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Functional production of catalytic domains of human MMPs in *Escherichia coli* periplasm

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Abstract

Because of their central roles in tumor growth and invasion, milligram level amounts of active MMPs are frequently required for cancer research and development of chemical or biological MMP inhibitors. Here we describe methods for functional production of catalytic domains of MMPs (cdMMPs) in *E. coli* periplasm without refolding or activation process. We demonstrate applications of this straightforward approach for cdMMP-9, cdMMP-14, and cdMMP-14 mutants.

Keywords

periplasmic expression

1 Introduction

MMPs (matrix metalloproteases) are multi-domain zinc-dependent endopeptidases that share a basic structural organization comprising propeptidic, catalytic, hinge, and hemopexin like domains [1] (Fig 1AB). Besides functioning in normal physiological processes, many MMPs correlate with cancer progression, including cancer cell invasion, proliferation and apoptosis, and tumor angiogenesis and vasculogenesis [2–4]. Taking these preclinical and clinical evidences together, some MMPs such as MMP-2/-9/-14 etc have been considered as important regulatory enzymes for cancer research and predominant therapeutic targets for cancer treatments [5–7]. Therefore, the consistent supply of active MMPs at mg scales is essential for cancer biology research and for the developments of novel diagnostic and therapeutic agents, *e.g.* identification of physiological substrates of MMPs, characterization of extracellular matrix remolding *in vitro* and *in vivo*, and screening for highly selective MMP inhibitors.

Recombinant overexpression of many human MMPs and their catalytic domains in *E. coli* exclusively results in the formation of inclusion bodies [8]. After solubilization and purification, the denatured MMPs can be refolded to their active form via multiple steps of gradient dialysis, which are often labor-extensive and time-consuming. Besides low yields (typically <35%) and uncontrollable lot-to-lot variation, during the refolding process, autoproteolysis occurs at both termini with various degrees thus generating heterogeneous

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mixtures [9]. Alternatively, MMP with its propeptidic domain, *e.g.* pro-MMP-14, has been periplasmically expressed in *E. coli* by fusing with a signal peptide [10]. However, after purification, pro-MMP-14 was treated with 4-aminophynylmercuric acetate (APMA) or trypsin to yield the functional enzyme [11]. As an organomercury compound, APMA is toxic, and complete activation of pro-MMP-14 using APMA is difficult [12]. In addition to cleavage of the propeptide, trypsin treatment also digests the C-terminus of MMP-14 at the putative furin cleavage site (RRKR/YAIQ) resulting in truncated products [11].

Taking advantages of a slow processing rate controlled by secretion machineries and multiple periplasmic molecular chaperons that enhance proper protein folding, we develop a method to directly express functional MMP catalytic domains in the periplasmic space of *E. coli* [13]. This straightforward method avoids the problematic activation processes and tedious refolding associated with inclusion bodies. Here we demonstrate this method for producing catalytic domains of both a secreted MMP (cdMMP-9) and a membrane-based MMP (cdMMP-14). We further expand this method to produce cdMMP-14 mutants. A facile approach to obtaining milligrams of active cdMMPs is beneficial for MMP related studies, particularly for the development of therapeutic drugs targeting important MMPs for cancer treatments.

2 Materials

2.1 Construction of cdMMP genes

1. Plasmid pMopac16 [14]
2. DNA fragments encoding cdMMPs (IDT)
3. Jude-I [DH10B F′[proAB $lacI^{Q}$ lacZ ΔM15 Tn10(Tet^{R})]] competent cells

2.2 Periplasmic expression and purification of cdMMPs

1. BL21 [*E. coli* B F^{-} *ompT gal dcm lon* $hsdS_{B}(r_{B}^{-}m_{B}^{-})$ [$malB^{+}$]$_{K\text{-}12}$(λ^{S})]
2. 2×YT/Chlor: 2×YT (BD Difco), 34 μg/ml chloramphenicol
3. LB/Chlor agar: LB (BD Difco), 1.5 g/L agar, 34 μg/ml chloramphenicol
4. Periplasmic buffer: 200 mM Tris-HCl, pH 7.5, 20% sucrose, 50 μg/ml lysozyme
5. 0.45 μm filter units, 90 mm, 500 ml
6. Ni-NTA agarose
7. Ni-NTA column equilibrium buffer: 50 mM Tris-HCl, pH 8.0, 300 mM NaCl
8. Ni-NTA column washing buffer: 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole
9. Ni-NTA column elution buffer: 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 200 mM imidazole
10. Ultrafiltration centrifugal units, 3 kDa MWCO
11. SnakeSkin dialysis tubing, 3.5 kDa MWCO, 16 mm

