Cathepsin B, the Lysosomal Thiol Proteinase of Calf Liver

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Cathepsin B from calf liver was obtained by a method involving preparation of a lysosomal-mitochondrial pellet and treatment of this pellet with acetone. The material was extracted with an acid buffer, pH 4.0, and then precipitated from the extract with acetone. The precipitate was dissolved in phosphate buffer, pH 7.4, and subjected to gel filtration on Sephadex G-200 and G-100. The cathepsin B emerged in a range of molecular weight much lower than 50 000 as a well-defined component. The purity of this material was checked by electrophoresis. To obtain maximum activity the enzyme had to be activated with a chelating agent and a reducing agent (i.e. EDTA and cysteine). A number of different substrates were used. The enzyme was active for the hydrolysis of both peptide bonds and ester bonds and had approximately equal reactivity in the two cases. The pH-dependence of the hydrolysis was the same with both substrates. The binding of the substrates was half-maximal at pH 4.5 and at pH 6.8. A thiol group occurred in the active centre but this group ought to have a much higher pK than that found in this enzyme.

It is believed that the cathepsins, i.e. the endogenous intracellular proteinases of animal origin, play an important role in cell physiology. The liver intracellular proteinases are the classical examples of lysosomal proteinases (de Duve, Wattiaux & Baudhuin, 1962) but in spite of this only the acid cathepsin D has been studied (Barrett, 1967). Although the activity of the thiol-dependent cathepsin B has been demonstrated in a great variety of tissues it has received little attention. The activity, as usually measured, is low and hence the isolation of the enzyme in quantity seems difficult. The cathepsins of spleen have been studied in some detail and classified according to their specificities by Tallan, Jones & Fruton (1952), who also separated cathepsin C. Cathepsin B has been partially purified from ox spleen (about 200-fold) (Greenbaum & Fruton, 1957). It was characterized as a 'trypsin-like' intracellular proteinase that hydrolyses benzoyl-L-arginine amide to benzoyl-L-arginine and ammonia (Fruton, Irving & Bergmann, 1941*a,b*).

In the present paper the purification of cathepsin B from calf liver is described and measurements of the activity towards a variety of different substrates are given.

MATERIALS

The following substrates were obtained from Mann Research Laboratories, New York, N.Y., U.S.A.: BANA,*

* Abbreviations: BANA, α -N-benzoyl-L-arginine naphthylamide; BAEE, α -N-benzoyl-L-arginine ethyl ester; LNA, L-leucine β -naphthylamide; ANA, L-arginine β -naphthylamide; TAME, toluene-*p*-sulphonyl-L-arginine methyl ester.

BAEE, LNA, ANA and TAME. Edestin was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex. Human serum albumin was obtained from KABI AB, Stockholm, Sweden. EDTA was obtained from Hopkin and Williams Ltd. and cysteine hydrochloride from E. Merck A.-G., Darmstadt, Germany. Sephadex was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

METHODS

Activity measurements. The hydrolysis of different substrates was measured at 40° in the pH range 4.5–8.0. For ester substrates the hydrolysis was followed by measuring proton release by using a pH-stat. The hydrolysis of naphthylamine derivatives was measured by diazotizing the naphthylamine released and converting it into an azo-dye.

The pH-stat equipment (Radiometer, Copenhagen, Denmark) consisted of a titrator (type TTT1c), a recorder and a syringe burette, which delivered 0.5 ml. at full deflexion of the recorder.

The glass-calomel electrodes were of type K100. Enzymic reactions were carried out in a 12 ml. beaker with a water jacket connected with a thermostat (Calora; Lorch A.-G., Hamburg, Germany). Throughout the investigations 1 mM-EDTA and 4 mM-cysteine were used as activators added to both the substrate solution and the enzyme solution.

To the reaction vessel were added 6 ml. of substrate solution and 3 ml. of 0.3 M-KCl, the temperature was equilibrated and the medium adjusted to the desired pH. The 1 ml. of prewarmed enzyme solution was added and the reaction was followed for about 20 min. The reaction velocity was measured from the recorder trace. It was a straight line for the time used. Generally 3–5% of the material was hydrolysed in this interval.

