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Immobilized palladium(II) ion affinity chromatography for recovery of recombinant proteins with peptide tags containing histidine and cysteine

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Fusion of peptide-based tags to recombinant proteins is currently one of the most used tools for protein production. Also, immobilized metal ion affinity chromatography (IMAC) has a huge application in protein purification, especially in research labs. The combination of expression systems of recombinant tagged proteins with this robust chromatographic system has become an efficient and rapid tool to produce milligram-range amounts of proteins. IMAC-Ni(II) columns have become the natural partners of 6xHis-tagged proteins. The Ni(II) ion is considered as the best compromise of selectivity and affinity for purification of a recombinant His-tagged protein. The palladium(II) ion is also able to bind to side chains of amino acids and form ternary complexes with iminodiacetic acid and free amino acids and other sulfur-containing molecules. In this work, we evaluated two different cysteine- and histidine-containing six amino acid tags linked to the *N*-terminal group of green fluorescent protein (GFP) and studied the adsorption and elution conditions using novel eluents. Both cysteine-containing tagged GFPs were able to bind to IMAC-Pd(II) matrices and eluted successfully using a low concentration of thiourea solution. The IMAC-Ni(II) system reaches less than 20% recovery of the cysteine-containing tagged GFP from a crude homogenate of recombinant *Escherichia coli*, meanwhile the IMAC-Pd(II) yields a recovery of 45% with a purification factor of 13.

Keywords: metal ion affinity chromatography; palladium(II); recombinant protein purification

INTRODUCTION

The development of novel tools for protein purification is always welcome at research laboratories. One of the most successful concepts developed in the field of affinity chromatography is its combination with peptide-based tags onto recombinant proteins (Hearn and Acosta, 2001; Terpe, 2003). The design of new affinity tags with novel features is an important field of research because of the high demand for cheap, efficient, and fast methods for purification of recombinant proteins (Widakowich *et al*., 2011).

At present, peptide tags are widely used to facilitate recombinant protein expression and purification. During the last decades, significant progress has been made in identifying the strengths and weaknesses of various labeling options (Lichty *et al*., 2005).

The use of inorganic ions immobilized on beaded agarose as affinity ligands was described in the 1970s. The first applications were fractionation of RNA and proteins of medical relevance such as fibroblast interferon (Shankar and Joshi, 1974; Edy *et al*., 1977). The technological breakthrough of this chromatography occurred in the late 1980s when this technique was used to purify a protein with a 6xHis tag added at its *N*- or *C*-terminal sequence. The combination of expression systems of recombinant tagged proteins with a robust chromatographic system has become an efficient and rapid tool to produce milligram-range amounts of proteins in research labs. The commercial availability of iminodiacetic acid (IDA) and nitrilotriacetic acid ion-chelating matrices has also contributed to expanding this technique significantly. The immobilized metal ion affinity chromatography (IMAC) has become the natural partner of 6xHis-tagged proteins.

Several applications of IMAC with metal ions have been reviewed in detail by several authors (Arnold, 1991; Gaberc-Porekar and Menart, 2005). The cations Cu(II), Ni(II), Co(II), and Zn(II) have been used to purify proteins containing His on their surface (Gaberc-Porekar and Menart, 2005). The degree of interaction depends on the strength of the cation interaction with the imidazole group of His. Thus, Cu(II) allows purifying proteins that contain only one His on its surface, whereas Ni(II) requires at least two spatially close His, and Co(II) also requires a spatial arrangement (conformation alpha helix 1–3) (Chaga, 2001). The Ni(II) ion is considered as the best compromise of selectivity and affinity

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for purification of a recombinant protein with His tag. From a lysate of *Escherichia coli*, a recombinant tagged protein can be purified up to 80–90%. Relatively high-ionic-strength buffers are usually used during the adsorption step to remove the contribution of electrostatic interactions from the chelator and/or cation.

Other strong Lewis acid cations such as Fe(III), Ca(II), and Yb(III) have also been studied. The Fe(III) ion in a slightly acid medium has specific affinity for phosphate groups (Grazyna *et al*., 1992), allowing purification of phosphorylated proteins. Similarly, the Al(III) cation has affinity for carboxylate (Gaberc-Porekar and Menart, 2001). However, in the bioprocess area, these features have not been extended to affinity chromatography systems because of protein phosphorylation difficulties or the high incidence of carboxylate moieties in proteins.

Other metal ions can also complex amino acids and potentially be used to purify proteins. These cations, which include Cu(I), Ag(I), Hg(I), Cd(II), Pt(II), and Pd(II), are classified as soft Lewis acids and have not been used for protein purification. One exception is phenylmercuric agarose (Affi-Gel 501 from Bio-Rad, discontinued), which has been used to isolate sulfhydryl-containing proteins (Beadling *et al*., 1993). However, the ligand is an organomercurial compound instead of a chelated mercuric ion. Most of these ions have redox properties. However, for the particular case of Pt(II) and Pd(II), they can generate stable complexes with organic ligands having weak Lewis acid (S) or mixed (O, S, N) moieties. In this work, we explore the replacement of cysteines (Cys) into the His tag of a recombinant protein to analyze the adsorbing/desorbing properties to IMAC-Pd(II) system. Cys was selected as an alternative amino acid to build a novel tag with a modified specificity for protein adsorption. Generally, Cys has usually been avoided to use in affinity tags as a consequence of it high instability. However, the reactivity of sulfhydryl groups can be useful to perform chemical modifications of the tagged protein or used as a linker for further immobilization protocols.

