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HISTONE HYDROLASE ACTIVITY OF RAT LIVER LYSOSOMAL CATHEPSIN B2

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SUMMARY

1. Histone hydrolase activity of rat liver is mainly localized in the light mitochondrial–lysosomal fraction and is due mainly to cathepsin B2.

2. The rate and extent of histone hydrolysis by cathepsin B2 were higher than those by cathepsins A, C (EC 3.4.4.9) and D (EC 3.4.4.23) and trypsin.

3. The specific activity of cathepsin B2 was much higher on histones than on the basic proteins lysozyme, cytochrome *c* and protamine sulfate.

4. The DEAE-cellulose column chromatography elution profile, pH-profile, stability properties and the effect of halide ions, sulfhydryl reagents and inhibitors were similar for histone and benzoylarginine amide hydrolase activities, indicating that the activities are due to the same enzyme.

5. The products of cathepsin B2 hydrolysis of arginine-rich histones were separated by Sephadex G-25 column chromatography. Some evidence of endopeptidase activity was shown by the presence of products with molecular weights approximating those of amino acids through hexapeptides.

INTRODUCTION

Cathepsin B and related enzymes[1–3] have been detected in tissues and subcellular fractions by their sulfhydryl activated hydrolysis of synthetic substrates. Cathepsin B has been assayed by its proteinase activity on edestin[4], gelatin[5] and trypsinogen[6]. Otto[7] showed that cathepsin B1 catalyzes the hydrolysis of benzoylarginine-*p*-nitroanilide, and that it is different from the enzyme that hydrolyzes benzoylarginine amide, cathepsin B2. The hydrolysis of trypsin substrates by the cathepsin B group of enzymes suggests their preferential hydrolysis of basic proteins. Greenbaum[8] recommended studying the activity of cathepsin B on proteins to better understand their role as a protease or peptidase.

Histones and other nucleoproteins are involved in control of DNA template activity, and the ability of lysosomes to stimulate mitosis[9–12] implies the function of lysosomal cathepsins in the hydrolysis of histones and other basic proteins.

Information on the hydrolysis of these substrates by lysosomal cathepsins is required in order to elucidate these possible roles. This study reports the properties of cathepsin B2 and other cathepsins as histone hydrolases.

MATERIALS AND METHODS

Preparation of lysosomal fractions

Liver lysosomal fractions were prepared from male Sprague–Dawley rats according to the procedure described by Ragab *et al.*[13]. For subcellular distribution studies, the fractionation was carried out according to the procedure of de Duve and coworkers[14].

Preparation of cathepsins A, B2, C and D

Cathepsin D (EC 3.4.4.23) was prepared according to the procedure of Goettlich-Riemann and workers[15]. The method of Liao-Huang and Tappel[16] was used to prepare cathepsin C (EC 3.4.4.9) from rat liver lysosomal fractions. For the preparation of cathepsins A and B2, lysosomal fractions were frozen and thawed 10 times and centrifuged at 100 000 $\times$ *g* for 60 min. The supernatant was dialyzed against 1 mM sodium acetate buffer (pH 5.0). The fraction of the supernatant that precipitated at 30–60% $(NH_4)_2SO_4$ saturation was redissolved and dialyzed against 1 mM sodium acetate buffer (pH 5.0) and was then applied to a DEAE-cellulose column equilibrated with the same buffer. Protein was eluted stepwise with 5, 10, 20 and 50 mM NaCl. The main fractions of cathepsin A (eluted at 50 mM) and cathepsin B2 (eluted at 20 mM) were rechromatographed separately, and the rechromatographed fractions were used in these studies. Cathepsin B2 was also prepared by a modification of this procedure using a continuous NaCl gradient at pH 6.8 on DEAE-cellulose followed by Sephadex G-100 chromatography[3].

Cathepsin B2 assay

Cathepsin B2 activity was determined with 0.1 M benzoylarginine amide in 0.1 M citrate–phosphate buffer (pH 5.4), 10 mM dithioerythritol and 20 mM NaCl, essentially according to the procedure of Greenbaum and Fruton[1]. The NH_3 released was removed from the reaction mixture as described by Seligson and Seligson[17] and determined colorimetrically either by phenol–hypochlorite reagent[18] or by the ninhydrin method of Moore and Stein[19] as adapted in this laboratory[20]. For halide activation studies, the Cl^- from benzoylarginine amide·HCl was precipitated with a slight excess of $AgNO_3$ and the excess Ag^+ was precipitated with dithioerythritol.

