

Multi-step processing of procathepsin L in vitro

Kazumi Ishidoh*, Eiki Kominami

Department of Biochemistry, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

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Abstract The proteolytic processes involved in the conversion of procathepsin L to cathepsin L on a negatively charged surface, dextran sulfate, were studied. Upon incubation for 30 min at 37°C, pH 5.5 with dextran-sulfate and dithiothreitol, purified procathepsin L showed maximal activation and, correspondingly, the complete conversion to the 30 kDa, single chain mature form of enzyme was observed. In contrast, incubation under the same conditions on ice rather than at 37°C for 30 or 60 min resulted in partial proteolysis to produce a 31 kDa form without a significant increase in activity. Amino terminal amino acid sequence analyses showed that the 30 kDa form obtained by incubation at 37°C corresponds to the purified form of mature cathepsin L with a 2 amino acid extension at the amino terminal, and that the 31 kDa form generated by incubation on ice possesses a 6 amino acid amino terminal extension, suggesting that the activation and processing of procathepsin L are different processes, and that 4 amino acid residues (Glu-Pro-Leu-Met) at the carboxyterminal in the propeptide function to prevent the activation of processed cathepsin L.

Key words: Proteolytic processing; Procathepsin L; Cathepsin L; Proteinase activity

1. Introduction

Cathepsin L is the most potent cysteine proteinase and plays a major role in the lysosomal proteolysis of both endocytosed and endogenous proteins [1]. cDNA cloning and amino acid sequence analysis show cathepsin L to have a long prepro-sequence in front of the mature enzyme [2,3]. Pulse-chase experiments with different types of cells suggest that the enzyme is translated in a prepro-form, is then processed into the pro-enzyme in the rough endoplasmic reticulum, and then localizes in lysosomes as the active enzyme [4,5]. It has been reported that 1,10-phenanthroline and pepstatin partially inhibit the processing of the pro-form to the mature enzyme and it was speculated that metallo-proteinases or as an aspartic proteinase such as cathepsin D are involved in processing [6,7]. An in vitro study of purified procathepsin L isolated from conditioned medium of cultured murine fibroblasts showed the enzyme to become autocatalytically activated at pH 3.0 [8], a finding also demonstrated for guinea pig sperm cathepsin L [9]. On the other hand, considerable amounts of procathepsin L are secreted into the medium in *v-ras*-transformed fibroblasts and cells stimulated by growth factors, and this secreted procathepsin L is thought to be proteolytically processed to the active enzyme [8,10], but the mechanism is unknown. Thus, it remains to be demonstrated how the activation and processing of cathepsin L occur both intracellularly and extracellularly.

Mason and Massey showed that procathepsin L is autoactivated by contact with negatively charged materials such as dextran sulfate at the physiological lysosomal pH at 5.5 [11]. This system is thought to most closely resemble to the physiological state. Previously, we obtained procathepsin L-specific antibodies that recognize procathepsin L but not cathepsin L [12]. In this study using two antibodies, procathepsin L-specific antibodies and antibodies against mature cathepsin L which

recognize both procathepsin L and mature cathepsin L, we show that the negatively charged surface-mediated processing of procathepsin L occurs in at least two steps.

2. Materials and methods

The materials used in this study were obtained as follows: Z-FR-MCA and AMC from Peptide Institute (Osaka); dextran sulfate and CM-Sephadex C-50 from Pharmacia LKB Biotechnology (UK); ECA immunoblot detection kit from Amersham; BCA protein assay reagent from Pierce; Biogel P-60 from BioRad, and other reagents from Wako Pure Chemical Industries (Osaka). The generation of the anti-cathepsin L and procathepsin L-specific antibodies was described previously [12].

