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CRYSTALLIZATION AND AMINO ACID COMPOSITION OF CATHEPSIN B FROM RAT LIVER LYSOSOMES

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SUMMARY

Cathepsin B (EC 3.4.22.1) from rat liver was crystallized and its amino acid composition was determined. The purified enzyme formed spindle-shaped crystals and its homogeneity was proved by ultracentrifugical analysis. Its $S_{20,w}$ value was 2.5 S and its molecular weight was calculated to be 24,000 from the result of sedimentation equilibrium analysis. Amino acid analysis showed that it contained glucosamine and galactosamine. The activity of the protease was maximal at pH 6.0 with α-N-benzoyl-DL-arginine p-nitroanilide as substrate. The apparent Kms for α-N-benzoyl-DL-arginine p-nitroanilide and α-N-benzoyl-DL-arginine-2-naphthylamide were 1.4×10^{-2} M and 2.0×10^{-3} M, respectively

Otto et al. (1-4) showed that the enzymic activity of "cathepsin B", which was originally defined as the enzyme deamidating α-N-benzoyl-L-arginine amide in the presence of cysteine (5,6), was attributable to the action of more than one enzyme. It is now known that the "cathepsin B" fraction contains cathepsin B, lysosomal carboxypeptidase B (7),cathepsin L (8,9) and cathepsin H (10). Cathepsin B, now generally thought to be a lysosomal thiol protease, hydrolyzes BAPA and BANA as well as BAA, and has a molecular weight of about 25,000. Its distribution has been

Abbreviation: BAPA, α-N-benzoyl-DL-arginine p-nitroanilide; BANA, α-N-benzoyl-DL-arginine-2-naphthylamide; BAA, α-N-benzoyl-L-arginine amide.

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examined in various tissues of rats and other animals and it has been partially purified (7, 11-15).

This paper reports the isolation, crystallization and some properties, including the amino acid composition, of cathepsin B from rat liver lysosomes.

MATERIALS AND METHODS

Wistar strain rats, weighing 200-250 g and fed on standard diet, were used as a source of enzyme. BAPA and BANA were obtained from Sigma Chemical Co. p-Toluene sulfonic acid and 3-(2-amino ethyl) indole were from Pierce Chemical Co. The cleavage of BANA was measured by coupling the 2-naphthylamide with fast garnet GBS salt, as described by Barrett (16). The cleavage of BAPA was measured by the procedure of Otto and Bhakdi (3). Protein concentrations were determined by the method of Lowry et al. (17), using bovine serum albumin as the standard. Quantitative amino acid analysis was performed by the standard method (18), except that of glucosamine, galactosamine and tryptophan were measured by the method of Liu and Chang (19) using a solution of 3 N p-toluene sulfonic acid containing 0.2% 3-(2-amino ethyl)indole.

RESULTS AND DISCUSSION

Enzyme purification – Cathepsin B was purified to the crystallization step by the published procedures(2,3) principally with certain modifications and additional procedures.

Step 1. The crude lysosomal fraction from 500 rat livers was suspended in 0.05 M acetate buffer, pH 5.0, containing 0.1 M sodium chloride and 5 mM 2-mercaptoethanol and subjected to seven cycles of freezing and thawing. The mixture was centrifuged at 105,000 x g for 30 min and the precipitate was discarded.

Step 2. Cold acetone was added to the supernatant from step 1 to 45% (v/v) with stirring over a period of 5 min and then the mixture was rapidly centrifuged at 8,000 x g for 5 min. The resulting precipitate was discarded. Further cold acetone was added to the supernatant to give a final concentration of 75% (v/v). The resulting precipitate was collected by centrifugation and dissolved in a minimum volume of 0.05 M acetate buffer, pH

5.0, containing 0.1 M sodium chloride and 5 mM 2-mercaptoethanol. The preparation was centrifuged briefly to remove insoluble material and the supernatant was passed through a Sephadex G-25 (6 x 20 cm) column equilibrated with 0.05 M acetate buffer, pH 5.0, containing 0.1 M sodium chloride and 5 mM 2-mercaptoethanol. The enzyme solution was concentrated by ultrafiltration (Diaflo Ultrafilter with an UM-10 membrane, Amicon Co.).

