Cathepsin L Plays an Important Role in the Lysosomal Degradation of L-Lactate Dehydrogenase

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A cystatin α-sensitive cysteine proteinase that plays an important role in the lysosomal inactivation and degradation of L-lactate dehydrogenase (LDH) was purified by column chromatography from an ammonium sulfate precipitate of lysosome extract prepared from rat livers. It was eluted with marked delay from cathepsins B and H in a Sephacryl S-200 column by its specific interaction with the gel, and then effectively separated from cathepsins B and H and other proteins. It was eluted with 0.5 M NaCl after washing with 0.2 M NaCl in a CM-Sephadex column, indicating that it showed the same elution behavior as cathepsin L from the CM-Sephadex column. It had activity to hydrolyze z-Phe-Arg-NH-Mec, a synthetic substrate for cysteine proteinases, including cathepsins B and L. The N-terminal sequences of the final preparation of LDH-inactivating enzyme were identical with those of rat cathepsin L. Inactivation and degradation of LDH by the final preparation were observed and effectively inhibited by a low level of cystatin α as well as a general cysteine proteinase inhibitor, leupeptin or (L-3-trans-carboxyoxirane-2-carbonyl)-L-leucine (3-methylbutyl)-amide (E-64-c). From these results, it is concluded that cathepsin L plays a critical role in the lysosomal degradation of native LDH.

Key words: L-lactate dehydrogenase; lysosomes; cathepsin L; cystatin α

Various species of cellular proteins, including L-lactate dehydrogenase (LDH), are thought to be ultimately degraded by the mechanism of autophagy by lysosomal proteinases, because autophagy is a non-selective bulk degradation process and thereby cytoplasmic proteins are autophagically sequestered into lysosomes.1-3) Many species of proteinases are present in lysosomes and degrade various proteins to amino acids completely.3) Many items of information about peptidases, including lysosomal proteinases, are now provided by the peptidase database, MEROPS (http://merops.sanger.ac.uk).4) Specific roles of lysosomal cysteine proteinases have been reported, for example, cathepsins L and S for antigen presentation,5) cathepsin L for some virus infections,6-8) cathepsin F for proteolytic modification of low density lipoprotein,9) and cathepsin K for degradation of kinin10) and collagen.11) But the specific roles of lysosomal proteinases in the degradation of intracellular proteins provided by autophagy are now understood only to a limited extent. Lysosomal cysteine proteinases are believed to play an important role in the intralysosomal degradation of various cellular proteins because their lysosomal degradation is markedly suppressed with cysteine proteinase inhibitors in vivo and in vitro.1,12-14) They also include a cysteine proteinase(s) that are believed to be important in the initial proteolytic attack in lysosomes, because various cytosolic enzymes, including both short-lived and long-lived enzymes, accumulate and retain their enzymatic activities in lysosomes prepared from rat livers after exposure to a cysteine proteinase inhibitor, leupeptin, in vivo.1,14) LDH is a long-lived enzyme and is exclusively degraded by autophagy.1) An interesting feature of the lysosomal degradation of LDH is that the degradation of its native form is markedly suppressed by a low level of cystatin α, suggesting that a cystatin α-sensitive cysteine proteinase plays an important role in the lysosomal degradation of native LDH.15) In this study, we purified and identified a cystatin α-sensitive cysteine proteinase as playing a critical role in the lysosomal degradation of LDH.