MATERIALS AND METHODS

Materials

The IMAC Sepharose fast flow was obtained from GE Biosciences (Buenos Aires, Argentina). According to the company, the chelating group corresponds to IDA. $NiCl_2$ and $PdCl_2$ were purchased from Sigma Chem. Co. (St. Louis, MO, USA). All other chemical reagents were of analytical grade purity. Double distilled water was used to prepare all the solutions.

Protein source

Escherichia coli strain and plasmid

The host strain used in this study was *E. coli* BL-21(DE) (Invitrogen). The recombinant plasmid pET-28a(+) expression vector for *N*-terminus tagged GFP expression was derived by inserting the GFP gene and the hexapeptide-encoding sequence used as a purification tag at the *N*-terminus of the GFP sequence. One clone containing GFP was prepared with the 6xHis tag (called GFPHis6), and other two clones were prepared by replacing the second, fourth, and sixth positions with Cys or Ser. This allowed obtaining the following tag sequences: HCHSHS (called GFPCys1) and HCHCHC (called GFPCys3) (Figure 1). Clones were confirmed by sequencing the starting material of the reading frame of GFP plasmid. We found 100% sequence identity.

Fermentation and culture conditions

The transformed *E. coli* strain was cultured under standard conditions in sterilized Luria broth agar medium at 37 °C for 18 h. A single colony was inoculated into a 500-ml shake flask containing 150 ml of Luria broth medium (tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l, and glucose 2 g/l) and 15 µl of 100 mg/ml ampicillin and incubated at 37 °C and 200 rpm for 10 h. This culture was used as pre-inoculum for GFP production.

The GFP was produced in a 5-l BioFlow III reactor (New Brunswich) containing 3 l of Riesenberg medium (Kweon *et al*., 2001) and 3 ml of 100 mg/ml ampicillin. The inoculum in the same medium was prepared from the pre-inoculum. The agitation speed was gradually increased to prevent the limitation of dissolved oxygen. During fermentation, aqueous ammonia (25%) was used to maintain the pH at 6.9. After the initial supply of glucose was consumed, a solution of glucose (400 g/l) and magnesium sulfate (15 g/l) was fed into the fermenter. When the OD reached 20.0, the culture was induced adding isopropyl-b-D-thiogalactopyranoside to a final concentration of 0.5 mmol/l. The culture was allowed to grow for another 4 h at 28 °C. All culture parameters (pH, pO2, temperature, agitation,

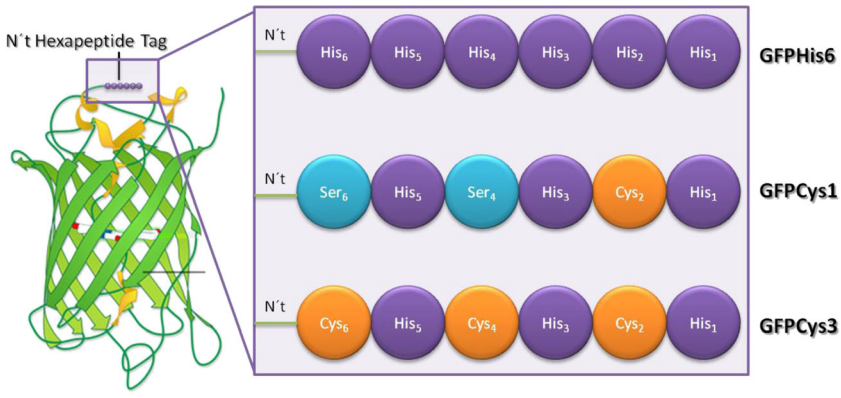


Figure 1. Schematic representation of the different variants of tagged GFP used in this work. Tags were inserted in the *N*-terminal of GFP sequence. The sub-index indicates amino acid sequence position. The black frame shows the highly relevant amino acid positions for the metal ion interaction (first, third, and fourth). The fourth position was the most important variant among three recombinant proteins (His, Ser, or Cys for GFPHis6, GFPCys1, or GFPCys3, respectively).

aeration, ammonia volume, and glucose feeding) were recorded and monitored.

The *E. coli* pellet was then harvested by centrifugation at 8 000 rpm for 20 min at 4 °C and stored at −20 °C until required for use.

Green fluorescent protein recovery and purification

Cells were disrupted by a Niro Soavi PANDA 2000 homogenizer device. The supernatant was clarified by centrifugation and deep filtration. After diafiltration (20×) with 30 mM phosphate buffer pH 7, GFP recombinant proteins were purified by IMAC-Ni(II) eluted with imidazole gradient (0–250 mM). Fractions were collected and analyzed for protein content by Bradford's method and determination of relative fluorescence units (RFU). The main elution peak was pooled and desalted in a Sephadex G-25 column and used for IMAC experiments.

The GFP variants were further purified in order to calculate the maximum specific fluorescence and determine GFP purity. GFP variants eluted from Ni(II)-IMAC columns were purified by ion-exchange chromatography (DEAE Sepharose FF, GE Healthcare) equilibrated with 50 mM Tris-HCl buffer pH 7.5 and eluted in a linear gradient of NaCl in 20 column volume (CV) (from 0 to 0.5 M). Subsequently, fluorescent eluted proteins were conditioned in 50 mM phosphate buffer pH 6.0 by size exclusion chromatography (PD-10, GE Healthcare). Maximum specific fluorescence was determined from fluorescence and absorbance at 280 nm measurements of pooled GFP variants peak. Extinction coefficient at 280 nm of 20 000 $M^{-1} \cdot cm^{-1}$ for all GFP variants was used (Jung et al, 2005). Maximum specific fluorescence of 1 160 000 RFU/mg, 450 000 RFU/mg, and 340 000 RFU/mg were the yields for GFPHis6, GFPCys1, and GFPCys3, respectively. Purity of GFP variants used for adsorption–desorption experiments were 65%, 60%, and 58% for GFPHis6, GFPCys1, and GFPCys3, respectively.

