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Function of the Propeptide Region in Recombinant Expression of Active Procathepsin L in *Escherichia coli*[1]

Tetsuya Ogino,* Toshio Kaji,* Mitsuhiko Kawabata,* Kazumasa Satoh,* Koji Tomoo,* Toshimasa Ishida,*,[2] Hiroshi Yamazaki,† Kazumi Ishidoh,‡ and Eiki Kominami‡

*Department of Physical Chemistry, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094; and †Department of Parasitology and ‡Department of Biochemistry, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421

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In order to determine the functional role of the procathepsin L propeptide region for the preparation of active recombinant rat cathepsin L (CL), cDNAs encoding two short-length propeptides (C-terminal 2 and 27 residues) and the full-length (96 residues) one plus the entire CL were expressed as two soluble fusion proteins with a fragment of maltose-binding protein and an insoluble fusion protein with glutathione-S-transferase in *Escherichia coli*, respectively. After refolding of the insoluble fusion protein, each gene product was purified to homogeneity by amylose or glutathione-Sepharose-4B affinity column, and digestion with factor Xa and α-thrombin under alkaline conditions (pH ~8.0) led to the elution of two pure short-length procathepsin Ls (PCLs) and a full-length one, respectively. The enzymatic activity, estimated by hydrolytic assaying of benzoxycarbonyl-Phe-Arg-7-(4-methyl)coumarylamide under acidic conditions (pH 5.5), indicated that the two short-length PCLs exhibited in a great loss of the activity, as compared with the full-length PCL. The CD spectra of the short-length PCLs were different from that of the full-length one. The present results clearly show that the full-length propeptide is essential for construction of the active tertiary structure of CL at the stage of recombinant protein expression, although the expression of CL itself in *E. coli* does not require the propeptide. Based on the tertiary structure of PCL, the propeptide region necessary for the construction of the CL active structure has been discussed.

Key words: activity, autoprocessing, CD spectra, fusion protein, propeptide, rat procathepsin L, recombinant protein expression.

Cathepsin L [EC 3.4.22.15] (hereafter abbreviated as CL) is the most active of the lysosomal cysteine proteases with regard to the ability to hydrolyze azocasein (*1*), elastin (*2*), collagen (*3*), and class II-associated Ii peptide (*4*), and plays a major role in intracellular protein proteolysis because it is most commonly found in lysosomes (*5*). Since evidence has been accumulating of its action in tumor invasion and metastasis (*6*), bone resorption (*7*), and rheumatoid arthritis (*8*), the structure-activity relationship of CL has been receiving increasing attention for the characterization of its biological function and the development of a specific inhibitor.

CL is synthesized as a preproenzyme in the cell, which is processed to a proenzyme and then to a mature form, *i.e.* either a single polypeptide or two-chains of an heavy chain involving an active cysteine residue and a light chain, which are linked by a disulfide bond (*1*). In order to obtain a ready source of easily purifiable cathepsin L for studies on its structural/biological function, some systems for gene expression have been constructed (*9-11*). For the efficient preparation of active recombinant CL, generally, a cDNA clone has been expressed as procathepsin L (PCL) and then converted to the mature form through autoprocessing. Although the propeptide region of PCL has been proposed to be important for gene expression, folding, and/or processing (*9, 12*), it remains to be determined for which biological process the propeptide is absolutely necessary. In this study, thus, the function of the propeptide, to prepare an active recombinant CL, was investigated by means of recombinant protein expression, folding, and processing/activity examination of three kinds of rat PCLs, *i.e.*, two short-length propeptides (C-terminal 2 and 27 residue propeptides), and a full-length one (96 residues) plus the entire CL.

