COMPARATIVE STUDY OF ACID SH-DEPENDENT PROTEINASES FROM HOG KIDNEY CORTEX

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The study of proteolytic enzymes from the hog kidney cortex has already revealed an SH-dependent proteinase, hydrolyzing hemoglobin in an acid medium [1]. To identify the enzyme, it was decided to compare its properties with those of tissue SH-dependent enzymes already known: lysosomal carboxypeptidase B (cathepsin B-2), cathepsin B [5], and cathepsins H and L, recently discovered in rat liver [10].

Since the above-mentioned enzymes have not been described in the renal cortex, their isolation and characteristics were interesting on their own account, and the investigation described below was conducted for that purpose.

EXPERIMENTAL METHOD

The enzymes were isolated from the cortex of the hog kidney at 4°C. After freezing and thawing four times the tissue was homogenized and then extracted with 1 mM aqueous solution of EDTA in a ratio of 1:2. The residue was removed by centrifugation at 5000g; ballast proteins were precipitated at pH 3.5. The protein fraction precipitated by ammonium sulfate at 40-70% saturation was isolated from the supernatant. After desalination through Sephadex G-50 the resulting fraction was subjected to gel filtration through Sephadex G-100 (column 3 × 90 cm), equilibrated with 0.005 M Na-phosphate buffer, pH 6.74, containing 1 mM EDTA and 0.02% NaCl. Covalent chromatography of active fractions was then carried out on Hg-Sepharose 4B (organomercurial Sepharose), prepared by Barrett's method [6]. Columns (1.2 × 20 cm) with Hg-Sepharose were equilibrated with 0.05 M Na-phosphate buffer, containing 1 mM EDTA and 40 mM KCl (pH 6.7 for lysosomal carboxypeptidase B and pH 6.5 for cathepsins B and H). The absorbed protein was eluted with the above buffer with the addition of 10 mM dithiothreitol and 100 mM KCl. Subsequent rechromatography through Sephadex G-100 (1.6 × 90 cm column) was carried out under the conditions described above for the first gel filtration. Proteolytic activity was determined from hydrolysis of acid-denatured hemoglobin at pH 4.0 [4], of Nα-benzoyl-DL-arginine-p-nitroanilide·HCl (BAPA) at pH 5.8 [9], and of Nα-benzoyl-DL-arginine-β-naphthylamide·HCl (BANA) at pH 6.0 [8]. Aminopeptidase activity was determined from hydrolysis of Nα-leucine-β-naphthylamide·HCl (LNA) at pH 6.0 [8]. Amidase activity was determined by a microdiffusion method [2] at pH 5.4 with Nα-benzoyl-L-argininamide·HCl (BAA) as the substrate.* Protein was determined by Lowry's method [11].

EXPERIMENTAL RESULTS

During gel filtration of the protein fraction through Sephadex G-100 activity relative to hydrolysis of hemoglobin in an acid medium manifested in the presence of EDTA and dithiothreitol (SH-dependent proteolytic activity) was found in three main peaks (Fig. 1). The SH-proteinase discovered by the writers previously occurred in the first peak (region of proteins with mol. wt. of about 100,000) [1]. The hemoglobin-hydrolyzing activity of the second peak (mol. wt. 50,000) coincided with the peak of activity for hydrolysis of BAA, and was probably due to lysosomal carboxypeptidase B (cathepsin B-2). Besides hemoglobin-hydrolyzing activity, high activity for hydrolysis of BAPA, BANA, and LNA also was found in the fractions of the

*Leupeptin and pepstatin were generously provided by Professor Umezawa from the Institute of Microbiological Chemistry, Tokyo, Japan.

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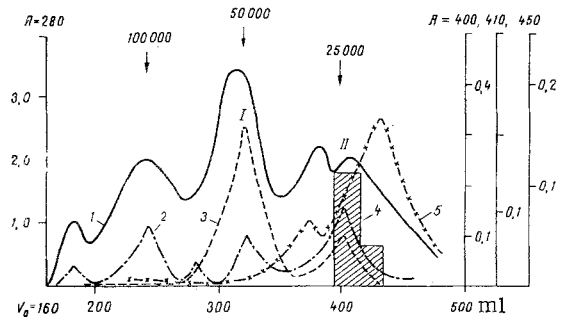

Fig. 1

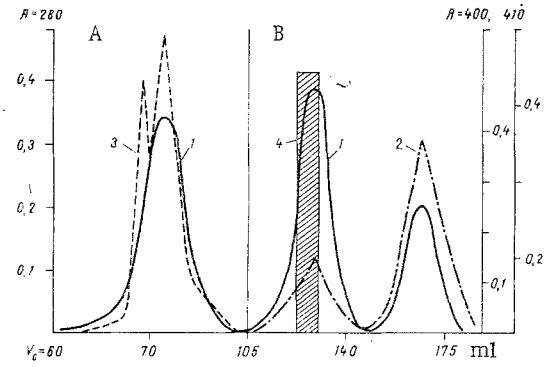

Fig. 2

Fig. 1. Gel filtration of protein fraction precipitated at 40-70% saturation with $(NH_4)_2SO_4$, through Sephadex G-100. Quantity of protein applied 1.5 g. Rate of elution 14 ml/h. I) Fraction used to purify lysosomal carboxypeptidase B; II) fraction (shaded zone) used to purify cathepsin B. 1) A-280; 2) hydrolysis of hemoglobin (pH 4.0; A-280); 3) BAA (pH 5.4; A-400); 4) BAPA (pH 5.8; A-410); 5) Leu-BNA (pH 6.0; A-450). Crosses — 450 — optical density at 250 nm.