Immobilized metal ion affinity chromatography experiments

Adsorptive/desorptive protocols

The IMAC Sepharose fast flow resin (1 ml) was packed in polypropylene columns. Adsorptive studies were performed according to the following procedure described in CV. The chromatographic matrix was washed with 0.1 N HCl (2 CV) and then charged with 10 mM $PdCl_2$ solution or 65 mM $NiCl_2$ solution, according to the experiment. Subsequently, the column was washed with distilled water (3 CV) and equilibrated with 50 mM phosphate buffer, pH 6.0 (5 CV). After adsorption of one of the recombinant GFP variants (GFPHis6, GFPCys1, and GFPCys3) diluted in 50 mM phosphate buffer, pH 6.0, we performed a washing step with the same buffer (3 CV). The elution step was carried out with 10 CV eluting solution, and fractions were collected. Loss of Pd(II) cation from IMAC-Pd(II) columns was analyzed by measuring the Pd(II) content in the collected fractions by thiocyanate reaction (Antico *et al.*, 1994).

Adsorption experiments

Five IMAC-Pd(II) or IMAC-Ni(II) columns were used as adsorptive matrices to analyze the average protein adsorption using GFPHis6, GFPCys1, and GFPCys3. Adsorbed protein was determined by Bradford's method by subtracting the non-adsorbed proteins (present in the washing step fractions) from the total loaded protein.

Desorption experiments

Solutions of the following chemical reagents at 1 M concentration were prepared in 50 mM phosphate buffer, pH 6.0: imidazole, NaCl, thiourea, urea, guanidinium chloride, potassium thiocyanate, b-mercaptoethanol, ethanolamine, cysteamine, ethylenediamine, ethanol, ethylene glycol, dithiothreitol, thioglycolic acid, glycine, and oxalic acid. The elution efficiency of every solution was studied for GFPCys1 and GFPCys3 from one IDA-Ni(II) or IDA-Pd(II) column. Protein recovery was estimated by RFU in the elution fractions.

Gradient elution profile of tagged green fluorescent protein

Gradient elution chromatography was performed for GFPHis6, GFPCys1, and GFPCys3 on AKTA prime plus (GE Biosciences) using a 1-ml IDA-Pd(II) column and 0.1 M thiourea in 50 mM phosphate buffer, pH 6.0, as elution buffer between 0% and 100% in 20 CV. Then, 5 ml of protein solutions (according to Section on Immobilized metal ion affinity chromatography experiments) were injected. Online 280 nm absorbance was analyzed, and RFU of collected fractions were measured.

Protein recovery studies

Five columns containing IDA-Pd(II) matrices were used to estimate the average percentage of recovered protein over all the 0.1-M thiourea step elution. This procedure was carried out for three recombinant GFP variants. The robustness of this process was determined by performing six consecutive chromatographic runs onto three different columns using GFPCys1 as sample protein. No additional cleaning protocol was performed between runs.

GFPCys3 purification using IMAC-Pd(II) or IMAC-Ni(II)

The homogenate prepared as described in Section on Immobilized metal ion affinity chromatography experiments was used for GFPCys3 purification on AKTA prime plus (GE Biosciences) using a 1-ml IDA-Pd(II) or IDA-Ni(II) columns. For IDA-Pd(II) purification, the elution buffer was 0.1 M thiourea in 50 mM phosphate buffer pH 6.0 between 0% and 100% in 20 CV. For the IDA-Ni(II) process, the same conditions were used replacing 0.1 M thiourea by 0.5 M imidazole. Detection was performed online at 280 nm absorbance and by RFU measurements of collected fractions.

Protein analysis and quantification

The samples were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis 15% gels on the basis of the procedure of Laemmli. Each gel was run with a 20 mA current with a standard power supply and stained with Coomassie brilliant blue method.

The RFU were determined in a 2-μl sample in the Nanodrop 3300 and relativized to the initial RFU loaded in the column. Samples were diluted with 50 mM phosphate buffer, pH 6.0, or the corresponding eluent solution. In all cases, the RFU correspond to the fluorescence emission at 506 nm.

Protein concentrations were determined from UV absorbance at 280 nm and Bradford's method using Coomassie dye (purchased

from Bio-Rad (Rockford, IL, USA)). Calibration curves were made using the corresponding protein (diluted in 50 mM phosphate buffer, pH 6.0, or the corresponding eluent solution). Extinction coefficients at 280 nm were 1 ml · $mg^{-1} \cdot cm^{-1}$ and 20 000 $M^{-1} \cdot cm^{-1}$ for crude extract and GFP variants, respectively (Jung et al, 2005).

RESULTS AND DISCUSSION

The IMAC has become one of the most successful laboratory-scale purification systems of recombinant proteins. The versatility of this system, on the basis of the chemistry of inorganic ion complexation, allows original alternatives of specific chemical interactions that can be exploited for selective purification of proteins. In this work, this particularity is explored through Pd(II) cation as an alternative in conjunction to original amino acid tags introduced to a recombinant protein.

