A Nuvia™ IMAC Resin–Based Purification Workflow for the Production of Highly Pure Recombinant Proteins

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Tech Note

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Abstract

A three-column workflow has been developed for the purification of a histidine-tagged recombinant protein from *E. coli* extract. The macroporous Ni^{2+}-charged Nuvia IMAC Resin provides efficient capture of the target protein molecules with excellent selectivity, binding capacity, and recovery. The subsequent mixed-mode chromatography steps, performed with Nuvia™ cPrime™ Resin followed by CHT™ Ceramic Hydroxyapatite Media, eliminate leached Ni^{2+} ions and offer further clearance of residual impurities from host cells.

Introduction

Immobilized metal affinity chromatography (IMAC) is one of the most popular tools for the preparation of recombinant proteins in a single step. Proteins naturally rich in histidine or cysteine residues, as well as those with histidine residue clusters engineered to their N- or C-terminus via recombinant DNA technology, form strong chelation complexes with transition metals, such as Ni^{2+}, Cu^{2+}, Co^{2+}, or Zn^{2+}, immobilized on chromatography matrices. After impurities are washed off, the bound target protein molecules are selectively eluted from the IMAC column with buffers containing metal chelator imidazole. As with other affinity chromatography techniques, purification with IMAC is robust and requires minimal optimization of conditions. It is typically performed at physiological pH and shows good tolerance for a wide variety of chemicals (Bio-Rad bulletin 6859), which is crucial to maintaining the structural integrity and biological activity of target protein molecules. Proteins prepared with IMAC are often pure enough for structural and functional characterization studies.

In recent years, the use of IMAC has been extended to the manufacture of protein therapeutics (Bhattacharyya et al. 2004), vaccines (Angov et al. 2003, Darko et al. 2005, Ockenhouse et al. 2006, Stoute et al. 2007), and adeno-associated virus (AAV) (Koerber et al. 2007). However, protein pharmaceuticals are held to extremely high purity standards to ensure drug efficacy and the safety of patients. In addition to initial target extraction from crude bioprocess mixtures, further polish purification is necessary for sufficient clearance of contaminants, such as host cell proteins (HCPs) and DNA, and leachables from chromatography media. In this study, we demonstrate a three-column worfklow for the purification of a histidine-tagged protein (Figure 1). Nuvia IMAC, a rigid macroporous high-capacity resin designed for process production at high flow rates, was used to capture histidine-tagged protein from *E. coli* lysate. Further purification was facilitated by chromatography with two mixed-mode media, Nuvia cPrime and CHT Ceramic Hydroxyapatite. This purification sequence resulted in effective removal of hazardous contaminants such as endotoxins and leached Ni^{2+} and the significant reduction of host cell proteins and DNA.

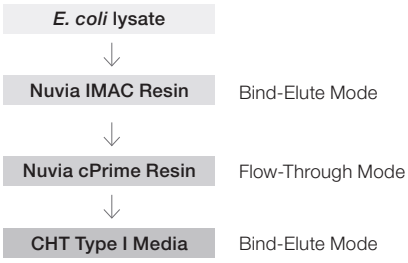

Fig. 1. Purification workflow for a histidine-tagged target protein from *E. coli* lysate.

Material and Methods

General

E. coli cell lysate containing the target histidine-tagged protein was prepared by the Center for Biocatalysis and Bioprocess, University of Iowa. All chromatography was conducted on a BioLogic DuoFlow QuadTec™ 10 System (Bio-Rad Laboratories). Chromatography media Nuvia IMAC, Nuvia cPrime, and CHT Type I, 40 µm (Bio-Rad) were packed and regenerated following manufacturer's instructions (bulletins 10044307, 10023853, and 6086, respectively). Protein fractions were analyzed by SDS-PAGE using Criterion™ Precast Gels (Tris-HCl 4–20% linear gradient), Bio-Safe™ Coomassie Stain, and Gel Doc™ EZ Gel Documentation System (all Bio-Rad). The clearance of HCPs and double-stranded DNA (dsDNA) was determined by *E. coli* HCP ELISA Kit (Cygnus Technologies) and ddPCR™ *E. coli* Residual DNA Quantification Kit (Bio-Rad), respectively. Endotoxin contamination in protein samples was detected with the Endpoint Chromogenic LAL Assays (Lonza). Leached Ni^{2+}

was quantified by Exova using inductively coupled plasma mass spectrometry (ICP-MS). Protein concentration was quantified with Bradford Protein Assay (Bio-Rad).

