Simplified, Enhanced Protein Purification Using an Inducible, Autoprocessing Enzyme Tag

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Abstract

We introduce a new method for purifying recombinant proteins expressed in bacteria using a highly specific, inducible, self-cleaving protease tag. This tag is comprised of the Vibrio cholerae MARTX toxin cysteine protease domain (CPD), an autoprocessing enzyme that cleaves exclusively after a leucine residue within the target protein-CPD junction. Importantly, V. cholerae CPD is specifically activated by inositol hexakisphosphate (InsP₆), a eukaryotic-specific small molecule that is absent from the bacterial cytosol. As a result, when His₆-tagged CPD is fused to the C-terminus of target proteins and expressed in Escherichia coli, the full-length fusion protein can be purified from bacterial lysates using metal ion affinity chromatography. Subsequent addition of InsP₆ to the immobilized fusion protein induces CPD-mediated cleavage at the target protein-CPD junction, releasing untagged target protein into the supernatant. This method condenses affinity chromatography and fusion tag cleavage into a single step, obviating the need for exogenous protease addition to remove the fusion tag(s) and increasing the efficiency of tag separation. Furthermore, in addition to being timesaving, versatile, and inexpensive, our results indicate that the CPD purification system can enhance the expression, integrity, and solubility of intractable proteins from diverse organisms.

Introduction

The availability of simple, reliable, and cost-effective methods for recombinant protein purification is critical for the work of high throughput structural and proteomic centers and many individual researchers alike. While the addition of affinity tags such as poly-His and glutathione transferase (GST) to target proteins has greatly simplified purification strategies, it is often difficult to obtain soluble recombinant protein [1]. As a result, intractable affinity-tagged target proteins are often fused to small proteins such as NusA and SUMO to improve their solubility, expression, and stability [2].

Since these tags can alter the biological activity of target proteins and interfere with protein crystallization studies, many biological and biomedical applications require that the tag be removed from the target protein. Most commonly used methods involve the addition of exogenous site-specific proteases to cleave the affinity tag off the target protein at engineered sites [2]. Unfortunately, high levels of endoprotease must often be applied for extended periods of time, and this can result in undesirable cleavages within the target protein. Furthermore, these endoproteases are costly, often exhibit poor solubility, and require the inclusion of additional chromatography steps to remove the exogenous protease.

To circumvent these disadvantages, we have developed an on-bead cleavage purification system in which a site-specific affinity-tagged protease is fused directly to the target protein. This approach condenses affinity purification, cleavage, and tag separation into a single step, simplifying protein purification procedures and increasing purification yields. The key element of this purification method is the Vibrio cholerae MARTX toxin cysteine protease domain (CPD) [3]. The CPD exhibits several properties that facilitate its development into an inducible, autocleaving protease tag. First, the CPD is a highly specific protease that cleaves exclusively after Leu residues [4]. Second, the CPD is inducible, as it is specifically activated 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 can be purified from bacterial lysates in a protease-inactive form using imidazolae affinity chromatography (IMAC). Addition of InsP₆ to an immobilized, C-terminally His₆-tagged fusion protein induces autoprocessing at the P1 Leu cleavage site (P1 refers to the residue N-terminal to the scissile bond), which is located at the 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. Third, as an
untagged proteins, suggesting that it will have widespread utility in rapid purification of both soluble and intractable, recombinant, and integrity of target proteins. Thus, this method facilitates the affinity tags [2] in that it can increase the expression, solubility, and efficiency [4,5]. This property should limit fusion protein cleavage autoprocessing enzyme, the CPD exhibits poor transcleavage efficiency [4,5], which should limit its ability to cleave target proteins at heterologous sites. Indeed, mutation of the P1 Leu to an Ile residue is sufficient to prevent CPD-mediated transcleavage, a finding that is explained by the observation that the P1 Leu residue fits snugly into the S1 substrate binding pocket in the crystal structure of the P1 Leu azaepoxide inhibitor modified V. cholerae CPD [4]. Nevertheless, since other site-specific proteases used to remove fusion tags have been observed to cleave target proteins at secondary sites [2], we examined whether the CPD would spuriously cleave target proteins. Specifically, we tested whether the CPD would cleave an intrinsically disordered protein after Leu residues within the target protein. We used the intracellular domain (ICD) of the cytokine receptor gp130 as a test substrate, since it is unstructured in solution by NMR [11] and contains multiple Leu residues that might serve as cleavage substrates [12]. The ICD-CPD-His6 fusion protein was expressed and purified from E. coli lysates using IMAC, and CPD-mediated cleavage of the immobilized fusion protein was activated by InsP6 addition. As shown in Figure 4, autoprocessing occurred exclusively at the ICD-CPD interdomain junction, with a single protein equivalent to the size of His6-tagged ICD being released into the supernatant fraction. These results strongly suggest that the CPD will not promiscuously cleave target proteins.