Step 3. The enzyme solution was divided in half and applied to two identical Sephadex G-75 columns (3.0 x 100 cm) previously equilibrated with 0.05 M acetate buffer, pH 5.0, containing 0.1 M sodium chloride and 5 mM 2-mercaptoethanol, and the columns were eluted with the same buffer. The fraction that contained BAPA hydrolyzing activity and had a molecular weight of about 25,000 was excluded from the gel, collected and concentrated to a small volume by ultrafiltraiton using an Amicon UM-10 membrane. In this step, the enzyme solution was separated from lysosomal carboxypeptidase B and cathepsin D.

Step 4. The enzyme solution was applied to a DEAE Sephadex A-50 column (1.8 x 20 cm) equilibrated with 0.02 M acetate buffer, pH 5.0, containing 0.01 M sodium chloride and 5 mM 2-mercaptoethanol. The column was washed with the same buffer and then eluted stepwise with 0.05 M and 0.1 M sodium chloride in 0.02 M acetate buffer, pH 5.0, containing 5 mM 2-mercaptoethanol. The BAPA hydrolyzing activity was eluted in two fractions: One fraction was not adsorbed at low ionic strength and contained cathepsin H activity(10), which has low sensitivity to inhibition by leupeptin as described by Davidson et al. (20); the other fraction was eluted with 0.05 M sodium chloride and contained cathepsin B, cathepsin L (new cathepsin) and leucine-2 2-naphthylamide hydrolyzing enzyme.

Table 1. Purification of cathepsin B from rat liver

Purification step	Total volume	Total protein	Total activity	Specific activity	Purity
	ml	mg	units	units/mg	-fold
1) Lysosomal extract	910	10,700	32.21	0.003	1
2) Acetone treatment	36	685	24.00	0.035	12
3) Sephadex G-75	18	324	26.43	0.082	27
4) DEAE Sephadex A-50	40	24	9.66	0.403	134
5) CM Sephadex C-50	1	23	9.60	0.417	139
6) Crystallization	1	5.6	2.65	0.473	158

One unit of activity is defined as the amount of enzyme which liberates 1 μmole of p-nitroaniline per min from BAPA

Step 5. The enzyme solution eluted with 0.05 M sodium chloride was applied to a CM Sephadex C-50 column (1.8 x 15 cm) equilibrated with 0.02 M acetate buffer, pH 5.0, containing 5 mM 2-mercaptoethanol and 0.05 M sodium chloride. The column was washed with the same buffer and then eluted stepwise with 0.1 M, 0.2 and 0.5 M sodium chloride in 0.02 M acetate buffer, pH 5.0, containing 5 mM 2-mercaptoethanol. The enzyme was eluted with 0.1 M sodium chloride and concentrated to over 20 mg protein per ml in a collodion bag. In this step, the leucine-2-naphthylamide hydrolyzing enzyme was eluted with 0.05 M sodium chloride and cathepsin L with 0.5 M sodium chloride.

Step 6. Solid ammonium sulfate was added gradually to the concentrated solution until just before a slight turbidity appeared in the cold and the solution was then stood for several days in the cold. The spindle-shaped crystals that appeared are shown in Fig. 1. Representative results on the purification of cathepsin B from rat liver are summarized in Table 1.

