Partial Purification of Cathepsin B in the Bovine Ciliary Body and Iris

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Cathepsin B in the bovine ciliary body and iris was studied biochemically using α-N-benzoyl-D,L-arginine-2-naphthylamide as substrate. The enzyme was purified to 210-fold from the autolyzed extract. The partially purified enzyme had a pH optimum at 6.0 and a molecular weight of 27,000. The apparent Km value for the substrate was 1.6 mM. The enzyme was activated by disodium ethylenediamine tetraacetate, cysteine, and dithiothreitol. The enzyme activity was inhibited strongly by leupeptin and partially by hyaluronate and chondroitin sulfate A. Invest Ophthalmol Vis Sci 24:682–686, 1983

Cathepsin B is known to be a thiol-dependent lysosomal proteinase[1] that can degrade collagens,[2,3] connective tissue proteins,[4] and some native enzymes.[5] Using α-N-benzoyl-D,L-arginine amide as substrate, we previously demonstrated that the ciliary body and iris had the highest activity of cathepsin B among bovine ocular tissues.[6] A more specific substrate, α-N-benzoyl-D,L-arginine-2-naphthylamide, was introduced recently and is now widely used for the assay of cathepsin B.[1]

The present study was undertaken to examine the enzyme properties in the partially purified preparation from the bovine eye.

Materials and Methods

Chemicals

α-N-benzoyl-D,L-arginine-2-naphthylamide, mersalyl acid, concanavalin-A Sepharose, pepstatin, and human umbilical cord hyaluronate were obtained from Sigma Chemical Company, St. Louis, MO; 4-amino-2',3-dimethylazobenzene and α-naphthylamine hydrochloride from Wako Pure Chemical Company (Japan); leupeptin from Protein Research Foundation (Japan); dithiothreitol and chondroitin sulfate A from Seikagaku-Kogyo Company (Japan); cysteine from Kyowahakko Company (Japan); ribonuclease A, chymotrypsinogen A, ovalbumin, Sephadex G-100, and DEAE-Sephadex A-25 from Pharmacia Fine Chemicals (Sweden).

Purification of Cathepsin B

About 60 adult bovine eyes were maintained at 4 C from the time of slaughter through all subsequent steps. The ciliary body and iris were dissected in ice-cold 20 mM potassium phosphate buffer (pH 6.8) containing 1 mM disodium ethylenediamine tetraacetic acid (EDTA), frozen and thawed five times, and homogenized in a Waring blender. The homogenate was centrifuged at 9,000 g for 15 min to obtain the supernatant (crude extract).

The crude extract was adjusted to pH 4.5 with 1 M acetic acid, incubated at 37 C for 2 hrs, and then centrifuged at 20,000 g for 15 min. The pellet was discarded and the supernatant was adjusted to pH 6.8 with 1 M potassium phosphate buffer (pH 7.0) (autolyzed extract). To the autolyzed extract, solid ammonium sulfate was added to 30% saturation (176 g/l), with constant mechanical stirring. After centrifugation, the precipitate was discarded, and the supernatant was brought to 60% saturation by adding solid ammonium sulfate (198 g/l). The precipitate was collected by centrifugation, dissolved in a small amount of 20 mM potassium phosphate buffer (pH 6.8) containing 1 mM disodium EDTA, and dialyzed overnight against the same buffer-EDTA solution, and ammonium sulfate fraction was prepared. To the ammonium sulfate fraction was added acetone to 40% (v/v), with constant mechanical stirring. After centrifugation, the precipitate was discarded and the supernatant was brought to 65% acetone (v/v) by the addition of acetone. The precipitate was collected by centrifugation, dissolved in a small amount of 20 mM

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Table 1. Purification of Cathepsin B from Bovine Ciliary Body‡

Fraction	*Volume (ml)*	*Total Protein (mg)*	*Total* Activity*	*Specific† Activity*	*Recovery (%)*	*Purification (n fold)*
Autolyzed extract	120	1644	26.6	0.016	100	1
Ammonium sulfate fraction (30–60% sat.)	26	553.8	34.6	0.062	130	3.9
Acetone fraction (40–65%)	4	80	23.7	0.30	89	18.8
Sephadex G-100 fraction	60	16.8	13.3	0.80	50	50
DEAE-Sephadex fraction	100	2.2	7.4	3.36	27.8	210

* μ moles 2-naphthylamine released/37° C, 30 min.
† μ moles 2-naphthylamine released/37°C, 30 min/mg protein.
‡ Enzyme assay and partial purification procedures were described in **Materials and Methods.**

potassium phosphate buffer (pH 6.8) containing 1 mM disodium EDTA, dialyzed overnight against the same buffer-EDTA solution, and the acetone fraction was obtained.

