SEPARATION OF A NEW PROTEASE FROM CATHEPSIN B1 OF RAT LIVER LYSOSOMES

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1. Introduction

The intracellular proteases known to be capable of hydrolyzing high molecular weight proteins are lysosomal cathepsin D (EC 3.4.4.23) and B1 (EC 3.4.4.-) [1–5] and seryl-proteases (Group Specific Proteases; GSP), which we recently found in mitochondrial membranes [6–8]. Of these, cathepsin B1 and GSP are probably most important for intracellular protein degradation under physiological conditions, because they can hydrolyze certain native proteins under weakly acidic and weakly alkaline conditions, respectively, whereas cathepsin D is only active under strongly acidic conditions and only attacks denatured proteins. Cathepsin B1 has been defined as a protease possessing the following characteristics [2–5,9]: (1) activity with some high mol. wt. proteins and α-*N*-benzoyl-DL-arginine-*p*-nitroaniline (BAPA), (2) a mol. wt. of about 25 000, (3) a pH optimum of 5.0–6.0 and (4) inhibition by leupeptin. However, we observed that the activities of crude cathepsin B1 on BAPA and on an enzyme protein used as substrate were affected differently by heat treatment. Therefore, we purified cathepsin B1 to find out whether these activities were due to the same protein. As described in this paper, using circulating long column chromatography of Sephadex G-75 as the final purification step, we separated into two proteases from the most pure B1 fraction which has been published [19], the first peak is active toward BAPA and aldolase, and the second peak is active toward glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), ornithine-ketoacid aminotransferase (OTA, EC 2.6.1.13), casein and denatured hemoglobin but not toward BAPA or aldolase. On the basis of the above definition of cathepsin B1, the later enzyme can be regarded as a new protease. The possibility that intracellular proteases may show some group specificities on intracellular substrate proteins is also discussed.

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2. Materials and methods

2.1. *Materials*

Male Wistar strain rats weighing 200–250 g and fed on standard diet were used. BAPA was obtained from Sigma Chemical Co. Ltd. G6PD from yeast and aldolase (EC 4.1.2.13) from rabbit skeletal muscle were purchased from Boehringer, Mannheim GmbH. Crystalline OTA from rat liver and its apoenzyme were prepared by the methods of Matsuzawa et al. [10]. Acid denatured bovine hemoglobin was prepared by the method of Schlamowitz et al. [11]. Protease inhibitors of bacterial origin (chymostatin, pepstatin and leupeptin) were kindly supplied by Dr Aoyagi and Dr Umezawa (Institute of Microbial Chemistry, Tokyo). Phenyl-methyl sulfonylfluoride (PMSF) was purchased from Sigma Chemical Co. Sephadex G-75 and DEAE Sephadex A-50 were from Pharmacia.

2.2. *Enzyme assay*

G6PD was assayed by measuring the rate of decrease in absorbance of NADPH at 25°C [2]. Aldolase and OTA were measured by the methods of Taylor [13] and Katunuma et al. [14], respectively. BAPA hydrolyzing activity was assayed by the method of Otto and Bhakdi [2,4]. The inactivations of G6PD and

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OTA were measured as follows. The reaction mixtures contained 100 μmol of potassium phosphate buffer (KPB), pH 6.0 with 5 μmol of β-mercaptoethanol, 0.5–1.0 units (0.05–1.0 mg protein) of G6PD or OTA as substrate and a suitable amount of the preparation in a final volume of 1.0 ml. Mixtures were incubated at 37°C for 15–30 min, and then 200 μl samples were removed to measure the remaining activity of the substrate enzymes. One unit of inactivating enzyme was defined as the amount showing a first-order rate constant k min^{-1} of 1. Proteolytic activities on casein and acid-denatured hemoglobin were assayed by measuring liberation of free amino acids into the TCA soluble fraction. The reaction mixture contained 100 μmol of sodium acetate buffer, pH 5.0, with 5 μmol of β-mercaptoethanol, 10 mg of substrate protein and a suitable amount of the protease preparation in a final volume of 1.0 ml. Mixtures were incubated at 37°C for 30 min and then 1.0 ml of 10% TCA was added, the mixtures were centrifuged and the amino acid concentrations in the supernatants were assayed by the ninhydrin method [15].

2.3. *Studies on the effects of inhibitors*

The standard mixture for assay of protease was incubated with and without various concentrations of the test inhibitors and the percentage of remaining activity was calculated as 100·B/A, where A = activity without inhibitor; B = activity with inhibitor.

2.4. *Protein determination*

Protein concentrations were determined by the method of Lowry et al. [16] using crystalline bovine serum albumin as the standard.

