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Purification and Refolding of Recombinant Human proMMP-7 (pro-Matrilysin) Expressed in *Escherichia coli* and Its Characterization

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Human matrix metalloproteinase-7 (MMP-7=matrilysin) was overproduced in *Escherichia coli* as a recombinant zymogen (31 kDa), the C-terminus of which bears artificial hexa-histidines. Most of the enzyme was isolated from the insoluble fraction of the cell lysate and purified by a single step using Ni-NTA resin after solubilization of the precipitates with 8 M urea solution. The resin-bound recombinant protein was refolded into a form that is activatable by *p*-amino-phenylmercuric acetate in an autocatalytic manner. The activated enzyme cleaved a synthetic peptide substrate at the reported site for MMP-7. Digestion of carboxymethylated transferrin (a natural substrate of MMP-7) by the recombinant proteinase generated fragments with the same peptide map as in the case of native purified MMP-7. The autocatalytic activation and enzyme reaction were entirely dependent on the presence of calcium and zinc ions. The enzyme activity to cleave carboxymethylated transferrin was inhibited by tissue inhibitors of metalloproteinases-1 and -2, MMP-specific inhibitors. The activity of the recombinant MMP-7 was also inhibited by a synthetic peptide derived from a part of the cysteine switch that maintains the zymogen in an inactive state. Thus, we report here a simple means of preparing a large quantity of recombinant proMMP-7 that can be used to study the activation mechanism and to screen synthetic inhibitors.

Key words: Ni-NTA resin, MMP inhibitors, recombinant matrylisin, recombinant MMP-7, zymogen activation.

Extracellular matrix is important as a scaffold for tissue structure and also as an environment regulating cell function in tissues. Matrix metalloproteinases (MMPs) are zinc-binding endopeptidases that degrade a variety of extracellular macromolecules including collagens, glycoproteins and proteoglycans (*1*). Through these activities, MMPs play essential roles in the tissue remodeling that occurs during various physiological processes such as morphogenesis, wound healing, ovulation, implantation, and uterus involution (*2, 3*). Aberrant function of MMPs causes the severe tissue damage found in pathological processes such as tumor invasion (*4-6*), tissue ulceration, and rheumatoid arthritis (*7*). To remodel tissues in a coordinated manner, the activities of MMPs are regulated strictly at multiple steps, such as gene expression (*8-10*), secretion, activation of zymogens (*11*), and inhibition by specific inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (*12*). Thus, it is important to clarify the regulation of the enzyme activities to achieve a better understanding of the mechanisms of tissue remodeling and to aid development of inhibitors to control tissue damage. A simple means of providing a large quantity of MMPs will facilitate the biochemical analysis of the enzymes and allow an efficient screening system to be developed for obtaining better therapeutic inhibitors.

Although several systems can express recombinant proteins, it is unpredictable whether or not the recombinant protein retains the native character (*13*). Expression of recombinant proteins in *Escherichia coli* is convenient due to its low cost and easy handling (*14*). However, expressed recombinant proteins in the cells frequently form insoluble inclusion bodies that sometimes resist refolding into the native form. Because of this difficulty, only limited success has been reported in the expression of functional MMPs in *E. coli* (*15, 16*).

We chose MMP-7 (matrilysin) to express in *E. coli*, because it is the smallest molecule in the family (*17*) and is frequently expressed in human malignant tumor cells (*18-20*). Here we report expression of the zymogen of MMP-7 (proMMP-7) in *E. coli* and its refolding *in vitro*.

MATERIALS AND METHODS

Plasmid Construction—The bacterial expression plasmid pTH-72 was constructed with 0.4 kb of *Bgl*II-*Sal*I frag-

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ment of pT7-7 (*21*), and 28 bp of hexahistidines-TAG (Amber) (*22*) oligonucleotides inserted into 2.4 kb of the *Hin*dIII-*Bgl*II fragment of pSP72. Plasmid pTH-72 has a tandem repeat of T7 promoters that is followed by a ribosome binding site (rbs), a translational initiation codon (ATG), multiple cloning sites, hexahistidines coding sequence, and an in-frame termination codon. The cDNA for human proMMP-7 was prepared by polymerase chain reaction (PCR) using a set of primers (5′ primer: GGCGGATCCATGCTGCCGCTGCCTCAGGAG, 3′ primer: GCCGTCGACTTTCTTTCTTGAATTACTTCT) based on the reported sequence and the template was poly(A)$^+$ RNA isolated from a human rectal carcinoma cell line, CaR-1 (*18, 23*). The resulting 0.8 kb PCR fragment was digested with *Bam*HI/*Sal*I and cloned into the *Bam*HI/*Sal*I sites of the pTH-72 vector (pTH-MMP7).

Expression of Human Recombinant proMMP-7 (r-proMMP-7) Protein in E. coli—Plasmid pTH-MMP7 or control plasmid (pTH-72) was transfected into *E. coli* strain BL21(DE3), which carries an integrated copy of the T7 RNA polymerase gene under the control of the inducible lacUV5 promoter (*14*). Transformed cells were grown in Luria broth with 200 μg/ml ampicillin at 30°C to a cell density of $A_{590}=0.4$–0.6. The recombinant protein was induced by adding 0.1 mM isopropyl-1-β-D-galactopyranoside (IPTG) according to Studier *et al.* (*14*) and incubation was continued for 6 h at 30°C.

