Published in final edited form as:

Protein Expr Purif. 2011 September ; 79(1): 88–95. doi:10.1016/j.pep.2011.04.022.

Purification of proteins containing zinc finger domains using Immobilized Metal Ion Affinity Chromatography

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Abstract

Heterologous proteins are frequently purified by Immobilized Metal Ion Affinity Chromatography (IMAC) based on their modification with a hexa-histidine affinity tag (His-tag). The terminal His-tag can, however, alter functional properties of the tagged protein. Numerous strategies for the tag removal have been developed including chemical treatment and insertion of protease target sequences in the protein sequence. Instead of using these approaches, we took an advantage of natural interaction of zinc finger domains with metal ions to purify functionally similar retroviral proteins from two different retroviruses. We found that these proteins exhibited significantly different affinities to the immobilized metal ions, despite that both contain the same type of zinc finger motif (i.e. CCHC). While zinc finger proteins may differ in biochemical properties, the multitude of IMAC platforms should allow relatively simple yet specific method for their isolation in native state.

Keywords

IMAC; zinc finger proteins; M-PMV; HIV-1; Gag polyprotein

Introduction

Immobilized Metal Ion Affinity Chromatography (IMAC) is a widely used technique for purification of proteins with engineered histidine tags or with natural surface-exposed histidine residues. Typically, this system utilizes interactions between His residues and Ni²⁺ ions, although many different divalent metal ions such as Cu²⁺, Co²⁺, Fe²⁺ and Zn²⁺ can interact with His residues. These metal ions are immobilized using various metal-chelator systems, the most common being iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) [1]. Purification of proteins using such a system is often achieved using the terminally added poly-histidine tag, since most proteins do not have significant affinity for these ions. However, terminal modification of the protein with the His affinity tag may negatively

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affect the structure or function of the protein with changes to its conformation, solubility, and biological activity, as it was previously demonstrated [2,3]. Due to these negative effects, different methods for the specific removal of these tag sequences have been developed. These include chemical cleavage and designing protease cleavage sites into the chimeric protein and using commercially available proteases to remove the tag [4,5]. Cleavage of the protein using chemicals is largely non-specific, and the protein is likely to be denatured and multiply cleaved [6]. The use of proteases to remove the tag sequence generally necessitates additional purification steps to remove the protease or cleaved tag [4]. Alternately, if available, domains offering specific binding properties as natural intrinsic histidine domains or exposed histidines, may be exploited to purify the protein of interest in its native state by IMAC [7]. Also the natural affinity of the zinc finger domain to divalent metal ions may be utilized to specifically purify proteins containing this domain [7]. These domains are present in many nucleic acid binding proteins and form a specific tertiary structure that allows multiple cysteine or histidine residues to coordinate the metal ion [8]. The zinc finger domains may be further classified by the number of cysteine or histidine residues responsible for this interaction, for example C₂H₂ indicates that two cysteines and two histidines are involved. The first identified and still one of the best characterized zinc finger proteins is the transcription factor TFIIIA, which contains multiple C₂H₂ zinc finger domains [9].

All retroviruses contain the *gag* gene that encodes a structural polyprotein responsible for the assembly of immature retroviral particles. During virus maturation, the Gag polyprotein is cleaved by viral protease to yield the mature structural proteins. While every retrovirus encodes a unique set of these proteins, all retroviruses encode the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins. The MA protein forms the outermost shell of the mature virus and interacts with the inner membrane of the viral envelope. The CA protein forms an inner shell surrounding the ribonucleoprotein complex, which consists of viral genomic RNA and NC protein [10]. NC proteins of various retroviruses are rich in basic amino acids, contain one or two zinc finger domains and mediate binding, dimerization, and incorporation of genomic RNA into the assembling virion [11,12]. Retroviral NC zinc finger domains have a Cys-X₂-Cys-X₄-His-X₄-Cys (CCHC) structure, where X indicates any amino acid. The majority of these 'X' residues are not conserved either among retroviruses or between the two motifs of a given NC, while the cysteine and histidine residues are strictly conserved in order to maintain the ability to coordinate Zn²⁺ ions [13,14].

Several publications recommend the use of IMAC to purify untagged metal binding proteins using their affinity to the column matrix through their metal binding domains as stretch of histidine residues or exposed histidines. DeJong and Roeder published the use of Ni-resin for purification of TFIIA protein containing an intrinsic histidine rich region [15]. Also Ni-IDA column was used for purification of HIV-1 integrase through its zinc-binding motif [16]. To date, zinc finger proteins have largely been purified by methods involving affinity tags or based on nonspecific interaction [17,18,19,20]. However, the structure and function of retroviral Gag proteins is not conducive to sequence manipulation. Therefore, instead of the more traditional methods of purifying these proteins, we took advantage of the presence of the zinc finger domains in NC for specific purification of the retroviral CA-NC portion of the Gag polyprotein using IMAC. This strategy was employed to purify the CA-NC proteins from the betaretrovirus Mason-Pfizer monkey virus (M-PMV) as well as the lentivirus human immunodeficiency virus type 1 (HIV-1). Despite the fact that both M-PMV and HIV-1 have CCHC zinc-finger motifs, we found significant differences in their affinity to the metal immobilized on the column matrix. In spite of this, we proved the method to be specific, efficient and easy to carry out. The advantage of this method is that the purified protein remains in its native state for subsequent use in functional studies in the *in vitro* system of virus-like particles assembly [21]. Moreover, protein purified by this method has

no detectable nucleic acids contamination, which is important for nucleic acid-interaction studies where such contamination can affect further use of this protein.