Procathepsin L was purified from *v-Ha-ras* transformed NIH3T3 cell conditioned medium as described previously [12]. Purified procathepsin L was stored at pH 7.5 at –80°C. Cathepsin L was purified from the same cells as follows. Cells grown until near confluence were collected with a cell scraper, washed twice with phosphate-buffered saline, and suspended in 10 volumes of 50 mM sodium acetate buffer (pH 5.5) containing 1 mM EDTA. After cell disruption by sonication, the homogenate was centrifuged at 18,500 × *g* for 10 min at 4°C, and the supernatant was applied to a Biogel P-60 gel filtration column previously equilibrated with 50 mM sodium acetate buffer (pH 5.5), 1 mM EDTA, 200 mM NaCl. Active fractions were combined and concentrated by ultrafiltration using a YM10 membrane. After dialysis against 50 mM sodium acetate buffer (pH 5.5) containing 1 mM EDTA, the sample was fractionated on CM-Sephadex C-50 with a linear gradient from 100 mM to 400 mM NaCl in 50 mM sodium acetate buffer (pH 5.5) containing 1 mM EDTA. Active cathepsin L was eluted at approximately 250 mM NaCl. Further purification was carried out with ConA coupled to Affigel P-10. Cathepsin L was eluted with 0.5 M α -methyl-mannoside in 50 mM sodium acetate buffer (pH 5.5) containing 1 mM EDTA and 0.2 M NaCl.

Processing of procathepsin L on the negatively-charged surface was performed according to the method of Mason and Massey [11]. In short, 1 mg of purified procathepsin L, 500 ng of dextran sulfate in 50 μ l of 100 mM sodium acetate buffer (pH 5.5), and 1 mM dithiothreitol were incubated at 37°C for 30 min or on ice for 30 min or 60 min, and then aliquots of the reaction mixtures were subjected to enzyme assay as follows. Assay mixtures containing 100 μ l of 100 mM sodium acetate buffer (pH 5.5), 4 mM cysteine, 100 mM Z-FR-MCA were preincubated at 37°C for 5 min and the reaction was initiated by the addition of 2.5 μ l procathepsin L. To avoid activation of procathepsin L during incubation, the enzyme reaction was stopped after 2 min by the addition of SDS at a final concentration of 5%. The mixture was then diluted with 2 ml of 0.1 M Tris-HCl (pH 9.0) and the released AMC was measured by fluorescence spectrophotometry at an excitation wave length of 370 nm and an emission wave length of 460 nm. For SDS-PAGE and immunoblot analyses, the reaction was stopped by the

*Corresponding author.

Abbreviations: SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; Z-FR-MCA, carbobenzoxy-L-phenylalanyl-L-arginine 4-methyl-coumarinyl-7-amide; AMC, 7-amino-4-methyl-coumarin; SDS, sodium dodecyl sulfate.

addition of leupeptin at a final concentration of 10 mM. SDS-PAGE was performed according to the method of Laemmli [13] and immunoblot analyses were carried out by the method of Towbin et al. [14].

For amino terminal amino acid sequence analysis, the proteins were subjected to electrophoresis on 15% acrylamide gels containing 1% SDS, and transferred electrophoretically onto Pro Blot membrane. After stained with Coomassie brilliant blue G-250, the proteins were cut out with a razor and destained with 50% methanol. After drying in a freezer, the samples were analyzed on a protein sequencer, Applied Biosystem Model 477A.

3. Results

3.1. *In vitro* activation and processing of procathepsin L

Cathepsin L has been purified from various mammalian tissues including liver, brain, and spleen, and from *E. coli* carrying an expression vector bearing the cDNA for cathepsin L [15–17]. The specific activities of these purified samples determined

using the synthetic substrate, Z-FR-MCA have been reported to range between 22.5 and 34.4 nmol of AMC released/min/mg protein. Our purified preparation of cathepsin L from *v-Ha-ras* transformed NIH3T3 cells has a specific activity of 97.5 nmol of AMC released/min/mg protein, which is at least three times higher than that of the purified enzyme reported previously (Fig. 1a, Control).

Purified procathepsin L is apparently homogeneous and detected as a single band with a molecular weight of 39 kDa by both the procathepsin L-specific antibody and the anti-cathepsin L antibody that recognized both procathepsin L and mature cathepsin L (Fig. 1b, lane 2). The specific activity of procathepsin L was estimated to be 10.10 ± 0.92 nmol of AMC released/min/mg protein (Fig. 1a, column 1), ten times lower than that of purified cathepsin L. AMC release was linear for the first 3 min, but hyperbolic after 5 min (data, not shown). To avoid

