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Characterization of metal affinity of green fluorescent protein and its purification through salt promoted, immobilized metal affinity chromatography

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Abstract

Immobilized metal affinity chromatography (IMAC) was investigated as a method of recovery for green fluorescent protein (GFPuv). It was found that in the absence of genetic modification to enhance metal affinity, GFPuv displayed strong metal affinity to Cu(II) and Ni(II), and weak or negligible affinity to Zn(II) and Co(II). Changes in the mobile phase NaCl concentration during Ni(II)-IMAC strongly affected purity and yield of GFPuv, with fine resolution under higher NaCl concentrations. Finally, IMAC via Cu(II) and Zn(II) with intervening diafiltration was used to recover GFPuv with high yield and purity.

Keywords: Immobilized metal affinity chromatography; Salt effects; Green fluorescent protein; Proteins

1. Introduction

The 237 amino acid long green fluorescent protein (GFP) was first extracted from the jellyfish *Aequorea aequorea* for the study of bioluminescence in photobiology [1]. Endowed with unique autofluorescence [2], GFP recently has become a powerful biochemical tool used to study gene expression [3,4], cell localization [5], stress responses in bacterial fermentation [6], and dynamics of protein expression [6,7], not to mention its use in the development of imaging technology [8]. Considerable activity also has been described in which GFP protein fusions (e.g., Arg_n or His_6) served as models to characterize affinity tail efficacy. Examples include the use of GFP fusion proteins to report upon interactions directed toward mica-ion-exchange [9], ceramic hydroxyapatite [10], TNB–thiol gel and oxotechnetate complex [11], and chelated metal ions in various chromatographic and two-phase extraction systems [12–14].

A GFP mutant designated GFPuv was the focus of this study, as GFPuv is extensively used in the aforementioned applications. This GFP variant was created by DNA shuffling which resulted in 45-fold greater fluorescence signal relative to wild type GFP,

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while possessing the same fluorescence characteristics (excite at 395 nm and emit at 509 nm) [15]. Furthermore, GFPuv typically has greater expression rates, intracellular yield, and intracellular solubility [15]. Germane to this paper is the characterization of GFPuv recovery via immobilized metal affinity chromatography (IMAC) without the use of an affinity tail such as His_6. Although the recovery of His_6GFP via Ni(II)-IMAC has been reported [16], it is instructive to consider recovery in light of the protein's nacent metal binding ability and not through enhancement of metal affinity. GFPuv contains 10 histidine residues which could interact favorably with chelated metals such as Cu(II), Ni(II), Co(II), or Zn(II).

Past efforts to isolate GFPuv from cell lysates via multiple chromatographic steps [17], or alternatively via the combination of organic extraction followed by chromatography [18] did not recognize the potential use of IMAC in the recovery scheme. IMAC offers several advantages including low cost of ligand, amenability to scale up, ease of stationary phase regeneration, and fine resolution based on amino acid residues topography [19,20]. In order to assess the potential utility of IMAC in the isolation of GFPuv, experiments were carried out in both batch protein uptake and column chromatography. Chromatography conditions were then manipulated to optimize yield and purity. Specifically, we report on the role NaCl plays during adsorption/elution of GFPuv with various metal ions [M(II)] as affinity ligands, and present a new method for its isolation via one- or two-step IMAC.

2. Experimental

2.1. Assessment methods

Measurement of the relative fluorescence intensity (RFI or RFU) was taken by a Mini fluorometer (RF-150, Shimadoza) with filters appropriate for quantifying GFPuv (Omigafilters, USA). During IMAC, total protein was monitored on-line at 280 nm (LKB-Uvicord, Pharmacia Biotech, USA) and each fraction was confirmed by off-line measurements at 280 nm (Beckman spectrometer). Bradford method (Bio-Rad, USA, Protein Assay Kit) was used to identify total protein in selected fractions. In analysis of both fluorometer and spectrometer data, samples were diluted into the relative measurable range. Silver-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, Bio-Rad Ready Gel) was used to assess purity in a visual fashion.

2.2. Fermentation and preparation of cellular lysate

Plasmid pBAD-GFP hosted by *Escherichia coli* JM105 was kindly given by Dr. Govind Rao (University of Maryland, Baltimore, MD, USA). Fermentation was carried out in Bioflo IIc (New Brunswick, USA) containing sterilized 1 l Lauria–Bertinelli broth with 100 μg/ml ampicillin at 30°C. An overnight culture was used to inoculate the reactor. Arabinose at a final concentration of 0.2% was added to induce GFPuv expression when the absorbance (at 600 nm) of cells reached 0.5. Cells were harvested after 10 h post induction when the $A_{600\ nm}$ was over 3. The green color of cells laden with GFPuv became obvious at that time under room lighting.