Purification of Human r-proMMP-7 Protein from E. coli—Cells were collected by centrifugation and the expressed protein was extracted as described (*23*). Briefly, cells were suspended in 33 mM Tris-HCl (pH 8.0)/2.5 mM EDTA/10 mM β-mercaptoethanol and 1 mg/ml lysozyme. After disruption by freezing and thawing (three times), DNA was digested with DNase I (0.125 mg) in the presence of 10 mM $MgCl_2$. Cell debris was removed by centrifugation and EDTA and Triton X-100 were added to final concentrations of 15 mM and 1%, respectively. The r-proMMP-7 was recovered as insoluble inclusion bodies. The precipitate was collected and solubilized in 8 M urea/10 mM Tris-HCl (pH 8.0)/100 mM Na-phosphate/100 mM β-mercaptoethanol after centrifugation at 5,000$\times g$ for 10 min. The solubilized protein was mixed with Ni-NTA resin (QIAGEN, USA) equilibrated with the same buffer (*22*). After incubation at 4°C, unbound proteins were washed out with 8 M urea/10 mM Tris-HCl (pH 6.3)/100 mM Na-phosphate. None of the proteins other than r-proMMP-7 was detected in the resin-bound fraction by SDS-polyacrylamide gel electrophoresis (*24*).

Refolding of the r-proMMP-7 on Ni-NTA Resin—Recombinant proMMP-7 bound to Ni-NTA resin was incubated at 4°C for 12 h in 6 M urea/10 mM Tris-HCl/100 mM Na-phosphate (pH 6.3) to allow partial refolding. The beads were then collected by centrifugation, washed and resuspended in 50 mM Tris-HCl. This resin-bound enzyme was used as refolded r-proMMP-7. To refold soluble r-proMMP-7, the protein bound to Ni-NTA resin was eluted with 6 M urea/1% Triton/10 mM Tris-HCl/100 mM Na-phosphate (pH 4.5). The pH was adjusted to 7.5, and the eluate was dialyzed against TNCB buffer [50 mM Tris-HCl (pH 7.5)/150 mM NaCl/10 mM $CaCl_2$/0.05% Brij 35/0.02% NaN_3] containing 1% Triton X-100 and 6 M urea. The urea concentration was reduced to 3, 1.5, 0.5, and 0 M in a stepwise manner, then the Triton concentration was reduced to 0.1% by dialysis.

Gel Electrophoresis and Zymography—SDS-polyacrylamide gel electrophoresis (PAGE) was done in a Tris-glycine buffer system as described using a 3% stacking gel and 12.5% separation gel. Zymography was performed as described (*18, 25*). Briefly, samples were mixed with SDS sample buffer in the absence of a reducing agent, incubated for 20 min at 37°C and separated on a 12.5% polyacrylamide gel containing 1 mg/ml of casein or gelatin. After electrophoresis, gels were soaked in 2.5% Triton-X100 for 1 h then digested by incubating the gel in 50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl, 10 mM $CaCl_2$, and 0.02% NaN_3 at 37°C for 24 h. The gels were stained with 0.1% Coomassie Brilliant Blue R250, and the location of proteolytic activity was detected as clear bands in a background of uniform staining.

Digestion of Carboxymethylated Transferrin (Cm-Tf) with Native and Recombinant MMP7—Purified proMMP-7 from CaR-1 culture media (*23*) or soluble r-proMMP-7 was activated with 1 mM *p*-aminophenylmercuric acetate (*p*-APMA) and incubated with 20 μg of Cm-Tf. After incubation of the mixture for 1 h at 37°C, the reaction was terminated with 20 mM EDTA and the products were analyzed by SDS-PAGE (*26*).

RESULTS

Expression of r-proMMP-7 in E. coli—MMP-7 cDNA was subcloned into a bacterial expression vector to produce the recombinant protein in *E. coli*. The vector has a T7 promoter to express the cDNA. For translation of the cDNA in *E. coli*, the original initiation codon and the following signal peptide were substituted with a bacterial translation signal and the following 9 amino acids derived

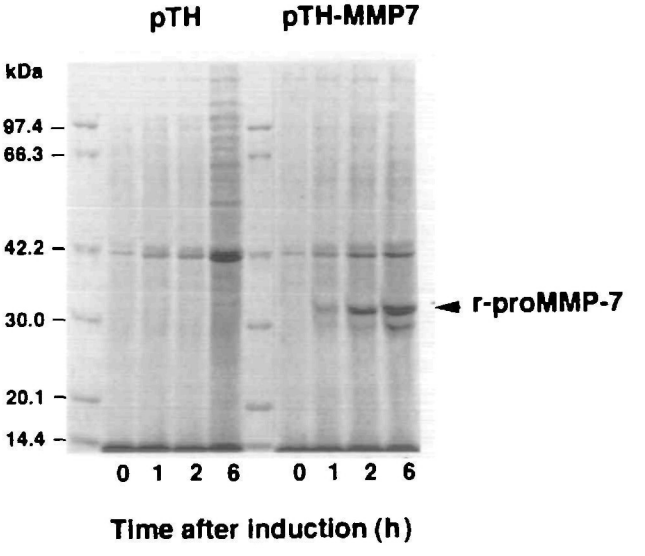

Fig. 1. **Polyacrylamide gel electrophoretic analysis of the induction of r-proMMP-7 protein.** *E. coli* BL21(DE3) was transformed by the expression plasmid pTH or pTH-MMP7. After extraction as described in "MATERIALS AND METHODS," aliquots of the extracts were denatured and reduced. The samples were resolved on 12.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate, then stained with Coomassie Brilliant Blue R-250. The location of the r-MMP-7 protein is indicated by an arrowhead.

