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Methodology paper

Affinity purification of histidine-tagged proteins

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Abstract

Expression of recombinant proteins is a standard technique in molecular biology and a wide variety of prokaryotic as well as eukaryotic expression systems are currently in use. A limiting step is often the purification of the expressed recombinant protein, particularly if mammalian expression systems that yield low expression levels are employed. Here, we discuss the advantages and restrictions of tagging recombinant proteins with histidines and purifying them by Ni^{2+}-NTA chromatography.

Abbreviations: GST – glutathione S-transferase, NTA – nitrilotriacetic acid, His – histidine, PAGE – polyacrylamide gel electrophoresis

Introduction

Different classical separation procedures can be employed for purification of a recombinant protein that are based on its physicochemical properties such as its charge, size or hydrophobicity. These procedures are often time-consuming and laborious and consequently simple and rapid alternatives for purification have recently been developed. Such procedures make use of a particular property of a protein moiety or short amino acid sequence that is fused to the recombinant protein as a 'tag' [1]. Examples of techniques exploiting this strategy are: (a) the glutathione S-transferase (GST) fusion system used together with glutathione-Sepharose beads [2], (b) the protein A fusion system combined to immunoglobulin columns [3], (c) epitope-tagging used together with specific antibodies [4] and (d) the histidine-tagging technique for metal chelate affinity chromatography [5]. In this review, we will focus on Ni^{2+}-NTA chromatography.

The Ni^{2+}-NTA purification system: general considerations

Immobilized metal-affinity chromatography was initially used for the fractionation of proteins rich in residues contributing to metal binding, essentially histidines and reduced cysteines [6]. The initial reports used iminodiacetate which coordinates first row transition metals through three sites. The Cu^{2+} cation having four coordination sites appeared to yield insufficiently strong binding of proteins. Ni^{2+} which has six coordination

sites, was only weakly bound to the matrix and was washed out during loading of a protein extract. Recent development of nitrilotriacetic acid (NTA), which has four chelating sites, solved this problem [5]. It allows a stable interaction between Ni^{2+} and column matrix leaving two metal coordination sites free to interact with functional groups of proteins (see Fig. 1).

A stretch of 6 histidine residues (His-tag) linked to the N- or C-terminal part of the recombinant protein is sufficient to allow a high affinity interaction with the Ni^{2+}-NTA resin ($Kd = 10^{-13}$, pH 8.0) [7]. The Ni^{2+}-NTA resin has also a high capacity (5 to 10 mg protein per ml of resin). Bound proteins are subsequently eluted from the resin upon protonation of the interacting amino acid side chains or by displacement with other metal binding ligands such as imidazole, a precursor of histidine.

Several cloning vectors for different expression systems have been described, some of which are commercially available. These include vectors for bacteria (Qiagen or Invitrogen), vaccinia virus [8] and transient [9] as well as stable expression in mammalian cells (Invitrogen). The vectors are designed to permit fusion of the His-tag either to the N- or C-terminus, with or without a protease cleavage site allowing the removal of the foreign amino acids. For example, thrombin cleavage

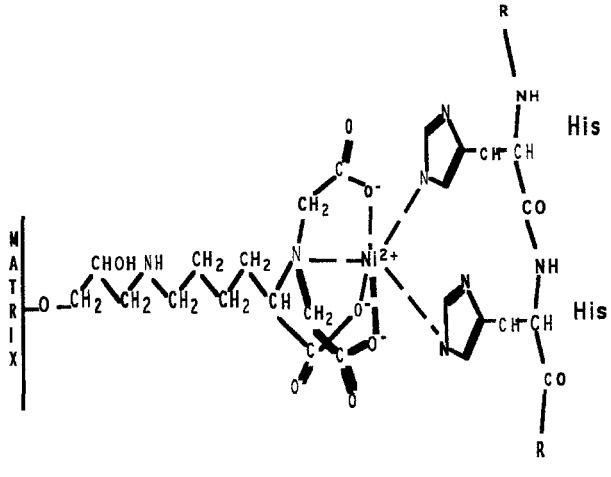


Fig. 1. Interaction between a His-tagged protein and the Ni^{2+}-NTA resin. NTA, linked to Sepharose CL-6B, is commercially available from Qiagen/Diagen.

was used by Nikolow and coworkers to remove the His-tag from recombinant bacterially expressed TATA binding protein used for crystallographic studies [10].