The assay of enzyme activity by release of naphthylamine followed the method of Goldberg & Rutenburg (1958)

as adapted for use on a small scale by Sylvén & Boiss-Svensson (1965). Samples (50 μ l.) of substrate were mixed with enzyme (or blank) samples (10 μ l.) (containing 6 mM-EDTA and 24 mM-cysteine) and incubated at 40°, usually for 1 hr. The reaction was stopped by adding 50 μ l. of 40% (w/v) trichloroacetic acid. The naphthylamine released was then determined by diazotization and coupling to form an azo-dye, whose E_{580} was measured.

Determinations of the proteolytic activity against urea-denaturated edestin were made by the method of Ottoson & Sylvén (1960) at 40°. The digested material was precipitated with 5% (w/v) trichloroacetic acid. The precipitate was spun down and the supernatant was taken for determinations of the amount of peptide left with the Folin-Ciocalteu reagent in a Beckman DU spectrophotometer by measuring the E_{650} .

The activated enzyme was used as soon as possible (less than 2 hr.) after activation. In some experiments 10 times as much cysteine was tried without any alteration in the activity. If less than 2 mM-cysteine was used the activity decreased. The amount of EDTA could not be varied much on account of the buffer capacity of the substance.

Column chromatography. Sephadex-gel-filtration columns were prepared as recommended by the manufacturers. They were equilibrated with the elution buffer before use and flow rates were controlled by a peristaltic pump.

Gel electrophoresis. A Shandon preparative polyacrylamide-gel electrophoresis apparatus was used. Enzyme solution (0.05 ml.) was layered on the top of a 3 cm. column (10% gel) followed by 50 mM-tris-HCl buffer, pH 7.2. The duration of the run was 10 hr. The eluate was taken out in 2 ml. fractions.

Paper electrophoresis. Paper-electrophoresis experiments were performed with an LKB instrument. The time used for electrophoresis was 4–6 hr. with 200 v and 36 ma. The papers were stained for protein with Amido Black 10B as described by Grassmann & Hannig (1952).

RESULTS

Purification of cathepsin B. For the preparation of cathepsin B the following buffers were used: buffer A, 50 mM-tris-HCl, pH 7.4, containing 0.25 M-sucrose and 1 mM-EDTA; buffer B, 50 mM-sodium formate buffer, pH 3.7, containing 20% (w/v) of acetone and 1 mM-EDTA; buffer C, 50 mM-tris-HCl buffer, pH 7.4, containing 1 mM-EDTA.

Fresh calf liver (1 kg.) obtained from the slaughterhouse was cut into pieces and put into 2 l. of cold buffer A. The solution was homogenized by means of a Polytron (Mobil Aarau A.G., Selzach, Switzerland). A fraction consisting of nuclei and debris was removed from the homogenate by centrifugation for 10 min. at 2000 rev./min. (700g) in a Wifug centrifuge. The supernatant was re-centrifuged in a Spinco model L ultracentrifuge (rotor 21) at 8000 rev./min. (7000g) for 60 min. to produce a lysosomal-mitochondrial pellet. This pellet was treated with 1 l. of cold acetone (4°). The mixture was stirred and left for 1 hr. The fluid was filtered off and the solid residue was washed with

acetone and then air-dried on a filter paper. The yield was 46 g. This material could be stored for several months without loss of activity.

The acetone-dried powder was extracted with buffer B. The yield from this extraction was very dependent on the pH of the buffer. Maximum yield was obtained at pH 4. During the extraction the pH of the solution increased and it was therefore found better to start at a slightly lower pH, if necessary with adjustment of the pH afterwards. The acetone-dried powder was first extracted with 300 ml. of buffer B for 90 min. After decantation the sediment was re-extracted for 19 hr. with 200 ml. of buffer B. The sediment was spun down. The two supernatants were pooled and poured into cold acetone (4 vol.). The precipitate was collected by centrifugation and drained (6 g. was obtained). Part of the drained material (2 g.) was resuspended in 50 ml. of buffer C. The suspension was stirred for 4 hr. and then centrifuged. The clear supernatant was concentrated by ultrafiltration to 5 ml. (to put on a Sephadex column) in a Diaflo no. 5 cell with filter UM-1 (Amicon Corp., Lexington, Mass., U.S.A.), which retains material of mol.wt. over 10 000. It was found that an excellent separation could be obtained by using a set of columns. The set consisted of one column containing Sephadex G-200 (41 cm. $\times$ 2.5 cm.) and two columns containing Sephadex G-100 (each 41 cm. $\times$ 2.5 cm.). The Sephadex was equilibrated with 1% sodium chloride. The separation was started on the column containing Sephadex G-200. The first 88 ml. was collected separately. This contained most of the material passing directly through the column. After the first 88 ml. had emerged, the column was coupled to the other columns of Sephadex G-100. The solution emerging from the last column was passed through a Uvicord 1 spectrophotometer (to measure E_{254}) and then to a fraction collector; 5 ml. fractions were collected (Fig. 1).

