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RAT MAMMARY GLAND IN CULTURE SECRETES A STABLE HIGH MOLECULAR WEIGHT FORM OF CATHEPSIN L

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Culture medium from rat mammary gland explants was analyzed for the presence of cysteine proteinases. In addition to a putative precursor of the lysosomal enzyme cathepsin B, a cysteine proteinase with enzymatic properties similar to those reported for cathepsin L was found. Further evidence of the cathepsin L-like nature of this activity was provided by its high sensitivity towards the diazomethane inhibitors Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 and towards leupeptin. The secreted form of cathepsin L is distinguished from the lysosomal form by its increased stability at alkaline pH and by its larger molecular size. It may thus represent an incompletely processed precursor form of the lysosomal enzyme.

The cysteine proteinase cathepsin L is thought to be of major importance in lysosomal protein catabolism because of its high degradative activity on cellular and matrix proteins (1,2,3). The enzyme has been purified from rat (1), rabbit (4) and human (5) liver and molecular weights of 25,000-29,000 have generally been reported. In all species studied cathepsin L displays a high degree of instability at neutral or alkaline pH. No extracellular cathepsin L activity has been described previously.

Cathepsin B, the other major lysosomal cysteine proteinase, has been shown to be secreted from human and animal tumors (6,7) and from mammary gland explants (8) as a stable, high molecular weight form displaying activity against synthetic substrates. A stable latent form of this enzyme has been characterized in ascitic fluid from cancer patients (9). As these larger molecular weight forms are immunologically related to cathepsin B (10,11), they are thought to be abnormally secreted precursor forms of the lysosomal enzyme.

Abbreviations: -CHN_2, diazomethane; βNA, β-naphthylamide; TCA, trichloroacetic acid; Z, carbobenzoxy.

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In this report we present evidence that cathepsin L in addition to cathepsin B, is secreted from mammary gland explants as a stable, high molecular weight form.

Materials and Methods

Materials: Leupeptin and pepstatin were provided through the U.S.-Japan Medical Science Program. Z-Phe-Phe-CHN_2[1] and Z-Phe-Ala-CHN_2 were kindly provided by Dr. E. Shaw and Z-Arg-Arg-βNA was synthesized as described by Knight (12).

Culture Media: Mammary glands were dissected from lactating Wistar rats 10-12 days post partum and explant cultures established as described for mouse mammary gland (8). Cultures were maintained in Dulbecco's modified minimum essential medium supplemented with porcine insulin (5 μg/ml) and antibiotics (penicillin G, 50 U/ml and streptomycin, 50 μg/ml). Culture media collected at days 4, 6 and 8 were pooled and stored frozen until used.

Gel filtration chromatography: Culture medium was dialyzed against two changes of 20 vol column buffer (20 mM sodium MES, 0.2 M NaCl, pH 7.0) and concentrated 15 fold by ultrafiltration through a Millipore PSAC membrane. Concentrated medium (1.5 ml) was applied to an Ultrogel AcA54 column (1.6 x 90cm) equilibrated with column buffer. Ovalbumin (0.4 mg) was added to the sample as an internal marker and its elution position was determined by Laurell rocket immunoelectrophoresis. The elution positions of molecular weight standards and rat liver cathepsin B were determined on separate runs. A supernatant from rat liver homogenate was used as a source of rat cathepsin B.

Enzyme assays: Acid proteinase activity was measured using acid denatured hemoglobin as substrate at pH 3.9 (6). Cathepsin L activity was determined using either azocasein (0.5 mg/assay) or ^{125}I-labeled azocasein (5 μg containing 1-1.4x10^5dpm) as substrate. The incubation buffer was 50 mM MES, containing 1 mM EDTA, 2 mM cysteine and 1 μg/ml pepstatin (final concentrations), to inhibit cathepsin D activity which is also present in the culture media. The final assay volume was 250 μl. Incubations were for 1 to 4 h at 40°C and reactions were stopped by the addition of 1 ml cold 3% TCA. When ^{125}I-labeled azocasein was used, cold carrier was added just prior to the addition of TCA. TCA soluble degradation products were determined by reading the absorbance at 366 nm when azocasein was used or counting an aliquot in a γ-counter.

Cathepsin B activity was determined using Z-Arg-Arg-βNA as substrate as described previously (9).

Inhibition studies: Inhibition of cathepsin B and L activities by the diazomethanes, Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 and by leupeptin were measured by preincubation of enzyme samples with the inhibitors in the presence of 2 mM cysteine for 15 min at 40°C. Reactions were started by the addition of substrate. Effects of other serine or cysteine proteinase inhibitors were determined in a similar fashion.

pH stability: The stability of cathepsin L in concentrated culture medium was determined by preincubating samples for the indicated times in 50 mM Tris-HCl buffer, pH 8.0 at 40°C and then determining residual activity.

