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Human Liver Cathepsin D

Purification, Crystallization and Preliminary X-ray Diffraction Analysis of a Lysosomal Enzyme

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The two-chain form of active cathepsin D, a glycosylated, lysosomal aspartic proteinase, has been isolated from human liver. Isoelectric focusing revealed two major species of enzyme that differed by approximately 0·2 pI unit. Crystals suitable for X-ray diffraction analysis were prepared from acidic solutions using precipitation with ammonium sulfate. The hexagonal crystals diffracted X-rays to beyond 3·1 Å resolution and belonged to space group $P6_1$ (or $P6_5$) with cell constants $a = b = 125{\cdot}9$ Å, $c = 104{\cdot}1$ Å, $\gamma = 120{\cdot}0°$. The crystals likely contain two molecules in the asymmetric unit, giving a solvent content of 56% (v/w). Biochemical analysis of crystals indicated that both isoforms were present in approximately equimolar proportions. Full structure determination of the enzyme is underway.

Keywords: cathepsin D; aspartic proteinase; lysosomal enzyme; purification; crystallization

1. Introduction

Cathepsin D (EC 3.4.23.5) is a highly abundant lysosomal endoprotease that is distributed widely throughout mammalian tissues and is thought to play a housekeeping role in protein catabolism *via* the degradation of intracellular and extracellular endocytosed proteins (Barrett, 1977). Cathepsin D activity has been associated with several biological processes of possible therapeutic significance. Pro-cathepsin D is reported to be mitogenic to breast cancer cells *in vitro* (Vignon *et al.*, 1986). The enzyme can degrade extracellular matrix components after autoactivation at acidic pH (Briozzo *et al.*, 1988), and cathepsin D-mediated proteolysis has been linked to connective tissue diseases (Woessner, 1971). Elevated levels of cathepsin D in primary breast cancer tissues have been correlated with increased risk of metastasis and shorter relapse-free survival in breast cancer patients (for a review, see Rochefort, 1990). A role for cathepsin D in antigen processing has been suggested, based on the processing site selectivity of the enzyme (van Noort & van der Drift, 1989), and on the observation that MHC‡ class II molecules may interact with cathepsin D-processed peptides in precursor lysosomes (Guagliardi *et al.*, 1990; Peters *et al.*, 1991).

Inhibition studies (Barrett, 1977) along with amino acid sequence determinations of cathepsin D from porcine spleen (Shewale & Tang, 1984), human kidney (Faust *et al.*, 1985) and rat liver (Fujita *et al.*, 1991), indicated that the enzyme is a member of the pepsin family of aspartic proteinases (Tang & Wong, 1987). Five immunologically distinct enzymes of this family have been found in humans, and these are pepsin, gastricsin, cathepsin D, cathepsin E and renin. High-resolution crystal structures have been reported for three fungal and three mammalian aspartic proteinases: penicillopepsin (James & Sielecki, 1983), rhizopuspepsin (Suguna *et al.*, 1987), endothiapepsin (Blundell *et al.*, 1985), porcine pepsin (Abad-Zapatero *et al.*, 1990; Sielecki *et al.*, 1990; Cooper *et al.*, 1990), recombinant bovine chymosin (Gilliland *et al.*, 1990; Newman *et al.*, 1991), and recombinant human renin (Sielecki *et*

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‡ Abbreviations used: MHC, major histocompatibility; MPR, mannose 6-phosphate receptors; Con A, concanavalin A; PAGE, polyacrylamide gel electrophoresis.

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al., 1989). The structure and function of aspartic proteinases has been reviewed by Davies (1990).

