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PURIFICATION AND PROPERTIES OF AN ACID PROTEASE FROM HUMAN ASCITIC FLUID

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Summary

An acid protease was isolated from the ascitic fluid of a patient with ovarian cancer. It was purified about 400-fold to homogeneity by ammonium sulfate fractionation, gel filtration on Sephadex G-200 and DEAE-cellulose column chromatography. Its molecular weight was calculated to be 28 000 by gel filtration, and its isoelectric point was found to be pH 4.1. It showed similar activities on acid-denatured bovine serum albumin and on acid-denatured bovine hemoglobin, and its optimal pH for both substrates was 3.0. Sulfhydryl compounds and metal ions had no apparent effects on this enzyme, but pepstatin was strongly inhibitory.

Introduction

Various lysosomal hydrolases derived from the host or tumor cells have been found in the ascitic fluid of animals bearing ascitic tumors [1]. Acid proteases such as cathepsin D are also thought to be secreted from leucocytes and cancer cells into the ascitic fluid [1,2], and these proteases have been suggested to play a role in establishment of the pathophysiological condition of tumor-bearing hosts [3]. However, little is known about the exact enzymatical characters and pathological roles of proteases in the ascitic fluid, especially in humans.

During studies on acid proteases in the ascitic fluid of patients with peritonitis carcinomatosa, we found very high acid protease activity in the ascites of a case of ovarian cancer. We purified this protease to homogeneity, characterized it, and compared it with the other acid proteases described so far.

Materials and Methods

Ascitic fluid and chemicals

The ascitic fluid used for purification of the enzyme was obtained from a 48-

year-old patient with ovarian cancer. This cancer was diagnosed as a mucous cystadenocarcinoma and it disseminated in the abdominal cavity, causing peritonitis carcinomatosa.

Bovine hemoglobin and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Sephadex G-200 was a product of Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose (DE52) was purchased from Whatman Co. Ltd., Maidstone, Kent, U.K. Ampholine was a product of LKB Produkter, Stockholm, Sweden. Fluorescamine was purchased from Hoffman-La Roche Inc., Nutley, N.J., U.S.A. Pepstatin, leupeptin, chymostatin and antipain were generous gifts from Dr. H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan. All other chemicals used including synthetic substrates were commercial products of reagent grade.

Protease assay

Protease activity was determined essentially based on the Anson's method [4], with slight modification as follows; reaction mixture was composed of 0.5 ml 2.5% hemoglobin, 0.3 ml 0.2 M citrate phosphate buffer (pH 2.8) and 0.2 ml enzyme. Reaction was proceeded for 60 min at 37°C and stopped by addition of 1 ml 10% trichloroacetic acid. The trichloroacetic acid soluble product of enzyme reaction was determined by fluorescence at 475 nm using fluorescamine. Tyrosine was used as a standard. One unit of enzyme was defined as the amount which yielded the same fluorescence as 1 nmol of tyrosine at above condition.

Protein determination

In the early stages of enzyme purification, protein was determined by the method of Lowry et al. [5]; in later stages when the protein concentration was low, the fluorescamine method was used [6]. Bovine serum albumin was used as a standard in both methods.

Emzyme purification

The ascitic fluid was stored at −20°C and was thawed and dialysed against 0.15 M NaCl before use. All purification procedures were carried out at 4°C unless otherwise stated. Insoluble materials were removed by centrifugation at 27 000 $\times g$ for 20 min. Then solid $(NH_4)_2SO_4$ was added to the supernatant to give 60% saturation, and the precipitate was collected by centrifugation, dissolved in a small volume of 0.15 M NaCl and dialyzed against 0.15 M NaCl.

The dialysed material was applied to a Sephadex G-200 column and eluted with 0.15 M NaCl. Fractions in the main peak of acid protease activity were combined and dialysed against 20 mM sodium phosphate buffer (pH 6.9).

The dialysed preparation was applied to a DE52 column (1 × 15 cm), equilibrated with 20 mM sodium phosphate buffer (pH 6.9). The column was washed with 5 vols. of the same buffer, and then eluted with a linear gradient of 0 to 0.3 M NaCl in the same buffer and 1-ml fractions were collected. Fractions containing the activity were combined and dialysed against 20 mM sodium phosphate buffer (pH 6.9). After this step of purification, protein was determined by the fluorescamine method.

Dialyzed enzyme from the first DE52 column was applied to a DE52 column

under the same conditions, and the preparation eluted from the second DE52 column was used for enzyme characterization.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed by the method of Ferris et al. [7] using 5% gel and 90 mM veronal buffer (pH 8.6).

Isoelectric focusing in polyacrylamide gel

This was performed in 5% polyacrylamide slab gel in an LKB-Multiphore apparatus essentially as described by Righetti and Drysdale [8]. After electrofocusing for 2 h the gel was immersed in 0.5% hemoglobin solution in 0.2 M citrate/phosphate buffer (pH 3.0) for 30 min, removed, incubated for 2 h at 37°C in a moist chamber, and then stained with 0.2% Coomassie Brilliant Blue R-250 in a mixture: water/ethanol/acetic acid (8 : 3 : 1, v/v) and destained in the same mixture.