Fig. 2. Rechromatography of lysosomal carboxypeptidase B (A) and cathepsin B (B) through Sephadex G-100. 1) A-280; 2) hydrolysis of hemoglobin (pH 4.0; A-280); 3) BAA (pH 5.4; A-400); 4) BAPA (pH 5.8; A-410). Remainder of legend as to Fig. 1.

third peak (mol. wt. about 25,000), indicating the presence of cathepsins B, H, and L, evidently, in that peak.

To purify the lysosomal carboxypeptidase B further, fractions of the second peak were used. As a result of chromatography on Hg-Sepharose and subsequent rechromatography through Sephadex G-100 an enzyme preparation was obtained with activity increased 9000 times (Table 1). On rechromatography through Sephadex G-100 the lysomal carboxypeptidase B was eluted in one symmetrical peak, in which two peaks of activity were found (Fig. 2A). Each of these active fractions of lysosomal carboxypeptidase B on electrophoresis in polyacrylamide gel at pH 8.9 gave one diffuse zone connected with activity toward BAA, and contained no other enzymes as impurities. The molecular weight of the lysosomal carboxypeptidase B, determined by chromatography in a thin layer of Sephadex G-100 (superthin) [3], was 50,000.

Purification of the fractions of the third peak at the stage of chromatography on Hg-Sepharose removed cathepsin D (SH-independent activity for hydrolysis of hemoglobin in an acid medium) and inert proteins present as impurities in the unadsorbed fraction. On elution of the adsorbed fraction, containing SH-dependent proteinases, cathepsin B (activity toward BAPA) and cathepsin L left the column a little earlier than cathepsin H (activity toward BANA and LNA). By subsequent rechromatography through Sephadex G-100, cathepsin B could be separated from the SH-enzyme (Fig. 2B), which hydrolyzes hemoglobin but not BAPA, BANA, or LNA. Since cathepsin L from rat liver possesses similar specificity [10], it was postulated that this proteinase is cathepsin L. As the result of purification, activity of cathepsin B for hydrolysis of BAPA was increased by 240 times. The preparation of cathepsin B thus obtained gave three zones on electrophoresis in polyacrylamide gel connected with activity toward BAPA. The molecular weight of the cathepsin B was 26,000.

The action of various activators and inhibitors on activity of the isolated enzymes was studied. All the enzymes tested were SH-dependent, but they differed in their sensitivity to the action of certain inhibitors. For instance, cathepsin H was only half as sensitive to the action of leupeptin as cathepsin B, in agreement with results obtained by other workers [10].

These results are evidence that lysosomal carboxypeptidase B (cathepsin B-2) and cathepsin B isolated from the cortex of the hog kidney are identical with the corresponding enzymes obtained from other biological objects [5]. Enzymes in all probability identical with cathepsins H and L, recently described in rat liver [10], were found in the renal cortex. The SH-

TABLE 1. Purification of Lysosomal Carboxypeptidase B and Cathepsins B and H from the Hog Kidney Cortex

Stage of purification	Total protein, mg	Yefel of protein	Activity								
			carboxypeptidase B			cathepsin B			cathepsin H		
			specific	total	yield	specific	total	yield	specific	total	yield
Extract	306 000	100	0,001	306	100	10,2*	3 121 200	100	28	8 568 000	100
Precipitation of proteins at pH 3.5	44 880	14,6	0,065	2917,2†	953	36,2	1 624 656	52	30	1 346 400	15
40-70% saturation with $(NH_4)_2SO_4$	1 260	0,41	0,51	642,6	210	204	257 040	9,2	254	320 040	3
Sephadex G-100 fractions											
I	40	0,013	3,2	128	41,8						
II	35	0,011				940	32 900	1,0	664	23 240	0,27
Hg-Sepharose fractions											
I	12	0,004	5,8	69,6	22,7						
II	7,26	0,0023				1040	7 550	0,24			
III	4,6	0,0015							1920	8 832	0,10
Rechromatography: carboxypeptidase B:											
I	0,3		8,2	2,5	0,2						
II	0,9	0,0003	9,8	8,9	2,88						
Cathepsin B	0,9	0,0003				2400	2 160	0,07			

*The high initial specific activity of the tissue extract for hydrolysis of BAPA was due to the presence of other enzymes besides cathepsin B in the extract (cathepsin H, BANA-amidohydrolase), capable of hydrolyzing the synthetic substrate of cathepsin B. This purification of cathepsin B by 240 times was therefore greatly underestimated.

†This sharp increase in amidase activity on acidification of the extract suggests that an inhibitor of enzymes hydrolyzing an amide substrate was present in the extract. On the other hand, it may be that the extract contained the inactive precursor of carboxypeptidases, which was activated in the course of isolation. These hypotheses have no experimental proof.

Legend. Yield of protein (in %) and enzyme activity (of carboxypeptidase B and cathepsins B and H, respectively, in micromoles ammonia/mg protein/h, nanomoles p-nitroaniline/mg protein/h, and nanomoles β-naphthylamine/mg protein/30 min) given per kg tissue.

proteinase discovered by the writers was shown to differ from lysosomal carboxypeptidase B and cathepsins B, H, and L in molecular weight, for it is eluted during gel filtration in the high-molecular-weight zone. In addition the "high-molecular-weight" enzyme does not hydrolyze synthetic substrates of cathepsin B. Further investigations are needed for the more precise identification and classification of the "high-molecular-weight" enzyme.

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