

Chapter 2

Affinity Purification of Heme-Tagged Proteins

Wesley B. Asher and Kara L. Bren

Abstract

Protein affinity purification techniques are widely used for isolating pure target proteins for biochemical and structural characterization. Herein, we describe the protocol for affinity-based purification of proteins expressed in *Escherichia coli* that uses the coordination of a peptide tag covalently modified with heme *c*, known as a heme-tag, to an L-histidine immobilized Sepharose resin. This approach provides an affinity purification tag visible to the eye, facilitating tracking of the protein. In addition, we describe methods for specifically detecting heme-tagged proteins in SDS-PAGE gels using a heme-staining procedure and for quantifying the proteins using a pyridine hemochrome assay.

Key words Affinity protein purification, Affinity tag, Heme-tag, Visible-tag, L-histidine immobilized Sepharose chromatography, Protein quantification, Visible tracking

1 Introduction

Protein purification using affinity chromatography is an indispensable tool in biochemistry and molecular biology [1–3]. The addition of affinity tags to the amino- (N) or carboxyl- (C) terminus of a target protein for purification purposes can dramatically reduce the amount of preparation time, reduce the number of purification steps needed, and increase the yield of pure protein [1–4]. Recently, we developed peptide fusion tags covalently modified with heme *c* chromophores, known as heme-tags, that purify proteins expressed in *Escherichia coli* using an L-histidine immobilized Sepharose (HIS) affinity resin [5]. The heme-tag-HIS method combines the ease of affinity-based purification with the convenience of visible detection for protein tracking purposes, as the heme chromophore grants the fusion protein with an intense red or red-brown color. Visible detection significantly reduces the time and effort associated with protein expression and handling during all purification and chromatographic steps [4]. Because of these

Table 1
Heme-binding peptides used to mature heme-tags for HIS affinity purification of the test protein *Pseudomonas aeruginosa* azurin

Heme-binding peptides	Amino acid sequence
Hm14	GATSC <u>AA</u> CHADSER
Hm16	RES <u>DACAACH</u> SRGSTG
Hm17	GATSER <u>DCAACH</u> ADSER

advantages, fusion tags have been developed for visual tracking purposes [4, 6], but unlike heme-tags, cannot be used for affinity purification procedures.

Mature heme-tags are composed of a heme-binding peptide (HBP) that is linked to the heme chromophore by two stable thioether bonds [7]. See Table 1 for the amino acid sequences of HBPs used for HIS affinity purification. HBPs are typically small, consisting of 14–20 amino acid residues. The critical element of a HBP is a Cys-X-X-Cys-His (CXXCH) heme-binding motif within the sequence, with X representing any amino acid residue [8]. The CXXCH motif is recognized by the *E. coli* cytochrome *c* (cyt *c*) maturation (Ccm) apparatus, which facilitates the covalent addition of the Cys-thiol groups from the CXXCH motif to the two vinyl groups of heme [9]. In our tag designs, the heme iron is coordinated by four pyrrole nitrogens of the porphyrin ring and by the histidine residue of the CXXCH motif (serves as a fifth coordinating ligand), leaving the sixth coordination site open for coordinating exogenous ligands (Fig. 1a) [5]. The basis of the heme-tag-HIS affinity purification method is the reversible coordination between the heme-iron open coordination site and the side chain of histidine immobilized on Sepharose beads (Fig. 1b). After purification, the protein can be eluted from the column using an imidazole-containing buffer, a low pH (≤ 5) buffer, or a high pH (≥ 8) buffer (see Note 1) [5].

The Ccm system is responsible for attaching heme to the HBP and is composed of eight membrane proteins (Ccm A-H) that reside in the *E. coli* cytoplasmic membrane facing the periplasmic space where the heme attachment reaction occurs (Fig. 1c) [9]. Therefore, maturation of proteins fused with HBPs by the Ccm system must also contain an N-terminal signal sequence peptide for recognition by the secretory system and transport to the periplasm (see Note 2) [7]. Secretory peptidase removes the signal peptide after the protein is translocated to the periplasm [10]. Table 2 lists several signal sequences used for exporting recombinant proteins expressed in *E. coli* to the periplasmic space. We have used pelB, malE, and Pa-Azu signal peptides to successfully mature heme-tagged proteins using the Ccm system for heme attachment (see Note 3).

