Protein Expression and Purification 94 (2014) 85–94

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Purification of a recombinant human growth hormone by an integrated IMAC procedure

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ARTICLE INFO

Article history:
Received 2 October 2013
and in revised form 1 November 2013
Available online 22 November 2013

Keywords:
Human growth hormone
IMAC adsorbents
N-terminally tagged proteins
Tag removal
1,4,7-Triazacyclononane (tacn)
Bis(1,4,7-triazacyclononyl)propane
Novel affinity tags

ABSTRACT

In this study, integration of three discrete process aspects of the IMAC purification of *Escherichia coli* expressed recombinant proteins has been investigated. To this end, novel N-terminally tagged human growth hormone variants (tagged-vhGHs) have been expressed in *E. coli* by tank fermentation and captured directly from the cell lysate by a new IMAC approach. The chelating ligands used were 1,4,7-triazacyclononane (tacn) and bis(1,4,7-triazacyclononyl)-propane (dtnp) with copper(II) as the immobilised metal ion. The N-terminal tags were specifically selected for their potential to bind to these immobilised complexes and also for their ease of removal from the tagged protein by the dipeptidyl peptidase, DAP-1. Low levels of detergents in the binding buffer did not dramatically affect the purification, but increased concentrations of NaCl in the loading buffer improved the binding performance. The same IMAC systems, operated in the 'negative' adsorption chromatographic mode, could be used to obtain the purified mature human growth hormone variant, as assessed by MALDI-TOF and N-terminal sequencing studies, following removal of the affinity tag by the dipeptidyl peptidase 1. Western immunoblot analysis of the eluted fractions of both the tagged and de-tagged vhGH demonstrated significant clearance of *E. coli* host cell proteins (HCPs). Further, these IMAC resins can be used multiple times without the need for metal ion re-charging between runs. This study thus documents an integrated approach for the purification of specifically tagged recombinant proteins expressed in genetically modified *E. coli*.

Introduction

Improvements to affinity chromatographic methods have become extremely important with the advent of proteomics, structural biology, metabolic engineering, systems pathway analysis and biopharmaceutical discovery [1]. Because of its applicability as a platform technology [2–5], immobilised metal ion affinity chromatography (IMAC)[1] has emerged over the past decade as one of the most widely used methods at the laboratory scale to isolate target proteins from fermentation broths, mammalian cell culture supernatants or other biological sources. In IMAC, protein purification is achieved by the formation of co-ordination bonds between the chelated metal ions and functional groups of surface accessible amino acids, often introduced into a target protein as an N- or C-terminal peptide 'tag'.

Typically in IMAC, borderline metal ions, as classified according to the Pearson acid–base concept [6], i.e. Ni^{2+}, Cu^{2+}, Zn^{2+} or Co^{2+} ions, are used. Due to their affinity for these borderline metal ions, surface accessible histidine residues of proteins are often involved in such coordinative interactions with the immobilised chelated metal ion [4,7–9]. Because of their relatively low abundance in naturally-occurring proteins, the inclusion of multiple histidines into a tag sequence N- or C-terminally fused to the target protein by recombinant DNA procedures has become a widely used approach to generate greater selectivity vis-à-vis the adventitious binding of host cell proteins (HCPs). The metal binding properties of histidine residues are conferred by the imidazole ring with elution of the fusion protein therefore readily achieved via competition with a suitable buffer additive, such as imidazole or histidine, or a decrease in the pH, resulting in protonation of the imidazolyl moiety.

Commonly used metal chelating resins for IMAC applications involve tridentate (*e.g.* iminodiacetic acid-IDA) or tetradentate (*e.g.* nitriloacetic acid-NTA) ligands. With such structurally unconstrained chelating ligands, the metal ion stability constants (β-values) for protein binding can be higher than for ligand binding

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[1] *Abbreviations used:* IMAC, immobilised metal ion affinity chromatography; HCPs, host cell proteins; IDA, iminodiacetic acid; NTA, nitriloacetic acid; MIT, metal ion transfer; tacn, 1,4,7-triazacyclononane; CIP, clean-in-place; SA, sinapinic acid; HCP, host cell proteins.

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http://dx.doi.org/10.1016/j.pep.2013.11.002

and are strongly influenced by pH or salt effects arising from the choice of the composition of the loading or elution buffer [8,10,11]. As a consequence, metal ion transfer (MIT) or metal ion leakage effects can occur, which can lead to disruption of the stability of the target proteins, the requirement for more demanding analytical and characterisation procedures, and the need to introduce additional unit operations in the downstream process to remove the contaminating metal ion [2,12,13]. Unlike other tridentate ligands, the structurally constrained metal chelating ligand, 1,4,7-tri-azacyclononane (tacn) exhibits much higher metal ion stability constants than IDA or its analogues for Ni^{2+} and Cu^{2+} ions (*e.g.* at least 100 fold larger), without sacrificing co-ordination site availability for protein interaction [14]. Tacn ligands can be readily linked synthetically to form higher homologues and analogues, such as the bis(tacn)s (Fig. 1), with even higher metal ion stability constants, *e.g.* with β-values of 10^{14} to 10^{20} [15]. Besides their ability to be structurally 'tuned' to generate ligand families of wide binding versatility, tacn-based ligands can be readily prepared by green chemical routes, exhibit very low toxicity, are easily immobilised onto support materials, are stable to a wide range of chemical conditions and are compatible with clean-in-place (CIP) procedures.