3. Results and discussion

3.1. *Purification and separation of the BAPA hydrolyzing and glucose-6-phosphate dehydrogenase inactivating enzymes*

The procedure used for purification and separation of the two kinds of protease from the cathepsin B1 fraction of rat liver lysosome is summarized in table 1. The light mitochondrial fraction (Lysosomal fraction) was prepared from rat liver by the method of Ragab et al. [17] and disrupted by freezing and thawing several times. The 105 000 g supernatant was fractionated with 45–75% acetone in acetone–dry ice. The G6PD inactivating activity increased markedly in this step. The precipitate was dissolved in a small volume of 0.05 M KPB, pH 6.0 containing 0.1 M NaCl and 5 mM β-mercaptoethanol and applied to a Sephadex G-75 column (30 × 100 cm). The column was eluted with the same buffer and the fractions with BAPA hydrolyzing activity and a mol. wt. of about 25 000 were collected and concentrated to a small volume in an Amicon UM-10 membrane ultrafiltration cell. The concentrated solution was

Table 1
Purification and separation method of the BAPA-hydrolyzing and G6PD-inactivating enzymes

	Total protein	BAPA			G6PD		
		Total activity	Specific activity	Purification (fold)	Total activity	Specific activity	Purification (fold)
	(mg)	(*p*-Nitroaniline formed μmoles/min)	(*p*-Nitroaniline formed μmoles/min/mg)		Unit (× 10^{-2})	Unit (× 10^{-2})/mg	
105 000 g sup.	16 762	12.78	0.0008	1	2585.44	0.163	1
Acetone (45–75%)	534	21.12	0.0396	49.5	8796.0	16.47	101.0
1st Sephadex G-75	143.5	10.57	0.0737	92.1	3598.7	25.08	153.9
DEAE Sephadex A-50	35.0	9.50	0.271	338.7	3677.3	105.06	644.5
3rd Sephadex G-75 (see fig.1)							

then applied to a column (1.8 X 20 cm) of DEAE Sephadex A-50 equilibrated with 0.02 M acetate buffer, pH 5.0 containing 5 mM β-mercaptoethanol. The column was eluted stepwise with concentrations of 0, 0.05, 0.1 and 0.5 M sodium chloride in 0.02 M acetate buffer, pH 5.0 containing 5 mM β-mercaptoethanol. The fraction responsible for hydrolyzing activity of BAPA, casein and denatured hemoglobin and for inactivating activity of G6PD and aldolase was eluted with buffer containing 0.1 M NaCl. This fraction is identical with peak II on DEAE cellulose column chromatography reported by F. Davidson et al. [19]. On the other hand, the fraction corresponding to peak I reported by them which shows hydrolytic activity only on BAPA but not on aldolase, casein or denatured hemoglobin is eluted at zero NaCl concentration on our DEAE Sephadex A-50. P. Distelmaier et al. [20] reported the separation of B1, B2 and other cathepsin B-like proteases using synthetic substrates on Sephadex G-75. They observed that B1 peak was responsible for the hydrolysis of both BAPA and aldolase. This peak appears to be a mixture of peak I and peak II reported by Davidson et al. [19]. But no further fractionation has been attempted. Since the peak I enzyme has no hydrolytic activity on any high mol. wt. proteins tested, i.e. aldolase, G6PD, ornithine aminotransferase, casein and denatured hemoglobin, we tried to separate the fraction corresponding to peak II reported by them (0.1 M NaCl fraction on our column) into two peaks. In our final circulation Sephadex G-75 column system, the first peak is reactive toward BAPA and aldolase, and the second peak is reactive toward G6PD, OTA, casein and denatured hemoglobin but not toward BAPA or aldolase. Therefore, peak II reported by Davidson et al. was the mixture of two different proteases as will be mentioned in the next paragraph. The 0.1 M NaCl fraction was concentrated (35 mg/3.0 ml) and applied to a Sephadex G-75 circulating column system (2.5 X 90 cm) equilibrated with 0.05 M KPB, pH 6.0 containing 0.1 M NaCl and 5 mM β-mercaptoethanol and circulated 3–4 times using the same buffer. Fig.1 shows the elution profiles of BAPA-hydrolyzing activity and G6PD-inactivating activity and of absorption at 280 nm. As shown in table 1 and fig.1, the BAPA-hydrolyzing activity in fraction I was clearly separated from the G6PD inactivating activity in fraction III. As in the past, if