Some proteins were first to emerge, followed by three other peaks as measured with the method of Lowry, Rosebrough, Farr & Randall (1951) at 650 nm. First emerged small amounts of leucine aminopeptidase, revealed by the activity against LNA. This activity was suppressed by EDTA. This peptidase was followed by another peptidase, which was activated by cysteine. Some unknown proteins followed. The third peak contained cathepsin D and subsequently cathepsin B followed as a broader peak. Cathepsin D has mol.wt. 50 000–55 000 (Barrett, 1967). Cathepsin B has about half this molecular weight, and emerged free from cathepsin D. The fraction containing cathepsin B was the only one that hydrolysed BANA. The curve in this region was identical with that given for activity against LNA and they agreed with the curve for protein in this range.

Fig. 1. Gel filtration of the material precipitated by acetone and then dissolved in buffer at pH 7.4. A series of Sephadex G-200 and G-100 columns was used (see the text). Protein eluted was determined by the method of Lowry *et al.* (1951) (E_{650} ; —) and activity against LNA in the presence of cysteine and EDTA is given (E_{560} ; ○). (a), Leucine aminopeptidase; (b), position of cathepsin D; (c), cathepsin B.

The fraction containing cathepsin B was collected, dialysed against distilled water and freeze-dried. About 20 mg. was obtained, i.e. 60 mg./kg. of liver.

The material used in this investigation was obtained by the above procedure. It was preserved in a desiccator in the cold. Weighed amounts dried over phosphorus pentoxide were used to calibrate determinations with the method of Lowry *et al.* (1951) for protein contents in samples used.

Degree of purification. The specific activities, given in arbitrary units/mg. of substance (measurements performed with BANA as substrate), were: liver homogenate, 0.002; acetone-dried powder, 15; Sephadex filtrate, 3300. This represents a purification from the acetone-treated powder of greater than 200-fold. The yield is 30% of that obtained in the acetone-dried powder.

Experiments were performed with a Shandon preparative polyacrylamide-gel electrophoresis apparatus to study the homogeneity of the cathepsin. Phosphate buffers with pH between 6 and 7 were used. In all cases only one component appeared (Fig. 2), as revealed by protein determinations by the method of Lowry *et al.* (1951). However, the enzyme activity had disappeared during the process, although the amount of protein obtained was the same as that put on the gel.

Paper-electrophoresis experiments were performed at different pH values with an LKB instrument. Only one component was seen in the range studied (pH 4.0–7.5). The measurements indicated an isoelectric point in the vicinity of pH 5.

The preparation did not hydrolyse glycyl-L-phenylalanine amide, which is the specific substrate

Fig. 2. Polyacrylamide-gel electrophoresis of cathepsin B performed on a Shandon preparative gel-electrophoresis apparatus. Conditions: 3 cm. column, 10% gel, 50 mM-tris-HCl buffer, pH 7.2. Protein was determined by the method of Lowry *et al.* (1951).

Table 1. *Proteolytic activity of cathepsin B with different activators*

Buffers (0.01 M-acetate and 0.01 M-citrate) were used at pH 6.0, with 4 mM-cysteine and 2 mM-EDTA. The substrate was 2% edestin.

Buffer	Activator	Activity (increase in E_{650} /hr.)
Acetate	No addition	0.092
	EDTA	0.092
	Cysteine	0.073
	Cysteine + EDTA	0.315
Citrate	No addition	0.092
	Cysteine	0.225

of cathepsin C. After treatment with iodoacetic acid the proteolytic activity disappeared. Cathepsin D is undisturbed by such treatment. When Mn^{2+} ions were added the cathepsin B activity disappeared (and also that towards LNA), but it could be restored by treatment with EDTA.