from the vector plasmid. A histidine hexamer (H6-tag) was added to the C-terminus of the protein to facilitate purification of the recombinant protein using Ni-NTA resin (*27*). The plasmid was transfected into *E. coli* BL21(DE3) cells that have a lysogenic T7 RNA polymerase gene under the control of lac promoter. Without IPTG, no T7 RNA polymerase was expressed in the cells, and therefore, no MMP-7 proteins were detected in the cell lysates (Fig. 1).

The addition of IPTG to the culture media induced a major protein of 31 kDa in the cell lysate within 2 h as shown in Fig. 1. The induced protein was thought to be proMMP-7 from its molecular weight. Expression of the recombinant proMMP-7 reached 20–30% of the total proteins in the cell lysate.

To examine whether exposing the cells to IPTG induces proteinase activities, cells exposed to IPTG for different periods were used and analyzed by casein and gelatin zymography. Caseinolytic activity appeared after IPTG-induction, but not without the treatment (Fig. 2A). The activity corresponded to the protein band of 31 kDa in Fig. 1. Gelatinolytic activity was also detected by zymography (Fig. 2B), but was weaker than the activity against casein, as reported with native MMP-7 (*28*).

Purification—To purify the expressed r-proMMP-7, cell lysates were fractionated and proteins were analyzed by SDS-PAGE. As shown in Fig. 3, most of the r-proMMP-7 was detected in the precipitate as insoluble inclusion bodies. The precipitate was solubilized with 8 M urea containing 1% Triton and incubated with Ni-NTA resin. Almost all the r-proMMP-7 bound to the resin, as no r-proMMP-7 was detectable in the unbound fraction. The

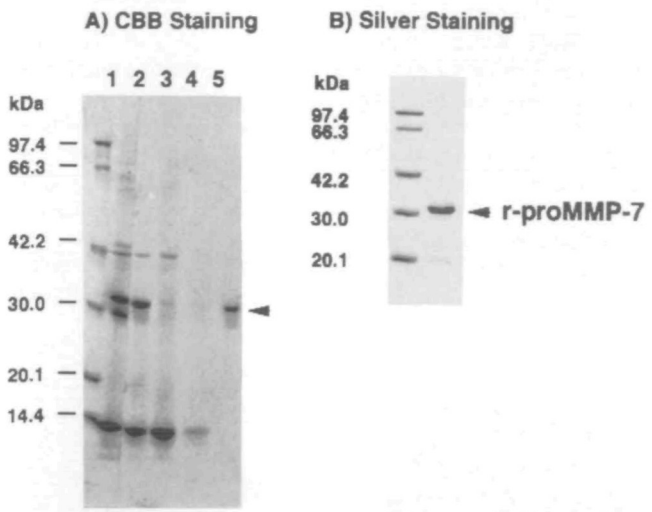

Fig. 3. **Polyacrylamide gel electrophoretic separation of r-proMMP-7 at each purification step with Ni-NTA resin.** Denatured and reduced samples from each purification step were resolved on a 12.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate, and stained with Coomassie Brilliant Blue R-250 Lane 1, *E coli* extract after 6 h IPTG induction, lane 2, insoluble proteins (precipitates of centrifugation at 5,000×*g* for 10 min); lane 3, unbound fraction after incubation with Ni-NTA resin in 8 M urea/10 mM Tris-HCl (pH 8.0)/100 mM Na-phosphate/100 mM β-mercaptoethanol; lane 4, washed fraction with 8 M urea/10 mM Tris-HCl (pH 6.3)/100 mM Na-phosphate/100 mM β-mercaptoethanol, lane 5, Ni-NTA resin retaining r-proMMP-7 protein.

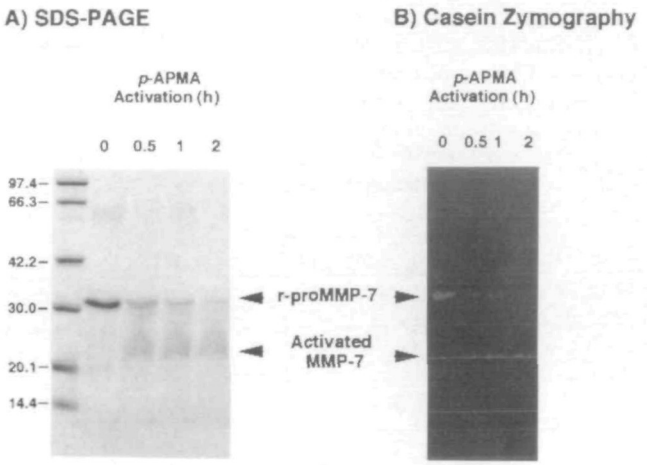

Fig. 4. ***p*-Aminophenylmercuric acetate (*p*-APMA) activation of r-proMMP-7 on Ni-NTA resin.** The r-proMMP-7 bound resin was incubated with refolding buffer and resuspended in 50 mM Tris-HCl (pH 8.0). The samples were incubated with 1 mM *p*-APMA at 37°C for the periods indicated. Thereafter, they were analyzed by means of SDS-PAGE (A) and casein zymography (B)

