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TITLE:

Bacterial Expression and Purification of Human Matrix Metalloproteinase-3 Using Affinity Chromatography

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KEYWORDS:

Matrix metalloproteinase, MMP-3cd, Bacterial expression, Solubilization and refolding of human proteins in E. coli, His-tag affinity protein purification.

SUMMARY:

His-tag purification, dialysis, and activation are employed to increase yields of soluble, active matrix metalloproteinase-3 catalytic domain protein expression in bacteria. Protein fractions are analyzed via SDS-PAGE gels.

ABSTRACT:

Matrix metalloproteinases (MMPs) belong to the family of metzincin proteases with central roles in extracellular matrix (ECM) remodeling and degradation, as well as interactions with several growth factors and cytokines. Secreted as inactive zymogens, MMPs are primarily responsible for extracellular matrix turnover; however, when dysregulated, they are also responsible for several diseases such as cancer, neurodegenerative diseases, and cardiovascular disease. Human MMPs have been the center of attention for decades while studying these diseases and developing therapeutics that target them efficiently. However, even the catalytic domain of most MMPs cannot be expressed in *Escherichia coli* (*E. coli*) in soluble form due to lack of posttranslational machinery, whereas mammalian expression systems are usually costly and have lower yields.

To study the MMP mechanism in solution, more facile and robust methods are needed for the production of active, soluble MMPs. Though relatively pure, MMP inclusion bodies must undergo the tedious and laborious process of extensive purification and refolding, significantly reducing the yield of MMPs in native conformation. This paper presents a protocol using Rosetta2(DE3)pLysS (hereafter referred to as R2DP) cells to produce active matrix metalloproteinase-3 catalytic domain (MMP-3cd), which contains an N-terminal His-tag for use in affinity purification. R2DP cells enhance the expression of eukaryotic proteins through a

chloramphenicol-resistant plasmid containing codons normally rare in bacterial expression systems. Compared to the traditional cell line of choice for recombinant protein expression, BL21(DE3), purification using this new strain improved the yield of purified Hisx6-pro-MMP-3cd. Upon activation and desalting, the pro domain is cleaved along with the N-terminal His-tag, providing active MMP-3cd for immediate use in countless *in vitro* applications. This method does not require expensive equipment or complex fusion proteins and, instead, rapidly facilitates testing needed for the growing development of MMP inhibition-based therapeutics.

INTRODUCTION:

Most complex recombinant proteins undergo elaborate posttranslational modifications after expression, requiring highly assisted protein folding and co-factors to be functional[1]. Producing high yields of active protein remains a significant challenge due to high costs and the lack of robust purification methods, even for smaller-scale laboratory experiments[2-3]. MMPs, human endopeptidases with large molecular weight, are overwhelmingly expressed as insoluble inclusion bodies when recombinantly expressed in *E. coli.* Extraction of soluble MMP protein leads to a laborious, time-consuming solubilization and refolding process[4].

MMPs have critical roles in both physiological and pathogenic processes. In humans, they are a family of 23 zinc endopeptidases, categorized by structure and substrate specificity, and differentially expressed in spite of a highly conserved catalytic domain[5-6]. MMPs are secreted as inactive zymogens, regulated via posttranslational activation and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs)[7-10]. Though initially recognized for their role in ECM turnover, MMPs are substantially involved in development, morphogenesis, tissue repair, and remodeling[8]. When dysregulated, MMPs have been notably linked to cancer along with neurodegenerative, cardiovascular, and fibrotic disease, among other illnesses[5,7].

The development of large-scale MMP production methods is critical to ensure the success of future studies of MMP mechanisms through biochemical and cell-based assays. Traditionally, MMP overexpression is carried out in *E. coli*, human plasma, or mammalian cells[11]. Various MMPs have been previously expressed in bacteria, including Hisx6-tagged MMPs, without altering MMP activity[12-15]. However, these methods include tedious, long steps that might be difficult to replicate. Mammalian cells can be used to express milligram quantities of many different human proteins while ensuring the proper posttranslational modifications[16].

Although the mammalian expression system is an ideal choice to produce human proteins, the main disadvantages of this method are low initial yields, expensive growth media, long wait times to reach stable expression lines, and risk of contamination with other species such as fungi or bacteria[2,11]. MMP production in mammalian cell lines yields impurities from associated cellular proteins such as TIMPs or fibronectins[11]. Unlike the slow cell growth observed in mammalian cells, the bacterial expression system offers large-scale protein production in a short period along with simpler media and growth requirements. However, due to the lack of other associated cellular proteins (i.e., TIMPs) in bacterial expression systems, active MMPs at higher concentrations are subject to degradation through autoproteolysis, resulting in poor MMP yield[17].

This paper describes a detailed method for bacterial expression of recombinant pro-MMP-3cd-Hisx6 utilizing *E. coli* as an expression host due to its accessibility, simplicity, and success in producing milligram quantities of MMPs[2-3,18]. However, *E. coli* lack the protein folding machinery and posttranslational processing required for recombinant MMPs and other complex proteins. Many *E. coli* strains have been engineered to overcome these limitations[16,19-20]. For instance, R2DP cells enhance eukaryotic expression by supplying a chloramphenicol-resistant plasmid containing codons rarely used in *E. coli*.

As described in the protocol, after overexpression of relatively pure inclusion bodies from the pET vector (**Figure 1**) in R2DP cells, Hisx6-pro-MMP-3 catalytic domain (MMP-3cd) proteins are extracted and denatured[4]. Affinity tag chromatography is utilized to purify His-tagged pro-MMP-3cd[3,19]. Upon refolding and dialysis, the MMP-3cd zymogens are activated by 4-aminophenylmercuric acetate (APMA), and SDS-PAGE analysis is used to evaluate yields and the need for further purification[5,21]. This protocol will describe expression, purification, and activation of soluble, active MMP-3cd as an example. However, it may be used as a rough guide for other MMPs and human proteases with similar activation mechanisms (**Figure 2**). If not purifying MMP-3cd, the reader is advised to determine optimal buffer compositions and methods for their target protein before attempting this protocol.