Activity determinations. The prepared cathepsin B showed a low activity when measured directly. The addition of EDTA or the use of a citrate buffer did not alter the activity in most cases, although in some cases there was a slight increase. Cysteine, in the absence of a chelating agent, increased the activity in the range pH 4–5. Cathepsin B is thus not fully activated by EDTA or cysteine alone. However, the combination of a substance that forms complexes with metal ions and a reducing agent such as cysteine greatly enhances the activity. In this respect cathepsin B resembles papain (Kimmel & Smith, 1957). Results are given in Table 1.

Stability. In the inactive form the enzyme seemed to be stable for a long time (tested up to 12 months), showing no loss of activity when re-activated. In the activated state it could not be stored for more than 1–2 days without loss of activity. If the preparation was freeze-dried with a small amount of cysteine, the pH-dependence of the enzyme activity was altered; the activity in the region pH 6–7 almost disappeared. In the measurements performed here, the enzyme was used directly after activation with cysteine and EDTA.

Effect of pH on the enzyme activity. In the cases where the substrate had the same ionization over the whole pH range studied, the shape of the curve when the value of k_{+2}/K_m (taken as k_{+1} ; see Discussion) is plotted as a function of pH is given in Fig. 3. The activity is half-maximal at pH_a 4.5 and pH_b 6.8, and reaches a maximum value at pH 5.7. The same curve is obtained when the protein substrates albumin and edestin are used, as well as with the synthetic substrate BANA. The

esterase effect, as measured on BAEE and TAME, followed the same trace, indicating that the same groups are responsible for the activity in all cases.

The two substrates LNA and ANA showed different kinds of pH-activity curves (see Fig. 4). These curves can be interpreted to mean that the α -amino group of these substrates has pK approx. 8.0. The form having the α -amino group un-ionized is hydrolysed in the region pH 6–8, whereas the ionized form is mainly hydrolysed in the region pH 4–6. The enzyme is less active against the latter form of the substrate.

Substrate concentration. The dependence of the velocity on the substrate concentration was determined. The method of Lineweaver & Burk (1934) was employed to evaluate the Michaelis parameters K_m and V . The results are given in Table 2. For BANA, LNA and ANA the graphs were made at different pH values (Fig. 5). The value of V was independent of pH and was the same for all three substrates.

Effect of cysteine on the activity. With measure-

Fig. 3. Relative values of k_{+1} as a function of pH. The curve drawn is calculated for two titratable groups with pK 4.6 and 6.7. Values obtained with edestin ($\blacktriangle$) and BAEE ($\circ$) are given. The same curve fits also for values obtained with serum albumin, BANA and TAME.

Fig. 4. Relative values of k_{+1} as a function of pH for LNA ($\blacksquare$) and ANA ($\circ$).

Fig. 5. Lineweaver-Burk plots of the hydrolysis of BANA ($\circ$) at pH 6, LNA ($\square$) at pH 7 and ANA ($\blacktriangle$) at pH 7. The results were obtained at the same concentration of cathepsin B.

Table 2. Kinetic constants for cathepsin B action

K_m is given as the molar concentration; maximum rate V as moles of substrate hydrolysed/min./mg. of enzyme. The units of k_{+1} and k_{+2} are derived from K_m and V .

Substrate	K_m	$1/V$	pH	k_{+1}	k_{+2}
BAEE	0.0175	10.2	6.0	559	98
BANA	0.0200	7.0	6.0	714	143
ANA	0.0200	7.0	7.0	714	143
LNA	0.0280	7.0	7.0	510	143
TAME	0.0263	24.0	6.0	158	42

ments with enzyme solutions that had been treated with a higher concentration of cysteine and preserved for a longer time the pH-activity curves obtained were anomalous. The same phenomenon was found when the enzyme was freeze-dried in the presence of cysteine. The activity decreased in the region pH 5.5–8 and the maximum activity seemed to occur at a lower pH than expected. No good reproducibility could, however, be obtained between different preparations.

DISCUSSION

In all cases where the rate of hydrolysis was measured as a function of substrate concentration and pH, the effect of substrate concentration could be described by the Michaelis-Menten equation derived from:

as judged by linearity of plots of $1/v$ versus $1/s$.