To prepare cell extracts, the reactor content was centrifuged (Beckman Model J2-21 M/E) and the supernatant was removed and discarded. Cell pellets were suspended with 0.05 *M* phosphate–0.25 *M* NaCl buffer (pH 7.5) and mashed by French press (Carver) to release the intracellular protein. Cell debris were removed by centrifugation. Aliquots of centrifuged extracts were filtered by presterilized filter (Millipore) to further remove remaining cell debris. The filtration was repeated if necessary to clarify the solution until the extract was homogenous and clear. Final crude GFPuv extracts were stored at −20°C.

2.3. Immobilized metal affinity chromatography–batch and column experiments

To qualitatively assess GFPuv adsorption to IMAC media charged with different metal ions, equilibrium batch uptake of GFPuv was assessed in following manner. A 2-ml volume of Chelating Sepharose 6B resin (Pharmacia) was charged with divalent copper, nickel, zinc and cobalt ions by thoroughly washing a sample of resin with Milli-Q

water, saturating it with appropriate metal salt (e.g., 5 mg/ml $CuSO_4$ in water), and washing with Milli-Q water. Metal charged resins were equilibrated with 0.05 *M* phosphate–0.25 *M* NaCl buffer at pH 7.5 (3 ml). A 1-ml aliquot containing GFPuv was then added to each tube and rotated for 5 min in the tube spinner (Bellco Glass). After settling for at least half an hour, the liquid and solid phases had clearly separated. GFPuv was visualized by illuminating tubes with a hand UV lamp (Spectronics) at 365 nm. When compared to appropriate controls, GFPuv was found either in upper solution (unbound) or bound to bottom resin. Photos of tubes were taken by Kodak digital camera (DC 260) with color filters.

IMAC columns (Pharmacia C 10/10) of 5 ml resin volume were prepared via a similar regiment, but with Chelating Sepharose Fast Flow 6B (Pharmacia) as the stationary phase. A 3-ml volume of clarified cell extracts was injected after 5 ml loading of running buffer (pH 7.6). Different buffers were prepared according to different NaCl concentration of salt to examine the salt effect on protein adsorption. Isocratic washing continued until 10 column volumes of buffer passed and the background protein elution was low as evident by $A_{280\ nm}$. Columns were then developed with a falling pH gradient. Typically 10 column volumes were used for a wash. In all experiments flow-rate was maintained at 0.5 ml/min. The chromatography process was monitored by using a hand UV lamp to locate GFPuv and photos were taken at critical steps. When appropriate, diafiltration was performed using a molecular mass cut-off (MWCO) of 1000 (Millipore) for adjusting buffer composition between chromatography steps.

3. Results

3.1. Assessment methods

The dependence of GFPuv fluorescence on pH was established as a precaution. RFI of a GFPuv sample (0.3 ml) was recorded before and after it was mixed with 0.05 *M* phosphate buffers (final volume 10 ml) that varied in pH from 4 to 13. The RFI was measured again after one half hour passed at room temperature. Experimental data showed no loss of RFI between pH 6 and 8.5, while unstable RFIs were recorded when the pH was greater than 8.5 or below 6. A dramatic drop in RFI occurred when the pH was out of the range of 4 to 10, accompanied by crude particle precipitation. The results are in accordance with reported description of GFPuv physical characteristics [1,21]. To maintain the accuracy in calculations of yield and purity, all IMAC runs were controlled within pH range of 6 to 8.

3.2. Chromatography

3.2.1. Batch uptake [Cu(II), Ni(II), Co(II) or Zn(II)]

Batch protein uptake provided a convenient evaluation on protein adsorption at initial stages, since equilibrium experiments are easy to interpret and not affected by flow or column artifacts. With specific regard to GFPuv, autofluorescence allows for qualitative results simply by observing fluorescence under the illumination of a UV lamp at 365 nm (Fig. 1) in either the liquid buffer phase or bottom solid phase. As shown in Fig. 1, GFPuv has strongest affinity to copper ion since the buffer phase of this system is free of fluorescence. The distribution of green fluorescence also demonstrated its relative strong binding to nickel ion, partial binding to zinc ion and almost negligible affinity towards cobalt ion.