It is well-known that the Pd(II) cation produces stable complexes with organic ligands having weak Lewis acid (S) or mixed (O, S, N) moieties. The Pd(II)-aqua complexes, which bind to the side chains of Met and His of peptides and proteins, can catalyze regioselective hydrolytic cleavage of the adjacent peptide bond (Chen *et al*., 1996, 1998). However, this property can be minimized when Pd(II) is surrounded by N and/or S chelating moieties.

Many immobilized chelating agents, such as thiols, guanidine, salicyl, and 8-hydroxyquinoline, among others, have been described for Pd(II) (Gulko *et al*., 1972; Myasoedova *et al*., 1985; Antico *et al*., 1994; Iglesias *et al*., 1999) and studied for ion recovery. Most of them have been developed for hydrometallurgy purposes where its specific recovery from acid liquors containing other metal ions is still a challenge.

The Pd(II) cation immobilized onto a chromatographic matrix has been proposed to purify amino acids and proteins by Díez *et al*. (1995). The amino acid, glycine, has been used as a model to adsorb and desorb to 8-hydroxyquinoline-Pd(II) chromatographic resin (Díez *et al*., 1995). In that study, glycine was eluted by lowering the pH to less than 1 to reach 90% yield. Because hydrochloric acid was more efficient than sulfuric acid to reach high recovery yield, the authors concluded that the chlorine ion also contributes to the elution process. In another study, phosphine sulfide derivative was also prepared as a Pd(II) chelator in an IMAC matrix for amino acid interactions. Two hydrophobic amino acids, tryptophan and tyrosine, were studied in the adsorption mode. However, the polystyrene-based matrix used in that report became a highly hydrophobic material, which should be used in water/dioxane solvent to enhance the metal adsorption rate (Marques *et al*., 1999).

Iminodiacetic acid, one of the most used IMAC ligands, involves three interaction sites with the chelated ion, one amino and two oxygen atoms. The affinity of IDA–metal ion complex has a pK range of 5.54–10.40 (IDA-Fe(II) and IDA-Cu(II), respectively). In case of IDA-Pd(II), the pK is 9.62, a value that is higher than IDA-Ni(II) (pK 8.20) and only lower than IDA-Cu(II) (Gonzalez-Vilchez and Castillo, 1975).

Heavy metal ions are established human carcinogens. As for the other metals, the use of palladium that is biologically available in the form of metal ions involves health risks (Kielhorn *et al*., 2002). Metal ion leakage is considered in many cases to have little or no influence on the outcome of the purification protocol (Chaga, 2001). However, there is a potential risk of toxicity in case of metal ion leaking from the column. It is well described that leaching of metal ions from IMAC columns is governed by the type of ion, the type of chelating compound involved, and the sort of elution. One advantage of Pd(II) over other metal ions is its stronger affinity with the chelating moiety IDA (Gonzalez-Vilchez and Castillo, 1975).

On the other hand, His is the amino acid with the strongest affinity for metal ions. More than one decade ago, soluble 6xHis-Pd(II)-IDA ternary complexes were studied by magnetic resonance spectroscopy (Chen *et al*., 1999; Ward *et al*., 2001).

Besides, there is a large amount of evidence about the existence of coordination compounds involving Pd(II) and Pt(II) with organic molecules. One of the most relevant complexes is the chemotherapy drug named cisplatinum (Siddik, 2003). The presence of palladium complexes with sulfur-containing peptides has been demonstrated by mass spectrometry (Luo *et al*., 1999). This interaction may take different forms depending on whether the sulfhydryl group is a thioether or a disulfide. On glutathione, the thiolate anion can be complexed with two Pd(II) cations; in case of a peptide with S-methyl-cysteine (SMC), Pd(II) coordinates thioether and amide nitrogen located in the *C*-terminus, to form a six-member ring. Oxytocin, a cyclic peptide, showed the presence of a 1:1 complex, forming a chelate ring of five junctions through an amino group and a sulfur atom of the Cys at the *N*-terminus in acid medium (Luo *et al*., 1999). Interestingly, in all the cases studied, Pd(II) always had at least one coordination ligand to sulfur. On the other hand, Pd(II) complexes could be used as synthetic specific peptidases by the interaction of metal ion with His and Met residues (Milovic *et al*., 2003).

Crystallographic analysis of tetranuclear Pd(II)-Cys complexes has shown that the four metal atoms have similar coordination spheres, with somewhat distorted square-planar geometry. Each cysteine amino group coordinates to one of the metal atoms, and each thiolate anion of the cysteine bridges two metal atoms, so that each metal atom has two bridging thiolate ligands (Chen *et al*., 1998).

More recently, it has been reported that the ternary complex with SMC-Cys-Pd(II) is significantly more stable than those of other amino acids with N or O as donor sites or ornithine with N, as a donor. This is consistent with the high affinity of both S and N atoms to Pd(II) coordination. Cys complexed at pH 2, with a formation percentage of 98% (Shehata *et al*., 2008). The preference of Pd(II) to coordinate with S-donor ligands has been recently demonstrated by Shoukry, (2009). With regard to inorganic ions, it is important to mention that the chloride ion can coordinate Pd(II) in acid solutions and that their presence could modulate the interaction with proteins.

