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Thioredoxin fusion construct enables high-yield production of soluble, active matrix metalloproteinase-8 (MMP-8) in *Escherichia coli*

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ABSTRACT

Matrix metalloproteinases (MMPs) are crucial proteases in maintaining the health and integrity of many tissues, however their dysregulation often facilitates disease progression. In disease states these remodeling and repair functions support, for example, metastasis of cancer by both loosening the matrix around tumors to enable cellular invasion and by affecting proliferation and apoptosis, and they promote degradation of biological restorations by weakening the substrate to which the restoration is attached. As such, MMPs are important therapeutic targets. MMP-8 participates in cancer, arthritis, asthma and failure of dental fillings. MMP-8 differs from other MMPs in that it has an insertion that enlarges its active site. To elucidate the unique features of MMP-8 and develop selective inhibitors to this therapeutic target, a stable and active form of the enzyme is needed. MMP-8 has been difficult to express at high yield in a soluble, active form. Typically recombinant MMPs accumulate in inclusion bodies and complex methods are applied to refold and purify protein in acceptable yield. Presented here is a streamlined approach to produce in *Escherichia coli* a soluble, active, stable MMP-8 fusion protein in high yield. This fusion shows much greater retention of activity when stored refrigerated without glycerol. A variant of this construct that contains the metal binding *cla*MP Tag was also examined to demonstrate the ability to use this tag with a metalloprotein. SDS-PAGE, densitometry, mass spectrometry, circular dichroism spectroscopy and an activity assay were used to analyze the chemical integrity and function of the enzyme.

1. Introduction

Matrix metalloproteinases are a class of proteins responsible for degrading extracellular matrix (ECM) proteins to support tissue remodeling and repair [1,2]. MMPs are of interest because of their complex biological roles and because of their participation in a variety of disease states, including cancer, metastasis and tumorigenesis, which is caused by the inappropriate up- or down-regulation of MMPs and/or their activity [3]. Elevated activity leads to loosening of the supporting matrix, which facilitates dissemination of cancer cells to enable metastasis [4]. Many MMP inhibitors have been created, but most have failed in clinical trials because of the complex set of contributions MMP activity exerts on disease progression, and as such, these enzymes remain the subject to much study [5,6].

MMPs utilize a zinc-bound metal active site and are secreted as glycosylated zymogens, with a prodomain bound to the metal active site [2]. Catalysis is activated by proteolytic cleavage of the prodomain, which exposes the active site. These enzymes are often difficult to produce recombinantly in a soluble form because of their complex composition, and generating them typically requires numerous steps, which include expression as proenzymes, refolding and use of several diverse purification approaches [7–9]. MMPs also are difficult to produce because of their proteolytic function, which permits autoproteolysis to occur [10]. Significant efforts have resulted in the production of a few MMPs in the amounts necessary to study their structure in atomic detail [11–14]. Crystal structures of MMP-8 have been solved (PDB: 3DNG, 3DPE, 1ZVX) [15,16] utilizing the complex protein production methods, and moreover, require the addition of an inhibitor to prevent autoproteolysis when concentrated [17]. The ability to obtain high-resolution information about the protein enables design of selective inhibitors and provides the ability to characterize other ligand and protein-

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protein interactions that regulate enzyme activity [18–20].

MMP-8 plays a role in cancer, arthritis, and asthma and promotes premature degradation of dental fillings by degrading the underlying collagen matrix within the dentin of demineralized tooth [21,22]. While the fold of MMP-8 is related to other MMPs, it differs from them in the active site because it contains an insertion that creates a significantly larger binding pocket [23]. The ability to study this enzyme and its unique active site in atomic detail in solution, without using an inhibitor would be extremely valuable for simplifying production and understanding how to design more potent and selective inhibitors of this particular MMP [24,25].

Here, we present a method to express an active, stable form of matrix metalloproteinase-8 (MMP-8) in *Escherichia coli* in sufficient amounts to enable structural evaluation. Several fusion constructs were generated that did not result in high yield of soluble, active, stable protein, but one construct met these criteria. This MMP-8 fusion includes two tags (thioredoxin and S Tag) to aid in folding and stability and a polyhistidine tag for affinity purification. Using this system, over 100 mg of MMP-8 can be expressed as a fusion protein in the soluble fraction of the cell lysate and purified quickly using immobilized metal affinity chromatography (IMAC) to recover appreciable amounts of catalytically active enzyme that retains full activity when stored refrigerated in simple buffer. Recognition sites for removal of the thioredoxin and thioredoxin-S Tag fusion partners were engineered into the construct. In addition, a spacer sequence also containing the metal-binding *cla*MP Tag was inserted between the fusion partner and the enzyme. An additional aim of this study was to demonstrate compatibility of use of the metal abstraction peptide (MAP) technology inline with a metalloprotein [26]. Genetic engineering of MAP into a plasmid creates, the *cla*MP Tag, a linker-less carrier for many transition metals with utility in healthcare applications [27]. The *cla*MP Tag is a tripeptide consisting of the amino acid sequence Asn-Cys-Cys, which scavenges small transition metals from chelating agents [28]. Because of the uniquely beneficial properties of the *cla*MP Tag, which includes extraordinarily tight binding, resistance to metal release upon dilution at serum pH and specific release in acidic conditions as in endosomes, the *cla*MP Tag is being investigated as a method for targeted delivery of metals for therapeutic and diagnostic applications by designing targeting proteins to contain the metal bound tripeptide [27]. The *cla*MP Tag has not been investigated previously in a system, such as matrix metalloproteinases, in which a structural and/or catalytic metal-binding site is present. Analysis of the activity and stability of these two fusion proteins and their cleaved products is presented herein.

