Process Biochemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Dual column approach for the purification of zinc finger proteins by immobilized metal affinity chromatography

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ARTICLE INFO

Keywords:
Zinc finger
Protein purification
Immobilized metal affinity chromatography
IMAC

ABSTRACT

The feasibility of using zinc fingers as the affinity tag for the purification of recombinant proteins with immobilized metal affinity chromatography (IMAC) was investigated. It was shown that upon the release of pre-existing metal ions from the model zinc-finger fusion protein by dialysis with buffer containing 500 mM NaCl, the recovery of the zinc-finger fusion protein was increased from 47% to 87%. The purity of the zinc-finger fusion protein was further improved with a pre-precipitation step at pH 5.0. The adsorption capacities for the zinc-finger fusion protein were in the order of Ni(II) > Cu(II) > Zn (II) > Co(II). Based on the low affinity of Zn (II)-loaded IMAC adsorbent for the model protein, a dual column process with Zn(II)-IMAC as the negative column for the removal of unwanted proteins and Ni(II)-IMAC as the positive column for the adsorption of the zinc-finger fusion protein was proposed, giving a purity of 98%. Purification of the zinc-finger fusion protein under denaturing conditions was also demonstrated. The results of this study suggest that it is possible to purify zinc finger proteins with IMAC without the need for external affinity tags, which is particularly useful for the purification of zinc finger proteins for structural analysis.

1. Introduction

Immobilized metal affinity chromatography (IMAC) has been widely used as a group specific affinity method for the purification of recombinant proteins since its introduction more than three decades ago [1]. The low dissociation constants between the chelated transition metal ions and the proton-donating imidazole rings of polyhistidine tags endow IMAC a superior selectivity for His-tagged proteins [2,3]. Compared to other biospecific chromatographic methods, IMAC exhibits the advantages of higher binding capacities and higher recovery yields under both native and denaturing conditions [4–6], making it an ideal approach for one-step purification of recombinant proteins on bench and industrial scales [7–11]. In addition to downstream processing, the applications of IMAC in chemical biology and proteomics have also been explored [12,13].

Although oligohistidine-tags of various lengths have been shown effective for mediating IMAC adsorption [14,15], results of a recent thermodynamic study suggest that hexahistidine (His) tags exhibit the strongest affinity toward Ni(II)-NTA IMAC resin [16]. Since His tags are much smaller compared to other affinity tags employed for protein purification [17–19], the fusion of His tags to the N- or C-termini generally does not affect the folding and thus the biological functions of recombinant proteins [20,21]. Upon recovery the His tags can be readily cleaved enzymatically with endo- or exo-proteases [18,22] and removed from the desired proteins by another round of IMAC, resulting in crystallization-grade proteins [18,23].

Nevertheless, changes in conformations and thus biological activities upon the fusion of His tags to either *N*- or *C*-termini of recombinant proteins have been documented. For example, it has been reported that the fusion of a His tag to the *C*-terminus of the *β*-lactamase from a thermophilic *Bacillus licheniformis* resulted in a modification of cleavage site of the signal peptide at the *N*-terminus [24]. Altered binding properties due to conformational change upon the fusion of His tags to recombinant proteins have also been observed [25,26]. Furthermore, incomplete cleavage of His tags from oligomeric proteins could lead to low yield of tag-free proteins [27]. Therefore, it may not always be advantageous to introduce His tags to recombinant proteins, especially for proteins exhibiting metal-binding affinity.

Zinc finger proteins are proteins that contain zinc-binding motifs or zinc fingers -functional, independently folded zinc-coordinated domains mediating nucleic acid-binding, protein-protein interaction, and membrane association [28]. A variety of repeating substructures exhibiting zinc affinity have been identified since zinc finger's discovery three decades ago [29,30]. Based on the main chain conformation and

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https://doi.org/10.1016/j.procbio.2018.08.025
Received 1 June 2018; Received in revised form 24 July 2018; Accepted 21 August 2018

the secondary structure around the zinc-binding domains, zinc finger structures have been classified into eight fold groups [31]. Potential applications of zinc finger proteins have been reviewed [32].

Due to zinc finger proteins' intrinsic propensity to coordinate with zinc ions, it might be possible to purify native zinc finger proteins without fusion tags using IMAC [11]. Voráčková and coworkers have demonstrated that different host proteins containing the same type of zinc finger motif may exhibit different affinities for metal ions and that it is possible to purify zinc finger proteins with the appropriate metal ions [33]. Based on our unpublished results, however, the purities of zinc finger proteins thus obtained were generally lower than those with His tags, probably due to the relatively weak affinities of zinc fingers for chelated metal ions compared to His tags. To improve the utility of IMAC for the purification of zinc finger proteins, a systematic investigation was conducted in this study with an engineered fragment of M. HhaI cytosine methyltransferase containing a Zif268 (1AAY) zinc finger [34]. The Zif268 zinc finger belongs to the classical C2H2 finger family that typically contains a repeated 28–30 amino acid sequence with two conserved cysteines and two conserved histidine residues [31]. It was found that upon optimization a dual column IMAC technique is effective for the purification of the model zinc-finger fusion protein

2. Experimental

2.1. Expression of the engineered zinc-finger fusion protein

E. coli K-12 strain ER2267 harboring pDIMN8 with gene encoding M. HhaI cytosine methyltransferase fragment was grown in Luria-Bertani medium (USB) at 37 °C [34]. When the OD_{600} of the culture reached 0.6, the expression of recombinant methyltransferase fragment, the model zinc-finger fusion protein, was induced by adding arabinose (Sigma-Aldrich) to a final concentration of 10 mM. Upon induction, the culture was further incubated at 37 °C for another 12 h with shaking. Cells pellets, collected by centrifugation at 10,000 x g at 4 °C for 10 min, were re-suspended in 50 mM phosphate buffer, pH 8.0 containing 100 mM NaCl and 50 mg/l lysozyme (Sigma-Aldrich) and subsequently lysed by ultrasonication in an ice bath. The crude cell lysate containing the zinc-finger fusion protein was collected by centrifugation at 10,000 × *g* for 15 min and room temperature.

