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Simplified Protein Purification Using an Autoprocessing, Inducible Enzyme Tag

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Summary

The development of affinity tags has greatly simplified protein purification procedures. A variety of affinity tags are now available to improve expression, solubility, and/or tag removal. In this chapter, we describe a method for purifying recombinant proteins expressed in *Escherichia coli* that uses a highly specific, inducible, C-terminal autoprocessing protease tag. This method streamlines affinity purification, cleavage, and tag separation into a one-step purification procedure, avoiding the need to remove fusion tags from target proteins with exogenous proteases. In addition to accelerating protein purification, we show that this method can enhance the expression, stability, and solubility of select proteins.

1. Introduction

The development of affinity tags such as poly-His and glutathione transferase (GST) has greatly simplified recombinant protein purification, while the development of fusion tag has improved the expression, solubility and stability of target proteins ([1,2]). Despite these advances, tags can alter the biological activity of target proteins and interfere with protein crystallization studies. To overcome this problem, fusion tags are often coupled to protease cleavage sites so that the tag can be removed from target proteins upon addition of site-specific proteases [1,2]. Unfortunately, these endoproteases can be costly, poorly soluble, and inefficient, which can lead to spurious cleavage and require additional chromatography steps to remove these proteases.

This report describes the use of an inducible, self-cleaving protease tag (CPD) to simplify and accelerate the purification of recombinant proteins from bacteria (**Figure 1**, [3]). The CPD tag consists of the *Vibrio cholerae* MARTX toxin cysteine protease domain (CPD) [4] fused to a C-terminal hexahistidine tag. The CPD is a highly specific protease that cleaves exclusively after Leu residues upon activation by the eukaryotic-specific small molecule inositol hexakisphosphate (InsP₆) [5,6]. Since InsP₆ is absent from bacterial cells [7,8], full-length CPD-His₆ fusion proteins are purified from bacterial lysates in a protease-inactive form using imidazole affinity chromatography (IMAC). Addition of InsP₆ to the immobilized, C-terminally His₆-tagged fusion protein induces autoprocessing after a Leu residue within the engineered target protein-CPD junction (**Figure 1**). This processing event

releases the untagged target protein into the supernatant, while the C- terminally His₆-tagged CPD remains immobilized on the Ni²⁺-NTA resin.

We have previously used the CPD tag to purify a wide range of both soluble and insoluble proteins; in a number of cases, we showed that the CPD can increase the expression, solubility, and stability of fusion proteins ([3]). Indeed, Wright *et al.* recently used the CPD to purify recombinant antimicrobial peptides, which can be difficult to produce in large quantities because of their intrinsic toxicity to *E. coli* ([9]). Here, we describe the use of the CPD tag to purify CspBA, a serine protease from the Gram- positive pathogen *Clostridium difficile*, and mouse macrophage metalloelastase (MMP- 12) from *E. coli*. Although both these proteins exhibit poor solubility when expressed as a C-terminal His₆-tag fusion, when fused to the CPD autoprocessing tag, they can be purified as soluble proteins (unpublished data and [10,3], **Figure 2**). We also use the CPD tag to improve the purification of biotin ligase from *Escherichia coli*, which is used to biotinylate recombinant proteins at engineered sites ([11,3]).

2. Materials

Prepare all solutions using ultrapure water (deionized water purified to a sensitivity of 18 MΩ) and molecular biology grade reagents. Prepare and store all reagents at room temperature unless specified otherwise.

To facilitate the production of CPD fusion proteins, we previously constructed a suite of pET-based expression vectors that allow the linker peptide to be varied and also permit the addition of C-terminal HA-tag to the protein of interest. These vectors are readily available from Addgene (Cambridge, MA, USA). In this report, we provide a detailed protocol for purifying proteins using the pET22b-CPD_{SalI} expression vector.