Fusion of Target Proteins to the CPD Can Increase Their Expression and Purity

We noticed that the expression of the ICD-CPD-His6 fusion protein was at least three-fold higher than the ICD-His6 protein in individual research labs and high-throughput structural and proteomic centers.

Results

Development of the One-Step CPD Purification System

In order to produce CPD fusion proteins, we first constructed CPD expression vectors (pET-CPD expression vectors) using the pET expression vector backbone. DNA encoding the CPD was cloned into the SalI restriction site (Figure 2) such that the fusion protein produced upon IPTG induction of E. coli harboring the pET-CPD expression vector carries the P2-P1 residues of the native CPD (Ala-Leu, respectively) and the P4-P3 residues encoded by the SalI site (Val-Asp, respectively) (Figures 1 and 2). The P1 residue refers to the amino acid N-terminal to the scissile bond, while the residue N-terminally adjacent to the P1 residue is termed P2, and so on. When InsP6 is added to induce CPD-mediated autocleavage of the fusion protein, the untagged target protein is released from the resin and carries four additional C-terminal residues (Val-Asp-Ala-Leu); the His6-tagged CPD remains bound to the resin (Figure 1). The Val-Asp-Ala-Leu C-terminal addition can be reduced to two amino acids (Glu-Leu) by cloning into the SacI site, or to a single amino acid (Leu) by cloning into the BamHI site and adding a Leu codon to the 3’ cloning primer (Figure 2).

To demonstrate the feasibility of this system, we first expressed and purified green fluorescent protein (GFP) as a fusion to CPD-His6 using IMAC. As anticipated, addition of increasing amounts of InsP6 stimulated the release of GFP from the Ni²⁺-NTA resin in a dose-dependent manner (Figures 3A and B), while the His6-tagged CPD remained bound to the Ni²⁺-NTA agarose beads (bead eluate, Figure 3A).

The CPD Cleaves Exclusively at the Fusion Protein Junction

We have previously shown that V. cholerae CPD is positioned to undergo autocleavage at a proximal N-terminal leucine and that it exhibits significantly reduced transcleavage efficiency [4,5], which should limit its ability to cleave target proteins at heterologous sites. Indeed, mutation of the P1 Leu to an Ile residue is sufficient to prevent CPD-mediated transcleavage, a finding that is explained by the observation that the P1 Leu residue fits snugly into the S1 substrate binding pocket in the crystal structure of the P1 Leu azaepoxide inhibitor modified V. cholerae CPD [4]. Nevertheless, since other site-specific proteases used to remove fusion tags have been observed to cleave target proteins at secondary sites [2], we examined whether the CPD would spuriously cleave target proteins. Specifically, we tested whether the CPD would cleave an intrinsically disordered protein after Leu residues within the target protein. We used the intracellular domain (ICD) of the cytokine receptor gp130 as a test substrate, since it is unstructured in solution by NMR [11] and contains multiple Leu residues that might serve as cleavage substrates [12]. The ICD-CPD-His6 fusion protein was expressed and purified from E. coli lysates using IMAC, and CPD-mediated cleavage of the immobilized fusion protein was activated by InsP6 addition. As shown in Figure 4, autoprocessing occurred exclusively at the ICD-CPD interdomain junction, with a single protein equivalent to the size of His6-tagged ICD being released into the supernatant fraction. These results strongly suggest that the CPD will not promiscuously cleave target proteins.