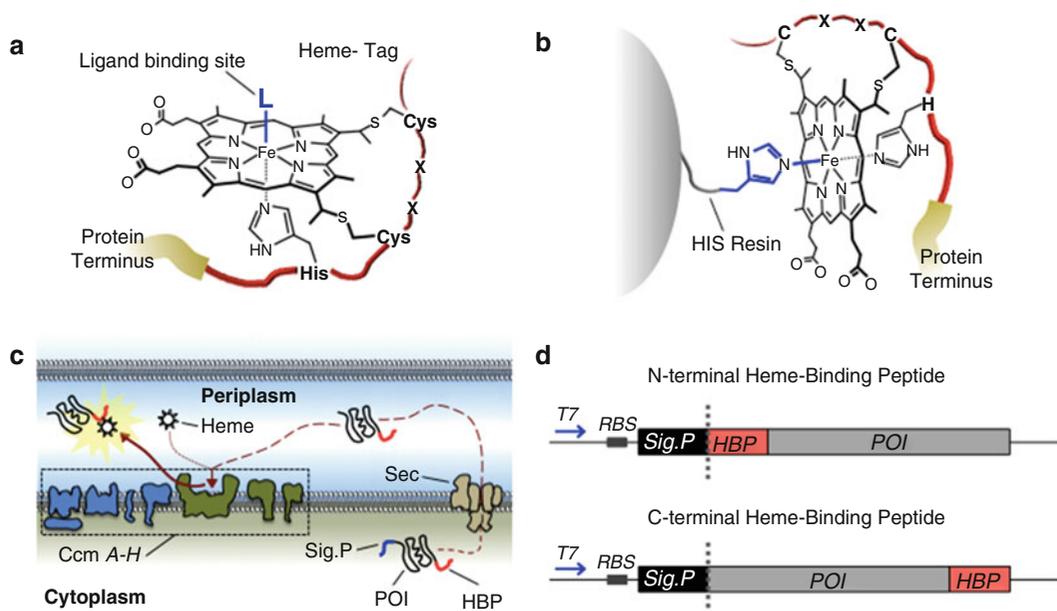


Fig. 1 Schematic overview of the maturation and HIS affinity purification of heme-tagged proteins. **(a)** Mature heme-tag emphasizing the open coordination site for binding exogenous ligands (L). **(b)** Immobilized heme-tagged protein on the HIS affinity resin. **(c)** Transport of a POI containing a C-terminal heme-binding peptide (red) by sec translocase (Sec) from the cytoplasm to the periplasm where heme attachment occurs by the Ccm A-H system. The N-terminal signal sequence is shown in blue and is removed from the POI by secretory peptidase in the periplasmic space. **(d)** DNA segments of the pET expression vectors showing the general coding region for POIs (gray) fused with N-terminal (top) and C-terminal (bottom) heme-binding peptide (HBP, red) sequences. Both designs include the N-terminal signal sequence peptide (Sig. P, black) and the point of cleavage (gray dotted line) by secretory peptidase. T7, blue arrow: T7 promoter, RBS, square: ribosomal binding site

Table 2

List of representative signal sequences for protein translocation to the periplasm

Signal sequence	Amino acid sequence	References
pelB (pectate lyase B)	MKYLPTAAAGLLLLAAQPAMA	[10]
ompA (outer-membrane protein A)	MKKTAIAIAVALAGFATVAQA	[10]
CSP (synthetic signal sequence)	MKKKLLALALLLFNGAQ	[11]
StII (heat-stable enterotoxin 2)	MKKNIAFLASMFVFSIATNAYA	[10]
MalE (maltose binding protein)	MKIKTGARILALSALTTMMFSASALA	[10]
Pa-Azu (<i>P. aeruginosa</i> azurin)	MLRKLAAVSLLSLLSAPLLA	[12]
Modified-cyt c_{550} (<i>P. versutus</i>)	MKISYATLAALSLALPAGA	[13]

Herein, we outline the maturation of heme-tagged proteins by the concurrent overexpression of HBP-tagged proteins (see Fig. 1d for general construct designs) using the pET-expression vector series (Novagen) with the overexpression of the *Ccm A-H* cassette

from the pEC86 [14] vector. Using the pET17b vector for expression, we have successfully expressed and purified the test protein azurin from *Pseudomonas aeruginosa* fused at the C-terminus with the Hm14 and Hm17 HBP sequences, and at the N-terminus with the Hm16 sequence (*see* Table 1 for HBP sequences). In all three constructs we use the Pa-Azu signal sequence [12] for translocation of the protein to the periplasm. These plasmid constructs have the general design as shown in Fig. 1d for C- and N-terminally tagged proteins of interest (POIs), and are available upon request (*see* Note 4). Several cloning strategies are available for fusing the HBP's in Table 1 and the signal peptides in Table 2 to a POI in a variety of *E. coli* expression vectors. However, it is beyond the scope of this chapter to describe detailed molecular cloning strategies and instead we refer the reader to protocols focusing on these methods [15].

In this chapter, we outline the procedures used for expressing heme-tagged proteins in *E. coli* using the Ccm system and for their subsequent affinity purification using the HIS method. Likewise, we describe the protocol for synthesizing the HIS medium from functionalized Sepharose beads. In addition to expression and purification, we describe methods for SDS-PAGE detection of heme-tagged proteins using standard Coomassie Blue staining and a procedure for specifically detecting in-gel heme *c* proteins, referred to as heme staining [16]. The heme staining method can reveal heme-tagged proteins in non-purified samples and confirms that the target protein contains covalently bound heme. Lastly, we detail a spectrophotometric approach for quantifying heme-tagged proteins using the pyridine hemochrome assay (PHA) [17].

2 Materials

Prepare all solutions and buffers using ultrapure water unless stated otherwise. Consult the materials safety data sheets for all chemicals used and follow the procedures for safe handling and disposal. The pEC86 vector coding the *Ccm A-H* gene cassette can be requested from Prof. Linda Thöny-Meyer (EMPA Materials Science and Technology, St. Gallen, Switzerland).