On the basis of this experimental evidence, it appears that sulfur-containing amino acids are able to generate high-affinity ternary chelates with Pd(II). The amino acid side chains with ion-interaction capabilities are generally classified in three major categories: (i) non-coordinating (e.g., Ala, Val, Leu, Ile, Phe, and Trp); (ii) weakly coordinating (e.g., Ser, Thr, Tyr, Lys, Arg, Asp, Glu, Asn, Gln, Arg, and Met); and (ii) strongly coordinating (e.g., His and Cys). However, this classification is rather arbitrary because of the high metal ion dependence of the coordination ability (Sóvágó and Osz, 2006).

Histidine is the amino acid with the strongest affinity for metal ions, and the affinity tag peptide containing the hexamer 6xHis is the most used in the IMAC system. However, not all His interact with the chelated ion, because it has only three or four free

coordination bonds for the interaction. Thus, the His at the first, third, and fourth positions along the peptide are the most relevant of the 6xHis hexamer (Chen *et al*., 2000). UV–vis spectra analysis of 6xHis tag-Cu(II)-IDA complexes shows, in the range of pH 5–7, two chelate ring attachments and three at the range of 7–8 (Chen *et al*., 2000).

In this work, Cys was selected as an alternative amino acid to build a novel tag with a modified specificity for protein adsorption. Luo *et al*. (1999) demonstrated that palladium complexes with Cys-containing peptides, such as oxytocin, complex with amino and sulfur atoms of the Cys in acid medium. Other related molecules studied by these authors showed that Pd(II) has at least one coordination ligand to sulfur. Additionally, a more recent study has determined the formation constant for complexes (*N*,*N*-diethylethylendiamine)-Pd(II) with free amino acids. Cys and His showed the maximum values of affinity constant, followed by other natural amino acids (Pro, Iso, Gly, Ala, Lys, and Ser) with lower constants in the same order of magnitude (Shoukry, 2009).

According to spectroscopic studies in solution, two consecutive amino acids (third and fourth position) in the 6xHis hexamer are highly relevant in the ion interaction (Chen et al, 1999). We designed two different tags containing His-X, where X corresponds to Cys or Ser in these positions (Figure 1). Because the second position has no evident interaction with the ion, the His-X frame can be moved two positions upstream keeping the same chelating properties. Ser was selected because of the structural similarity to Cys and the presence of an O atom, to study the influence of S in the ternary complex formation.

Six amino acid tags were linked to the *N*-terminal position of GFP expressed in recombinant *E. coli*. This protein allows easy detection at low concentrations (even in a crude homogenate mixture) and monitoring the presence of GFP for every purification step. Because GFP does not have affinity to divalent cations, binding and elution of tag fusion proteins are not affected (Lee, 2009). In addition, GFP is stable in the pH range of IMAC purification process (between 6 and 10) and in the presence of organic solvents and chaotropic reagents (Champbell and Choy, 2001). Adsorption of a protein to the IMAC support is performed at a pH at which imidazole nitrogens in histidyl residues are in the non-protonated form normally in neutral or slightly basic medium (Gaberc-Porekar and Menart, 2001). The reactivity of a sulfhydryl group is related to its pKa, because its deprotonated form (thiolate = RS^-) is more nucleophilic and reacts faster with oxidants than the protonated form (R-SH). The sulfhydryl groups of most cysteines (linked to a polypeptide backbone or free cysteine) possess low reactivity at neutral or slightly acid pH, which is related to the fact that their pKa is around 8.5 (Benesch and Benesch, 1955). In contrast, some redox proteins possess reactive cysteines that are stabilized in the thiolate form by basic amino acids residue in their microenvironment, in most cases lysines or arginines (Copley *et al*., 2004). In conclusion, reactive cysteines in proteins are kept in a reactive form (thiolate = RS^-) by structural interactions with other amino acids (Netto *et al*., 2007). Therefore, chromatographic runs were performed at slightly acidic conditions (pH 6.0) to avoid Cys oxidation, as well as Pd(II) ion is more stable in acid medium, and to prevent denaturation of GFP. However, lower affinity to imidazole was expected.

As Pd(II) is stable in acidic medium, IMAC Sepharose FF (GE Healthcare) was previously washed with acidic solution to avoid ion precipitation before ion loading. The orange solution of Pd(II) was loaded into the column. When Pd(II) is chelated, the matrix turns from white to pale orange. An equivalent molar solution of Pd(II) has a stronger color than a Ni(II) ion solution, thus making it easier to follow the chelation process. Pass-through of free Pd(II) was determined to ensure full ion loading. No free Pd(II) ion was detected in the column output during the washing step in buffer pH 6.0.

The three tagged GFP variants were run into an adsorption experiment using chromatographic columns containing immobilized Ni(II) and Pd(II) ions. The protein adsorptive step was performed five times, using 1-ml columns, to have a statistical value. Table 1 shows the percentages of adsorbed protein to the IMAC-Ni(II) and IMAC-Pd(II) systems using recombinant tagged GFP proteins.

The GFPHis6 protein had 100% adsorption to the IMAC-Ni(II) column and 84% to the Pd(II)-loaded column, suggesting a lower affinity of Pd(II) to His than to Ni(II) cation. As mentioned earlier, much evidence supports this interaction. Both Cys-containing tagged proteins (where the second, fourth, and sixth positions were changed by Cys or Ser) show high adsorption levels for both ions (more than 72%) but lower than GFPHis6. This behavior can be explained considering that both tag variants have one relevant His_4 (fourth position, which has been identified as ion ligand by spectroscopic analysis (Chen *et al*., 2000)) less than the classical 6xHis-tag. In the case of Ni(II), Cys-containing motifs such as Cys-X-X-Cys were not capable of binding this metal ion (Volz *et al*., 1998). GFPCys3 exhibits adsorption percentages statistically similar to those of GFPCys1 to the IMAC-Pd(II) column (Student's *t*-test, $\alpha = 0.05$) and slightly lower for the Ni(II) loaded column.

