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Refolding and Recovery of Recombinant Human Matrix Metalloproteinase 7 (Matrilysin) from Inclusion Bodies Expressed by *Escherichia coli*[1]

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The recombinant prepro-form of human matrix metalloproteinase 7 (matrilysin or MMP-7) was overexpressed in *Escherichia coli* as insoluble inclusion bodies. The recombinant protein was refolded by 100-fold dilution after solubilization with 6 M guanidine HCl. The refolding was monitored by the recovery of matrilysin activity. The addition of either 1.0 M arginine or 0.1% Brij-35 promoted remarkably the refolding. The refolding was dependent on pH and temperature, with lower temperature (< 10°C) and pH 6-8 preferable. Glutathione had no effect on refolding, and it was excluded from the refolding conditions. Starting with inclusion bodies (2.0 g, wet) containing 360 mg protein, 29.5 mg of pro-matrilysin (30 kDa) was obtained after refolding with 1.0% Brij-35 at pH 7.5 and 4°C for 12 h. Pro-matrilysin (24.0 mg) was purified to homogeneity by cation-exchange HPLC with a 15-fold increase in purity and an activity yield of 81.3%. Pro-matrilysin was converted entirely to matrilysin (19.0 kDa; 15.2 mg) by activation with a mercuric reagent. The activity (k_{cat}/K_m) of matrilysin was 1.7×10^5 $M^{-1}\cdot s^{-1}$.

Key words: inclusion bodies, matrilysin, matrix metalloproteinase, proteolysis, refolding.

Matrix metalloproteinases (MMPs) comprise a family of zinc endopeptidases that degrade extracellular matrix and basement membrane components. These enzymes are believed to take part in processes involving physiological and pathological degradations, such as development, differentiation, tissue morphogenesis, wound healing, ovulation, rheumatoid arthritis, and tumor invasion (*1, 2*).

Matrilysin (MMP-7) [EC 3.4.24.23] is the smallest MMP family member, and lacks a carboxy-terminal hemopexin-like domain conserved in common MMPs. The molecular mass of the latent pro-form of matrilysin is 28 kDa and that of its mature form is 19 kDa (*3, 4*). Human prepro-matrilysin is composed of 267 amino acid residues, including 17 residues in the signal peptide and 77 residues in the propeptide (*3*). It is known that the pro-enzyme is activated automatically by cleavage of the pro-peptide under physiological conditions. The pro-peptide domain contains a single cysteine residue that is involved in the cysteine-switch mechanism of activation (*5*). X-ray crystallographic analysis of recombinant human matrilysin produced from CHO cells demonstrated that matrilysin is composed of a five-stranded β-sheet and three α-helices, and contains a zinc ion essential for enzyme activity, as well as a 2nd zinc ion and two calcium ions that are regarded as necessary for enzyme stability (*6*). Matrilysin degrades gelatins of type I, III, IV, and V, fibronectin, proteoglycan, laminin, and type IV basement membrane collagen (*4, 7*). It has been reported that human matrilysin is over-expressed in cancer cells of various organs including prostate (*8*), colorectum (*9*), brain (*10*), and stomach (*11*), and it is thought to play important roles in tumor invasion and metastasis.

Preparing a large quantity of human matrilysin at reasonable cost is indispensable for studying its structure-and-function relationship and for the development of inhibitors that could be useful for cancer therapy. The matrilysin cDNA has been expressed in COS cells (*3*), NSO mouse myeloma cells (*12*), and CHO cells (*13*). High-level expression of the human pro-matrilysin cDNA was achieved in *Escherichia coli*, although the polypeptide expressed was formed as insoluble inclusion bodies and no activity was detected (*14*). Refolding of the pro-form of stromelysin, another MMP member, from inclusion bodies formed in *E. coli* cells has been reported (*15*). The insoluble protein was solubilized with 6 M guanidine hydrochloride (Gdn-HCl) and 0.3 M dithiothreitol (DTT). The pro-enzyme was refolded by diluting the solution with 100 volumes of 50 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 5 mM $CaCl_2$, 0.05% Brij-35, 0.02% NaN_3, 0.1 mM Zn-acetate, and 10 mM oxidized GSH, and it was activated with *p*-aminophenyl mercuric acetate (APMA). This procedure has also been applied to the refolding of pro-matrilysin (*16*). Pro-matrilysin bearing an artificial tag of hexa-histidines has been reported to refold on a nickel-

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Abbreviations: APMA, *p*-aminophenylmercuric acetate; DTT, dithiothreitol; Gdn-HCl, guanidine hydrochloride; GSH, glutathione; 2-MET, 2-mercaptoethanol; MOCAc-PLGL(Dpa)AR, (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diamino-propionyl]-L-Ala-L-Arg-NH_2; MOCAc-PLG, MOCAc-L-Pro-L-Leu-Gly.