Material and methods

Preparation of DNA constructs

The preparation of an expression plasmid (pSIT- Δ ProCA-NC) encoding the CA-NC portion of M-PMV Gag, but lacking the N-terminal proline, has been previously described [22]. Similar vector for the expression of the CA-NC portion lacking the N-terminal proline from the HIV-1 molecular clone NL4.3 (pET22b- Δ ProCA-NC) was constructed using standard techniques. The CA-NC region of HIV-1 *gag* was PCR amplified from pNL4.3 and cloned into the pET22b vector from Novagen® (Merck). All cloning steps were carried out by established techniques that were described elsewhere [23]. The cloning strategies and details of the PCR primers can be obtained upon request from the authors. No mutations were introduced by the cloning strategy as was verified by DNA sequencing. The correct molecular sizes of expressed proteins were confirmed by SDS-PAGE and the N-termini were verified by N-terminal sequencing by Edman method.

Bacterial expression of M-PMV and HIV-1 genes

Luria-Bertani medium containing ampicillin (final concentration of 100 μ g/ml) was inoculated with *Escherichia coli* BL21(DE3) cells carrying the appropriate Δ ProCA-NC construct. Expression was induced by the addition of isopropyl- β -D-thiogalactopyranoside to a final concentration of 0.4 mM when the culture reached O.D.₅₉₀ ~ 0.4 – 0.6. The bacteria were harvested 4 h post-induction by low-speed centrifugation.

Purification of M-PMV and HIV-1 Δ ProCA-NC proteins

The following buffers were utilized in the isolation of Δ ProCA-NC proteins from bacteria. Lysis buffer was prepared with 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mg/ml lysozyme, 0.05 % 2-mercaptoethanol, 100 μ g/ml phenylmethylsulfonyl fluoride (PMSF), and 1.2 ml of Complete™ protease inhibitor mix (Roche, Basel, Switzerland). Wash buffer with 1 M salt was prepared with 50 mM Tris-HCl pH 8.0, 1 M NaCl, 1 mM EDTA, and 0.5 % Triton X-100. Wash buffer with 1.5 M salt was prepared with 50 mM Tris-HCl pH 8.0, 1.5 M NaCl, 1 mM EDTA, 0.5 % Triton X-100, and 0.05 % 2-mercaptoethanol. Column loading buffer was prepared to contain 50 mM sodium phosphate buffer pH 7.5 and 0.5 M NaCl. Storage buffer was prepared with 50 mM sodium phosphate buffer pH 7.5, 0.5 M NaCl, 0.01 % 2-mercaptoethanol, and 1 μ M ZnCl₂.

The bacterial pellet obtained from low speed centrifugation of 1 liter of bacterial culture was resuspended in 30 ml of lysis buffer and stirred at room temperature for 30 minutes and then sonicated on ice 4 $\times$ 10 seconds at 27 W, using a Sonicator 3000 (Misonix, Farmingdale, NY, USA). Sodium deoxycholate was then added to the lysates to a 0.1 % final concentration. This mixture was incubated at 4 °C for 30 minutes, after which the lysates were centrifuged at 10,000 $\times$ g for 10 minutes at 4 °C. The supernatant was carefully removed and stored on ice. The pellet was subjected to three additional wash steps, the first with wash buffer contained 1 M salt and the other two in wash buffer contained 1.5 M salt. For each of these, 10 ml of buffer was used to resuspend the pellet. The resuspended mixture was centrifuged at 10,000 $\times$ g for 10 minutes at 4 °C. For each of these centrifugations, the supernatants were carefully removed and stored on ice for later use. Following all centrifugation steps, the recovered Δ ProCA-NC protein containing supernatants were dialyzed against column loading buffer overnight at 4 °C.

HiTrap™ Chelating HP columns and IMAC Sepharose 6 Fast Flow columns (GE Healthcare, Little Chalfont, UK) containing 5 ml of resin were prepared according to the manufacturer's instructions. The resins were charged with 2.5 ml of a metal salt solution (either containing 0.1 M ZnSO₄ or 0.1 M NiSO₄) and equilibrated with column loading buffer. The dialyzed sample containing ΔProCA-NC protein was loaded onto the column and following binding was washed with 50 ml of column loading buffer. Bound proteins were eluted with 35 ml column loading buffer containing 2 M NH₄Cl, collecting 2.5 ml fractions. The presence of ΔProCA-NC protein was determined by SDS-PAGE electrophoresis and Coomassie staining. The fractions found to contain the ΔProCA-NC protein were combined and dialyzed against appropriate buffer overnight at 4 °C. Protein samples to be used for *in vitro* assembly were dialyzed against storage buffer and concentrated to 1 – 2 mg/ml using Centrplus® membranes (Millipore, Billerica, MA, USA), aliquoted, and stored at –20 °C. Samples to be used for atomic absorption spectrometry (AAS) were dialysed against column loading buffer two additional times for approximately 12 hours each at 4 °C. Nucleic acid contamination was determined using the RiboGreen® RNA Quantitation Kit, according to the manufacturer's protocol (Molecular Probes, Carlsbad, CA, USA). All purification procedures for both M-PMV and HIV-1 ΔProCA-NC proteins were repeated independently at least in triplicates.