[Place Figure 1 here]
[Place Figure 2 here]

PROTOCOL:

1. MMP expression

1.1. Cloning and transformation of pET-Hisx6-pro-MMP-3cd into R2DP cells

1.1.1. Digest the pET plasmid (see the **Table of Materials**) with NdeI and BamHI restriction enzymes in Digest Buffer (see the **Table of Materials**). In a total reaction volume of 40 µL, add 4 µL of Digest Buffer, 33 µL of plasmid, and 1.5 µL of each restriction enzyme and allow the reaction to proceed for ~2 h until completion at 37 °C.

1.1.2. Perform a PCR reaction on the MMP-3cd sequence to insert an N-terminal His-tag. Use 25 µL of PCR Mix (see the **Table of Materials**), 2.5 µL of primers (**Supplemental Figure S1**), and 1.25 µL of the insert sequence. Add sterile water to a final reaction volume of 50 µL.

1.1.3. Run the PCR product and digested vector on a 1% agarose gel. Purify the gel bands using a Gel Recovery Kit (see the **Table of Materials**) per the manufacturer's protocol.

1.1.4. Clone the amplified PCR product into the digested vector between the NdeI and BamHI restriction sites using DNA Assembly Mix (see the **Table of Materials**). Use online tools to determine the required volume of the insert and cut vector for a total reaction volume of 15 µL.

1.1.5. Per number of assembly reactions, thaw 50 µL aliquots of high-transformation efficiency cells (see the **Table of Materials**) on ice until thawed. Prewarm SOC Growth Medium (see the **Table of Materials**) to 37 °C and LB-ampicillin (LB Amp) plates (see the **Table of Materials**).

1.1.6. Add 1–2 µL of the pET-pro-MMP-3cd assembly reaction to each 50 µL aliquot. Incubate on ice for 30 min.

1.1.7. Heat-shock the cells by incubating at 42 °C for 30 s. Incubate on ice for 2 min.

1.1.8. Add 950 µL of SOC growth medium to each transformant mixture. Shake for 1 h at 250 rpm and 37 °C.

1.1.9. Plate 250 µL of the transformants on LB Amp plates and incubate overnight at 37 °C.

1.1.10. Inoculate each isolated colony in 10 mL of LB Amp medium. Shake overnight at 250 rpm and 37 °C.

1.1.11. Extract plasmid DNA per manufacturer's protocol for the miniprep kit (see the **Table of Materials**). Confirm the sequence of the construct using T7 forward and reverse primers (**Supplemental Figure S1**).

1.1.12. Thaw one 50 µL aliquot of R2DP cells (see the **Table of Materials**) on ice for 2–5 min. Prewarm SOC growth medium to room temperature and LB Amp Cam^R plates to 37 °C (see the **Table of Materials**).

1.1.13. Add 1 µL of sequence-confirmed pET-Hisx6-pro-MMP-3cd to the 20 µL aliquot. Stir gently to mix and return the tube to ice.

1.1.14. Incubate the tube on ice for 5 min.

1.1.15. Heat-shock the cells by incubating at 42 °C for exactly 30 s. Do not shake.

1.1.16. Place the cells on ice for 2 min.

1.1.17. Add 950 µL of room-temperature SOC medium to the transformant mixture. Shake for 1 h at 250 rpm and 37 °C.

1.1.18. Plate the transformants on LB Amp Cam^R plates and incubate overnight at 37 °C.

1.2. Growth and induction

1.2.1. Inoculate a single, isolated colony of pET-Hisx6-pro-MMP-3cd transformant from an LB Amp Cam^R plate in 5 mL of LB Amp Cam^R media at 37 °C. Shake at 250 rpm overnight (~16 h).

Save aliquots from each culture and prepare 40% (v/v) glycerol (see the **Table of Materials**) stocks if desired.

1.2.2. Per overnight culture, inoculate a 1 L flask containing 500 mL of LB Amp Cam^R medium to an optical density at 600 nm (OD_{600}) of 0.05–0.1.

NOTE: This should return the cells to logarithmic growth.

1.2.3. Measure the OD_{600} at several time points, typically for 3–4 h, until it falls between 0.4 and 0.6.

1.2.4 Before induction, aliquot a fraction of culture into a 1.5 mL microfuge tube (see the **Table of Materials**) and label it **Un-induced Fraction**. Store it at -80 °C. If not running an SDS-PAGE gel, skip this step and proceed to step 1.2.5.

1.2.5. Induce the cultures to a final concentration of 1 mM using 1 M isopropyl-ß-D-thiogalactopyranoside (IPTG) stock (see the **Table of Materials**). Continue to incubate in the 37 °C shaker for an additional 3–4 h.

NOTE: During expression, the reader should determine the optimal OD_{600} at the time of induction and the IPTG concentration. If the yield drops substantially after purification, the imidazole concentration in purification buffers may require adjustment, or the cell pellet may need to be sonicated further.

1.2.6. Before centrifuging the cultures, aliquot a fraction of culture into a second 1.5 mL microfuge tube and label it **Induced Fraction**. Store it at -80 °C. If not running an SDS-PAGE gel, skip this step and proceed to step 1.2.7.

1.2.7. Centrifuge the cells in 250 mL conical bottles (see the **Table of Materials**) at 13,000 × *g* and 4 °C for 10 min.

1.2.8. Repeat step 1.2.7 until the cultures are completely pelleted.

NOTE: PAUSE: Cell pellets can be frozen at -80 °C and thawed later for further processing. Otherwise, skip this step and proceed to step 1.3.1.

1.3. Inclusion body extraction and solubilization

NOTE: Prepare 10 M urea freshly and no earlier than one day in advance, stirring thoroughly until dissolved completely. Do not heat or autoclave urea; store it at room temperature.

1.3.1. Resuspend the pellet (from step 1.2.8) in lysis buffer (see the **Table of Materials**). Per gram of pellet, add 3 mL of lysis buffer and resuspend by vortexing or pipetting. Shake overnight at 4 °C.

1.3.2. Add 1.25 mL of 10% (w/v) sodium deoxycholate (see the **Table of Materials**) per 1 L of culture. Shake at room temperature for 30 min at 150 rpm.

1.3.3. Add 10 µL of DNase I (see the **Table of Materials**) per 1 L of culture. Shake at room temperature for 30 min at 150 rpm.

1.3.4. Centrifuge for 10 min at 13,000 × *g* and 4 °C.

1.3.5. Set aside a fraction of **Lysed MMP** for gel analysis. Store it at -80 °C. If not performing gel analysis, proceed to step 1.3.6.