nitrilotriacetic acid resin after solubilizing the inclusion bodies with 8 M urea and 0.1 M 2-mercaptoethanol (2-MET) (*17*). Human pro-matrilysin was produced in *E. coli* as an N-terminal fusion protein with ubiquitin, and the insoluble protein was solubilized and refolded (*18*). It was demonstrated that the kinetics and intermediates of the activation of the fusion protein with APMA are similar to those of pro-matrilysin produced in CHO cells. Most recently, it was reported that human matrilysin devoid of its pro-peptide was expressed in *E. coli* and purified to homogeneity by heparin chromatography after refolding of the Gdn-HCl solubilized protein (*19*), indicating that the pro-peptide is not essential for proper folding or stability. Notwithstanding the previous efforts, the procedures reported so far for the refolding of recombinant proteins from inclusion bodies are still based on empirical trials, and efficient procedures are not always formulated.

In this paper, we describe the effects of a non-ionic detergent, Brij-35, and charged amino acids, especially L-Arg, on the refolding and recovery of human matrilysin from inclusion bodies formed in *E. coli* cells, and propose an effective procedure to prepare active matrilysin in good yield.

MATERIALS AND METHODS

Materials—Recombinant *E. coli* cells expressing human prepro-matrilysin bearing the signal peptide of *E. coli* alkaline phosphatase were obtained according to the method reported by Kihira *et al.* (*16*). The cells were treated with a Dyno-Mill (W.A. Bachofen Manufacturing Engineers, Switzerland) and then suspended in 1 M sucrose. The suspension was centrifuged at 7,940 × *g* and the precipitate was washed with 2% Triton X-100 containing 10 mM EDTA·2Na (pH 8.0). The washing and centrifugation were repeated three times, and the resulting wet precipitate was denoted as the inclusion body preparation. It is believed that the signal peptide of the prepro-matrilysin can be removed when the expressed protein is transferred into the inner space of *E. coli* cells, and that pro-matrilysin forms the insoluble inclusion bodies. The protein content in 1 g of the inclusion body preparation was determined to be 180 mg by the method of Lowry *et al.* (*20*) with bovine serum albumin (BSA) as the standard. (7-Methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diamino-propionyl]-L-Ala-L-Arg-NH_2 [MOCAc-PLGL(Dpa)AR, Lot 480429] and MOCAc-L-Pro-L-Leu-Gly (MOCAc-PLG, Lot 471218) were purchased from the Peptide Institute (Osaka). The concentrations of MOCAc-PLGL(Dpa)AR and MOCAc-PLG were determined spectrophotometrically using the molar absorption coefficients $\varepsilon_{410} = 7{,}500\ M^{-1}\cdot cm^{-1}$ and $\varepsilon_{342} = 12{,}900\ M^{-1}\cdot cm^{-1}$ (*21*). EDTA·2Na (Lot CJ034) and CAPSO (Lot HT189) were obtained from Dojindo (Kumamoto, Japan). All other chemicals were of reagent grade and purchased from Nacalai Tesque (Kyoto).

Refolding and Purification of Pro-Matrilysin—Two grams of the inclusion body preparation containing 360 mg protein was solubilized in 6 ml of 0.1 M Tris-HCl buffer (pH 7.5) containing 6 M Gdn-HCl and 0.1 M DTT with gentle stirring for 2 h at 25°C. The solution was diluted with 100 volumes of refolding buffer (50 mM Tris-HCl, 0.1 mM Zn-acetate, 0.2 M NaCl, 10 mM $CaCl_2$, and 0.02% NaN_3, pH 7.5) added with Brij-35 (0-2.0%) or L-Arg (0-1.0 M), and the aggregates formed were removed by paper filtration. The filtrate was dialyzed against 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ and 0.02% NaN_3 (buffer A) at 4°C, and then was concentrated 10-fold by ultra-filtration using a centrifugal concentrator, Amicon Centriprep-10 (Millipore, Bedford, MA), at 3,000 × *g* at 4°C. The concentrated solution (800 μl) was applied to cation-exchange HPLC on a TSKgel SP-5PW column [7.5 mm (inner diameter) × 75 mm] (Tosoh, Tokyo) equilibrated with buffer A. A linear gradient was generated from 0 M to 0.5 M NaCl at time 25 min over 30 min at a flow-rate of 1 ml/min at 25°C. Pro-matrilysin was eluted at about 0.28 M NaCl and collected for further analysis. The concentration of pro-matrilysin was determined using the molar absorption coefficient at 280 nm of 41,000 $M^{-1}\cdot cm^{-1}$ calculated from the amino acid composition (*3*) with a Shimadzu UV-2200 spectrophotometer (Kyoto).