Size-exclusion gel chromatography

Size-exclusion gel chromatography was performed on Superdex 200 10/300GL (1 × 24 ml; GE Healthcare, Little Chalfont, UK), Biologic DuoFlow fast protein liquid chromatography (FPLC) system (Bio-Rad, Hercules, CA, USA) equipped with QuadTec detector. Approximately 2 mg of each purified protein were loaded on the column equilibrated with the storage buffer (50 mM sodium phosphate buffer pH 7.5, 0.5 M NaCl, 0.01 % 2-mercaptoethanol, and 1 μM ZnCl₂) as a mobile phase at flow rate of 0.5 ml/min. BSA and carbonic anhydrase were used as molecular weight standards.

SDS-PAGE and Western blotting

Protein samples were separated on 10 % SDS-PAGE gels. The protein purity was assessed by densitometric analysis of Coomassie brilliant blue stained SDS-PAGE gels using the TotalLab electrophoresis analysis system (TotalLab Ltd., Auckland, New Zealand). Content of purified protein in final sample was quantified using the Rolling ball background algorithm on 1D SDS-PAGE gels. Protein peaks were detected automatically and the percentage of the total protein content contained within given peaks was calculated, with this value representing the protein purity of a sample. This calculated protein purity represents the average value for three independently purified proteins. For Western blotting, proteins were transferred to nitrocellulose membranes using the wet transfer technique (Mini Trans-Blot Cell®, Bio-Rad, USA). Membranes were probed using rabbit anti-M-PMV CA or anti-HIV-1 CA polyclonal antibodies. These antibodies were generated using recombinant CA proteins as antigens, with the antibodies used in these experiments coming from the fourth bleed of each animal. Horseradish peroxidase conjugated goat anti-rabbit secondary antibody (Promega, Madison, WI, USA) was bound to primary antibodies and chemiluminescence was used to assess binding, luminescence was directly detected using a Fuji LAS-1000 CCD camera (Fuji, Tokyo, Japan).

Assembly of retroviral particles *in vitro*

Purified proteins were used for the *in vitro* assembly as previously described [21]. The formation of particles was confirmed by transmission electron microscopy using a JEOL JEM-1010 (JEOL, Tokyo, Japan).

Atomic absorption spectrometry (AAS)

The zinc or nickel content in 5 ml of protein samples (protein concentration about 0.5 mg/ml) was measured by atomic absorption spectrometer using the Spectra AA300 (Varian, Inc., Palo Alto, CA, USA). The Δ ProCA-NC containing samples were acidified by addition of 200 μ l nitric acid (p.a.) per 5 ml of sample and the protein concentration was determined by Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA). The detection limit of Zn and Ni ions using this method is 0.05 mg/ml.

Results

Production and pre-purification of Δ ProCA-NC proteins

Purification of the zinc-finger proteins using IMAC columns followed the strategy shown in Figure 1. The first step was to produce the viral Δ ProCA-NC proteins, for this *E. coli* producing either M-PMV or HIV-1 Δ ProCA-NC were harvested and lysed 4 hours after induction. Following an initial low-speed centrifugation of the bacterial lysate, it was observed that the majority of the Δ ProCA-NC protein was present in the pellet, while a smaller portion remained in the supernatant (Fig. 2). The pellet was resuspended three times in high salt buffers. The first wash was performed with 1 M NaCl, while the following two washes were performed with 1.5 M NaCl. Each wash step was followed by centrifugation of the suspension. These pre-purification washes resulted in three fractions of soluble Δ ProCA-NC proteins: supernatants S1, S2 and S3 from washes 1, 2 and 3, respectively (Fig. 2). Both M-PMV and HIV-1 Δ ProCA-NC proteins were produced and pre-purified by the same procedure with nearly identical yields. Therefore only the gel documenting protein material in individual steps of HIV-1 purification is shown (Fig. 2). For protein purification by affinity chromatography on metal chelating columns, the Δ ProCA-NC-containing S1, S2, and S3 fractions were consolidated and dialysed against the column loading buffer.

Purification of Δ ProCA-NC proteins using affinity chromatography

Supernatants (S1+S2+S3) obtained in the previous steps were loaded on IMAC columns charged with either Zn^{2+} or Ni^{2+} ions. The columns were tested following charging with two metal ions because individual zinc-finger proteins are known to have unique affinities to different metal ions. Following binding of input pre-purified lysates and washing of the column, captured proteins were eluted from the IMAC columns using a buffer containing 2 M ammonium chloride. We also tested the elution of purified proteins using decreasing pH of acetate buffer. However, the elution occurred at pH 4.0 that might affect the protein native structure. Therefore we rather applied the high ionic strength based elution. The efficacy of the columns in capturing either M-PMV or HIV-1 Δ ProCA-NC proteins was analyzed by polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3).

