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Purification of Cathepsin D from Guinea Pig Peritoneal Macrophages

TSUTOMU OIKAWA* and TAKAO IWAGUCHI

Division of Cancer Therapeutics, The Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113, Japan

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Aspartic proteinase was purified from guinea pig peritoneal macrophages (MØs) by pepstatin-Sepharose, Sephadex G-150 and diethylaminoethyl-cellulose column chromatographies. The purified enzyme was homogeneous on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. In isoelectric focusing, the enzyme was resolved into four peaks which had pIs of 6.04, 6.53, 7.11 and 7.75. The molecular weight of the enzyme was estimated to be 43000 by gel filtration and 45000 by SDS-polyacrylamide gel electrophoresis. The enzyme was adsorbed on a column of concanavalin A-Sepharose and was eluted with α-methyl mannoside, indicating that it is a glycoprotein. The pH optimum of the enzyme for hydrolysis of acid-denatured hemoglobin was around 3.2. The enzyme was completely inhibited by pepstatin, but not by other chemicals tested, including antipain and leupeptin. Since the properties of the enzyme from MØs resemble those of cathepsins D isolated from the lysosome of other tissues, it can be concluded that this enzyme is cathepsin D (EC 3.4.23.5).

Keywords——cathepsin D; aspartic proteinase; affinity chromatography; peritoneal macrophage; pepstatin; glycoprotein; guinea pig

Cathepsin D (EC 3.4.23.5) has been purified from various sources,[1] and its characteristics have been investigated for the purpose of elucidating the functions of this enzyme in the body. However, there are few reports which deal with cathepsin D from macrophages (MØs). Recent findings have indicated that MØs play an important role in biological defense actions, including the immune response.[2] The information on the cathepsin D from MØs is fragmentary because it is difficult to obtain sufficient amounts of MØs for the isolation of this enzyme; however, Kato *et al.* found cathepsin D activity in the lysates of rat peritoneal MØs,[3] and Dingle *et al.* described the intracellular degradation of proteoglycan by cathepsin D in the alveolar MØs.[4]

In a preliminary experiment, peritoneal MØs from guinea pig were found to show the highest activity toward acid-denatured hemoglobin (Hb) at pH 3.5; this activity amounted to more than 2 times that in guinea pig spleen, which has the highest activity among various tissues. In addition, to our knowledge, no protease from MØs has yet been purified to homogeneity on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. In the present study, we purified the cathepsin D from guinea pig peritoneal MØs and determined some of its properties. The purified enzyme was homogeneous as judged by SDS-polyacrylamide gel electrophoresis. This is the first paper to report on the purification of cathepsin D, one of the lysosomal proteases, from MØs.

Materials and Methods

Materials——AH-Sepharose, Sephadex G-150 and concanavalin A-Sepharose were purchased from Pharmacia. Diethylaminoethyl (DEAE)-cellulose (DE 52) was a product of Whatman. Microbial protease inhibitors, including pepstatin, were generous gifts from Drs. H. Umezawa and T. Aoyagi of the Institute of Microbial Chemistry, Tokyo. Ampholine was obtained from LKB and bovine serum Hb from Worthington. Pepstatin-Sepharose was

prepared as described by Kregar *et al.*[5]

Cathepsin D Assay——The cathepsin D activity was determined in an assay mixture (0.2 ml, final volume) containing the enzyme, 2% (w/v) acid-denatured Hb as the substrate, and 50 mM sodium citrate buffer, pH 3.5. The mixture was incubated at 37 °C for 30 min or less, and the 2.62% (w/v) trichloroacetic acid-soluble products were assayed by the method of Lowry *et al.*[6] One unit of the activity was defined as the amount of enzyme which liberated the equivalent of 1 μg of Tyr per min under the conditions described above. Protein concentration was determined by the Lowry method with bovine serum albumin (BSA) as a standard.[6]

Preparation of MØs——Twenty ml of sterile liquid paraffin was *i.p.* injected into a female guinea pig of the Hartley strain, weighing about 500 g. After 4 d, peritoneal exudate cells were collected by removing them with 200 ml of sterile Hanks solution,[7] and they were incubated with 10 ml of Tris-HCl buffered 0.747% (w/v) NH_4Cl, pH 7.4, to remove contaminating erythrocytes, then washed 3 times with sterile saline. The cells thus obtained were used as the MØs preparation without further purification in this study because present-day methods cannot completely remove the contaminating cells, including lymphocytes. The yield was about 3×10^8 cells from a single guinea pig, consisting of about 85% MØs, 8% lymphocytes and 7% other cells such as polymorphonuclear cells (PMNs), as judged from observations with both the Wright–Giemsa stain and non-specific esterase activity staining by the method of Willcox *et al.*[8]