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E. coli lysates (Figure 4, compare + lanes). This result suggested that the CPD might generally enhance target protein expression and/or solubility levels. To test this hypothesis, we compared the expression and solubility of CPD fusions to several other target proteins carrying either a His6-tag and/or GST-fusion tag (Figures 5-8 and Table 1). In all cases, the presence of the CPD-His6 fusion tag increased the expression and solubility of target proteins. For example, fusion of the CPD-His6 tag to biotin ligase (BirA) from *E. coli* raised BirA expression levels by three-fold over the GST-BirA construct [13] (Figure 5 and Table 1).

The CPD purification system also enhanced the expression and purity of a previously uncharacterized SUMO/Sentrin-specific peptidase 1 (SENP1) from the parasitic pathogen *Plasmodium falciparum*, the causative agent of malaria (Figure 6) [14]. Although PISENP1 carrying an N-terminal His6-tagged can be readily expressed and purified from *E. coli*, a number of contaminating bands are present, and the N-terminal His6-tag must be removed by the addition of thrombin followed by multiple chromatography steps (Table 2). In contrast, when PISENP1 is expressed as a fusion to CPD-His6 and released as untagged PISENP1 upon InsP6 addition, only one minor contaminant co-purifies with PISENP1 (Figure 6B). This variant is easily removed using gel filtration chromatography (Figure 6C), and the untagged PISENP1 is of sufficient purity that we have used it to obtain diffraction-quality crystals (E. Ponder, unpublished results). Notably, although the heterologous expression of *P. falciparum* proteins in *E. coli* is typically challenging [15], we have observed that this system can enhance the expression and purification of other parasitic proteins from *P. falciparum* and a related apicomplexan parasite *Toxoplasma gondii*.

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**Figure 2. Schematic of pET-CPD expression vectors.** Bent arrow, T7 promoter, Oval (RBS), ribosome binding site, green rectangle, target protein, grey rectangle, CPD, *V. cholerae* MARTX (aa. 3440–3650), darker grey rectangle, ΔP1-CPD, *V. cholerae* MARTX (aa. 3442–3650), darkest grey rectangle, ΔP2-CPD, *V. cholerae* MARTX (aa. 3444–3650), black rectangle, His6-tag, white rectangle, HA-tag. The dotted vertical line and arrow indicate the CPD cleavage site. Residues added onto the C-terminus of the target protein following CPD-mediated cleavage, and the relevant restriction sites are shown (residues encoded by the restriction sites that are appended to the C-terminus of target proteins are underlined). The composition of the residues added to the C-terminus of the target protein can be varied depending on the cloning site and pET-CPD vector used. It should be noted that the P1 Leu shown for pET22b-CPD_BamHI-Leu must be encoded in the 3′ cloning primer of the target gene (i.e. add a Leu codon to the end of the target insert). Both pET22b and pET28a vector backbones were used to construct the CPD expression vectors.

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Figure 3. Purification of GFP using the CPD-His$_6$ tag. (A) SDS-PAGE analysis of GFP purification using Coomassie stain. GFP-CPD-His$_6$ bound to Ni$^{2+}$-NTA resin was incubated with increasing amounts of InsP$_6$ for 2 hrs at 4°C. GFP released into the supernatant was collected (InsP$_6$ supernatant); Ni$^{2+}$-bound proteins were then eluted from the resin by the addition of 200 mM imidazole (Imidazole elution). Collected fractions were analyzed by SDS-PAGE. (B) Visual analysis of GFP released into the supernatant fraction upon InsP$_6$ addition to immobilized GFP-CPD-His$_6$ fusion protein.
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Figure 4. The CPD does not cleave within an intrinsically unstructured protein. gp130 intracellular domain (ICD)-CPD-His$_6$ or gp130(ICD)-His$_6$ bound to Ni$^{2+}$-NTA resin was incubated with 100 μM InsP$_6$ for 2 hr at room temperature; the resin was washed four times, followed by elution of Ni$^{2+}$-bound proteins by 200 mM imidazole. Purification fractions were analyzed by SDS-PAGE followed by Coomassie staining. CL, cleared lysate, FT, flowthrough, IP6, elution from InsP$_6$ incubation.
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Fusion of Target Proteins to the CPD Can Improve Their Stability and Solubility