NOTE: After centrifugation, the pellet may be stringy and not compactly packed, making it risky to discard the supernatant. If this is the case, then skip step 1.3.6 and proceed to step 1.3.7.

1.3.6. Discard the supernatant from the centrifuged samples.

NOTE: The protocol can be paused at this point and the cell pellets frozen at -80 °C and thawed later. Otherwise, skip this step and proceed to step 1.3.7.

1.3.7. Resuspend the pellet in 100 mL/L culture of Inclusion Body Buffer (see the **Table of Materials**) by pipetting up and down.

1.3.8. During sonication, keep the samples on ice to prevent overheating. Sonicate each sample for 6 cycles of 15 s, output 5, and 50% pulse. Allow 15 s rest periods for cooling between cycles.

1.3.9. Transfer the samples into 50 mL conical tubes (see the **Table of Materials**). Centrifuge for 10 min at 13,000 × *g* and 4 °C.

1.3.10. Set aside a fraction of **Sonicated MMP** for gel analysis. Store it at -80 °C. If not performing gel analysis, proceed to step 1.3.11.

1.3.11. Check the pellet. If stringy, repeat steps 1.3.8–1.3.10. If the pellet is compact, discard the supernatant and proceed to step 1.3.12.

NOTE: Sonication in Inclusion Body Buffer can be repeated to recover more protein from the lysed cell debris. However, too much sonication can cause shearing, which harms MMP yield. The protocol can be paused at this stage, and the cell pellets can be frozen at -80 °C and thawed later.

1.3.12. Resuspend each pellet from a 1 L culture in 5 mL of Solubilization Buffer (see the **Table of Materials**) by pipetting. Incubate for at least 30 min on ice to allow the proteins to solubilize.

1.3.13. Set aside a fraction of **Solubilized MMP** for gel analysis. Store it at -80 °C. If not performing gel analysis, proceed to step 1.3.14.

1.3.14. Centrifuge the cells for 10 min at 13,000 × *g* and 4 °C. DO NOT DISCARD THE SUPERNATANT.

1.3.15. If a pellet forms after centrifugation, pour the supernatant into a separate 50 mL conical tube. Resuspend the pellet in another 5 mL of Solubilization Buffer (per 1 L of culture) by pipetting up and down.

1.3.16. Centrifuge for 10 min at 13,000 × *g* and 4 °C. DO NOT DISCARD THE SUPERNATANT.

1.3.17. Repeat steps 1.3.14 and 1.3.15 until a pellet no longer forms after centrifugation or only gray precipitate remains. Pool the supernatants. Discard or store the pellet at -80 °C for additional sonication.

2. MMP purification and refolding

2.1. His-tag affinity purification

2.1.1. Per the manufacturer's protocol, fill a gravity-flow column (see the **Table of Materials**) with well-mixed Ni-NTA resin (see the **Table of Materials**). Allow the resin to settle and separate from the storage buffer such that a distinct line forms between the two layers.

NOTE: Never allow the resin to dry, as air will penetrate the resin and harm protein yield. In between uses, perform the resin regeneration procedure described in section 2.2.

2.1.2. Allow the storage buffer to drain. Fill the column with two resin-bed volumes of HT Equilibration Buffer.

2.1.3. Drain the HT Equilibration Buffer and discard. As the column is draining, centrifuge the protein extract at maximum speed for 1 min and filter-sterilize using a 0.22 µm filter (see the **Table of Materials**).

2.1.4. Swap the waste container for a 50 mL conical tube labeled **HT Flowthrough**. Add the prepared protein extract to the column.

2.1.5. Reapply the flowthrough to maximize binding.

2.1.6. Set aside a fraction of **Flowthrough Fraction** for gel analysis. Store it at -80 °C. If not performing gel analysis, proceed to step 2.1.7.

2.1.7. Immediately wash the resin with 15 mL of HT Wash Buffer (see the **Table of Materials**). Collect the flowthrough in 15 mL conical tubes (see the **Table of Materials**) labeled **HT Wash**.

NOTE: Absorbance values at 280 nm (A280) were obtained via spectrophotometry and used along with molecular weight and extinction coefficient, ε, to estimate protein concentrations. For denatured Hisx6-pro-MMP-3cd, the molecular weight is 29.86 kDa, and ε is 34.38 M^{-1} cm^{-1}.

2.1.8. Blanking against HT Wash Buffer, measure and record the A280. Repeat steps 2.1.7 and 2.1.8 with additional wash fractions. Once the A280 approaches baseline and contamination has been minimized, proceed to step 2.1.9.

2.1.9. Set aside a fraction of **Wash Fraction** for gel analysis. Repeat for multiple wash fractions. Store the fractions at -80 °C. If not performing gel analysis, proceed to step 2.1.10.

2.1.10. Immediately elute His-tagged proteins by adding 5 mL of HT Elution Buffer (see the **Table of Materials**). Collect the flowthrough as 0.5–1 mL fractions in microfuge tubes labeled **HT Elution**.

2.1.11. Set aside a fraction of **Elution Fraction** for gel analysis. Repeat for multiple elution fractions. Store the fractions at -80 °C. If not performing gel analysis, proceed to step 2.1.12.

2.1.12. If the A280 is >0.3 mg/mL, dilute the fraction with HT Equilibration Buffer (see the **Table of Materials**).

NOTE: The eluted fraction must be diluted to an A280 of 0.3 mg/mL or less to prevent precipitation during dialysis. The protocol can be paused here and the pooled fractions frozen at -80 °C and thawed later. Otherwise, skip this step and proceed to step 2.2.1.

2.2. Resin regeneration

2.2.1. Wash the resin with ten resin-bed volumes of HT Regeneration Buffer (see the **Table of Materials**) and ten resin-bed volumes of sterile water.

2.2.2. Store the resin as a 50% slurry in 20% (v/v) ethanol.

3. Protein refolding

NOTE: For smaller volumes, dialysis cassettes can be used at a lower risk of sample loss. Dialysis tubing is required if larger volumes are used (see the **Table of Materials**).