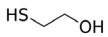
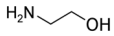
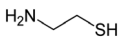
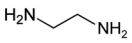
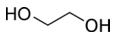
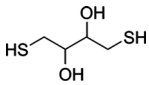
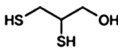
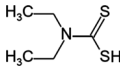
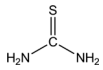
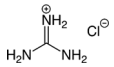
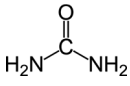
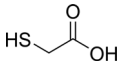
In the next step, we studied the elution conditions under a mild environment compatible with proteins. It is well known that strong acid media can be used as eluent to recover Pd(II) from ion-adsorptive matrices. However, such condition is not friendly to work with protein samples. A number of small organic molecules able to displace the protein but not the metal ions from the matrix was examined for GFPCys1 elution from the IDA-Pd(II) or IDA-Ni(II) columns. The compounds were selected according to their different functional groups, keeping structural chemical similarities, which are grouped in Table 2. Low-molecular-weight reagents containing N, O, and S in linear or cyclic assembly were studied. In addition, NaCl was examined because of the ability of chloride complex of Pd(II) ion. Some compounds, such as dithiocarbamic or thioacetic acid, were not analyzed as eluents because they are immiscible or water-insoluble at pH 6.0. Others, such as thioglycolic acid and potassium thiocyanate, block GFP fluorescence detection and interfere with Bradford's reaction. Dithiothreitol is considered toxic upon inhalation, which brings about some restriction to work on the table.

Table 1. Average adsorption ($n = 5$) of recombinant tagged GFP proteins to IDA-Ni(II) and IDA-Pd(II) matrices

	Immobilized metal ion	
Protein	Ni(II)	Pd(II)
GFPHis6 (HHHHHH)	100.0 ± 0.1	82.6 ± 0.8
GFPCys1 (HCHSHS)	86.8 ± 1.4	80.1 ± 10.0
GFPCys3 (HCHCHC)	71.6 ± 5.9	72.4 ± 5.4

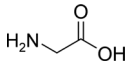
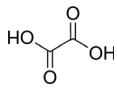
Values correspond to percentage of total protein loaded (±1 SD).

Table 2. Eluent screening for GFPCys1 desorption from IDA-Pd(II) and IDA-Ni(II) columns

Compound	Chemical formula	Characteristics	Metal ion Pd (II)	Metal ion Ni (II)
Imidazole			s	+
Sodium chloride	NaCl		—	—
β-mercaptoethanol		uo	s	—
Ethanolamine			—	—
Cysteamine		uo	+; s	+; *
Ethylene diamine			—	+
Ethanol			—	—
Ethylene glycol			—	—
Dithiothreitol		uo	+	+
Dimercaptopropanol		l; uo	Nd	Nd
Diethyl dithiocarbamic acid		l; uo	Nd	Nd
Thiourea			+	—
Guanidinium chloride			—	—
Urea			—	—
Potassium thiocyanate	KSCN		Na	Na
Thioglycolic acid		uo	Na	Na

(Continues)

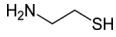
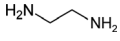
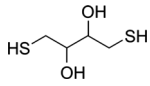
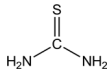
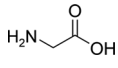
Table 2. (Continued)

Compound	Chemical formula	Characteristics	Metal ion Pd (II)	Metal ion Ni (II)
Glycine			—	+
Oxalic acid			—	—
Thioacetic acid		l; uo	Nd	Nd

s, it scavenged the ion from the column; Na, not analyzed; it interfered with the fluorescence measurement and/or Bradford assay; Nd, not determined; *, the color of the column changed to violet/indigo; l, insoluble; uo, unpleasant odor.

As a consequence of its weakly coordinating property, the amino acid Ser was chosen as Cys replacement in the tag. It is expected that GFPCys1 has lower affinity binding than GFPCys3 in the IMAC columns. Hence, only effective eluents for GFPCys1

Table 3. Eluent screening for desorption of GFPCys3 protein from IDA-Pd(II) and IDA-Ni(II) columns

Compound	Chemical formula	Characteristics	Metal ion Pd (II)	Metal ion Ni (II)
Imidazole			s	+
Cysteamine		uo	+; s	+; *
Ethylendiamine			—	+
Dithiothreitol		uo	+	+
Thiourea			+	—
Glycine			—	—

s, it scavenged the ion from the column; *, the color of the column changed to violet/indigo; uo, unpleasant odor.

from the IDA-Pd(II) and IDA-Ni(II) columns were further analyzed for GFPCys3 protein (Table 3). As expected, almost all compounds able to elute GFPCys1 from the IDA-Pd(II) or IDA-Ni (II) columns were also able to elute GFPCys3.

Imidazole, the most used eluent of 6xHis-tagged protein to IMAC-Ni(II), was able to elute both Cys-tagged GFP from Ni(II)- and Pd(II)-loaded columns. However, imidazole strips the ion from the latter column, thus contaminating the eluted proteins.

