Increased Activity of a Neutral Protease in Cytosol from Rat Hepatoma Induced by N-2-Fluorenylacetamide

Kenji Wada, Hidenori Matsui, and Kinji Tsukada

Department of Pathological Biochemistry, Medical Research Institute, Tokyo Medical and Dental University, Kandasurugadai, Chiyoda-ku, Tokyo 101, Japan

ABSTRACT

A protease active with N-α-benzoyl-DL-arginine-p-nitroanilide with an optimum pH of 7.3 has been found in the cytosol of rat liver. The activity of this protease increased in N-2-fluorenylacetamide-induced hepatoma as well as in fetal liver. It has been purified from normal liver and hepatoma about 200-fold. Its molecular weight is estimated by gel filtration to be about 200,000 in each tissue. The protease activity is unaffected by chymostatin, pepstatin, soybean trypsin inhibitor, and p-chloromercuribenzoate. Antipain, leupeptin, tosyl-L-lysine chloromethyl ketone, and phenylmethylsulfonyl fluoride inhibit the protease activity. This protease appears to be a serine protease.

INTRODUCTION

The intracellular breakdown of proteins probably involves the participation of proteases and peptidases, although its degradation mechanism and regulation still remain to be clarified. Many intracellular proteases have been isolated from various tissues and characterized. In mammalian tissues, acid proteases are found in high concentration in lysosomes (3, 4). The rate of degradation of cytosol proteins was found to be less than 10% that of whole homogenate (5, 6), but it seems likely that nonlysosomal protease may play an important role in the degradation of certain cell proteins. Several proteases are known to exist in liver; however, few have been purified and characterized. Proteases with their major activity at neutral pH have been demonstrated in nuclear (6, 10) and lysosomal (8, 18) fractions. Recently, several proteases, including high-molecular-weight neutral proteases (7, 17) and Ca2+-dependent neutral protease (19), are known to exist in the cytosol in mammalian cells. In the course of studies on the distribution and properties of a neutral, trypsin-like protease isolated to homogeneity from rat intestine (22), the considerable rate of BAPA3 hydrolysis at pH 7.3 was found in the high-speed supernatant fraction from a rat hepatoma induced by a hepatocarcinogen. This communication describes that protease activity from rat hepatoma induced by 2-FAA increased in the cytosol fraction and also describes a method for the partial purification of this protease as well as some properties of this protease.

MATERIALS AND METHODS

Materials

BAPA, 2-FAA, TLCK, PCMB, PMSF and TPCK were obtained from Sigma Chemical Co., St. Louis, Mo. Protease inhibitors from cultured broth of Actinomycetes were supplied by Research Resources, Ministry of Education, Science and Culture, Japan. DEAE-cellulose and Sephadex G-150 (superfine) were products of Pharmacia, Uppsala, Sweden. Soybean trypsin inhibitor was purchased from Boehringer/Mannheim, Mannheim, Federal Republic of Germany. Azocoll, azocasein, and elastin-orcein were from Calbiochem-Behring Corp., La Jolla, Calif. All other reagents were of analytical grade.

Animals

The rats used were albino Wistar strain. The gestational age of fetuses was calculated from the mating date, known within 12 hr. The gestational period for this strain of rats was 22 days. Partial hepatectomy refers to the removal of about 70% of the liver (left lateral and median lobes) (11).

Treatment of Animals

Male Wistar rats (Fuji Animal Farm, Tokyo, Japan) weighing 150 to 200 g were used. The basal diet (Oriental Yeast Co., Tokyo, Japan) was the same as described previously (15). Animals were fed for 3 months with the basal diet containing 0.025% 2-FAA followed by a basal diet after this treatment. The rats were sacrificed about 6 months after the start of the experiment and about 5 months from the end of the 2-FAA diet. The liver of each rat was examined macroscopically and divided into nonhepatomatous and hepatoma areas. Tissue samples were taken from an area adjacent to tissues used for biochemical studies. For histological studies, tissues were fixed in 10% neutral buffered formaldehyde solution and stained with hematoxylin and eosin. Histologically, hepatoma areas showed findings of typical hepatocellular carcinoma. In this experiment, hepatoma areas were used as hepatoma for the enzyme assay.

Determination of Protease Activity

Activity in BAPA was measured at 410 nm in 0.05 m potassium phosphate buffer (pH 7.3) as described by Erlanger et al. (9). The assay mixture consisted of 0.45 ml of enzyme, 0.5 ml of 2 m M BAPA, and 0.05 ml of 1 m M potassium phosphate buffer (pH 7.3). After incubation at 37° for 1 to 10 hr, the reaction was terminated by addition of 0.1 m l of 10% sodium lauryl sulfate and 0.4 m l of 0.5 M Tris-HCl (pH 10.0). Blank determinations were carried out in the same way except that distilled water replaced enzyme solution. A major absorption difference of 8000 m m -1 cm -1 was used for all calculations. A unit of enzyme activity is defined as μmol of product liberated per hr.

Protein Determination

Protein was determined by the method of Lowry et al. (14) with bovine serum albumin taken as the standard.

Polyacrylamide Gel Electrophoresis

Disc electrophoresis was performed at pH 4.5 and 7.5% polyacryl-
Increased Activity of a Protease in Hepatoma

RESULTS AND DISCUSSION

The protease activity from cytosol fraction of normal liver and hepatoma was assayed at various pH values. Chart 2 shows the protease activity in soluble fractions from hepatoma induced by 2-FAA. At pH 7.3, the activity of the protease increased at about 5 months, when the tissue is shown to be hyperplastic and premalignant, after onset of feeding 2-FAA, and markedly increased 8 months after treatment. A neutral protease activity in regenerating rat liver 24 and 48 hr after operation increased approximately 2-fold compared with that from normal liver (Table 1). On the 19th day of gestation, markedly increased protease activity was observed in fetal rat liver just as it was in hepatoma (Table 1). The effects of leupeptin, chymostatin, TLCK, TPCK, and PMSF on each crude enzyme preparation in Table 1 were shown to be almost same as those of partially purified protease as described in Table 3.