3.1. Dialysis

NOTE: The optimum protein concentration for dialysis is ~0.3 mg/mL. If significant precipitation occurs during dialysis, reduce the urea concentration gradient between each dialysis using a stepwise dialysis method and add more intermediate steps (e.g., from 6 M to 5 M, and then 5 M to 4 M, rather than skipping the 5 M stage). As freeze-thaw cycles damage the cellular and protein structure, it is vital to minimize pauses in the protocol.

3.1.1. Per the manufacturer's protocol, use the appropriate amount of dialysis tubing according to the volume of **Elution Fraction** samples.

3.1.2. Submerge the eluted MMP fractions in dialysis tubing in 1 L of Dialysis Buffer 1 (see the **Table of Materials**). Stir the tubing and its contents on a magnetic stirrer for no less than 8 h at 4 °C.

3.1.3. Transfer to 1 L of Dialysis Buffer 2 (see the **Table of Materials**). Stir the tubing and its contents on a magnetic stirrer for no less than 8 h at 4 °C.

3.1.4. Transfer to 1 L of Dialysis Buffer 3 (see the **Table of Materials**). Stir the tubing and its contents on a magnetic stirrer for no less than 8 h at 4 °C.

3.1.5. Transfer the sample into new 1.5 mL microfuge tubes and label them as **Dialyzed MMP**.

3.1.6. Examine the tube for any precipitate. If precipitate has formed, centrifuge the sample for 1 min at 13,000 × *g* and 4 °C.

3.1.7. Transfer the supernatant into new 15 mL conical tubes and label them as **Refolded MMP**.

3.1.8. Set aside a fraction for gel analysis and label it as **Refolded MMP**. Store it at -80 °C. If not performing gel analysis, proceed to step 3.1.9.

NOTE: If yields are low, the precipitate can be dissolved in HT Equilibration Buffer and steps in section 3.1 repeated with dialysis tubing. If gel analysis is not to be performed or if the protocol must be paused here, freeze the samples at -80 °C and thaw them later. If yields are in the desired range, proceed to step 3.2.1.

3.2. Reconcentration

NOTE: The extinction coefficients for refolded and denatured Hisx6-pro-MMP-3cd are expected to be the same; hence, A280 calculations are not affected.

3.2.1. Reconcentrate the sample up to 0.5 mg/mL. Use a 400 mL stirred cell (see the **Table of Materials**) to concentrate the sample to 15 mL and a 50 mL reconcentration tube to concentrate further if needed.

NOTE: If a precipitate forms, it can be pelleted and dissolved in HT Equilibration Buffer. Then, repeat sections 3.1 and step 3.2.1. Otherwise, continue to step 3.2.2.

3.2.2. Set aside a fraction for gel analysis and label it as **Concentrated MMP**.

NOTE: The protocol can be paused here and the samples frozen at -80 °C and thawed later.

4. Activation

4.1. 4-Aminophenylmercuric acetate (APMA) activation

NOTE: APMA is highly toxic. Make a fresh stock solution of 20 mM APMA before activation, and always work under a fume hood when using APMA. Discard the APMA waste into its container.

4.1.1. Per 1 mL aliquot of MMP (1 mg/mL), add 50 µL of 20 mM APMA (see the **Table of Materials**) to reach a final APMA concentration of 1 mM. Incubate overnight at 37 °C.

4.1.2. If a precipitate forms, centrifuge it at maximum speed for 10 min at 4 °C. Store the supernatant in a 1.5 mL microfuge tube labeled **Activated MMP**. Discard the precipitate into a container marked for APMA waste.

4.1.3. Set aside a fraction for gel analysis and label it **Activated MMP**.

NOTE: The protocol can be paused here and the samples frozen at -80 °C and thawed later. If not performing gel analysis, proceed to step 4.2.1. After activation, the molecular weight and extinction coefficient of MMP-3cd are 19.40 kDa and 28.42 M^{-1} cm^{-1}, respectively.

4.2. Desalting

4.2.1. Remove APMA from the activated MMP-3cd sample with a 2 mL desalting column (see the **Table of Materials**), following the manufacturer's protocol.

4.2.2. Set aside a fraction for gel analysis and label it **Desalted MMP**. Store it at -80 °C. If not performing gel analysis, proceed to section 4.3 with the remaining samples.

NOTE: The protocol can be paused here and the samples frozen at -80 °C and thawed later.

4.3. Running the SDS-PAGE gels

4.3.1. Run all protein fractions on SDS-PAGE gels: Un-induced Fraction, Induced Fraction, Lysed MMP, Sonicated MMP, Solubilized MMP, Flowthrough Fraction, Wash Fraction, Elution Fraction, Refolded MMP, Concentrated MMP, Activated MMP, and Desalted MMP.

4.4. Long-term storage of MMP-3

4.4.1. Add 0.05% (v/v) nonionic surfactant (see the **Table of Materials)** to the desalted MMP-3cd samples and store them at -80 °C.

REPRESENTATIVE RESULTS:

When running samples on SDS-PAGE, because the protein is expressed in the form of insoluble inclusion bodies, the lysed and sonicated fractions should contain little to no Hisx6-pro-MMP-3cd extract, as the protein has not yet been resolubilized in urea. **Figure 3** compares the His-tag purification elution fractions of Hisx6-pro-MMP-3cd from BL21(DE3) cells and R2DP cells. Elution fractions were pooled separately for both BL21(DE3) and R2DP cells before dialysis. Fractions from each step were run after the proteins were desalted (**Figure 4**). All Hisx6-pro-MMP-3cd samples display a band at 29.86 kDa, and active MMP-3cd displays a band at 19.40 kDa upon removal of the His-tag and pro-peptide (**Figure 4** and **Supplemental Figure S1**).

The total yield of protein in mg/L of *E. coli* culture was determined after purification and desalting of BL21(DE3) and R2DP cultures (**Table 1**). Using R2DP cells yields substantially higher levels of MMP expression. Whereas regular BL21(DE3) cells yielded only 3.5 mg of purified Hisx6-pro-MMP-3cd per liter of culture, R2DP cells produced 45 mg /L culture. Similarly, yields of functional, desalted MMP-3cd increased from 0.13 mg/L culture to 6.2 mg/L culture for BL21(DE3) and R2DP cells, respectively. Human pro-MMP-3cd overwhelms the cellular machinery of the standard BL21(DE3) strain because of its size (approximately 30 kDa) and the elaborate posttranslational modifications required that are exclusive to eukaryotes. The R2DP strains are BL21(DE3) derivatives, designed to enhance the expression of eukaryotic proteins. The R2DP strain carries tRNAs for AGA, AGG, AUA, CUA, GGA, CCC, and CGG, which are rarely used in *E.coli* but abundant in the pro-MMP-3cd DNA sequence. This is potentially a key factor in the increased levels of protein expression observed in R2DP cells.

