

Note

Expression in *Escherichia coli*, Refolding, and Purification of the Recombinant Mature Form of Human Matrix Metalloproteinase 7 (MMP-7)

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In the latent pro-form of matrix metalloproteinase 7 (MMP-7), the cysteine residue in the pro-peptide binds the active-site zinc ion. Hence, recombinant active MMP-7 was prepared from pro-MMP-7 by modification of this cysteine residue with a mercuric reagent. In this study, mature MMP-7 was expressed in *Escherichia coli* as inclusion bodies, solubilized, and refolded with 1 M L-arginine. The purified product was indistinguishable from the one prepared from pro-MMP-7 as assessed by hydrolysis of (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N³-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-Ala-L-Arg-NH₂.

Key words: arginine; *Escherichia coli*; metalloproteinase; matrix metalloproteinase (MMP)-7; refolding

Human matrix metalloproteinase 7 (MMP-7, matrilysin) [EC 3.4.24.23] is the smallest matrix metalloproteinase (MMP). It lacks the carboxy-terminal hemopexin-like domain conserved in common MMPs. It is believed to play an important role in tumor invasion and metastasis.^{1,2} It contains a zinc ion essential for activity and other zinc and calcium ions that are considered necessary for stability.³ Like all other MMPs, it has the consensus sequence HEXXHXXGXXH, in which three histidine residues chelate an active-site zinc ion, and a methionine-containing turn (Met-turn). It exhibits a broad bell-shaped pH-dependence with pK_e values of about 4 and 10, indicating that at least two ionizable groups are involved in the catalytic mechanism.^{4–7}

An efficient production method for recombinant human MMP-7 is necessary for basic study of this enzyme and the development of inhibitors. MMP-7 is synthesized as an inactive preproenzyme (267 amino acid residues) including 17 residues in the pre-peptide and 77 residues in the pro-peptide. The pro-peptide domain contains a single cysteine residue (Cys70), which binds the active-site zinc ion and renders pro-MMP-7 inactive. This mechanism is called the cysteine-switch mechanism of activation.⁸ Under physiological

conditions, the pro-form (28 kDa) is activated into the mature form (19 kDa) automatically by cleavage of the peptide bond linking Glu77 and Tyr78 (the numbering of amino acid residues of pro-MMP-7 is applied to mature MMP-7 beginning at the N-terminal Tyr78). In the production of recombinant MMP-7, pro-MMP-7 is expressed in *E. coli* as inclusion bodies, solubilized, and refolded. Refolded pro-MMP-7 is then converted to mature MMP-7 by modification of Cys70 with a mercuric reagent, such as *p*-aminophenylmercuric acetate.^{9,10} The drawback of this method is that mercuric reagent is dangerous and their disposal is strictly regulated.

Generally, the pro-domain of protease acts as a molecular chaperone, and is sometimes necessary to produce a recombinant catalytically active enzyme. Both pro- and mature forms of MMPs are expressed in *E. coli* for this purpose.¹¹ Cha *et al.* expressed mature human MMP-7 in *E. coli* as inclusion bodies, from which the recombinant protein was solubilized with 4 M guanidine HCl and refolded by dialysis to remove guanidine HCl, but the activity (*k*_{cat}/*K*_m) of this product was about 50% of that of the one produced from pro-MMP-7.⁶ We have reported that of various additives, arginine is the most effective at suppressing protein aggregation during the refolding process of pro-MMP-7.¹⁰ Today, arginine is widely used in the refolding of proteins.^{12,13} This is explained by its affinity for the aromatic groups present in proteins.¹³ In this study, we used arginine in the refolding process of mature MMP-7 expressed in *E. coli*.

The expression plasmid for mature MMP-7 (pET-MMP-7) was constructed by the insertion of amplified MMP-7 DNA into the *Nde*I and *Eco*RI sites of pET-22b(+) (Merck, Tokyo). As shown in Fig. 1A, pET-MMP-7 contains initiator codon ATG followed by a DNA sequence encoding the mature sequence (Tyr78–Lys250) and stop codon TAG. BL21(DE3) cells were transformed with pET-MMP-7. Ampicillin was used at 50 µg/ml. For seed culture, 2 ml of L broth was inoculated with glycerol stock of the transformants and

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Abbreviations: AMPSO, 3-[(1,1-dimethyl-2-hydroxy-ethyl) amino]-2-hydroxypropane sulfonic acid; DMSO, dimethyl sulfoxide; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; *K*_e, proton dissociation constant; MES, 2-(*N*-morpholino)ethanesulfonic acid; MMP, matrix metalloproteinase; MOCAC-PLG, (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly; MOCAC-PLGL(Dpa)AR, (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N³-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-Ala-L-Arg-NH₂