Depending on the use intended for the recombinant protein, it may be judicious to add the minimum number of foreign amino acids, that is the 6 His residues. In this case, the tag can be added to the cDNA using either PCR or inserted into an existing expression vector using a small fragment encoding 6 histidines. It should be mentioned that a triplet encoding histidine (CAC or CAG) which is placed immediately adjacent to the ATG might create a sub-optimal sequence for initiation of translation in mammalian cells [11]. For secreted proteins, the tag should not be placed at the N-terminus because it would be removed together with the leader sequence. If the protein is subject to proteolytic degradation, it is worth considering that degradation products bearing the His-tag will also be enriched.

Using Ni^{2+}-NTA under denaturing conditions

Interaction with the Ni^{2+}-NTA matrix does not require a particular conformation of the protein. Therefore proteins can be purified under denaturing conditions. This procedure is particularly useful for the purification of bacterially expressed proteins which are often obtained as aggregates, as observed in the example shown in Figure 2. Moreover, the presence of 6 M guanidiniumhydrochloride (GuHCl) allows an efficient inactivation of all contaminating phosphatases and proteolytic enzymes, thereby facilitating, for example, the purification of phosphoproteins without detectable dephosphorylation [8]. Proteins are eluted under these conditions from the column by reducing the pH to 4.5. This will protonate the histidines and disrupt their interaction with the metal. Before eluting His-tagged proteins, it is worth washing the column at pH 6 in order to remove weakly interacting proteins.

Technical details of the procedure to be followed for purification of proteins under denaturing conditions using Ni^{2+}-NTA are given in the

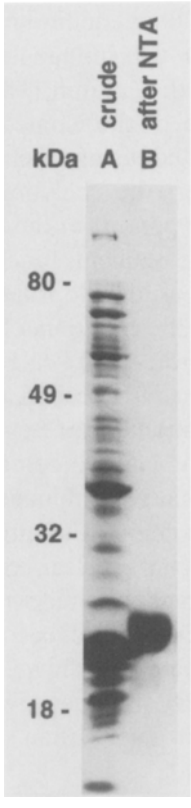


Fig. 2. Purification under denaturing conditions of a bacterially expressed N-terminal fragment of mouse p59. Coomassie stained PAGE showing (A) the proteins present in inclusion bodies and (B) the NTA purified recombinant N190.
Methods: Bacteria transformed with pJHC-N190 were grown up until OD_{600} = 0.6, then induced during 1 h with 0.5 mM IPTG using standard techniques [12, 20]. Bacteria were lysed in 0.1 culture volume of 50 mM Tris-Cl pH 8, 50 mM NaCl, 1 mM PMSF, 10 % glycerol using a freeze-thawing procedure. After centrifugation at 100,000 g, the pellet was resuspended in buffer G (6 M GuHCl, 0.1 M Na-phosphate, 10 mM Tris), pH 8 (lane A) and applied in batch on Ni^{2+}-NTA during 1 h. The loaded resin was washed using buffer G pH 6 and bound proteins were eluted with buffer G pH 4 (lane B). GuHCl was removed using reverse phase chromatography as described [21].

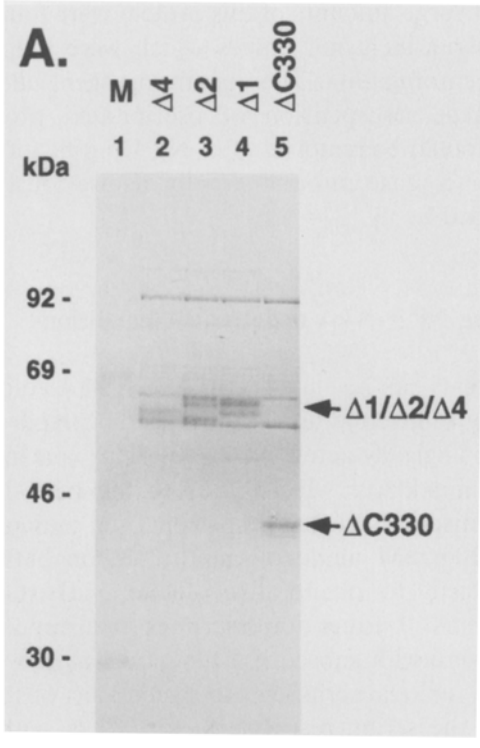
following example. To produce antibodies against mouse p59, an immunophilin which is believed to be analogous to heat shock protein 56, a fragment encoding the N-terminal 190 amino acids with a His-tag was expressed in bacteria [12]. The recombinant protein was purified in the presence of 6 M GuHCl as shown in Figure 2. Probably due to its high expression level, large amounts of this protein were found in bacterial inclusion bodies which were prepared by centrifugation. The denaturing agent allowed efficient resuspension of precipitated proteins and could be removed after NTA purification by reverse phase chromatography, as previously described by us [8].