2.1 Cloning Reagents

1. Cloning primers: The forward primer should carry a 5' NdeI restriction site sequence (CATAG) diluted to 25 μM in water; the reverse primer should carry a 5' SalI restriction site sequence (*see Note 1*)
2. Deoxyribonucleotide (dNTP) mix: 10 mM stock diluted in water (Invitrogen, Carlsbad, CA, USA).
3. Phusion DNA polymerase (New England Biolabs, Ipswich, MA, USA).
4. NdeI (New England Biolabs, Ipswich, MA, USA)
5. XhoI (New England Biolabs, Ipswich, MA, USA)
6. Antarctic Phosphatase (New England Biolabs, Ipswich, MA, USA)

¹We usually add an additional 4 nt upstream of the restriction site sequence to ensure efficient digestion. To construct a CPD fusion in pET22b-CPD_{SalI}, the forward primer should carry an NdeI restriction site, while the reverse primer should carry a SalI restriction site. This will produce a fusion protein carrying a VDAL linker sequence; CPD-mediated autoprocessing will leave a VDAL C-terminal tag on the target protein. CPD expression vectors are available from Addgene that provide alternate 5' (NcoI) and 3' cloning sites (SacI, BamHI). Changes in the 3' restriction site will alter the C-terminus of the target protein produced upon CPD autoprocessing

7. T4 DNA ligase (New England Biolabs, Ipswich, MA, USA).
8. IPTG-inducible expression vector containing CPD-His₆ fusion tag (available from Addgene, Cambridge, MA, USA).
9. Tris-borate-EDTA buffer (TBE): 0.1M Tris pH 8.0, 90 mM boric acid, 1 mM EDTA (see **Note 2**).
10. EDTA solution. 0.5 M stock solution in water. Autoclave to sterilize. (see **Note 3**).
11. NaOH pellets (Fisher Scientific, Dallas, TX, USA)
12. 0.75% agarose gel in TBE: 1 g molecular biology grade agarose (Fisher Scientific, Dallas, TX, USA) in 100 mL TBE buffer. Microwave to melt and add 100 μ L Sybr SAFE (Invitrogen, Carlsbad, CA, USA).
13. Fermentas PCR purification kit (Fermentas, Glen Burnie, MD, USA)
14. Fermentas miniprep kit (Fermentas, Glen Burnie, MD, USA)

2.2. *E. coli* Expression Reagents

1. LB Media: 5 g NaCl, 5 g yeast extract, 10 g tryptone per liter H₂O (Fisher Scientific, Dallas, TX, USA)
2. 2YT Media: Measure 5 g NaCl, 10 g yeast extract, 15 g tryptone (Fisher Scientific, Dallas, TX, USA) into a 2.8 L Fernbach flask then add 1 L water. Autoclave to sterilize.
3. Ampicillin, 1000X stock (Fisher Scientific, Dallas, TX, USA). 100 mg/mL solution in water. Filter sterilize and store at 4°C. (see **Note 4**)
4. Isopropyl β -D-1-thiogalactopyranoside (IPTG, Fisher Scientific, Dallas, TX, USA). 1 M solution in water. Measure 1.19g IPTG in a 15 mL conical tube. Add H₂O up to final volume of 5mL. Filter sterilize and store at 4°C (or –20°C). (see **Note 5**).
5. DH5 α chemically competent cells: prepared in house or purchased from (Invitrogen, Carlsbad, CA, USA)
6. BL21(DE3) chemically competent cells: prepared in house or purchased from (Invitrogen, Carlsbad, CA, USA).
7. 4X FSB (final sample buffer): for 50 mL stock, in a fumehood, mix 20 mL glycerol, 10 mL 1 M Tris pH 6.8 (SDS-PAGE stacking buffer), 10 mL β -mercaptoethanol, 6 g sodium dodecylsulfate (SDS, use mask to weigh and avoid

²10X TBE stock: 108 g Tris Base, 55 g boric acid, 40 mL 0.5 M EDTA pH 8.0 (or 9.3 g Na₂-EDTA) in 1 L H₂O (final volume). TBE can be used at 1X or 0.5X for electrophoresis.

³EDTA will not dissolve at high concentrations unless it is ~pH 8.0. To make a 1L stock, dissolve 186.6 g EDTA in water (bring volume up to 800 mL). Add ~20 g NaOH pellets, stir to dissolve, and add more NaOH as needed to bring pH to 8.0. Add water to give final volume of 1 L. Autoclave to sterilize.

⁴Store filter-sterilized ampicillin at 4°C. It is stable for 1-2 months.