The initial strategy for purification of the Δ ProCA-NC proteins employed the HiTrapTM Chelating HP column containing iminodiacetic acid (IDA) as a chelating group. We observed that while M-PMV and HIV-1 Δ ProCA-NC proteins are structurally very similar and both contain two zinc finger domains, they had different affinities to this resin based on the metal ion used to charge the column. We observed that the M-PMV Δ ProCA-NC protein efficiently bound the HiTrapTM column when charged with Zn^{2+} , while under the same conditions the HIV-1 Δ ProCA-NC protein passed through the column with no appreciable retention (Fig. 3A). However, when Ni^{2+} was used to charge the HiTrapTM column resin, a different phenomenon was observed. In contrast to what was observed with the use of Zn^{2+} , the HIV-1 Δ ProCA-NC protein was efficiently retained on the Ni^{2+} charged HiTrapTM resin. Similar to the Zn^{2+} charged HiTrapTM column, the M-PMV Δ ProCA-NC protein was efficiently bound by the nickel ions. No detectable protein was observed in the flow-through fraction and a bit surprisingly the protein was not removed from the resin following the 2 M

NH₄Cl elution. Under these conditions the use of a stronger elution buffer, containing 100 mM imidazole, was necessary for successful elution of the M-PMV Δ ProCA-NC protein from the resin.

In order to streamline the purification process of the Δ ProCA-NC proteins from both HIV-1 and M-PMV, it would be ideal to charge the IMAC column with the same metal ion for both purifications. As there was a strict dichotomy for the type of metal ion used to charge the HiTrapTM column for purification of M-PMV or HIV-1, we also tested the IMAC Sepharose 6 Fast Flow column for its ability to capture both M-PMV and HIV-1 under similar conditions. According to the manufacturer, this column contains a stronger chelating group than iminodiacetic acid. Unfortunately, the structure of the IMAC Sepharose 6 Fast Flow column chelating group is confidential and the column manufacturer declined to comment on the number of bonds involved in chelating the metal ions.

Purification of Δ ProCA-NC proteins using the IMAC Sepharose 6 Fast Flow column was performed using a similar protocol to the HiTrapTM Chelating HP column. In accordance to what was observed using the HiTrapTM column, the M-PMV Δ ProCA-NC was successfully captured by the Sepharose 6 column where Zn²⁺ and also Ni²⁺ were immobilized (Fig. 3B). As was observed with the HiTrapTM column the use of Ni²⁺ for charging the Sepharose 6 column necessitated the use of stronger 100 mM imidazole-containing elution buffer in order to release the M-PMV Δ ProCA-NC protein. In contrast to what was observed using the HiTrapTM column, charging of the Sepharose 6 column with Zn²⁺ resulted in retention of the HIV-1 Δ ProCA-NC protein. Similarly to what was observed with the HiTrapTM column, the HIV-1 protein was also captured on Ni²⁺ charged Sepharose 6 columns.

As it is clear in the Figure 3, while the protein samples loaded onto both IMAC columns looked very similar, the amount and identity of contaminating proteins in the elution fractions differed between the methods used for purification. The amount of residual bacterial proteins in the eluate is inversely correlated with the amount of unbound proteins in flow-through fraction. Generally, the purity of the eluted Δ ProCA-NC protein from the HiTrapTM column was higher than that observed in the case of Sepharose 6 column purification.

Biochemical analysis of purified Δ ProCA-NC proteins

Eluates containing Δ ProCA-NC protein were dialysed overnight at 4 °C against protein storage buffer. The purified, dialyzed proteins were concentrated to approximately 1 mg/ml. The composition and purity of M-PMV or HIV-1 Δ ProCA-NC proteins in the final preparations were assessed by Coomassie staining of SDS-PAGE gels as well as Western blot analysis (Fig. 4). Coomassie staining demonstrates that the dominant protein recovered following the entire purification protocol corresponds to the Δ ProCA-NC proteins (Fig. 4A). Densitometry analysis was performed on the Coomassie stained SDS-PAGE gels to quantify the purity of the protein preparations. It was observed that the use of the HiTrapTM column resulted in purity of Δ ProCA-NC proteins exceeding 95 % or 88 % when zinc or nickel ions were chelated, respectively. While, the Sepharose 6 column resulted in Δ ProCA-NC protein purity exceeding 75 % or 84 % when zinc or nickel ions were chelated, respectively (Fig. 4A). The standard deviations of purities calculated from three independent purifications did not exceed 6 % for all the proteins reported in this paper.