In addition to augmenting the expression of target proteins, CPD-His₆ fusions protected target proteins from proteolytic degradation. This effect was observed when the CRAC-activation domain (CAD) of the ER calcium sensor STIM1 was fused to the CPD (Figure 7). CAD is a small 107 aa polypeptide that activates Ca²⁺ release-activated Ca²⁺ (CRAC) channels by binding to the CRAC channel protein Orai1 [16]. Until now, large-scale expression and purification of this important regulatory domain has proven difficult due to its apparent instability even when fused to GST (Figure 7). However, using the CPD system, we were able to obtain significant quantities of a CAD-containing polypeptide (CAD128), which has subsequently been used in high-throughput screens for Orai1-CAD binding partners (A.M. Sadaghiani).

Finally, the CPD purification system also increased the solubility of difficult-to-express proteins. Fusion of the mouse macrophage metalloelastase (MMP12) to CPD-His₆ facilitated its purification from the soluble fraction of E. coli lysates, whereas His₆-tagged MMP12 remained largely insoluble (Figure 8A). The currently used method for purification of His₆-tagged MMP12 is a laborious procedure that requires solubilization of MMP12 inclusion bodies, refolding over multiple days, followed by anion and cation exchange chromatography [17]. The CPD purification system dramatically simplifies this purification procedure, allowing soluble, active MMP-12 to be isolated in approximately 7 hours (Figs. 8B and C, Table 3). We have used this improved purification protocol to rapidly express, purify and analyze MMP12 mutant proteins.

Discussion

We have developed a novel one-step purification system that accelerates untagged recombinant protein purification from bacterial systems. By directly fusing an affinity-tagged, site-specific protease to a target protein, the CPD system ensures rapid and efficient removal of the fusion tag in a cost-effective manner. As a result, the CPD system overcomes many of the disadvantages associated with the exogenous addition of site-specific proteases, like thrombin and TEV protease, to remove fusion tags. These disadvantages can include their expense, generally low activity [2,18], sensitivity to buffer conditions, and cleavage of target proteins at spurious sites [2]. In contrast, the CPD rapidly completes tag removal within two hours of addition (Figures 3–8), since the CPD is present at a 1:1 ratio to the target protein and poised to undergo the autocleavage reaction [5]. Furthermore, the responsiveness of the protease specifically to InsP₆ provides the user with complete control over the timing and conditions of fusion tag removal, while the autoprocessing nature of the CPD confers a high degree of specificity to fusion tag removal [4,5]. Specifically, the protease is poised to undergo autocleavage upon InsP₆ addition and exhibits poor transcleavage efficiency, as evidenced by the lack of CPD-mediated cleavage within any of the target proteins tested (Figures 3–8), including an intrinsically unstructured protein (Figure 4).

While purification systems based on fusing a protease to target proteins have previously been developed [9,10], our demonstration that the CPD can enhance the expression, solubility, and stability of target proteins (Figures 4–8) suggests that the CPD system likely represents an improvement over existing methods like the intein-chitin-binding-domain (CBD) [9,10] and sortase-His₆, one-step purification systems [9]. Although these self-cleaving systems simplify the purification of well-expressed proteins, the large size of the intein-CBD fusion tag can decrease target protein solubility [2], while sortase-His₆ fusion tags do not increase target protein solubility [9]. Furthermore, unlike self-cleaving elastin-like polypeptide (ELP) tags [19], CPD fusion proteins do not need to be subjected to temperature cycles, pH shifts, or high salt concentrations, a feature that is critical for the purification of intractable proteins. Based on the properties reported here, the CPD could replace the intein-tag in the self-cleaving-ELP system and potentially improve the solubility of ELP-tagged proteins while retaining their self-cleavability [19].