FIGURE AND TABLE LEGENDS:

Figure 1: Plasmid map of the protein expression vector pET-Hisx6-pro-MMP-3cd. An N-terminal Hisx6-tag sequence is cloned into the pET-3a-based vector, including pro-MMP-3cd, to yield the pET-Hisx6-pro-MMP-3cd construct. The pET vector also includes an ampicillin resistance gene to ensure plasmid stability.

Figure 2: Bacterial expression of pro-MMP-3cd, purification, refolding, and activation. 1.1: pET-Hisx6-pro-MMP-3cd plasmid was transformed into BL21(DE3) or R2DP Cells. 1.2: Pro-MMP-3cd protein expression was induced using IPTG. 1.3: Chemical lysis and sonication are used to extract Hisx6-pro-MMP-3cd proteins that are mainly insoluble and found in the inclusion bodies. Urea was used to denature and solubilize protein from inclusion bodies. 2.1. Denatured Hisx6-pro-MMP-3cd protein was purified via affinity chromatography purification. 3. The eluted Hisx6-pro-MMP-3cd was slowly refolded during dialysis through gradual removal of urea from the buffer. 4. Finally, refolded MMP-3cd protein was activated using APMA by removing the N-terminal pro-peptide domain. APMA is later removed from the solution through desalting. The numbers correspond to protocol sections describing these steps. Abbreviations: MMP-3 = Matrix metalloproteinase-3; APMA = 4-aminophenylmercuric acetate.

Figure 3: SDS-PAGE gel of purified Hisx6-pro-MMP-3cd from BL21(DE3) and R2DP cells. (A)The first eight elution fractions of Hisx6-pro-MMP-3cd in BL21(DE3) cells. (**B**) The first eight elution fractions of Hisx6-pro-MMP-3cd in R2DP cells. Following extraction and solubilization of MMP inclusion bodies in urea, Hisx6-pro-MMP-3cd samples were purified through Ni-NTA

chromatography column using batch-gravity flow technique. Gels are truncated to show only the elution fractions. Initially, due to high concentrations of protein, fractions 1–5 are 1 mL. Later fractions (6–8) are between 5 and 8 mL each. The Hisx6-pro-MMP-3cd band is observed at ~30 kDa. Abbreviations: MMP-3 = Matrix metalloproteinase-3; SDS-PAGE = sodium dodecylsulfate polyacrylamide gel electrophoresis; Ni-NTA = nickel–nitrilotriacetic acid; FT = flowthrough.

Figure 4: Proteolytic cleavage of His-tag and pro-domain upon activation of MMP-3cd. The induced, refolded, concentrated, activated, and desalted fractions of MMP-3cd in R2DP cells are shown. After dialysis, Hisx6-pro-MMP-3cd is concentrated and activated using APMA. Upon activation, the molecular weight of the activated MMP-3cd band is approximately 20 kDa, as opposed to the His-tagged zymogen, which remains at ~30 kDa. Impurities are removed in the activation and desalting stages. Abbreviations: MMP-3 = Matrix metalloproteinase-3; APMA = 4-aminophenylmercuric acetate.

Table 1: Table of volumes and concentrations across stages of MMP-3cd purification. Hisx6-pro-MMP-3cd was expressed either in 2 L of a culture of BL21(DE3) or in 2 L of R2DP cells. Volume, concentration, and yield (mg per liter) culture are reported for BL21(DE3) and R2DP cells. Yield (mg per liter of culture) was obtained by dividing the total yield of protein (mg) obtained by volume of culture, which was 2 L for both the BL21(DE3) and R2DP cases. Protein amounts yields are reported for two stages: Hisx6-pro-MMP-3cd following Hisx6-tag purification and active MMP-3cd after desalting.

Supplemental Figure S1: Sequences of T7 primers and MMP-3cd protein before and after activation.

DISCUSSION:

This protocol describes a detailed method to express and purify Hisx6-pro-MMP-3cd in R2DP *E. coli* cells, along with the activation and refolding of MMP-3cd. As the protocol takes several days to complete, careful planning is critical to minimize the loss of functional MMPs due to multiple freeze-thaw cycles. As R2DP cells are the key improvement in this method, it is paramount to optimize expression yield, as large quantities can be lost through refolding and activation. During expression, the operator should determine the optimal OD_{600} and IPTG concentration prior to induction. After His-tag purification, if yield drops substantially, then MMPs are possibly not binding to the resin, or the cell pellet may need to be sonicated further.

If significant precipitation occurs during dialysis, reduce the changes in urea concentration between stages in stepwise dialysis by adding more stages (e.g., from 6 M to 5M, and then 5 M to 4 M, rather than skipping the 5 M stage). Once refolded, and particularly after activation, MMPs are substantially more prone to precipitation or degradation through autoproteolysis[17]. After pH and salt concentrations of all buffers have been optimized and desired tests have been performed, all steps following dialysis should be completed with urgency.

The large-scale production of soluble, human, recombinant MMPs remains a challenging task. Mammalian cells can express functional MMPs at high costs and long wait times, whereas *E. coli*

rapidly produce high quantities of MMP inclusion bodies that must be purified and refolded[11,16]. R2DP cells significantly increase the yield of MMP inclusion bodies, enabling a more cost-effective and productive MMP refolding process. However, *E. coli* lack the posttranslational machinery needed to fold MMPs, and though engineered strains show greatly improved expression levels, only some intermediates are properly folded upon denaturant removal[4]. Consequently, occasional precipitation of MMPs is still expected during refolding and activation. These results show that a significant portion of purified Hisx6-pro-MMP-3cd yield is lost after refolding, activation, and desalting.

These stages can be further optimized by adding more dialysis stages along with testing concentrations of MMP-3cd and APMA. However, per liter of culture, the yield of functional MMP-3cd is 49-fold higher in R2DP cells than BL21(DE3) cells. Additionally, the proportion of functional, desalted MMP-3cd recovered from purified Hisx6-pro-MMP-3cd rose from 3.7% for BL21(DE3) cells to 14% for R2DP cells. Therefore, R2DP cells offer a viable alternative to current MMP production options, such as expression in mammalian cells, offering more competitive yields per liter of culture.