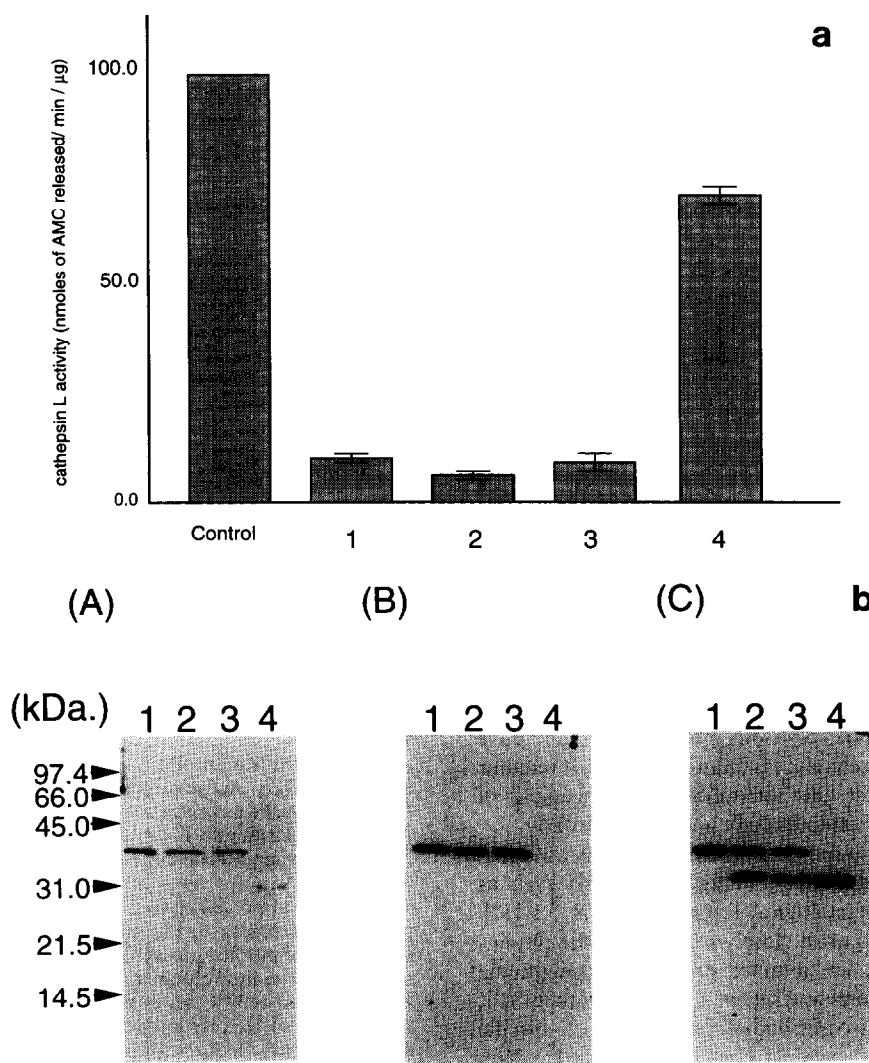


Fig. 1. Activation and processing of procathepsin L in the presence of dextran sulfate. (a) Purified procathepsin L (column 1) was incubated on ice for 30 min (2) or 60 min (3) or at 37°C for 30 min (4) in the presence of dextran sulfate and dithiothreitol. After incubation, aliquots of the mixtures were subjected to enzyme assay. Proteolytic activity was measured during 2 min of incubation to avoid activation of procathepsin L. Control shows the activity of purified cathepsin L. (b) The same reaction mixtures after activation on ice or 37°C were subjected to SDS-PAGE and immunoblotting after the addition of leupeptin. Electrophoresis was performed in a 15% gel containing 1% SDS and 2-mercaptoethanol and the proteins were visualized with silver staining (A) or detected by immunoblotting using procathepsin L-specific antibody (B) or anti-cathepsin L antibody (C). Molecular markers used were: 97.4 kDa, phosphorylase b; 66.0 kDa, bovine serum albumin; 45.0 kDa, ovalbumin; 21.5 kDa, carbonic anhydrase; 14.5 kDa, soybean trypsin inhibitor.

activation of procathepsin L, the activity was measured during 2 min incubation. This value is the maximal activity of procathepsin L, since the possibility that purified procathepsin L contains amounts of active cathepsin L undetectable on SDS-PAGE and immunoblotting with anti-cathepsin L antibody can not be ruled out. Mason and Massey [12] also reported that procathepsin L has little activity and that this activity is responsible for the initiation of activation, based on the incorporation of radioactive inhibitor into procathepsin L.