Capture purification of histidine-tagged protein by immobilized metal affinity chromatography using Nuvia IMAC Resin

Nuvia IMAC Resin was packed in a 2.2 x 25 cm column and equilibrated with 10 column volumes (CV) of 50 mM sodium phosphate, 10 mM imidazole, 300 mM sodium chloride, pH 7.5 (buffer A). *E. coli* cell lysate was centrifuged at 30,000 rpm for 5 min to collect the supernatant, which was then applied onto the Nuvia IMAC Column at 150 cm/hr. The loaded column was washed with 5 CV of buffer A followed by 5 CV of 50 mM Tris-HCl, 5 mM sodium phosphate, pH 7.5 (buffer B). The bound target protein was eluted with 10 CV of 50 mM Tris-HCl, 5 mM sodium phosphate, 125 mM imidazole, pH 7.5 (buffer C). Manufacturer's instructions (Bio-Rad bulletin 10044307) were followed to regenerate the column.

Intermediate polish purification by hydrophobic cation exchange chromatography using Nuvia cPrime Resin

The eluate from the Nuvia IMAC column was loaded at 300 cm/hr directly onto a 5 ml Nuvia cPrime Column (1.25 x 4 cm) pre-equilibrated with 10 CV of buffer B. The target histidine-tagged protein was recovered in the flow-through fractions. This column was then regenerated with 5 CV of 50 mM Tris-HCl, 5 mM sodium chloride, pH 7.5 (buffer D), 5 CV of 50 mM sodium phosphate, 2 M sodium chloride, pH 7.5 (buffer E), followed by 5 CV of 1 N sodium hydroxide.

Further purification by ceramic hydroxyapatite media chromatography

CHT Ceramic Hydroxyapatite Type I, 40 µm Media was employed for further purification of target protein. A design of experiment (DoE) study was performed to screen for the optimal elution condition. The effects of sodium phosphate and sodium chloride concentrations and buffer pH were evaluated using a two-level full factorial screening design as suggested by JMP Software (jmp.com) (Table 1). Mini Bio-Spin® Columns, each containing 100 µl of CHT Media pre-equilibrated with buffer B, were used in this screening study. Pre-equilibrated CHT Media was mixed at room temperature for 3 min with agitation in a spin column with 500 µl of load, which contained ~0.4 mg histidine-tagged protein purified using the above described Nuvia cPrime chromatography step. Unbound material was removed at the end of the incubation by centrifugation at 1,000 x g for 1 min. Elution of target protein was achieved by incubation with each elution buffer to be tested for 10 min with agitation, followed by centrifugation at 1,000 x g for 1 min. These eluates were analyzed by SDS-PAGE. The target protein yield, as reflected by the target protein band density, and target protein purity were quantified using Image Lab™ Software (Bio-Rad). A standard least squares model was employed to obtain the response surfaces using JMP Software.

Table 1. Design of experiment (DoE) setup.

Experiment	Elution Buffer Sodium Phosphate Concentration, mM	Elution Buffer Sodium Chloride Concentration, M	Elution Buffer pH
1	150	2	6.5
2	50	2	6.5
3	50	0	7.5
4	150	0	6.5
5	50	0	6.5
6	**100**	**1**	**7**
7	150	2	7.5
8	**100**	**1**	**7**
9	50	2	7.5
10	150	0	7.5

Note: Experiments 6 and 8 are the center points

For scale-up purification, a 1 ml CHT Type I, 40 µm Column (0.56 x 4.0 cm) was equilibrated with 10 CV of buffer B. The partially purified histidine-tagged target protein from the previous two steps was applied onto this CHT Column. The loaded column was washed with 10 CV of 50 mM sodium phosphate, 400 mM sodium chloride, pH 7.5 (buffer F). Target protein was eluted by 10 CV of 50 mM sodium phosphate, 1.2 M sodium chloride, pH 7.5 (buffer G). The used column was regenerated by 2 CV of 50 mM Tris-HCl, 5 mM sodium phosphate, 2 M sodium chloride, pH 7.5 (buffer H) followed by 5 CV of 1 N sodium hydroxide.

Results

The open pores of Nuvia IMAC allow efficient mass transfer under dynamic conditions. Its hydrophilic inert surface results in minimal nonspecific binding through charge or hydrophobic interactions. As demonstrated in Figure 2, the histidine-tagged target protein was efficiently captured from the *E. coli* lysate by chelation with Nuvia IMAC Resin and eluted with over 95% recovery and near homogeneous purity as visualized by SDS-PAGE. The 10% breakthrough dynamic binding capacity of target protein was estimated as 50–60 mg per ml of resin (data not shown).