Growing attention toward MMPs as potential targets for therapeutics has been met with rapid innovations in protein engineering for improving binding, inhibition, and selectivity of MMP therapeutics[22]. Consequently, once-ambitious prospects in the field of MMP therapeutics are steadily becoming more attainable[6]. The need for fast, reliable methods to recover soluble, active MMPs undoubtedly will become more imperative with time.

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DISCLOSURES:
The authors declare that they have no competing financial interests.

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Figure

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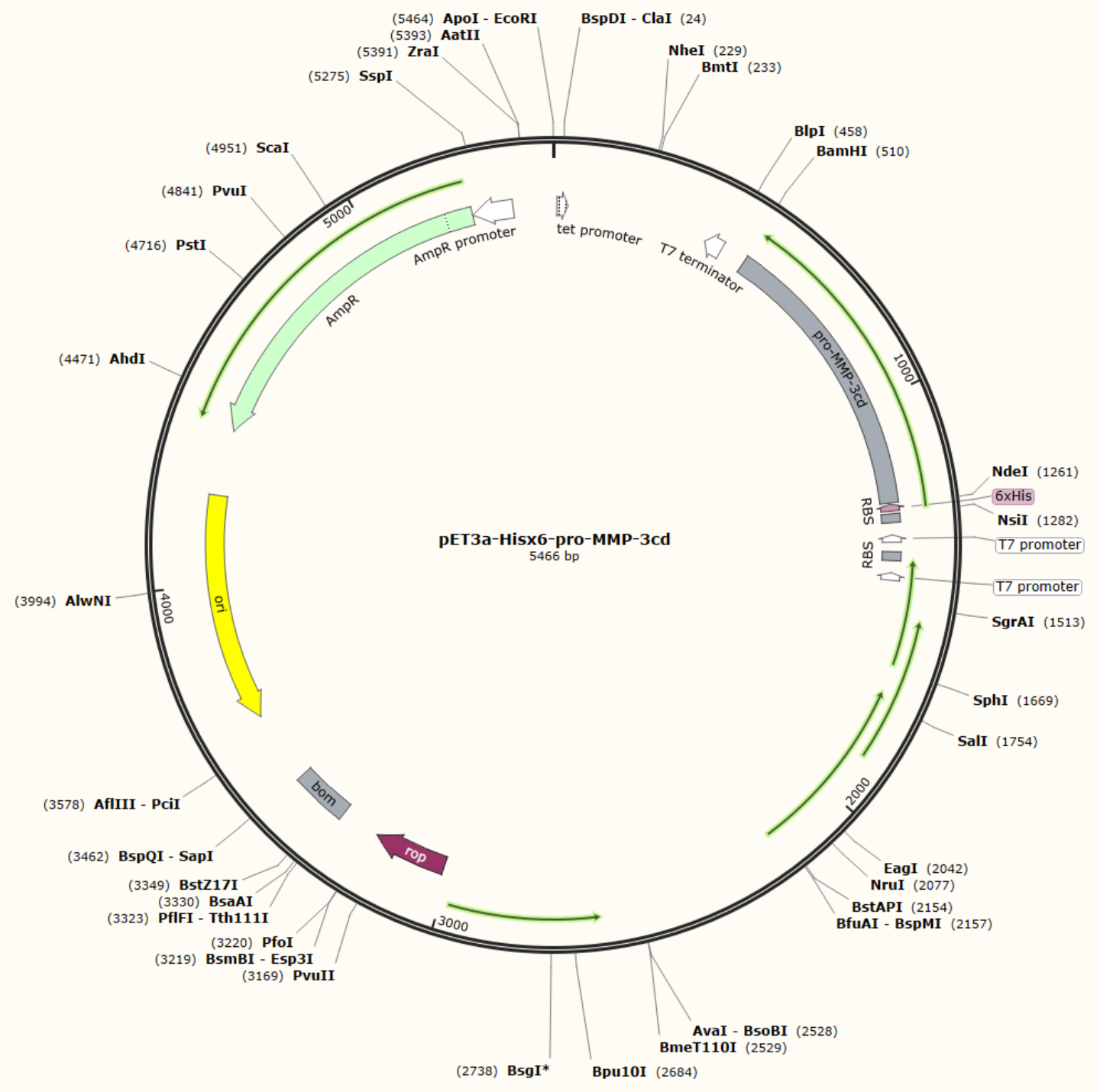
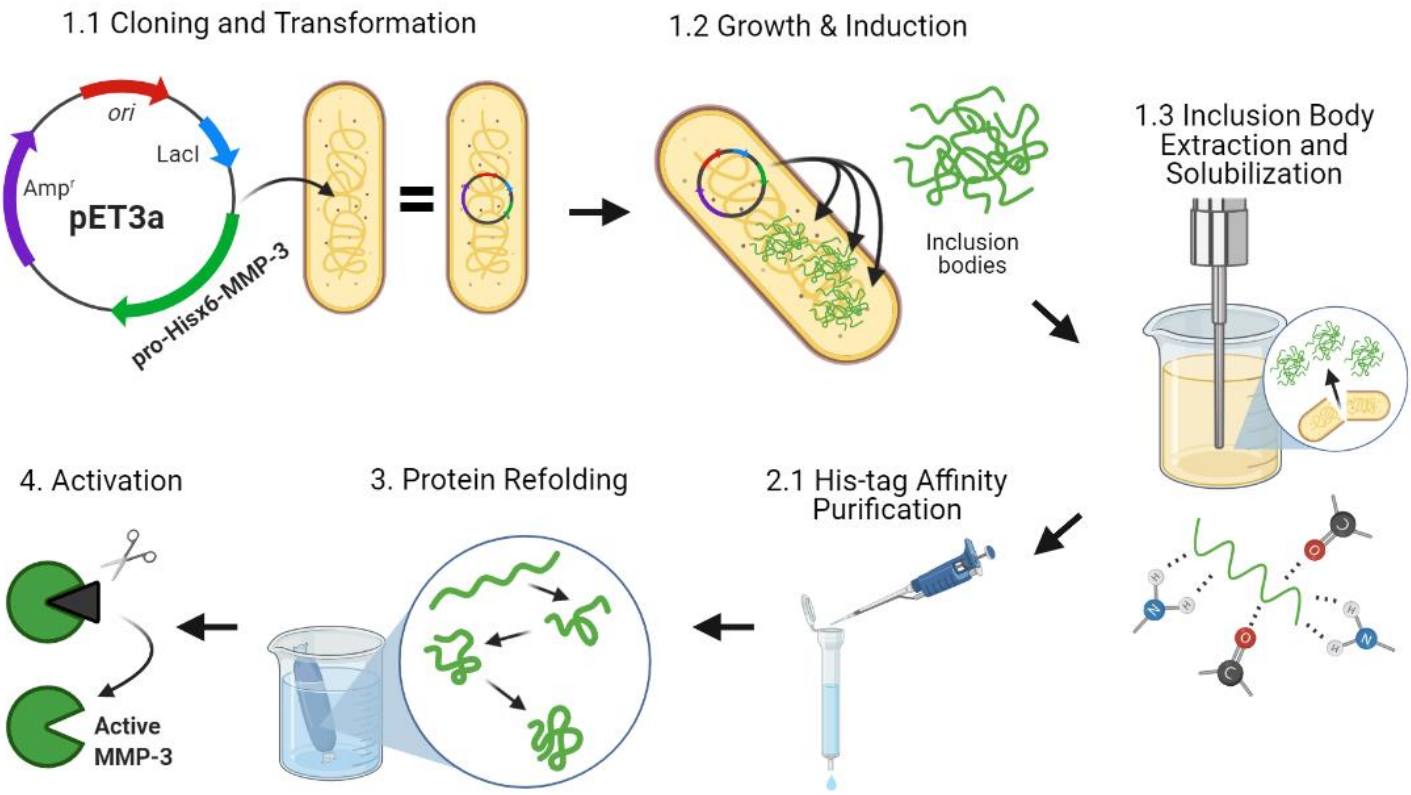
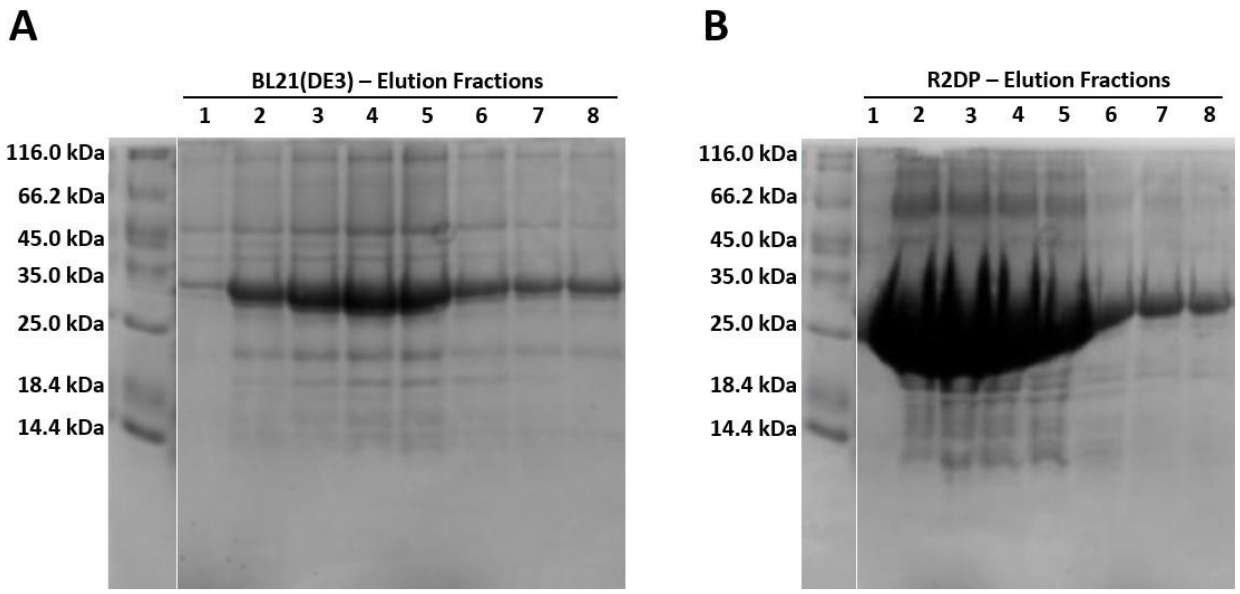