2.1 Expression of Heme-Tagged Protein in *E. coli*

2.1.1 Protein Expression in *E. coli*

1. pET-17b or other suitable expression plasmid encoding the POI tagged with an HBP (*see* Table 1 for sequences) and with an N-terminal signal sequence (*see* Table 2) following the general design shown in Fig. 1d.
2. pEC86 vector (chloramphenicol resistant) coding *Ccm A-H*.
3. Chemically competent One Shot® BL21 Star™ (DE3) *Escherichia coli* (Invitrogen) or other suitable *E. coli* expression host (*see* Note 4).

4. Liquid Luria–Bertani (LB) medium: add 10 g Bacto-Tryptone, 5 g Bacto-Yeast extract, and 10 g NaCl to 900 mL of ultrapure water. Adjust pH to 7.3 with NaOH if needed. Adjust to final volume of 1 L. Sterilize by autoclaving and store at 4 °C.
5. LB agarose plates: add 10 g Bacto-Tryptone, 5 g Bacto-Yeast Extract, 10 g NaCl, and 18 g Bacto-Agar to 500 mL of ultrapure water. Adjust pH to 7.3 with NaOH if needed. Adjust to final volume of 1 L. Sterilize by autoclaving, cool to ~45–50 °C, and add ~25 mL of LB agarose to 8 cm plates and allow to solidify. Store the plates at 4 °C.
6. 50 mg/mL ampicillin (Amp) solution: Filter-sterilize and aliquot to a desired volume. Store aliquots at –20 °C. Once an aliquot of Amp solution is thawed, store at 4 °C and discard after 2 weeks. Use at 50 µg/mL. Note: prepare, store, and use antibiotic solutions according to literature standards if using an expression vector with antibiotic resistance other than Amp.
7. 50 mg/mL chloramphenicol (CM) solution: Dissolve in ethanol. Prepare CM solution as needed. Store at 4 °C and use at 50 µg/mL within 2 weeks.
8. Amp supplemented LB agar plates: add 30 µL of 50 mg/mL Amp on top of the agar and spread over the entire surface aseptically.
9. Amp-CM supplemented LB agar plates: add 30 µL of 50 mg/mL Amp on top of the agar and spread over the entire surface aseptically. Follow by adding 30 µL of 50 mg/mL CM in ethanol and spread over the entire surface aseptically.
10. Incubated orbital shaker.
11. 100 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG): Add 2.38 g of IPTG to 100 mL of ultrapure water, filter-sterilize, and store at 20 °C in the dark (*see Note 5*).
12. Refrigerated (4 °C) centrifuge.
13. Ultraviolet–Visible (UV–Vis) spectrophotometer.
14. Plastic or quartz cuvettes.

2.1.2 Osmotic Shock Procedure

1. Sucrose buffer: 50 mM Tris–HCl, 1 mM EDTA, 20 % sucrose, pH 7.5–8. Prepare a 0.5 M stock solution of EDTA, pH 8.0 in ultrapure water and adjust pH with NaOH. Use the EDTA stock solution to prepare the sucrose buffer.
2. Osmotic shock buffer: Ice-cold (0–4 °C) 5 mM MgSO₄ in water.
3. Method for concentrating protein in solution. Ex. EMD Millipore Amicon® Ultra centrifugal filters (0.5–15 mL capacity) or Amicon® stirred cells (4–400 mL capacity).
4. PD-10 desalting and buffer exchange columns (GE Healthcare).

2.1.3 Whole Cell/ Cytoplasm Lysis Procedure

1. Cytoplasmic extraction buffer: 50 mM Tris-HCl, 4 mg/mL chicken egg white lysozyme (EMD Millipore).
2. DNAase I solution (NEB).
3. Triton™ X-100, molecular biology grade.
4. Method for concentrating protein in solution. Ex. EMD Millipore Amicon® Ultra centrifugal filters (0.5–15 mL capacity) or Amicon® stirred cells (4–400 mL capacity).
5. PD-10 desalting and buffer exchange columns (GE Healthcare).

2.2 Preparation of L-Histidine Immobilized Sephacrose Resin

1. 25 mL *N*-hydroxysuccinimide (NHS)-Activated Sepharose™ 4 Fast Flow (GE Healthcare).
2. 2.5 × 10 cm glass chromatography column. Ex. Econo-Column® Bio-Rad glass columns (*see Note 6*).
3. L-histidine coupling buffer: 10 mg/mL L-histidine, 200 mM NaHCO₃, 500 mM NaCl, pH 8.3. Prepare ~15 mL and store at room temperature.
4. 1 mM HCl in water: Prepare ~500 mL of the HCl solution for use and chill on ice before procedure (0–4 °C).
5. pH indicator strips (pH range 1–14).
6. Open air orbital shaker.
7. Bottom top 0.1 μm filtration unit, ≥250 mL capacity (e.g., Thermo Scientific Nalgene MF75™ 0.1 μm PES Filter Units).
8. Blocking buffer: 500 mM ethanolamine, 500 mM NaCl, pH 8.3. Prepare ~15 mL of block buffer for use.
9. High pH wash buffer: 100 mM Tris-HCl, pH 8–9.
10. Low pH wash buffer: 100 mM sodium acetate buffer, 500 mM NaCl, pH 4–5.