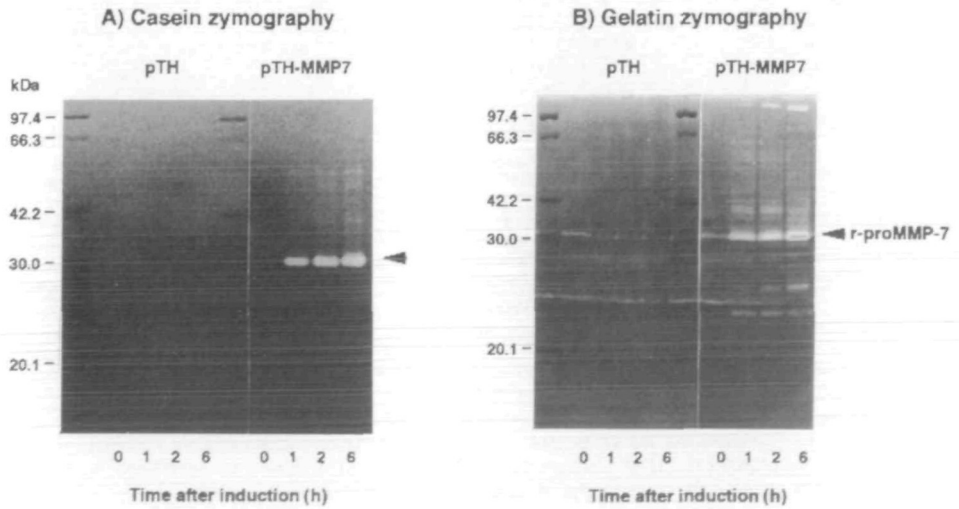

Fig 2. **Detection of caseinolytic and gelatinolytic activities of the r-proMMP-7 after SDS polyacrylamide gel electrophoresis.** The extracts (1/100 volume of that used in Fig. 1) were mixed with non-reducing SDS/gel sample buffer and applied without boiling to a 2.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate and 1 mg/ml casein (A) or gelatin (B). After electrophoresis, the gel was washed, incubated in the zymography buffer for 24 h at 37°C and stained with Coomassie Brilliant Blue R-250 The location of the r-MMP-7 protein is indicated by an arrow head

protein bound to the resin was solubilized with SDS-sample buffer and resolved by SDS-PAGE. The r-proMMP-7 bound to the resin was detected as a single band in the gel, as shown in Figs. 3 and 4. Thus, the binding of the recombinant protein to the resin through the H6-tag at the C-terminus was specific enough to allow single-step purification of the product, affording 8 mg of protein per liter of bacterial culture.

Refolding of r-proMMP-7—The r-proMMP-7 bound to the resin was not eluted with buffer containing 0.5 M imidazole, that competes for binding to the Ni ion on the resin. Therefore, we examined the direct refolding of the proteinase on the Ni-NTA resin. The resin was resuspended in refolding buffer (6 M urea/10 mM Tris-HCl/100 mM Na-phosphate at pH 6.3) and incubated for 12 h at 4°C to allow gradual refolding, then the r-proMMP-7-bound resin was collected and resuspended in 50 mM Tris-HCl (pH 8.0) buffer. The recovered r-proMMP-7 contained a low ratio of activated species and most of it was in the pro-enzyme form when analyzed by zymography (Fig. 4). The pro-enzyme was activated by *p*-APMA with a decrease in molecular weight, leading to the activated 21 kDa form during the incubation (Fig. 4A). The *p*-APMA-induced processing was entirely dependent on Ca^{2+} and Zn^{2+} ions (data not shown) indicating that the activation was mediated by the endogenous metalloproteinase activity. The two major species retained the caseinolytic activities, as shown in Fig. 4B.

The resin-bound r-proMMP-7 was eluted at pH 4.5 (6 M urea/1% Triton/10 mM Tris-HCl/100 mM Na-phosphate). The pH was adjusted to 7.5, then the solution was dialyzed against TNCB buffer containing urea and Triton. Refolding proceeded during dialysis against TNCB buffer with gradient concentrations of urea (3 to 0 M) and Triton (1 to 0.1%). The refolded proteinase contained a substantial amount of the activated 21 kDa form (~20%), which was thought to be activated during elution and refolding. Addition of *p*-APMA to the eluate converted the r-proMMP-7 into the activated 21 kDa form (data not shown).

