Elevated Activity and Increased Mannose-6-phosphate in the Carbohydrate Moiety of Cathepsin D from Human Hepatoma

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ABSTRACT

A significant elevation of cathepsin D activity was observed in six human hepatoma tissues as compared to 12 normal human livers. In isoelectric focusing experiments, cathepsin D purified from normal liver exhibited three different forms, with isoelectric points of 5.6, 6.1, and 6.7, while cathepsin D purified from hepatoma contained another five to six more acidic forms in addition to the forms observed in normal liver cathepsin D. When the tumor enzyme was treated with endo-β-N-acetylglucosaminidase H followed by isoelectric focusing, the acidic components disappeared and were converted to forms identical to those of the normal liver cathepsin D. Determination of the mannose-6-phosphate content showed that hepatoma cathepsin D contains twice as much mannose-6-phosphate as normal liver cathepsin D. Peptide mapping and amino acid analysis showed that the protein moiety of cathepsin D from hepatoma is almost identical with that from normal liver. These findings indicate that the appearance of acidic variants in hepatoma cathepsin D is mainly due to changes in the oligosaccharide chains of the enzyme, which are closely associated with the increase of mannose-6-phosphate in the tumor enzyme.

INTRODUCTION

Cathepsin D (E.C. 3.4.23.5) is a lysosomal acid protease widely distributed in eukaryotic and prokaryotic cells. Since the name cathepsin D was first given to the enzyme by Press et al. (1) in 1960, many purification studies have been reported (see review in Ref. 2), and charge heterogeneity in cathepsin D molecules has been demonstrated in the bovine spleen (1), human liver and chicken liver (3), and porcine spleen (4). It is widely known that many lysosomal enzyme activities are elevated in malignant neoplasms. The level of cathepsin D activity has also been reported to be elevated in human ovarian carcinoma tissues (5). However, few studies have been carried out on the properties of purified cathepsin D from human malignant neoplasms. Our previous study on arylsulfatase B, one of the lysosomal enzymes, from human lung carcinoma indicated its increased activity level and the appearance of an acidic variant form which is closely associated with phosphorylation (6). Such variant forms were also observed in arylsulfatase B in chronic myelogenous leukemia (7), arylsulfatase A in human lung carcinoma (8), β-hexosaminidase in human lung carcinoma (9), and β-glucuronidase in human lung carcinoma (10). It is necessary to examine whether such tumor-associated changes in human lysosomal hydrolases occur in general. In the present study, we investigated the activity levels of cathepsin D in human hepatoma and changes in the properties of purified tumor enzymes, and we discovered that there is also an increase in acidic variants of cathepsin D from human hepatoma.

MATERIALS AND METHODS

Chemicals. Pepstatin was obtained from the Peptide Institute (Osaka, Japan), and pepstatin-aminohezyl-Sepharose 4B gels were prepared according to the method of Huang et al. (4). Protease-free neuraminidase from Arthrobacter ureafaciens was purchased from Nakarai (Kyoto, Japan); endoglycosidase H4 of Streptomyces griseus was from Seikagaku Kogyo (Tokyo, Japan); and acid phosphatase (type III) of potato, Man6-P, bovine milk β-galactosyltransferase, yeast phosphoglucone isomerases, and L-(1-tosylamino-2-phenylethyl)chloromethyl ketone derivative of trypsin were from Sigma Chemical Co. (St. Louis, MO). Yeast glucose-6-phosphate dehydrogenase and yeast phosphomannose isomerase were the products of Boehringer Mannheim (West Germany). Sodium [1-23]iodide (100 mCi/ml) and UDP[-11H]galactose (11.4 Ci/mm) were purchased from New England Nuclear (Boston, MA). Other chemicals were of analytical grade.

Liver Tissues. Normal human livers which were histologically free from pathological change were obtained at autopsy. Human hepatoma tissues were obtained at surgery and autopsy. The hepatoma tissues used were all characterized histopathologically as hepatocellular carcinoma. These tissues were frozen immediately after delivery and stored at -80°C until use.

Tissue Extracts. Approximately 1 to 2 g of the liver tissues were homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) in 5 volumes of 10 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose. After centrifugation at 500,000 x g for 30 min, the supernatant was assayed for enzyme activity.