The high degree of sequence identity (45 to 49%) shared between cathepsin D and renin, pepsin and chymosin suggests that the overall three-dimensional structure of cathepsin D should be very similar to the crystal structures of other aspartic proteinases. However, cathepsin D has two interesting characteristics that distinguish it from other enzymes of its class. First, it contains two phosphorylated oligosaccharide chains that target the enzyme *via* mannose 6-phosphate receptors (MPR) into lysosomes (Maguchi *et al.*, 1988; Takahashi *et al.*, 1983). A key event in lysosomal targeting is the recognition of a protein structural determinant on lysosomal hydrolases by a phosphotransferase enzyme that selectively phosphorylates high mannose oligosaccharides (Kornfeld & Mellman, 1989). The recognition domain on cathepsin D has been mapped by molecular modeling and mutagenesis studies using chimeric enzymes (Baranski *et al.*, 1991). Cathepsin D can also migrate to lysosomes *via* a less well-characterized, MPR-independent mechanism in certain cell lines (Rijnboutt *et al.*, 1991). Second, cathepsin D, like many lysomal enzymes, is found predominantly in a two-chain form, whereas most other eukaryotic aspartic proteinases function as single-chain enzymes (Tang & Wong, 1987). The two non-covalently associated polypeptide chains of active cathepsin D may be the result of autoprocessing of a single chain precursor (Huang *et al.*, 1979; Conner & Udey, 1990). Cleavage occurs within a processing region that appears as an insertion in the cathepsin D gene sequence and that is removed during enzyme maturation. The processing regions in cathepsin D vary in length and amino acid composition from species to species as does the relative proportion of the one and two-chain forms (Yonezawa *et al.*, 1988; Fujita *et al.*, 1991). A structural model has been proposed for the processing region in human cathepsin D (Yonezawa *et al.*, 1988).

Determination of the three-dimensional structure of cathepsin D should reveal structural features related to the various biological properties of this enzyme, including phosphotransferase recognition, lysosomal targeting and proteolytic processing. In addition, knowledge of cathepsin D structure will facilitate the structure-based design of specific inhibitors of this enzyme and could lead to novel chemotherapeutic agents for certain forms of malignant, immunological or connective tissue diseases. This paper presents the purification, crystallization and preliminary X-ray crystallographic analysis of cathepsin D from human liver.

2. Materials and Methods

Bovine hemoglobin (2× crystallized), diisopropylfluorophosphate, pepstatin A-agarose, and methyl-α-D-mannopyranoside were purchased from Sigma Chemical Co. Concanavalin A(Con A)-Sepharose and protein markers for SDS/PAGE and isoelectric focusing were obtained from Pharmacia Fine Chemicals. Diethylaminoethyl cellulose (DE 23) was obtained from Whatman LabSales. All other chemicals were analytical grade.

(a) *Purification of cathepsin D from human liver*

Human liver was obtained by autopsy from an adult male (age 52 years) within 3 h of death due to heart failure and was stored at −70°C. Cathepsin D was purified from liver at 4°C using a modification of the procedure described by Ikeda *et al.* (1989) for cathepsin D from human spleen. Human liver (380 g) was homogenized using a Waring Blender in 380 ml of 0·1 M-potassium phosphate (pH 6·5) 2 mM-Na_2EDTA, 2 mM-diisopropylfluorophosphate, 0·2% (v/v) Triton X-100. The homogenate was centrifuged at 35,000 revs/min for 40 min at 5°C (Spinco L-8 ultracentrifuge, Beckman Ti-45 rotor). The pellet was homogenized in 380 ml of 0·05 M-potassium phosphate (pH 6·5), 1 mM-Na_2EDTA, 0·1% Triton X-100 and centrifuged in the same conditions. The supernatants were combined and shaken for 90 min with 800 ml of DE 23 that had been equilibrated with 0·05 M-potassium phosphate (pH 6·5). The resulting suspension was filtered over glass, and KCl and $MnCl_2$ were added to the filtrate to final concentrations of 500 mM and 3 mM, respectively. After adjustment of the pH to 7·4 with 1·0 M-Tris-base, a slight precipitate appeared and was removed by centrifugation at 68,500 ***g*** for 40 min at 4°C. The supernatant was applied to a 2·6 cm × 11 cm column of Con A-Sepharose equilibrated with buffer A (0·05 M-Tris·HCl (pH 7·4) 0·5 M-KCl) at a flow-rate of 60 ml/h. The column was washed using buffer A and cathepsin D was eluted using buffer A containing 0·4 M-methyl-α-D-mannopyranoside. Fractions containing proteolytic activity were pooled and adjusted to pH 4·0 by addition of glacial acetic acid. Precipitated proteins were removed by centrifugation at 16,000 revs/min for 30 min at 5°C (RC-5 centrifuge, Sorval SS-34 rotor). The supernatant was applied to a 1·6 cm × 14 cm column of pepstatin A-agarose that had been equilibrated with 0·05 M-sodium acetate (pH 4·0), 0·5 M-KCl at a flow-rate of 10 ml/h. The column was washed for 65 h, with 0·005 M-sodium acetate (pH 4·0), 0·5 M-KCl at a flow-rate of 30 ml/h, and then by 0·1 M-Tris·HCl (pH 7·1), 0·5 M-KCl until the A_{280} of the eluate was less than 0·05. Cathepsin D was eluted with 0·2 M-Tris·HCl (pH 8·6), 0·5 M-KCl and immediately adjusted to pH 5·0 by adding 1·0 M-sodium acetate buffer (pH 4·0). The enzyme was dialyzed against 5 changes of deionized water, lyophilized, and stored at 4°C.