12. Dialysis buffer: 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.1 mM $ZnCl_2$

2.3 Characterizations of cdMMPs

2.3.1 Activity measurements by FRET

1. Peptide M-2350: Mca-Lys-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg-NH_2 (Bachem)
2. Peptide XV: QXL®520-γ-Abu-Pro-Glu-Gly-Leu-Dab(5-FAM)- Ala-Lys - NH_2 (AnaSpec Inc)
3. GM60001 (EMD Millipore)
4. nTIMP-2, prepared as details described in Ref #15.
5. Black flat-bottom polystyrene NBS 96-well microplate
6. 20% DMSO
7. Assay buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.1 mM $ZnCl_2$

2.3.2 Analysis by gel permeable chromatography

1. Ovalbumin (43 kDa)
2. Lysozyme (14 kDa)
3. Superdex 75 10/300 GL size-exclusion column (GE Healthcare)
4. Equilibrium buffer: 50 mM HEPES pH 7.5, 150 mM NaCl

3 Methods

3.1 Construction of cdMMP genes

1. Synthesize DNA fragments encoding catalytic domains of human MMP-14 (Ile114-Pro290) and human MMP-9 (fibronection domain removed, Ile107-Val216 fused with Glu391-Tyr443) (Fig 1CD).
2. Following standard molecular biology techniques, clone cdMMP genes to the periplasmic expression plasmid pMopac16 [14], which carries a *Lac* promoter, a pelB leader peptide, and a C-terminal His-tag (Fig 1CD).
3. Construct cdMMP-14 mutant genes by overlapping PCRs, in which the site-specific mutagenesis is introduced by overlapped primers.
4. Confirm all cloning results by DNA sequencing (*see* Note 1). Transform constructed cdMMP periplasmic expression plasmids to Jude-I component cells. Incubate on LB/Chlor agar plates.

[1]In addition to DNA sequencing, correct in-frame cdMMP clones can also be identified by measuring their proteolytic activities (16, also see **Subheading 3.3.2**)

3.2 Periplasmic expression and purification of cdMMPs

1. Inoculate a single colony of Jude-I or BL21 cells carrying pMopac-cdMMP to 5 ml 2×YT/Chlor. Culture at 30 °C overnight (*see* Note 2).
2. Add 5 ml overnight pre-culture to 500 ml 2×YT/Chlor. Cultures overnight at 30 °C without IPTG (*see* Note 3).
3. Measure OD_{600}. Centrifuge cells at 4,500 ×*g*, 4 °C for 15 min. Decant supernatant.
4. Add periplasmic buffer (*see* Note 4) at a volume of 20 μl per OD_{600}. Suspend collected cells by vortex.
5. Incubate for 5 min at room temperature with gentle shaking.
6. Add ice-cold DDW at volume of 20 μl per OD_{600}. Incubate on ice for 10 min with gentle shaking.
7. Centrifuge 4,500 ×*g*, 4 °C for 15 min. Filtrate supernatant through 0.45 μm membranes. Osmotic shocked supernatants are ready for cdMMP purification.
8. Purify cdMMPs by Ni-NTA resin. Use 20 and 200 mM imidazole in 50 mM Tris-HCl, pH 8.0, 300 mM NaCl as the washing and elution buffers.
9. Concentrate eluted samples using ultrafiltration units (3 kDa MWCO) by centrifugation at 4,000×*g* 4 °C for 15 min.
10. Dialyze concentrated cdMMP samples at 4 °C overnight (*see* Note 5).
11. In the next day, measure cdMMP concentrations. Analyze by SDS-PAGE (typical results shown in Fig 2). Store in 20 % glycerol at −80 °C.

3.3 Characterizations of cdMMPs

K_m and k_{cat} values of purified cdMMPs and their mutants can be measured (**Subheading 3.3.1**). In addition to enzymatic kinetics, the periplasmic preparation without purification can be directly applied for activity tests (**Subheading 3.3.2**), which are particularly useful in screening of MMP inhibitors [16].

3.3.1 Enzymatic kinetics measurement

1. Dissolve fluorogenic substrate peptide M-2350 in 20% DMSO to concentrations of 62.5–2000 μM.
2. Add 50 μl 1–10 nM cdMMP to a 96-well black assay plate.

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[2]Expression of cdMMP-9 in Jude-I resulted in truncated products likely due to endogenous proteases. Using protease deficient host BL21 reduced the amount of unwanted truncations. In addition, although cdMMPs do not have disulfide bonds, we found that co-expression of molecular chaperon DsbC (a disulfide isomerase) significantly improved the yields of cdMMPs (Fig 2B), a similar observation for TIMPs [15].

[3]Although cdMMPs are regulated under a *Lac* promoter, experimental results indicate that the highest activity was achieved without IPTG induction [13].