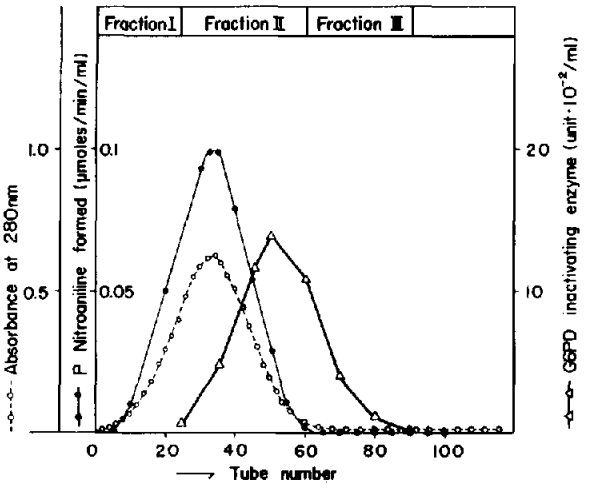

Fig.1. The elution profiles of circulating Sephadex G-75 column chromatography. The concentrated sample which was eluted from DEAE Sephadex A-50 was applied to a Sephadex G-75 circulating column system (2.5 X 90 cm) equilibrated with 0.05 M KPB, pH 6.0 containing 0.1 M NaCl and 5 mM β-mercaptoethanol and circulated 3–4 times using the same buffer. The thin solid line (•———•) shows the BAPA hydrolyzing activity, the thick solid line (^———^) illustrates the G6PD inactivating activity and the dotted line (o- - - -o) indicates the protein concentration.

cathepsin B1 is defined as the enzyme responsible for hydrolysis of BAPA and having mol. wt. about 25 000, B1 is separated into two kinds of enzymes as followes. One is eluted with low ion concentration buffer from DEAE or DEAE Sephadex A-50 column and shows hydrolytic activity only on BAPA but not on high mol. wt. protein, the other is the protease which is reactive toward BAPA and aldolase and was separated from the third enzyme by our circulating Sephadex G-75 column. Therefore, the third protease which was separated from the second enzymes mentioned above by our column and was not active to BAPA but reactive toward G6PD, OTA, casein, denatured hemoglobin might be regarded as a new protease.

3.2. *Specificity of susceptibility for substrate enzymes*

The eluate from the circulating Sephadex column was divided into three fractions as shown at the top of fig.1 and each was concentrated to a small volume in an ultrafiltration cell. The substrate specificities of these fractions are compared in table 2. Fraction I

Table 2
Substrate specificities of three fractions from circulating Sephadex G-75 column

Substrate	Method and Unit	Fraction I	II	III
BAPA	*p*-Nitroaniline formed (μmoles/min/mg)	0.226	0.182	0.050
Aldolase	U ($\times 10^{-2}$)/mg	452.0	n.d.	100
Apo-OTA	U ($\times 10^{-2}$)/mg	7.0	44.4	165.6
G6PD	U ($\times 10^{-2}$)/mg	0.0	41.9	155.2
Acid-denatured hemoglobin	Amino acids released (μmoles/min/mg)	0.035	0.257	1.115
Casein	Amino acids released (μmoles/min/mg)	0.030	0.202	0.656

Fraction I, II and III are the concentrated samples of the tube number 1–4, 5–60 and 61–90 of circulating Sephadex G-75 column chromatography in fig.1, respectively.

(Peak 1) contained BAPA-hydrolyzing and aldolase-inactivating activities, but no G6PD and OTA-inactivating or caseinolysis and denatured hemoglobin-hydrolyzing activity. Fraction III (Peak 2) showed almost no BAPA-hydrolyzing and aldolase-inactivating activities, but it was the only fraction showing G6PD- and OTA-inactivating activity and proteolytic activities on aciddenatured hemoglobin and casein.

Thus, the peak 1 enzyme participates in degradation of aldolase and BAPA whereas the peak 2 protease shows proteolytic activity on G6PD, casein and hemoglobin. Cathepsin B1 activity is usually assayed by measuring hydrolysis of BAPA. However, our results show that this assay requires reconsideration. The seryl proteases bound to the mitochondrial membrane, which were reported by one of the authors, participate in proteolytic inactivation of serine dehydratase, cystathionase, OTA and δ-aminolevulinate synthetase [6–8]. Thus, it seems likely that the various intracellular proteases each degrade certain groups of intracellular enzyme proteins and as suggested previously intracellular proteases may show group specificity in their actions on substrate enzymes [7,8].