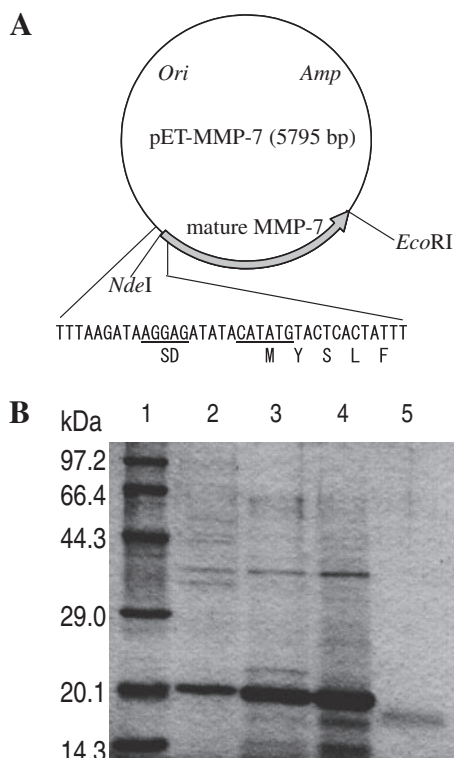


Fig. 1. Preparation of Recombinant Mature MMP-7.

A, Structure of pET-MMP-7. SD indicates the Shine-Dalgarno sequence. The *NdeI* site is underlined. B, Commassie Brilliant Blue-stained 12.5% SDS-polyacrylamide gel showing marker proteins (lane 1), inclusion bodies (lane 2), the centrifuged supernatant after fractionation by ammonium sulfate at 20% saturation (lane 3), the centrifuged pellet after fractionation by ammonium sulfate at 60% saturation (lane 4), and active fractions of heparin affinity chromatography, which was purified MMP-7 (lane 5). Molecular mass marker kit was purchased from Takara Bio (Ohtsu, Japan).

grown with shaking at 37 °C for 8 h. The culture was then diluted 1:100 in 200 ml of L broth and grown with shaking. After IPTG induction, growth was continued for 16 h. The cells were harvested by centrifugation (5,000 × *g*, 30 min), and suspended with 1 M sucrose. After centrifugation, the pellet was suspended with 10 ml of 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 10% sucrose, 1 mM EDTA, and 0.5% Triton X-100. This step was repeated once. After centrifugation, the pellet was suspended with 20 ml of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5 mM PMSF, and 0.1 mg/ml lysozyme, and disrupted by sonication. The lysate was centrifuged (10,000 × *g*, 30 min) and the pellet was collected. The inclusion body preparation thus obtained was solubilized with 10–20 volumes of 0.1 M Tris-HCl, 6 M guanidine HCl, and 0.1 M DTT at pH 7.5 with stirring overnight at 25 °C. The solution was diluted with 100 volumes of refolding buffer (50 mM Tris-HCl, 10 mM CaCl₂, 0.1 mM Zn(CH₃COO)₂, and 1.0 M L-arginine at pH 7.5), and left for 4 h at 4 °C. MMP-7 activity hydrolyzing MOCac-PLGL(Dpa)AR was detected in this diluted solution, indicating that MMP-7 was refolded from inclusion bodies. When refolding buffer without L-arginine was used, no activity was detected (data not shown). Saturated ammonium sulfate solution was added to the refolded solution to a final concentration of 20% saturation. After centrifugation, solid ammonium sulfate was added to the supernatant to

a final concentration of 60% saturation. After centrifugation, the pellet was dissolved in 50 mM HEPES buffer (pH 7.5), 10 mM CaCl₂, and 0.05% v/v Brij-35 (buffer A) at 4 °C, and dialyzed against the same buffer. The solution was applied to the column (10 mm inner diameter × 45 mm) packed with a heparin agarose (Sigma, St. Louis, MO) equilibrated with buffer A. The fractions containing MMP-7 were eluted with buffer A, containing 1.0 M NaCl, and collected. The MMP-7 concentration was determined spectrophotometrically using a molar absorption coefficient at 280 nm, ϵ_{280} , of 31,800 M⁻¹ cm⁻¹.⁵⁾ Starting with 100 ml of culture, about 2 mg of purified MMP-7 was recovered. On SDS-PAGE under reducing conditions, the MMP-7 preparation thus obtained yielded a single band with a molecular mass of 19 kDa (Fig. 1B). NH₂-terminal sequence determination by Edman degradation revealed that it lacked the expected three N-terminal amino acid residues, Met-Tyr-Ser, which is coincident with a previous report by Cha *et al.*⁶⁾ that denatured MMP-7 during the refolding process had the expected N-terminal sequence of Met-Tyr-Ser-Leu-Phe, while the correctly refolded one lacked three N-terminal amino acids, Met-Tyr-Ser. It is thought that Met-Tyr-Ser was cleaved during the purification process.