Indeed, a considerable strength of this method is that the CPD remains active over a wide range of conditions. CPD-mediated...
cleavage is complete within 1–2 hrs at temperatures between 4°C and 37°C, requires only micromolar concentrations of the small molecule InsP₆ (an abundant and inexpensive reagent), and occurs efficiently both in the presence of standard protease inhibitor cocktails and in the absence of salt. This latter property carries the additional advantage of allowing the user to determine the buffer system in which to elute the target protein, eliminating the need for desalting or buffer exchange steps that can reduce protein

Figure 6. Comparison of His₆-tag removal from *Plasmodium falciparum* SENP1 using thrombin relative to the CPD. (A) Coomassie stain of SDS-PAGE analysis of *P. falciparum* SENP1 (PISEN1, 25 kDa) purified using either the CPD-His₆ or His₆-affinity tags. PISEN1-CPD-His₆ or His₆-PISEN1 bound to the Ni²⁺-NTA resin was incubated with 100 μM InsP₆ for 2 hr at room temperature; the resin was washed three times, and wash fractions were collected. Ni²⁺-bound proteins were eluted by adding 200 mM imidazole. +, IPTG induced culture, CL, cleared lysate, E, imidazole elution prior to InsP₆ addition, IP6, elution following InsP₆ incubation. (B) UV trace PISEN1 further purified by gel filtration chromatography following His₆-tag removal. Inset, Coomassie stain of gel filtration fractions of PISEN1 purifications. Thrombin refers to PISEN1 purified following thrombin-mediated removal of the N-terminal His₆-tag, while InsP₆ refers to InsP₆-induced, CPD-mediated removal of the C-terminal CPD-His₆-tag. The residues added to the resulting PISEN1 protein are shown: GSHM is added to the N-terminus of PISEN1 following thrombin cleavage, while VDAL is added to the C-terminus of PISEN1 following InsP₆-activated CPD cleavage. (C) Coomassie stain of SDS-PAGE analyses of fractions taken during His₆-PISEN1 purification prior to thrombin incubation (–), following 12 hr thrombin incubation (+), and following subtractive IMAC to remove uncleaved His₆-PISEN1 (Ni²⁺-NTA). The yield of PISEN1 diminished with each experimental manipulation.

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yields. In addition, we have created a number of vector backbones that can be used to vary the residues that are appended to the target protein following CPD-mediated cleavage, which can range from a single amino acid residue to an HA epitope tag (Figure 2). Thus, the CPD system allows for considerable flexibility in optimizing purification procedures, as is often necessary for uncharacterized target proteins.

This versatility, combined with our observation that it can improve the solubility and integrity of difficult-to-express proteins (Figures 5 to 8), suggests that it will have widespread utility in biological research. The simplicity of this system will also make it amenable for large-scale proteomic, structural genomic, and commercial applications by eliminating the cost and complexity associated with exogenous site-specific proteases, potentially permitting its use in robotic systems for constructing protein arrays for screening purposes.

Materials and Methods

Bacterial Growth Condition

Overnight bacterial strains were grown at 37°C in Luria-Bertani (LB) broth. Antibiotics were used at 100 μg/mL carbenicillin for pET22b vectors expressed in E. coli.

Strain Construction

Primers used are listed in Table S1; strains constructed are listed in Table S2 in the Supporting Information. For construction of pET-CPDsal1 vectors, DNA encoding Vibrio cholerae MARTX toxin amino acids 3440-3650 from Vibrio cholerae N16961 was PCR amplified from genomic DNA using primers #5 and #2, and the resulting PCR fragment was cloned into the XhoI and SacI sites of pET22b-CPDSalI. For construction of the pET-CPDBamHI-Leu vector, DNA encoding Vibrio cholerae MARTX toxin amino acids 3440-3650 from Vibrio cholerae N16961 was PCR amplified from genomic DNA using primers #5 and #2, and the resulting PCR fragment was cloned into the Sall and XhoI sites of the pET22b and pET28a expression vectors, respectively. For construction of the pET-CPDBamHI vector, DNA encoding Vibrio cholerae MARTX toxin amino acids 3440-3650 from Vibrio cholerae N16961 was PCR amplified from genomic DNA using primers #6 and #2, and the resulting PCR fragment was cloned into the BamHI and XhoI sites of pET22b.