Properties of the enzyme — The crystallized enzyme gave a single

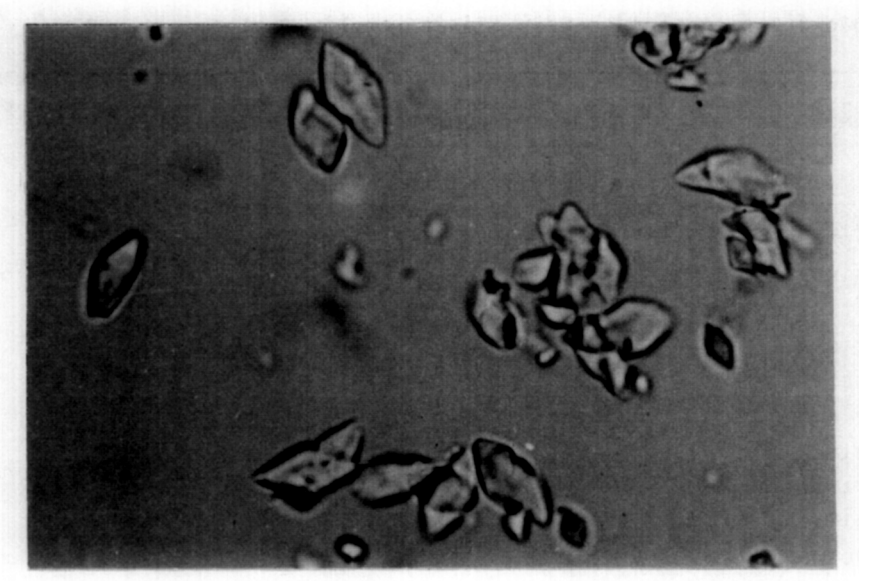

Figure 1. Photomicrograph of crystals of cathepsin B from rat liver. (Magnification X 400)

symmetrical boundary on ultracentrifugation, and the sedimentation constant was calculated to be 2.5 S at 6.5 mg protein per ml in 0.02 M acetate buffer, pH 5.0, containing 0.1 M sodium chloride. The molecular weight was found to be 24,000 by the sedimentation equilibrium method, 26,000 by Sephadex G-75 column chromatography and 22,000 by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

On polyacrylamide gel disc electrophoresis at pH 4.5, the enzyme migrated as a single protein, but at pH 8.3, it showed a minor component besides the major one. Similar results were obtained on the mother liquor. The activity of the crystalline protease was maximal at pH 6.0 with BAPA as substrate. The apparent Km values for BAPA and BANA were 1.4×10^{-2} M and 2.0×10^{-3} M, respectively. in 0.1 M potassium phosphate buffer, pH 6.0. The protease inactivated aldolase and glucokinase, as described by Otto (21), and also apo-ornithine aminotransferase and apo-cystathionase (22).

Table 2. Amino acid composition of cathepsin B from rat liver

Amino acid	Residues after		Calculated number of residues
	24 h	48 h	
Tryptophan	6.6[a]		6.6
Lysine	7.6	7.9	7.8
Histidine	4.1	5.2	4.7
Arginine	5.3	6.3	5.8
Aspartic acid	18.9	19.2	19.1
Threonine	10.3	10.5	10.4
Serine	16.8	15.8	16.3
Glutamic acid	19.0	20.0	19.5
Proline	14.2	11.3	12.8
Glycine	30.2	28.3	29.3
Alanine	11.9	11.6	11.8
Half-cystine	9.4[b]		9.4
Valine	10.9	12.2	11.6
Methionine	3	2.4	3.6[c]
Isoleucine	8.2	11.8	11.8
Leucine	5.9	8.1	8.1
Tyrosine	8.7	9.2	9.0
Phenylalanine	7.0	6.7	6.9
Glucosamine	7.8[a]		7.8
Galactosamine	7.9[a]		7.9
Total			220.2

Values are residues/mole (molecular weight, 25,000)

a; Hydrolyzed in 3 N p-toluene sulfonic acid containing 0.2% 3-(2-amino-ethyl) indole at 110°C for 24h
b; Oxidized with performic acid before hydrolysis in 6N-HCl at 110°C for 24h
c; Values extrapolated to zero-hour hydrolysis

It also hydrolyzed acid-denatured hemoglobin, casein and bovine serum albumin and was inhibited by monoiodoacetate, leupeptin, antipain, N-α-p-tosyl-L-lysine chloromethyl ketone HCl and L-1-tosylamide-2-phenylethylchloromethyl ketone HCl as described previously (23)