From the results described above, we concluded that the r-proMMP-7 was refolded efficiently as judged in terms of the functions of the cysteine switch maintaining the latency of the zymogen and the catalytic domain required for autocatalytic processing. Plasmin and neutrophil elastase reportedly activate proMMP-7 by cleaving serine proteinase-sensitive sites in the propeptide domain (*20*). Indeed, exposing r-proMMP-7 to these enzymes also activated the proenzyme (data not shown).

Cleavage Specificity—To study the cleavage specificity by rMMP-7, an oligopeptide (DNP-Pro-Leu-Gly-Ile-Ala-

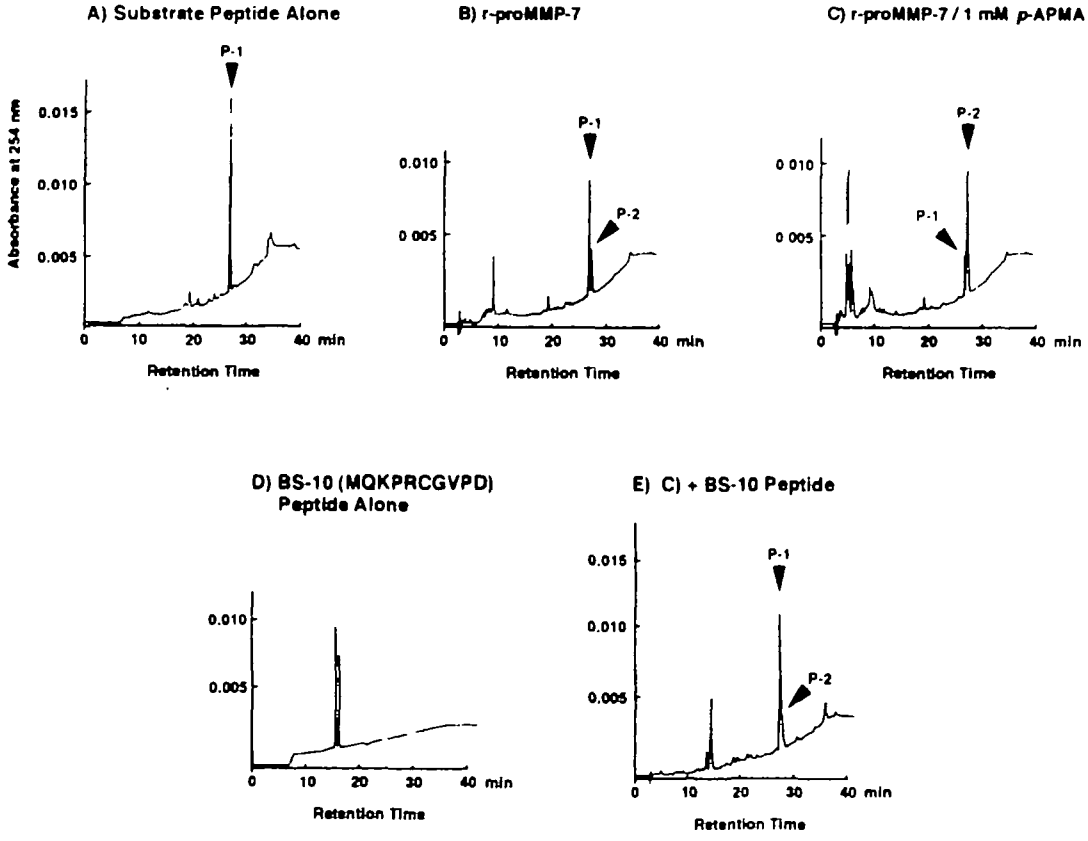

Fig. 5. **Digestion of DNP-peptide with r-MMP7.** The substrate DNP-peptide (DNP-Pro-Leu-Gly-Ile-Ala-Gly-Arg) was incubated in 40 μl of 50 mM Tris-HCl (pH 8.0)/200 mM NaCl/5 mM $CaCl_2$/5 μM $ZnCl_2$ at 37°C for 5 h. A half of the reaction volume was applied to the HPLC column [CAPCELLPAK C18 (4.6 I.D. × 250 mm] and separated for 30 min with a linear gradient (0 to 50%) of acetonitrile in 0.1% TFA at 40°C. The peptides were detected by measuring UV absorption at 254 nm. Incubation was performed with (A), the buffer alone; (B), r-proMMP-7; (C), 1 mM *p*-APMA activated r-proMMP-7; (D), BS-10 peptide (Met-Gln-Lys-Pro-Arg-Cys-Gly-Val-Pro-Asp) alone without substrate; and (E), 1 mM *p*-APMA activated r-proMMP-7 and 10 mM BS-10 peptide. P-1 and P-2 indicate the substrate DNP-peptide and the cleaved peptide (DNP-Pro-Leu-Gly), respectively, that were confirmed by amino acid analysis of each peptide.