Taking into account ethylene-based compounds, mercaptoethanol was effective for the IDA-Pd(II) column, ethylene diamine for the IDA-Ni(II) column, and cysteamine, which has both heteroatoms N and S, for both immobilized metal ion columns. A similar behavior was found for dithiothreitol, the only disulfur compound analyzed. Among the urea-like molecules (urea, guanidinium, and thiourea), only thiourea was able to remove Cys-tagged GFP from the IDA-Pd(II) column but not from the IDA-Ni(II) column. Among acidic compounds, 1 M glycine was a good eluting solution of GFPCys1 from the IDA-Ni(II) column but not of GFPCys3. This agrees with the expected higher affinity of the extra thiol-Pd(II) interaction instead of the hydroxyl group of Ser.

Among the compounds studied, thiourea and dithiothreitol were the only ones able to elute both Cys-tagged GFP from both columns with more than 20% efficiency, without stripping the metal ion from columns or interfering with protein detection. Interestingly, thiourea eluted Cys-tagged GFP from the IDA-Pd (II) matrix but not from the IDA-Ni(II) one. This behavior makes this molecule an attractive specific eluent. In addition, it is not toxic and is one of the few odorless sulfur-containing compounds. Thiourea in 3 M HCl solution has been previously reported as eluent of Pd(II) ion from amine mesoporous silica to ion recovery (Ebrahimzadeh *et al*., 2010). Also, thiourea (1.5% w/v) in acidic medium has also been used for Pd(II) ion recovery from activated carbon fiber column (Lin *et al*., 1995). However, it has been never used as protein or amino acid eluent at near neutral pH.

Figure 2 shows that the chromatographic profiles of GFPHis6, GFPCys1, and GFPCys3 runs in a IDA-Pd(II) column when applying a gradient from 0 to 0.1 M thiourea in 20 CV. Fractions collected in the elution step were analyzed by RFU to specifically detect GFP. Very similar behaviors were observed for three recombinant proteins. The maximum peak of GFP elution was obtained at only 25 mM thiourea concentration.

With the aim to observe the robustness of the IDA-Pd(II) chromatographic system, six consecutive chromatographic runs, using three columns, were performed with 0.1 M thiourea step elution. Because of the similar performance of both Cys-tagged GFP, only GFPCys1 was used for this assay. RFU were measured for each collected fraction for the presence of GFP and the amount of total recovered protein quantified by Bradford's method during the elution step for each chromatography. The recovery observed after the six successive chromatographies

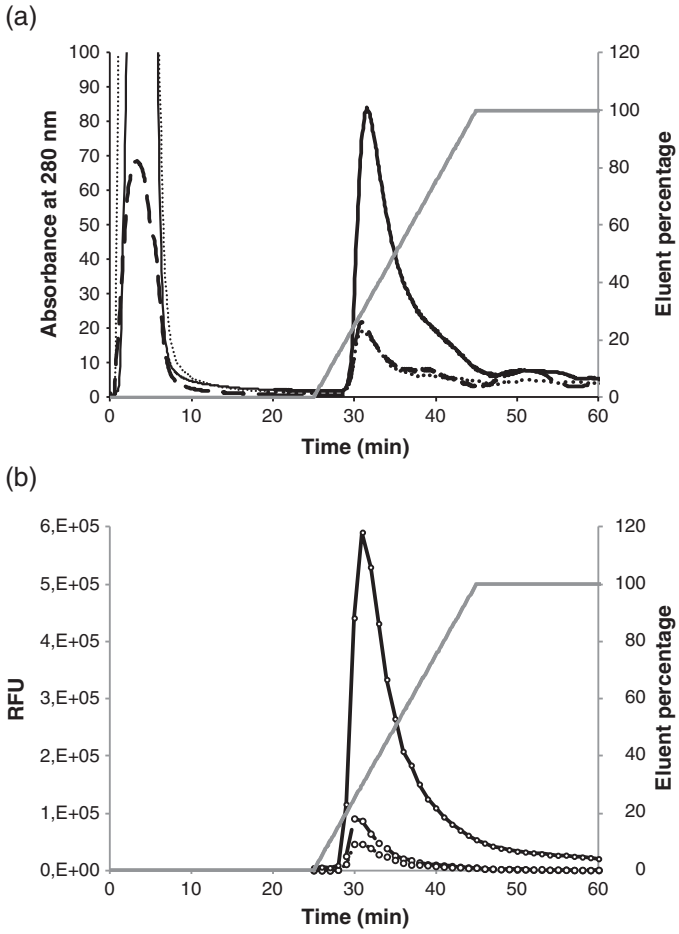


Figure 2. IDA-Pd(II) chromatographic runs performed for tagged GFPs. Plots of (a) absorbance (280 nm) and (b) RFU obtained in the gradient chromatographic run of GFPHis6 (solid black line), GFPCys1 (dashed black line), and GFPCys3 (dotted black line) eluted with 0.1 M thiourea (solid gray line).