[4]EDTA treatment, a step of standard periplasmic fraction protocol [18], should be avoided due to its ability to chelate Ca^{2+} and Zn^{2+} which are essential for MMP structure and activity.

[5]Dialysis is required to remove imidazole (a weak inhibitor of MMPs) for downstream applications.

3. To start the reaction, add 1 μl substrate peptide stock solutions into wells to give final concentrations of 1.25–40 μM.
4. Monitor the hydrolysis of peptide M-2350 with excitation at 328 nm and emission at 393 nm.
5. Triplcate the experiments. Fit Michaelis-Menten equation to find K_m and k_{cat} (Fig 3).

3.3.2 Periplasmic FRET assays

1. Suspend 2–3 OD_{600} overnight cultures (**Subheading 3.2**) in 100 μl periplasmic buffer. Incubate on ice for 5 min.
2. Add same volume ice-cold DDW. Incubate on ice for 10 min to release periplasmic fraction.
3. Centrifuge 13,000 ×*g*, RT for 2 min.
4. Add 50–100 μl periplasmic fraction to a 96-well black assay plate.
5. To start the reaction, add peptide XV to a final concentration of 1 μM (*see* Note 6). Monitor hydrolysis with excitation at 490 nm and emission at 520 nm.
6. For inhibitor screening, add 0–1000 nM compound inhibitors (*e.g.* GM60001) or 0–8,000 nM Fabs (*e.g.* 3A2, 17) into the periplasmic fraction before adding the peptide substrate (*see* Note 7).

3.3.3 Analysis by gel permeable chromatography

1. Equilibrate superdex 75 10/300 GL size-exclusion column with 50 mM HEPES pH 7.5, 150 mM NaCl.
2. Inject 200 μl 500 μg/ml cdMMP-9 at a flow rate of 0.5 ml/min.
3. Monitor chromatograms of absorbance at 280 nm (Fig 4).
4. Estimate the molecular mass of cdMMP-9 based on the retention times of ovalbumin (43 kDa) and lysozyme (14 kDa).

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[6]Certain FRET substrates (*e.g.* M-2350 peptide, Bachem) cannot be used for periplasmic activity assays due to its proteolysis by homologous endopeptidases present in *E. coli* periplasm.
[7]Other than adding purified Fabs into periplasmic fraction, the Fab clone of interest can also be co-expressed with cdMMP for inhibitor screening [13].

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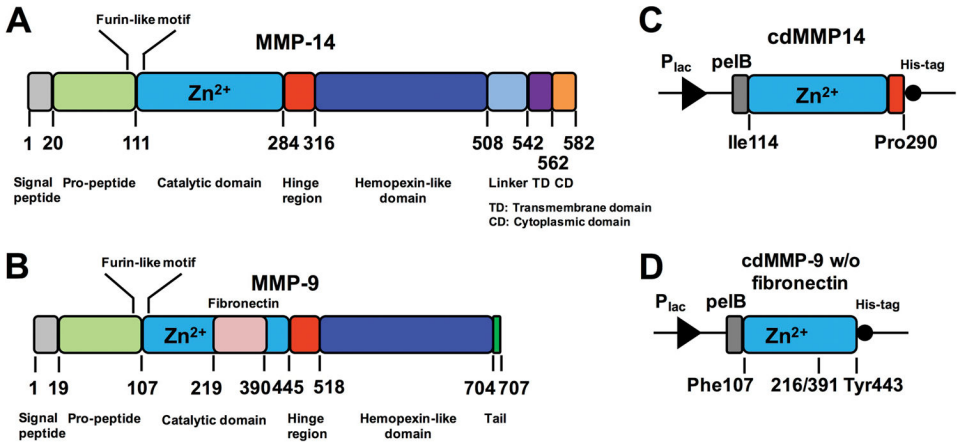

Figure 1.

Gene structures for (A) full length MMP-14 and (B) full length MMP-9, and periplasmic expression cassettes for (C) MMP-14 catalytic domain and (D) MMP-9 catalytic domain without fibronectin domain. Plac promoter and pelB leader peptide were used for cdMMP expression.