Subcellular Fractionation——Subcellular fractions were obtained as described by Chodirker *et al.*[9] with a minor modification. MØs (8×10^8 cells) were suspended in 40 ml of 0.25 M sucrose containing 250 units/ml of heparin and then homogenized in a Potter-Elvehjem homogenizer (3 strokes at 1000 rpm) connected to a Braun homogenizing motor. The homogenate was subjected to differential centrifugation and separated into 3 fractions: nuclei fraction ($800 \times g$, 15 min), granule fraction ($25000 \times g$, 30 min) and postgranule fraction. Acid phosphatase was determined by the method of Walter and Schütt.[10] Cathepsin B was measured according to the method of Barrett.[11] Succinic-cytochrome c reductase was determined by the method of Tisdale.[12]

SDS-Polyacrylamide Gel Electrophoresis——SDS-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn.[13]

Isoelectric Focusing——Isoelectric focusing was carried out using a 110 ml-column with carrier ampholine of pH 5—8 and 3.5—10 (4 : 1) at a final concentration of 1% (v/v), and focusing was performed at 4 °C for 48 h with a potential of 680 V. Fractions of 2 ml were collected, and the activities and the pH values at 4 °C were measured.

Results and Discussion

Subcellular Distribution of Cathepsin D

The granule fraction from MØs was rich (72% of total activity) in cathepsin D, also having high contents of acid phosphatase, cathepsin B and succinic-cytochrome c reductase. This is in accordance with the enzyme distribution in human leucocytes.[1b]

Purification and Properties of Cathepsin D from MØs

All procedures for the purification described below were carried out at 4 °C. MØs (2×10^{10} cells) were suspended in 860 ml of distilled water and then freeze-thawed 6 times according to the method of Suzuki and Murachi.[14] The cell lysates were ultracentrifuged at $105000 \times g$ for 60 min. Approximately 70% of cathepsin D activity in the lysates was recovered in the supernatant. The supernatant obtained was used as the crude enzyme solution. It was dialyzed against 120 l of 100 mM sodium acetate buffer, pH 3.5, containing 1 M NaCl and ultracentrifuged at $105000 \times g$ for 60 min. The supernatant was applied to a column (0.9×10 cm) of pepstatin-Sepharose equilibrated with the dialysis buffer. The column was washed with over 20 volumes of the dialysis buffer, over 5 volumes of 6 M urea in the same buffer, and more than 5 volumes of the starting buffer. Cathepsin D was finally eluted with 100 mM $NaHCO_3$, pH 8.6, containing 1 M NaCl, and 4-ml fractions were collected at a flow rate of 20 ml/h. Cathepsin D fractions were combined and concentrated in a collodion bag (Sartorius GmbH). The concentrated fractions were then chromatographed on a column (2.0×85 cm) of Sephadex G-150 equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 100 mM NaCl, and 2.8-ml fractions were collected at a flow rate of 10 ml/h. The column was calibrated using BSA (Sigma, molecular weight of 67000), egg albumin (Sigma, molecular weight of 43000) and α-chymotrypsinogen A (Sigma, molecular weight of 25700). Cathepsin D was eluted as a single peak, corresponding to a molecular weight of 43000.

TABLE I. Purification of Cathepsin D from Guinea Pig Peritoneal Macrophages

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude enzyme	830	59800	72	100
Pepstatin-Sepharose	1.2	9250	7520	15
Sephadex G-150	0.88	7960	9070	13
DEAE-cellulose	0.54	5730	10600	10

Fractions with activity were collected and dialyzed against 10 mM Tris-HCl buffer, pH 8.3, then adsorbed on a DEAE-cellulose column (0.9 × 12 cm) equilibrated with the dialysis buffer. The DEAE-cellulose column was eluted with a 200 ml linear gradient of 0 to 100 mM NaCl in the same buffer, and 2.6-ml fractions were collected at a flow rate of 10 ml/h. Cathepsin D was eluted as a single peak at 35 mM NaCl and was purified 150-fold with an overall yield of 10%. A typical purification is summarized in Table I. The recovery of the activity on the column of pepstatin-Sepharose appeared to be low. This result, however, is similar to that reported by Afting and Becker,[15] who used 6 M urea washing in a similar column in the course of the purification of cathepsin D from pig myometrium. They proposed that the apparent low recovery from the column was not due to partial inactivation of the enzyme caused by washing with 6 M urea, since there was no difference in recovery with and without this washing, but was the result of separation of the "activator-proteins" at this step, as suggested by Huang and Tang.[16] There was also no difference in recovery regardless of 6 M urea washing in our experiment. On the other hand, Yamamoto *et al.* obtained a high recovery of cathepsin D from rat spleen at this step.[1d] The reason for this difference remains unclear.