Using Ni^{2+}-NTA under native conditions

Proteins can be eluted from Ni^{2+}-NTA columns using mild conditions, allowing the preparation of biologically active molecules. This is achieved with imidazole which binds to the Ni^{2+}-NTA and displaces the tagged protein. The presence of 50–100 mM imidazole in the elution buffer is sufficient to quantitatively elute 6 His-tagged proteins. Furthermore, it does not appear to interfere with subsequent functional assays such as *in vitro* transcription experiments, as observed with the serum response factor [8] or with the glucocorticoid receptor [13]. It is important to adjust the pH of imidazole solutions to neutral values.

The high binding affinity of Ni^{2+}-NTA resin allows efficient coupling even in very dilute solution such as secreted proteins in culture media. The column should be loaded slowly (3 to 4 column volumes per hour). Alternatively a batch procedure, which might be more efficient if large volumes have to be handled, can be used. The matrix is then added to the extract and incubated at 4 °C for 2 h on a rotating wheel. The NTA beads can be recovered by centrifugation at 1,000g for 2 min. To reduce non-specific interactions, it is possible to include low concentrations of imidazole (up to 8 mM), non ionic detergents (up to 0.1% Triton X-100 or Nonidet P-40) or high salt concentrations (up to 1 M) in column washing solutions. In addition, we recommend to determine the optimum quantity of matrix to be used since His-tagged proteins when saturating the matrix will prevent binding of weakly interacting proteins.

Elution of bound proteins from Ni^{2+}-NTA, either in column or batch procedure, should be



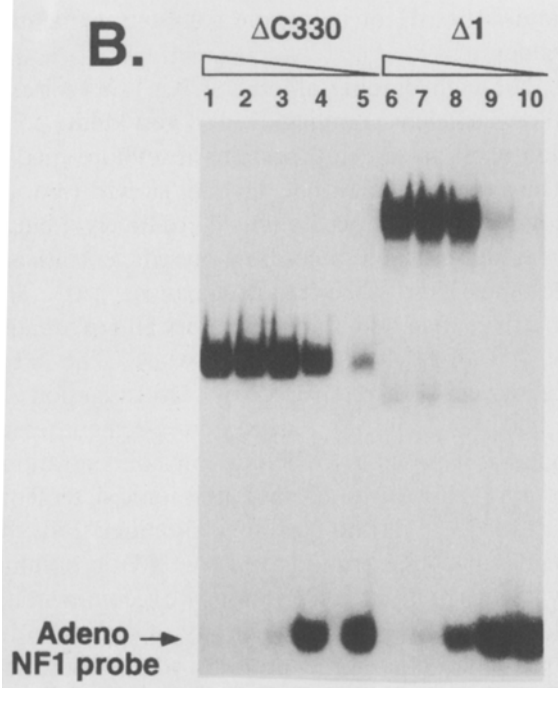
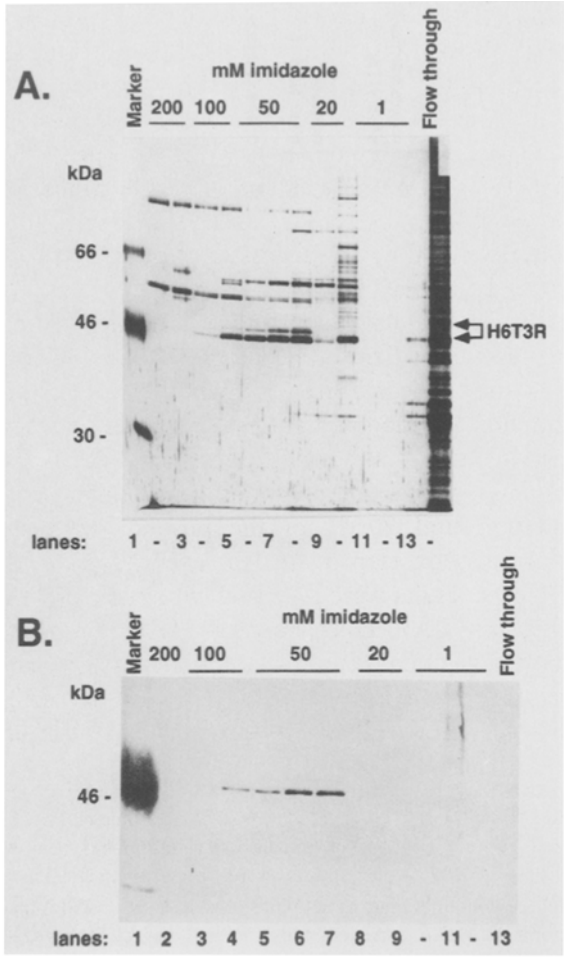