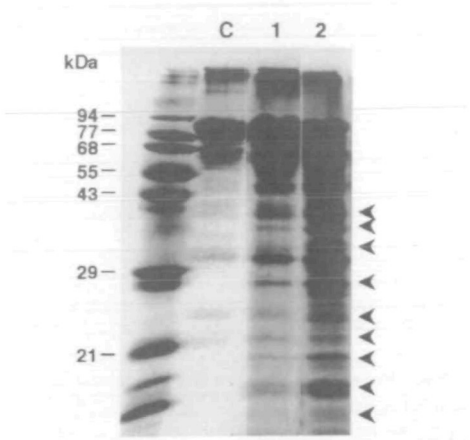

Fig 6. **Digestion profiles of Cm-Tf with native and r-MMP7 enzymes.** Cm-Tf (20 μg) was incubated with *p*-APMA-activated r-MMP-7 (lane 1) or native MMP-7 (lane 2) enzyme at 37°C for 1 h The reaction was terminated with EDTA at a final concentration of 20 mM The samples were reduced, boiled and resolved in 12.5% polyacrylamide gel containing 0 1% sodium dodecyl sulfate. After gel electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. Lane C is the same amount of Cm-Tf incubated without enzyme for 1 h Arrowheads indicate the MMP-7-specific cleaved fragments.

Gly-Arg-COOH) that was designed based on the cleavage site of the type I collagen α-chain by MMPs was used as a substrate (*29*). The peptide was incubated with purified rMMP-7 and the product was analyzed by high-performance liquid chromatography (HPLC). The peptide alone was eluted at 26.7 min (P-1) after elution through a reverse-phase column (Fig. 5A). Incubation of the peptide with the r-proMMP-7 generated an additional small peak (P-2) at 27.3 min (Fig. 5B). On the other hand, the *p*-APMA-treated enzyme had less P-1 and more P-2 (Fig. 5C). The latter was thought to be a cleavage product because of the characteristic absorbance of the cleaved dinitrophenyl(DNP)-peptide OD_{365}. An amino acid analysis of P-2 identified it as the N-terminal fragment cleaved between Gly-Ile of the peptide. The specific activity of the activated enzyme was calculated as 560.7 nmol cleavage/min/mg pro-enzyme.

Activation of the enzymes by *p*-APMA is caused by the elimination of the amino acid sequences responsible for the cysteine switch in the propeptide domain. To confirm this, a synthetic decapeptide (MQKPRCGVPD) corresponding to the cysteine switch region was added to the reaction mixture. As shown in Fig. 5 (D and E), cleavage of the substrate peptide was inhibited by the decapeptide, and the inhibition was dose-dependent (data not shown).

To confirm the substrate specificity of the rMMP-7, we further examined the digestion profiles of carboxymethylated transferrin (Cm-Tf) (*26*) as a representative natural substrate and compared the products with those in the case of authentic native MMP-7 isolated from the culture medium of CaR-1 cells (*23*). Cm-Tf was incubated with *p*-APMA-activated recombinant or native MMP-7 at 37°C and the products were compared by SDS-PAGE (Fig. 6). The profiles of the digests produced by recombinant and native MMP-7 were identical, indicating that both enzymes have the same substrate specificity. TIMP-1 and TIMP-2 specifically inhibit MMPs by forming a 1:1 complex. Both TIMP-1 and -2 inhibited the above enzyme reaction (data not shown).

DISCUSSION

Recombinant proMMP-7 was expressed in *E. coli* as insoluble inclusion bodies, solubilized with 8 M urea solution and purified by a simple one-step method using Ni-NTA-resin. Although imidazole is commonly used to elute recombinant proteins tagged with histidine residues from Ni-NTA-resin (*27*), it was ineffective for r-proMMP-7 elution. However, the resin-bound r-proMMP-7 was eluted at low pH (4.5) in the presence of 6 M urea and 1% Triton. The eluted protein was refolded by successively decreasing the urea and Triton concentrations by dialysis. Partial activation of proMMP-7 occurred during the elution and the refolding processes, and the final preparation contained ~20% of the activated 21 kDa species. The C-terminal His6-tag conferred an advantage not only for purification but also for refolding of the protein. The resin-bound r-proMMP-7 was refolded efficiently during a 12-h incubation at 4°C in the solution containing 6 M urea. The urea was eliminated easily by precipitation and by resuspending the resin in a different buffer. Most of the refolded recombinant proteinase was in the 31 kDa zymogen form and only a small amount of the activated species of 21 kDa was present. Gradual refolding in the urea solution in the absence of Ca^{2+} and Zn^{2+} is thought to be effective in preventing autoactivation. Refolding of the recombinant protein with its C-terminus immobilized to the resin may enhance effective refolding by preventing undesirable folding from the C-terminus. Thus, the present purification and refolding procedures are especially useful for preparing r-proMMP-7.

To evaluate the functional refolding, we examined autocatalytic activation (*20*) of the proenzyme as shown in Fig. 4. The latency of MMP zymogens is maintained by a mechanism called "the cysteine switch" (*11*). The switch is composed of two conserved regions of MMP: the PRCGX-PD sequence in the propeptide domain and the catalytic zinc-binding domain. In the latent enzymes, the cysteine residue in the PRCGXPD masks the zinc atom at the catalytic site. Processing of the propeptide domain by other proteases or chemical modification of the cysteine residue unveils the endogenous proteolytic activity and induces autocatalytic processing that excises the entire propeptide domain to generate the fully active form (activation) (*20*). Thus, functional proMMPs should maintain their latency by this mechanism and undergo autocatalytic activation upon modification of the cysteine residue with *p*-APMA. This is, therefore, a good indication of functional recovery of the recombinant enzyme. The processing of r-proMMP-7 caused by *p*-APMA did indeed result in the enzymatic activation, as demonstrated in Fig. 5.