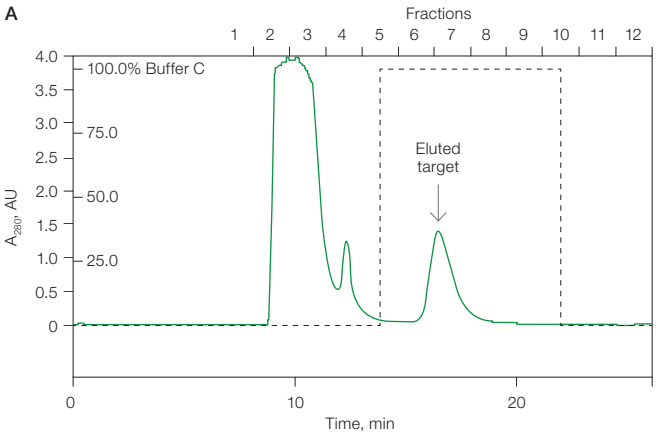

Fig. 2A. Capturing histidine-tagged protein from *E. coli* cell lysate using a Nuvia IMAC Column. Chromatogram of histidine-tagged protein purification on a Nucia IMAC Column. A_{280} (—); % buffer C (--); AU, absorbance units.

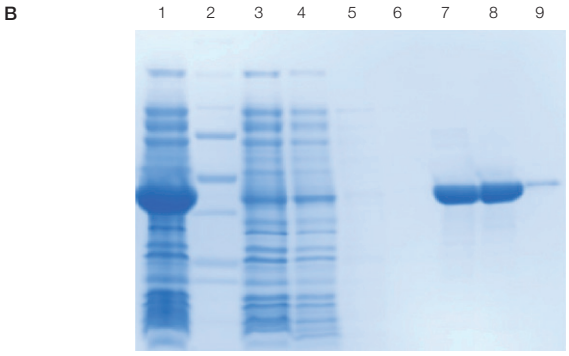

Fig. 2B. Capturing histidine-tagged protein from *E. coli* cell lysate using a Nuvia IMAC Column. SDS-PAGE analysis of column fractions. Lane 1, *E. coli* cell lysate; lane 2, Precision Plus Protein™ Standards; lanes 3–9, column fractions 3–9. Target protein containing fractions 7 and 8 were pooled and subjected to next purification step.

The predicted pI of the target protein, calculated based on its polypeptide sequence, is ~6. The protein is thus expected to carry a net negative charge at pH ~7.5. We tested multiple ion exchange resins for polish purification (data not shown). The hydrophobic cation exchange resin Nuvia cPrime was eventually chosen, as it predominantly binds basic host cell proteins while leaving the acidic target protein in the flow-through fractions (Figure 3). In addition, the Nuvia cPrime Resin ligand contains an electron-rich carboxylic moiety (Figure 4), which makes this resin an excellent scavenger of the Ni^{2+} ions leached from the IMAC resin at pH 7.5. The conductivity of preequilibration buffer used for the Nuvia cPrime Column has a significant effect on the Ni^{2+} clearance efficiency (Table 2). Buffer B is preferred, as it ensures maximum retention of Ni^{2+} by Nuvia cPrime Resin.

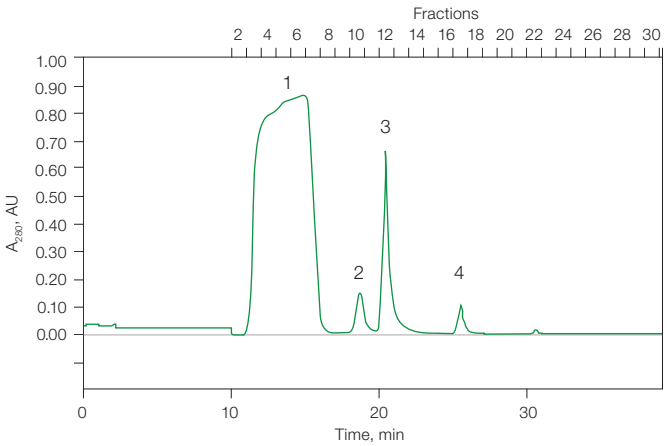

Fig. 3. Intermediate polishing purification of histidine-tagged protein in flow-through mode using a Nuvia cPrime Column. Peak 1, histidine-tagged target protein; peak 2, impurities eluted by buffer D; peak 3, impurities eluted by buffer E; peak 4, impurities stripped by 1 N NaOH. A_{280} (—); AU, absorbance units.