⁵IPTG is stable for at least 1 month at 4°C. IPTG can also be aliquoted in 0.5 mL volumes and stored at –20°C.

aerosolizing, *see* **Note 6**), 20 mg bromophenol blue. Add water to final volume of 50 mL and dissolve. Aliquot and store in freezer for long-term storage (*see* **Note 7**).

2.3. Protein purification reagents

1. NaCl solution. 5 M stock solution in water. Weigh 292.1 g of NaCl and pour into 2 L beaker. Bring water to 1L and stir to dissolve. Autoclave to sterilize. (*see* **Note 8**).
2. Tris pH 7.5 solution. 1 M stock solution in water. Dissolve 121.1 g Tris Base in 800 mL water. Add 65 mL concentrated HCl. Adjust pH to reach 7.5 and add water until final volume reaches 1 L. Autoclave to sterilize or filter-sterilize. (*see* **Note 9**).
3. Imidazole solution pH 8.0. 1 M stock solution in water. Dissolve 68.08 g imidazole in 0.8 L H₂O, and add NaOH until pH reaches 8.0; bring H₂O to 1 L volume. Filter-sterilize and store at room temperature or 4°C.
4. Lysis buffer (LIB): 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5, 15 mM imidazole, 10% glycerol (*see* **Note 10**). Store at 4°C. To minimize degradation of fusion proteins, add Halt protease inhibitor cocktail 1:100 dilution (Pierce, Rockford, IL, USA, *see* **Note 11**).
5. Ni-NTA agarose (Qiagen, Valencia, CA).
6. Inositol hexakisphosphate (InsP₆) solution. 25 mM stock in water. Dissolve 25 mg InsP₆ (EMD Millipore, Billerica, MA, USA) also called phytic acid, (Sigma Chemical Company, St. Louis, MO, USA) in 1.5 mL sterile H₂O to give a final concentration of 25 mM. Aliquot into 100 µL fractions to freeze (*see* **Note 12**).
7. Elution buffer (HIB): 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5, 250 mM imidazole, 10% glycerol ± complete protease inhibitor (Pierce, Rockford, IL, USA, *see* **Notes 10 and 11**).

3. Methods

3.1. Clone gene encoding target protein into CPD fusion vector

1. Design cloning primers to amplify target gene sequence (*see* **Note 1**).
2. Prepare working stocks of primers. Resuspend primers from lyophilized stocks by spinning down primers at 13,000 x g for 30 s and adding TE buffer (10 mM Tris pH 8.0, 0.5 mM EDTA, *see* **Note 13**) to give final concentration of 250 µM. Let

⁶SDS is an irritant and should not be inhaled.

⁷Storage of FSB at room temperature will result in oxidation of the reducing agent over time. We store FSB at room temperature for a maximum of 2 weeks.

⁸We find that heating the solution to 65°C ensures that all the salt dissolves.

⁹The pH of Tris solutions is temperature dependent. We prepare our Tris solutions at room temperature.

¹⁰Due to the high viscosity of the glycerol, we use a 50% glycerol stock to make up the LIB buffer. For applications where reducing conditions are necessary, add β-mercaptoethanol to 2 mM.

¹¹We typically make a 1 L lysis buffer stock; for a given purification, we add protease inhibitor to 50 mL of lysis buffer. The protease inhibitor will retain activity for a few days, but we use lysis buffer with freshly added inhibitor. Note - commercially available protease inhibitors do not affect CPD autoprocesing.

¹²InsP₆ will tolerate multiple freeze-thaws.

primers hydrate for 1-2 min before vortexing them vigorously for 10-15s. Quick spin to collect primer stock at bottom of tube. Dilute primer stock 1:10 in water to give a 25 μ M working stock.