In order to capture the full potential of these tacn-based IMAC adsorbents, recently we reported the generation of several unique sets of peptides that selectively displayed strong binding affinities for borderline metal ions. Further work based on bio-panning random phage display libraries against Ni^{2+}-tacn and the bis(tacn) analogues, Ni^{2+}-dtne and Ni^{2+}-dtnp, led to the discovery of additional peptides of unique sequences that could be ranked in subsequent screening studies according to their ease of cleavability by proteases/peptidases [16].

The application of tacn-related IMAC resins in the downstream processing of tagged recombinant proteins has been further investigated with Cu^{2+} as the chelated metal ion. In this study Cu^{2+} ions were employed as the coordinating ion because the NT1- and NT1A-tags bind to Cu^{2+}- and Ni^{2+}-charged tacn-derived resins with similar efficiencies, and secondly to avoid exposure to the significant skin allergic properties of Ni^{2+} ions [13,17]. Moreover, in stream tag removal was investigated since this often is an important requirement to validate the full functionality of a recombinant protein undergoing structure–function studies or during evaluation of its therapeutic potential [18–21]. Due to its therapeutic and commercial importance [22,23], a human growth hormone variant, containing examples of these new tags (namely NT1-HHHNSWDHDINR or its $Ile^{10}\rightarrow Ala^{10}$ analogue, NT1A-HHHNSWDHDANR) inserted as N-terminal sequences, was expressed in *Escherichia coli* cells by tank fermentation and purified by this IMAC approach. The NT1/NT1A tags were specifically selected to validate their predicted propensity to be specifically excised by dipeptidyl peptidase 1 (EC 3.4.14.1) (DAP-1), an enzyme with several advantages over other proteolytic enzymes for tag removal [24–26], from this recombinant fusion protein thus generating the mature human growth hormone variant. Moreover, the versatility of this IMAC system was evaluated by varying a number of parameters, such as resin type and elution conditions, in order to establish the scope and a range of working protocols of this integrated metal ion affinity chromatography procedure. Examples documenting this scope are reported herein.

Materials and methods

Synthesis and immobilisation of tacn and dtnp

1,4,7-Triazacyclononane (tacn) and bis(1,4,7-triazacyclononyl)propane (dtnp) were prepared as described previously [14,15,27,28]. The ligands were immobilised onto epoxy-activated Sepharose™ FF with several modifications to our method reported previously [29]. Thus, suction-dried Sepharose™ FF (100 ml) was activated using epichlorohydrin (60 ml) and 2 M NaOH (100 ml) at 28 °C for 21 h. Solutions of the ligand salts were dissolved in water (100 ml) and adjusted to pH 12 with 2 M NaOH to a concentration of 0.2 M. Suction-dried activated Sepharose™ FF (100 ml) was added and the suspension gently agitated at 28 °C for 4.5 days. Immobilised tacn adsorbents were also prepared by mixing the suspension at 60 °C for 24 h. The ligand densities of the immobilised matrices were determined by elemental analysis (The Campbell Micro-analytical Laboratory, Department of Chemistry, University of Otago, Dunedin, New Zealand) and typically corresponded to 330 μmol/g dry gel and 210 μmol/g dry gel or the tacn- and dtnp-resins respectively.

Construction of the expression vector containing the NT1 and NT1A-tagged variant human growth hormone gene

Amplification of the human growth hormone variant (vhGH) cDNA by polymerase chain reaction (PCR) methods used the forward primer (TAAAGTCGACTTCCCAACCATTCCCTTA) and the reverse primer (TAAAAGCTTTTAGAAGCCACAGCTGCCCTC). The forward primer incorporated a *Sal*I restriction site (underlined) and the reverse primer incorporated a *Hin*dIII site (underlined) for downstream cloning steps. The PCR product was digested with SalI and HindIII and cloned into *Sal*I/*Hin*dIII digested pBluescript SKII+ vector. In order to generate the vhGH construct tagged with NT1 or NT1A at the N-terminus, pTrc99a containing the NT1-GST or NT1A-GST sequence [30] was digested with *Sal*I and *Hin*dIII to remove the GST sequence. This sequence was replaced by directional subcloning with the vhGH sequence excised from pBluescript SKII+ following *Sal*I/*Hin*dIII digestion.

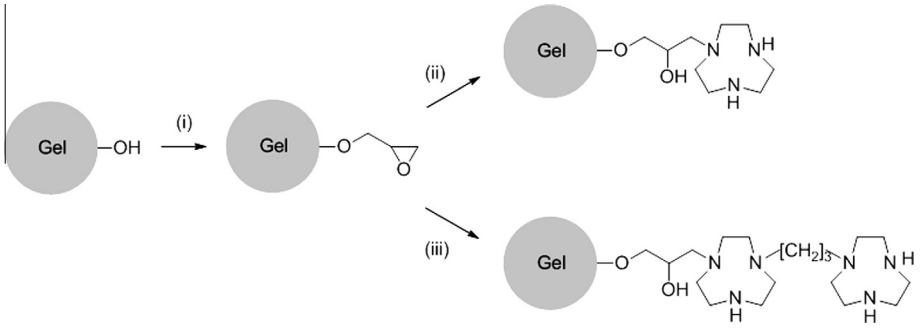

Fig. 1. Structures of 1,4,7-triazacyclononane (tacn) and bis(1,4,7-triazacyclononyl) propane (dtnp) and procedures employed to generate the respective IMAC resins. (i) Activation of Sepharose™ FF with epichlorohydrin; (ii) immobilisation of tacn; and (iii) immobilisation of dtpn.