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Some Characteristics of Cathepsin B and α-*N*-Benzoylarginine--β-Naphthylamide Hydrolase From Bovine Lymph Nodes

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Some properties of cathepsin B and α-*N*-benzoylarginine-β--naphthylamide (BANA) hydrolase from bovine lymph nodes have been studies. α-*N*-benzoylarginine-β-naphthylamide was a sensitive substrate for both enzymes. Leucine-2-naphthylamide was cleaved only by BANA hydrolase. Degradation of low molecular weight substrates was optimal at pH = 6.0. At this pH value, the enzymes were most stable. Cathepsin B inactivated aldolase, was inhibited by 1 μM leupeptin and by thiol blocking compounds. BANA hydrolase was not inhibited by 1 μM leupeptin but showed that it required thiol compounds and EDTA for full activation. It was concluded that BANA hydrolase is very similar or identical to cathepsin H from rat liver lysosomes.

Intracellular proteinases of bovine lymph nodes have been studied in our laboratory in light of the important role of lymph tissue in defense mechanisms within mammalian organisms. In addition to cathepsins D (EC 3.4.23.5) and S (EC 3.4.22.—), cathepsin B (EC 3.4.22.1) has also been found in lymph nodes[1,2]. The last, along with other thiol dependent lysosomal proteinases, are thought to play an important role in the degradation of native and denatured proteins[3]. Cathepsin B may also be involved in the generation of active peptides from their precursors[4,5]. Further studies of thiol proteinases from lymph nodes have led to a novel method which has enabled us to simultaneously isolate pure cathepsin B and another α-*N*-benzoylarginine-β-naphthylamide (BANA) hydrolase[6]. The aims of the present work were to determine some characteristics of those enzymes not previously isolated from lymph tissues and to identify BANA hydrolase.

MATERIALS AND METHODS

Bovine lymph nodes were used for the isolation of enzymes as previously described[6].

Enzymatic Assay

Cathepsin B activity was measured by the method of Barrett[7] with α-*N*-benzoyl--DL-arginine-β-naphthylamide. HCl (Bz-Arg-2-NNap, Sigma, USA) was used as substrate. Free naphthylamine was determined colorimetrically, after coupling with Fast Garnet GBC (Sigma, USA). One unit of activity hydrolyses 1 nmol of substrate per minute under the conditions of the reaction. Enzyme activity was also measured toward Leu-2-naphthylamide and the amount of released 2-naphthylamine

was determined as previously mentioned. When α-*N*-benzoyl-DL-arginine-4-nitroanilide (Bz-Arg-NPhNO_2, Sigma, USA) was used as the substrate, the modified method of Otto[8] was employed. 0.1 ml of preactivated enzyme with cysteine and EDTA was incubated for 60 min with 1 ml of substrate solution (0.6 mM substrate in phosphate buffer, pH = 6.0). Reaction was stopped by the addition of 0.2 ml of Tris-phosphate buffer, pH = 10. The amount of released 4-nitroaniline was determined by measuring the absorbance at 410 nm.

Proteolytic activity toward hemoglobin was measured by the method of Anson[9] and toward casein by the method of Langner[10].

Assay of Other Enzymes.

Aldolase (Sigma, USA) from rabbit muscle was measured by the Beisenherz method[11] using Test Combination Aldolase Kit No. 123838 (Behringer, GFR). Urease (Merck, GFR) was assayed by the method of Berthelot[12]. Lactate dehydrogenase (Behringer, GFR) was measured using LDH Monotest Kit No. 124885[13], glutamate dehydrogenase, according to Test Combination GLDH Kit No. 124320[13], and galactose dehydrogenase by Test Combination Galactose Kit No. 15921 (all kits from Behringer, GFR)[13]. Inactivation was determined following the incubation of the substrate enzyme with cathepsin B for 15 min at 37 °C in a citrate buffer of pH 5.2, containing 0.2 mM EDTA and 0.1 mM DTE.

Nitrogen was determined by the modified Kjeldahl method[14]. The amount of protein was estimated by multiplying the value by 6.25.

Specific Extinction Coefficient.

Pure enzyme preparations were lyophilized, dried in a desiccator over $CaCl_2$ and weighed. They were then dissolved in a 1 M acetate buffer, pH = 2.5, or in 0.8 M $NaHCO_3$, pH = 8.0, at an approximate concentration of 0.25 mg/ml. Absorbance of the solution was measured at 280 nm and the specific extinction coefficient was calculated:

$$A_{280}^{1\%} = \frac{A^{280 \text{ in 1 cm cuvette}} \cdot 10}{\text{conc. of protein in mg/ml}}$$

Isoelectric Focusing.