The temperature dependence of the recovery of matrilysin activity was examined by incubating the inclusion body preparation solution containing 6 M Gdn-HCl plus 0.1 M DTT with the refolding buffer (pH 7.5) at various temperatures (4-37°C). The pH dependence of the recovery was examined at 4°C in refolding buffers as below: 50 mM succinate buffer (pH 4.3-5.5), 50 mM MES (pH 5.5-7.0), 50 mM Tris (7.0-9.0), and 50 mM CAPSO (pH 9.0-10.0), all containing 0.1 mM Zn-acetate, 0.2 M NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, and either 1.0% Brij-35 or 1.0 M L-Arg.

Activation of Pro-Matrilysin and the Enzyme Assay—Pro-matrilysin recovered from inclusion bodies was incubated with 1 mM APMA in 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 M NaCl, 10 mM $CaCl_2$, and 0.02% NaN_3 (buffer B) at 37°C for 2 h, unless otherwise indicated. When the activation process was followed by SDS-PAGE, the activation of pro-matrilysin by APMA was stopped by the addition of 2% SDS containing 8% glycerol and 4% 2-MET.

The enzyme activity was measured with a fluorescent substrate, MOCAc-PLGL(Dpa)AR (*21*). It is known that the peptide bond between Gly and Leu residues is cleaved by matrilysin, and the amount of the product MOCAc-PLG was estimated by the fluorescence intensity by comparison with the fluorescence intensity of authentic MOCAc-PLG solution. The substrate was dissolved in dimethyl sulfoxide (DMSO) to 0.290 mM. The matrilysin-catalyzed hydrolysis of the substrate was performed by mixing 1,222 μl of buffer B, 8 μl of the substrate solution, and 20 μl of matrilysin sample at 37°C. The concentrations of the substrate, APMA, and DMSO were 1.85 μM, 16 μM, and 0.6%, respectively. Substrate hydrolysis was measured by following the increase in the fluorescence intensity at 393 nm at excitation 328 nm with a JASCO FP-777 fluorescence spectrophotometer (Tokyo). Hydrolysis was carried out under pseudo-first order conditions, where the substrate concentration is lower than the Michelis constant, K_m, because of the sparing solubility of the substrate and the large fluorescent intensity of the product. The enzyme activity was evaluated by the specificity constant, k_{cat}/K_m (*22*).

SDS-PAGE—SDS-PAGE was performed in a 15-25% gradient gel system (Multigel 15/25, Lot 205RCV, Daiichi Pure Chemicals, Tokyo) under reducing conditions according to the method of Laemmli (*23*). A constant current of 40

mA was applied for 1 h. Proteins were reduced by treatment with 2.5% 2-MET at 100°C for 10 min. Proteins were stained with Coomassie Brilliant Blue R-250. The molecular mass marker kit consisting of rabbit muscle phosphorylase *b* (97.4 kDa), BSA (66.3 kDa), rabbit muscle aldolase (42.4 kDa), bovine erythrocyte carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa), and hen egg-white lysozyme (14.4 kDa) was a product of Daiichi Pure Chemicals (Tokyo).

RESULTS

Effect of Brij-35 and L-Arg on the Recovery of Matrilysin Activity—The recovery of matrilysin activity from unfolded pro-matrilysin by the refolding procedures was evaluated by the matrilysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR. Figure 1 shows that the recovery of matrilysin activity by the refolding procedure at pH 7.5, 4°C, for 12 h depends on the concentrations of Brij-35 and L-Arg, the activity increasing as the concentrations increase. The Brij-35 concentration dependence gave a saturation curve, whereas the L-Arg concentration dependence curve was sigmoidal. The activities reached a maximum at 1.0% Brij-35 and 0.8–1.0 M L-Arg. The maximum activity at 1.0% Brij-35 was 1.60 nM/s, slightly higher than the activity (1.34 nM/s) with 1 M L-Arg. The concentrations of Brij-35 and L-Arg giving half maximum activity were 0.21% and 0.57 M, respectively. The effects of other charged amino acids on the recovery of enzyme activity were also examined at pH 7.5, 4°C, for 12 h. The activities recovered with 1.0 M D-Arg, L-Lys, L-Glu, and L-Asp were 1.31, 0.19, 0.01, and 0.01 nM/s, respectively. The control value obtained without additives was 0.002–0.003 nM/s. The effect of D-Arg was essentially the same as that of L-Arg, while that of L-Lys was considerably less than that of L-Arg. L-Glu and L-Asp had only a small effect. This indicates that a non-ionic detergent, Brij-35, and positively charged amino acids, Arg and Lys, are effective for the recovery of matrilysin activity compared with negatively charged amino acids, and that Arg is much more effective than Lys. Although Brij-35 and Arg are different in structure, 1.0% Brij-35 and 1.0 M Arg produced the highest effect. Among the additives examined, 1.0% Brij-35 was the most effective for the recovery of matrilysin activity from inclusion bodies.