2.2. Preparation of IMAC resins

Ni-NTA resin (Qiagen, Germany) was used for the purification of the zinc-finger fusion protein, unless specified otherwise. To prepare IMAC adsorbents with other metal ions, 25 ml of Ni-NTA resin (Qiagen, Germany) was washed sequentially with 25 ml of DI water and 25 ml of 50 mM phosphate buffer, pH 8.0 containing 100 mM EDTA (USB) to dissociate the chelated Ni(II). After rinsing with DI water, the bare IMAC resin was equilibrated with 25 ml of metal ion solution, containing 100 mM $CoSO_4$, $CuSO_4$, $Fe_2(SO_4)_3$, or $ZnSO_4$, depending on the metal ion under study, and then rinsed with equal volume of DI water. The metal ion-loaded NTA IMAC resin was then stored in 30% ethanol solution at 4 °C before use.

2.3. Optimization of the IMAC process

To remove the chelated metal ions from the zinc-finger fusion protein, crude cell lysate was mixed with equal volume of 50 mM phosphate buffer, pH 8.0 containing 0.9 M and 1.9 M NaCl, giving a final NaCl concentration of 500 mM and 1000 mM, respectively. The mixtures were then dialyzed (MWCO 12000–14000, Spectra, USA) extensively against 50 mM phosphate buffer, pH 8.0 containing 500 mM or 1000 mM NaCl accordingly. To adjust the concentrations of NaCl (ranging from 100 mM to 1000 mM) and imidazole (Sigma, USA, ranging from 0 to 20 mM) and pH (ranging from 5 to 8) of the cell lysate, crude cell lysate was dialyzed against phosphate buffer with the desired concentrations of NaCl and imidazole at the designated pH. The dialyzed cell lysate was clarified by centrifugation at 10,000 × *g* for 10 min, if precipitated was observed.

Unless specified otherwise, for all IMAC purification 5 ml cell lysate was loaded to IMAC column containing 2.5 ml resin and allowed to sit at 4 °C for 3 h. The column was then washed with buffer of composition identical to that of the cell lysate and subsequently eluted with phosphate buffer containing 250 mM imidazole. For protein purification under denaturing conditions, cell lysate containing urea (Showa, Japan) at concentrations ranging from 2 to 8 M was incubated at 4 °C for 2 h before loading.

For the dual column process, cell lysate or clarified cell lysate obtained after pre-precipitation at pH 5.0 was loaded to a Zn(II)-IMAC column. The flow-through fraction from the Zn(II)-IMAC column was then used as the feed to a Ni(II)-IMAC column.

2.4. Analysis

Protein concentrations of all samples were analyzed by Bradford method with protein dye (Bio-Rad, USA) at 595 nm [35]. SDS-PAGE with 12% polyacrylamide gel stained with Commassie blue was used for the analysis of protein purities and recoveries [36]. Image analysis was performed with the GelAnalyzer software (GelAnalyzer.Com).

3. Results and discussion

3.1. Purification of the zinc-finger fusion protein with IMAC

To enable effective adsorption of zinc finger proteins via the formation of coordinate bonds between the zinc-binding motifs of the proteins and the metal ions chelated on IMAC adsorbents, it might be necessary to remove the pre-existing metal ions in zinc finger proteins. It has been shown that the coordinated ions can be released and removed by dialysis against buffer containing 1.0 M NaCl [33]. We thus examined the effect of dialysis on the adsorption of the zinc-finger fusion protein in IMAC. As shown in Fig. 1, upon extensive dialysis against the dialysis buffer containing 500 mM NaCl, the amount of the zinc-finger fusion protein adsorbed and recovered from Ni-NTA column was significantly increased. Results of image analysis indicated that the recovery of the zinc-finger fusion protein was increased from 47% (lane 4) to 87% (lane 8). However, further increase in NaCl concentration did not lead to higher recovery (data not shown), suggesting that extensive dialysis in the presence of 500 mM NaCl is sufficient to release the pre-

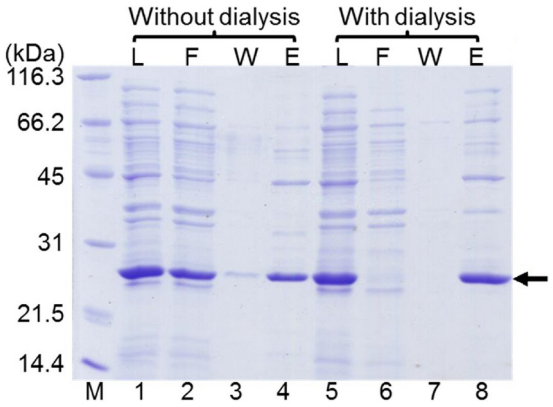

Fig. 1. Effect of dialysis on the adsorption of the zinc-finger fusion protein in IMAC. Crude cell lysate and cell lysate dialysis against 50 mM phosphate buffer, pH 8.0 containing 500 mM NaCl were loaded to a Ni-NTA IMAC column, respectively. Lane M: molecular weight markers, lanes 1 & 5: cell lysate, lanes 2 & 6: flow-through fractions, lanes 3 & 7: wash fractions, and lane 4 & 8: eluted fractions.