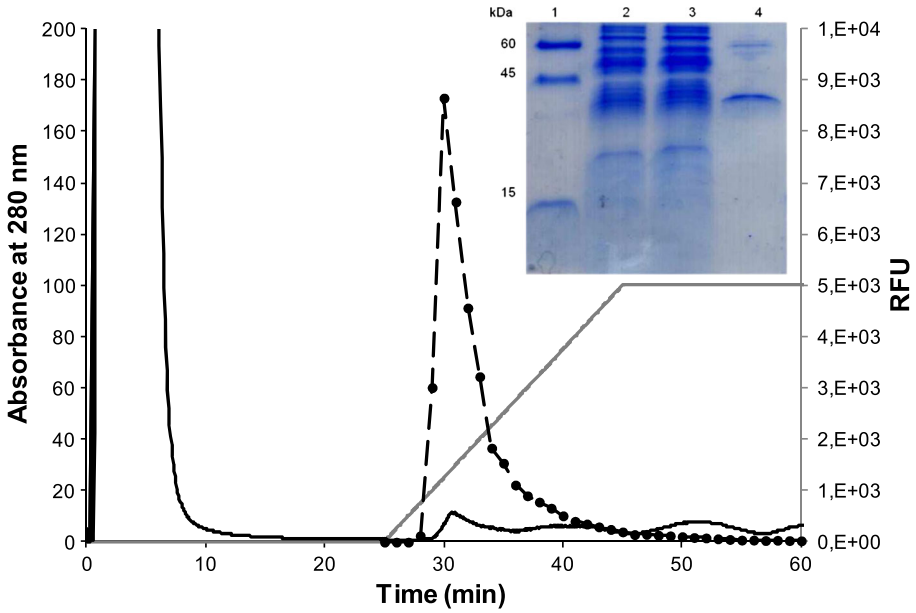


Figure 3. Chromatographic profile and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis corresponding to GFPCys3 purification from recombinant *Escherichia coli* homogenate using IMAC-Pd(II) (column size 1 ml) equilibrated in 50 mM phosphate buffer pH 6.0 and eluted with a linear gradient of thiourea (0–0.1 M, solid gray line) in the same buffer at a flow rate 1 ml/min. The solid black line corresponds to detection at 280 nm and dashed black line to RFU. SDS-PAGE lines: 1, molecular weight marker (60, 45, and 15 kDa); 2, sample; 3, wash; 4, elution.

Table 4. Purification of GFPCys3 from recombinant *Escherichia coli* homogenate using IMAC-Pd(II) or IMAC-Ni(II)

Sample	Total proteins[a] (mg)	Total fluorescence[b] (RFU)	Specific fluorescence (RFU/mg)	Recovery (%)	Purification factor
Escherichia coli homogenate expressing GFPCys3	12.15	211 742	17 427	—	—
IMAC-Ni(II) purified	0.50	38 361	76 722	18.1	4.4
IMAC-Pd(II) purified	0.41	95 603	233 178	45.2	13.4

RFU, relative fluorescence units.
[a]Total protein was obtained by absorbance measurement at 280 nm, eluted volume and extinction coefficients at 280 nm of 1 ml · mg^{-1} · cm^{-1} and 20 000 M^{-1} · cm^{-1} for crude extract and GFP variants, respectively (Jung et al, 2005).
[b]Total fluorescence was obtained as a result of multiplying fluorescence intensity of *Escherichia coli* soluble fraction by its volume.

was statistically similar ($\alpha = 0.05$), proving to be a robust system. On the other hand, five parallel chromatographies of each recombinant GFP variant were performed. Total protein recovery throughout the elution step (Bradford) was 101 ± 19, 110 ± 8, and 83 ± 18 for GFPHis6, GFPCys3, and GFPCys1, respectively.

Finally, the ability of IMAC-Pd(II) to purify a recombinant protein from a crude sample was determined by loading a clarified crude homogenate of a recombinant *E. coli* expressing GFPCys3 at pH 6.0. Sulfhydryl group of free cysteine has a relatively high pKa (8.5), and as a consequence, it is relatively inert for redox reaction in physiological conditions (Wood *et al*., 2003). Figure 3 shows the purification process using an elution gradient of 0.1 M thiourea. The elution profile shows a main peak of GFPCys3 at 25 mM thiourea. The corresponding sodium dodecyl sulfate polyacrylamide gel electrophoresis of the selected fractions demonstrates a good purification record (Figure 3).

In Table 4, the purification records of GFPCys3 from a clarified crude homogenate of recombinant *E. coli* expressing GFPCys3 are compared. Recovery of the recombinant protein with the Cys3 tag by standard IMAC-Ni(II) was as low as 20%, but it is recovered to 45% for the IMAC-Pd(II) system. Purification factor around 13 is reached for Pd(II) ion, which it is comparable with the purification of 6xHis-tagged proteins by IMAC-Ni(II) or IMAC-Cu(II) (Choe *et al*., 2002).

CONCLUSION

The affinity tag peptide containing the hexamer 6xHis is largely the most used tag for protein purification by IMAC-Ni(II) columns. However, the development of new amino acid tags would have alternative expression and purification systems, which would enlarge the usefulness of this chromatography.

The combination of IMAC-Pd(II) with Cys-containing tagged proteins has been demonstrated to be possible under experimental conditions similar to those of standard IMAC-Ni(II).

Cys-containing tagged GFP bounds to IMAC-Pd(II) under near physiological conditions. Thiourea solution, in millimolar concentration, was enough to elute the studied tagged proteins from the Pd(II)-loaded columns with a purification factor of 13. These features approach an ideal affinity tag system able for

effective binding and elution under mild conditions of the desired protein. The use of Cys-containing tags can be exploited for protein to be immobilized in an oriented mode, in this way, Cys amino acids could be very useful for further applications of these proteins to immobilization systems, containing epoxy-activated groups or gold surfaces as well as novel click chemistry reactions (Jones et al, 2009), under mild experimental conditions.

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