The pET22b-GFP-CPD construct was cloned by PCR amplifying GFP from pEGFPN3 (Clontech) using primers #7 and #8. To construct the pET22b-gp130(ICD)-CPD vector, amino acids 642-918 of gp130 corresponding to the intracellular domain were PCR amplified using primers #9 and #10 and pET21a-gp130(ICD) as a template. The pET22b-BirA-CPD vector was constructed by PCR amplifying the bist1 gene from a pGEX4T1-BirA template using primers #9 and #10. The pET22b-STIM1(CAD)-CPD plasmid was constructed by PCR amplifying DNA encoding amino acids 342–469 of STIM1 using pET22b-STIM1(CAD)-CPD constructs was constructed by PCR amplifying the catalytic domain of mouse MMP12 (amino acids 29–267) using pET41a-mMMP12 as a template and primers #13 and #14. The pET22b-nMMP12-CPD construct was constructed by PCR amplifying the catalytic domain of mouse MMP12 (amino acids 29–267) using pET41a-nMMP12 as a template and primers #13 and #14. In all cases, the resulting PCR products were cloned into the NdeI and Sall sites of pET22b-CPDsal1.

Protein Expression and Purification

For purification of His6-tagged CPD fusion proteins, overnight cultures of the appropriate strain were diluted 1:500 into 1 L 2YT
When an OD_{600} of 0.6 was reached, IPTG was added to 250 μM, and cultures were grown for 3–4 hrs at 30°C. Cultures were pelleted, resuspended in 25 mL lysis buffer [500 mM NaCl, 50 mM Tris-HCl, pH 7.5, 15 mM imidazole, 10% glycerol] and flash frozen in liquid nitrogen. Lysates were thawed, then lysed by sonication and cleared by centrifugation at 15,000 × g for 30 minutes. His_{6}-tagged CPD fusion proteins were affinity purified by incubating the lysates in batch with 0.5–1.0 mL Ni-NTA Agarose beads (Qiagen) with shaking for 2–4 hrs at 4°C. The binding reaction was pelleted at 1,500 × g, the supernatant was set aside, and the pelleted Ni^{2+}-NTA agarose beads were washed three times with lysis buffer. In some cases, 10% of the Ni^{2+}-NTA beads containing immobilized CPD-His_{6} fusion proteins were removed, pelleted and then His_{6}-tagged fusion protein eluted using high imidazole buffer [500 mM NaCl, 50 mM Tris-HCl, pH 7.5, 175 mM imidazole, 10% glycerol].

To liberate untagged target proteins into the supernatant fractions, 300–500 μL lysis buffer was added to the Ni^{2+}-NTA beads containing CPD-His_{6} fusion proteins and the indicated amount of inositol hexakisphosphate (InsP_{6}, Calbiochem) was added. In general, on-bead cleavage was allowed to proceed by

**Figure 8. The CPD improves the solubility of mouse macrophage metalloelastin (MMP12) relative to a His_{6}-affinity tag.** (A) SDS-PAGE analysis of His_{6}-tagged MMP12 using Coomassie stain. MMP12 was fused to His_{6}-tagged CPD, and the expression of the fusion protein relative to His_{6}-tagged MMP12 was compared. Asterisk indicates a putative chaperone protein that co-purifies with MMP12_{VDAL}. The diagonal arrows indicate a His_{6}-tagged truncated MMP12 product that is also observed during MMP12 purification from inclusion bodies [17]. His_{6}-tagged proteins bound to the Ni^{2+}-NTA resin were incubated with 50 μM InsP_{6} for 1 hr at room temperature, and the resin was washed three times, followed by elution of Ni^{2+}-bound proteins by 200 mM imidazole. CL, cleared lysate, +, IPTG induced culture, FT, flowthrough, IP6, elution from InsP_{6} incubation, E, imidazole elution prior to InsP_{6} addition. (B) Additional purification of MMP12_{VDAL} by gel filtration chromatography. Inset, Coomassie stain of SDS-PAGE analysis of gel filtration fractions of MMP12_{VDAL}. (C) MMP12 activity assay. The activity of MMP12 purified under denaturing conditions and refolded (MMP12 (Refolded)) and MMP12_{VDAL} purified using the CPD system (CPD method) against a standard fluorogenic substrate were compared. Comparable rates of fluorogenic substrate cleavage are observed for MMP12 purified by the CPD method relative to the refolding method.