The purification results from hepatoma tissues are summarized in Table 2. The enzyme was purified about 200-fold with a yield of 12%, but it was not homogeneous, judging from the results that the activity and absorbance at 280 nm did not coincide in the fractions from Sephadex G-150 (data not shown). From analytical disc electrophoresis of the partially purified protease on polyacrylamide gel, several bands were detected by protein staining, one of which was also active on BAPA (data not shown). The protease was also purified about 200-fold from normal rat livers with the yield of about 10%. With the partially purified protease, the rate of degradation of the substrate proceeded linearly for more than 4 hr and was

### Table 1

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Protease activity (mU/mg protein)</th>
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<tbody>
<tr>
<td>Normal liver</td>
<td>0.56 ± 0.07a</td>
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<tr>
<td>Regenerating liver</td>
<td></td>
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<tr>
<td>6 hr after operation</td>
<td>0.63 ± 0.05</td>
</tr>
<tr>
<td>24 hr after operation</td>
<td>1.19 ± 0.09</td>
</tr>
<tr>
<td>48 hr after operation</td>
<td>1.20 ± 0.10</td>
</tr>
<tr>
<td>Fetal liver, 3 days before birth</td>
<td>2.92 ± 0.26</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>8.21 ± 0.58</td>
</tr>
</tbody>
</table>

*a Mean ± S.D.
proportional to the amount of the protease added to the assays. The molecular weight of the protease from the cytosol of hepatoma and liver was estimated by gel filtration on Sephadex G-150 column as approximately 200,000 in each tissue, by the method of Andrews (1), as shown in Chart 3. The pH dependence of the activity of partially purified protease was similar to that of crude preparations as shown in Chart 2. Some reagents for general protein substrates, e.g., azocoll, azocasein, and elastin-orcein, were not hydrolyzed by the protease.

The thermal stability of the partially purified protease from hepatoma was determined by assaying the activity remaining after incubation for 30 min at various temperatures between 30° and 70° in 0.05 M buffer, pH 5.0 or 7.0, indicated in Chart 4A. The results indicated that the activity of the protease was stabilized at pH 5.0 compared to pH 7.0, although the activity was almost completely destroyed at 70° in both pH. When the protease was heated at 50° in 0.05 M potassium phosphate buffer (pH 7.0) with the indicated concentrations of ammonium sulfate, the activity of the protease was prevented from destroying by the addition of 0.2 M ammonium sulfate as shown in Chart 4B. Almost the same results for thermal stability were obtained from the partially purified protease from normal rat liver.

The effects of protease inhibitors are presented in Table 3. Among the protease inhibitors obtained from cultured broths of Actinomycetes, the protease was inhibited only by antipain and by also leupeptin, which is a strong competitive inhibitor of proteolysis by plasmin, trypsin, or papain (2). The protease was also inhibited by a specific inhibitor of trypsin, TLCK, and could be identified as serine protease as it was inhibited by PMSF. The protease was not inhibited by soybean trypsin inhibitor, PCMB, or TPCK. Apparently, no different properties were observed between the normal and neoplastic hepatic enzymes, except that the specific activity of the latter showed a remarkably higher increase than that of the former.

Leupeptin markedly inhibited tumorigenesis in mouse skin induced by a single, noncarcinogenic dose of 7,12-dimethylbenzanthracene followed by repeated application of croton oil, and it also inhibited the activity of p-toluenesulfonyl-L-arginine methyl ester esterase in the skin of animals treated with croton oil (12). It was reported that synthetic inhibitors of proteases markedly depressed tumorigenesis in mouse skin induced by painting the skin with 7,12-dimethylbenzanthracene and croton oil (13, 21). Therefore, they suggested that certain proteases might be involved in tumorgenesis.

In contrast to other intracellular proteases in liver, this enzyme is shown to be located in cytosol. On the basis of evidence presented in this study, this protease is shown to be distinct from lysosomal proteases as to optimum pH and the effect of several protease inhibitors. The protease, cathepsin B active with BAPA in lysosomal fraction from rat liver, was crystallized by Towatari et al. (20). The activity of this protease was maximal at pH 6.0 and decreased markedly above pH 7.0. It is a thiol protease with a molecular weight of 26,000 by gel filtration (20). The protease described here is distinct from...
other proteolytic enzymes in liver cytosol that have been reported recently (7, 17). The protease reported by Rose et al. (17) is a high-molecular-weight (M.W. >400,000) protease active with labeled globin at pH 7.5 in soluble fractions from mouse tissues and is most active in liver. This protease is inhibited by PCMB and is not inhibited by TLCK and TPCK (17). The enzyme reported by DeMartino and Goldberg (7) is alkaline endoprotease with an apparent molecular weight of 550,000 the activity of which is stimulated by ATP and other nucleotides as well as by PP.

We have shown here that the neutral protease activity increased in hepatoma as well as in fetal liver, but the protease may be characteristic of the cancer cells. These proteases may be related to growth, highest in hepatoma (8.21) and next in fetal liver (2.92), regenerating liver (1.20) compared to normal liver (0.56). The identification of this enzyme may lead to an indicator enzyme for liver carcinogenesis.

REFERENCES

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