MATERIALS AND METHODS

Materials—Enzymes (T4 DNA ligase, T4 polynucleotide kinase, restriction endonucleases, RNase A, α-thrombin,

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[2] To whom correspondence should be addressed.
Abbreviations: β-ME, β-mercaptoethanol; CD, circular dichroism; CL, cathepsin L; DTT, dithiothreitol; GST, glutathione-S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; PCL, procathepsin L; P2CL, C-terminal 2 residues of propeptide plus entire CL; P27CL, C-terminal 27 residues of propeptide plus entire CL; P96CL, full-length (96 residues) propeptide plus entire CL; Z-Phe-Arg-MCA, benzyloxycarbonyl-Phe-Arg-7-(4-methyl)coumarylamide.

and factor Xa) were purchased from Takara Shuzo, Toyobo, Bethesda Research Laboratories, P-L Biochemicals, Novagen, or New England Biolabs. As the bacterial strain for the transformations, *Escherichia coli* BL21 (Novagen) was finally used. The pGEX-4T-2 [a glutathione-S-transferase (GST) gene fusion plasmid] and pMAL-p2 [a maltose binding protein gene fusion plasmid] were purchased from Pharmacia Biotech. and New England Biolabs, respectively. The affinity resins for purification [glutathione-Sepharose 4B and amylose resins] were purchased from Pharmacia Biotech. and New England Biolabs. Sequencing kits were obtained from Toyobo. Gene mutation was carried out using the site-directed mutagenesis system, MutanTM-K (Takara Shuzo). The markers for molecular weight estimation were obtained from Bio-Rad. Other commercially available materials used were of reagent grade or higher quality. The cDNA for rat PCL was prepared according to the previous paper (*13*), and some restriction sites for the construction of an expression vector were created by means of site-directed mutagenesis.

Construction of an Expression Vector—To insert rat full-length PCL cDNA into the multi-cloning site of the pGEX-4T-2 plasmid, restriction sites for *Sal*I and *Not*I were constructed at both the N- and C-terminal sites of the cDNA by the PCR method. The cDNA was then phosphorylated with ATP (10 nM) using T4 polynucleotide kinase (5 units) in 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$, 10 mM β-ME, and 1 mM spermine (total volume, 10 ml) at 37°C for 60-90 min. The gene was then annealed by heating to 75°C for 15 min and gradual cooling to room temperature over a period of 4 h in the presence of 66 mM Tris-HCl buffer (pH 7.6) containing 6.6 mM $MgCl_2$ and 0.5 mM ATP. On the other hand, the pGEX-4T-2 plasmid was cleaved with restriction enzymes *Sal*I (20 U) and *Not*I (20 U); the cleavage was performed in 10 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 7 mM $MgCl_2$, 7 mM β-ME, and 0.01% Triton (for *Not*I) at 37°C for 2-12 h, and the reaction was stopped by heating to 65°C for 5 min. The phosphorylated cDNA of PCL (0.5 pM) was then added to 66 mM Tris-HCl buffer (pH 7.6) containing 6.6 mM $MgCl_2$, 0.5 mM EDTA, and 10 mM β-ME, and then religated with the plasmid by means of T4 DNA ligase (200 U) at 16°C for 4 h, the pGEX-4T-P96CL vector thus being formed (Fig. 1a); the base sequence of the P96CL region was confirmed by the standard dideoxy-sequencing procedure.

For the construction of expression vectors for the short-length (2 and 27 residues) PCLs, the pMAL-p2 plasmid was used. The *Eco*RI-*Hin*dIII or *Pst*I-*Hin*dIII restriction sites of the plasmid and cDNA were utilized to construct the expression vector, pMAL-p2-P27CL (Fig. 1b) or pMAL-p2-P2CL (Fig. 1c), for the C-terminal 27 or 2 residue propeptide plus the entire CL, respectively, the gene manipulation being performed in the same manner as for the full-length CL, according to the previously reported procedure (*13*).

Recombinant Protein Expression—Calcium chloride-treated *E. coli* cells (100-200 μl), which had been prepared according to the Morrison method (*14*), were transformed with a vector [pGEX-4T-P96CL] (10-20 μl), and then grown in 2×YT medium containing 100 μg/ml ampicillin (1% seeding in 3 ml medium) at 37°C in a jar fermenter. The gene was induced by the addition of IPTG (0.1 mM). Induction was continued for 12 h, after which the cells were harvested. The cells were disrupted by sonication at 20 kHz and 100 W (30 s, 10 times; Model UD-200, Tomy). The pellet and supernatant were separated by centrifugation (15,000 rpm at 4°C for 30 min). On the other hand, the *E. coli* cells transformed with the pMAL-p2-P2CL or -P27CL vector were plated on ampicillin (20-50 mg/ml) containing M9 casamino acid medium (300 ml) at 37°C in a jar fermentor. After fermentation for 3 h, the gene was induced by the addition of 0.4 mM IPTG. Induction was continued for 8 h, and then the cells were harvested and disrupted.