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nutating the beads in the presence of 50–100 μM InsP₆ for 1–2 hr at either room temperature or 4°C. The beads were pelleted at 1,500 g, and the supernatant fraction was removed. The beads were then washed 3–4 times with 300–500 mL lysis buffer, and supernatant fractions retained. His₆-tagged proteins remaining on the beads (i.e. cleaved CPD-His₆) were eluted using high imidazole buffer [500 mM NaCl, 50 mM Tris-HCl, pH 7.5, 175 mM imidazole, 10% glycerol] in 300–500 mL volumes. The elution was repeated 3–4 times, and eluate fractions were collected.

Purification of His₆-tagged proteins lacking the CPD was performed in parallel.

This general procedure was followed with the following exceptions: for purification of MMP12 constructs, the cultures were grown at 16°C for 8 hr after IPTG induction, and 1 mM tris(2-carboxyethyl)phosphine (TCEP) was added to the lysis buffer to prevent misfolding of the protein. PfSENP1 and BirA protein purifications were performed exclusively at room temperature, since at 4°C, protein aggregation was observed. For removal of the His₆-tag from His₆-PfSENP1, thrombin beads (Calbiochem) that had been washed in PBS were added to the eluted His₆-PfSENP1, which had been buffer exchanged into PBS according to the manufacturer’s instructions. Thrombin cleavage was allowed to proceed with shaking overnight for 12 hr at room temperature.

Aliquots were taken before and after thrombin addition to monitor cleavage efficiency. Thrombin cleaved, untagged PfSENP1 was enriched by performing a subtractive Ni²⁺-NTA pull-down. Untagged PfSENP1 from both methods was then buffer-exchanged into gel filtration buffer (50 mM NaCl, 20 mM Tris pH 8.0). Protein purifications were analyzed by SDS-PAGE and Coomassie staining using GelCode Blue (Pierce). Purified protein concentrations of purified were determined by Bradford assay (Pierce).

Purification of MMP12-His₆

MMP12-His₆ was purified as previously described [17] with the following modifications. The cell pellet was resuspended in 100 mM NaCl, 100 mM Tris pH 8.0, 5.0 mM EDTA, 0.5 mM DTT, 100 μg/mL lysozyme and stirred for 2 hr. The cells were sonicated then centrifuged at 10,000 rpm for 10 min. The resulting inclusion bodies were washed two times and then resuspended in 50 mL 6M guanidine hydrochloride, 10 mM Tris pH 8.0 by stirring at 4°C overnight. The mixture was centrifuged at 15,000 rpm for 30 min, and 2 mL aliquots of supernatant were prepared. The supernatant was diluted 1:100 into denaturing buffer [6M Urea, 50 mM Tris pH 8.0, 10 mM CaCl₂, 30 mM NaCl, 5 mM DTT] to a final concentration of 0.1–0.2 mg/mL.

Table 1. Target proteins expressed and purified by CPD purification method.

| Target protein                  | Yield* (mg/L culture) | Yield (nmol/L culture) | Activity                                           |
|---------------------------------|-----------------------|------------------------|----------------------------------------------------|
| GFP₃₉₅₋VDAL (CPD method)        | 3.3                   | 105                    | Fluorescence at 511 nm                             |
| gp130(ICD)₃₉₅₋VDAL (CPD method) | 5.9                   | 188                    | n/a                                                |
| gp130(ICD)-His₆                 | 3.7                   | 115                    | n/a                                                |
| BirA₃₉₅₋VDAL (CPD method)       | 10.9                  | 202                    | Biotinylates LHHILDAQKMWWNH BirA biotinylation site |
| GST-BirA-His₆                   | 12.0                  | 90                     | Biotinylates LHHILDAQKMWWNH BirA biotinylation site |
| PISENP1₃₉₅₋VDAL (CPD method)    | 2.0                   | 67                     | Cleaves PISUMO                                     |
| SISENP1                           | 1.4                   | 46                     | Cleaves PISUMO                                     |
| STIM1(CAD)₃₉₅₋VDAL              | 2.1                   | 148                    | Binds Orai1                                        |
| GST-STIM1(CAD)-His₆             | 2.5                   | 62                     | n/a                                                |
| MMP12₃₉₅₋VDAL (CPD method)      | 1.4                   | 47                     | Cleaves fluorogenic peptide substrate Mca-PLGLDL(Dpa)AR |
| MMP12 (refolded)                | 23                    | 767                    | Cleaves fluorogenic peptide substrate Mca-PLGLDL(Dpa)AR |

*Protein yield per litre of culture.