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Abbreviations: LDH, L-lactate dehydrogenase (EC 1.1.1.27); z, benzylxycarbonyl; Mec, 4-Methylcoumaryl; E-64-c, (L-3-trans-carboxyoxirane-2-carbonyl)-L-leucine (3-methylbutyl)-amide; CA-074, (L-3-trans-(propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline; Mes, 2-Morpholinoethanesulfonic acid; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Materials and Methods

Reagents. (l-3-trans-carboxyoxirane-2-carbonyl)-l-leucine(3-methylbutyl)lamide (E64-c), (l-3-trans-(propylcarbamoyl)oxirane-2-carbonyl)-l-isoleucyl-l-proline (CA-074), z-Arg-Arg-NH-Mec (z, benzoxycarbonyl; Mec, 4-Methylcoumarin), and Arg-NH-Mec were from the Peptide Institute (Osaka, Japan). 4-Nitrocatechol sulfate was from Nakalai Tesque (Osaka, Japan). Sephacryl S-200 HR, CM-Sephadex C-50, and Percoll were from Amersham Biosciences (Tokyo). Cystatin α was purified from a recombinant strain of E. coli.16)

Purification of LDH. LDH was purified from the cytosol fraction of rat livers by the method of Scopes,17) with slight modifications. A post-microsomal fraction was prepared from a homogenate of rat livers and used as the starting material. The post-microsomal fraction was subjected to ammonium sulfate fractionation. The 45–60% saturated ammonium sulfate fraction was applied to a Sephadex G-25 column (250 ml bed volume) equilibrated with 10 mM 2-morpholinoethanesulfonic acid (Mes)–Tris buffer, pH 6.5. The protein-containing fraction eluted in the void volume was collected and then applied to a CM52 column (40 ml bed volume) equilibrated with 10 mM Mes–Tris buffer, pH 6.5. After the column was washed with the same buffer solution, LDH bound to the column was eluted with 10 mM Mes–Tris buffer, pH 6.5, supplemented with 0.2 mM NADH. The fractions containing LDH were pooled, concentrated by ultrafiltration on a YM10 membrane (Amicon Grace Japan, Tokyo), and then applied to a Sephacryl S-200 column (2.5 x 92 cm) equilibrated with 0.2 mM NaCl. Fractions containing LDH were pooled and concentrated. Approximately 5 mg of LDH was recovered in the final preparation from 50 g of rat liver. The enzyme was stored at −30 °C in the presence of 20% glycerol until use, and was stable in this condition.

Preparation of lysosomes and their extracts. Male Wistar rats weighing 200 to 250 g were fasted overnight. Under ether anesthesia, the livers or kidneys were excised and rinsed several times with ice-cold 0.25 M sucrose. Lysosomes were prepared from the livers or kidneys by Percoll density gradient centrifugation, as described elsewhere, with a slight modification.18) The modification was introduced in the preparation of the mitochondrial-lysosomal fraction from the post-nuclear supernatant after Ca²⁺ treatment. The post-nuclear supernatant was supplemented with approximately 3% Percoll containing 0.25 M sucrose to sediment the mitochondrial-lysosomal fraction loosely by centrifugation, and then to suspend the sediment without disrupting the lysosomes. The final lysosomal sediment was suspended in 20 mM sodium acetate, pH 5.0, mixed with an equal volume of 50% glycerol, and stored at −30 °C until use. A freeze-thawed fraction of lysosomes prepared from 30 rats was diluted to a final volume of 15 ml, supplemented with 0.5 mM NaCl, 1 mM EDTA, 5 mM 2-mercaptoethanol and 20 mM sodium acetate buffer, pH 5.0, and stirred for 30 min at ice-cold temperature. The lysosomal suspension was centrifuged at 48,300 x g (max) for 30 min at 4 °C, and the lysosomal extract of the supernatant was collected. The lysosomal extract was dialyzed against buffer A (20 mM sodium acetate buffer, pH 5.0, 1 mM EDTA, 5 mM 2-mercaptoethanol) containing 0.1 M NaCl.