2.3 HIS Affinity Purification of Heme-Tagged Protein

1. Wash/binding buffer: 50 mM sodium phosphate (NaP_i), pH 7.0 (*see Note 7*). Prepare 1 L of buffer and store at 4 °C until use.
2. Imidazole elution buffer: 300 mM Imidazole, 50 mM NaP_i, pH 7.0. Prepare 500 mL of solution and store at 4 °C.
3. Low pH elution buffer: 50 mM NaP_i, pH 4–5.
4. High pH elution buffer: 50 mM Tris-HCl, pH 8–9.
5. Method for concentrating protein in solution. Ex. EMD Millipore Amicon® Ultra centrifugal filters (0.5–15 mL capacity) or Amicon® stirred cells (4–400 mL capacity).
6. PD-10 desalting and buffer exchange columns (GE Healthcare).

2.4 SDS-PAGE Analysis of Heme- Tagged Protein

2.4.1 SDS-PAGE

1. SDS-PAGE gel electrophoresis unit. Ex. Hoefer SE 250 or 260 Mighty Small II mini gel electrophoresis unit and dual gel caster.
2. 30 % acrylamide/bis-acrylamide solution (Bio-Rad).

3. 1.5 M Tris-HCl, pH 8.8.
4. 1.0 M Tris-HCl, pH 6.8.
5. 10 % Sodium dodecyl sulfate (SDS) solution.
6. 10 % ammonium persulfate (APS) solution. Store at 4 °C and use within 1 month.
7. *N,N,N',N'*-tetramethyl-ethylenediamine (TEMED).
8. 10× Tris-glycine reservoir/running buffer: 30 g Tris-base, 144 g glycine, 10 g SDS per 1 L of solution. Add 100 mL of 10× running buffer to 900 mL ultrapure water to prepare 1 L of 1× running buffer.
9. SDS sample buffer: 1.6 mL ultrapure water, 625 μL 1.0 M Tris-HCl (pH 6.8), 5 mL 50 % glycerol, 2.0 mL 10 % SDS solution, 250 μL 0.5 % Bromophenol Blue. Add 50 μL β-mercaptoethanol (βME) to 950 μL of SDS sample buffer before use (*see Note 8*).
10. Molecular weight markers: Pre-stained Bio-Rad Precision Plus Protein™ all blue standards (10–250 kDa).

2.4.2 Heme Staining

1. Gel fixing solution: 12.5 % trichloroacetic acid (TCA) solution. Prepare ~150–200 mL of the TCA solution and store at 4 °C. The solution can be reused multiple times.
2. Heme staining solution: dissolve 0.2 g *o*-dianisidine in 15–20 mL glacial acetic acid (*see Note 9*). Add the dissolved *o*-dianisidine solution to 20 mL of 500 mM sodium citrate buffer (pH 4.4), 400 μL 30 % hydrogen peroxide, 160 mL ultrapure water (~200 mL total volume of heme staining solution).
3. Open air orbital shaker.

2.4.3 Coomassie Blue Staining

1. Coomassie blue staining solution: 1 % Coomassie brilliant blue R250, 40 % methanol, 10 % glacial acetic acid. Store at room temperature.
2. Destaining solution: 40 % methanol, 10 % glacial acetic acid. Store at room temperature.

2.5 Pyridine Hemochrome Assay for Protein Quantification

1. Pyridine-base Solution: 100 mM NaOH (dissolve NaOH pellets in 50 mM NaP_i), 20 % pyridine. Prepare solution just before use.
2. Saturated dithionite solution: ≥1.0 M sodium dithionite (dissolved in 50 mM NaP_i). Prepare immediately before use.
3. UV-Vis spectrophotometer.
4. Quartz or plastic cuvettes.

3 Methods

3.1 Expression of Heme-Tagged Protein in *E. coli*

The procedure for expressing heme-tagged proteins below is based on the maturation of heme-tagged azurins using the pET17b expression vector [5]. The *E. coli* expression hosts used with the pET expression vectors (ex. pET17b) are lysogens of bacteriophage DE3 and contain a chromosomal copy of a DNA fragment containing the *lacI* gene coding for the *lac* repressor and the T7 RNA polymerase (T7 RNAP) gene regulated by an isopropyl- β -D-thiogalactopyranoside (IPTG) inducible *lacUV5* promoter. When supplemented in the growth media, IPTG binds and deactivates the *lac* repressor, allowing for increased production of T7 RNAP in the cell, which in turn binds to the strong T7 promoter of the pET vector controlling the overexpression of the HBP-tagged protein. It is important to note that expression schemes may vary depending on the protein being tagged. Several expression variables: temperature, shaking speeds, aeration, and the amount of IPTG used for induction, may need to be optimized to determine which conditions are most suitable for a given POI. In addition, the predicted solubility of the heme-tagged protein should be analyzed (*see Note 10*), as the target protein may form inclusion bodies in the cytoplasm, preventing transport to the periplasmic space. We highly recommend following the general expression strategies outlined in the Novagen pET systems manual before developing an optimized expression scheme for heme-tagged protein production (*see Note 11*).