(b) *Assay of cathepsin D activity*

The enzymatic activity of cathepsin D was measured using a slight modification of the procedure of Anson (1939). A portion (0·5 ml) of a 2% (w/v) bovine hemoglobin solution in 0·2 M-glycine·HCl buffer (pH 3·5) was incubated for 5 to 30 min with 5 to 50 μl of enzyme solution at 37°C. Proteolysis was terminated by addition of 2·5 ml of 5% (w/v) trichloroacetic acid. Samples were centrifuged for 10 min at 13,600 ***g***. Blank assays were run under similar conditions but with the enzyme solution addition after the trichloroacetic acid. One unit of activity was defined as the amount of enzyme that produced an increase in the supernatant A_{280} of 1·0/min. Protein concentrations were determined by the method of Bradford (1976) using a Bio-Rad Protein Assay Kit.

(c) *Electrophoretic analysis*

Polyacrylamide gel electrophoresis was performed using the method of Laemmli (1970). Gels were run under

reducing conditions in an 8 to 25% polyacrylamide linear gradient (PhastSystem, Pharmacia). Protein bands were visualized with silver staining. Protein standards for molecular mass determinations and isoelectric focusing were purchased from Pharmacia. Isoelectric focusing was performed using PhastGel IEF media (Pharmacia) that covered the pH ranges 3 to 9 and 5 to 8. Protein bands were visualized with silver staining.

(d) *Crystal growth and stabilization*

Crystallization conditions were screened in hanging drop experiments (McPherson, 1982). Lyophilized cathepsin D was dissolved in deionized water (20 mg/ml) and centrifuged for 15 min at 13,600 ***g*** at 4 °C. The clear supernatant was mixed 1 : 1 (v/v) with well solution that contained the appropriate buffer and precipitant. The best crystals were obtained from drops in which 5 μl of enzyme solution was mixed with an equal amount of 50 mM-sodium acetate buffer (pH 5·1) containing 62 to 65% saturated ammonium sulfate. Crystals attained maximal size (0·5 mm × 0·2 mm × 0·2 mm) after 3 to 4 weeks at 20 °C. Crystals of cathepsin D were stabilized in 75% saturated ammonium sulfate, 50 mM-sodium acetate (pH 5·1) for 1 week prior to data collection.

(e) *X-ray diffraction data collection*

X-rays were produced from a Rigaku R-200 generator operating with fine focus at 50 kV and 100 mA. CuKα X-rays were selected with a graphite monochromator and the beam was collimated to 0·3 mm with a double pinhole. X-ray diffraction data were collected using a Siemens area detector. The crystal to detector distance was 14·0 cm and 2θ was set to 0·0°. Each frame consisted of a 0·25° oscillation. The program XENGEN (Howard *et al.*, 1987) was used to process the data.

3. Results and Discussion

(a) *Purification of cathepsin D from human liver*

Cathepsin D has been purified from a wide variety of mammalian tissues, including porcine spleen (Huang *et al.*, 1979), rat spleen (Yamamoto *et al.*, 1979), pig myometrium (Afting & Becker, 1981), monkey skeletal muscle (Tanji *et al.*, 1991), human brain (Azaryan *et al.*, 1983), human gastric mucosa (Pohl *et al.*, 1981), human placenta (Contractor *et al.*, 1982), human leukocytes (v.Clausbruch & Tschesche, 1988), human spleen (Ikeda *et al.*, 1989) and human liver (Barrett, 1970, 1979; Maguchi *et al.*, 1988). In this study, cathepsin D was purified from human liver using the procedure described by Ikeda *et al.* (1989) with the addition of an initial batch adsorption step on DEAE cellulose at pH 6·5. This step did not result in an increase in the specific activity of enzyme but did remove most of the nucleic acids and some acidic proteins (Table 1). To decrease the likelihood of deglycosylation, the active fractions eluted from the Con A-Sepharose column were acidified and used immediately for pepstatin A-agarose chromatography. This purification procedure resulted in an overall 938-fold purification of the enzyme based on specific activity measurements (Table 1), and an overall yield of 44·1%. A total of 23 mg of enzyme was obtained from 380 g of liver tissue.