Two milliliters of acetone fraction were applied to a column (1.5 by 96 cm) of Sephadex G-100 equilibrated with 20 mM potassium phosphate buffer (pH 6.8) containing 1 mM disodium EDTA. The column was eluted with the same buffer-EDTA solution at a flow rate of 18 ml per h, and the fractions of 3.3 ml were collected. The fractions containing enzyme activity (fraction nos. 13–21) were combined to prepare the Sephadex G-100 fraction. To 15 ml of Sephadex G-100 fraction, 10 μl of 2-mercaptoethanol was added and applied to a column (0.9 by 27 cm) of DEAE-Sephadex A-25 equilibrated with 20 mM potassium phosphate buffer (pH 6.8) containing 1 mM disodium EDTA and 0.1 mM 2-mercaptoethanol. The column was washed with 50 ml of the same buffer-EDTA-mercaptoethanol solution and eluted with 80 ml of sodium chloride at a continuous linear gradient established between 0 and 0.5 M, containing the same buffer—EDTA-2-mercaptoethanol solution. The fractions of 2 ml were collected. The fractions containing enzyme activity (fractions nos. 10–22) were combined and dialyzed overnight against 20 mM potassium phoshphate buffer (pH 6.8) containing 1 mM disodium EDTA, obtaining DEAE-Sephadex fraction.

Enzyme Assay

Cathepsin B [E.C. 3.4.22.1.] activity was determined by a slight modification of the method described by Barrett.[1] Samples of enzyme solution were incubated in a total volume of 0.5 ml for 30 min at 37°C in 0.1 M potassium phosphate buffer at pH 6.0, containing 1.4 mM cysteine and 0.7 mM disodium EDTA, with 0.5 mg α-N-benzoyl-D,L-arginine-2-

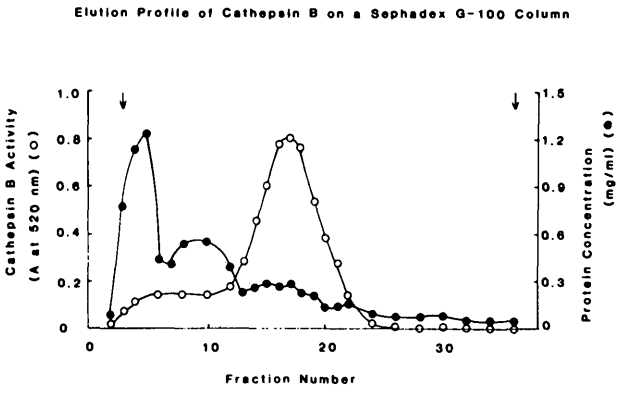

Fig. 1. Elution profile of cathepsin B in the ciliary body and iris on a Sephadex G-100 column: 100 μl of each fraction were incubated at 37 C for 30 min in 0.1 M potassium phosphate buffer (pH 6.0) containing 1.4 mM cysteine and 0.7 mM disodium EDTA with 0.5 mg α-N-benzoyl-D,L-arginine-2-naphthylamide as substrate in a total volume of 0.5 ml (○). Twenty to 50 μl of fraction were used for protein determination (●). Fraction nos. 13–21 were combined as the Sephadex G-100 fraction. Blue dextran was eluted at No. 3 (↓), and pyridoxal phosphate was eluted at No. 36 (↓).