In addition to separating the viral Δ ProCA-NC proteins from other proteins in the whole cell lysate, removal of nucleic acids is extremely important for many subsequent experiments where nucleic acid contamination could affect further functional studies. Protein purification using IMAC columns has been shown to effectively remove contaminating nucleic acids from the protein sample [24]. The nucleic acid contamination of Δ ProCA-NC protein

preparations was measured for pre-purified protein samples as well as final protein preparations. Before the loading on the IMAC column, the pre-purified protein samples contained about 50 ng of nucleic acids per 1 µg of protein. Following IMAC purification the amount of contaminating nucleic acids was about only 1 ng of nucleic acid per 1 µg of protein. The final nucleic acid contamination found in the ΔProCA-NC protein samples is very close to the detection limit of the RiboGreen® kit used in these studies, demonstrating the efficient removal of contaminating nucleic acids from the protein samples.

Another factor that might affect the purification procedure could be a tendency of the purified protein to multimerize. Thus we performed size-exclusion chromatography to determine whether the proteins are monomeric and the zinc finger motifs cannot be sterically hindered. The elution profile confirmed that the vast majority (over 95 %) of purified proteins was in monomeric state upon studied conditions (data not shown).

While it appears that the ΔProCA-NC protein preparations are highly enriched for viral zinc finger protein, Western blotting was performed to demonstrate that the 32 and 35 kDa bands observed in Figure 4A were the desired ΔProCA-NC proteins (Fig 4B). As expected, blotting with anti-M-PMV and anti-HIV-1 CA polyclonal antibodies revealed that the major 35 and 32 kDa bands correspond to viral ΔProCA-NC proteins. In addition to these major bands, immunoblotting revealed several minor bands that were not apparent in the Coomassie stained SDS-PAGE gels. Higher molecular weight bands were observed in purified ΔProCA-NC protein preparations from both viruses. According to their sizes, these bands most likely represented SDS-resistant ΔProCA-NC dimers and their amount accounted for less than 5 % of total weight of purified proteins as determined by size-exclusion chromatography. Also present in all HIV-1 ΔProCA-NC protein purifications were lower molecular weight bands of approximately 24 kDa in size. This corresponds to the molecular weight of the HIV-1 CA protein, so these bands likely represented cleavage products of the HIV-1 ΔProCA-NC polypeptide.

While it was found that the IMAC columns can be used for the ΔProCA-NC proteins purification, it is important to confirm that the resulting protein remained folded in its native conformation. We have previously shown that bacterially expressed viral ΔProCA-NC proteins are able to self-assemble *in vitro*, with M-PMV ΔProCA-NC forming spherical virus-like particles and HIV-1 ΔProCA-NC forming tubular particles [21]. We tested the purified ΔProCA-NC proteins for their ability to form appropriate structures in an *in vitro* assembly assay. Confirming the native physiological conformation and activity of purified protein, M-PMV ΔProCA-NC assembled into spherical structures (Fig. 5A) and HIV-1 ΔProCA-NC assembled into tubular structures (Fig. 5B) in the presence of genomic RNA of phage MS2 [21]. Thus, IMAC purification yields highly enriched preparations of viral ΔProCA-NC proteins in their native conformation with little nucleic acid contamination.

Determination of zinc presence in purified proteins

To test the hypothesis that the interactions between immobilized metal ions on the IMAC column and our proteins are mediated through the zinc fingers we measured the metal ion content in protein fractions by Atomic absorption spectrometry (AAS). The results are summarized in Table 1. Theoretically, two metal ions should be conjugated by one protein molecule, as retroviral NC proteins contain two zinc finger domains. The metal ion content in protein samples prior to loading on the IMAC column showed that both M-PMV and HIV-1 ΔProCA-NC proteins contain approximately 1 of Zn²⁺ or Ni²⁺ ion per 10 protein molecules. This result suggests that the majority of the zinc finger domains in the pre-purified input were lacking coordinated Zn²⁺ or Ni²⁺ ions, and thus were likely not present in true zinc finger conformation. We reasoned that the lack of coordinated metal ions in their zinc finger domains allowed the ΔProCA-NC proteins to interact with immobilized ions on

the IMAC column. To explore this possibility, we tested the binding capability of M-PMV Δ ProCA-NC to the IMAC column following dialysis against column loading buffer containing Zn^{2+} ions. The zinc content following this dialysis step was found to be 15 Zn^{2+} ions per 10 M-PMV Δ ProCA-NC molecules. The protein that incorporated zinc ions into its zinc finger domains during the dialysis was not able to adsorb on IMAC column. Also no retention of M-PMV Δ ProCA-NC occurred without charging the IMAC resin with the metal ions (data not shown).