Effect of Temperature on the Recovery of Matrilysin Activity—The recovery of matrilysin activity from unfolded pro-matrilysin during incubation with 1.0% Brij-35 or 1.0 M L-Arg at pH 7.5 was followed at 4, 15, and 25°C (Fig. 2). The activity increased progressively with increases in the incubation time, reaching a maximum within 12 h in every case. The incubation time required to reach 50% maximum activity was less than 30 min in all cases. The maximum recovery was dependent on temperature of the refolding procedures, and decreased with increasing temperature (Fig. 3), suggesting that refolding may proceed more effectively at lower temperatures. The recovery with 1.0% Brij-35 showed a maximum at 4–15°C, and decreased

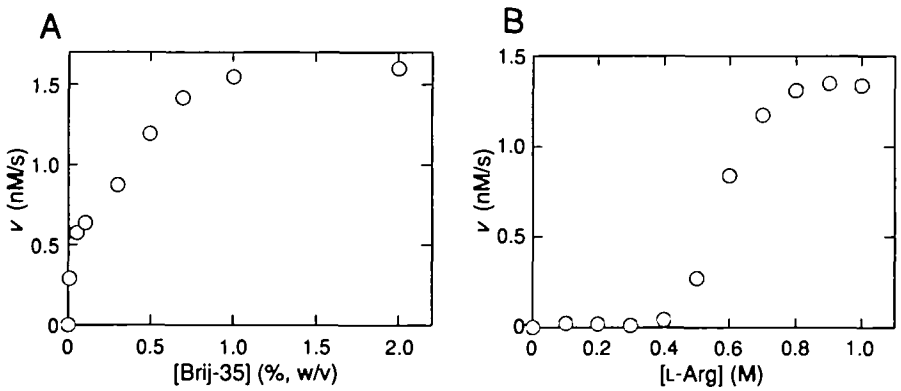

Fig. 1. **Effect of Brij-35 and L-Arg on the recovery of matrilysin activity from the inclusion body preparation.** Pro-matrilysin refolding from inclusion bodies was carried out by incubating the inclusion body preparation in refolding buffer (50 mM Tris-HCl, 0.1 mM ZnOAc, 0.2 M NaCl, 10 mM $CaCl_2$, and 0.02% NaN_3, at pH 7.5) containing Brij-35 (panel A) or L-Arg (panel B) at the concentration indicated at 4°C for 12 h. The inclusion body preparation treated by the refolding procedures was activated by incubating with 1 mM APMA at pH 7.5, 37°C for 2 h. The degree of refolding was estimated by the recovery of matrilysin activity as measured by the hydrolysis of MOCAc-PLGL(Dpa)AR in assay buffer (50 mM Tris-HCl, 0.2 M NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, and 0.06% DMSO, pH 7.5) at 37°C. Initial concentrations of inclusion bodies and MOCAc-PLGL(Dpa)AR were 1.61 μg/ml and 1.85 μM, respectively.

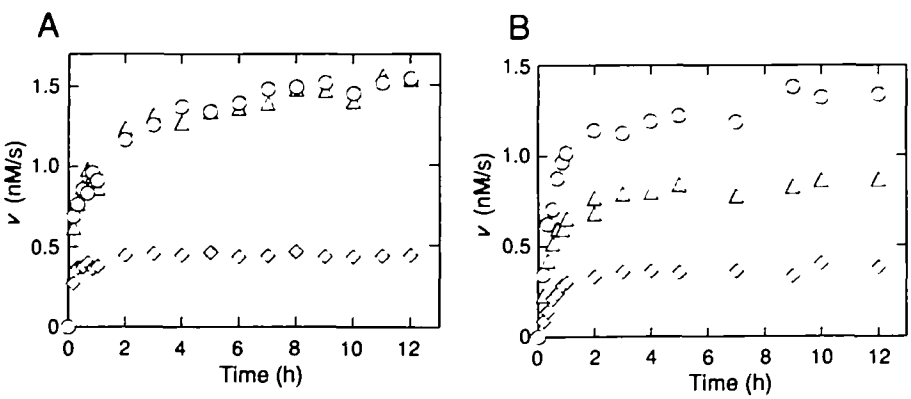

Fig. 2. **Time course of the recovery of matrilysin activity from the inclusion body preparation.** Refolding of the pro-matrilysin from the inclusion body preparation was carried out in refolding buffer (pH 7.5) containing 1.0% Brij-35 (panel A) or 1.0 M L-Arg (panel B) at 4°C (○), 15°C (△), or 25°C (◇) for the period indicated. All other conditions were the same as those described in the legend to Fig. 1.

sharply with the increase in temperature from 15 to 37°C. On the other hand, the recovery with 1.0 M L-Arg decreased progressively with increasing temperature from 4 to 37°C. At 15°C, the recovery with 1.0% Brij-35 was almost 2 times that with 1.0 M L-Arg. In both cases, only slight pro-matrilysin refolding occurred at temperatures above 37°C. The recovery with 1.0 M L-Arg appears to be even higher than that with 1.0% Brij-35 at temperatures below 0°C. As long as the refolding procedures are performed between 4 and 15°C, 1.0% Brij-35 appears to be more suitable than 1.0 M L-Arg.