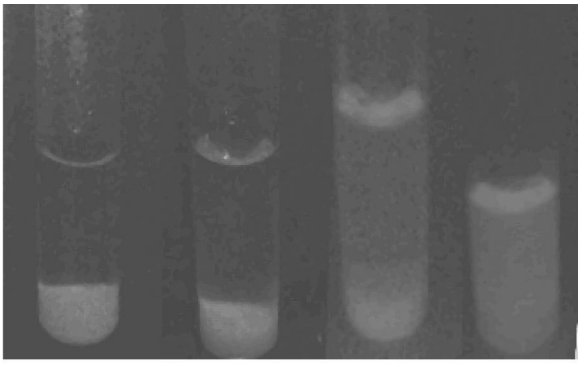

Fig. 1. Equilibrium batch protein uptake. Each tube contained 1 ml cell extract containing GFPuv, 3 ml phosphate buffer (top phase), pH 7.5, 2 ml IMAC resin (bottom phase) charged with, from left to right, Cu(II), Ni(II), Zn(II) and Co(II). Absence of fluorescence in upper phase indicated GFPuv adsorption.

3.2.2. Column experiments with different metal ions [Cu(II), Ni(II), or Zn(II)]

Chromatography runs employing Cu(II), Ni(II), or Zn(II) as affinity ligands were performed with falling pH primarily used as the method of elution in a 0.25 *M* NaCl and 0.05 *M* Na_2HPO_4–NaH_2PO_4 buffer. GFPuv was strongly adsorbed during Cu(II)-IMAC with no elution evident by pH 6.0 (data not shown). Since falling pH was not able to elute GFPuv, the addition of imidazole was next examined as a method of GFPuv elution. When an imidazole gradient supplied by 5 m*M* and 70 m*M* imidazole solutions was employed, a well defined peak of both fluorescence and $A_{280\ nm}$ was observed (Fig. 2). Weak binding to Zn(II) under flow conditions were indicated by substantial GFPuv leakage during the isocratic wash, although the peak of fluorescence came slightly later than the peak of the total protein (Fig. 3).

Chromatography using Ni(II) was most dynamic and warrants further discussions. An irregular fluorescence peak was obtained with GFPuv leakage when the mobile phase contained 0.25 *M* NaCl (Fig. 4). Experiments also showed that prolonging the isocratic step (>10 column volumes) would cause GFPuv to continually leak at very low levels, but starting pH elution would cause significant GFPuv elution. This ill-conditioned elution is inherent to the binding characteristics between GFPuv and Ni(II) at conditions of low NaCl concentration since similar

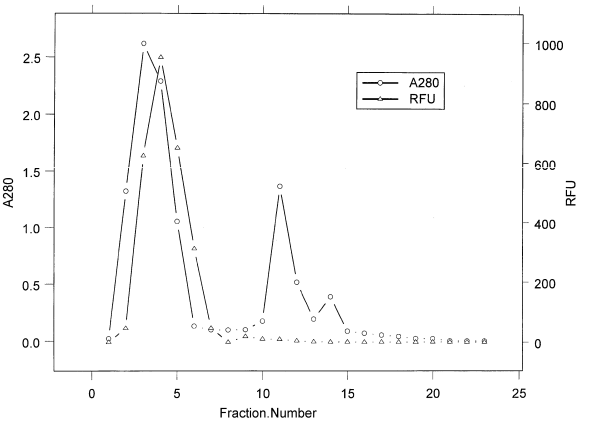

Fig. 3. Zn(II)-IMAC with 0.25 *M* NaCl in mobile phase. Fractions 1–10 phosphate buffer, pH 7.5. Fractions 11–20 EDTA elution (step to 50 m*M*).

profiles were obtained when the following were varied: different batches of cell extract, sample volumes, steepness of pH gradient, and shortening of the isocratic washing. Although GFPuv partially purified by using 0.25 *M* NaCl buffer, the allocation of target protein in a wide range of pH unavoidably caused a trade-off between yield and purity as collection had to be made among all eluted fractions between pH 7.5 and 6.0, where various contaminant proteins were present. This judgment was confirmed by SDS–PAGE (Fig. 5), where a strong GFPuv band with relative clear background showed in two middle fractions (top of the peak at pH 6.5). The distribution