Purified cathepsin D revealed an electrophoretic banding pattern on SDS/PAGE (Fig. 1(a), lane 5) that is characteristic of the two-chain form of the enzyme (Maguchi *et al.*, 1988). The 30,000 M_r band, corresponding to the heavy chain, was quite homogeneous. The light chain appeared to migrate as a doublet of 14,000 M_r and 15,000 M_r peptides, and this may reflect differences in oligosaccharide composition.

(b) *Charge heterogeneity of human liver cathepsin D*

Analytical isoelectric focusing on polyacrylamide gels of human liver cathepsin D revealed two major bands, with isoelectric points of 6·6 and 6·85 (Fig. 1(b)). It should be noted that Barrett (1979) and Maguchi *et al.* (1988) observed three major isoforms of human liver cathepsin D, which they designated α, β and γ in order of increasing pI. The two species in our preparation apparently correspond to the β and γ isoforms. The more acidic α isoform was present in only minor amounts, and probably was adsorbed during the DEAE cellulose step in our procedure. Alternatively, its absence may reflect organ donor differences. Hemogram analysis (Foltmann *et al.*, 1985) indicated that all three isoforms of cathepsin D were active proteolytically against bovine hemoglobin (data not shown).

(c) *Preliminary X-ray diffraction analysis of crystals*

A 3·1 Å X-ray diffraction data set (1 Å = 0·1 nm) was collected from a single 0·18 mm × 0·40 mm crystal of native cathepsin D. A trigonal or hexagonal lattice was expected on the basis of the hexagonal morphology of the crystals (Fig. 2). The data could be readily indexed assuming either $\bar{3}$ or 6/m Laue symmetry, and initial R_{sym} scaling factors were approximately 12% for both cases. Data could also be reduced in Laue groups $\bar{3}$m or 6/mmm but scaled

Table 1
Purification of cathepsin D from human liver

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification (fold)
(1) Homogenate	49,322	142	0·0029	100	1
(2) DE-23 batch adsorption	39,000	113	0·0029	80	1
(3) Con A-Sepharose	296	112	0·378	79	130
(4) Pepstatin A-agarose	23	62	2·72	44	938