3.1.1 Protein Expression in *E. coli*

1. Transform both the pET expression plasmid coding the heme-tagged protein and the pEC86 plasmid coding Ccm A-H into One Shot[®] BL21 Star[™] (DE3) *E. coli*. Add 5–10 ng of both plasmids per one aliquot of *E. coli* cells for the transformation. Use LB agar plates supplemented with Amp and CM for producing single colonies overnight.
2. Inoculate 5–10 mL of LB broth containing Amp and CM with a single colony of transformed *E. coli* and grow for ~8 h in an orbital shaker at 37 °C and 200–230 rpm.
3. Inoculate 1 L of liquid LB containing Amp and CM with the 5–10 mL of starter growth. Place foil over the top of the growth flask to reduce aeration (*see Note 12*). Grow for a desired amount of time before induction with IPTG. For most POIs, the growth is allowed to reach logarithmic phase (optical density at 600 nm of ~0.5) before induction with IPTG. However, this is dependent on the POI. Consult the Novagen pET systems manual for more details.
4. Grow (foil covered flask) for ~16 h uninduced at 25–28 °C with shaking at 125 rpm. Add 4 mL 100 mM IPTG and grow for an additional 4–6 h at the same conditions (*see Note 13*).

5. Harvest the cells by centrifugation at $10,000 \times g$ for 20 min at 4 °C. Pour off the supernatant. The pellet should exhibit a dark brown/red color, immediately confirming that expression of the heme-tagged protein occurred (*see Note 14*).

3.1.2 Osmotic Shock Procedure

The *E. coli* cell pellets can be lysed using several standard protocols. Here, we describe two procedures: an osmotic shock procedure for obtaining only proteins from the periplasmic space (where the heme-tagged protein resides) and a whole cell/cytoplasmic lysis procedure, both of which will allow for purification of the heme-tagged protein by the HIS method. In some cases, the whole cell/cytoplasmic lysis results in a higher heme-tagged protein yield (but with a higher level of contamination) than the osmotic shock procedure.

1. For periplasmic lysis, resuspend the pellet in 25–35 mL of sucrose buffer. Slowly stir the solution at room temperature for 15–20 min until the pellet fully resuspends. Centrifuge the solution at $10,000 \times g$ for 20 min at 4 °C to collect the cells. Carefully remove the supernatant (*see Note 15*).
2. Resuspend the pellet in 25–35 mL of cold osmotic shock buffer and stir gently at 4 °C for 30 min to disrupt the outer membrane of the cells, releasing the periplasm.
3. Centrifuge the solution to pellet the shocked cells and carefully isolate the periplasmic fraction/supernatant containing the heme-tagged protein. The supernatant should have a dark brown-red color. Concentrate the periplasmic fraction to a final volume of less than 10 mL (preferably lower if possible). Use a PD-10 desalting column to exchange the periplasmic fraction into 50 mM NaPi, pH 7.0. Keep the periplasmic sample at 4 °C.

3.1.3 Whole Cell/Cytoplasmic Lysis Procedure

1. For whole cell/cytoplasmic lysis, resuspend the pellet in 25–35 mL of cytoplasmic lysis buffer containing ~20 µL Triton™ 100-X. After thorough resuspension, add ~2 units of DNAase I to the solution and mix by gently inverting the centrifuge tubes containing the resuspended cells 3–4 times (*see Note 16*).
2. Incubate for 1–1.5 h at 30 °C and gently invert the tubes to mix the solutions every 15–20 min during incubation. Centrifuge the solution to pellet the ruptured cells and carefully isolate the supernatant containing the heme-tagged protein. The supernatant should have a dark brown-red color.
3. It is recommended to partially clarify the lysate obtained using the whole cell/cytoplasm lysis procedure before any further chromatography steps. In the case for heme-tagged azurin, we apply the lysate to an ion-exchange resin and wash with an

appropriate buffer before eluting with a one-step NaCl buffer [5]. Concentrate the partially clarified lysate to a final volume of less than 10 mL (preferably lower if possible). Use a PD-10 desalting column to exchange the clarified lysate buffer to 50 mM NaP_i, pH 7.0. Keep the clarified lysate sample at 4 °C.

3.2 Preparation of L-Histidine Immobilized Sepharose Resin

The procedure for preparing the HIS resin is largely adapted from the manufacturer's instructions for reacting primary amine (–NH₂) containing ligands with NHS-activated Sepharose™ 4 Fast Flow (GE Healthcare Life Sciences Instructions 71-5000-14AD). The NHS-ester of the medium reacts specifically with primary amines forming a stable covalent linkage between the Sepharose beads and the ligand. Because histidine is a small ligand with an unobstructed –NH₂ group, the coupling efficiency will be >90 %. Thus, quantitative determination of the coupling efficiency (often done when large proteins are coupled to the resin) is not necessary.