With the substrates BANA, LNA and ANA the maximum velocity constant was independent of pH and the same in all cases. This indicates that the ionization of the enzyme-substrate complex does not vary. The variation of the activity with pH is dependent on the ionization of the enzyme and of the substrate. The shape of the activity curve can be described by:

$$\frac{V}{K_m} = \frac{k_{+1}k_{+2}e}{k_{-1} + k_{+2}} \left(\frac{1}{1 + [H^+]/K_a + K_b/[H^+]} \right) \times \left(\frac{1}{1 + K_s/[H^+]} \right)$$

where K_a and K_b are the ionization constants of the enzyme having influence on the kinetics (Alberty, 1956). K_s has to be included in the cases where the substrate alters its ionization constants in the pH range studied.

The theoretical curve in Fig. 3 was calculated for two titratable groups of pK_a 4.6 and pK_b 6.7 with the maximum value of k_{+1} being equal to 1.00. The pK values were calculated from:

$$K_a = H_a + H_b - 4\sqrt{(H_a H_b)}$$

$$K'_a K_b = H_a H_b$$

(see Alberty, 1956).

In Table 2 the constants for the substrates used are given. The values of k_{+1} and k_{+2} are relative values referred to 1 mg. of enzyme/ml. They are evaluated with the assumption that $k_{-1} \ll k_{+2}$ and therefore $K_m = k_{+2}/k_{+1}$. This evaluation is done to show a similarity with papain. For a discussion of this assumption see Sluyterman (1964). The affinities of the enzyme for BANA and BAEE are approximately equal, as judged from their k_{+1} values, as are the breakdown rates and the pH-

dependence of the activity. The same active areas on the enzyme may therefore be used for the decarboxylation in both cases. In all cases, with the exception of TAME, the rates of splitting of the substrate as measured by k_{+2} are nearly equal, indicating that this step may be rate-limiting in these measurements. TAME, which has a lower affinity for the enzyme, may have a step other than that relating to k_{+2} as the rate-limiting step.

The affinities of the substrate for the enzyme are approximately the same for ANA and LNA as for BANA. At the optimum pH, where the results were recorded, the majority of the two substrates (ANA and LNA) have α -amino groups un-ionized. In this region therefore ANA closely resembles BANA with respect to charge and it is thus possible to understand the similarities in their affinities. With LNA there is no charged group on the substrate at pH 7. The reason for this substance being a good substrate is open to question.

The two pK values (4.6 and 6.7) obtained deserve some comment, since an identification by titration alone is not completely certain.

A method to activate the enzyme is clearly indicated since the active centre contains a thiol group. If *p*-hydroxymercuribenzoate is added to the enzyme solution the activity disappears, showing that a thiol group is associated with the activity. In the cases where treatment of the enzyme caused a fall in activity between pH 5.5 and 8, there appears to be a special reaction giving rise to an inhibition, and the product of this reaction is stable only above pH 5.5.

The pK value of 6.7 is lower than the expected value for a thiol group. With papain the titratable thiol group has pK 8.0 (Sluyterman, 1964), but here the value is in a region where histidine has its pK . It should be remembered, however, that other groups have pK values in this region (see Dixon & Webb, 1958). Only very tentatively can the value be ascribed to a histidine group.

The reason that histidine is discussed here is that, in papain, a histidine residue participates in the reaction in addition to the thiol group (Husain & Lowe, 1968*a,b*). Further studies, by other means, are necessary to confirm the occurrence of a histidine group taking part in the reaction sequence.

Cathepsin B is trypsin-like in that both enzymes attack bonds to the same amino acid residues in a protein. If albumin is first digested with trypsin and this partially degraded albumin is then used as a substrate for cathepsin B, no further hydrolysis is detected as measured by the titration method. The pH range of the activity, however, is very different in the two cases. The activity of cathepsin B decreases in the same region where the activity of trypsin begins.

A comparison with papain shows that both

enzymes have a thiol group at the active centre, and cathepsin B might, like papain, have a histidine residue involved in the mechanism of action. However, the specificities of the two enzymes are not comparable in all respects, papain being less specific. For papain the esterase effect is much stronger than the amidase effect, but for cathepsin B the two activities are nearly equal. The less common substrate LNA is split about 50 times as rapidly by cathepsin B as by papain. Although the principle of the reaction might be the same in both cases there must be alterations in structure that modify the reaction so that the two proteins are not in detail comparable.

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