Results and Discussion

Gel filtration analysis of concentrated culture medium from lactating rat mammary gland for acid proteinase activity showed a much broader peak than was expected for cathepsin D, with a significant amount of activity in the lower molecular weight region. When this acid proteinase activity was analyzed in the presence of pepstatin to inhibit cathepsin D a separate component was found. This activity was greatly enhanced if

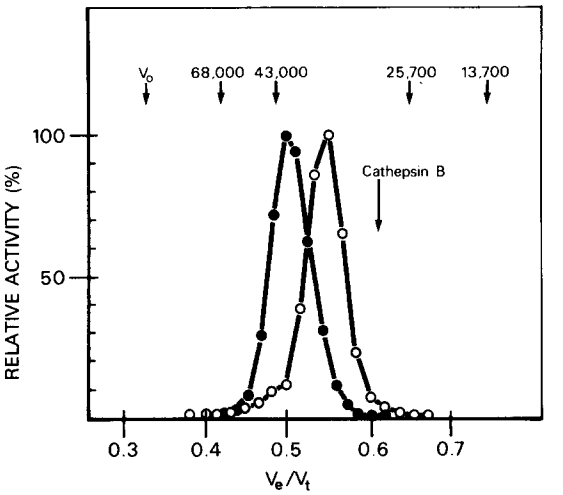

Figure 1: Elution profile of cathepsin B and cathepsin L-like activities from rat mammary gland culture medium. Concentrated culture medium was analyzed by gel filtration chromatography (Ultrogel AcA54). Fractions were assayed for cathepsin B-like activity (—●—) using Z-Arg-Arg-βNA as substrate and for cathepsin L-like activity (—○—) using ^{125}I-labeled azocasein in the presence of pepstatin (1 μg/ml). Elution positions of molecular weight markers and rat liver cathepsin B were determined in a separate run.

cysteine was included in the assay mixture suggesting the action of a cysteine proteinase. The pepstatin resistant activity thus measured eluted as a symmetrical peak more retarded than stable cathepsin B measured using Z-Arg-Arg-βNA as substrate (Fig. 1). The shapes of the activity peaks indicate that the cysteine proteinase displays essentially no activity towards the synthetic cathepsin B substrate. The stable cathepsin B activity present in the culture medium does not show significant activity when azocasein is used as substrate, as indicated by the clear separation of the two activities. This latter observation is consistent with the conclusion that the stable active form of cathepsin B is only partially active and does not cleave protein substrates (Mort and Recklies, manuscript submitted for publication). Using azocasein as the substrate the pH optimum for the pepstatin resistant proteinase was between 5 and 5.5.

The pepstatin resistant proteolytic activity was characterized as being of the cysteine proteinase class by its susceptibility to iodoacetic acid or mercurials and its insensitivity to EDTA, phenylmethylsulfonyl fluoride and soybean trypsin inhibitor. The absence of activity against the synthetic substrate Z-Arg-Arg-βNA suggests that the secreted proteinase may be cathepsin L-like since both cathepsin B and cathepsin H hydrolyze this substrate whereas cathepsin L does not (2). In order to further characterize the enzyme, its inhibition by the selective inhibitors, Z-Phe-Phe-CHN_2, Z-

Phe-Ala-CHN_2 and leupeptin was studied. The diazomethane inhibitors were developed by Green and Shaw (13) as specific cysteine proteinase inhibitors and differential sensitivities of cathepsins B and L to them have been reported for enzyme preparations from rat (14) and human liver (5). The inhibitor profiles for these compounds are shown in Figure 2 using peak fractions from the gel filtration analysis for stable cathepsin B and cathepsin L-like activities (Fig. 1). The two enzyme forms were differentially inhibited. The cathepsin L-like activity had a higher affinity for all of the compounds with 50% inhibition being observed at 10^{-8}M for Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 and 10^{-9}M for leupeptin. The same degree of inhibition of the cathepsin B-like species requires 1.5-2 orders of magnitude higher concentrations similar to values reported for the purified lysosomal proteinases (1,5,14). In particular lysosomal cathepsin L exhibits the same affinity for both Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 whereas cathepsin B,