1. Pour the 25 mL NHS-Activated Sepharose™ 4 Fast Flow suspended in isopropanol from the manufacturer's container onto the membrane of a 250 mL capacity bottom top 0.1 µm filtration unit.
2. Vacuum-filter to remove the isopropanol (*see Note 17*) and immediately resuspend the beads in ~150 mL of ice-cold 1 mM HCl in the absence of vacuum suction. Use a small stir rod to suspend the beads in the HCl solution. Vacuum-filter to remove the HCl solution and repeat the HCl wash step two additional times.
3. After the last wash step with HCl, immediately resuspend the beads in 12.5 mL of the histidine coupling buffer (to achieve the recommended 0.5:1 coupling solution to medium volume ratio) and pour the suspension into a sterile 100–125 mL beaker. Adjust the pH of the resuspended medium to ~8.0–8.3 using NaOH. Use pH indicator strips to measure approximate pH. Cover the beaker and gently agitate the coupling buffer/medium suspension using an open-air orbital shaker for 3 h. After coupling is complete, pour the newly synthesized HIS medium onto a new filter device and apply vacuum to remove the coupling buffer.
4. Resuspend the HIS medium in 12.5 mL of blocking buffer to block any remaining NHS-ester groups. Follow the same protocol for incubation as described with the L-histidine coupling buffer. After the blocking reaction, remove the blocking buffer by vacuum filtration.
5. To wash the HIS resin, resuspend the medium in 100 mL high pH wash buffer, vacuum-filter, and then resuspend the medium in 100 mL low pH. Vacuum-filter and repeat the high pH/low pH cycle two additional times.

- Decant or slowly pour the washed HIS medium into a 2.5×10 cm glass chromatography column. Wash the HIS column three times with 20 % ethanol and store in the same solution at room temperature.

3.3 HIS Affinity Purification of Heme-Tagged Protein

- Pre-equilibrate the HIS column with 100 mL 50 mM NaP_i , pH 7.0. Carefully load the concentrated periplasmic fraction or partially clarified lysate containing the heme-tagged protein on the HIS column (*see Note 18*). After the lysate has loaded on the resin, carefully add 2 column volumes (CV, ~50 mL) of wash buffer onto the resin (*see Note 19*). After 0.5 CV of buffer elutes, a bright red band will form at the top of the resin and a green band will begin migrating down the column (*see Fig. 2, Note 20*). The red band is the heme-tagged protein coordinated to the HIS resin, and the green band contains a degraded heme by-product associated with overexpressed heme-containing proteins. The green by-product does not coordinate the HIS resin and therefore migrates with the *E. coli* containing proteins during the wash step.
- After 1 CV (~25 mL) of wash buffer elutes from the HIS resin, the green band will be at the bottom of the column and in the process of eluting. Collect a small fraction of the green band as it begins to elute from the column (~0.9–1 CV of wash buffer), and a fraction after the addition of 2.0 CV of wash buffer for analysis.
- After washing the column with a total of 2 CV of wash buffer, add 50 mL of imidazole-, low pH-, or high pH-elution buffer. The red band at the top of the column will immediately begin to elute from the column. Collect the entire red fraction from the column. Concentrate the pure heme-tagged protein after elution and use a PD-10 desalting column to exchange the fraction into an appropriate buffer (ex. 50 mM NaP_i , pH 7.0).

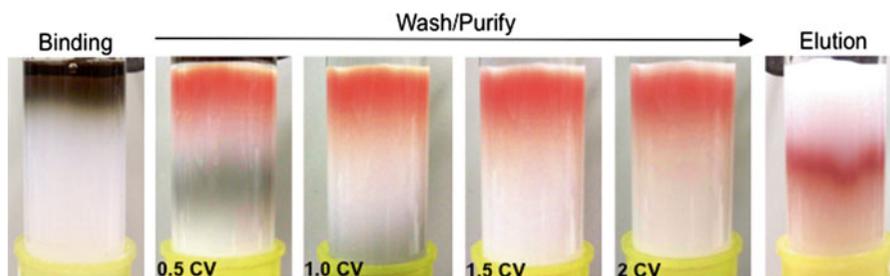


Fig. 2 Affinity purification of azurin tagged at the N-terminus with the Hm16 sequence using the HIS resin. Loading of partially clarified lysate containing Az-Hm16 on the HIS resin (Binding), Az-Hm16 binding to the HIS resin after the addition of 0.5, 1.0, 1.5, and 2 CV (~12.5, 25, 37.5, and 50 mL, respectively) of wash buffer (Wash/Purify), and after ~1.5 CV of binding buffer containing 300 mM imidazole (Elution). A *green band*, representing the contaminating proteins, migrates down the column as shown after 0.5 CV wash buffer, and begins to elute at ~1 CV

3.4 SDS-PAGE Analysis of Heme- Tagged Protein

Here, we outline SDS-PAGE using the Hoefer electrophoresis unit; however, any commercial system can be used. The protocol for using the Hoefer duel gel caster and the assembly of the gel sandwich stack consisting of a glass and alumina plate separated by two spacers is thoroughly outlined in the SE 245 duel gel caster user's manual (*see Note 21*).

