COMPARATIVE INVESTIGATION OF LIVER CATHEPSINS OF NORMAL RATS AND RATS WITH SARCOMA AND OF CATHEPSINS OF RAT SARCOMA

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UDC 612.351.015.13 :616-006
.3.04-008.931 : 577.156

The authors have purified and described the properties of two proteolytic enzymes from the tissues of a transplantable rat sarcoma [1, 2]. Among the proteinases from various tissues and organs of mammals which have been studied and described, no enzyme could be found identical in its properties to either of the sarcoma cathepsins. It seemed interesting to determine whether the enzymes studied were characteristic of tumor tissue or whether they are also present in the organs of healthy animals. However, the cathepsins of normal tissues have received comparatively little study, and there is no generally accepted classification of these enzymes. Substantial differences are also possible in the complement and properties of the proteinases from the same organ in animals of different species. For this reason, analysis of data in the literature could not provide an unequivocal answer to the question for the investigation of which the present investigation was undertaken.

EXPERIMENTAL METHOD

Rats were used in the experiments on the 18th-19th day of growth of the sarcoma (strain M-1). The proteolytic activity of the enzyme preparations was determined by Anson's method [4], and the protein concentration in the solutions from the optical density at 280 mμ and by Lowry's method [6]. The specific proteolytic activity of the cathepsins relative to 1 mg protein was expressed as values of increase of optical density at 280 mμ after incubation of the enzyme for 1 h at 37° with a 0.5% solution of hemoglobin in 0.1M citrate buffer, pH 3.8. The purity of the enzyme preparations was determined by starch gel electrophoresis [8] and also by sedimentation in an ultracentrifuge.

During the study of the substrate specificity of the cathepsins, enzymic hydrolysis of proteins was carried out in 0.1 M citrate buffer, pH 3.8. The sample contained 30 mg substrate and 300 μg enzyme. At the end of hydrolysis the samples were divided into two equal parts for determination of the N- and C-terminal amino acids liberated in the process of hydrolysis. The N-terminal amino acids of the peptides were determined by Sanger's method [7], and the C-terminal amino acids by the method of Akabori and Ohno as modified by Bradbury [5]. Characteristics of the substrate proteins were given in the authors' previous paper [2].

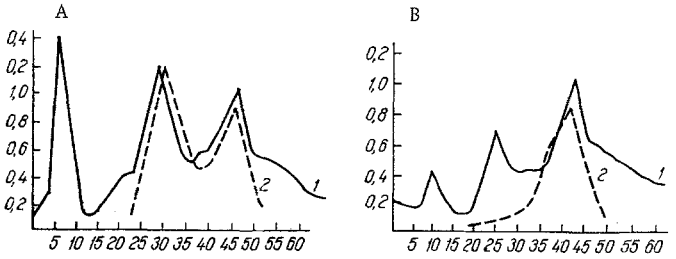

Fig. 1. Protein concentration (1) and proteolytic activity (2) of acetone fraction of extract of sarcoma (A) and liver (B) during chromatography on DEAE-cellulose. Abscissa—sample No. (volume of each sample 4 ml); ordinate—extinction at wavelength 280 mμ. Elution carried out in a concentration gradient of NaCl (0.75 M solution), volume of mixer 300 ml, volume of fractions 5 ml.

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Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 64, No. 11, pp. 76-80, November, 1967. Original article submitted December 25, 1966.