Separation of LDH-inactivating enzyme by Sephacryl S-200 column chromatography. The lysosomal extract was supplemented with 70% saturated ammonium sulfate and lysosomal enzymes were precipitated by centrifuging at 14,600 x g for 20 min. The precipitate was dissolved in 3 to 4 ml of buffer A containing 0.1 M NaCl, and then recentrifuged to remove insoluble materials. The clear supernatant was collected and then applied to a Sephacryl S-200 column (2.5 x 92 cm) equilibrated with buffer A, containing 0.1 M NaCl. Fractions of 2.9 ml were collected and the activities of their lysosomal cysteine proteinases and LDH-inactivating enzyme were measured in the presence or absence of a cysteine proteinase inhibitor, CA-074 or cystatin α, as described below.

CM-Sephadex C-50 column chromatography of fractions B and C from Sephacryl S-200 column. The CM-Sephadex C-50 column (bed volume, 20 ml) was equilibrated with buffer A containing 0.05 M NaCl. A concentrated sample of fraction B (4.0 ml, A₂₈₀ = 2.55) or fraction C (11.0 ml, A₂₈₀ = 0.472) from a Sephacryl S-200 column was applied. The column was eluted stepwise with buffer A containing first 0.1 M NaCl, followed by 0.2 M NaCl and finally 0.5 M NaCl. Fractions of 2.9 ml of the eluate were collected and the activities of LDH-inactivating enzyme and cysteine proteinases were measured.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the final preparation of LDH-inactivating enzyme from CM-Sephadex C-50 column. The LDH-inactivating enzyme (400 µl, A₂₈₀ = 0.10) purified by CM-Sephadex C-50 column chromatography of fraction C derived from a Sephacryl S-200 column was supplemented with rat serum albumin (51 µg) as a carrier protein and trichloroacetic acid (10%) and stored at ice cold temperature overnight. The proteins were collected by centrifugation and residual trichloroacetic acid was removed by extraction with ether three times. The protein precipitate was analyzed by SDS–PAGE using a 10% polyacrylamide slab gel according to the method reported by Laemmli.19) The resolved protein bands were visualized by staining with Coomassie brilliant blue R-250.

The amino-terminal sequences of LDH-inactivating...
enzyme. The final preparation of LDH-inactivating enzyme derived from CM-Sephadex C-50 column was resolved with a carrier protein by SDS–PAGE on a slab gel containing 10% polyacrylamide. The resolved protein bands were transferred electrophoretically onto a polyvinylidene difluoride (PVDF) membrane. The membrane was thoroughly rinsed with water, stained for 1 min with Coomassie brilliant blue R-250 dissolved in 50% methanol containing 10% acetic acid, and stained with 50% methanol. The 24 kDa band derived from the purified enzyme was cut out and its amino terminal sequences were determined, as described by Matsudaira.20)

Analytical procedures. The activity of LDH was measured according to Stolzenbach21) by determining the decrease in absorbance at 340 nm of NADH at 25 °C. Arylsulfatase was assayed using 4-nitrocatechol sulfate as the substrate.22) The activity of cysteine proteinases was measured using synthetic substrates, Arg-NH-Mec for cathepsin H,23) and z-Arg-Arg-NH-Mec for cathepsin B23) or z-Phe-Arg-NH-Mec for lysosomal general cysteine proteinases including cathepsins C,24) B, and L.23) The reaction mixtures (500 μl each), containing synthetic substrates (20 nmol each), 10 mM 2-mercaptoethanol, 1 mM EDTA, 100 mM sodium acetate buffer, pH 5.0, and lysosomal cysteine proteinases were incubated for 0–20 min at 37 °C. The reaction was stopped with 1 ml of 0.1 M sodium chloroacetate containing 0.1 M sodium acetate, pH 4.3. The fluorescence intensity of the liberated 7-amino-4-methyl coumarin was measured according to Stolzenbach21) by determining the decrease in absorbance at 340 nm of NADH at 25 °C. Arylsulfatase was assayed using 4-nitrocatechol sulfate as the substrate.22) The activity of cysteine proteinases and LDH-inactivating enzyme of the extracts of lysosomes from Sephacryl S-200 column