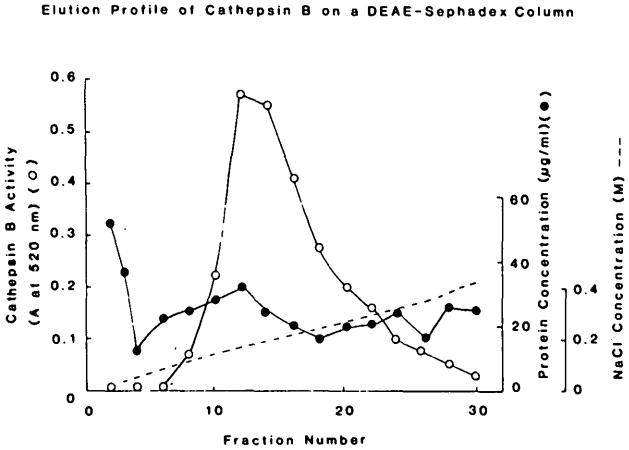

Fig. 2. Elution profile of cathepsin B in the ciliary body and iris on a DEAE-Sephadex column: 200 μl of each fraction were incubated at 37 C for 30 min in 0.1 M potassium phosphate buffer (pH 6.0) containing 14 mM cysteine and 0.7 mM disodium EDTA with 0.5 mg α-N-benzoyl-2-naphthylamide as substrate in a total volume of 0.5 ml (○). One hundred microliters of fraction were used for protein determination (●). Fraction nos. 10–22 were combined as the DEAE-Sephadex fraction.

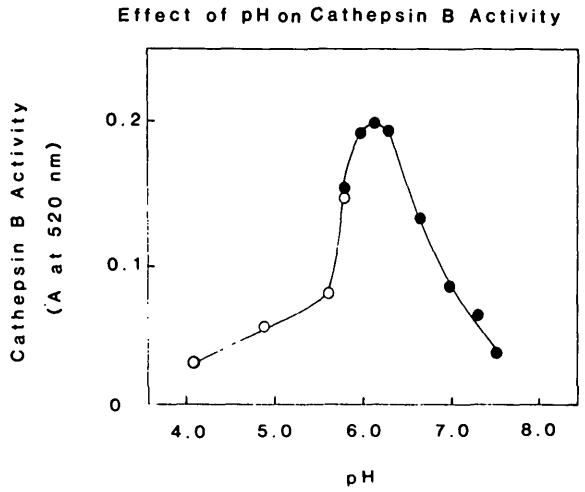

Fig. 3. Effect of pH on partially purified cathepsin B of DEAE-Sephadex fraction from the bovine ciliary body and iris: 2.2 μg protein of partially purified cathepsin B was incubated at 37 C for 30 min in 1.4 mM cysteine and 0.7 mM disodium EDTA with 0.5 mg α-N-benzoyl-D,L-arginine-2-naphthylamide as substrate. Buffers used were 100 mM citrate-sodium citrate buffer (pH 4.1–5.8) (○), and 100 mM potassium phosphate buffer (pH 5.8–7.5) (●).

naphthylamide as substrate. Incubation was terminated by the addition of mersalyl acid. The 2-naphthylamide released was colorimetrically determined after coupling with 4-amino-2',3-dimethylazobenzene.

Protein Determination

Protein content was determined by the method of Lowry et al.[7] Specific activity was expressed as the enzyme activity per milligram of protein.

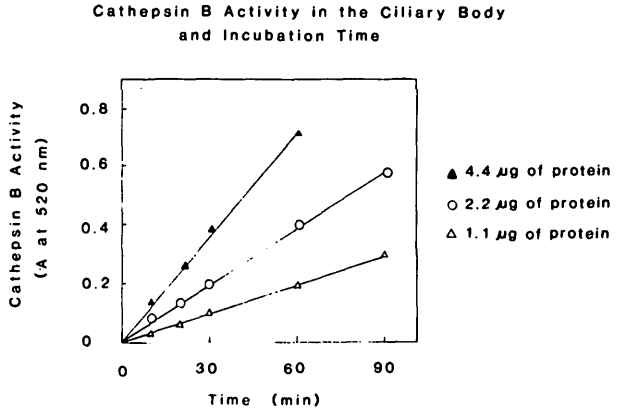

Fig. 4. Cathepsin B activity in the ciliary body and iris and incubation time: 4.4 μg protein (▲), 2.2 μg protein (○), and 1.1 μg protein (△) of partially purified cathepsin B of DEAE-Sephadex fraction were incubated at 37°C in 0.1 M potassium phosphate buffer (pH 6.0) containing 1.4 mM cysteine and 0.7 mM disodium EDTA, with 0.5 mg α-N-benzoyl-D,L-arginine-2-naphthylamide as substrate.