Fig. 3. Deletion mutants of nuclear factor 1 expressed in HeLa cells using recombinant vaccinia viruses. Panel A: Coomassie stained SDS-PAGE of Ni^{2+}-NTA enriched proteins. Panel B: DNA binding activity of two NF1 mutants.

done slowly to allow equilibrium to be reached, that is to allow the imidazole to occupy the binding sites on the column. This helps to minimize the volume of the eluate. Bound proteins can also be eluted using chelating agents like EDTA. However, the chelating substance and the metal will be present in the eluate and might interfere with subsequent functional assays or with the activity of metalloproteins.

To determine the function of various regions from nuclear factor 1 (CTF/NF1), we produced mutant proteins using vaccinia virus vectors and purified the recombinant proteins in native conditions using Ni^{2+}-NTA columns. Coomassie staining shows that recombinant products represent around 20 % of the proteins after one fractionation step of nuclear extracts (Fig. 3A). Similar results were obtained with other proteins (see Fig. 4A). DNA-binding of NF1 deletion mutants is unaffected as shown by mobility shift assay (Fig. 3B). By this assay, we estimated that an enrichment of more than 100 fold of the

Methods: HeLa S3 cells (10^6 cells/ml) were infected for 20 h with recombinant vaccinia viruses at a multiplicity of infection of 5. Nuclear extracts were prepared according to Dignam et al. [22] in 20 mM Hepes pH 7.9, 5 mM $MgCl_2$, 420 mM NaCl, 5 mM β-mercaptoethanol, 10 mM NaF, 0.1 mM PMSF, 1 μM pepstatin, 1 μM leupeptin and 0.13 mM bestatin. 1 ml Ni^{2+}-NTA agarose (Qiagen) was added to the extract from 3 liters of cells and incubated on a rotating wheel for 2 h at 4°C. The resin was packed into a column and washed at 0.2 ml/min with 5 column volumes of buffer D300 (20 mM Hepes pH7.9, 5 mM $MgCl_2$, 10 mM NaF, 15% glycerol and 300 mM NaCl). The resin was further washed with 3 column volumes of buffer D100 (as buffer D300 but with 100 mM NaCl) and with 3 column volumes of D100 containing 20 mM imidazole. The His-tagged receptors were eluted in several fractions of 0.5 column volume with D100 containing 100 mM imidazole. The peak fraction (1 ml), shown in panel A, was estimated to contain more than 100 mg NF1 derivative per ml as deduced from the mobility shift assay (panel B) using an oligonucleotide derived from the sequence of the adenovirus origin of replication and proceeding as previously described [23]. We added 1μl (lanes 1 and 6), 0.1μl (lanes 2 and 7), 0.01μl (lanes 3 and 8), 0.001μl (lanes 4 and 9) and 0.0001μl (lanes 5 and 10) from the peak fraction in a binding reaction. The NF1 deletion mutants described in this example lack the amino acids 239 to 255, 258 to 282, 313 to 338, 314 to C-terminus for Δ1, Δ2, Δ4 and ΔC330, respectively.



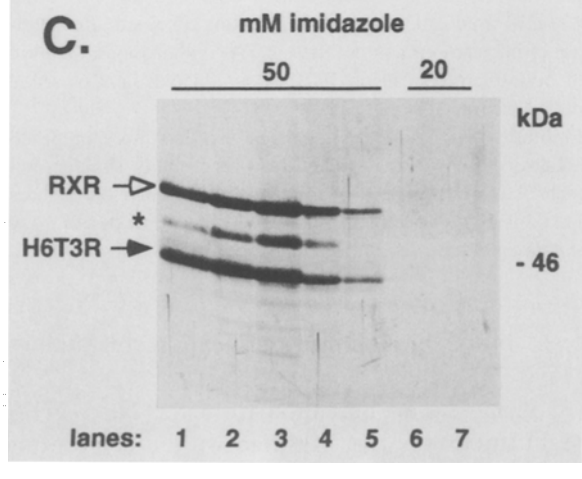


Fig. 4. Co-purification thyroid hormone and retinoid X receptors. Panel A: Silver stained SDS-PAGE of fractions obtained from the purification of His-tagged thyroid hormone receptor (H6T3R) using Ni^{2+}-NTA chromatography. Panel B: Analysis by Western blotting using a T3R specific antibody (generously obtained from H. Beug) of the same fractions as in panel A. Panel C: Copurification of the retinoid X receptor (RXR) together with H6T3R. The star indicates an RXR degradation product.