Assay of Cathepsin D Activity. Proteolytic activity was determined with human hemoglobin as the substrate in a modification of the method of Anson (11). The incubation mixture in a micro-test tube (1.5 ml, Eppendorf) contained 0.5 mM sodium formate buffer, pH 3.3, 100 μl; 3.0% human hemoglobin solution, 100 μl; and the enzyme sample, 50 μl. After incubation at 37°C for 20 min, 1.0 ml of 3% trichloroacetic acid was added to precipitate proteins. Following centrifugation at 10,000 x g for 1 min in a microcentrifuge (KUBOTA KM-15000), the absorbance of the supernatant was measured at 280 nm.

The control experiments were carried out with incubation mixtures to which enzyme samples were added after the addition of trichloroacetic acid. One unit of enzyme activity is defined as the supernatant giving 1.0 net extinction value of 1.0.

All quantitative determinations were carried out in the linear range of the assay, up to a net absorbance of 0.3.

Protein was determined according to the method of Lowry et al. (12), with bovine serum albumin as a standard.

Purification of Cathepsin D from Normal Human Liver and Hepatoma. All the procedures were carried out at 4°C.

In Step 1, tissues were minced and homogenized in 4 volumes of 20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, using a Brinkmann homogenizer. The homogenate was centrifuged at 5000 x g for 30 min, and the supernatant was obtained.

In Step 2, solid ammonium sulfate was added to the supernatant to give 60% saturation, and the precipitate formed was dissolved in 20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl.

In Step 3, to the above solution, one volume of acetone (−20°C) was added, with stirring, over a period of 30 min. The solution was centrifuged, and the pellet was suspended in 20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. Triton X-100 was added to the solution to a final concentration of 0.1% (w/v). The solution was stirred for 2 h and centrifuged at 5000 x g for 30 min. The supernatant was dialyzed against 0.1 M acetate buffer, pH 5.5, containing 10% (v/v) glycerol. The solution was desalted by passage through a Sephadex G-50 column (100 x 2 cm) equilibrated with 0.1 M acetate buffer, pH 5.5, containing 10% (v/v) glycerol.

The abbreviations used are: endoglycosidase H, endo-β-N-acetylgalcosaminidase H; Man6-P, mannose-6-phosphate; GlicNac, N-acetylgalacosamine.

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against deionized water and adjusted to pH 3.5 by adding 0.1 N HCl. The precipitate was then removed by centrifugation.

In Step 4, the solution was applied to a pepstatin-aminohexyl-Sepharose 4B column (12 cm × 2 cm) which had been equilibrated previously with 50 mM sodium acetate buffer, pH 3.5, containing 0.2 M NaCl. After washing of the column with the same buffer, cathepsin D was eluted with 50 mM Tris-HCl buffer, pH 8.5, containing 0.2 M NaCl, as described by Huang et al. (4). Cathepsin D-active fractions were combined, dialyzed against deionized water, and then lyophilized.

In Step 5, lyophilized powder was dissolved in a minimum volume of 20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, and applied to a Sephadex G-100 column (118 cm × 2.2 cm) which had previously been equilibrated with the same buffer. The active fractions were pooled and dialyzed against deionized water.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and mercaptoethanol was performed according to the method of Laemmli (13).

Immunodiffusion. Antiserum were raised against cathepsin D purified from normal human liver in rabbits. IgG was obtained by 40% ammonium sulfate precipitation and anion exchange chromatography on DEAE-cellulose. Ouchterlony’s immunodiffusion (14) was performed in 1.2% agarose.

Isoelectric Focusing on Polyacrylamide Slab Gel. Purified cathepsin D was applied to a vertical slab gel in a dual electrophoresis cell (Bio-Rad), using 0.2% carrier ampholites (LKB, Ampholine) ranging from pH 3.5 to 10. Prefocusing was performed at 200 V for 1 h at 4°C, followed by focusing with stepwise increases of voltage from 200 to 700 V at a rate of 100 V per 30 min. The voltage was maintained at 700 V for the final 2 h, giving a total of 6 h as described by Stevens et al. (15).

After electrophoresis, the protein in the gel was first fixed by soaking the gel in a solution of 5% sulfosalicylic acid-10% trichloroacetic acid for 30 min at 60°C. The gels were then transferred to a staining solution of Coomassie Brilliant Blue G250 as was done by Vesterberg et al. (16). When the amount of cathepsin D applied was very low, silver staining was carried out. For this procedure, the gel was shaken in ethanol:acetic acid:water (25:8:65, v/v/v) for more than 10 h to remove amipholie after the fixation. Silver stain “DAIICHICHI” (Daichi Pure Chemicals, Tokyo, Japan) was used for silver staining.