The whole cell proteins were analyzed by 15% SDS-PAGE and Western blotting using antibodies to CL.

Purification of a Fusion Protein and Isolation of PCL. Full-Length PCL—The pellets were resuspended in the dissolution buffer [8 M urea, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 50 mM NaCl, 10 mM DTT] and then stood for 20 h at 4°C. After centrifugation at 15,000 rpm for 1 h, the combined supernatants were subjected to renaturation according to either of the following two different methods. One was the dropwise method, where the supernatants were dropwise (2 ml/1 h) added to 300-400 volumes of the renaturation buffer [50 mM potassium phosphate (pH 10.7), 5 mM EDTA, 1 mM reduced glutathione, 0.1 mM oxidized glutathione], stirred overnight at 4°C, adjusted to pH 8.0 with HCl, and then subjected to DEAE anion exchange chromatography with a buffer comprising 50 mM potassium phosphate (pH 8.0), 5 mM EDTA, and 2 mM cysteine. The other one was the dialysis method, where the solubilized denatured protein was dialyzed in a tube against a buffer comprising 100 mM Tris-HCl (pH 8.6), 0.015% Triton X-100, 3 mM cysteine, and 0.1 mM cystine, followed by stirring overnight at 4°C. The renatured protein solution was then applied at the flow rate of 10 ml/h to a 2 ml glutathione-Sepharose 4B affinity column equilibrated with buffer A [140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4] and washed with the same buffer until the optical density returned to the base line. The fusion protein, GST-P96CL, was then eluted with buffer B (buffer A+100 mM glutathione). On the other hand, the GST-bound column was reacted with 0.5-2.0 units/mg of α-thrombin in 20 mM Tris-HCl (pH 8.4) buffer containing 150 mM NaCl and 2.5 mM $CaCl_2$ at 20°C for 20 h, and then P96CL was eluted with 50 mM Tris-HCl buffer (pH 8.0) and purified by ion-exchange chromatography on a Mono-Q column.

Short-Length PCLs—Bacteriolysis of the cultured cells was carried out for 1 h at 37°C by adding lysozyme (60 μg/ml), followed by sonication. After 30 min centrifugation at 8,000 rpm, the supernatant was obtained by filtration and then subjected to affinity-chromatography (amylose resin); a buffer consisting of 20 mM Tris-HCl (pH 7.5) and 0.5 M NaCl was used for the evaluation, and the same buffer containing a 10 mM maltose was used for the elution. The digestion of the fusion protein with factor Xa was performed for 24 h at 25°C in the elution buffer, which contained the same volume of water, 2 mM $CaCl_2$, and factor Xa at the weight ratio of 1/400 with respect to the fusion protein. The purification of the isolated P2CL or P27CL was performed in the same way as for the full-length PCL.

Enzyme Assaying of CL—Since the recombinant PCL is known to cause autohydrolysis under acidic conditions, its

enzyme activity was determined by assaying of the isolated recombinant PCL with Z-Phe-Arg-MCA (*1*). The assay was performed at 37°C using a system comprising 20 mM acetate-EDTA buffer (pH 5.5) containing 8 mM cysteine, 50 mg/ml fusion protein, and 40 mM Z-Phe-Arg-MCA; although the reaction proceeded faster at pH 4.0, the experiment was performed at pH 5.5 to obtain reliable data.

Circular Dichroism Measurements—Circular dichroism (CD) spectra were recorded with a Jasco J-20C spectropolarometer with a DP-500N data processor using 2-mm path-length cells. The sample temperature in the cuvette was regulated with a circulating water bath and was kept at 25°C. The recombinant PCLs, dissolved in 20 mM phosphate buffer (pH 7.0), were preincubated for several hours before recording the spectra. The concentrations of PCLs were adjusted to about 20 μM. The results were expressed in terms of molar ellipticity [θ].