TABLE 1. N-Terminal Amino Acids Liberated by Action of Liver and Sarcoma Cathepsins on Proteins (in μg Amino Acids/15 mg Protein)

Substrate	Enzyme	Aspartic and glutamic acids	Glycine	Alanine	Serine	Threonine	Valine-leucine	Phenyl-alanine
Serum albumin	Liver cathepsin	50	40	66	25	24	176	40
	Sarcoma cathepsin II	75	20	20	12	7	150	–
Insulin	Liver cathepsin	13	65	13	14	–	97	50
	Sarcoma cathepsin II	60	125	25	40	–	250	10
Hemoglobin	Liver cathepsin	28	75	36	–	17	160	–
	Sarcoma cathepsin II	34	14	70	42	24	150	42

TABLE 2. N- and C-Terminal Amino Acids Liberated by Action of Liver Cathepsin on β-Chain of Horse Globin (in μg/15 mg Protein)

Amino acids	Aspartic and glutamic acids	Glycine	Alanine	Serine	Threonine	Phenyl-alanine	Valine	Leucine
C-terminal	20	80	15	30	10	25	60	15
N-terminal	65	16	70	33	18	37	84	20

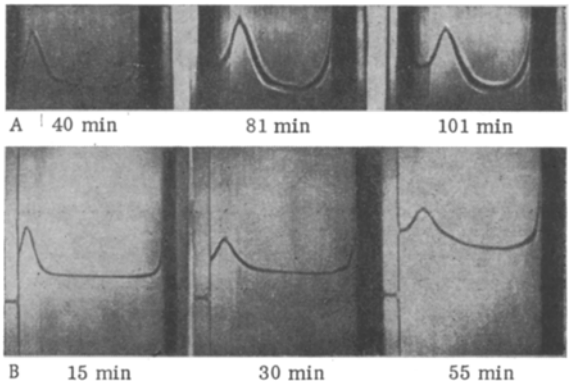

Fig. 2. Sedimentation diagrams of sarcoma cathepsin II (A) and of rat liver cathepsin (B). A) Sedimentation in a Svedberg ultracentrifuge, 0.05% solution of protein in 0.07M phosphate buffer, pH 7.4, speed of rotation of rotor 56,680 rpm, temperature 18°; B) sedimentation in Hitachi ultracentrifuge, 0.04% solution of protein in 0.07M phosphate buffer, pH 7.4, speed of rotor 59,780 rpm, temperature 19°.

To study the activation and inhibition of their enzymic action the cathepsins were kept for 30 min in a solution with the corresponding reagent, the concentration of which was $1 \cdot 10^{-4}$ M, and then the proteolytic activity was measured.

EXPERIMENTAL RESULTS

Isolation. Cathepsins from the liver of normal rats and of rats with sarcoma were isolated by the method developed for sarcoma cathepsins [2], incorporating fractionation of the proteins by organic solvents and chromatography on DEAE-cellulose. The specific proteolytic activity in liver extract of normal animals was 50-60% of the specific activity of the sarcoma extracts, while for the liver of rats with sarcoma this index was slightly higher – 70-80%. During fractional precipitation of the liver proteins with ethyl alcohol and acetone the distribution of specific proteolytic activity among the fractions was very similar to that obtained for sarcoma. The fraction precipitated by acetone in a concentration of 25-70 V% had a specific proteolytic activity which was increased by 10-12 times (sarcoma) and by 6-8 times (liver). During

chromatography of the acetone fraction of liver extract on DEAE-cellulose, one protein peak with proteolytic activity was observed in the eluate, whereas in the case of sarcoma there were two active components in this fraction, from which cathepsins I and II were isolated by rechromatography (Fig. 1). As may be seen in Fig. 1, liver cathepsin was eluted from the ion-exchange medium by the same salt concentration as sarcoma cathepsin II. By rechromatography on DEAE-cellulose a highly purified preparation of liver cathepsin was obtained, fully comparable to the sarcoma cathepsin II obtained by the preparative method. The preparation sedimented in the ultracentrifuge as one peak, and during electrophoresis by Smities' method it gave one clearly defined band. The specific proteolytic activity and yield of the enzyme were the same as for sarcoma cathepsin II. The enzyme activity in the liver of the animals with sarcoma was somewhat higher, and the quantitative yield of the preparation was 20-25% greater. This fact confirms earlier investigations showing an increase in proteolytic activity in different animal organs during the development of a malignant tumor in vivo [3].