Effect of pH on the Recovery of Matrilysin Activity—The recovery of matrilysin activity from unfolded pro-matrilysin is dependent on pH of the refolding procedures with either 1.0% Brij-35 or 1.0 M L-Arg (Fig. 4). Refolding of the unfolded pro-matrilysin from the inclusion bodies was examined in the pH range of 4 to 10, at 4°C for 12 h, and the matrilysin activity was measured at pH 7.5, 37°C. A bell-shaped pH-dependence of recovery was observed with both additives, with the effective pH range wider with 0.1% Brij-35 than with 1.0 M L-Arg. The optimal pH was 6.5-8.5 with 1.0% Brij-35 and 6.5-7.5 with 1.0 M L-Arg. The pK_a values of the residues controlling refolding in the presence of 1.0% Brij-35 were estimated to be 4.7 and 9.0, while those in the presence of at 1.0 M L-Arg were 5.2 and 8.2. In both cases, the number of groups with acidic pK_a values was estimated to be 1.5, and those with basic pK_a values 2.0 from the Dixon plots (not shown). The acidic pK_a values (4.7 and 5.2) are close to those of the carboxyl groups of Asp and Glu residues and the carboxyl terminus, and the basic pK_a values (9.0 and 8.2) are close to those of the amino groups of Lys residues and the amino terminus. Acidic and basic groups might be involved cooperatively in the refolding processes. The acidic pK_a shifted to the acidic side by 0.5 pH unit and the alkaline pK_a shifted to the alkaline side by 0.8 pH unit when 1.0% Brij-35 was used in place of 1.0 M L-Arg. Because of the wide pH-range with 0.1% Brij-35 compared with 1.0 M L-Arg, the former additive might be more suitable for the refolding of matrilysin.

Purification of Pro-Matrilysin—SDS-PAGE of the inclusion body preparation solubilized in 0.1 M Tris-HCl buffer (pH 7.5) containing 6 M Gdn-HCl and 0.1 M DTT showed the presence of 31- and 30-kDa proteins (Fig. 5). The 31-kDa protein is prepro-matrilysin, and the 30-kDa protein might be a pro-matrilysin species formed by limited proteolysis of the 31-kDa protein during the formation of inclusion bodies and/or during solubilization. The inclusion body preparation was subjected to the refolding procedures in the presence of 1.0% Brij-35 or 1.0 M L-Arg, and then was applied to cation-exchange HPLC on a TSKgel SP-5PW column. HPLC of the solution treated with 1.0 M L-Arg was difficult because of the high ionic strength. In order to bind the pro-matrilysin species to the gel, the L-Arg concentration must be below 1 nM, and repeated dialysis and gel-filtration procedures were needed. On the other hand, the solution treated with 1.0% Brij-35 was

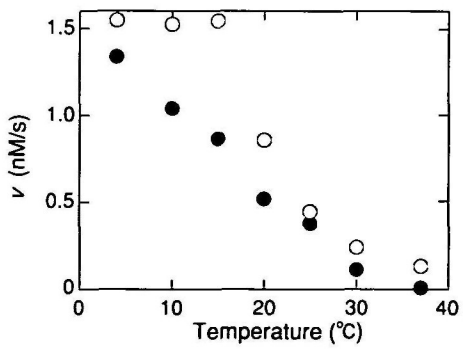

Fig. 3. **Temperature dependence of the recovery of matrilysin activity from the inclusion body preparation.** The refolding of pro-matrilysin from the inclusion body preparation was carried out in refolding buffer (pH 7.5) containing 1.0% Brij-35 (○) or 1.0 M L-Arg (●) for 12 h at the indicated temperature. Other conditions were the same as those described in the legend to Fig. 1.

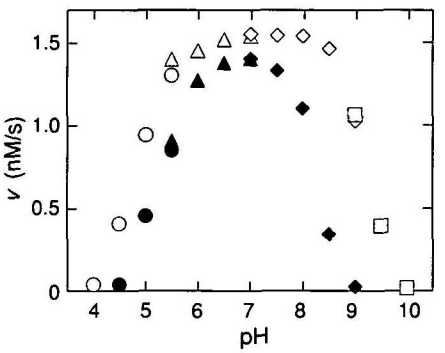

Fig. 4. **pH-dependence of the recovery of matrilysin activity from the inclusion body preparation.** The refolding of pro-matrilysin from the inclusion body preparation was carried out in various 50-mM buffers containing 0.1 mM ZnOAc, 0.2 M NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, and 1.0% Brij-35 (open symbols) or 1.0 M L-Arg (closed symbols) at 4°C. Buffers were as follows: succinate (○), pH 4.0-5.5; MES (△), pH 5.5-7.0; Tris (◇), pH 7.0-9.0; and CAPSO (□) pH 9.0-10.0. Other conditions were the same as those described in the legend to Fig. 1.