RESULTS

Construction of a PCL Expression Vector—The cDNA used for recombinant rat PCL consists of a pre-peptide (N-terminal 17 residues), a pro-peptide (96 residues), and the full sequence of mature CL (221 residues). The expression system for the full-length PCL gene was constructed as a fusion with the GST protein using the pGEX-4T-2 plasmid and host bacterial strain BL21. To construct the expression vector (pGEX-4T-P96CL), the plasmid was digested with *Sal*I-*Not*I restriction enzymes, and then recovered by 1% (w/v) agarose gel electrophoresis. The plasmid and a cDNA fragment of PCL were then religated

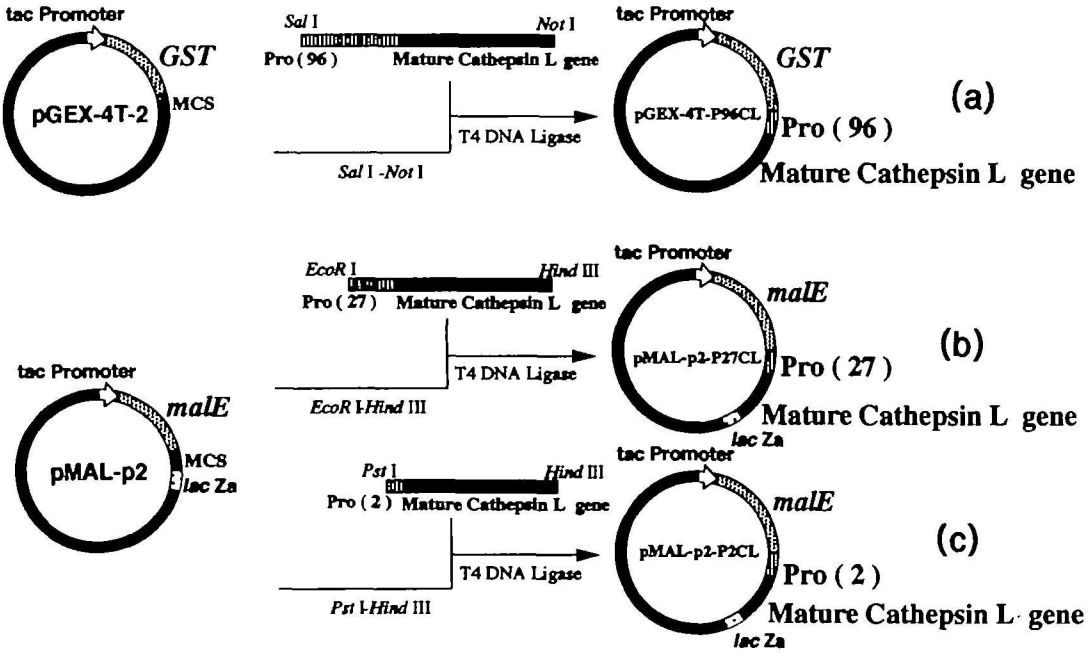

Fig. 1. **Expression plasmid construction of the full-length PCL as an insoluble fusion protein with GST, pGEX-4T-P97CL (a), and short-length PCLs as soluble fusions with the maltose-binding protein, pMAL-p2-P27CL (b), and pMAL-p2-P2CL (c).**