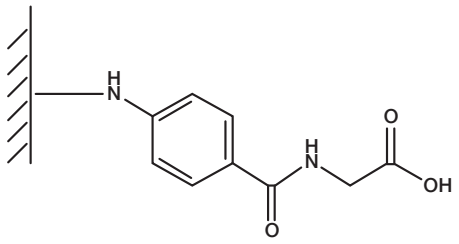

Fig. 4. Ligand structure of Nuvia cPrime Resin.

Table 2. Nuvia cPrime chromatography in flow-through mode: Correlation of buffer conductivity and Ni^{2+} clearance efficiency.

Buffer Composition	Buffer Conductivity, mS/cm[a]	Ni^{2+}, µg/g[b]
50 mM sodium phosphate, 25 mM NaCl, pH 7.5, buffer I	14.56	0.120
50 mM Tris-HCl, pH 7.5, 5 mM sodium phosphate, buffer B	7.02	0.014

[a] Determined at 23°C on a BioLogic DuoFlow QuadTec 10 System.
[b] ICP-MS analysis, per gram of solution. Detection limit is 0.002 µg/g.

For the second polish step we used CHT Media, a naturally occurring mixed-mode chromatography media that provides unmatched clearance of process-related impurities (Figure 5). CHT has been widely used in the downstream processing of therapeutic biologics (Gagnon et al. 2006). In the purification workflow we present here, CHT offers additional clearance of endotoxins, proteins, and nucleic acids from expression host cells (Table 3). The residual Ni^{2+} ions in the Nuvia cPrime Column flow-through pool were effectively eliminated by CHT, most likely through their strong interaction with the phosphoryl groups on this chromatography media (Figure 6). The eluate from the CHT Media contained less than 151 endotoxin units per mg of purified protein, or less than 8 EU/50 µg dose of vaccine, which was significantly lower than the acceptable criteria of 350 EU per 50 µg dose for 70 kg human body weight (Angov et al. 2003). Additionally, the low conductivity buffer employed for the flow-through operation of the Nuvia cPrime Column allowed direct loading of the target protein-containing material onto the CHT Column. Therefore, feedstream manipulation was minimized, which greatly reduced the overall time and cost associated with target protein production.

Table 3. Summary of process impurity clearance.

Samples from Purification Steps	Endotoxin, EU/mg[a]	Ni^{2+}, µg/g[b]	dsDNA, ng/mg[c]	HCP, ng/mg[d]
E. coli lysate	1.05×10^5	—	1.34×10^4	2.87×10^5
Nuvia IMAC Column	5.13×10^3	1.400	4.63×10^2	1.11×10^4
Nuvia cPrime Column	$<3.60 \times 10^3$	0.014	2.42×10^2	6.03×10^2
CHT Column	$<1.51 \times 10^2$	Not detected	Not detected	1.46×10^2

[a] LAL assay, per milligram of protein.
[b] ICP-MS analysis, per gram of solution. Detection limit is 0.002 µg/g.
[c] ddPCR analysis, per milligram of target protein. Detection limit is 1.2 pg/ml.
[d] ELISA, detection limit is 1.6 ng/ml.

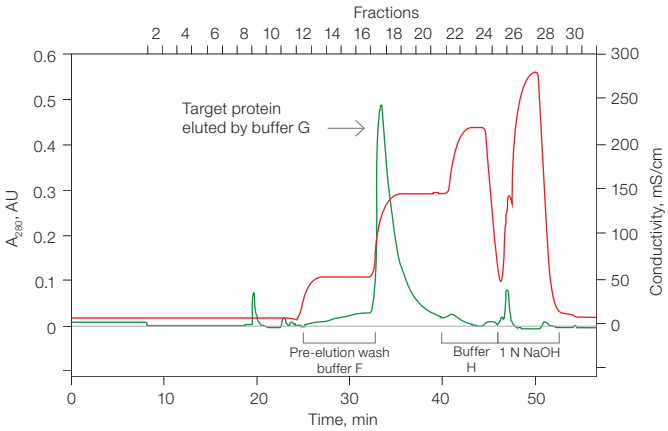

Fig. 5. Further purification of target protein using a CHT Column.
Target protein eluted from the CHT Column was collected in fractions 18–22. A_{280} (—); conductivity (—); AU, absorbance units.

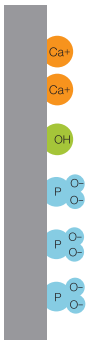

Fig. 6. Schematic representation of functionalities of CHT Media.

Conclusions

We employed three chromatography columns in sequence to obtain highly purified histidine-tagged recombinant protein from *E. coli*. This workflow requires minimum feed conditioning during transitions between chromatography steps, which greatly minimizes labor and the consumption of materials. This robust purification process provides the product quality, productivity, and economics demanded by today's downstream manufacturing.

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