This method was performed on the Desaga apparatus (Desaga, GFR) as described by the manufacturer. 5% polyacrylamide gel plates (9 × 16 cm) containing carrier Ampholines (LKB, Sweden) hawing pH ranges of 2—11 were used. Voltage was gradually increased from 200 to 1180 V during the 90 min focusing time, whereas current dropped from an initial 50 mA to 7 mA. Gels were washed with 10% trichloroacetic acid in order to remove Ampholines, and were subsequently stained with Coomassie brilliant blue G 250 (Serva, GFR). A mixture of 8 standard proteins with known isoelectric points (Serva, GFR) was run parallel in the same gel. pI values of proteins were determined by measuring the pH in the gel using a microcombination electrode (Desaga, GFR).

N-terminal Amino Acid Analysis.

N-terminal amino acid was determined by the dansylation method (15). After the hydrolysis of dansylated proteins, amino acid derivatives were identified by thin layer chromatography on micropolyamide F-1700 sheets (Schleicher-Schüll, GFR). Chromatograms were viewed under UV light at 254 nm and 366 nm (Camag, Switzerland). Dansyl amino acid standards were obtained from Calbiochem, Switzerland.

Amino Acid Analysis.

Amino acid composition was determined by the method of Moore and Stein[16] on a Jeol JLC-BC2 amino acid analyzer.

Molecular Weight

Molecular weight was determined by the method of Weber and Osborn[17] using sodium dodecyl sulphate gel electrophoresis.

Circular Dichroism Measurements.

CD spectra were recorded on a Jobin Yvon Dichrograph III (France), using 0.5 mm cuvettes. Amounts of alpha-helix, beta structure and random coil were calculated using the method of Chen et al[18].

RESULTS

The summarized purification procedure yielding electrophoretically pure cathepsin B and BANA hydrolase is shown in Figure 1. Isoelectric focusing of cathepsin B showed the presence of one strong band of pI between pH = 5.0 and 5.1, with minor impurities, whereas BANA hydrolase consisted of two equally intense bands of pI = 7.1 and 7.35 (Figure 2). The specific extinction coefficient of cathepsin B is 18.5, and 14.0 for BANA hydrolase. Leucine was identified as the *N*-terminal amino acid of cathepsin B. The amino acid composition of both enzymes is given in Table I. The number of residues was calculated by assumming a molecular weight of 26,300 for cathepsin B and

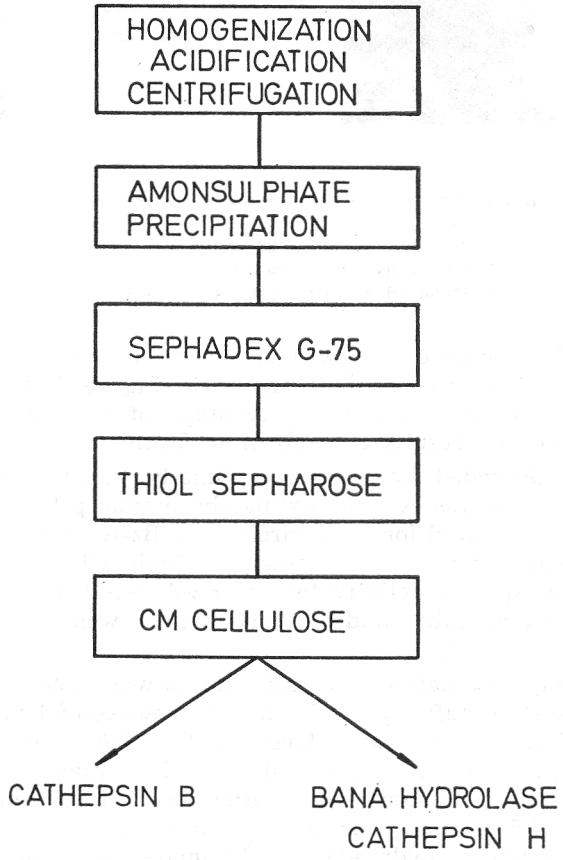

Figure 1. Flow diagram summarizing the purification procedure of cathepsin B and of BANA hydrolase.

