM1, 40; EMG1,30; IL-4, 15.5; IL-7,18; MAPKAPK5,55; ETS1,55; SMARCD1,60; CREB1,50; p38a, 40; NFκB1A, 40; IL-6,21; IFNα, 20; PIM2, 40; BIRC5, 30; Jun, 50; Lef1, 55; JNK1, 45; CM-CSF, 15; IFNγ, 17; TNFα, 17; FYN, 40; CDC2, 35; YY1, 66; SMAD2, 55. *M*, markers (kDa).

of protein for animal immunization, structural, or pharmacokinetic studies. Single proteins can be purified using standard ÄkTA or FPLC systems, and an ÄkTA system for slightly increased throughput has been developed (ÄkTAxpress). However, systems with a significantly higher throughput, lower complexity, and more dedicated to one-step (mainly IMAC) affinity purification have been reported (Steen *et al.*, 2006; Strömberg *et al.*, 2005) and are in use in high-throughput projects such as the human protein atlas project (Hober and Uhlén, 2008).

2.8. Special applications: Purification of membrane proteins

Membrane proteins have received the highest attention of all protein classes in the past few years due to their enormous importance as drug targets. In fact, more than 50% of all currently commercialized drugs as well as those under development target membrane proteins (Drews, 2000). Furthermore, membrane proteins account for approximately 30% of the human

proteome. However, in contrast to soluble proteins, very little is known about the biology and structure of membrane proteins which is reflected by the underrepresentation of membrane protein structures in public databases such as the pdb ($<$1%). While structure determination remains a challenge, purification of membrane proteins by a more or less standardized IMAC procedure has recently become significantly simpler by the development of new detergents and consequent screening for the most suitable detergent for both resolubilization and purification (Eshaghi *et al.*, 2005; Klammt *et al.*, 2005; Lewinson *et al.*, 2008). We have screened more than 50 of the most frequently used detergents for their IMAC compatibility and their resolubilization and Ni-NTA purification efficiency testing more than 10 bacterial and human membrane proteins and have reduced the number to seven powerful detergents as follows: Cymal 6 (Cy6), *n*-nonyl-β-D-glucopyranoside (NG), *n*-octyl-β-D-glucopyranoside (OG), *n*-decyl-β-D-maltopyranoside (DM), *n*-dodecyl-β-D-maltoside (DDM), FOS-choline-16 (FC16), and *N*,*N*-dimethyldodecylamine-*N*-oxide (LDAO). In screenings, we achieved a positive result for efficient resolubilization from *E. coli* or insect cell membrane fractions and IMAC purification for at least one detergent in each case. In Fig. 27.8, the detergent screening and Ni-NTA purification of His-tagged human Caveolin 1 is shown as an example.

IMAC compatible detergents are listed in Table 27.2 whereas this list may not be complete. Nevertheless, binding of a His-tagged membrane protein to IMAC resins seems not to work in combination with certain
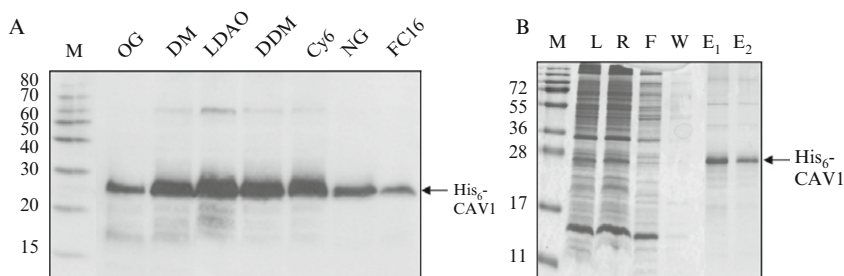

Figure 27.8 Detergent screening and IMAC purification of His$_6$-tagged human Caveolin 1 expressed in *E. coli* C41(DE3) in the presence of LDAO. The gene optimized for expression in *E. coli* was synthesized and cloned into pQE-T7 with an N-terminal His$_6$ tag. (A) Detergent screening; the membrane fraction from 200 ml *E. coli* culture divided into seven portions was mixed with detergent as indicated, centrifuged at $20,000 \times g$ and aliquots of detergent-soluble protein analyzed by Western blot (Penta anti-His antibody). Detergents are given in the text. (B) Caveolin 1 purification upscale; His$_6$-tagged Caveolin 1 (CAV1) was purified from 200 ml *E. coli* culture volume via Ni-NTA Superflow in the presence of 30 mM LDAO. Fractions were analyzed by SDS–PAGE and Coomassie staining: M, markers (kDa); L, *E. coli* total lysate; R, supernatant from resolubilization (loaded onto IMAC column); F, IMAC flow-through fraction; W, wash fraction; E, elution fractions.

detergents, including some of the listed ones even though resolubilization is fine. These phenomena depend on the protein–detergent combination and seem to arise from the detergent micelle around the protein partially or completely hiding the His tag. The use of longer tag sequences such as a H_{10} tag has become popular and seems to overcome such limitations of membrane protein recovery and improve affinity to IMAC resins (Byrne and Jormakka, 2006; Grisshammer and Tucker, 1997; Mohanty and Wiener, 2004; Rumbley et al., 1997).