The lysosome extract prepared from rat livers was applied to a Sephacryl S-200 column and the activities of cysteine proteinases and LDH-inactivating enzyme were monitored. As Fig. 1 shows, three fractions (A, B, and C) containing cysteine proteinase activities were clearly separated and LDH-inactivating activities were found slightly in fraction B and mainly in fraction C. Fraction A apparently contains a lysosomal high molecular cysteine proteinase of cathepsin C (dipeptidyl-peptidase1) hydrolyzing z-Phe-Arg Mec, which was eluted near the void volume fractions containing 150–200 kDa proteins.24,26) About 30 kDa of proteins were eluted in fraction B. Cathepsins B, H, and L were expected to be eluted in this fraction, because their molecular masses are all approximately 30 kDa.23) Activities hydrolyzing Arg-Mec by cathepsin H were eluted in fraction B. Activities hydrolyzing z-Phe-Arg-Mec (a suitable substrate for cathepsins B and L) were eluted in fraction B, and almost all activities disappeared in the presence of CA-074, a specific inhibitor of cathepsin B, showing that the activities hydrolyzing z-Phe-Arg-Mec eluted in fraction B were due almost entirely to cathepsin B. A cysteine proteinase hydrolyzing z-Phe-Arg-Mec was also eluted in fraction C, in which low molecular materials such as amino acids are eluted. It was then effectively separated from cathepsins B and H and other proteins, indicating that it was eluted with marked delay by its specific interaction with the gels. Its activity was not affected in the presence of CA-074, indicating that cathepsin B was not eluted in fraction C. The LDH-inactivating enzyme eluted in fractions B and C and activities hydrolyzing z-Phe-Arg-Mec in fraction C were all inhibited by a low level of cystatin α. A marked delay in the elution from a Sephacryl S-200 column of a lysosomal cysteine proteinase hydrolyzing z-Phe-Arg-Mec was reproductive, and it was also observed with an extract of kidney lysosomes (Fig. 2).

CM-Sephadex C-50 column chromatography of fractions B and C from Sephacryl S-200 column

Ion exchange column chromatography using CM-Sephadex C-50 has often been used to separate cathepsin L or S from other cathepsins, and the elution behaviors
of cathepsins B, H, S, and L from the column have been confirmed.23,27,28) Fractions B and C from the Sephacryl S-200 column were, therefore, analyzed by CM-Sepha-
dex C-50 column chromatography. Fraction B was first applied to the column to reconfirm the elution behaviors of cathepsins B, H, and L from the column. As shown in Fig. 3, cathepsin H, hydrolyzing Arg-Mec, was eluted in the flow-through fractions with buffer containing a low concentration of salt. The activities hydrolyzing z-Phe-Arg-Mec were then eluted with buffer solution containing 0.1–0.2 M NaCl after cathepsin H, and they were completely inhibited by adding CA-074, a specific inhibitor of cathepsin B, indicating that the activities

SDS–PAGE analysis of LDH-inactivating enzyme

The LDH-inactivating enzyme derived from a CM-Sephadex C-50 column was precipitated by adding trichloroacetic acid with a carrier protein and the
precipitated samples were analyzed by SDS–PAGE. As Fig. 5 shows, a 24 kDa protein band was resolved in a sample, and the molecular mass was identical with that of rat cathepsin L, reported previously. 23,28) The amino-terminal sequences of LDH-inactivating enzyme were deduced by 19 residues with a protein sequencer, and it was compared with those of cathepsins L reported previously by Towatari et al. 29) and other rat cysteine proteinases provided by the peptidase database, MEROPS.4) The amino-terminal sequences of the LDH-inactivating enzyme were identical with those of rat cathepsin L (Fig. 6). The MEROPS database shows that the amino-terminal sequences of rat cathepsin L are different from other rat cysteine proteinases.