3.4.1 SDS-PAGE

1. Use the Hoefer duel gel caster manual to properly set up the caster for pouring the gels.
2. Using a small ruler, measure 3 cm below the top of the glass plate and draw a line at this point.
3. To prepare the 12 % resolving gel, combine 1.6 mL of ultrapure water, 1.3 mL of 1.5 M Tris-HCl pH 8.8, 2.0 mL 30 % acrylamide/bis-acrylamide solution, 50 μ L 10 % SDS, and 50 μ L 10 % APS in a 15 mL conical tube. Mix by inverting the tube several times.
4. Next, add 2 μ L of TEMED to the mixture, invert the tube to mix, and quickly pour the resolving gel solution up to the marked line (made in **step 2**) in the gel sandwich stack and overlay with ultrapure water. Allow 20–25 min for the resolving gel to polymerize, at which point the water can be poured off the top of the gel.
5. Prepare the 5 % stacking gel by combining 1.4 mL ultrapure water, 0.25 mL 1.0 M Tris-HCl pH 6.8, 0.33 mL 30 % acrylamide/bis-acrylamide solution, 20 μ L 10 % SDS, and 20 μ L 10 % APS in a 15 mL conical tube. Add 2 μ L of TEMED to the mixture, quickly invert the tube to mix, and pour the stacking gel solution on top of the polymerized resolving gel up to the top of the plates. Immediately insert the comb and allow 30 min to polymerize.
6. After the stacking gel has polymerized, carefully remove the comb and assemble the gel into the Hoefer SE 250 or 260 Mighty Small II mini gel electrophoresis unit. Add 1 \times electrophoresis running buffer to the upper and lower chambers. Dilute aliquots of fractions collected during the HIS purification process 1:2 with SDS-sample buffer. Heat the samples at 95–100 $^{\circ}$ C for 5 min. Load the samples and the pre-stained molecular weight standard solution onto the gel. Run the gel for ~30–45 min at 200 mV, or until the bromophenol blue runs off the bottom of the gel.
7. Remove the gel stack from the electrophoresis unit and disassemble the gel sandwich stack by carefully separating the plates. Remove the gel between the plates and immerse in 200 mL of ultrapure water with gentle agitation using an orbital shaker for 30 min.

3.4.2 Heme Staining

1. Immerse the washed gel in 200 mL of gel fixing solution and gently agitate for 30 min.
2. After the TCA wash, repeat the 30 min water wash in **step 6** from Subheading 3.4.1.
3. Immerse the gel in heme staining solution with agitation. Bright green bands will appear in the gel within 15–30 min. Remove the gel and wash in ultrapure water before handling and imaging. *See Fig. 3* for an example of expected results.

3.4.3 Coomassie Blue Staining

1. Prepare an identical gel with the same samples using **steps 1–6** from Subheading 3.4.1 for Coomassie blue staining. Immerse the gel in Coomassie blue stain for 30 min with agitation.
2. Immerse the gel in destaining solution with agitation for 20 min. Remove the stain, add fresh destaining solution, and agitate the gel for 2 h or until the background is completely removed. *See Fig. 3* for an example of expected results.

3.5 Pyridine Hemochrome Assay for Protein Quantification

1. Prepare samples for the pyridine hemochrome assay as follows: add 80 μ L of HIS purified heme-tagged protein in 50 mM NaP_i to 910 μ L pyridine/base solution and mix by vortexing.
2. Prepare the saturated sodium dithionite solution and immediately add 10 μ L of this solution to the samples prepared in **item 1** of Subheading 2.5 (*see Note 22*). Quickly mix by vortexing, transfer to a quartz cuvette and obtain the UV-Vis absorption spectrum of the samples at room temperature using a spectrophotometer (*see Note 23*).

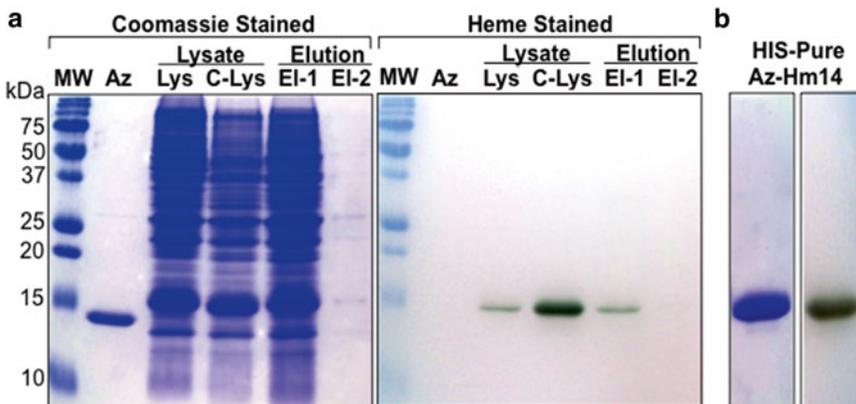


Fig. 3 12 % SDS-PAGE gel showing results from the HIS purification of azurin (Az) tagged at the C-terminus with Hm14 (Az-Hm14). **(a)** Both gels contain identical samples. The *left* gel was stained with Coomassie blue and the *right* using heme stain. *MW*: pre-stained molecular weight markers (10–250 kDa). *Az*: purified wild type Az (~14 kDa) not fused with any tag sequence. *Lys*: non-clarified lysate containing Az-Hm14. *C-Lys*: lysate clarified using an initial anion-exchange chromatography step. *El-1*: Fraction collected after ~0.9 CV of wash buffer had eluted from the column during HIS purification of Az-Hm14. *El-2*: Fraction taken after 2.0 CV of wash buffer eluted. **(b)** Coomassie and heme-stained gels showing pure Az-Hm14 after elution from the HIS resin

3. Repeat **steps 1–2** of Subheading 3.5 for the same sample to obtain data in triplicate.
4. Using the Beer-Lambert law ($A = \epsilon \cdot l \cdot c$, where A is absorbance, ϵ is the extinction coefficient, l is cuvette path length, and c is concentration), the concentration of the heme-tagged protein solution can be calculated using the extinction coefficient of $30.27 \text{ mM}^{-1} \text{ cm}^{-1}$ at 550 nm for heme c containing proteins.