Cathepsin D assay

Cathepsin D was assayed by a modification of the method of Anson[21]. The reaction mixture consisted of 0.20 ml of 3% acid-denatured hemoglobin in 0.1 M sodium acetate buffer (pH 3.8) and usually 0.1 ml of enzyme in a total volume of 0.5 ml. After 30 min of incubation the reaction was stopped by addition of 0.5 ml 20% trichloroacetic acid; after centrifugation the supernatant portion was assayed by reaction with ninhydrin[19].

Cathepsin A assay

Cathepsin A activity was assayed according to the procedure of Iodice *et al.*[22] with *N*-carbobenzoxy-α-L-glutamyl–L-phenylalanine (Schwarz-Mann). The phenylalanine released was measured by the ninhydrin reagent[19].

Cathepsin C assay

Cathepsin C was assayed with glycyl–L-tyrosine amide (Schwarz-Mann) at pH 6.8 according to the procedure of Metrione *et al.*[23].

Acid phosphatase assay

The reaction mixture for acid phosphatase (EC 3.1.3.2) contained 0.4 ml of 0.125 M β-glycerophosphate, 0.1 ml of enzyme solution and 1.5 ml of 1 M sodium acetate buffer (pH 5.2). After incubation for 20 min at 37 °C, 0.4 ml of 25% trichloroacetic acid was added and the inorganic phosphate in the supernatant was determined by the method of Fiske and SubbaRow[24].

Hydrolysis of histones

Whole calf thymus histones (Type II) and arginine-rich histones (Type IV, Sigma Chemical Co.) were heated at 60 °C for 20 min to denature the protein and to inactivate the nucleohistone-associated protease[25,26]. The denatured protein was dialyzed against 0.1 M citrate–phosphate buffer (pH 5.4) and then diluted with the same buffer to a final concentration of 30–40 mg/ml. A typical 0.5-ml incubation mixture consisted of 2–3 μg of enzyme protein, 3–4 mg of substrate, 20 mM citrate–phosphate buffer (pH 5.4), 10 mM dithioerythritol and 20 mM NaCl. After incubation for 30–60 min at 37 °C, the reaction was stopped by addition of 0.5 ml of 20% trichloroacetic acid and ninhydrin-positive material was determined[19].

Hydrolysis of other basic proteins

Lysozyme (Worthington Biochemicals), cytochrome *c* (Sigma Chemical Co.) and protamine sulfate (Nutritional Biochemicals) were denatured with urea under alkaline conditions as described by Rick[27]. The substrates were dialyzed against distilled water, lyophilized and resuspended in 0.1 M citrate–phosphate buffer (pH 5.4) at a final concentration of 40 mg/ml. Lysozyme and protamine sulfate were not completely soluble in this buffer but a homogeneous suspension was obtained by homogenization in a glass and Teflon homogenizer. The assay conditions for hydrolysis of these basic proteins by cathepsin B2 were similar to those used for hydrolysis of histones. The basic proteins were also hydrolyzed with trypsin (EC 3.4.4.4, bovine pancreas trypsin, Type III two times crystallized, Sigma Chemical Co.) according to the method described by Rick[27].

Estimation of molecular size of hydrolysis products

Hydrolysis of arginine-rich histones was done in a 1.5-ml incubation mixture consisting of 9 μg of enzyme protein, 9 mg of histones, 50 mM citrate–phosphate buffer (pH 5.6), 10 mM NaCl and 7 mM dithioerythritol at 37 °C for 26 h. Appropriate blanks were carried out and 5 drops of toluene were added to the incubation mixtures to prevent microbial growth. The hydrolyzate (0.5 ml) was applied to a Sephadex G-25 column (1.2 cm × 85 cm) and eluted with 5 mM citrate–phosphate buffer