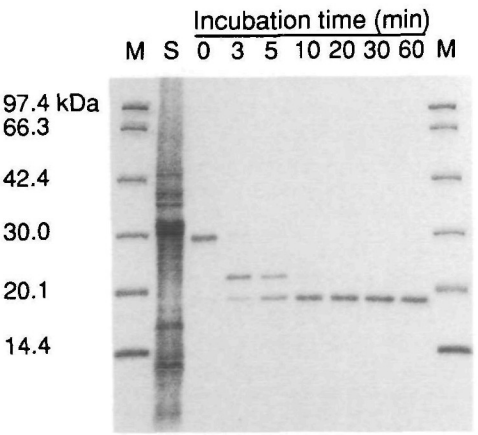

Fig. 5. **SDS-PAGE of pro-matrilysin activated with APMA.** Pro-matrilysin was purified by cation-exchange HPLC, and activated by incubation in 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 M NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, and 1 mM APMA at 37°C for the period indicated. The activation was stopped by adding 2% SDS containing 8% glycerol and 4% 2-MET. Each lane contains 0.46 μg pro-matrilysin. Lane M, molecular mass markers. Lane S, inclusion body preparation containing 2.36 μg protein, solubilized with 8 M urea plus 0.1 M DTT. Although 6 M Gdn-HCl was used to solubilize the inclusion body preparation throughout this study, the solubilization for lane S was done with 8 M urea instead of 6 M Gdn-HCl, because Gdn-HCl disturbs the migration of proteins in SDS-PAGE.

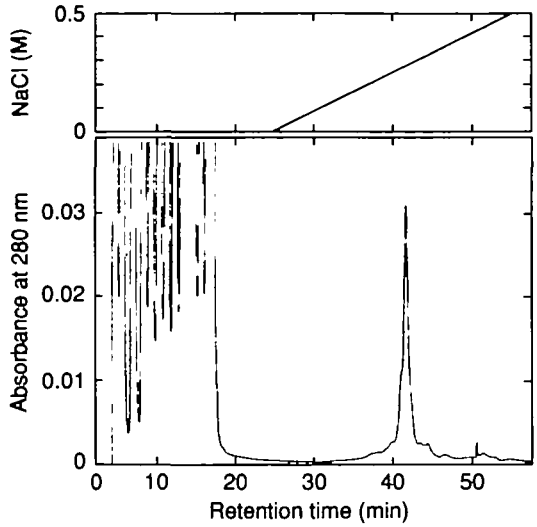

Fig. 6. **Purification of pro-matrilysin by cation-exchange HPLC on TSKgel SP-5PW.** The inclusion body preparation (wet, 250 mg) containing 45 mg protein was solubilized with 0.75 ml of 6 M Gdn-HCl plus 0.1 M DTT, and treated with 75 ml of refolding buffer containing 0.1% Brij-35 at pH 7.5, 4°C, for 12 h. The solution was dialyzed against buffer A, and then subjected to ultra-filtration. The resulting solution (7.5 ml) was subjected to HPLC. 800 μl of solution containing 4.8 mg protein was injected onto the column equilibrated with buffer A, and eluted with a linear gradient of NaCl from 0 M to 0.5 M generated from 25 min to 55 min in buffer A at 25°C. The elution was monitored by the absorbance at 280 nm. The flow-rate was 1.0 ml/min. The fraction eluting from 40 to 43 min was collected and shown to contain 0.32 mg purified pro-matrilysin. The HPLC conditions are described in the text.

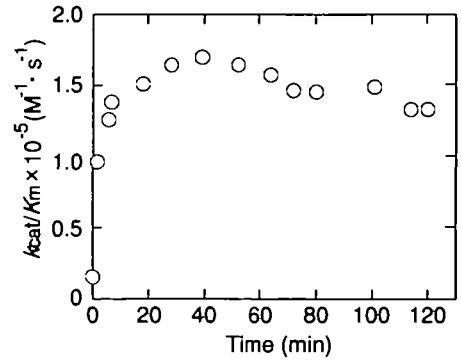

Fig. 7. **Activation of pro-matrilysin by APMA.** Pro-matrilysin (1.86 nM) purified by HPLC was incubated with 1 mM APMA in buffer B (pH 7.5) at 37°C. Twenty microliter aliquots of the reaction mixture were removed at the indicated times, and added to 1,230 μl of substrate solution. The concentrations of pro-matrilysin and MOCAc-PLGL (Dpa)AR during hydrolysis were 0.030 nM and 1.85 μM, respectively. The hydrolysis was carried out under pseudo-first order conditions, where the substrate concentration is lower than the Michelis constant, K_m, because of the sparing solubility of the substrate and the large fluorescent intensity of the product. The enzyme activity was evaluated by the specificity constant, k_{cat}/K_m.