Upon incubation at 37°C in the presence of dextran sulfate, procathepsin L is reported to be autocatalytically converted to the active form at pH 5.5 [11]. We chose two incubation temperatures, 37°C and 'on ice', in order to identify the relationship between activity and proteolytic processing. Incubation at 37°C, pH 5.5 for 30 min produced the maximal activation (Fig. 1a, column 4). The specific activity was determined to be 69.92 ± 2.52 nmol of AMC released/min/mg protein, about 70% of the activity of purified cathepsin L. In parallel with activation, the proteolytic maturation of procathepsin L to the mature single chain form of the enzyme with an apparent molecular mass of 30 kDa was observed (Fig. 1b, lane 4). Immunoblotting analyses with antibodies against both cathepsin L and procathepsin L confirmed that procathepsin L is converted completely to a 30 kDa form (Fig. 1b, lanes 3 and 4).

When the procathepsin L was incubated at pH 5.5 on ice for 30 min, the activity was somewhat lower (6.65 ± 0.61 nmol of AMC released/min/mg protein) than procathepsin L (Fig. 1a, column 2). Statistical analysis by the computer package ANOVA in Statview II revealed this difference to be significant at the 95% level. Incubation on ice for 60 min showed a specific

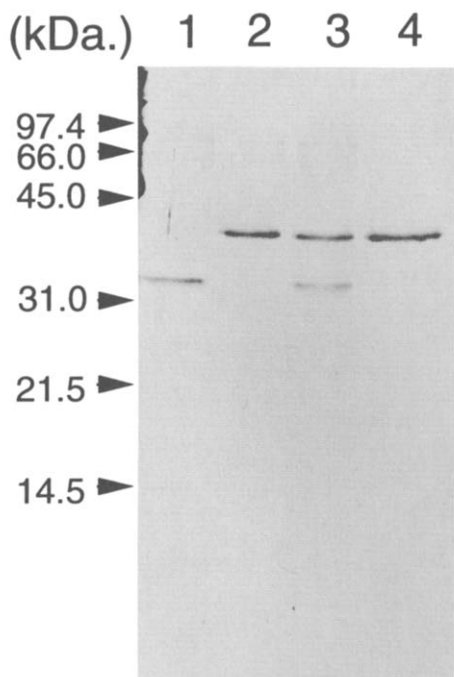


Fig. 2. Effects of leupeptin on the processing of procathepsin L. Procathepsin L was processed in dextran sulfate and dithiothreitol at pH 5.5 for 30 min in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 10 mM leupeptin. Incubation at 37°C (lanes 1 and 2) or on ice (lanes 3 and 4). Electrophoresis was performed as in Fig. 1 and proteins in the gel were stained with silver. For molecular markers, see the legend to Fig. 1.



Fig. 3. Amino terminal sequences of the processed forms of procathepsin L. The amino acid sequence of the carboxyl end region of the propeptide of mouse procathepsin L is shown. Numbers above the sequence start at the amino terminus of purified cathepsin L and negative numbers indicate the amino acid sequence of the propeptide region. The amino acid sequences of the 31 kDa and 30 kDa forms produced by incubation for 30 min on ice and at 37°C for 30 min, respectively, are shown by arrows. (A) 31 kDa form and (B) 30 kDa form.

activity of 8.9 ± 2.56 nmol of AMC released/min/mg protein, comparable to that of procathepsin L (Fig. 1 column 3). Further incubation at 37°C for 30 min after standing on ice for 30 min produced the maximal activation comparable to column 4 (data not shown).

The results after 30 and 60 min incubation on ice in the presence of dextran sulfate are interesting since the partial proteolytic processing occurred despite the fact that there was no activation. In addition to the non-proteolysed procathepsin L of 39 kDa, a 31 kDa protein that cross-reacted with the cathepsin L antibody, but not the procathepsin L-specific antibody, was detected (Fig. 1b, lanes 2 and 3).

Fig. 2 shows that the dextran sulfate-mediated processing of procathepsin L at 37°C and on ice is completely inhibited by the cysteine proteinase inhibitor leupeptin. Other inhibitors tested, PMSF, a serine proteinase inhibitor, pepstatin A, an aspartic proteinase inhibitor, and EDTA, a metalloproteinase inhibitor, had no effect on the processing of procathepsin L (data not shown). Purified preparations of procathepsin L contained no cathepsin B, H, S or C activities. The results suggest that processing of procathepsin L that occurs during incubation at 37°C and on ice is autocatalytic.