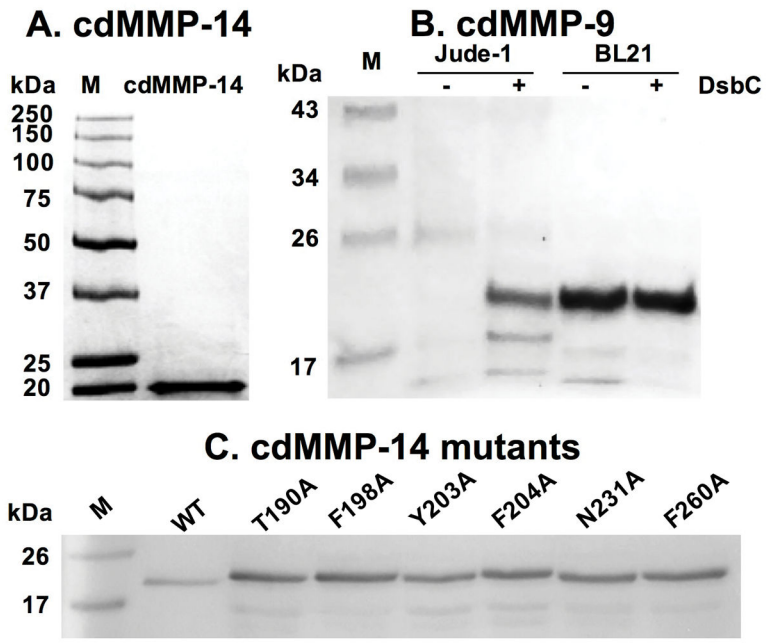

Figure 2.
SDS-PAGE of purified (A) cdMMP-14 (B) cdMMP-9 and (C) cdMMP-14 mutants.

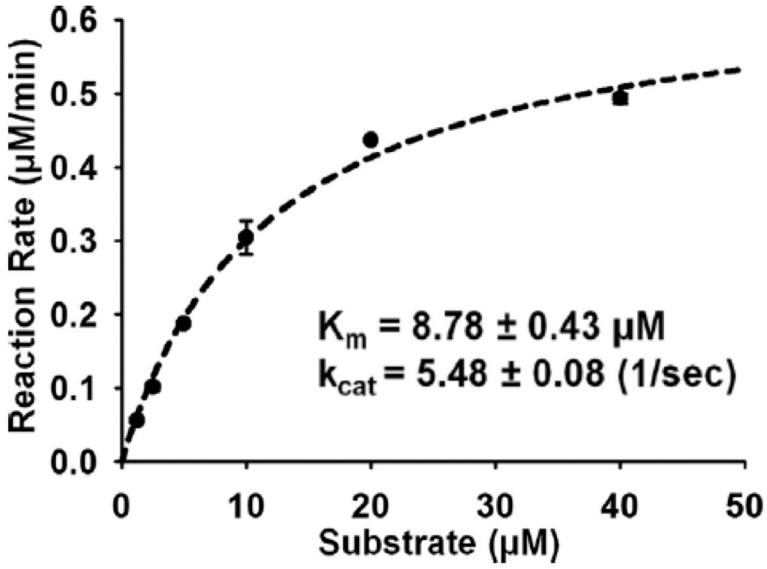

Figure 3.

Enzyme kinetics of cdMMP-14. 1–10 nM cdMMP-14 and 1.25–40 μM FRET peptide M-2350 were used for measurements of K_m and k_{cat}.

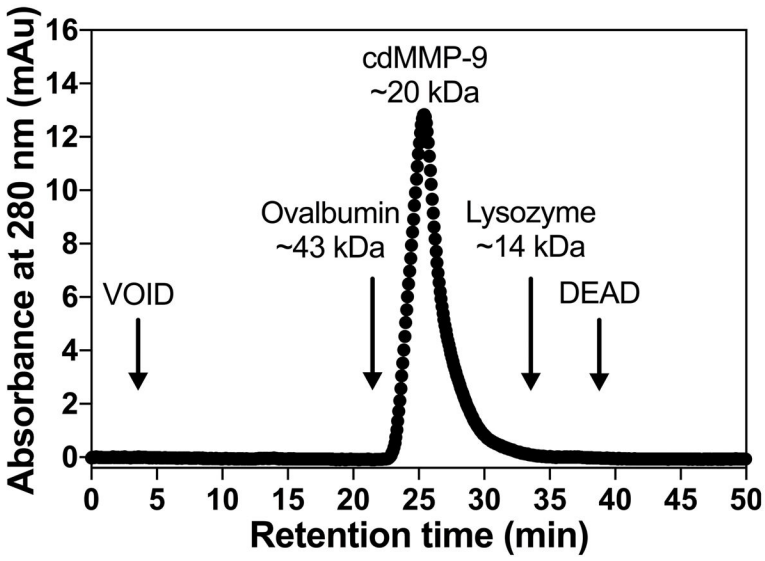

Figure 4.

Gel permeation chromatography result of purified cdMMP-9. Chromatograms were obtained by monitoring absorbance at 280 nm. The molecular mass of cdMMP-9 was estimated by its retention time and comparison with these of standard molecular mass markers, e.g. ovalbumin (43 kDa) and lysozyme (14 kDa).