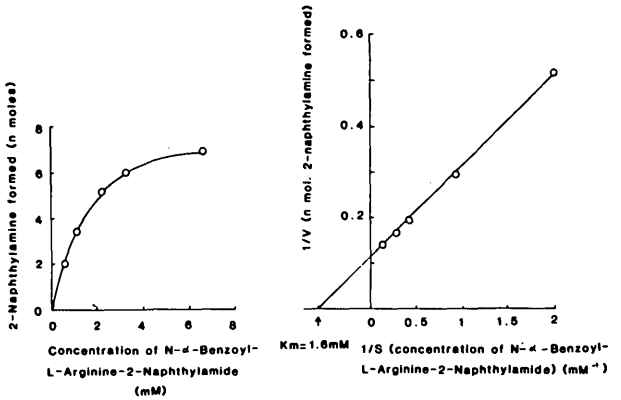

Fig. 5. Concentration of N-α-benzoyl-L-arginine-2-naphthylamide and cathepsin B activity in the ciliary body and iris: 2.2 μg protein of partially purified cathepsin B of DEAE-Sephadex fraction were incubated at 37 C for 30 min in 0.1 M potassium phosphate buffer (pH 6.0) containing 1.4 mM cysteine and 0.7 mM disodium EDTA.

Molecular Weight Determination

The apparent molecular weight of cathepsin B was determined according to the method of Andrews.[8] The acetone fraction was applied on a Sephadex G-100 column (1.5 by 96 cm) previously equilibrated with 20 mM potassium phosphate buffer (pH 6.8) containing 1 mM disodium EDTA, and the column was eluted with same buffer-EDTA solution. Ribonuclease A (molecular weight, 13,700), chymotrypsinogen A (molecular weight, 25,000) and ovalbumin (molecular weight, 43,000) were used as reference standards.

The Response to Concanavalin A

The Sephadex G-100 fraction (0.1 mg protein) was dialyzed for 3 hours against 4 mM $CaCl_2$-4 mM $MnCl_2$-4 mM $MnCl_2$-10 mM potassium phosphate buffer (pH 6.8) and applied on a concanavalin A-Sepharose column (1.5 by 20 cm) previously equilibrated with 4 mM $CaCl_2$-4 mM $MnCl_2$-4 mM $MnCl_2$-10 mM potassium phosphate buffer (pH 6.8). The column was washed with the starting buffer until the A 280 fell nearly to the starting value, and then the absorbed fraction was eluted with 250 mM α-methyl-D-mannoside in the same buffer.

Results

The results of partial purification are summarized in Table 1. The final preparation, DEAE-Sephadex fraction, was purified 210-fold over the autolyzed extract, with an overall recovery of 27.8%. Fig. 1 shows the elution profile of cathepsin B on Sephadex G-100

column chromatography, and Fig. 2 shows the elution profile of cathepsin B on DEAE-Sephadex column chromatography.

The effect of pH on the DEAE-Sephadex fraction is shown in Fig. 3. The optimal pH was near 6.0–6.2 when N-α-benzoyl-D,L-arginine-2-naphthylamide was used as substrate.

The enzyme activity was proportional to both enzyme concentration and incubation time (Fig. 4).

The apparent Km value of DEAE-Sephadex fraction for N-α-benzoyl-D,L-arginine-2-naphthylamide was estimated to be 1.6 mM (Fig. 5).

The molecular weight of the enzyme appeared to be 27,000 by the estimation of a gel filtration method (Fig. 6).

The enzyme activity in DEAE-Sephadex fraction was activated by disodium EDTA, cysteine, and dithiothreitol, and inhibited by leupeptin, which is an inhibitor of thiol proteinase. The enzyme activity was not affected by pepstatin, an inhibitor of carboxyl proteinase. The enzyme activity was partially inhibited by hyaluronate and chondroitin sulfate A (Table 2). The enzyme was unadsorbed by concanavalin A-Sepharose.