3. PCR amplify target gene sequence following manufacturer's directions for Phusion DNA polymerase.
4. Run PCR product on 0.75% agarose gel in TBE (*see Note 2*).
5. PCR purify PCR product using PCR purification kit according to manufacturer's instructions (Fermentas, Glen Burnie, MD, USA). Elute in 50 μ L water.
6. Add 6.3 μ L of buffer 4 (New England Biolabs, Ipswich, MA) and 6.3 μ L 10X bovine serum albumin (New England Biolabs, Ipswich, MA, supplied with restriction enzymes) to PCR purified product. Vortex briefly, quick spin, then add 1 μ L NdeI and 1 μ L SalI and digest for minimum of 1-2 hrs at 37°C.
7. Add 6.3 μ L of buffer 4 and 6.3 μ L 10X bovine serum albumin to 25-50 μ g of pET22b-CPD_{SalI} vector in 50 μ L water. Vortex briefly, quick spin, then add 1 μ L NdeI and 1 μ L SalI and digest for a **minimum** of 1-2 hrs. Add 1:10 dilution of 10X Antarctic phosphatase buffer, mix and add 1 μ L of Antarctic phosphatase to remove 5' phosphate groups for 0.5-1 hr at 37°C.
8. PCR purify restriction digests using PCR purification kit according to manufacturer's instructions (Fermentas, Glen Burnie, MD, USA). Elute in 50 μ L water.
9. Mix 0.5-1.0 μ g of digested pET22b-CPD_{SalI} plasmid (Addgene, Cambridge, MA) with digested PCR product (amount will vary depending on size of insert) and 2 μ L 10X T4 DNA Ligase buffer (New England Biolabs, Ipswich, MA) in a total volume of 19 μ L. Vortex briefly, quick spin, then add 1 μ L of T4 DNA ligase and incubate at 4-16°C for a minimum of 4 hr.
10. Add 50 μ L of DH5 α chemically competent cells directly to the ligation and transform according to manufacturer's directions.
11. After 20-24 hr growth, pick transformants and inoculate 3.5 mL of LB+Amp 100 μ g/mL for miniprep plasmid isolation.
12. Identify transformants containing gene of interest in pET22b-CPD_{SalI} by restriction digest (*see Note 14*).
13. Sequence positive transformants and transform resulting plasmid into BL21(DE3) expression cells (*see Note 15*).

¹³To make TE buffer, we add 0.5 M EDTA to the elution buffer (10 mM Tris pH 8.0) supplied in Fermentas PCR purification kits to give a final [EDTA] of 1 mM.

¹⁴We typically screen transformants using restriction digest with NdeI and XhoI, which will produce a product that consists of the gene of interest fused to the CPD (645 bp).

¹⁵It may be necessary to optimize the *E. coli* expression strain used to avoid problems such as toxicity (e.g. transform BL21(DE3) pLysS cells instead to reduce leaky expression prior to induction) or poor expression due to rare codon usage (e.g. transform into Rosetta strains to supply rare tRNAs).

3.2. Purification of target proteins using CPD autoprocessing fusion tag

Carry out all procedures at room temperature unless otherwise specified.

1. Inoculate 2.5 mL overnight culture LB media containing 100 µg/mL ampicillin with BL21(DE3) CPD expression construct (*see Note 16*).
2. Dilute overnight culture 1:500 into 1L 2YT media containing 100 µg/mL ampicillin (*see Note 17*).
3. Grow with vigorous shaking (225-250 rpm) at 37°C until OD₆₀₀ of 0.6-1.0 is reached (between 3-4 hrs).
4. *Optional*: Remove a 1 mL sample for uninduced whole cell lysate. Pellet sample at 13,000 x g for 1 min and remove supernatant. Resuspend pellet in 50-80 µL 1X FSB. Boil at 95°C for 3 min and save for SDS-PAGE.
5. Add IPTG to 250 µM (250 µL of 1M IPTG stock, *see Note 18*).
6. Grow induced cultures overnight shaking (225-250 rpm) at 18-20°C for 10-16 hr.
7. *Optional*: Remove 1 mL sample for +IPTG whole cell lysate prep. Pellet sample at 13,000 x g for 1 min and remove supernatant. Resuspend pellet in 100-200 µL 1X FSB. Boil at 95°C for 3 min and save for SDS-PAGE (**Figure 2**).
8. Pellet 1 L culture at 4,000 x g in 1 L screw cap bottle for 20 min.
9. Discard supernatant and partially resuspend pellet in 25 mL LIB by first scraping pellet with 10 mL pipet. Resuspend slurry by pipetting up and down with 10 mL pipet.
10. Transfer cells to 50 mL conical tube and flash freeze in liquid nitrogen (*see Note 19*).
11. Thaw sample in water then lyse by sonication (Branson sonifier 250, 3 cycles of 45 s at 40% with 3 - 5 min rest on ice between cycles using a 1/2" tip, *see Note 20*).
12. Transfer lysate from 50 mL conical to round Sorvall tube and spin at 15,000 x g for 30 min to clear lysate (*see Note 21*).