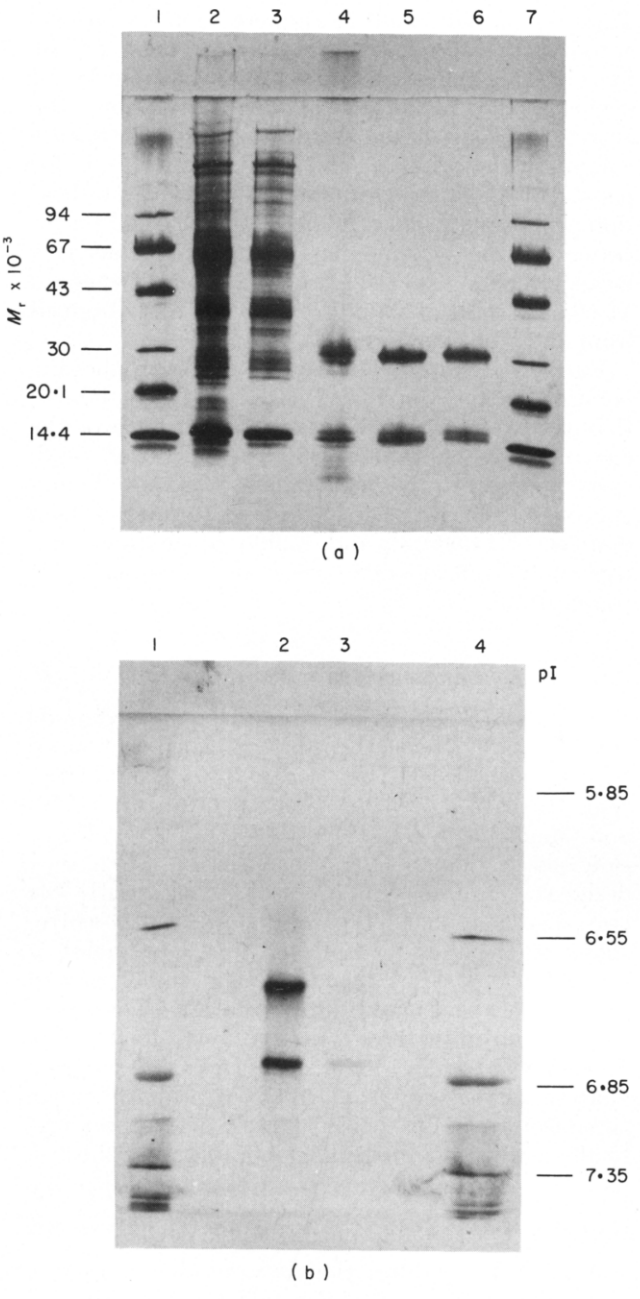

Figure 1. Electrophoretic analysis of human liver cathepsin D. (a) SDS/PAGE of cathepsin D after various stages of the purification. Lanes 1 and 7, molecular mass markers (LMW kit, Pharmacia); lane 2, liver homogenate; lane 3, material after DE 32 batch adsorption; lane 4, the eluate from Con A-Sepharose column; lane 5, cathepsin D after final purification; lane 6, cathepsin D from dissolved crystals. (b) Isoelectric focusing of cathepsin D in PhastGel IEF media covering the pH range 5 to 8. Lanes 1 and 4, pI markers; lane 2, cathepsin D from redissolved crystals; lane 3, purified enzyme. Silver staining was used to visualize bands in both cases.

poorly in both cases with R_{sym} values greater than 25%. This analysis indicated that the correct space group was $P6_1$ (or $P6_5$). The refined cell dimensions were $a = b = 125{\cdot}9$ Å, $c = 104{\cdot}1$ Å, $\alpha = \beta = 90{\cdot}0°$ and $\gamma = 120{\cdot}0°$. Assuming two cathepsin D molecules (M_r 44,000) in the asymmetric unit and a protein specific volume of 0·72 cm^3/g gives a solvent content of 56% (v/v) in the crystals and a V_M value of 2·70 $Å^3$/dalton.

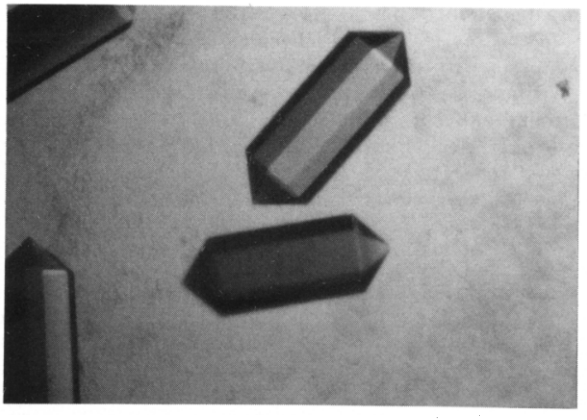

Figure 2. Crystals of human liver cathepsin D. Maximal crystal length is about 0·5 mm.

(d) *Characterization of cathepsin D isolated from crystals*

The β and γ isoforms of cathepsin D co-purified using the current purification procedure. The structural differences between these isoforms is unknown, but crystals grew readily out of the mixture. In order to assess whether the crystallization process was selective for a particular isoform, several crystals were washed, dissolved, dialyzed against water and subjected to isoelectric focusing analysis. The results indicated that the two isoforms were present in approximately the same proportions as were found in purified preparations of enzyme (Fig. 1(b), compare lanes 2 and 3). Hemogram analysis of dissolved crystals showed that both isoforms were enzymatically active (data not shown). Effords were underway to separate the β and γ isoforms for further biochemical and crystallographic analysis.

4. Conclusions

Human liver cathepsin D, a lysosomal enzyme, has been purified to near homogeneity. Crystals of the glycosylated enzyme preparation diffract to 2 Å resolution on stills. The hexagonal $P6_1$ (or $P6_5$) crystal form likely contains two independent molecules in the crystallographic asymmetric unit. Biochemical analysis of cathepsin D crystals revealed the presence of nearly equimolar amounts of the two major isoforms (β and γ) found in purified preparations. The structure of this enzyme should provide useful insights into the structural features that target enzymes to lysosomes as well as provide a high-resolution model for structure-based drug design efforts. The complete structure determination of these crystals is underway.

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