2. Materials and methods

2.1. Genetic engineering

Human matrix metalloproteinase-8 (MMP-8) in the pCMV6-XL4 vector was obtained (OriGene Technologies, Inc. Cat # SC 127843) and the catalytic domain was amplified with designed primers (Integrated DNA Technologies). Two constructs of MMP-8 were prepared, one containing solely MMP-8 and one bearing an additional N-terminal *cla*MP Tag (Asn-Cys-Cys) [27,29]. The primers contained a region matching the pET-32Xa/LIC vector (underlined), a portion matching the MMP-8 catalytic domain (italics), the *cla*MP Tag (bold) and an inserted linker (bold and underlined) between the *cla*MP Tag and MMP-8. 5′-GGT ATT GAG GGT CGC *AAT CCA GGA AAC CCC AAG TG*-3′ (forward primer no *cla*MP Tag), 5′-**TGC GGC TCT TCT GGC ATT GAG GGT CGC** *AAC CCC AAG TGG GAA*-3′ (first forward primer for *cla*MP-link insertion), 5′-GGT ATT GAG GGT CGC CCA GAT CTG GGT **AAC TGC TGC GGC TCT TCT GGC**-3′ (second forward primer for *cla*MP-link insertion), and 5′-AGA GGA GAG TTA GAG CCT TAT CCA TAG ATG GCC TG-3′ (reverse primer for both constructs). The MMP-8 PCR reaction was purified using QIAquick PCR Purification Kit (Qiagen) and was inserted into the pET-32Xa/LIC vector by ligation independent cloning (protocol provided by Novagen). *cla*MP-link-MMP-8 required two sequential PCR steps to incorporate the full-length linker. The PCR reaction was then purified using QIAquick PCR Purification Kit (Qiagen) and was inserted into the pET-32Xa/LIC vector by ligation independent cloning (protocol provided by Novagen). The inserted tag contained a *Bgl*II site so that the original Factor Xa recognition sequence could be removed by cleaving with this endonuclease. The new plasmid was cut with the restriction enzyme, *Bgl*II and was re-ligated with T4 DNA ligase.

Using the standard heat shock procedure, the reactions were transformed into DH5α *Escherichia coli* (*E. coli*) cell strain. Luria broth (LB) agar plates with 100 μg/mL ampicillin were used to select transformed cells. The plates were incubated overnight at 37 °C. Individual colonies were grown in 5 mL LB with 100 μg/mL ampicillin overnight at 37 °C and 250 rpm. The starter cultures were spun down at 1717× *g* for 15 min and the supernatant was discarded. A miniprep kit (Quiagen) was used to purify the plasmid DNA. DNA sequences were confirmed by UC Berkeley DNA Sequencing Facility.

Four additional constructs bearing a variety of tags that may improve folding and/or solubility were generated and tested for expression in *E. coli*. These constructs did not lead to accumulation of soluble protein and are described in Supplemental Information.

2.2. Expression of MMP-8

Plasmids were transformed into BL21 *E. coli* cell strain using standard heat shock techniques. The cells were plated on LB agar plates with 100 μg/mL ampicillin and incubated overnight at 37 °C. Cultures were started using a single colony to inoculate 50 mL of LB with 100 μg/mL ampicillin and grown for 16 h at 37 °C, 250 rpm in an orbital shaker. Twenty milliliters of starter culture was transferred to 1 L of LB with 100 μg/mL ampicillin in a 3 L fernbach flask. The cells were grown at 37 °C, 250 rpm until the OD_{600} reached approximately 0.7. One milliliter of 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) was used to induce the cells, which were harvested after four hours by centrifugation at 1391× *g* for 8 min. The cell pellets were stored at −80 °C until use.

2.3. Protein purification

All solutions were made to an ionic strength of 150 mM by adjusting the concentration of NaCl. A one-liter pellet was resuspended in 25 mL lysis buffer (50 mM Tris-Cl, 20 mM imidazole, 59 mM NaCl, 10 μM N-Isobutyl-N-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid (NNGH), pH 7.9) and three passes through a french press at 21,000 psi was used to lyse the cells. Lysates were centrifuged for 1 h at 21,000× *g* and 4 °C. The supernatant containing the protein was filtered through a 0.45 μm filter followed by a 0.2 μm filter and applied to a nickelated 5 mL Hi-Trap Chelating HP column (GE Lifesciences) equilibrated in lysis buffer. The column was washed with 50 mM Tris-Cl, 58 mM NaCl, 40 mM imidazole, 10 μM NNGH, pH 7.9 for 10 CV at a flow rate of 1.25 mL/min at 4 °C. The protein was eluted from the column using a linear gradient elution from 0 to 100% of 50 mM Tris, 37 mM NaCl, 500 mM imidazole, 10 μM NNGH, pH 7.9. The eluate was concentrated using Amicon Ultra 10 kDa MWCO (Millipore) concentrators to approximately 2 mL and injected on a HiLoad 26/600 Superdex 75 prep grade column (pack size 1 × 320 mL, GE Lifesciences #28-98930-34) equilibrated in 50 mM Tris-Cl, 60 mM NaCl, 10 μM NNGH, pH 7.9 to separate degradation fragments and eliminate the imidazole in the sample. The fractions were concentrated using an Amicon Ultra