The presence of an analogous enzyme was also sought in the spleen and kidneys of normal rats and rats with sarcoma. However, the scheme worked out for isolating sarcoma cathepsins proved unsuitable in this case. During fractionation of extracts of the kidneys and spleen with ethyl alcohol and acetone, the proteolytic activity was distributed completely differently, and no appreciable increase in enzyme activity took place in any fraction. Hence no proteolytic enzyme similar in its physicochemical properties to sarcoma cathepsin was present in these organs.

Sedimentation. The sedimentation coefficients of sarcoma cathepsin II and the liver cathepsins of normal rats and rats with sarcoma were determined. All three preparations had a sedimentation coefficient of 2.5 Svedberg units (Fig. 2). Sarcoma cathepsin I, unlike these enzymes, had a sedimentation coefficient of 3.3 Svedberg units.

Activation and inhibition of proteolytic activity, pH optimum of action. The liver proteinases, like the sarcoma cathepsins, were most active at pH 3.8. Reagents for sulfhydryl groups $-Hg^{++}$ ions and p-chloromercuribenzoate – inhibited the action of liver cathepsins by 50-70%, while cysteine and EDTA increased their activity by 23-27%. Similar results were obtained earlier for sarcoma cathepsin II. Sarcoma cathepsin I, on the contrary, was virtually not inhibited by thiol poisons and not activated by reducing or chelating agents [2].

Action on protein substrates. The action of liver cathepsin was studied on proteins of different amino-acid composition and molecular size: bovine serum albumin, bovine hemoglobin, and insulin. The β-chain of horse globin was also used. The results of quantitative determination of the N-terminal amino acids in enzyme hydrolysates of these proteins are given in Table 1. For comparison, some results obtained for sarcoma cathepsin II are given [4]. These results show that liver cathepsin, in the specificity of its action, has much in common with sarcoma cathepsin II. In all substrates, both enzymes liberated the largest amount of N-terminal valine and leucine. Many bonds incorporating the amino groups of alanine, glycine, glutamic acid, and aspartic acid were also ruptured. However, the action of these two similar proteinases showed certain differences. For instance, sarcoma cathepsin II did not liberate N-terminal phenylalanine in serum albumin, and in insulin it ruptured fewer phenylalanine bonds than liver cathepsin. In insulin and hemoglobin, sarcoma cathepsin II ruptured more peptide bonds incorporating the glycine-NH_2 group. In contrast to these enzymes, sarcoma cathepsin II ruptured more peptide bonds incorporating the glycine NH_2 group. In contrast to these enzymes, sarcoma cathepsin I ruptured, in the first place, the largest number of bonds formed by amino groups of dicarboxylic amino acids. During the action of this enzyme, in most substrates more than half of the peptide bonds to be hydrolyzed were bonds of glutamic and aspartic acids.

The results of the quantitative determination of N- and C-terminal amino acids liberated by the action of liver cathepsin on the β-chain of horse globin are given in Table 2. The order of the amino-acid residues in this protein is known. However, since the specificity of action of the enzyme, with respect both to the amino and the carboxyl components of the peptide bond is wide, it is difficult to identify with certainty the bonds which are ruptured; all that can be done is to postulate rupture of bonds of the gly–val, val–val, gly–ala, val–glut, and val–asp types. The specificity of action of liver cathepsin with respect to carboxyl was found to be slightly wider than that of sarcoma cathepsin II. Among the liberated C-terminal amino acids were large amounts of glycine and valine and rather less alanine, phenylalanine, and dicarboxylic amino acids. Despite the fact that the composition and relative proportions of N- and C-terminal amino acids liberated by the action of the two compared proteinases on proteins varied somewhat, the specificity of action of liver cathepsin and of sarcoma cathepsin II is apparently similar.

As mentioned above, little information on liver cathepsins is to be found in the literature. The insulin-splitting enzyme from bovine liver, described by Tomizawa and Halsey [9], has been studied in the greatest detail. This enzyme, active at pH 7.5, is quite different in its properties from the cathepsin described by the authors.

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