We have also determined the amount of metal ions in the purified protein sample after the chromatography to address the question whether the metal binding proteins could capture metal ions from the chromatographic column matrix and incorporate them into their structure (Table 1). In agreement with that concept we confirmed that both M-PMV and HIV-1 proteins scavenge Zn^{2+} and Ni^{2+} ions from charged IMAC column matrix during their elution. On average approximately 16 Zn^{2+} ions were presented per 10 molecules of the M-PMV protein purified with the HiTrapTM Chelating HP column as documented by AAS. The HIV-1 protein after its bacterial production contained only 1 Zn^{2+} ion per 10 protein molecules, however the Zn^{2+} content in flow-through fraction of the HiTrapTM column increased to 10 Zn^{2+} ions per 10 protein molecules. Since the HIV-1 protein passed through the column packed with the IDA matrix without any retention, it may be concluded that this protein can strip off the zinc ions from the column matrix and capture them in its structure, most probably by its zinc finger domains. On the other hand, the HIV-1 protein was successfully adsorbed on the IDA resin charged with nickel ions. The nickel content in the elution fractions after chromatography was only 2 Ni^{2+} ions per 10 molecules of the HIV-1 protein. The same value (2 Ni^{2+} ions per 10 protein molecules) was measured in elution fraction of M-PMV Δ ProCA-NC protein purified on IDA resin, following elution with imidazole-containing buffer.

As seen in Table 1, we confirmed that stripping of the metal ions from the resin during the protein purification is significantly reduced when using the IMAC Sepharose 6 Fast Flow column matrix compared to the HiTrapTM column. This result was not unexpected as the Sepharose 6 column is reported to have a stronger chelating group. The M-PMV protein eluted from the Sepharose 6 column contained 8 Zn^{2+} ions per 10 protein molecules, while eluted HIV-1 protein contained 9 Zn^{2+} ions per 10 molecules of protein. In contrast to the results with Zn^{2+} charged resin, following Ni^{2+} charging of the Sepharose 6 column the eluted fraction of both M-PMV and HIV-1 proteins did not exceed the limit of detection for this ion using AAS (approximately 0.2 ions per 10 protein molecules).

Discussion

Many proteins exhibiting endogenous affinities for metal ions can be purified using IMAC [25,26]. Here, we demonstrate that IMAC can be used to successfully purify retroviral structural proteins in their native state. These proteins contain two zinc finger motifs in their nucleocapsid domain, which specifically interact with zinc ions immobilized on the IMAC column matrix. We also demonstrated that an alternative divalent metal ion, nickel, can be coordinated by the retroviral zinc finger domains, at least in the context of the purification strategies employed here. As the Δ ProCA-NC proteins are being purified in order to study their assembly into virus-like particles *in vitro*, great emphasis has been placed on the purification of these proteins in their native state. To this end, we have avoided adding any affinity tag to these proteins, as it has been reported that such tags can significantly alter the bacterial expression and subsequent behavior of retroviral proteins [27] as well as the DNA binding properties of DNA-binding proteins [28]. A final consideration for the purification of retroviral proteins to be used for studying retroviral particle formation is importance of eliminating nucleic acids (NA) contaminants. In spite of the propensity for IMAC resins to

bind RNA and DNA [29], the procedures presented here resulted in protein preparations with very low RNA/DNA contamination (less than 1 ng NA per 1 µg of protein). This was achieved without the requirement of nuclease digestion, chemical treatment, or secondary purification [30], through the inclusion of 0.5 M NaCl in the column binding buffer.

The purification efficiency of IMAC protocols varies greatly based on the different combination of protein being purified as well as the specific chelating group present on the resin. Previously published results have demonstrated that a zinc ion charged NTA resin may be used for the purification of untagged M-PMV CA-NC proteins [31]. In agreement with these data we confirmed that the M-PMV ΔProCA-NC protein is efficiently bound to the HiTrap™ Chelating HP column (IDA resin) charged with zinc ions. In contrast, under the same experimental conditions HIV-1 ΔProCA-NC proteins failed to bind to the HiTrap™ column. It is known that the unique binding affinity of individual proteins to different metal ions is the primary factor in binding efficiency to IMAC columns [32]. Thus, we surmised that the difference in affinity between M-PMV and HIV-1 ΔProCA-NC proteins to the zinc charged HiTrap™ column was the result of an innate difference in affinity to this metal ion presented on column matrix. Using AAS we confirmed an increase in the amount of zinc ions in the HIV-1 flow-through fraction as compared to the same sample before application on column, suggesting that the HIV-1 zinc finger motifs form a stronger bond with the zinc ions than these ions form with the IDA of the HiTrap™ column resin. Ultimately, this results in passing of HIV-1 protein through this column without any retention. As the M-PMV ΔProCA-NC protein is efficiently bound by zinc charged HiTrap™ Chelating HP resin, this finding suggests that a stronger bond is formed between zinc and the zinc finger domains of HIV-1 ΔProCA-NC protein than that of the analogous M-PMV protein. Furthermore, elution of the M-PMV ΔProCA-NC protein from the nickel charged HiTrap™ column required the use of strong elution agent imidazole, instead of the 2 M NH₄Cl elution buffer in the case of zinc charged HiTrap™ column. This observation suggests that M-PMV ΔProCA-NC protein has higher affinity to nickel ions than to zinc ions. Additionally, the M-PMV ΔProCA-NC protein appears to have a higher affinity for nickel ions than HIV-1 ΔProCA-NC protein, as HIV-1 ΔProCA-NC bound to the nickel charged HiTrap™ columns and was eluted in 2 M NH₄Cl. This observation was supported by dialysis equilibrium experiments, which showed that HIV-1 ΔProCA-NC protein has a higher affinity for zinc ions and a lower affinity to nickel ions in comparison to M-PMV ΔProCA-NC protein (data not shown).