As shown in Fig. 1, the purified enzyme migrated as a single protein band on SDS-polyacrylamide gel electrophoresis, and was estimated to have a molecular weight of 45000. This result agreed well with that of gel filtration on Sephadex G-150 as described above. The molecular weight of cathepsin D from MØs is similar to those (molecular weight of range of 40000—50000) of most cathepsins D from various sources.[1a,b,d]

The enzyme was resolved by isoelectric focusing into 4 components with pIs of 6.04, 6.53, 7.11 and 7.75. The present result is comparable to those obtained for other cathepsins D reported so far, [1a,b,d,e] though it is not clear whether the multiple forms of our cathepsin D resulted from a difference in amino acid composition and/or sugar content.

To determine whether the purified enzyme contained carbohydrate, it (100 μg) was subjected to concanavalin A-Sepharose column (0.9 × 10 cm) chromatography, since cathepsin D was found to be a glycoprotein.[1d] The enzyme bound to the column equilibrated with 20 mM sodium phosphate buffer, pH 7.0, containing 500 mM NaCl, and was eluted with 200 mM α-methyl mannoside in the same buffer, suggesting that it is a glycoprotein. About 90% of the cathepsin D activity applied was recovered from the column.

The maximal activities were found at pH 3.2 toward acid-denatured bovine serum Hb, at pH 3.5 toward native Hb and at pH 3.0 toward native BSA in 50 mM sodium acetate–HCl buffer of various pH's (1.5—5.8) at 37 °C for 10 min, using a substrate concentration of 2% (w/v) in each case. The enzyme showed about 50% of the maximal activity even at pH 5.0, but no activity at pH 7.0. Ishikawa and Cimasoni reported that human mononuclear leucocytes contained only acid protease, while PMNs showed a prominent protease activity at neutral pH with a small peak in the acid range.[1b] The crude enzyme obtained from MØs in our study also showed no activity at neutral pH, indicating that our preparation contained little or no

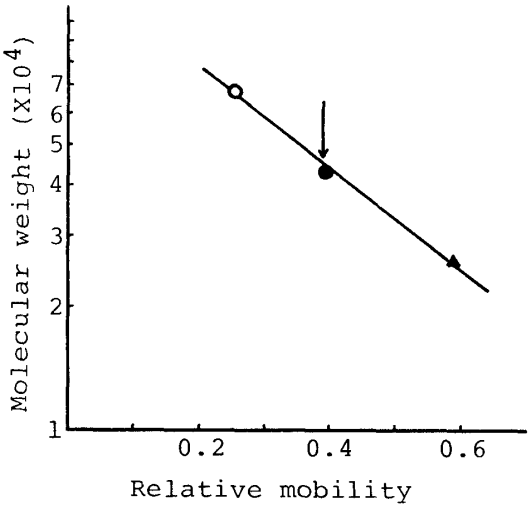

Fig. 1. SDS-Polyacrylamide Gel Electrophoresis of Cathepsin D

Samples (10 μg) were pretreated with 2% (v/v) 2-mercaptoethanol in the presence of 0.1% SDS for 5 min in a boiling water bath. After electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS at a constant current of 8 mA/column for 5 h, the protein in the gels was prefixed with 12.5% trichloroacetic acid for 1 h and stained with 0.25% Coomassie brilliant blue R-250. The molecular weight of cathepsin D (arrow) was estimated from the mobilities of marker proteins examined at the same time: BSA (○), egg albumin (●), α-chymotrypsinogen A (▲).

PMNs.

The enzyme reaction was carried out in the presence of various reagents under the standard assay conditions. The enzyme was completely inhibited by 0.15 mM pepstatin, but was not inhibited by the other reagents (1 mM), including NaCl, $FeCl_3$, *p*-chloromercuribenzoate, iodoacetic acid, phenylmethylsulfonyl fluoride, antipain and leupeptin; this behaviour is similar to that of most cathepsins D. The results described above strongly indicate that the aspartic proteinase isolated in this study is cathepsin D.

The preparation of MØs used in this study contained about 15% cells other than MØs. However, the activity of lymphocytes, comprising nearly 8% of the total peritoneal exudate cells, was estimated to be only 1/40 of that of all exudate cells on the basis of the activity of lymphocytes in the lymph-node. This result and the observed pH dependence of the activity strongly suggest that our cathepsin D originates mainly from MØs.

Further investigations are necessary to elucidate whether cathepsin D plays any physiological role in MØs.

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