¹⁶The culture only needs to be in stationary phase. We often inoculate from a freshly transformed plate in the morning and grow the culture for a minimum of 4-5 hr until it is in stationary phase before back-diluting into 2YT media.

¹⁷We often use pre-warmed 2YT media to reduce the time it takes for the expression culture to reach OD ~0.8. It is important to use a wide bottom flask to ensure maximum aeration of the culture and more efficient growth. Yields will be reduced if cultures are grown under oxygen-limiting conditions. We typically perform initial purifications on a 1 L scale to determine whether small or large scale (>2 L) preps are needed to obtain sufficient amounts of protein. The 1 L scale of preps allows us to purify 0.1 – 0.3 mg/L of poorly expressed proteins.

¹⁸We find that we get better yields when inducing at a higher OD, e.g. 1.0. However, it is sometimes desirable to cool down the cultures on ice prior to adding IPTG. This slows down translation and protein folding which can aid in the solubility of difficult-to-express proteins.

¹⁹The flash-freeze step improves bacterial cell lysis.

²⁰It is important not to over-sonicate the sample (the pitch of the sonication will increase sharply if this occurs) or have the sample heat up too much. If sample temperature increases significantly, increase the incubation on ice or decrease the sonication time.

²¹Transfer the cleared lysate immediately to a 50 mL conical to minimize transfer of unlysed cells and other debris.

13. During spin, wash 1.0 mL Ni-NTA agarose bead slurry with LIB buffer in 1.5 mL tube (pellet at 9,000 x g for 2 min, remove supernatant, add 0.5 – 1.0 mL lysis buffer) (*see Note 22*).
14. Decant cleared lysate into 50 mL conical tube. Remove 30 μ L cleared lysate sample and add to 10 μ L 4X FSB for SDS-PAGE analysis (**Figure 2**).
15. Transfer Ni-NTA agarose beads to cleared lysate and nutate or place on shaker (low speed) for 2 hr to overnight at 4°C (*see Note 23*).
16. Pellet binding reaction at 2,000 x g for 2 min at 4°C.
17. Wash beads in LIB (10 – 12 mL) and transfer to a 15 mL conical (*see Note 24*). Wash out 15 mL conical with MLIB buffer to minimize bead loss.
18. Pellet binding reaction at 2,000 x g for 2 min at 4°C.
19. Transfer beads to 1.5 mL tube (wash out 15 mL conical with MLIB buffer to minimize bead loss).
20. Wash beads 1x with 1 mL LIB (add 1 mL LIB, pellet binding reaction at 8,000 x g for 2 min at room temperature or 4°C, and remove supernatant)
21. Repeat wash step 2 more times.
22. Add 300 μ L of LIB to beads and resuspend by inversion; add InsP₆ to give a final concentration of 50 μ M (1:500 dilution, *see Note 25*).
23. Incubate shaking at room temperature for 1-2 hr (*see Note 26*)
24. Spin down beads and remove supernatant (containing cleaved protein of interest, *see Note 27*). Reserve a 30 μ L fraction for SDS-PAGE analysis (supernatant fraction 1, **Figure 2**).
25. Wash beads with 2-3 times more with 300 μ L LIB (nutate for 5-10 min, pellet at 8,000 x g for 2 min, transfer supernatant to a fresh tube (*see Note 28*). Remove 30 μ L of each supernatant fraction for SDS-PAGE analysis (**Figure 2**).
26. To assess the efficiency of cleavage, elute the His₆-tagged CPD attached to the Ni-NTA beads by adding 300 μ L of HIB buffer and repeating steps 24 and 25. The

²²Ensure that the Ni-NTA agarose slurry is fully resuspended: the beads settle quickly even after resuspension.

²³The longer the incubation time with the beads, the greater the yield. If the cleared lysate is not immediately transferred to the 50 mL conical tube, unlysed bacteria can sometimes be transferred and grow in the lysate during prolonged incubation. The cleared lysate will then appear cloudy when the beads are removed by centrifugation.