Table 2. Comparison of CPD-mediated and thrombin-mediated purification of PISENP1.

| PISENP1-CPD-His₆ | His₆-PISENP1 |
|------------------|-------------|
| Step 1           | Prepare soluble lysate (1 hr) |
| Step 2           | IMAC purification (2 hr)     |
| Step 3           | On-bead cleavage; collect supernatant (2 hr) |
| Step 4           | Concentrate protein (0.5 hr) |
| Step 5           | Gel filtration chromatography (1 hr) |
| Step 6           | Concentrate protein (0.5 hr) |
| Step 7           | Concentrate protein and buffer exchange (0.5 hr) |
| Step 8           | Gel filtration chromatography (1 hr) |
| Total time       | 5 hr                     |

| PISENP1-CPD-His₆ | His₆-PISENP1 |
|------------------|-------------|
| Step 1           | Prepare soluble lysate (1 hr) |
| Step 2           | IMAC purification (1 hr)     |
| Step 3           | Imidazole elution            |
| Step 4           | Buffer exchange and concentrate protein (0.5 hr) |
| Step 5           | Thrombin cleavage overnight (>12 hr) |
| Step 6           | Remove His₆-tag and uncleaved fusion with IMAC (1 hr) |
| Step 7           | Concentrate protein (0.5 hr) |
| Step 8           | Gel filtration chromatography (1 hr) |
| Total time       | >17.5 hr                  |
The protein was then dialyzed for 24 hr in 2 L refolding buffer 1 ([3 M Urea, 50 mM Tris pH 8.0, 10 mM CaCl$_2$, 30 mM NaCl, 5 mM DTT]). The partially refolded protein was then dialyzed in 4 L of refolding buffer 2 ([1 M Urea, 50 mM HEPES pH 7.4, 10 mM CaCl$_2$, 5 mM DTT]). The buffer exchanged protein was then purified using tandem 5 mL MonoQ and SP Sepharose (GE Healthcare) at 4°C. After loading the protein on the column, the column was washed with 50 mL of refolding buffer 2 without DTT at 1 M, 0.5, and 0 M urea, respectively. The protein was eluted from the SP column in 500 mM NaCl, 50 mM HEPES pH 7.4, 10 mM CaCl$_2$.

### Gel Filtration Chromatography

Untagged PISENP1 obtained from either thrombin or InsP$_6$-mediated cleavage was concentrated using a 10 kDa Centricon concentrator (Millipore) and buffer exchanged into 50 mM NaCl, 20 mM Tris pH 8.0 and purified on a Superdex 200 10/30 column (GE Healthcare) equilibrated in the same buffer. For MMP12, the gel filtration buffer contained 150 mM NaCl, 50 mM Tris pH 7.4, 10 mM TCEP.

### Activity Assays

Fluorescence of purified GFP at 511 nm was verified using a Molecular Devices fmax plate reader in black 96-well plates and monitored continuously in a fluorescent plate reader (Molecular Devices) using an excitation wavelength of 325 nm and an emission wavelength of 395 nm.

### Supporting Information

**Table S1** Primers used in Study. a Restriction enzyme sequences are underlined, and the HA tag is shown in italics. b RE - Restriction site

**Table S2** Strains used in study. 1. Skiniotis G and Lupardus, PJ. (2009) Cell 136: 737–748. 2. Ponder EL, et al. (2009) Nat Rev Mol Cell Biol. 9: 151–161. 3. Michell RH (2008) Inositol derivatives: evolution and functions. Nat Rev Mol Cell Biol 9: 151–161.

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