The cleavage site of the synthetic substrate by rMMP-7 was exactly the same as that reported for native MMP-7. Thus, the refolded rMMP-7 and native MMP-7 have the same substrate specificity. Specific activity of the recombinant enzyme (560.7 nmol cleavage/min/mg pro-enzyme) was about 50% of the reported value for the native enzyme (1,160 nmol cleavage/min/mg pro-enzyme) (*30*). A possible explanation for the difference is incomplete refolding of the recombinant enzyme. However, we think that the

difference arises from the instability of the activated enzyme. The MMP-7 activated by APMA tends to be degraded by its autoproteolytic activity. For this reason, the ratio of the activated form in the APMA-treated proenzyme is difficult to control, depending on the experimental conditions.

The cleavage specificity against the small synthetic peptide may reflect the intactness of only a part of the catalytic domain of the enzyme. MMPs have multiple interaction modes with substrate extracellular matrix macromolecules, such as the fibronectin-like domains of MMP-2 and -9 that bind gelatin (*31, 32*), and this kind of interaction is also an important determinant of the substrate specificity of the enzymes. Thus, it is important to confirm that the r-MMP-7 can digest natural macromolecular substrates with the same specificity as that of the native enzyme. Cm-Tf was used as a representative natural substrate and rMMP-7 digested it with the same specificity as native MMP-7. Based on these results, we concluded that the purified r-proMMP-7 was functionally refolded. Negro *et al.* have recently reported expression and refolding of recombinant TIMP-2 in *E. coli* by a very similar method to ours (*33*).

In this study, we established a simple method of preparing pure and functional r-proMMP-7 using an expression system in *E. coli*. The key feature of this method is that the latent form of r-proMMP-7 can be prepared with a simple one-step procedure and the amount of activated species in the preparation was minimal. The latent form of proMMP-7 immobilized onto the resin has several benefits for biochemical studies. For example, it is easy to add the enzyme to and withdraw it from a reaction mixture. Thus, the recombinant enzyme will facilitate studies to identify other tissue activators of proMMP-7, or to examine the intermolecular activation of other MMP zymogens by MMP-7. Preparation of a large quantity of pure rMMP-7 may also aid screening of inhibitors to find better therapeutics, as well as biochemical studies.