For determination of pH gradient, small pieces of the gel were cut out with a cork borer, and extracted with 1 ml water and the pH values of the extracts were measured.

Results

Enzyme purification

The purification is summarized in Table I. Two peaks of enzyme activity were observed on gel filtration of Sephadex G-200, as shown in Fig. 1. The major peak constituted 95% of the activity recovered from the column. The molecular weight of the enzyme in the major peak was determined to be 28 000 from the elution positions of molecular weight markers. In the first DE52 column chromatography, all the activity was eluted with 0.13 M NaCl as a single peak. In the second DE52 column chromatography, the enzyme was again eluted at the same NaCl concentration. The purification procedure achieved 1.7 mg enzyme protein with about 400-fold purification and a recovery of 34%.

Fig. 2 shows the result of polyacrylamide gel electrophoresis of the purified enzyme under conditions not causing denaturation. The protein was stained as a single band, and the enzyme activity coincided with the protein band, showing that the purified enzyme was homogeneous.

TABLE I

PURIFICATION OF HUMAN ASCITIC PROTEASE

Fraction	Total activity (units · 10^{-6})	Yield (%)	Total protein (mg)	Specific activity (units/mg protein · 10^{-3})
Ascitic fluid	14.60	100	1730	8.2
Ammonium sulfate (0—60%)	9.33	65.7	750	12.4
Sephadex G-200	5.58	39.3	53.7	104
First DE52 column	4.95	34.9	3.3	1500
Second DE52 column	4.86	34.2	1.7	2950

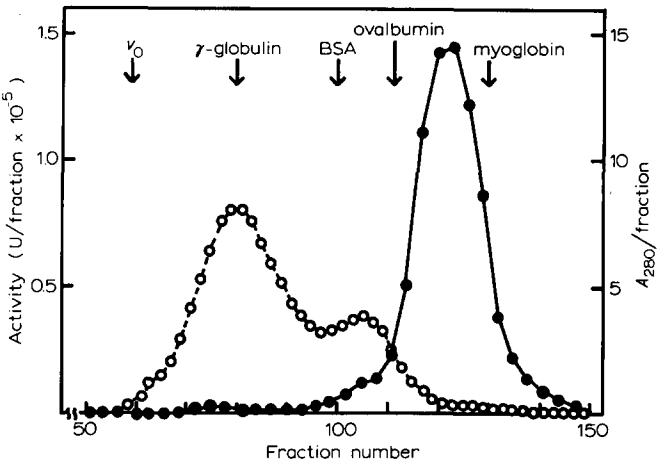

Fig. 1. Chromatographic profile of ascitic protease on a Sephadex G-200 column (4 X 65 cm). The column was eluted with 0.15 M NaCl at a flow rate of 16 ml/h, and fractions of 4 ml were collected. Arrows indicate the elution positions of molecular weight markers. ●———●, activity; ○- - - - -○, A_{280}. BSA, bovine serum albumin.

Isoelectric focusing in polyacrylamide gel

The purified enzyme gave a single band of activity upon isoelectric focusing in polyacrylamide gel, and its pI value was found to be 4.1.

Heat stability

The purified enzyme at a protein concentration of 1 μg/ml was heated at 56°C or 60°C in 5 mM sodium phosphate buffer (pH 7.0) and then the residual activity was measured.

The enzyme lost 50% of its activity in 15 min at 56°C and in 1 min at 60°C. It lost 70% of its activity in 60 min at 56°C, and 90% of its activity in 2 min at 60°C.

pH optimum

As shown in Fig. 3, the optimum pH for both acid-denatured hemoglobin

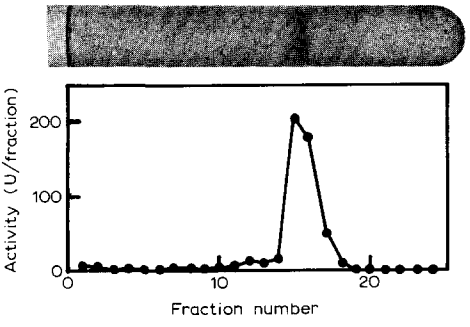

Fig. 2. Polyacrylamide gel electrophoretic pattern of purified ascitic protease. After electrophoresis, protein was stained with Coomassie Brilliant Blue R-250. A duplicate gel was cut into 2-mm sections and the enzyme activity of their extracts was assayed.