²⁴Leave sufficient buffer above beads to avoid aspirating Ni-NTA agarose beads during washes.

²⁵If cleavage is observed to be inefficient, it is possible to add InsP₆ up to 100 μ M.

²⁶We have observed for some proteins that cleavage at 4°C decreases their solubility. The CPD will autoproces at a wide range of temperatures (4 - 37°C), so the temperature used at this step depends on your specific application. In addition, we have performed the autoprocesing reaction in a wide range of buffer concentrations, including in the absence of salt (50 mM Tris pH 8.0). The composition of buffer used during the autoprocesing step should be user-determined based on the downstream applications for the purified protein.

²⁷Steps 23-25 can be repeated. The efficiency of protease cleavage depends on the accessibility of the linker region to the CPD. Some proteins that are highly folded at the C-terminus are hampered by this problem. In these cases, it may be necessary to extend the length of the linker region, for example by addition of an HA-tag using the HA-CPD constructs.

²⁸To quickly assess the efficiency of autoprocesing, we usually test the supernatant fractions for the presence of protein by adding 1 μ L of the supernatant fraction to 100 μ L of Bradford reagent (Pierce, Rockford, IL, USA) and observing the color change. The amount of protein detected in the supernatant will decrease with each successive wash.

elution time can be reduced to 5-10 min (*see Note 29*). Remove 30 μ L samples from the elution fractions for SDS-PAGE analysis (**Figure 2**).

27. To analyze the purification, add 10 μ L of 4X FSB to the 30 μ L fractions removed during the purification (+IPTG, cleared lysate, supernatant fractions 1-4, and elution fractions). Boil at 95°C and run on a 12% SDS-PAGE gel. Analyze by Coomassie staining according to manufacturer's direction (**Figure 2**, *see Note 30*).

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²⁹Immobilization of the CPD onto Ni-NTA agarose beads can reduce the efficiency of autocleavage. To avoid this issue, you can also elute the fusion protein with HIB buffer and then perform the InsP₆-induced autoprocessing reaction. Pool the collected fractions and buffer exchange using Amicon concentrators (EMD Millipore, Billerica, MA, USA) cleave with InsP₆, then perform a subtractive Ni-NTA pull-down to remove the His₆-tagged CPD (post-cleavage). Your protein of interest will be in the supernatant fraction.

³⁰The percentage SDS-PAGE gel that should be run depends on the size of the protein of interest. The CPD fusion tag will run ~24 kDa. We use GelCode Blue (Pierce, Rockford, IL, USA) to perform Coomassie staining, which has enhanced detection over traditional Coomassie staining.

Figure 1.

CPD autoprocessing purification system. (a) Schematic of purification scheme as described in text. (b) Schematic of CPD fusion protein and resulting protein post-CPD autoprocessing. (c) Schematic of pET-based cloning vector.

Figure 2.

Purification of recombinant proteins using the CPD fusion tag. SDS-PAGE analysis CPD fusion tag-purified proteins using Coomassie staining. (a) *Clostridium difficile* CspBA (CD2246). When expressed as a C-terminally His₆-tagged protein in *E. coli*, CspBA exhibits poor solubility (A. Shen, unpublished observations); when expressed as a CPD-His₆-tagged protein in *E. coli*, CspBA can be purified in a soluble form. (b) mouse macrophage metalloelastin (MMP-12). MMP-12 is insoluble as a His₆-tagged protein in *E. coli* ([10]). When fused to the CPD, active, untagged MMP-12 can be readily purified from *E. coli* ([3]). ** indicates the autoprocessed, active form of MMP-12. (c) *E. coli* biotin ligase BirA. His₆-tagged proteins were bound to Ni-NTA resin then incubated with 50 μM InsP_6 for 1 hr at room temperature; the resin was washed 2-3 times, followed by elution of Ni²⁺-bound proteins by 200 mM imidazole. +, IPTG- induced culture; Pre, imidazole elution prior to InsP_6 -mediated cleavage; CL, cleared lysate; SN; supernatant fraction resulting from InsP_6 -induced autoprocessing by the CPD.