3.3. *Effects of various reagents on the G6PD-inactivating enzyme*

As shown in table 3, addition of the SH reagent, β-mercaptoethanol, activated the G6PD-inactivating enzyme (Peak 2). This protease was strongly inhibited by leupeptin which is a specific inhibitor of cathepsin

Table 3
Effects of various reagents on the G6PD-inactivating enzyme

Reagent	Final concentration	Relative activity (%)
Control	–	100
β-Mercaptoethanol	1.0×10^{-3} M	236
β-Mercaptoethanol	5.0×10^{-3} M	309
Control (+5 mM β-mercaptoethanol)		100
Leupeptin	5.0×10^{-8} M	54
Leupeptin	1.0×10^{-7} M	0
Chymostatin	3.1×10^{-8} M	75
Chymostatin	3.1×10^{-7} M	44
Pepstatin	1.4×10^{-5} M	100
PMSF	1.0×10^{-5} M	99
PMSF	1.0×10^{-4} M	108

Table 4
Difference in heat stabilities of BAPA-hydrolyzing enzyme and G6PD-inactivating enzyme

Substrate	Heat treatment[a]		
	35°C	42°C	47°C
BAPA	100	95	85
G6PD	80	45	20

[a]Percentage activities remaining after heat treatment for 15 min are shown.

B group, but not by pepstatin, which strongly and specifically inhibits cathepsin D and pepsin. It was also inhibited by a low concentration of chymostatin. The BAPA hydrolyzing enzyme (Peak 1) was also strongly inhibited by leupeptin. Neither enzymes were affected by PMSF.

3.4. *Properties of the G6PD-inactivating enzyme*

The mol. wt. of the G6PD-inactivating enzyme and BAPA-hydrolyzing enzyme are both about 25 000, though the former is slightly smaller than the latter. Both enzymes are typical thiol-proteases. The heat stabilities of the two enzyme activities in the preparations from the DEAE Sephadex A-50 column were compared using BAPA and G6PD as substrates. Table 4 shows that the activity with BAPA was much more heat-stable than that with G6PD. The heat stabilities on the enzymes after separation were also very different. The optimum pH of the G6PD-inactivating enzyme was 5.0–6.0 and no activity was observed below pH 4.4 or above 6.5. The absence of activity at below pH 4.4 shows that this enzyme is different from cathepsin D.

References

[1] Coffey, J. W. and de Duve, C. (1968) J. Biol. Chem. 243, 3255–3263.
[2] Otto, K. (1967) Hoppe-Seyler's Z. Physiol. Chem. 348, 1449–1460.
[3] Otto, K. and Schepers, P. (1967) Hoppe-Seyler's Z. Physiol. Chem. 348, 482–490.
[4] Otto, K. and Bhakdi, S. (1969) Hoppe-Seyler's Z. Physiol. Chem. 350, 1577–1588.
[5] Otto, K. (1971) in: Tissue Proteinase (Barrett, A. J. and Dingle, T. J., eds.), pp. 1–28, North-Holland Publishing, Amsterdam.
[6] Katunuma, N., Kominami, E., Kobayashi, K., Banno, Y., Suzuki, K., Chichibu, K., Hamaguchi, Y. and Katsunuma, T. (1975) Eur. J. Biochem. 52, 37–50.
[7] Katunuma, N. (1975) Rev. Physiol. Biochem. Pharmacol. 72, 83–104, Springer-Verlag.
[8] Katunuma, N., Kominami, E., Banno, Y., Kito, K., Aoki, Y. and Urata, G. (1976) Adv. Enzyme Regul. 15, 325–346.
[9] Aoyagi, T., Takeuchi, T., Matsuzaki, A., Kawamura, K., Kondo, S., Hamada, M., Maeda, K. and Umezawa, H. (1969) J. Antibiot. (Tokyo) 22, 283–286.
[10] Matsuzawa, T., Katsunuma, T. and Katunuma, N. (1968) Biochem. Biophys. Res. Commun. 32, 161–166.
[11] Schlamowitz, M. and Peterson, L. U. (1959) J. Biol. Chem. 234, 3137–3145.
[12] Kornberg, A. and Horecker, B. L. (1955) Methods Enzymol. 1, 323–327.
[13] Taylor, J. F. (1955) Methods Enzymol. 1, 310–315.
[14] Katunuma, N., Okada, M., Matsuzawa, T. and Otsuka, Y. (1965) J. Biochem. 57, 446–449.
[15] Moore, S. and Stein, W. H. (1954) J. Biol. Chem. 211, 907–913.
[16] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[17] Ragab, H., Beck, C., Dillard, C. and Tappel, A. L. (1967) Biochim. Biophys. Acta 148, 501–505.
[18] Greenbaum, L. M. and Fruton, J. S. (1957) J. Biol. Chem. 226, 173–180.
[19] Davidson, E. and Poole, B. (1975) Biochim. Biophys. Acta 397, 437–442.
[20] Distelmaier, P., Hubner, H. and Otto, K. (1972) Enzymologia 42, 363–375.