A difference in affinity between HIV-1 and M-PMV ΔProCA-NC proteins for metal ions of different types is the most likely the cause of the different yields following IMAC purification. However, alterations in protein conformation may affect the accessibility of these proteins' zinc finger motifs for interaction with the metal ions on the column matrix. It is known that production of recombinant proteins places stress on bacterial cells, which can result in a portion of recombinant proteins being partially or completely miss-folded [33]. It is possible that these viral proteins are differentially sensitive to such perturbations. Another possible factor in the different purification behavior of M-PMV and HIV-1 proteins could be that these proteins have inherent structural differences. It is known that the secondary structures of both proteins are similar in that they both contain two CCHC boxes. However, these zinc finger domains differ in the amino acid composition inside the CCHC boxes and also the whole proteins contain different number of histidine, cysteine and tryptophan residues, which contribute to interaction with IMAC resins [34,35]. Furthermore, the length of the linker sequence between the zinc-finger domains differs between these proteins, with the zinc-fingers from M-PMV being separated by 15 AA and the zinc-fingers of HIV-1 being separated by 7 AA. As the protein binding to the IMAC column does not strictly depend on affinity between zinc finger domains and metal ion [36,37,38], all of these differences could be influencing the binding of proteins in their native conformation to the column matrices.

An advantage of utilizing the IMAC purification is that there are a number of commercially produced matrices with a diversity of unique chelating groups available. When combined with the possibility of selecting different metal ions, this enables one to optimize conditions for successful protein purification. In our case, the use of a stronger chelating group (in the Sepharose 6 Fast Flow column) allowed for the purification of the HIV-1 Δ ProCA-NC. This protein exhibited too high affinity for naturally interacting ion (Zn^{2+}) when the HiTrapTM column with IDA was used. In addition, using Ni^{2+} ions to charge the Sepharose 6 Fast Flow column did not result in the purified Δ ProCA-NC proteins being contaminated with non-natural ion (Ni^{2+}). Successful purification of metal-binding proteins using IMAC resin charged with metal ions other than that typically coordinated by the protein of interest has been previously reported [25,39,40]. The downside to using the higher affinity IMAC Sepharose 6 Fast Flow column is the resultant lower purification efficiency. This is likely caused by an enhanced nonspecific binding of bacterial cell proteins under these conditions.

For the successful utilization of IMAC, it is necessary to ensure that the zinc finger motifs in the input protein preparation lack zinc and nickel ions. Using our preparatory protocols, this requirement was achieved as the retroviral proteins contained about 1 to 2 of zinc ions per 10 protein molecules (Table 1). Furthermore, it is not usually recommended to purify proteins with metal-binding properties using IMAC due to possible metal ion transfer from column matrix to purified protein [41,1]. Using our purification strategy we did observe capture of both Zn^{2+} and Ni^{2+} by M-PMV and HIV-1 Δ ProCA-NC proteins except in case of Ni^{2+} charged Sepharose 6 column (Tab. 1). This was not surprising since retroviral zinc finger motif contains three cysteine residues and thiol groups have strong affinity for zinc and other ions and may thus scavenge them from IDA matrix [32,42]. Under all like conditions, metal ion contamination was found in eluates from the HiTrapTM column compared to that of the Sepharose 6 column (Tab. 1). In contrast to some other metal binding proteins, retroviral zinc finger proteins naturally contain zinc ions and the presence of these ions is necessary for the folding of these proteins into their native conformation as well as proper protein function.

When purified Δ ProCA-NC proteins were analyzed by SDS-PAGE (Fig. 4), several bands were observed that do not correlate to the expected molecular mass of the viral Δ ProCA-NC proteins (35 kDa for M-PMV and or 32 kDa for HIV-1). We would like to stress out that these bands are likely the products of proteolytic cleavage or multimeric forms of Δ ProCA-NC proteins. There exist endogenous cleavage sites for the retroviral protease between the CA and NC domains of Δ ProCA-NC fusion proteins [31] and these can be subject to cleavage by bacterial proteases during the purification process. The bands of smaller than expected size are likely cleavage products of Δ ProCA-NC and the major lower molecular weight band is of a size correlating to the viral CA protein. The band of larger than expected size is of a size consistent with dimers of these Δ ProCA-NC proteins (Fig. 4B). These retroviral proteins exhibit a naturally strong tendency toward forming homo-multimers. This affinity drives the retroviral particle assembly process, and likely also results in the formation of SDS-resistant dimers [43].