2.9. Special applications: Purification of zinc-finger proteins

Another big protein superfamily which—due to technical issues—shall be mentioned in the context of IMAC is the group of proteins containing zinc-finger motifs. The C2H2 zinc-finger transcription factors alone with over 600 members represent more than 2% of the human proteome (Knight and Shimeld, 2001). In these proteins, zinc ions are coordinated by a defined spatial organization of two cysteine and two histidine residues each per finger, and a single polypeptide usually contains four or five of these motifs. Purification of such a metalloprotein via a metal ion chelated in a similar, that is tetradentate, manner deserves some reflection regarding the best way of IMAC purification.

Despite the strong interaction of metal ions with tetradentate IMAC ligands, it may not be excluded that a metal from the resin could exchange with the zinc in the zinc finger. Nickel, the most frequently used metal in IMAC protein purification has quite similar physicochemical properties to zinc and might therefore well replace it in such a metal-binding motif, and the nickel concentration in an IMAC resin (approximately 15 mM) is usually considerably higher than the concentration of a His-tagged target protein (μM range). In order to analyze a potential metal ion exchange between matrix and metalloprotein, we expressed the His$_6$-tagged C2H2 zinc-finger containing transcription factor YY1 in E. coli and purified the protein in parallel using Ni-NTA and Zn-NTA. YY1 could be purified to high purity by both nickel- and zinc-based IMAC (Fig. 27.9A, lane E).

Both YY1 preparations were subjected to determination of nickel and zinc content by ICP-MS (intercoupled plasma mass spectrometry), a quantitative method frequently applied to detect trace metals in biological systems (Shi and Chance, 2008). The zinc-IMAC purified protein preparation contained 35.6 μM zinc corresponding to approximately six Zn^{2+} ions per YY1 polypeptide and some Ni^{2+} which can be attributed to residual traces from the buffers (Fig. 27.9B, right). The protein recovered by nickel-IMAC, however, contained more than 25 μM Ni^{2+} and 14.2 μM Zn^{2+} (Fig. 27.9B, left), corresponding to again approximately six Me^{2+} ions per YY1 polypeptide. The Me^{2+}:polypeptide molar ratio of 6 is higher than the four zinc-finger motifs in YY1 reported in databases (http://www.uniprot.org/uniprot/P25490) which may in part be explained with additional metal

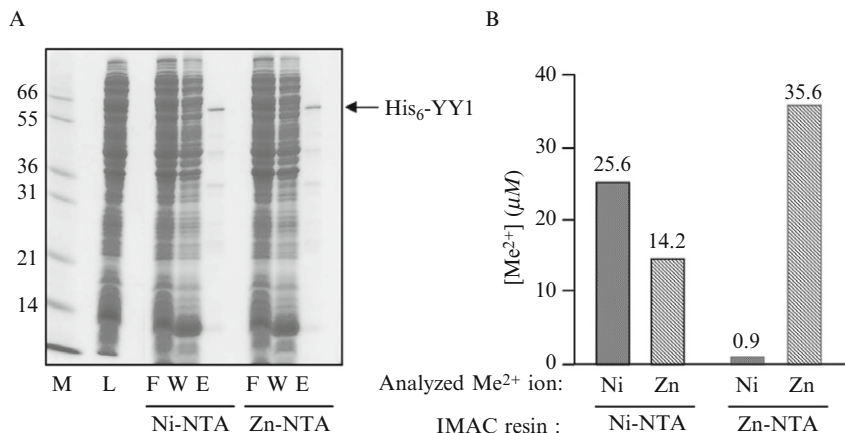A   B

Figure 27.9 Purification of His$_6$-tagged zinc-finger protein YY1 by Ni- and Zn-IMAC. (A) IMAC purification. His$_6$-YY1 was expressed in *E. coli* Bl21 (DE3) and a cleared lysate was generated as described below. NTA resin was treated with ZnCl$_2$ as described below to generate Zn-charged NTA (Zn-NTA). Protein purifications were performed using the same cleared lysate preparation under standard conditions (see Section 2.10) and aliquots of the chromatographic fractions (L, cleared lysate; F, flow-through fraction; W, wash fraction; E, elution fraction) analyzed by SDS–PAGE and Coomassie staining. Protein concentration of elution fractions were determined by the Bradford method to 0.3 mg/ml (6.7 μM, Ni-NTA) and 0.25 mg/ml (5.6 μM, Zn-NTA). (B) Quantitative detection of metals. Both elution fractions were then dialyzed for 16 h at 4 °C versus three changes of buffer NPI-10 to remove metal ions from the solution. Dialyzed proteins and fresh dialysis buffer were analyzed for nickel and zinc content by ICPMS at Wessling Laboratories (Bochum, Germany) and the values determined for the buffer subtracted from the protein analyses. Values (ppm, mg/l) were converted to molar units using the mean relative masses for Ni (58.7) and Zn (65.4).