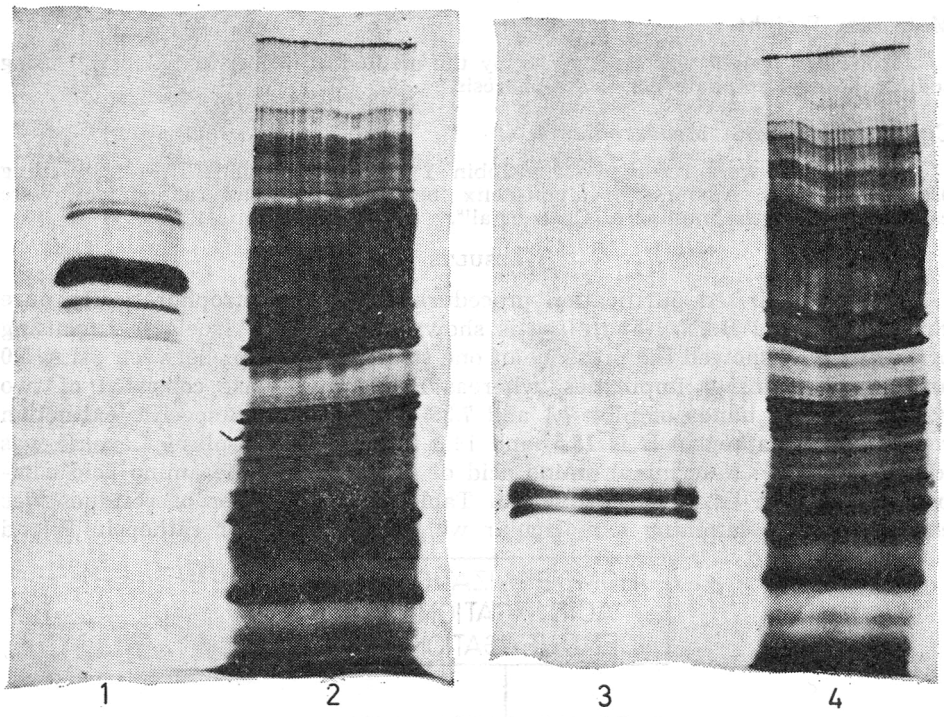

Figure 2. Isoelectric focusing of cathepsin B and of BANA hydrolase.
1. Cathepsin B (90 μg)
2. Standard proteins (pI 4.40—10.45)
3. BANA hydrolase (60 μg)
4. Standard proteins (pI = 4.40—10.45)

30,800 for BANA hydrolase. The CD spectrum in the far UV region for cathepsin B and BANA hydrolase is shown in Figure 3. From the experimentally determined elipticities, the percentage of the secondary structure was calculated and the results are shown in Table II.

Cathepsin B degraded hemoglobin optimally at pH = 4.0, and BANA hydrolase at pH = 6.5—7.0. An approximately similar pH optimum (pH = 6) for both enzymes was found for the hydrolysis of Bz-Arg-2-NNap. The specific activity toward some substrates is shown in Table III. Both enzymes have a high and similar specific activity toward Bz-Arg-2-NNap. Cathepsin B did not hydrolyze Leu-2-naphthylamide, which, in turn, was a good substrate for BANA hydrolase.

Cathepsin B (approximately 1 mg/ml) solution was rather stable on storage at —20 °C in an acetate buffer pH = 5.0 in the presence of 1 mM EDTA. After 5 months, the initial activity was reduced by 25%. BANA hydrolase was less stable. At 5 months, 50% of the activity was lost. Inactivation of the test enzyme was limited to a study with cathepsin B. More than 80% of the aldolase activity (8.5 mU/ml) was inactivated by purified cathepsin B. No inactivation of lactate dehydrogenase, glutamate dehydrogenase, galactose dehydrogenase and urease was noted.

TABLE I

Amino Acid Composition of Cathepsin B and Bana Hydrolase

Amino acid	Residues/molecule	
	Cathepsin B	BANA hydrolase
Asx	17	23
Thr	9	12
Ser	19	15
Glx	20	27
Pro	15	20
Gly	27	23
Ala	8	21
Cys	13	7
Val	13	13
Met	3	6
Ile	12	10
Leu	8	10
Tyr	8	10
Phe	7	11
Lys	13	18
His	9	5
Arg	7	7