3.2. Amino-terminal amino acid sequences of the processed forms

To compare the two processed forms of 31 kDa and 30 kDa generated by incubation on ice and at 37°C, respectively, the two proteins were subjected to amino acid sequence analysis. (Fig. 3). The amino-terminal sequence of the 31 kDa polypeptide obtained by autoproducting on ice was determined to be Glu-Pro-Leu-Met-Leu-Lys-Ile-Pro-Lys-Ser---, identical to the 6 amino acid residues upstream of the amino terminal isoleucine of purified mature mouse cathepsin L. In contrast, the amino-terminal sequence of the 30 kDa polypeptide produced by incubation at 37°C was found to be Leu-Lys-Ile-Pro-Lys-Ser-Val-Asp-Trp-Arg-Glu---, which corresponds to a two amino acid amino terminal extension of mature cathepsin L. The proteolytic activities of the two processed forms were quite different and full activation of the 31 kDa form was obtained by further incubation at 37°C for 30 min. Thus, a 4 amino acid (Glu-Pro-Leu-Met) amino terminal extension might be involved in inhibiting the expression of the Z-Phe-Arg-MCA hydrolyzing activity. A search with the computer package GeneWorks in the SWISS PROT amino acid sequence data bank produced more than forty proteins, including mouse and rat procathepsin L, that contain the 4 amino acid sequence Glu-Pro-Leu-Met. Among proteinases, only a metalloproteinase, thimet oligopeptidase (EC.3.4.24.15) contains this

amino acid sequence motif. None of the endogenous cysteine proteinase inhibitors, including members of the cystatin superfamily possess, this sequence.

4. Discussion

There have been several suggestions made about the functions of the propeptide of lysosomal cysteine proteinases. In experiments where the cDNA for mouse cathepsin L was expressed in *E. coli*, truncation of the pre-peptide region was shown to restore proteolytic activity after renaturation, whereas truncation of the pro-peptide region did not lead to activity recovery, suggesting that the pro-peptide is necessary for the three dimensional structure of the cysteine proteinase [17]. Tao et al. [18] constructed cathepsin L cDNAs encoding proteins with altered pro-regions and expressed them in COS cells. They showed that cathepsin L proteins with altered pro-regions display differences in protein folding, endoplasmic reticulum exit, stability and mannose phosphorylation. A recent report demonstrated that in addition to mannose 6-phosphate receptor-mediated transport of procathepsin L to lysosomes, another translocation signal to lysosomes is localized in some segment of the pro-peptide region [19]. A third function is inhibitory activity of the pro-peptide as discussed here. Purified procathepsin L has less than 10% of the activity of purified cathepsin L, but removal of the pro-peptide leads to almost full activity. This suggests that the full-length pro-peptide is potentially inhibitory.

Incubation of procathepsin L with dextran-sulfate on ice generates the processed form of procathepsin L with a 6 amino acid amino terminal extension on the mature cathepsin L. This form of the enzyme is inactive with two-thirds of the activity of purified procathepsin L. Further incubation at 37°C produced the active processed form. This form, produced by incubation at 37°C for 30 min, has a 2 amino acids extension at the amino terminal of the mature enzyme. This suggests that the removal of a four amino acid extension causes activation of the processed form. Cathepsin L cleaves at hydrophobic residues occupying sites S2 and S3 of peptide substrates [20]. The Leu-Met at the carboxyl end of the 4 amino acid extension sequence is identical to that present at one of the major cleavage sites of the peptide substrate, glucagon [21]. Thus, the four residue peptide might act as a 'pseudosubstrate' to inactivate of processed procathepsin L. Although the homology of the entire propeptide region of lysosomal cysteine proteinases is about 25%, this four residue peptide at the carboxyl end of the propeptide lacks sequence homology [22], also supporting the 'pseudo-substrate' model.

In this paper, we show that procathepsin L is activated in contact with dextran-sulfate at a physiological lysosomal pH of 5.5, and that the processing proceeds through the generation of an inactive processing intermediate, suggesting the occur-

rence of multiple autocatalytic cleavage steps. It is, however, uncertain whether the results obtained in vitro experiments can be reproduced in vivo. We are now conducting experiments using various antibodies that recognize specific cleavage sites of the propeptide to determine whether multiple autocatalytic processing of procathepsin L occurs in vivo and, if it does, where it occurs.

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