dialyzed against buffer A free of Brij-35, and applied successfully to HPLC after concentrating the dialyzed solution 10 times (Fig. 6). The dialyzed solution prepared from 0.25 g of inclusion body preparation (wet) containing 45 mg of protein was applied to HPLC. It was estimated that 3.7 mg of 30-kDa pro-matrilysin was contained in the dialyzed solution based on the matrilysin activity assuming that the activity resulted from the conversion of the pro-form to the active 19-kDa form (see below). The pro-matrilysin fraction (40-43 min) eluting at around 0.28 M NaCl was collected. It contained 3.0 mg of protein, and showed a single 30 kDa band on SDS-PAGE (Fig. 5).

Activation of Pro-Matrilysin—The pro-matrilysin fraction collected from cation-exchange HPLC was subjected to the activation procedure with APMA. The time dependence of the activation was monitored by SDS-PAGE (Fig. 5). At the incubation time indicated, the activation of pro-matrilysin with 1 mM APMA at pH 7.5, 37°C was stopped by adding 2% SDS containing 8% glycerol and 4% 2-MET. The 30-kDa band of pro-matrilysin disappeared progressively with time, and disappeared completely by 5 min. New bands at 22 and 19 kDa appeared correspondingly. The former, however, disappeared at 20 min, while the latter remained even after incubation for 60 min. The time-dependence observed in Fig. 5 suggests that the 30-kDa pro-matrilysin is converted first to the 22-kDa species, and then to the 19-kDa species, which is the active form of matrilysin. Here, it should be noted that the conversion of pro-matrilysin to the 22-kDa intermediate in the presence of APMA was complete in 3-5 min, and that the auto-catalytic conversion of the intermediate to the active form was also likely to finish within minutes. The first-order rate constants for both reactions were estimated to be 0.1-1 min^{-1}.

The activity of matrilysin in the hydrolysis of MOCAc-PLGL(Dpa)AR increased during the incubation of pro-matrilysin with APMA (Fig. 7). The activity (k_{cat}/K_m) increased rapidly, and 75% of the reaction was complete within 5-6 min. The activity then continued to increase gradually and reached the maximum level of 1.7×10^5 $M^{-1} \cdot s^{-1}$ after 30-40 min. The half-life, $t_{1/2}$, for the activity to reach half the maximum level was 1.2 min. The time-dependence shown in Fig. 7 suggests that there might be at least two phases in the activation of pro-matrilysin by APMA (see "DISCUSSION"). After the activity reaches a maximum at 30-40 min, it then decreases gradually with further incubation with APMA reaching 1.3×10^5 $M^{-1} \cdot s^{-1}$ at 120 min. This activity was then maintained even after incubation for 3 h. The time-dependent increase in the activity observed up to 40-50 min was similar to that of the appearance of the 19-kDa band of matrilysin (Fig. 5), suggesting that the 30-kDa pro-matrilysin is converted completely to the active form of matrilysin by the activation procedures.

DISCUSSION

Refolding of Pro-Matrilysin—A procedure introduced for the refolding of MMP-3 has thus far been commonly used to refold other MMPs (*15*). In this procedure, the refolding buffer is composed of 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 5 mM $CaCl_2$, 0.05% Brij-35, 0.02% NaN_3, 0.1 mM Zn-acetate, and 10 mM oxidized GSH. A shortcoming of this procedure is the high cost due to GSH when applied to the large-scale preparation of MMP. We have modified this refolding buffer for the refolding pro-matrilysin, and show that 1.0% Brij-35 or 1.0 M L/D-Arg can be used preferably, and that GSH can be omitted from the refolding

buffer. The activity of the matrilysin obtained by our procedure is even higher than that so far reported (*24*), suggesting that the absence of GSH does not interfere with the refolding. It is interesting that the effect of Lys is much lower than that of Arg, and that Asp and Glu have substantially no effect. L-Arg has been successfully applied to the refolding of recombinant human proteins from inclusion bodies, such as single-chain urokinase (*25*), Fab fragments (*26*), immunotoxins (*27*), γ-interferon (*28*), neurotrophin-3 (*29*), Bcl-2 protein (*30*), and diphtheria toxin fused with granulocyte-macrophage colony-stimulating hormone (*31*). The L-Arg concentration in these cases is 0.2-0.5 M, considerably lower than that (1.0 M) used in the present study. Arg carries a guanidino group that is similar to Gdn-HCl, and is known to have a weak labilizing effect on protein structure. It may labilize incorrectly folded or improperly associated protein molecules. This effect may be favorable for protein folding. In addition, Arg is used as a suppressor of the aggregation that occurs during the refolding of proteins (*26, 27, 29*). Presently, we have no evidence about the function of Arg or Brij-35 in refolding. Concerning the effect of Arg on the refolding of Fab fragments and immunotoxins, they are compact proteins composed mainly of seven-stranded β-sheet domains. Matrilysin is a single-domain protein composed of a five-stranded β-sheet and three α-helices. Arg may promote preferably the formation of β-structure. One possibility that Arg stabilizes the refolding structure by binding to the active site formed nascently cannot be ruled out, and the effect of L-Arg on the refolding of urokinase (*25*) might be an example of such a case. It is noted that the pK_a values for the residues controlling the refolding of matrilysin (Fig. 4) are close to those (4.60 and 8.65) estimated from the pH-dependence of the matrilysin activity (*24*). This suggests the possibility that the catalytic residues of matrilysin might participate in its refolding as well.