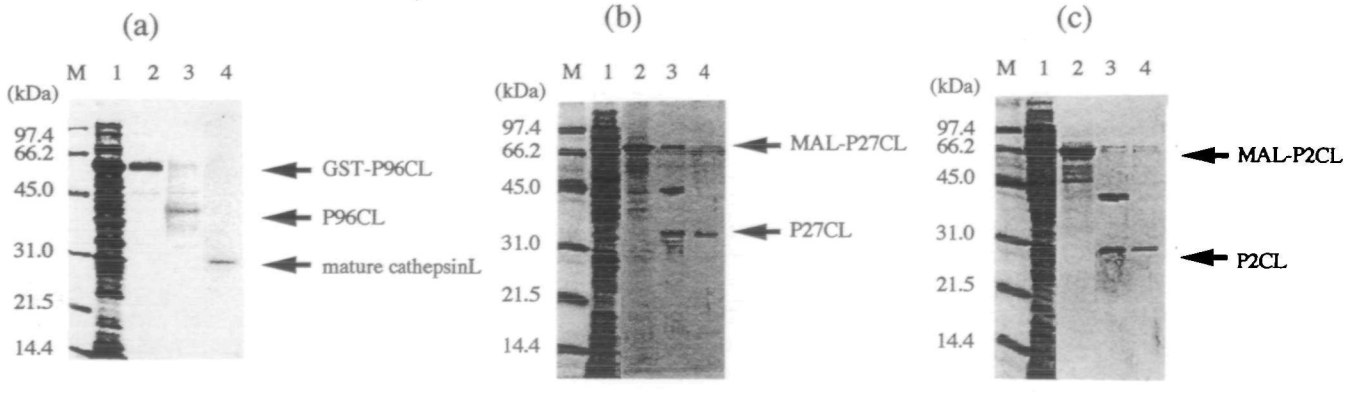

Fig. 2. **SDS-PAGE analyses of the P96CL (a), P27CL (b), and P2CL (c) fusion proteins, and their separation by enzymatic digestion.** Lane 1, whole cell extract; lane 2, fusion protein bound to a glutathione or amylose affinity column; lane 3, digestion of the fusion protein with α-thrombin or factor Xa; lane 4, mature CL produced on autoprocessing of the full-length PCL or the short-length PCLs at pH 5.5. The positions of the fusion, PCL, and CL are indicated by arrows, respectively.

using T4 DNA ligase (Fig. 1a). The *E. coli* BL21 strain cells were transformed with the vector on ampicillin-containing LB medium, the rat PCL cDNA being finally expressed as an insoluble fusion with the GST protein (Fig. 2a, lane 1).

On the other hand, two short-length PCLs were both expressed as soluble fusions with the maltose binding protein (Fig. 1, b and c). To construct a expression vector, the pMAL-p2 plasmid and cDNA were digested with *Eco*RI-*Hin*dIII or *Pst*I-*Hin*dIII restriction enzymes, and the respective fractions were recovered by 1% (w/v) agarose gel electrophoresis. The plasmid and the cDNA fragments were then religated using T4 DNA ligase. The resulting vectors, named pMAL-p2-P27CL or pMAL-p2-P2CL, encode a 27 and 2 propeptide sequence plus the entire CL, respectively. The *E. coli* BL21 strain was then transformed with these vectors on ampicillin-containing LB medium. The induction of the tac promoter by IPTG led to the emergence of a 72 kDa band for pMAL-p2-P27CL and a 68 kDa one for pMAL-p2-P2CL on 10% SDS-PAGE (Fig. 2, b and c, lane 1) of the supernatants; these bands correspond to the molecular weights of the mature CL plus the 27 and 2 propeptide residues, respectively.

Purification and Isolation of a Recombinant PCL—The full-length PCL expressed as an insoluble fusion protein was solubilized by the addition of urea and renatured by either of the dropwise or dialysis method; no notable difference was observed between the methods, although the former method was a little superior to the latter one. Purification of the renatured fusion protein was performed on a glutathione-Sepharose 4B affinity column. After the column had been thoroughly washed with buffer A, the purified fusion protein was obtained by elution with buffer B (Fig. 2a, lane 2). On the other hand, the bound fusion protein, GST-P96CL, was digested with α-thrombin on the

TABLE I. **Catalytic activity of recombinant PCL autoprocessed under pH 5.5.**

Enzyme	P96CL	P27CL	P2CL
μmol/min/mg[a]	18.38	0.02	0.01
Deviation of significance[b]	+	+	$F_s - F_c < 0.1\%$

[a]The value corresponds to μmol of MCA produced by the enzyme from 1 mg of Z-Phe-Arg-MCA in 1 min. The value was determined from the fluorescence intensity of MCA. [b]F_s and F_c represent the fluorescence intensities of the sample and standard, respectively. The limit of detention was $F_s - F_c < 0.1\%$ for the fluorometer used in this work, and + implies $F_s - F_c > 0.1\%$.