Methods: Recombinant vaccinia viruses expressing H6T3Rα and RXRα were obtained as previously reported [15]. Extract preparation and protein purification were performed as described in figure legend 3, except that 1 ml Ni^{2+}-NTA agarose (Qiagen) was added to the extract from 10 liters of cells. Western blotting was performed using standard techniques [24]. RXR was detected with a polyclonal antiserum raised against bacterially expressed protein (J. Pohl and H.G.S., unpublished).

overexpressed proteins was achieved in only one step. Endogenous CTF/NF1, which is abundant in a crude nuclear extract (not shown), was undetectable by mobility shift assay after this purification step (Fig. 3B, lane 1).

Co-purification of associated proteins

One of the advantages of the His-tagging system is that protein/protein interactions can be preserved during Ni^{2+}-NTA chromatography under native conditions. We and others have shown that thyroid hormone receptor (T3R) and retinoid X receptor (RXR) can form stable heterodimers in solution and upon binding to DNA (for review: [14]). Thus, expression and subsequent purification of His-tagged T3R (H6T3R) expressed in HeLa cells using recombinant vaccinia viruses also results in the co-purification of its heterodimer partner. The heterodimer partner can be either endogenous retinoid X receptor (RXR), present at low levels in HeLa cells, or vaccinia virus co-expressed RXR (Fig. 4).

H6T3R expressed in HeLa cells using recombinant vaccinia virus can be quantitatively bound under native conditions to the Ni^{2+}-NTA resin and eluted with 50 mM imidazole (Fig. 4A and B). Two polypeptides, detected by Western blotting, correspond to the phosphorylated and unphosphorylated forms of the recombinant thyroid hormone receptor. The low level of endogenous RXR cannot be detected by western blotting, but was shown to co-purify by gel retardation assays

[15]. Co-expression of H6T3R along with RXR and subsequent purification of H6T3R using Ni^{2+}-NTA results in co-purification of the recombinant RXR (Fig. 4C). RXR, in the absence of H6T3R, appears to have a low but appreciable affinity for Ni^{2+}-NTA and can be eluted with 20 mM imidazole ([15]; O. Lonnoy and H.G.S., unpublished data). From these experiments, we concluded that RXR can co-purify with H6T3R via protein-protein interactions mediated by the dimerization interfaces of both receptors.

Enrichment of His-rich cellular proteins

Certain cellular proteins have a high affinity for the Ni^{2+}-NTA resin. As shown in Fig. 4A (lanes 2–4), several proteins require high concentrations of imidazole to be released from the column. Upon fractionation of HeLa cytoplasmic or nuclear extracts on NTA columns, it was noticed that an octamer binding protein was enriched from nuclear extracts. As shown in Figure 5, this activity binds to the octamer site of the immunoglobulin gene heavy chain enhancer (lanes 4 and 5). Using specific antibodies, the octamer binding activity was identified as OCT6, previously believed to be expressed only in undifferentiated cells ([16] and data not shown). OCT6 contains a naturally occurring stretch of 6 histidines near the C-terminus (amino acids 436–441) as well as another His rich sequence (amino acids 208–216). Both stretches could mediate the high affinity binding of OCT6 to the NTA matrix [16]. The ubiquitous OCT1 protein which is much more abundant than OCT6 in HeLa nuclear extracts is essentially removed after NTA purification (compare lanes 4/5 and 6). The NTA matrix can therefore be considered as a very efficient tool for the purification of OCT6 from any source.