ions binding elsewhere on the protein. Nevertheless, the data from Fig. 27.9B suggest that in the case of YY1 there is a significant exchange of metal ions between the charged IMAC ligand and the zinc fingers. Metal-affinity purification of proteins with C2H2 zinc-finger motifs can be effectively performed but an IMAC ligand charged with zinc, if applicable, should be chosen in order to obtain a preparation with an intact and homogenous zinc-finger composition.

2.10. Protein purification protocols

In this section, the standard IMAC protocols will be described briefly. The procedures have been optimized for the use of tetradentate (i.e., Ni-NTA) resins but should be transferable to tridentate (IDA-based) resins as well. TED resins behave different and the manufacturers' recommendations should be followed (e.g., proteins elute at significantly lower imidazole concentrations and consequently imidazole level in wash buffers should be kept low).

Here, we will provide recommendations for agarose-based Ni–NTA resins (Superflow, Agarose) regarding purification, cleaning, and recharging. These can be regarded as an update of the more detailed description provided in the Ni–NTA handbook (QIAGEN, 2003). Buffers provided in the following may be supplemented according to the needs of the individual protein (e.g., in order to generate reducing conditions, to stabilize the protein with glycerol, or to provide the presence of cofactors).

2.10.1. Purification of His-tagged proteins under native conditions

1. Lyse cells using the basis buffer NPI-10 (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0) supplemented with a suitable lysis reagent.
   For *E. coli* lysis, we recommend to use standard hen egg white lysozyme at 1 mg/ml final concentration because lysis is extremely efficient (if cells had been frozen) and lysozyme is inexpensive. Lysozyme is reliably washed from IMAC resins and does not occur in elution fractions (Figs. 27.5A, 27.6A, and 27.8; Block *et al.*, 2008). Other suitable methods are based on detergents (e.g., 1 % (v/v) CHAPS or proprietary solutions) or physical treatment (sonication, high pressure/depressurization homogenization). For cultures derived from insect or mammalian cells, 1% Igepal CA–630 (former name NP–40) is recommended. For reduction of lysate viscosity, the addition of a nuclease is helpful, and Benzonase (3 units/ml bacterial culture) has been shown to be robust and its removal in wash steps from IMAC resins works reliably and can be checked by a commercial ELISA (Block *et al.*, 2008). Incubate the lysate on ice for 30 min.
2. Generate a cleared lysate by centrifugation for 30 min at $\geq 10{,}000 \times g$ and 2–8 °C. Collect the supernatant.
3. Load the cleared lysate onto the resin equilibrated with 5 bed volumes (bv) of NPI-10 and allow to flow through at approximately 1 ml/min (column of 1 ml bv) or let flow through (gravity flow application).
   A suitable linear flow rate during binding is 155 cm/h corresponding to 1 ml/min of a column with a bed diameter of ∼7 mm (1 ml HisTrap and Ni–NTA Superflow Cartridges). If applicable to the workflow, we recommend to perform binding in batch mode as this is most efficient with agarose-based resins in general; for this, add the required volume of lysate to the equilibrated resin and incubate for 1 h rotating end-over-end at 2–8 °C.
4. Wash the Ni–NTA column with 10 bv wash buffer NPI-20 (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole, pH 8.0).
   If binding had been performed in batch, pour the binding suspension into a suitable flow-through column.
5. Elute His-tagged protein with 5 bv of elution buffer NPI-500 (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 500 mM imidazole, pH 8.0).

2.10.2. Purification of His-tagged proteins under denaturing conditions

1. Lyse cells using the basis buffer B (100 mM NaH$_2$PO$_4$, 10 mM Tris–HCl, 8 M urea, pH 8.0).

   E. coli as well as most eukaryotic cells are efficiently lysed by 7–9 M urea but occasionally, His-tagged proteins forming inclusion bodies do not completely dissolve; in such cases, we recommend to replace the chaotrope urea by guanidinium hydrochloride (buffer A: 100 mM NaH$_2$PO$_4$, 10 mM Tris–HCl, 6 M Gu–HCl, pH 8.0). Reduction of lysate viscosity by the addition of Benzonase (3 units/ml final concentration) is also possible and effective under denaturing conditions but here the maximum urea concentration is 7 M (use of Gu–HCl is not possible in combination with Benzonase). Incubate the lysate for 30 min at ambient temperature.