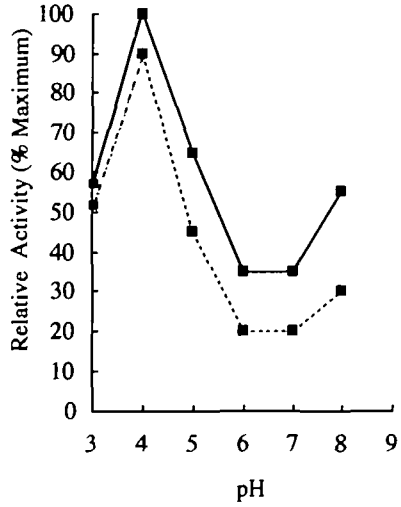

Fig. 3. **pH activity profiles of short-length PCLs for the hydrolysis of Z-Phe-Arg-MCA. P27CL (—) and P2CL (·····).** The activity of P27CL at pH 4.0 is taken as 100%, and the other values are given as relative values (%) with respect to it.

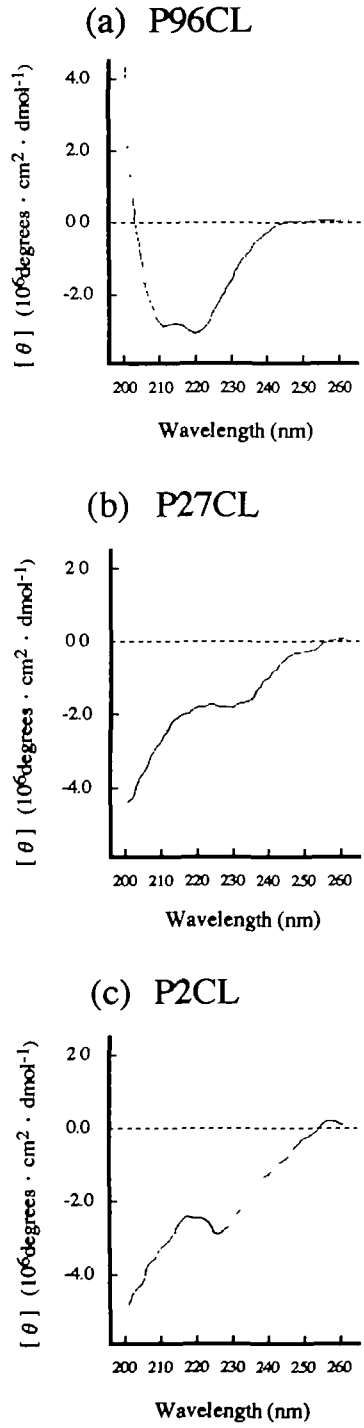

Fig. 4. **CD spectra of the purified recombinant full-length PCL (a) and two short length PCLs, *i.e.* 27 residue (b) and 2 residue (c) ones.** Samples were dissolved in 20 mM phosphate buffer (pH 7.0).

equilibrated affinity column and then eluted with buffer A (Fig. 2a, lane 3), where the scission of GST-P96CL was most effective in the case of 1.5 units/mg (ratio of α-thrombin per fusion protein). About 0.4 mg of pure recombinant PCL was obtained from 8.5 g *E. coli* cells. The 40-kDa form, which corresponds to the molecular mass of the full-length PCL, was obtained almost entirely as a 29-kDa band on ion-exchange chromatography at pH 5.5, which is of the molecular mass expected for the autoprocessed CL (Fig. 2a, lane 4).

On the other hand, the purification procedure for the short-length PCLs was carried out according to the scheme shown in Fig. 2, b and c (lanes 2-4). The soluble fraction obtained on centrifugation was purified by affinity chromatography on amylose resin (lane 2). After proteolysis with factor Xa (lane 3), the short-length PCLs were purified by ion-exchange chromatography on a Mono-Q column at pH 7.5 (lane 4). About 8 mg (P2CL) and 18 mg (P27CL) of the pure short-length PCLs were obtained from 5 g of *E. coli* cells.