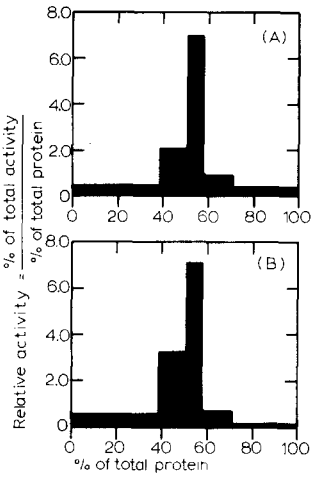


Fig. 1. Subcellular distribution profiles of (A) acid phosphatase and (B) histone hydrolase in rat liver. The percentage of total protein in each fraction is plotted in the order (1) nuclei and unbroken cells, (2) heavy-mitochondria, (3) lysosomes and light-mitochondria, (4) microsomes and (5) supernatant.

(pH 6.8) at 25 °C. The column was calibrated with oxidized glutathione, triglycine, glycine and glucagon. Blue dextran was used to determine the void volume. The hydrolysis products were detected by the ninhydrin procedure[19].

RESULTS

Subcellular localization of histone hydrolase activity

Fig. 1 shows the subcellular distribution of histone hydrolase activity and acid phosphatase, a lysosomal enzyme marker. The patterns of distribution are similar to those of other lysosomal acid hydrolases[9]. The lysosomal localization of cathepsin B in rat liver has been shown[28]. Additional evidence for lysosomal subcellular localization was obtained on lysosomal fractions[13]. The 14- and 20-fold purification of lysosomal cathepsin D over homogenate in two preparations compared well with the 14- and 16-fold purification of histone hydrolase activity.

TABLE I

DESCRIPTION OF PARTIALLY PURIFIED CATHEPSINS

Cathepsin	*Substrate*	*Specific activity*	*Purification over homogenate (-fold)*
A	Carbobenzoxyglutamylphenylalanine	8000*	971
B2	Benzoylarginine amide	2760**	1100
C	Glycyl-L-tyrosine amide	43800***	1500
D	Denatured hemoglobin	2417*	150

* nmoles amino group equivalents released per min per mg of protein.
** nmoles ammonia equivalents released per min per mg of protein.
*** nmoles tyrosine hydroxamate equivalents released per min per mg of protein.

Description of cathepsins used in this study

Table I gives the specific activities and purification of the cathepsins used for the most part of this study. Cathepsin D did not contain appreciable contamination of other cathepsins. Some cross-contamination of cathepsins A, B2 and C with one another was found. Preparations of each of these enzymes contained activities of the other two corresponding to 4–18% of the highest activities obtained. Cross-contaminations were greatest in cathepsins B2 and C. Cathepsin B2 fractions contained about 18% cathepsin C and cathepsin C contained about 7% cathepsin B2. The problem of cross-contamination was not resolved. The specific activity of cathepsin B1 was less than 0.1% of the cathepsin B2 activity.

TABLE II

ACTIVITIES OF CATHEPSINS A, B2, C AND D ON CALF THYMUS WHOLE AND ARGININE-RICH HISTONES

Incubations were done as described in Materials and Methods. Specific activity is expressed as nmoles amino group equivalents released per min per mg of protein and the relative activity as percentage, with cathepsin B2 specific activity set at 100%. The specific activities of cathepsins B2 and C were corrected for their mutual cross-contaminations.

Cathepsin	*Whole histones*		*Arginine-rich histones*	
	Specific activity	*Relative activity*	*Specific activity*	*Relative activity*
A	162	9	36	3
B2	1749	100	1007	100
C	1328	76	453	45
D	92	5	0	0

Hydrolysis of histones and other basic proteins by cathepsin B2 and other cathepsins

Table II shows the specific and relative activities of partially purified cathepsins on whole and arginine-rich calf thymus histones. Cathepsin B2 consistently showed the greatest extent of hydrolysis of whole and arginine-rich histones, followed by cathepsin C. The hydrolysis by cathepsin B2 was better differentiated from cathepsin C on arginine-rich histone, for which the specific activity of cathepsin C was only 45% of cathepsin B2 specific activity as compared with 76% on whole histones. The specific activity of each enzyme on arginine-rich histones was lower than for whole histones. When the partially purified cathepsins were incubated with protamine sulfate, lysozyme and cytochrome *c* for 60 min, only cathepsin B2 was able to hydrolyze protamine sulfate and cytochrome *c*. Hydrolysis of lysozyme occurred only after 3 h of incubation and the extent of hydrolysis was only 6% of the hydrolysis of whole histones. Insolubility of the denatured lysozyme was a problem.