REFERENCES

1. Birkedal-Hansen, H., Moore, W.G., Bodden, M.K., Windsor, L.J., Birkedal-Hansen, B., DeCarlo, A., and Engler, J.A. (1993) Matrix metalloproteinases: A review. *Crit. Rev. Oral. Biol. Med.* **4**, 197-250
2. Matrisian, L.M. (1990) Metalloproteinases and their inhibitors in matrix remodeling. *Trends Genet.* **6**, 121-125
3. Murphy, G., Cockett, M.I., Ward, R.V., and Docherty, A.J. (1991) Matrix metalloproteinase degradation of elastin, type IV collagen and proteoglycan. A quantitative comparison of the activities of 95 kDa and 72 kDa gelatinases, stromelysins-1 and -2 and punctuated metalloproteinase (PUMP). *Biochem. J.* **277**, 277-279
4. Basset, P., Bellocq, J.P., Wolf, C., Stoll, I., Hutin, P., Limacher, J.M., Podhajcer, O.L., Chenard, M.P., Rio, M.C., and Chambon, P. (1990) A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. *Nature* **348**, 699-704
5. Liotta, L.A., Steeg, P.S., and Stetler-Stevenson, W.G. (1991) Cancer metastasis and angiogenesis: An imbalance of positive and negative regulation. *Cell* **64**, 327-336
6. Stetler-Stevenson, W.G., Aznavoorian, S., and Liotta, L.A. (1993) Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annu. Rev. Cell. Biol.* **9**, 541-573
7. Woessner, J.F., Jr. (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J.* **5**, 2145-2154
8. Huhtala, P., Chow, L.T., and Tryggvason, K. (1990) Structure of the human type IV collagenase gene. *J. Biol. Chem.* **265**, 11077-11082
9. Pajouh, M.S., Nagle, R.B., Breathnach, R., Finch, J.S., Brawer, M.K., and Bowden, G.T. (1991) Expression of metalloproteinase genes in human prostate cancer. *J. Cancer Res. Clin. Oncol.* **117**, 144-150
10. Sato, H., Kita, M., and Seiki, M. (1993) v-Src activates the expression of 92 kDa type IV collagenase gene through the AP-1 site and the GT-box homologous to retinoblastoma control element. A mechanism regulating gene expression independent of that inflammatory cytokines. *J. Biol. Chem.* **268**, 23460-23468
11. Van Wart, H. and Birkedal-Hansen, H. (1990) The cysteine switch: A principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proc. Natl. Acad. Sci. USA* **87**, 5578-5582
12. Murphy, G. and Docherty, A.J. (1992) The matrix metalloproteinases and their inhibitors. *Am. J. Respir. Cell. Mol. Biol.* **7**, 120-125
13. Docherty, A.J. and Murphy, G. (1990) The tissue metalloproteinase family and the inhibitor TIMP: A study using cDNAs and recombinant proteins. *Ann. Rheum. Dis.* **49**, 469-479
14. Studier, F.W., Rosenberg, A.H., Dunn, J.J., and Dubendorff, J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes in *Methods in Enzymology* (Goeddel, D.V., ed.) Vol. 185, pp. 60-89, Academic Press, San Diego
15. Marcy, A.I., Eiberger, L.L., Harrison, R., Chan, H.K., Hutchinson, N.I., Hagmann, W.K., Cameron, P.M., Boulton, D.A., and Hermes, J.D. (1991) Human fibroblast stromelysin catalytic domain: Expression, purification, and characterization of a C-terminally truncated form. *Biochemistry* **30**, 6476-6483
16. Ye, Q.Z., Johnson, L.L., and Baragi, V. (1992) Gene synthesis and expression in *E. coli* for pump, a human matrix metalloproteinase. *Biochem. Biophys. Res. Commun.* **186**, 143-149
17. Quantin, B., Murphy, G., and Breathnach, R. (1989) Pump-1 cDNA codes for a protein with characteristics similar to those of classical collagenase family members. *Biochemistry* **28**, 5327-5334
18. Miyazaki, K., Hattori, Y., Umenishi, F., Yasumitsu, H., and Umeda, M. (1990) Purification and characterization of extracellular matrix-degrading metalloproteinase, matrin (pump-1), secreted from human rectal carcinoma cell line. *Cancer Res.* **50**, 7758-7764
19. McDonnell, S., Navre, M., Coffey, R.J., and Matrisian, L.M. (1991) Expression and localization of the matrix metalloproteinase pump-1 (MMP-7) in human gastric and colon carcinomas. *Mol. Carcinog.* **4**, 527-533
20. Crabbe, T., Willenbrock, F., Eaton, D., Hynds, P., Carne, A.F., Murphy, G., and Docherty, A.J. (1992) Biochemical characterization of matrilysin. Activation conforms to the stepwise mechanisms proposed for other matrix metalloproteinases. *Biochemistry* **31**, 8500-8507
21. Tabor, S. and Richardson, C.C. (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. USA* **82**, 1074-7078
22. Crowe, J., Dobeli, H., Gentz, R., Hochuli, E., Stuber, D., and Henco, K. (1994) 6xHis-Ni-NTA chromatography as a superior technique in recombinant protein expression/purification. *Methods Mol. Biol.* **31**, 371-387
23. Tsai, A.Y.M., Itoh, M., Streuli, M., Thai, T., and Saito, H. (1991) Isolation and characterization of temperature-sensitive and thermostable mutants of the human receptor-like protein tyrosine phosphatase LAR. *J. Biol. Chem.* **266**, 10534-10543
24. Gallagher, S.R. and Smith, J.A. (1989) One-dimensional gel electrophoresis of proteins in *Current Protocols in Molecular Biology* (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds.) 10.2.2-10.2.35, John Wiley & Sons, New York
25. Chin, J.R., Murphy, G., and Werb, Z. (1985) Stromelysin, a connective tissue degrading metallopeptidase secreted by stimulated rabbit synovial fibroblasts in parallel with collagenase. Biosynthesis, isolation, characterization and substrates. *J. Biol.*

Chem. **260**, 12367-12376
26. Okada, Y., Nagase, H., and Harris, J.E.D. (1986) A metalloproteinase from human rheumatoid synovial fibroblasts that digests connective tissue matrix components. *J. Biol. Chem.* **261**, 14245-14255
27. Janknecht, R., de Martynoff, G., Lou, J., Hipskind, R.A., Nordheim, A., and Stunnenberg, H.G. (1991) Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus. *Proc. Natl. Acad. Sci. USA* **88**, 8972-8976
28. Woessner, J.F.J. and Taplin, C.J. (1988) Purification and properties of a small latent matrix metalloproteinase of the rat uterus. *J. Biol. Chem.* **263**, 16918-16925
29. Masui, Y., Takemoto, T., Sakakibara, S., Hori, H., and Nagai, Y. (1977) Synthetic substrates for vertebrate collagenase. *Biochem. Med.* **17**, 215-221
30. Woessner, J.J. (1995) Matrilysin in *Methods in Enzymology* (Barrett, A.J., ed.) Vol. 248, pp. 485-495, Academic Press, San Diego
31. Goldberg, G.I., Marmer, B.L., Grant, G.A., Eisen, A.Z., Wilhelm, S., and He, C. (1989) Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteinases designated TIMP-2. *Proc. Natl. Acad. Sci. USA* **86**, 8207-8211
32. Collier, I.E., Krasnov, A.Y., Strongin, H., Birkedal-Hansen, H., and Goldberg, G.I. (1992) Alanine scaning mutagenesis and functional analysis of the fibronectin-like collagen binding domain from human 92 kDa type IV collagenase. *J. Biol. Chem.* **267**, 6776-6781
33. Negro, A., Onisto, M., Masiero, L., and Garbisa, S. (1995) Synthesis and refolding of human TIMP-2 from *E. coli*, with specific activity for MMP-2. *FEBS Lett.* **360**, 52-56