Inactivation and degradation of LDH by the purified cathepsin L

When the purified LDH was incubated at pH 5.0 in vitro with the final preparation of LDH-inactivating enzyme derived from a CM-Sephadex C-50 column, its activity decreased over time (Fig. 7), and its 35 kDa subunits largely disappeared (Fig. 8). The decrease in LDH activity and disappearance of the 35 kDa subunit of LDH were markedly suppressed by a low level of cystatin \( \text{C11} \) as well as a general cysteine proteinase inhibitor, E-64-c or leupeptin, indicating that cathepsin L has an important role in the lysosomal degradation of native LDH (Figs. 7 and 8).

Discussion

Many species of proteinases are present in lysosomes, and they are generally thought to degrade various species of cellular proteins to amino acids by their cooperative actions. An important role of lysosomal cysteine proteinases has been identified from the
observation that the lysosomal degradation of native LDH is almost completely suppressed by a cysteine proteinase inhibitor, leupeptin, E-64-c, or a low level of cystatin C15,30) One of the authors previously postulated that a cystatin/C11-sensitive cysteine proteinase other than cathepsins B, H, L, and C (previously referred to as cathepsin J) functions in the lysosomal degradation of native LDH.15,30) This hypothesis was based on the finding that LDH-inactivating enzyme was clearly separated from cathepsins H and C, the finding that the degradation of native LDH by partially purified LDH inactivating enzyme was not suppressed by CA-074 (a specific inhibitor of cathepsin B) and was markedly suppressed by a low level of cystatin C, and a report by Towatari et al.27) that neither cathepsin B or L purified from rat liver inactivates LDH in vitro. Our present study, however, indicates that the cystatin/C11-sensitive lysosomal cysteine proteinase that plays an important role in the lysosomal degradation of native LDH is cathepsin L. LDH-inactivating enzyme showed the same elution behavior as cathepsin L in CM-Sephadex C-50 column chromatography, which has often been used to purify cathepsin L. Hence we assume now that cathepsin L (purified as a new cathepsin) provided to the degradation of LDH by Towatari et al.,27) probably lost its activity for unknown reasons. Lysosomal accumulation of LDH in leupeptin-treated rat liver indicates that a cysteine proteinase(s) play an important role in vivo in the intralysosomal degradation of LDH. In this study, cathepsin L was completely separated from other lysosomal cysteine proteinases as a LDH-inactivating enzyme, suggesting that the role of cathepsin L is also important in vivo.
Cathepsin L may not be critical for the degradation of denatured LDH, because one of the authors has found that the overall degradation of native LDH by total lysosomal enzymes (disrupted lysosomes) was markedly different from the acid denatured form in the pH profile and the effect of cysteine proteinase inhibitors.\(^{15}\) The degradation rate of native LDH was higher at pH 4.0, but much lower above pH 5.0, than that of the acid-denatured form. The degradation of native LDH was markedly suppressed by a low level of cystatin \(\alpha\) as well as a general cysteine proteinase inhibitor, leupeptin or E-64-c, but that of acid-denatured LDH was only slightly suppressed by these inhibitors.\(^{15}\)

In this study, we found inadvertently that cathepsin L interacted with Sephacryl S-200 gel and was eluted with marked delay from the Sephacryl S-200 gel filtration column. This feature of cathepsin L is very useful in showing that cathepsin L is an LDH-inactivating enzyme. Interaction with Sephacryl S-200 gel and the delayed elution from the column of cathepsin L are, however, not found when the extracts of mitochondrial-delayed elution from the column of cathepsin L are, showing that cathepsin L is an LDH-inactivating enzyme. Interaction with Sephacryl S-200 gel and the effect of cysteine proteinase inhibitors.\(^{15}\) The structure of the sugar side chain of cathepsin L might interact with Sephacryl. It has been reported that heterogeneous delayed elution of lysosomal acid \(\alpha\)-glucosidase from Sephacryl S-200 gel might be due to the heterogeneity of the structure of its sugar side chain.\(^{31}\)

References