The basic proteins were hydrolyzed with trypsin to compare the relative specificity of this highly specific protease with that of cathepsin B2 (Table III). The specific activity of trypsin on all proteins except histones was higher than that of cathepsin B2. The relative activity of cathepsin B2 was highest on histones while trypsin hydrolyzed protamine sulfate most extensively.

TABLE III

HYDROLYSIS OF BASIC PROTEINS BY CATHEPSIN B2 AND TRYPSIN

The conditions of the assay are described in Materials and Methods. The specific activity is expressed as nmoles amino group equivalents released per min per mg of protein and the relative activity as percentage of the highest value, which is set at 100%.

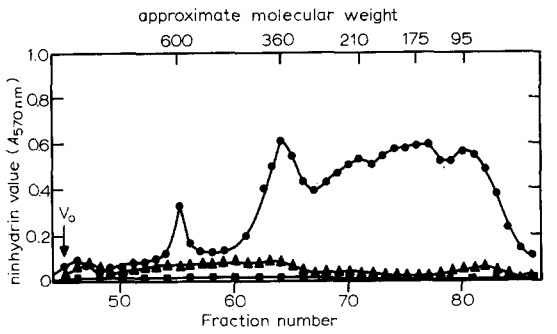
Protein substrate	*Trypsin*		*Cathepsin B2*	
	Specific activity	*Relative activity*	*Specific activity*	*Relative activity*
Whole histones	1270	37	1785	100
Lysozyme	200	6	0	0
Cytochrome *c*	828	24	153	9
Protamine sulfate	3500	100	597	33

Molecular size of histone hydrolysis products

Fig. 2 presents the elution pattern from Sephadex G-25 chromatography of the 26-h hydrolysis products of arginine-rich histones. The apparent molecular weights of the products varied from approximately those of amino acids through hexapeptides. The presence of small amounts of negatively charged fixed ions[29] may retard the elution of basic peptides so that the apparent molecular weights may be larger than what the data suggest. The data show the endopeptidase nature of cathepsin B2 on arginine-rich histones.

Properties of histone and benzoylarginine amide hydrolase activities of cathepsin B2

To determine further the properties of the enzyme with histone hydrolase



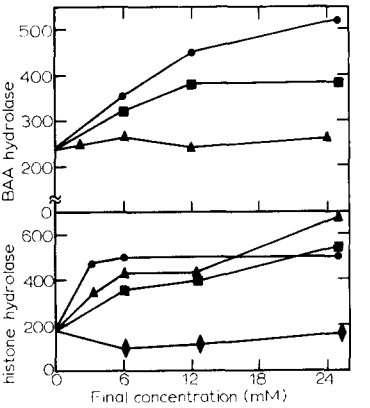


Fig. 2. Chromatographic estimation of the molecular weights of the hydrolysis products produced by action of cathepsin B2 on arginine-rich histones. The conditions for the hydrolysis and Sephadex G-25 column chromatography are described under Materials and Methods. Each fraction contained 0.8 ml. ●—●, enzyme + histones; ▲—▲, histone blank; ■—■, enzyme blank.

Fig. 3. Effect of halide ions on the hydrolysis of arginine-rich histones and benzoylarginine amide by cathepsin B2. The assays were done as described in Materials and Methods in the presence of 20 mM dithioerythritol. ●—●, NaI; ▲—▲, NaCl; ■—■, NaBr; ◆—◆, NaF. The specific activity of histone hydrolase is expressed as nmoles amino group equivalents released per min per mg of protein, and of benzoylarginine amide hydrolase as nmoles NH_3 released per min per mg of protein. BAA hydrolase = benzoylarginine amide hydrolase.