2. Generate a cleared lysate by centrifugation for 30 min at $\geq 10,000 \times g$ and room temperature. Collect the supernatant.

3. Load the cleared lysate onto the resin equilibrated with 5 bv of buffer B (or buffer A where applicable) and allow to flow through at approximately 1 ml/min (column of 1 ml bv) or by gravity flow.

   A suitable linear flow rate during binding is 155 cm/h corresponding to 1 ml/min of a column with a bed diameter of ∼7 mm (1 ml HisTrap or Ni-NTA Superflow Cartridges). If applicable to the workflow and the resin format used, we recommend to perform binding in batch mode as this is most efficient with agarose-based resins in general; for this, add the required volume of resin slurry to the lysate and incubate for 1 h rotating end-over-end.

4. Wash the Ni-NTA column with 10 bv wash buffer C (100 mM NaH$_2$PO$_4$, 10 mM Tris–HCl, 8 M urea, pH 6.3).

   If binding had been performed in batch, pour the binding suspension into a suitable flow-through column prior to the wash step. If lysate was generated using buffer A, wash and elution steps may be performed by switching to urea-based buffers or by continuing with Gu–HCl-based buffers with pH values adjusted accordingly.

5. Elute His-tagged protein with 5 bv of elution buffer E (100 mM NaH$_2$PO$_4$, 10 mM Tris–HCl, 8 M urea, pH 4.5).

2.11. Cleaning and sanitization

A simple and effective cleaning-in-place (CIP) method for Ni-NTA IMAC resins used to purify proteins from "standard" samples such as *E. coli* or human cell lysates or supernatants is contacting the resin with 0.5 M NaOH for 30 min (Schäfer *et al.*, 2000). The resins have been stored in up to 1 M NaOH for several months and shown to withstand these conditions without

compromising its performance even upon >100 cycles of purification/CIP cycles (data not shown). This CIP procedure reliably denatures and desorbs proteins originating from the loaded sample that might have bound unspecifically to agarose during purification and is generally suitable for sanitization (depyrogenation, viral, and microbial clearance; Levison *et al.*, 1995). Cleaning protocols may have to be adapted if more ''unusual'' samples such as lysates rich in lipids are loaded onto a column. Bases, acids, and other reagents that may be used for cleaning include ethanol (100%), isopropanol (30%, v/v), SDS (2%, w/v), acetic acid (0.2 M), NaOH (1 M), or detergents (see Table 27.2).

For repeated reuse of a Ni–NTA column, we recommend to perform the CIP description provided above followed by reequilibration. For long-term storage (several years), resin may be kept in either 30% (v/v) ethanol or, if an inflammable reagent is preferred, in 0.01–0.1 M NaOH. Storage in 10 mM NaN$_3$ is possible as well. It is usually not required to strip off the metal ions and recharge Ni–NTA, even after repeated reuse or long-term storage.

2.12. Simplified metal-ion stripping and recharging protocol

However, in cases where the resin has been seriously damaged or if binding capacity decreased over time, for example, by repeated loading of lipid–rich or samples containing chelating components, Ni–NTA may be easily stripped and recharged with nickel or a different metal ion. Starting with step 3, this simplified protocol is also suitable to initially charge NTA resin purchased uncharged.

1. Wash cleaned (see above) resin with 10 bv of deionized H$_2$O (dH$_2$O).
2. Strip off metal ions by passing 5 bv of 100 mM EDTA, pH 8.0 over the resin bed.
3. Wash resin with 10 bv of dH$_2$O.
4. Pass 2 bv of a 100 mM metal ion aqueous solution (e.g., NiSO$_4$ or NiCl$_2$) over the resin bed.
     Other metal ions that have been successfully and stably immobilized to NTA include copper (CuCl$_2$, CuSO$_4$), zinc (ZnCl$_2$, ZnSO$_4$), cobalt (CoCl$_2$, CoSO$_4$), and iron (FeCl$_3$, Fe$_2$(SO$_4$)$_3$).
5. Wash resin with 10 bv of dH$_2$O to remove any unbound metal ions.
6. Add storage buffer or equilibrate the column with at least 5 bv of starting buffer for immediate use.

3. CONCLUSIONS

We have presented the wide variety of applications the IMAC principle offers for research in general and for production of His-tagged proteins in particular. Its robustness and versatility are the reasons why IMAC has

become one of the most broadly used chromatographic methods. Modifica-
tions in production procedures for both resin and ligand materials as well as
optimized application protocols led to a significant improvement of IMAC
performance in the recent past, well reflected by, for example, the increase
of binding capacity of both NTA- and IDA-based matrices for His-tagged
proteins from 5–10 to 50 mg per ml resin bv. We anticipate a continued
methodological improvement and dissemination of the use of IMAC in the
field of purification of recombinant proteins, for example, regarding indus-
trial-scale production of biopharmaceuticals.

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