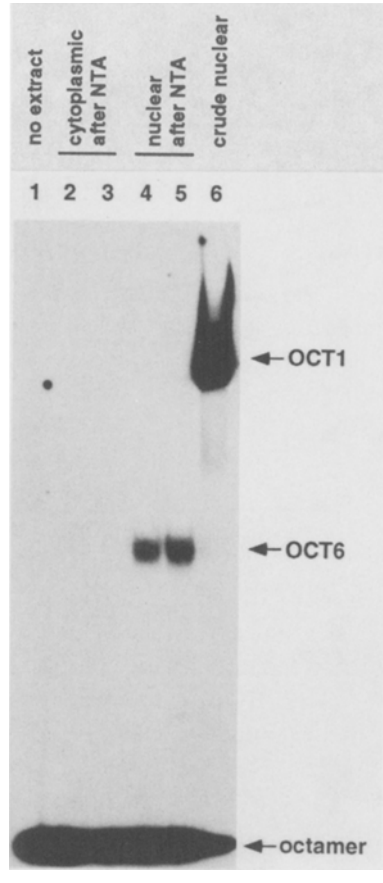


Fig. 5. Octamer binding activities present in Ni^{2+}-NTA fractionated HeLa extracts. Lane 1: no extract; lanes 2–3: fractions obtained after loading of cytosolic extracts on Ni^{2+}-NTA Sepharose and elution of bound proteins using 100 mM imidazole; lanes 4–5: fractions obtained after loading of nuclear extracts on Ni^{2+}-NTA Sepharose and elution of bound proteins using 100 mM imidazole; lane 6: crude nuclear extract.
Methods: Cytoplasmic and nuclear extracts were prepared according to Dignam et al. [22]. The mobility shift experiment was performed using previously described procedures [13]. The 1W octamer binding site is derived from the immunoglobulin heavy chain enhancer [25].

Discussion

Advantages

Metal chelate affinity chromatography, using Ni^{2+}-NTA resin offers a number of advantages over other bio-affinity purification techniques [17].

(a) Proteins can be eluted from the Ni^{2+}-NTA resin under mild conditions, without disrupting their folded conformation or possible protein-protein interactions and thus preserving their biological activity [8,13].

(b) The His-tag appears not to interfere with the

activity of the protein to which it is coupled [8, 9, 13, 18]. Thus normally it is not necessary to remove the tag from the recombinant protein. The removal of the His-tag is, however, possible if a protease cleavage site is inserted between the tag and the protein (for examples, see [10, 19]).
(c) The procedure allows quantitative binding and elution of tagged proteins.
(d) The Ni^{2+}-NTA procedure can also be used under denaturing conditions. This can be useful in cases where the tag is inaccessible from the surface of the molecule or if the protein is insoluble [7].
(e) Ni^{2+}-NTA resin is stable over a wide pH range (pH 2.5 to 13), in the presence of detergents (2% sodium dodecyl sulfate), disruptive eluents (8 M urea or 6M GnCl) or solvents (100% ethanol). The high stability of the resin allows regeneration and multiple use of the matrix. Compared to other affinity purification techniques, it is relatively inexpensive and can therefore be considered advantageous for large scale preparations.
(f) The Ni^{2+}-NTA resin is not affected by protease or nuclease activities present in the extracts. Therefore, in contrast to most other affinity purification procedures, it can be used as the first purification step. This allows the recovery of semi-purified and concentrated tagged-proteins in one step only.

Limitations

(a) Proteins are retained on Ni^{2+}-NTA columns according to the number of accessible histidines [17]. Although His is a lowly represented amino acid (2%), certain proteins bear naturally contiguous His residues. They will co-purify with the His-tagged product and contaminate the semi-purified recombinant product. However the likelihood that these natural proteins will have a lower selectivity of interaction with the Ni^{2+}-NTA resin can be exploited by more stringent washing procedures.
(b) In some cases, the recombinant protein does not interact in its native form with the Ni^{2+}-NTA resin. This may occur when the tag is inaccessible in the folded state of the protein. If an active molecule is required, one can try placing the tag at the other end of the protein. However, it is possible to purify the protein in any case under denaturing conditions. Refolding of denatured protein then becomes the limiting step.
(c) Buffer components affecting the interaction of metal with the column matrix should be avoided. Therefore, it is not advisible to use metal chelators (like EDTA) or strong reducing agent (like DTT). Under reducing conditions, the color of the column changes from blue to brown and the column loses its capacity. To minimize oxidation of the recombinant protein and to disrupt interacting cysteines, it is possible to use up to 10 mM β-mercaptoethanol.
(d) Due to its charge, the Ni^{2+}-NTA resin can also act as a weak ionic exchange column. This can however be overcome by washing the column with a buffer containing an elevated salt concentration (up to 1 M).

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