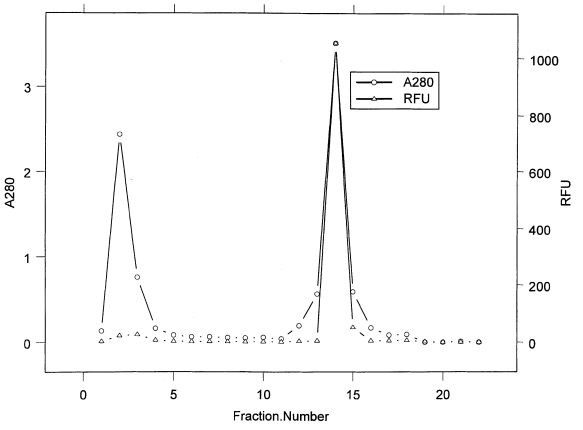

Fig. 2. Cu(II)-IMAC with 0.25 *M* NaCl in mobile phase. Fractions 1–10 phosphate buffer, pH 7.5. Fractions 11–20 imidazole gradient (5 to 70 m*M*).

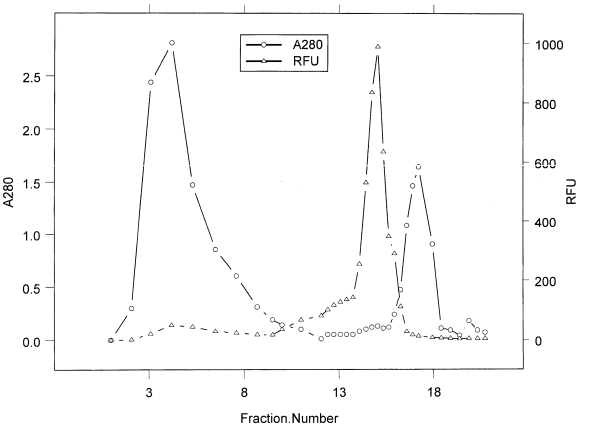

Fig. 4. Ni(II)-IMAC with 0.25 *M* NaCl in mobile phase. Fractions 1–10 phosphate buffer, pH 7.5. Fractions 11–20 pH elution (7.5 to 6.0).

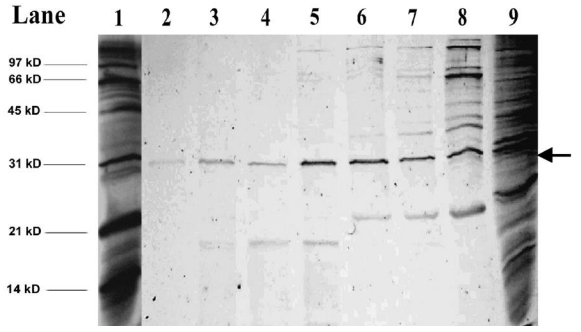

Fig. 5. SDS–PAGE of Ni(II)-IMAC with 0.25 *M* NaCl in mobile phase. Lanes 1 and 9 contain molecular mass standard and cell extract containing GFPuv (arrow indicates GFPuv mobility). Lanes 2–8 contain pooled Ni(II)-IMAC fractions from pH gradient. Lanes 5 and 6 correspond to the major peak of GFPuv at pH 6.5. kD=Kilodalton.

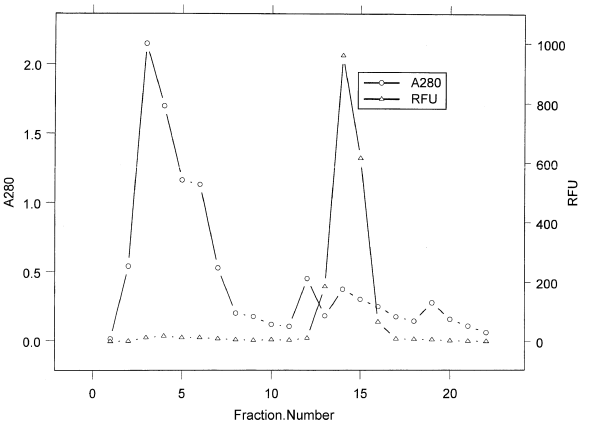

Fig. 6. Ni(II)-IMAC with 1.0 *M* NaCl in mobile phase. Fractions 1–10 phosphate buffer, pH 7.5. Fractions 11–20 pH elution (7.5 to 6.0).

of contaminant bands suggests different contaminants were eluted at different pH values, with the larger-molecular-mass species eluting later in the gradient. According to the yield and purification calculations (Table 1), to attain the highest purification (over 18-fold) the relative yield would be less than 20%.