activity and for comparison with benzoylarginine amide hydrolase activity, several parameters affecting the activities were investigated. The pH-profile of the two activities were similar. A slight difference in the pH optima was observed but the maximum hydrolysis of benzoylarginine amide and histones occurred at pH 5.2–5.5. Both activities were most stable at pH 5.4 when heated to 37 °C for 30 min. Under these conditions the two activities were more unstable at alkaline pH than at acid pH. Dithioerythritol increased the activity of histone hydrolase to 163% of the control at 7 mM final concentration, while maximum activation was obtained at 25 mM. Iodoacetamide at a concentration of $2 \cdot 10^{-4}$ M decreased the activity to 25% of the control. At −21 °C both histone and benzoylarginine amide hydrolase lost as much as 65% of their original activities within 3 weeks. In the presence of 0.15 M NaCl and 1 mM phosphate buffer, pH 6.8, at 3 °C, no loss of activity was observed for 7 weeks. The effects of halide ions on histone and benzoylarginine amide hydrolase were studied. Fig. 3 shows that for benzoylarginine amide hydrolase the order of activating effect by halides was $I^- > Br^- > Cl^-$ at all concentrations (0–25 mM). At concentrations up to 12 mM, the activating effect on histone hydrolase was $I^- > Cl^- > Br^- > F^-$; F^- was somewhat inhibitory.

Similarities in the behavior of the two activities were also observed in the batchwise elution pattern on DEAE-cellulose chromatography. The major benzoylarginine amide hydrolase and histone hydrolase peaks eluted at 20 mM NaCl. The apparent molecular weight of cathepsin B2 (see ref. 3) was confirmed to be about 52 000 by Sephadex G-100 chromatography.

DISCUSSION

The evidence presented here points to the lysosomal localization of histone hydrolase activity. The enzyme reported here is different from the nucleohistone-associated protease reported by Furlan *et al.*[25] and Bartley and Chalkey[26]. The lysosomal hydrolysis of histones is due mainly to cathepsin B2. Some of the quantitative interpretation of the results is limited by the presence in the enzyme preparations of small contaminations of cathepsins A, B2 and C with one another. The histone and benzoylarginine amide hydrolase activities were similar with respect to halide and sulfhydryl activation, iodoacetamide inhibition, effect of pH on activity, elution pattern on DEAE-cellulose chromatography and stability properties. This is the first report on the activation of benzoylarginine amide hydrolase activity of cathepsin B2 by halide ions. The similarities in the properties of cathepsins B2 and C are noteworthy, such as effects of activators and inhibitors, histone hydrolysis and stability properties.

Next to cathepsin B2, cathepsin C had the highest specific activity on whole histones. The difference in the rate of hydrolysis was more pronounced on arginine-rich histones. However, the specific activities on this substrate were lower than on whole histones for cathepsins B and C, indicating susceptible bonds in other histone fractions.

The endopeptidase nature of cathepsin B2 was shown on arginine-rich histones. Although there is no previous evidence for its endopeptidase property, the activity of cathepsin B2 on histones and other basic proteins had not been determined. The

presence of amino acid-size hydrolysis products could be due to the small amount of contamination by cathepsin C in addition to the action of cathepsin B2. Using the specific activities of cathepsin B2 and cathepsin C from Table II, calculations show that cathepsin C would contribute only 9% of the total NH_2 groups released from arginine-rich histones by a cathepsin B2 preparation contaminated with 18% (upper value) cathepsin C. Knowledge of the specificities of lysosomal proteases and peptidases has led to postulation of a pathway of protein catabolism[30] for which experimental support has been shown[15,16]. With the present evidence of the endopeptidase nature of cathepsin B2 on histones, a modification of the scheme is appropriate to include cathepsin B2 as an endopeptidase along with cathepsins D, E and B1. Lysosomes appear to have hydrolytic capacity to digest all types of proteins. The narrow endopeptidase specificity of cathepsins B2 and B1 complements, but does not overlap, the broad, pepsin-like action of cathepsin D.

The possible role of lysosomes in pathogenesis of cancer has been reviewed by Allison[9]. There is sufficient evidence to show that the activation of lysosomal enzymes can be a common mechanism set in motion in the cell by carcinogenic agents. The cathepsins, especially the cathepsin B group[2,3], by their hydrolysis of histones, may be involved in carcinogenesis in at least two ways: (a) in the stimulation of mitosis by lysosomes[10–12], cathepsins may serve to deproteinize the chromatin and to derepress DNA template activity and (b) by catheptic hydrolysis of the basic nucleoproteins, lysosomal deoxyribonuclease may then cause breaks in the DNA[31] or possible deletions in the chromosomes.

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