The pH-activity profiles as between the MMP-7 produced by the present method and that produced from pro-MMP-7¹⁰⁾ were compared. MOCac-PLGL(Dpa)AR (lot 491214) and MOCac-PLG (lot 510913) were purchased from the Peptide Institute (Osaka). Their concentrations were determined using $\epsilon_{410} = 7.5$ mm⁻¹ cm⁻¹ and $\epsilon_{324} = 12.9$ mm⁻¹ cm⁻¹ respectively.¹⁴⁾ The reaction was performed at 25 °C by mixing 1,222 μ l of the reaction buffer, 20 μ l of the enzyme solution, and 8 μ l of 234 μ M MOCac-PLGL(Dpa)AR dissolved in DMSO. The reaction buffers were 50 mM acetate-NaOH buffer at pH 3.6–5.8, 50 mM MES-NaOH buffer at pH 5.6–7.0, 50 mM HEPES-NaOH buffer at pH 6.8–8.6, and 50 mM AMPSO-NaOH buffer at pH 8.6–10.4, each containing 10 mM CaCl₂. The reaction was measured by following the increase in the fluorescence intensity at 393 nm with excitation at 328 nm with a JASCO FP-777 fluorescence spectrophotometer (Tokyo). The peptide bond of the Gly-L-Leu residues was cleaved by MMP-7, and the amount of the product, MOCac-PLG, was estimated by the fluorescence intensity in comparison with the fluorescence intensity of an authentic MOCac-PLG solution. Hydrolysis was carried out under pseudo first-order conditions, where the initial concentration of MOCac-PLGL(Dpa)AR (1.5 μ M) was much lower than K_m (60 μ M).¹⁵⁾ The Michaelis-Menten equation was expressed as $v_o = (k_{cat}/K_m)[E]_o[S]_o$, where v_o , k_{cat} , $[E]_o$, and $[S]_o$ are the initial reaction rate, the molecular activity, the initial enzyme concentration, and the initial substrate concentration respectively. The kinetic parameters, the intrinsic k_{cat}/K_m , $[(k_{cat}/K_m)_o]$, and the proton dissociation constants (K_{e1} and K_{e2}) for the pH-dependence of the activity were calculated from Eq. (1) by the non-linear least squares regression method with Kaleida Graph Version 3.5 (Synergy Software, Essex, VT):

$$(k_{cat}/K_m)_{obs} = (k_{cat}/K_m)_o / \{1 + ([H]/K_{e1}) + (K_{e2}/[H])\}$$

(1)

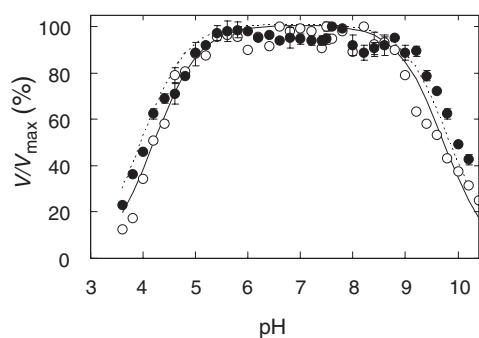


Fig. 2. Effect of pH on the MMP-7-Catalyzed Hydrolysis of MOCac-PLGL(Dpa)AR at 25 °C.

The initial enzyme and substrate concentrations were 11.4 nM and 1.5 μ M respectively for the MMP-7 produced by the present method (open circle) and 6.3 nM and 1.5 μ M respectively for the one produced from pro-MMP-7 (closed circle). The $v_{\max}$ values, which are the reaction rates at optimal pH levels, were 2.1 nM/s at pH 7.5 for the former MMP-7 and 1.5 nM/s at pH 7.5 for the latter. Solid and broken lines represent the best fit to Eq. (1).

In this equation, $[(k_{\text{cat}}/K_{\text{m}})_{\text{obs}}]$ and $[H]$ are the $k_{\text{cat}}/K_{\text{m}}$ value observed and the proton concentration respectively at the specified pH. As shown in Fig. 2, the pH-dependences of the MMP-7 produced by this method and the one produced from pro-MMP-7 were similar. The $[(k_{\text{cat}}/K_{\text{m}})_0]$, $\text{p}K_{\text{e1}}$, and $\text{p}K_{\text{e2}}$ values of the MMP-7 produced by the present method were $(1.20 \pm 0.01) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, 4.2 ± 0.0 , and 9.7 ± 0.0 respectively, and those of that produced from pro-MMP-7 were $(1.54 \pm 0.01) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, 4.0 ± 0.0 , and 9.8 ± 0.0 respectively.

We and others have reported the catalytic mechanism of MMP-7,^{4–7)} inhibition of MMP-7 activity by alcohols,¹⁵⁾ green tea catechins,¹⁶⁾ and lignans,¹⁷⁾ and the activation of MMP-7 activity by neutral salts.¹⁸⁾ The present production method is useful for site-directed mutagenesis experiments on MMP-7, which is currently under way to clarify the mechanisms. The present results also suggest the usefulness of L-arginine as an additive

in the refolding process of proteins, which is done by trial and errors in most cases.

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