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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.

Introduction

Immobilized metal ion affinity chromatography (IMAC)[1] 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 Ni^{2+} ions, although many different divalent metal ions such as Cu^{2+}, Co^{2+}, Fe^{2+} and Zn^{2+} 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 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 C_2H_2 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 C_2H_2 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

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[1] *Abbreviations used:* IMAC, immobilized metal ion affinity chromatography; His-tag, hexa-histidine affinity tag; IDA, iminodiacetic acid; NTA, nitrilotriacetic acid; MA, matrix; CA, capsid; NC, nucleocapsid; CCHC, Cys-X2-Cys-X4-His-X4-Cys; M–PMV, Mason–Pfizer monkey virus; HIV-1, human immunodeficiency virus type 1; PMSF, phenylmethylsulfonyl fluoride; AAS, atomic absorption spectrometry; FPLC, fast protein liquid chromatography; NA, nucleic acids.

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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 Zn^{2+} 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 [17–20]. 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 qhuman 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-ΔProCA-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-ΔProCA-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 μg/ml) was inoculated with *Escherichia coli* BL21(DE3) cells carrying the appropriate ΔProCA-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 $O.D._{590} \sim 0.4–0.6$. The bacteria were harvested 4 h post-induction by low-speed centrifugation.

Purification of M–PMV and HIV-1 ΔProCA-NC proteins

The following buffers were utilized in the isolation of ΔProCA-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 μg/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 μM $ZnCl_2$.

The bacterial pellet obtained from low speed centrifugation of 1 l of bacterial culture was resuspended in 30 ml of lysis buffer and stirred at room temperature for 30 min and then sonicated on ice 4 × 10 s 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 min, after which the lysates were centrifuged at 10,000*g* for 10 min 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*g* for 10 min 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 ΔProCA-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 $ZnSO_4$ or 0.1 M $NiSO_4$) 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 NH_4Cl, 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 Centriplus® 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 h 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),