Catalytic Activity of the Recombinant Rat CL—The catalytic activities of the two short-length PCLs and the full-length one autoprocessed under acidic conditions (pH 5.5) were measured against Z-Phe-Arg-MCA, an optimal synthetic substrate for CL, according to the method of Barrett and Kirschke (*1*). The results are given in Table I. In contrast with the full-length PCL, the short-length PCLs (both P2CL and P27CL) exhibited a great loss of activity. Since no notable difference was observed in the PAGE band position between the short-length PCLs isolated at pH 7.7 and processed at pH 5.5, this may be due to that autoprocessing had not taken place, as judged from the results of N-terminal amino acid analysis, although the pH-profile of activity (Fig. 3) showed signs of active CL (*9*).

CD Spectra—The CD spectra of the three kinds of PCLs are shown in Fig. 4; nearly the same spectra were obtained for the respective autoprocessed PCLs. It is obvious that the tertiary structures of the two short-length PCLs, although they have similar structures, are quite different from that of the full-length PCL. The latter spectrum would reflect the active structure of CL, because almost the same spectrum has been reported for the active form of recombinant human full-length PCL (C25S mutant) (*15*).

DISCUSSION

It has been reported that the propeptide region of PCL functions as a regulator of the catalytic activity and stability of CL in nonlysosomal compartments (*16, 17*), and is essential for the proper folding of CL in mammalian cells (*12*). On the other hand, it has also been reported that the propeptide is required for the renaturation of insoluble human CL expressed in *E. coli* (*9*). However, it remains unclear at which step the propeptide functions importantly in the processing of active CL, *i.e.*, the recombinant protein expression, the renaturation, or the autoprocessing of PCL. Therefore, we have attempted the preparation of proregion-altered recombinant PCLs and their autoprocessing to CL. With reference to the crystal structure of human recombinant full-length PCL (*18*), several proregion sizes of PCL were considered to correspond to the functional structural fragments, and three proregion-altered cDNAs were finally used in this study, based on the intended expression conditions. The full-length P96CL was expressed as an insoluble fusion with GST, which necessitated solubilization and consequent careful refolding to prepare the active enzyme. On the other hand, two short-length PCLs, P2CL and P27CL, were both secreted as soluble forms, which could be much more advantageous for preparation of the active enzyme than the insoluble form.

The present results showed that the full-length PCL, prepared by the usual renaturation operation, could be autoprocessed to the active CL under acidic conditions, while neither of the two short-length PCLs showed remarkable CL activity under the same conditions, in spite of the advantage of their soluble expression. On the other hand, the CD spectra showed the considerable difference between the tertiary structures of the two short-length PCLs and the full-length one. Judging from these results, it is obvious that the full-length propeptide region of PCL is necessary for the proper protein folding (including the formation of the proper secondary structures and their correct mutual orientation) at stage of the recombinant protein expression in *E. coli*, which leads to autoprocessing to active mature CL, although the importance of the propeptide at the step of renaturation of this insoluble full-length PCL could not be ruled out. On the other hand, it can be said from the present results that the propeptide moiety does not affect the expression of the recombinant protein itself apart from the active/inactive form.

The crystal structure of human recombinant full-length PCL (*18*) showed that the propeptide consists of two structural components: a long, extended portion (1-20 sequence), and a globular domain comprising sequence 25-96 (three α-helices, 21-28, 45-71, and 77-90, and an extended connecting loop, 37-40). The 17-25 region is located in the active-site region of CL, where the extended sequence lines the cleft along the S and S′ subsites and the contacts are extensive. Judging from the present results for P27CL, it can be said that the length of the propeptide, which covers the whole active site cleft of CL, is not enough for the tertiary structure of CL to be orientated correctly, and the globular domain consisting of propeptide sequence 25-96 is indispensable for construction of the CL active structure at the recombinant protein expression of its cDNA in *E. coli*, meaning that this globular domain functions as a stabilizer and/or promoter for the proper folding of CL. Interestingly, this discussion appears to agree well with results of CL inhibition by the propeptide fragment (*19*), *i.e.*, the propeptide segment (sequence 16-69), which lacks residues 1-15 of the PCL propeptide, exhibits good inhibitory activity, while the peptide sequence 1-44 contributes little to the inhibition.

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