Higher purity with accompanying yield was observed when the NaCl concentration was increased. GFPuv elution profiles resulting from the use of higher NaCl concentration (1 and 2 *M*) in the mobile phase are illustrated in Figs. 6 and 7. The higher NaCl content successfully sharpened the elution peak of GFPuv when 1 *M* NaCl was employed with GFPuv eluting as a sharp peak at pH 6.5. When 2 *M* sodium chloride was used, GFPuv elution peak shifted to pH 6.0, suggesting even stronger adsorption.

SDS–PAGE of final samples (Fig. 8) qualitatively show the purity of GFPuv from Cu(II)-IMAC (lane 3) and from Ni(II)-IMAC with salt-rich buffer (lanes 4 and 5). Both 1 *M* and 2 *M* NaCl provided favorable conditions for GFPuv recovery at high purity and yield. Yield and purification factors in Table 2 illustrate the poor selectivity (purification factor 3.3) of Cu(II)-IMAC and the improved selectivities/yields associated with increases in NaCl

Table 1
GFPuv yield and purification using Ni(II)-IMAC with 0.25 *M* NaCl

No. GFPuv pooled fractions	Yield (%)[a]	Purification factor[b]
1	18	18.5
2	34	17.7
3	46	16.7
4	63	16.1
7	82	11.2
8	89	9.7

[a] Yield was determined by calculating the ratio between pool and cell extract GFPuv contents (RFI×pool volume).

[b] Purification factor was determined by calculating the ratio between pool and extract GFPuv mass fractions (RFI/total protein).

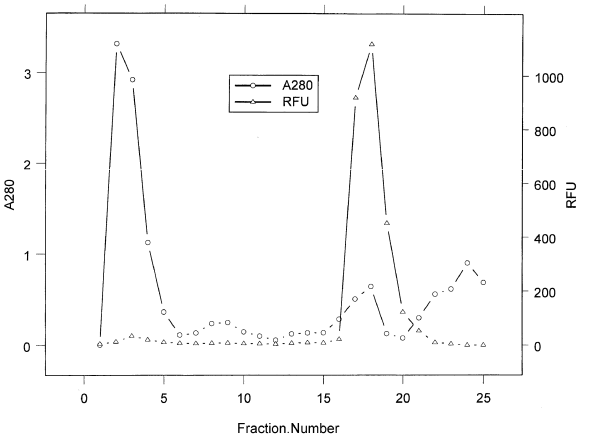

Fig. 7. Ni(II)-IMAC with 2.0 *M* NaCl in mobile phase. Fractions 1–10 phosphate buffer, pH 7.5. Fractions 11–20 pH elution (7.5 to 6.0). Fractions 21–25 phosphate buffer, pH 6.0.

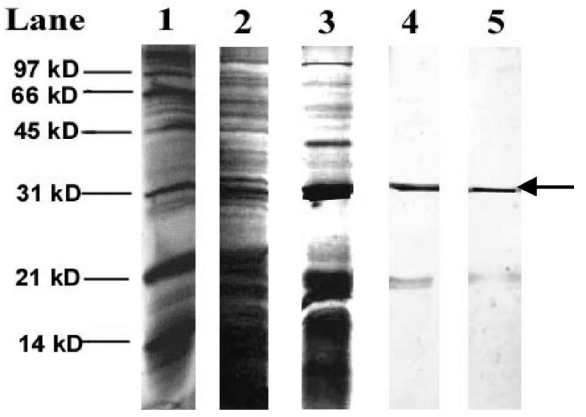

Fig. 8. SDS–PAGE of GFPuv obtained with variations in NaCl content and metal ion. Lanes 1 and 2 contain molecular mass standard and cell extract containing GFPuv (arrow indicates GFPuv mobility). Lanes 4 and 5 contain pooled GFPuv peak from Ni(II)-IMAC with 1.0 *M* and 2.0 *M* NaCl, respectively. Lane 3 contains product derived from Cu(II)-IMAC. kD=Kilodalton.

addition for single step Ni(II)-IMAC. It should be noticed that the adsorption and elution of total protein pool were also changed as evident by comparing the $A_{280\ nm}$ tracings (Figs. 6 and 7). Presumably, the low purification factor (18) albeit high yield (86%) was due to this increase in contaminant adsorption at 1 *M* NaCl conditions. However, when 2 *M* NaCl buffer was employed both the yield and purification were improved as evident by GFPuv elution with minimum background contaminants at pH 6.0 (Table 2).