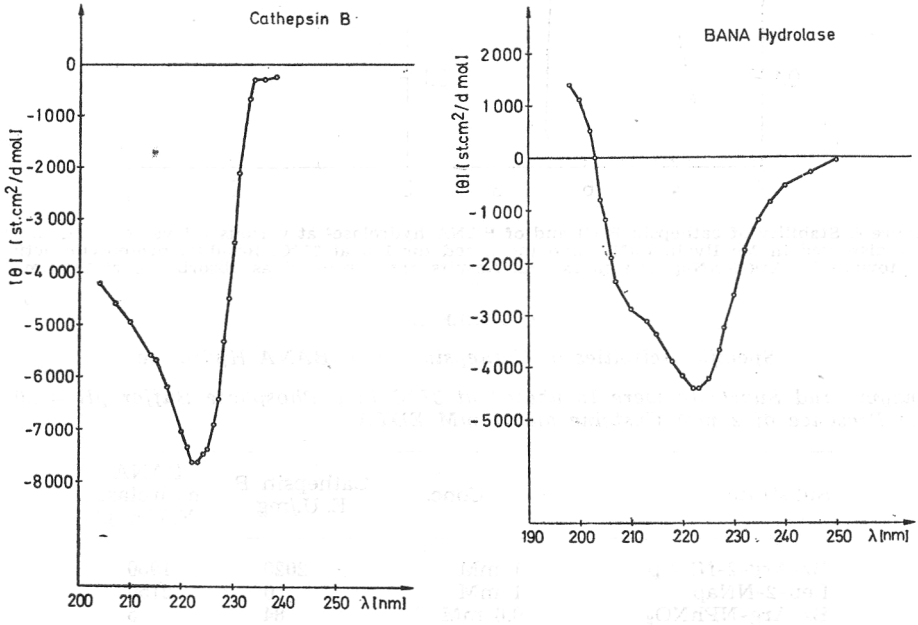

Figure 3. CD spectrum of cathepsin B (21.9 mg/100 ml in 0.02 M Na-acetate buffer, pH = 5.0) and of BANA hydrolase (9.8 mg/100 ml in 0.02 M Na-acetate buffer, pH = 5.0).

TABLE II

Secondary Structure of Cathepsin B and BANA Hydrolase

	Cathepsin B %	BANA hydrolase %
α-helix	12	18
β-structure	31	12
Unordered st.	57	70

The pH stability of the enzymes following 1 h incubation at 37 °C is shown in Figure 4. Both enzymes are maximally stable at pH = 6. Cathepsin B stability is decreased below pH = 5 and drops sharply above pH = 7, whereas BANA hydrolase is less sensitive to pH change.

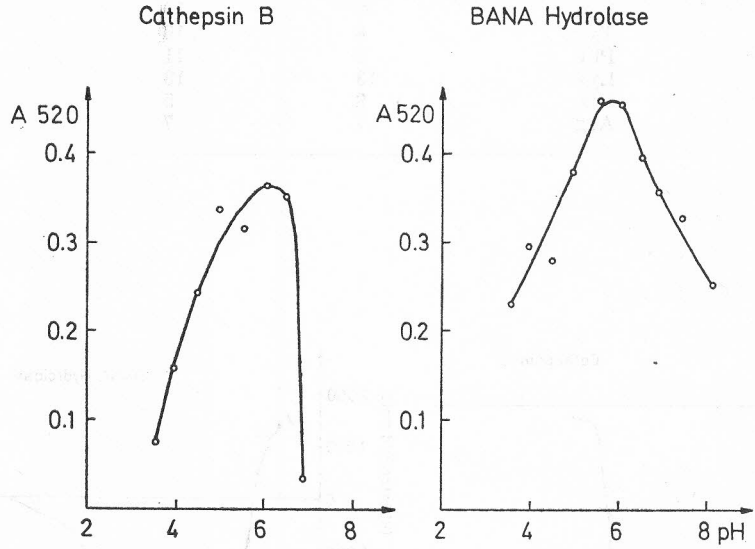

Figure 4. Stability of cathepsin B (1) and of BANA hydrolase[2] at various pH values. The enzyme was dissolved in Mc Ilvain buffer and incubated for 1 h at 37 °C. Residual proteolytic activity toward Bz-Arg-2-NNap was measured. Results are expressed as absorbance at 520 nm.

TABLE III

Specific Activities of Cathepsin B and BANA Hydrolase

Enzyme and Substrate were Incubated at 37 °C in a Phosphate Buffer pH = 6.0 in the Presence of 2 mM Cysteine and 1 mM EDTA.

Substrate	Conc.	Cathepsin B E. U./mg	BANA hydrolase E. U./mg
Bz-Arg-2-NNAp	1 mM	2020	1960
Leu-2-NNap	1 mM	0	2150
Bz-Arg-$NPhNO_2$	0.6 mM	84	5
Azocasein	3%	6	2

TABLE IV

Effects of Inhibitors and Activators on Cathepsin B and BANA Hydrolase The Enzymes were Preincubated with Eeach Compound at Various Concentrations for 5 min at 37 °C and Assayed Toward Bz-Arg-2-NNap. The Effect of EDTA on Enzyme Samples Previously Dialyzed Against Redistilled Water was Determined. Results Shown are the Mean Values of 3 Experiments.