Figure





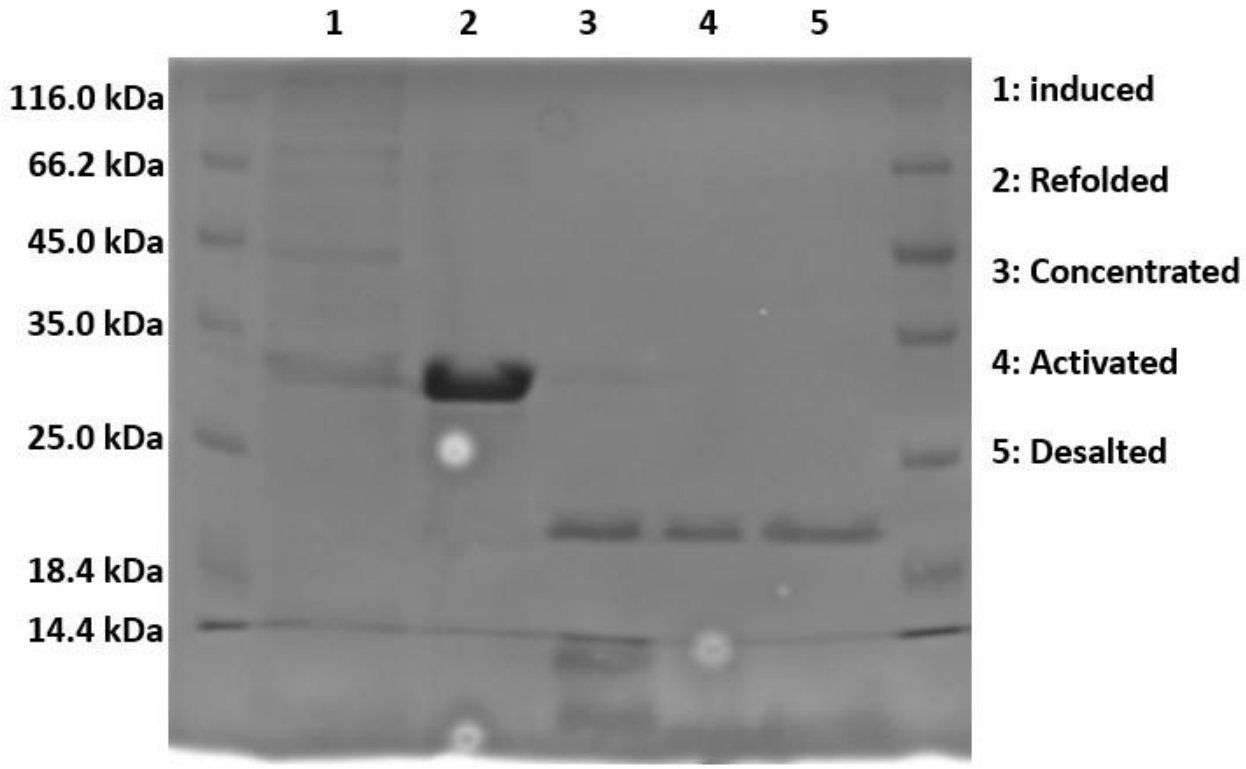


Table 1

Stages	BL21(DE3) cells			R2DP cells		
	Volume (mL)	Concentration (mg/mL)	Yield (mg/L culture)	Volume (mL)	Concentration (mg/mL)	Yield (mg/L culture)
Purification	23	0.30	3.5	42	2.1	45
Desalting	1.5	0.17	0.13	72	0.17	6.2

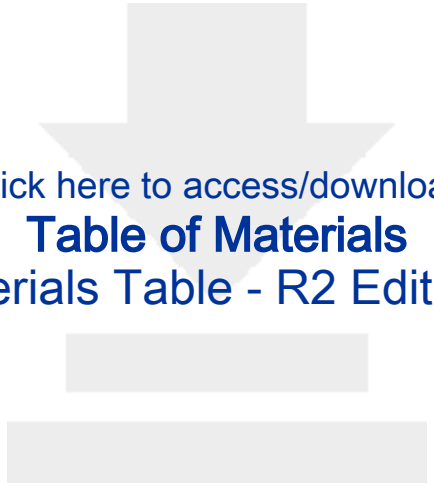


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Table of Materials

Materials Table - R2 Edit.xlsx

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
Authors have addressed most of the concerns. However, table 1 does not make sense. Also Figure legends are poorly described.
Response: Figure legends have been updated.
Major Concerns: The table shows a comparison of BL21 and R2CD. However, this table does not make sense. It would be best if you described what the starting culture volume for each in the legend is.
Response: As requested, this has been added to the legend.
Also, the final yield is low for both after desalting. We obtained at least 5-10 mg of the folded proMMP-3 catalytic domain in BL21(DE3) using the pET3a vector.
Response: More precipitation is common after activation of proMMP-3. Additionally, we expect that precipitation during dialysis can be fixed with more optimization, for example by increasing the number of stages in dialysis.
Why is the desalting volume of BL21 so small?
Response: After dialysis, it is necessary to re-concentrate the MMP-3cd to 0.3-0.5 mg/mL, which reduces the volume. Precipitation from dialysis and activation also reduces the volume.
Also, if the solution is 0.17 mg/ml and the volume is 1.5 ml, the total protein should be 0.255 mg. Why do you state that it is 0.13mg/L culture? Does this mean the culture was 2L?
Response: Correct. There are 2 L of culture, so the total yield in mg was divided by 2. This gives the new unit of “mg/L culture” shown in the table.
Why is the volume for R2DP bigger than the purification volume? Did you dilute them?
Response: Before dialysis, pro-MMP-3cd must be diluted to 0.3 mg/mL to prevent precipitation.
Also, if the concentration is 0.17 and the volume is 72 ml, the yield is 12.24mg. Is this mean that the starting culture was 4L?
Response: The R2DP culture was also 2 L. This has been updated.
Are these the same purification batch?
Response: We had a 2 L culture of BL21(DE3) cells, and a 2 L culture of R2DP cells. These were grown separately. All ensuing purification steps were also performed on each 2 L culture separately.
Please describe the condition in the legend. Otherwise, showing this table does not help. Also, discuss why there is a significant amount of protein loss after desalting. 22mg/l to 3.1 is a loss of over 85%. Even considering propeptide removal, this is a considerable loss.
Response: This has been mentioned in the discussion section as something that can be further optimized by troubleshooting the dialysis and activation stages.

Supplementary Figure S1.

Hisx6-pro-MMP-3cd protein sequence (29.86 kDa)

MHHHHHHMYP LDGAARGEDT SMNLVQKYLE NYYDLKKDVK QFVRRKDSGP
VVKKIREMQK FLGLEVTGKL DSDTLEVMRK PRCGVPDVGH FRTFPGIPKW
RKTHLTYRIV NYTPDLPKDA VDSAVEKALK VWEEVTPLTF SRLYEGEADI
MISFAVREHG DFYPFDGPGN VLAHAYAPGP GINGDAHFDD DEQWTKDTTG
TNLFLVAAHE IGHSLGLFHS ANTEALMYPL YHSLTDLTRF RLSQDDINGI
QSLYGPPPDS PET

Active MMP-3cd protein sequence (19.40 kDa)

FRTFPGIPKW RKTHLTYRIV NYTPDLPKDA VDSAVEKALK VWEEVTPLTF
SRLYEGEADI MISFAVREHG DFYPFDGPGN VLAHAYAPGP GINGDAHFDD
DEQWTKDTTG TNLFLVAAHE IGHSLGLFHS ANTEALMYPL YHSLTDLTRF
RLSQDDINGI QSLYGPPPDS PET

pET3a-Hisx6-MMP-3-FOR primer for cloning

TACTTTAGAGGAGATATAATGCATCACCATCACCATCATATGTATCCATTGGATG

pET3a-Hisx6-MMP-3-REV primer for cloning

CGGGCTTTGTTAGCAGCCGGATCCTCATCCATAGAGGGAC