Discussion

We previously showed the high specific activity of cathepsin B in the bovine ciliary body and iris using α-N-benzoyl-L-arginine amide as substrate.[6] The present study clarified some properties of the enzyme: the optimal pH (6.0), Km values for N-α-benzoyl-L-arginine-2-naphthylamide (1.6 mM), and molecular weight (27,000) in the bovine ciliary body and iris are

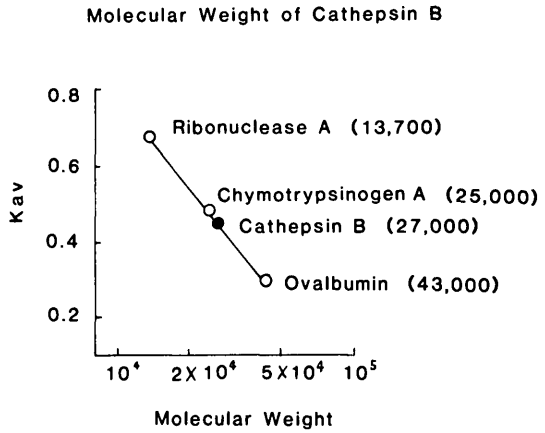

Fig. 6. Molecular weight of cathepsin B from the bovine ciliary body and iris. A column (1.5 by 96 cm) of Sephadex G-100 equilibrated with 20 mM potassium phosphate buffer (pH 6.8) containing 1 mM disodium EDTA was used to determine the molecular weight.

Table 2. Effect of Various Compounds on Cathepsin B from Bovine Ciliary Body*

Compounds	Absorbance at 520 nm†
Disodium EDTA (0.7 mM)	0.040 ± 0.010
Cysteine (1.4 mM)	0.280 ± 0.030
Disodium EDTA (0.7 mM) + cysteine (1.4 mM)	0.385 ± 0.035
Disodium EDTA (0.7 mM) + DTT (0.7 mM)	0.370 ± 0.035
Disodium EDTA (0.7 mM) + cysteine (1.4 mM) + pepstatin (10 nM)	0.375 ± 0.030
Disodium EDTA (0.7 mM) + cysteine (1.4 mM) + leupeptin (10 nM)	0.020 ± 0.010
Disodium EDTA (0.7 mM) + cysteine (1.4 mM) + human umbilical cord hyaluronate (0.17 mg/ml)	0.250 ± 0.035
Disodium EDTA (0.7 mM) + cysteine (1.4 mM) + chondroitin sulfate A (0.2 mg/ml)	0.210 ± 0.030

* DEAE-Sephadex fraction was dialyzed for 4 hours against 20 mM potassium phosphate buffer (pH 6.8). Dialyzed DEAE-Sephadex fraction (4.4 μ protein) was incubated at 37°C for 30 min in 0.1 M potassium phosphate buffer (pH 6.0) with 0.5 mg α-N-benzoyl-D,L-arginine-2-naphthylamide and with compound.

† Mean ± S.D. (N = 4)

quite similar to these in bovine liver and spleen, as reported by others.[1] The substrate used in the present study is active with cathepsin H as well as cathepsin B. Barrett and Kirschk[9] reported that cathepsin B does not bind to concanavalin A, while cathepsin H does. The Sephadex G-100 fraction was unadsorbed by concanavalin A-Sepharose, indicating cathepsin B. It is of particular interest that the enzyme was partially inhibited by exogenous hyaluronate and chondroitin sulfate A (Table 2). This finding suggests that the activation of the enzyme during autolysis may be partly due to the degradation of endogenous by hyaluronate and chondroitin sulfate. Hence, the enzyme released from the ciliary body may be partially inhibited by the vitreous body, as the ciliary body is in close contact with vitreous body hyaluronate.

Cathepsin B, a proteinase that degrades collagens and proteins,[1] should be considered in some pathophysiologies of the uvea, because (1) the enzyme is found in the aqueous humor from patients with Bahcet's disease[1,10]; (2) the enzyme may have an important role in tissue injury in uveitis; and (3) the enzyme may have potential to degrade not only native protein but also synthetic polymers, such as nylon.[11]

Key words: cathepsin B, bovine ciliary body and iris, partial purification

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