Effector	Conc. (mM)	Cath. B % act.	BANA hydrol. % cat.
None		1	19
Cysteine	2	100	100
EDTA	1	2	20
Cysteine + EDTA	2+1	100	100
Cysteine + EDTA	5+1	109	120
4-Chloromercuri-	0.1	72	90
benzoate	1	0	0
2.2'dipyridil disulphide	0.5	5	5
Tos-Phe-CH_2Cl	0.01	20	80
Iodoacetamide	1	5	10
Iodoacetic acid	1	0	0
Leupeptin	0.001	30	98

The effect of various potential inhibitors and activators is shown in Table IV. Inhibition with 4-chloromercuribenzoate, iodoacetic acid and iodoacetamide agrees well with the classification of cathepsin B and BANA hydrolase as thiol proteinases. Tos-PheCH_2Cl inhibited cathepsin B to a much higher extent than BANA hydrolase. Leupeptin at 10^{-6} M concentration did not inhibit BANA hydrolase, whereas cathepsin B activity was lowered by 70%.

DISCUSSION

Cathepsin B has been purified from a number of animal tissues, as well as from humans[3]. The existence of multiple forms of cathepsin B has been reported by many authors, although cathepsin B in a single form has been isolated from human placenta[19] and from rat liver lysosomes[20]. In the present study we have isolated cathepsin B which displayed one major protein band and some minor impurities upon isoelectric focusing. The isoelectric point of the major band agrees with the value for the major form obtained from human liver[21]. The specific extinction coefficient is very similar to the value reported by Barrett[21]. Leucine was indentified as the *N*-terminal amino acid, as also reported by Franklin and Metrione[22], and by Keilova and Tomašek[23]. Comparing our results on secondary structure obtained by CD measurement with those for papain[18] we can see that cathepsin B and BANA hydrolase contain less alpha-helix and beta structure. High proline content contributes to the low amount of alpha-helix. Our results on the specificity of cathepsin B toward low molecular weight substrates are in agreement with data reported in the literature[24,25]. Zero activity toward Leu-2-naphthylamide indicates the purity of our enzyme preparation[26]. Aldolase inactivation by cathepsin B has been used for the identification of cathepsin B[24]. The failure of the enzyme to inactivate lactate dehydrogenase and glutamate dehydrogenase has also been observed by Towatari et al.[27] whereas the effect upon urease and galactose

dehydrogenase has not been studied. The absolute requirements of free thiol groups for the enzyme activity were shown by the strong inhibitory effect of thiol blocking reagents, and natural aldehyde leupeptin.

Additional BANA hydrolyzing activity of a higher molecular weight than that of cathepsin B, but without aldolase inactivation capability, has been noted by Distelmeyer et al.[28] and investigated further by Davidson and Poole[29]. Kirschke et al.[30] reported on a thiol enzyme from rat liver lysosomes having a molecular weight of 28,000 with exo- and endo-peptidase activity and named it cathepsin H. Our results show that BANA hydrolase from bovine lymph nodes has properties similar or identical to those of cathepsin H, such as the isoelectric point, molecular weight, pH optimum toward Bz-Arg-2-NNap and specificity toward synthetic and protein substrates. BANA hydrolase was also inhibited with thiol blocking reagents and was not inhibited with 1 μM leupeptin, as cathepsin H. Lymph node BANA hydrolase shows more similarity with cathepsin H than with BANA hydrolase from rabbit lung[31].

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IZVLEČEK

Nekatere lastnosti katepsina B in α-*N*-benzoilarginin-β-naftilamid hidrolaze iz govejih limfnih žlez

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Študirali smo nekatere lastnosti katepsina B in BANA hidrolaze iz govejih bezgavk. Bz-Arg-2-NNap je bil občutljiv substrat za oba encima. Leu-2-naftilamid je hidrolizirala le BANA hidrolaza. Optimalni pH za razgradnjo nizkomolekularnih substratov je bil pH=6. Pri tej pH vrednosti sta bila encima tudi najbolj stabilna. Katepsin B je inaktiviral aldolazo. 1 μM leupeptin je inhibiral katepsin B, ne pa BANA hidrolaze. Oba encima so aktivirale tiolne spojine in EDTA, inhibirale pa so ju spojine, ki blokirajo -SH skupine. Ugotovili smo, da je BANA hidrolaza iz govejih limfnih žlez zelo podobna ali identična s katepsinom H iz lisosomov podganjih jeter.

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