Amino acid composition of the enzyme — As shown in Table 2, the most characteristic feature of the amino acid composition of the

enzyme is that there are two kinds of hexosamine, glucosamine and galactosamine. These findings probably imply that cathepsin B is a glycoprotein, like the other lysosomal enzymes that have been characterized. The existence of multiple forms of cathepsin B has already been reported and the presence of the N-terminal leucine in several forms of the bovine enzyme has been demonstrated by Franklin and Metrione (12) and Keilová and Tomasek (13). Since this charge heterogeneity of the enzyme was not changed by crystallization of the enzyme, the multiple forms may result from changes in the carbohydrate content of the enzyme during the solubilization step. This is important problem to settle in relation to the function of cathepsin B in lysosomes.

Amino acid analysis showed that there are 9.4 half cystine residues per mole (25,000 molecular weight), the contents of aspartic acid, glutamic acid and glycine are high and the content of methionine is low, as in cathepsin B from human liver (24). The present results show that this crystalline protease is the same enzyme as reviewed by Barrett previously (24).

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REFERENCES

1. Otto, K., and Schepers, P. (1967) Hoppe-Seyler's Z. Physiol. Chem. 348, 482-490
2. Otto, K. (1967) Hoppe-Seyler's Z. Physiol. Chem. 348, 1449-1460
3. Otto, K., and Bhakdi, S. (1969) Hoppe-Seyler's Z. Physiol. Chem. 350, 1577-1588
4. Barrett, A.J., and Dingle, J.T. (1971) Tissue Proteinases (Barrett, A.J., and Dingle, J.T., eds.) pp. ix-x. North-Holland Publishing Co., Amsterdam
5. Tallan, H.H., Jones, M.E., and Fruton, J.S. (1952) J. Biol. Chem. 194, 793-805

6. Greenbaum, L.M., and Fruton, J.S. (1957) J. Biol. Chem. 226, 173-180
7. McDonald, J.K., and Ellis, S. (1975) Life Sci. 17, 1269-1276
8. Towatari, T., Tanaka, K., Yoshikawa, D., and Katunuma, N. (1967) FEBS Lett. 67, 284-288
9. Kirschke, H., Langner, J., Weideranders, B., Ansorge, S., and Bohley, P. (1977) Eur. J. Biochem. 74, 293-301
10. Kirschke, H., Langner, J., Weideranders, B., Ansorge, S., Bohley, P., and Hanson, H. (1977) Acta Biol. Med. Germ. 36, 185-199
11. Snellman, O. (1969) Biochem. J. 114, 673-678
12. Franklin, S. G., and Metrione, R.M. (1972) Biochem. J. 127, 207-213
13. Keilová, H., and Tomasek, V. (1973) FEBS Lett. 29, 335-338
14. Barrett, A.J. (1973) Biochem. J. 131, 809-822
15. Ogino, K., and Nakashima, K.(1974) J. Biochem. 75, 723-730
16. Barrett, A.J. (1972) Anal. Biochem. 47, 280-298
17. Lowry, O. H., Rosebrough, N.J., Farr, A. L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275
18. Moore, S., and Stein, W.H. (1967) Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.) Vol. 19, pp.273-283, Academic Press, New York
19. Liu, T.Y., and Chang, Y.H. (1971) J. Biol. Chem. 246, 2842-2848
20. Davidson, E., and Poole, B. (1975) Biochim. Biophys. Acta 397, 437-442
21. Otto, K. (1971) Tissue Proteinases (Barrett, A.J., and Dingle, J.T., eds.)pp. 1-28, North Holland Publishing Co., Amsterdam
22. Towatari, T., Tanaka, K., Yoshikawa, D., and Katunuma, N. (1978) J. Biochem. in press
23. Swanson, A.A., Martin, B.J., and Spicer, S.S. (1974) Biochem. J. 137, 223-228
24. Barrett, A.J. (1977) Proteinases in Mammalian Cells and Tissues (Barrett, A.J., ed.) pp. 181-208, North-Holland Publishing Co., Amsterdam