4 Notes

1. The imidazole-containing elution buffer displaces the histidine residues of the HIS resin. We propose that the low pH (≤ 5) elution buffer protonates the histidine residues preventing coordination, and the hydroxide ions of the high pH (≥ 8) elution buffer displace the histidine residues of the resin [5].
2. We recently developed a new strategy for maturing heme-tagged proteins in the *E. coli* cytoplasm by the enzyme cytochrome c heme lyase (CCHL), eliminating the need for transporting the protein to the periplasmic space [18]. We are currently developing HBPs for maturation by CCHL that can be used for affinity purification by the HIS method.
3. There is no rule for choosing a signal sequence that will guarantee or maximize the level of transport to the periplasmic space for any specific target protein [10]. Several signal peptide sequences exist, yet the *pelB* and *ompA* sequences are frequently used in commercial vectors for the purpose of targeting a protein to the periplasm. In our constructs, we check the likelihood that the sequence we choose will be recognized for periplasmic transport and of proper cleavage by signal peptidase using the SignalP 4.0 server (<http://www.cbs.dtu.dk/services/SignalP/>) [19] and optimize the constructs accordingly.
4. If using an expression vector with a T7 promoter (as is the case for the pET series vectors), the *E. coli* host used for expression must contain a chromosomal copy of T7 RNAP. The host RNAP native to the organism will bind bacterial promoters (as in pEC86) but not the T7 promoter.
5. Solid IPTG should be stored in the absence of light in a desiccator at $-20 \text{ }^\circ\text{C}$.
6. Different column brands, sizes and types with a desired volume of HIS resin can be used. The 25 mL HIS resin packed in the $2.5 \times 10 \text{ cm}$ column is optimized for purifying protein from the 1 L scale of bacterial growth. After a purification run, the column can be washed with 5 CV of 50 mM NaP_i , 500 mM NaCl, pH 7.0 and stored in 20 % ethanol. After 10–15 purification runs, or until the flow rate is visibly slower than usual,

the column can be washed with 100 mM NaOH for cleaning purposes. Note that repeated washes with NaOH will reduce the binding capacity of the resin, as some histidine residues will be removed from the Sepharose. We typically dispose of the HIS resin after 1 year of use.

7. Wash buffers other than NaP_i that are not known to chelate/bind metals and that are near neutral pH may be used with the HIS resin.
8. β ME reduces the activity of the heme stain solution. Thus, it is recommended that β ME be omitted from the sample buffer if the POI is known to not form disulfide bonds in the native and unfolded state.
9. Allow ~5 min for *o*-dianisidine to dissolve with stirring using a glass rod.
10. Use bioinformatics programs for predicting solubility. Ex. PROSO sequence-based protein solubility evaluator (<http://mips.helmholtz-muenchen.de/proso/proso.seam>) [20].
11. The 11th edition of the pET systems manual is available from www.novagen.com under the Novagen technical bulletins category.
12. We have observed that reduced aeration increases the yield of heme-tagged proteins for unknown reasons [5]. Although the bacterial pellet size will be smaller, the yield of heme-tagged protein may significantly change. For example, we obtain ~1.5–2.0 mg/L bacterial culture for heme-tagged azurin with aeration (open container) versus 7.0–8.0 mg/L when foil is placed over the growth flask. It is advised to test both conditions when expressing any new heme-tagged protein.
13. This is the optimal time we found for heme-tagged azurins; optimal conditions may vary with POI.
14. As a negative control, the expression protocol used for a heme-tagged protein can be followed for the same protein in the absence of a HBP sequence. The pellet may have a green color (due to degraded heme species) if strategies to increase oxygenation (high shaking speeds, baffled flasks, open growth container) are used (*See Note 12*).
15. After incubation with the sucrose buffer, the pelleted cells will be loosely attached to the side of the container.
16. DNAase I can be denatured when vortexing or shaking vigorously.
17. Continued exposure to vacuum after the beads are dry could damage the resin. The Sepharose beads should form a pellet on the filter membrane that appears wet. Avoid pellets that look dry and cracked after the solutions are removed.

18. It is critical that the pH of the loaded lysate and the buffer in the pre-equilibrated column be near neutral for binding.
19. The lysate containing the heme-tagged protein will be dark brown in color (*see* Fig. 2, binding step) before adding the wash buffer. The brown color is indicative of an open coordination site at the heme-iron of the tags. After binding the HIS resin, the heme-tagged protein will change from brown to a bright red color (*see* Fig. 2, wash steps) due to the histidine residues of the HIS resin coordinating the tags.
20. The dark brown colored lysate will migrate down the column and elute at ~1 CV of wash buffer if the heme-tagged protein does not bind the HIS column.
21. The Hoefer SE 245 user's manual is online at <http://www.hoeferinc.com> under the mini vertical units section.
22. Add 15 μ L of saturated sodium dithionite solution to an identical sample. The heme-tag should be fully reduced if the spectra are identical.
23. Sodium dithionite is a strong reductant that reacts with air immediately upon exposure. Minimize the time between adding the sodium dithionite solution to the protein samples and taking the absorption spectra.

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