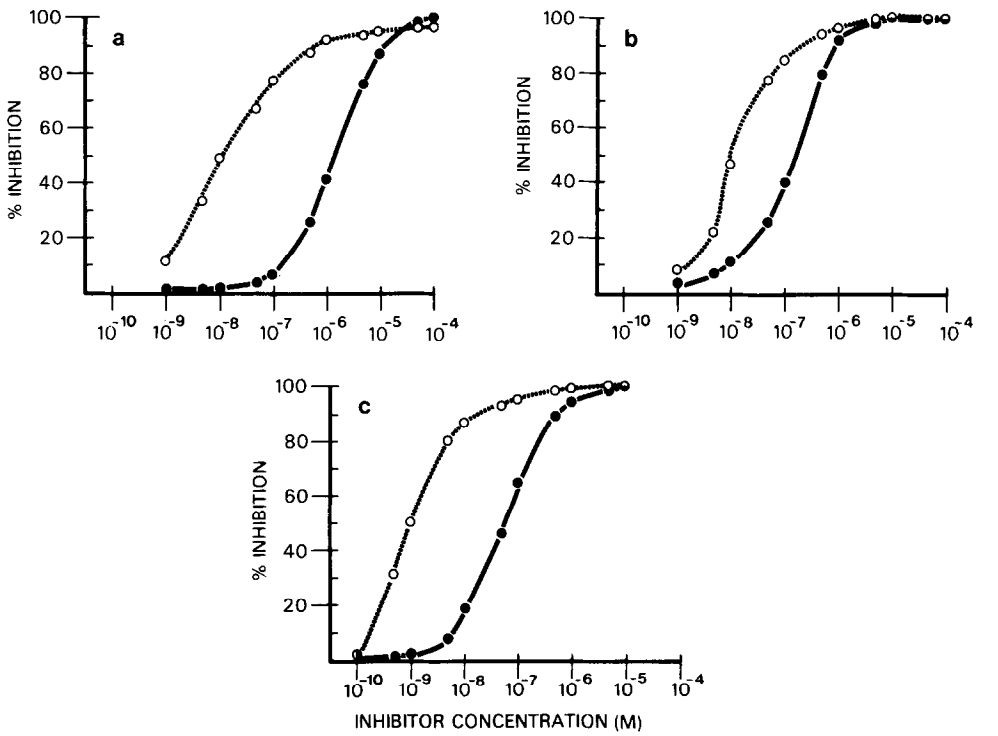

Figure 2: Effect of inhibitors on high molecular weight forms of cathepsin L (—○—) and cathepsin B (—●—). Peak fractions from the gel filtration experiment were used as enzyme sources. Aliquots were preincubated for 15 min at 40°C with Z-Phe-Phe-CHN_2 (panel a), Z-Phe-Ala-CHN_2 (panel b) or leupeptin (panel c) at the indicated concentrations, and the enzyme reaction started by the addition of substrate, Z-Arg-Arg-βNA for cathepsin B activity and ^{125}I-azocasein for cathepsin L. Data are presented as percent inhibition of the activity in the absence of inhibitor.

differentially affected by these inhibitors, has a relatively higher affinity for Z-Phe-Ala-CHN_2. This was also observed for the secreted cathepsin B.

The cathepsin L-like activity secreted from mammary gland explants differs from its lysosomal counterpart in two important aspects. The molecular size (Mr 35,000) as judged by gel filtration chromatography (Fig. 1) is markedly higher than that of rat lysosomal cathepsin L (Mr 24,000) (1). In addition, the activity described here is stable at alkaline pH. As this proteinase accumulates in the culture medium from mammary gland explants, it must be relatively stable under these conditions (pH 7.2-7.4, 37°C) for significant activity to be detectable. Using a stability assay at pH 7.0 or 8.0 and 40°C, similar to the conditions used for determining alkaline pH stability of the secreted cathepsin B species (9), it was found that the cathepsin L activity showed no loss of activity during the first 60 min of incubation at pH 8.0 (Figure 3). Some loss of activity was observed when samples were preincubated for longer than 60 min and this occurred more rapidly at pH 8.0 than pH 7.0, however this process is considerably slower than the denaturation reported for cathepsin L isolated from rat liver lysosomes (1). The cathepsin L-like activity was found to be unstable upon storage at pH 7.0. A concentrated preparation lost about 50% of its initial activity after storage at -20°C for 1 month. Whether the loss of activity is due to denaturation of the enzyme or to degradation by other proteinases present in the culture medium samples is not clear at present.

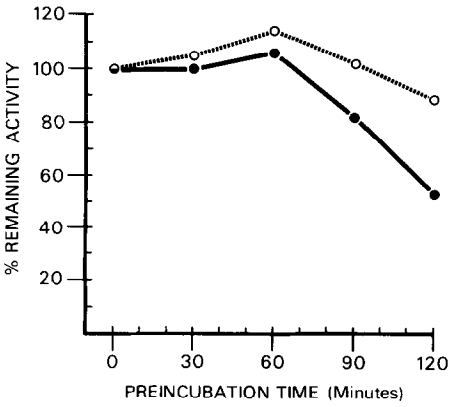

Figure 3: Alkaline stability of cathepsin L activity in culture medium. Aliquots of concentrated culture medium were incubated at 40°C and pH 7.0 (- -O- -) or pH 8.0 (—●—) for the indicated times and residual activity was determined in the presence of 2 mM cysteine and 1 µg/ml pepstatin using azocasein and substrate. Values are represented as percentage of the initial activity.

A similar high Mr cathepsin L activity was also detected in culture media from lactating mouse mammary gland. The secretion and partial processing of cathepsin L seems to be similar to that of cathepsin B which has been characterized extensively in mouse mammary gland (8), and in this system these two lysosomal proteinases appear to be synthesized and processed in a coordinated fashion.

Thus, both with respect to enzymatic activity and sensitivity to inhibitors the secreted proteinase behaves like cathepsin L. The secreted form appears to be larger and relatively stable at neutral pH, and thus would survive for some time in an extracellular environment. Since the secreted enzyme is proteolytically active, it could be of considerable importance in tissue remodeling and in destruction of extracellular matrix in inflammatory processes.

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