3.2.3. IMAC [Cu(II) and Ni(II)]

Two-step IMAC was then attempted. The cell extract was first applied to Cu(II)-IMAC and eluted with imidazole gradient after isocratic washing. Next GFPuv fractions were diafiltrated with phosphate buffer (pH 7.5) using a membrane with a MWCO of 1000 to remove imidazole and free copper ions. The permeate was fed to Ni(II)-IMAC with buffer conditions of 1 *M* or 2 *M* NaCl. The purification factors of Table 2 indicate that, with the aid of Cu(II)-IMAC as a lead step, a higher resolution of GFPuv can be achieved for 1 *M* and 2 *M* columns with similar purity. This result suggested that the lower purification with 1 *M* NaCl was mainly caused by proteins with low Ni(II) affinity whose binding were promoted by NaCl addition, and not by band broadening of GFPuv. The yield loss due to the additional diafiltration was less than 5%.

4. Discussion and conclusions

Although GFPuv contains 10 histidine residues, only a moderate metal affinity was found [strong interaction to Cu(II), less to Ni(II), weak or negligible to Zn(II) and Co(II)]. Inspection of the three-dimensional structure of GFP from *Aequorea aequorea* (PDB code 1EMF) stored in the database of the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) suggests that of the 10 His residues, five are involved in secondary structure (Table 3) and appear to be less solvent exposed. Presumably, several histidines at the ''top'' of the β-can could favorably interact with metal ions. In particular, His_{77}, His_{81}, and His_{231} are within 7.5

Table 2
Summary of GFPuv recovery

M(II)	[NaCl] (*M*)	Gradient	Yield (%)[a]	Purification factor[a]
Single-step IMAC				
Cu(II)	0.25	Imidazole	97	3.3
Ni(II)	0.25	pH	18	18.5
Ni(II)	1.0	pH	86	15.1
Ni(II)	2.0	pH	92	22.1
Use of two IMAC steps				
Cu(II)/Ni(II)	1.0	Imidazole/pH	79	28.7
Cu(II)/Ni(II)	2.0	Imidazole/pH	86	30.4

[a] Yield and purification factor as per Table 1 footnote.

Table 3
Histidine topography of GFP from *Aequorea aequorea*[a]

Surface exposed histidines	
His_{25}	
His_{139}	Nearest His neighbor is His_{25} (11 Å)
His_{77}/His_{81}	Top of β-can; amino acid sequence HMKRH
His_{231}	C-Terminus of protein; within 7 Å of His_{77}/His_{81}
Other histidines	
$His_{148}/His_{169}/His_{181}/His_{199}$	Imidazole side ring projects into β-can
His_{217}	Semi-buried

[a] Structure data are available at www.ncbi.nlm.nih.gov

Å of each other. It is likely that members of this triplet are responsible for adsorption during Ni(II)-IMAC, since affinity towards this metal ion depends on the cooperation of proximal His residues [19,20,22–25]. With respect to Cu(II) adsorption, the aforementioned triplet, in addition to His_{25} and His_{139} residues are solvent exposed and may thus form the basis for adsorption. To conclude, the native metal affinity of GFPuv is sufficient to provide for purification via Cu(II) and Ni(II), and with further investigation could form the basis for a naturally derived IMAC affinity tail [30].

Metal–protein interactions can also be modified by the operational conditions, especially the salt content in chromatography buffer [19,20]. In this study, high NaCl content of the mobile phase has several possible effects on protein binding: (i) promoting the hydrophobic interactions [20,26–28]; (ii) enhancing GFPuv dimer formation and thus multi-layer binding [29]; and (iii) stabilizing the protein–metal complex when Cl^- is involved in binding [29]. Regardless of which effect(s) played dominant roles in the attenuation of Ni(II)-IMAC, selectivity can be favorably manipulated by regulating the salt types and concentrations in running buffer. Chromatography results with NaCl concentrations of 0.25, 1 and 2 *M* demonstrate forced retention of both GFPuv and total protein pool for Ni(II)-IMAC, but to differing degrees.

During all experiments, total fluorescence calculations confirmed that activity of GFPuv was well maintained, indicating that IMAC under appropriate conditions (metal ion chosen, mobile phase composition) is useful for GFPuv recovery. Under optimized conditions, yield and purity were comparable to or greater than values reported in other investigations of GFPuv recovery [17,18]. Presumably, GFPuv has native metal affinity that may be exploited in the recovery of this protein, or may be used to develop a new metal affinity tail. Finally, the use of IMAC with an intervening diafiltration step demonstrated the possibility of using two IMAC steps in a recovery scheme.

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