In summary, IMAC purification strategies are suitable for the purification of retroviral zinc finger proteins in their native state, with the binding of these proteins to the column matrix being probably mediated by their zinc finger motif. We repetitively obtained purified M-PMV and HIV-1 Δ ProCA-NC proteins containing minimal nucleic acid contamination and in a suitable conformation for downstream applications. We were able to achieve the highest purity of M-PMV protein using the HiTrapTM Chelating HP column with immobilized zinc ions (94 % purity). In contrast, the use of the Sepharose 6 Fast Flow column charged with nickel ions was found to be the most suitable for purification of the HIV-1 protein (86 % purity). A higher purity of the HIV-1 protein (94 %) was obtained with the HiTrapTM

Chelating HP column charged with nickel ions. However, this resulting protein was contaminated by nickel ions (1 ppm) making it less suitable for downstream applications. It is likely that other proteins that contain zinc finger can also be successfully purified by IMAC without the addition of any exogenous affinity tag. However, the work presented here shows that careful empiric evaluation of metal ion and chelator combinations as well as purification buffers is necessary for each protein.

Acknowledgments

We thank Michaela Rumlova for kind providing expression plasmids and polyclonal anti-capsid protein antibodies. We thank Tibor Fuzik for help with preparation of figures. This study was supported by the grants from the Czech Ministry of Education 1M6837805002, ME 904 and MSM 6046137305 and National Institutes of Health, United States grant CA 27834.

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Figure 1.
M-PMV and HIV-1 ΔProCA-NC purification scheme.

Figure 2.

SDS-PAGE analysis of HIV-1 Δ ProCA-NC pre-purification fractions. 10 % SDS-PAGE gel, stained with Coomassie brilliant blue.

Lane M – Broad Range (Bio-Rad) molecular weight marker; lane E – uninduced *E. coli* whole lysate; lane I – induced *E. coli* whole lysate; lane S – supernatant after initial lysis and centrifugation; lane P – pellet after initial centrifugation; lane W1 – supernatant obtained after the first pellet wash with buffer contains 1 M NaCl; lane W2 - supernatant obtained after the second pellet wash with buffer contains 1.5 M NaCl; lane W3 - supernatant obtained after the third pellet wash with buffer contains 1.5 M NaCl; lane P2 – pellet remained after all three high-salt washes.

Figure 3.

SDS-PAGE analysis of M-PMV and HIV-1 Δ ProCA-NC protein purification steps using the HiTrapTM Chelating HP or IMAC Sepharose 6 Fast Flow column charged with Zn²⁺ or Ni²⁺. 10 % SDS-PAGE gel stained with Coomassie brilliant blue.

Panel A – purification of proteins using the HiTrapTM Chelating HP column; Panel B – purification of proteins using the IMAC Sepharose 6 Fast Flow column.

Lanes M – Broad Range molecular weight marker; lanes I – protein sample loaded to IMAC columns; lanes FT – flow-through of IMAC columns; lanes E – protein eluted from the IMAC columns using buffer containing 2 M NH₄Cl; lanes E2 – protein eluted from the IMAC columns using buffer containing 100 mM imidazole.

Figure 4.

Electrophoretic analysis of purified M-PMV and HIV-1 Δ ProCA-NC proteins.

Panel A – 10 % SDS-PAGE gel, stained with Coomassie brilliant blue; Panel B - Western blot analysis using anti-capsid protein rabbit antibody (M-PMV and HIV-1) and HRP-conjugated anti-rabbit antibody.

Panel A - Broad Range molecular weight marker (Bio-Rad); Panel B - Prestained Broad Range molecular weight marker (Bio-Rad). M-PMV or HIV-1 proteins were purified using appropriate IMAC column charged with Zn^{2+} or Ni^{2+} ions as indicated. The lane corresponding to HIV-1 protein purified on zinc charged HiTrap™ Chelating HP column is missing in the figure, because this protein passed through this column without any retention. The arrows indicate Δ ProCA-NC protein multimers or cleavage products.

HiTrap™ HiTrap™ Chelating HP column; Sepharose 6: IMAC Sepharose 6 Fast Flow column

Figure 5. Electronmicroscopic images of particles assembled from the M-PMV (panel A) and HIV-1 (panel B) Δ ProCA-NC proteins purified using the HiTrapTM Chelating HP column (M-PMV) or IMAC Sepharose 6 Fast Flow column (HIV-1). Negative staining was performed by 4 % sodium silicotungstate, pH 7.4. Scale bars represent 200 nm.

Table 1

Content of zinc and nickel ions in ΔProCA-NC samples following IMAC purification.

	M-PMV		HIV-1	
	initial sample	fraction	initial sample	fraction
HiTrap™	Zn	10:16	10:1	10:10 ^a
	Ni	10:2 ^b	10:1	10:2
Sephacrose 6	Zn	10:8	10:2	10:9
	Ni	under limit ^b	under limit	under limit

The values represent molar ratios between proteins and metal ions. Content of metal ions was measured in elution fraction or flow-through fraction. Metal ions were determined using atomic absorption spectrometry.

^a: Content of metal ions was measured in flow-through fraction. Viral ΔProCA-NC protein did not bind to the column under given conditions.

^b: Content of metal ions was measured in elution fraction using buffer containing 100 mM imidazole instead of 2 M ammonium chloride.

All samples were quantitated in triplicate and in all cases the relative standard deviation did not exceed 10 %.

HiTrap™ HiTrap™ Chelating HP column; Sepharose 6: IMAC Sepharose 6 Fast Flow column