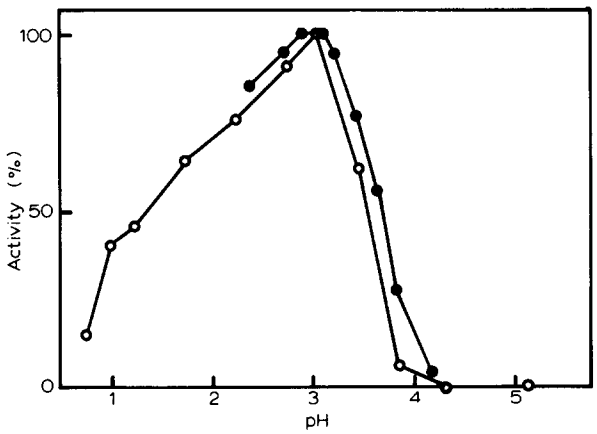

Fig. 3. Effect of pH on the activity of purified ascitic protease with acid-denatured hemoglobin (●———●) and acid-denatured albumin (○———○). Buffers used were; pH 0.8—2.3, appropriate concentrations of HCl and pH 2.4—5.1, 0.1 M citrate/phosphate buffer.

and acid-denatured bovine serum albumin was pH 3.0. Considerable activity for bovine serum albumin was still detected at pH 1.0, indicating that the enzyme was stable at low pH values, but no activity was observed above pH 4.2 with either substrate.

Effects of various ions and inhibitors

The enzyme activity was not affected by the following compounds: KCN, $MgCl_2$, $HgCl_2$, $CaCl_2$, $MnCl_2$, $CoCl_2$, $ZnCl_2$, $CdCl_2$, $FeCl_3$, and EDTA at concentrations of 10 mM; 1 mM EGTA; β-mercaptoethanol, iodoacetamide, iodoacetic acid and diisopropyl fluorophosphate at concentrations of 10 mM; 0.2 mM *p*-chloromercuric benzoic acid; leupeptin, antipain and soybean trypsin inhibitor at concentrations of up to 1 mg/ml, 1 mg/ml and 0.5 mg/ml, respectively. However, pepstatin was strongly inhibitory and its ID_{50} on the purified enzyme was $5 \cdot 10^{-2}$ μg/ml. About 40% of the activity was inhibited by 100 μg/ml of chymostatin. The effects of these compounds were tested at an enzyme concentration of 300 units/ml.

Activities with various substrates

The apparent K_m values of the purified enzyme for acid-denatured hemoglobin and acid-denatured bovine serum albumin were 0.67 mg/ml and 1.38 mg/ml, respectively. The V value for both acid-denatured hemoglobin and acid-denatured bovine serum albumin was 300 nmol tyrosine equivalent per 60 min/100 ng enzyme. No activity was detected with the following synthetic substrates: *N*-acetyl-L-phenylalanyl-L-diiodotyrosine [9], carbobenzoxy-L-glutamyl-L-tyrosine [10], *N*-α-benzoyl-L-argininamide [10] and glycyl-L-phenylalanine amide [10].

Discussion

The enzyme purified in the present work is very similar to human cathepsin D [11] in its enzymatic properties, such as its optimal pH, high activity on

hemoglobin, absence of requirement for metals or sulfhydryl-compounds and strong inhibition by pepstatin.

The molecular weight determination was only performed at the early stage of purification and sodium dodecyl sulfate polyacrylamide gel electrophoresis was not carried out on the purified enzyme. However, its estimated molecular weight of 28 000 seems significantly smaller than those of human cathepsin D from liver, leucocytes and erythrocytes which are reported to be 45 000, 42 000 and 55 000, respectively [11—13]. In addition, the isoelectric point of the enzyme from ascitic fluid is 4.1, whereas those of the multiple forms of human liver cathepsin D are 5.5, 6.0 and 6,5, respectively [11]. Moreover, a characteristic property of all preparations of cathepsin D [14] including that of human origin [11] is that activity is much lower on acid-denatured bovine serum albumin than on acid-denatured hemoglobin. However, the human ascitic enzyme purified in the present work showed similar activities on these two substrates, and thus it seems different from cathepsin D so far reported.

Cathepsin E, another intracellular acid protease is also present in polymorphonuclear leucocytes, and it hydrolyses bovine serum albumin better than hemoglobin [14]. However, the molecular weight of this enzyme is reported to be much larger than that of cathepsin D, e.g. 305 000 for the enzyme from bovine spleen [15].

The molecular weight of human gastricsin, another well known acid protease, is reported to be 31 400 [16], which is close to that of the enzyme purified in this work. Moreover, like the ascitic enzyme, gastricsin has a pH optimum of 3.0 [17]. However, the ascitic enzyme was stable at pH 8.6, whereas gastricsin and also human pepsin are unstable in alkaline conditions [18].

Thus the enzyme purified in the present work seems to be different from the acid proteases reported so far. Another possibility is that this enzyme may be an active product of proteolysis of a known acid protease during secretion from cells or during its accumulation in the ascitic fluid. However, it should be mentioned that we also found low but definite acid protease activity in the ascitic fluid of other patients with ovarian cancer. This acid protease showed the same behavior on Sephadex G-200 column chromatography and on isoelectric focusing as the purified enzyme described in this paper (data not shown). These data suggest that this type of enzyme purified in the present work, is usually present in the ascitic fluid of ovarian cancer patients and that it may play a role in the accumulation of ascitic fluid as suggested by Greenbaum et al. [3].

Acknowledgements

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