Activation of Pro-Matrilysin—The AMPA-mediated activation of human pro-matrilysin (30 kDa) expressed by NSO mouse myeloma cells has been studied extensively (*24*). It has been shown that an intermediate (21 kDa) is produced as a result of cleavage of the peptide bond between Glu63 and Ile64 of pro-matrilysin, followed by auto-cleavage at Glu77-Tyr78 to produce the active form (19 kDa). The time-dependent conversion of pro-matrilysin to the active form (Fig. 5) suggests that the APMA-mediated activation of human pro-matrilysin expressed in *E. coli* proceeds by the same mechanism as that in myeloma. The matrilysin activity increased immediately upon incubating pro-matrilysin with APMA, and 75% of the reaction was complete within 5-6 min, although it took an additional 30-40 min to reach the maximum (Fig. 7). This time-dependence suggests that there might be at least two phases in the activation of pro-matrilysin by APMA. One possibility is that there might be at least two forms of 30-kDa pro-matrilysin, properly-folded and improperly-folded forms, and the former is activated more rapidly than the latter. If this assumption is correct, then 75% of the pro-matrilysin molecules might be in the properly-folded form to be activated by APMA, while 25% are improperly folded and are converted to the properly-folded form during the 2nd phase ($t_{1/2} = 20$ min). On the other hand, it is possible that the 22-kDa intermediate might bear the enzyme activity. In this case, the activity of $1.0\text{-}1.4\times10^5$ $M^{-1}\cdot s^{-1}$ observed in the first phase may be due to the intermediate, while the 1.7×10^5 $M^{-1}\cdot s^{-1}$ activity is due to the active form. After reaching the maximum at 30-40 min, the activity decreases to 1.3×10^5 $M^{-1}\cdot s^{-1}$ with further incubation. This decrease might be caused by the inhibition of matrilysin by APMA and/or by auto-digestion of the active matrilysin.

In summary, starting with 2.0 g of inclusion body preparation (wet) containing 360 mg protein, 29.5 mg of pro-matrilysin (30 kDa) was recovered in the solution obtained after the refolding procedures with 1.0% Brij-35 at pH 7.5 and 4°C for 12 h. By subjecting the solution to HPLC on TSKgel SP-5PW, the 30-kDa pro-matrilysin (24.0 mg) was purified to homogeneity accompanied by a 15-fold increase in purity at a yield of recovery of 81.3%. The 30-kDa pro-matrilysin was converted entirely to the 19-kDa active form of matrilysin by activation with APMA, and 15.2 mg of matrilysin was obtained. The activity k_{cat}/K_m of the purified matrilysin was 1.7×10^5 $M^{-1}\cdot s^{-1}$, although it decreased to 1.3×10^5 $M^{-1}\cdot s^{-1}$ upon prolonged treatment with APMA.

Effect of High Salt Concentration on Metalloproteinases—Matrilysin, together with other members of the MMP family, shares some amino acid sequence homology with thermolysin around thermolysin residue Glu143 (*32*). This residue is thought to be involved in catalysis by a general base mechanism. His231 is considered to play a role in stabilizing the tetrahedral intermediate during thermolysin catalysis. An equivalent of the thermolysin residue cannot be readily identified in matrilysin (*32*), and confirmation of the catalytic mechanism must await further structural and kinetic studies. We have reported that both the activity and stability of thermolysin are remarkably enhanced in the presence of high concentrations (1-5 M) of salts (*22, 33-37*), and suggested that thermolysin is not only a thermophilic but also a halophilic enzyme. These properties of thermolysin provide appropriate probes with which to study the catalytic mechanism of this enzyme as well as the effect of site-directed mutagenesis (*38, 39*) and chemical modification (*40*). In the present study, we have shown that the refolding of matrilysin is promoted by high concentrations of Arg, suggesting that matrilysin may be stable in concentrated salt solutions. The pH-dependence and temperature-dependence of pro-matrilysin refolding by Brij-35 and Arg are likely to be similar to those of the salt-induced activation of thermolysin (*35*), suggesting that there might be a common principle between them. The catalytic mechanism of matrilysin might be studied by comparing data with those obtained with thermolysin.

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