Treatment of Hepatoma Cathepsin D with Exogenous Hydrolyases. It was examined whether the potato acid phosphatase is active or not on sugar phosphates. Five hundred nmol of Manα6-P were incubated with 0.5 unit of potato acid phosphatase in 100 mM sodium acetate buffer, pH 4.8, at 37°C for 24 h followed by assay of inorganic phosphate by the method of Fiske and Subbarow (17). Through the treatment with this phosphatase, more than 85% of inorganic phosphate was liberated from Manα6-P, indicating validity of the potato phosphatase on sugar phosphate. The purified cathepsin D (250 µg) was incubated with 0.5 unit of potato acid phosphatase in 100 mM sodium citrate buffer, pH 4.8, at 37°C for 24 h.

For treatment with endoglycosidase H, 0.02 unit of the endoglycosidase H was added to 250 µg of cathepsin D in 20 mM potassium phosphate buffer, pH 6.0, in a small dialysis bag. Incubation was done at 37°C for 24 h with concomitant dialysis against the same buffer. The reaction mixture in the bag was then dialyzed against deionized water at 4°C.

Neuraminidase treatment was carried out according to the method already reported (7, 8, 10). The purified cathepsin D (250 µg) was incubated with 0.1 unit of Arthrobacter neuraminidase in 50 mM sodium citrate buffer pH 5.0, at 37°C for 24 h.

The hydrolyase-treated cathepsin D was subjected to isoelectric focusing on polyacrylamide slab gel.

Assay of Manα6-P. Manα6-P was estimated enzymatically according to the method of Gewehr (18) and Askin and Kopepe (19). Cathepsin D (270 µg from an individual normal liver; 195 µg from an individual hepatoma) was hydrolyzed in 400 µl of 2 M trifluoroacetic acid at 100°C for 6 h. After evaporation to dryness, the hydrolysate was dissolved in 7 ml of 50 mM Tris-HCl buffer, pH 7.6, containing 3 mM MgCl₂, and then centrifuged.

An aliquot (3.2 ml) of the clear supernatant was put in both sample and reference cuvets. To the cuvets were added sequentially, 30 µl of 5 mM NADP, 30 µl of glucose-6-phosphate dehydrogenase (140 units/ml), and 15 µl of phosphoglucose isomerase (1000 units/ml). Fluorescence measurement was performed at 25°C using a Shimadzu RF-503 difference spectrophotometer equipped with a home-made mixing device with a temperature-controlled circulating bath as previously described (20, 21). The fluorescence was excited at 340 nm, and the light emitted was detected at 455 nm. The reaction was started by the addition of 15 µl of phosphomannose isomerase (300 units/ml) to the sample cuvet and 15 µl of water to the reference cuvet, and the change in the fluorescence was recorded. The change became constant within 40 min of incubation time. Manα6-P was used as a standard.

Galactosylation of Cathepsin D. Galactosylation of cathepsin D was carried out according to the method of Brohe et al. (22) to test if a phosphodiesterase (Manα6-P-GlcNAc) is present in the cathepsin D. Cathepsin D (1 nmol) from normal human liver or hepatoma was incubated in 100 µl of 50 mM Tris-HCl buffer, pH 7.4, containing 50 mM MnCl₂, 100 µM UDP[3H]galactose (100,000 dpm), and 0.005 unit of galactosyltransferase at 37°C for 1 h. The reaction was stopped by adding 150 µl of 0.1 N EDTA. Five hundred µl of 15% trichloroacetic acid were then added to precipitate the proteins. The precipitate was washed with distilled water 4 times, dissolved in a scintillation fluid, and assayed for radioactivity in a liquid scintillation spectrometer. Ovalbumin was used instead of cathepsin D as an acceptor for a positive control.

Two Dimensional Peptide Mapping. Radioiodination and the mapping of tryptic peptides of cathepsin D (heavy chain and light chain) recovered from polyacrylamide gel were carried out by the method of Elder et al. (23) with some modifications (24). In brief, the stained protein band was cut from the sodium dodecyl sulfate-polyacrylamide electrophoresis gel. Then the polypeptide was radioiodinated with 112I by the chloramine-T method (25). The gel slice was washed, dried, and supplemented with 50 µg/ml of L-(1-lysylamine-2-phenyl-ethyl)chloromethyl ketone derivative of trypsin in 0.5 ml of 50 mM NH₄HCO₃ (pH 8.4). After incubation for 24 h at 37°C, the supernatant, which contained most of the tryptic peptides, was lyophilized. The residue was redissolved in 20 µl of acetic acid:formic acid:water (15:5:80, v/v/v). A 5- to 10-µl portion of this solution was spotted on a silica gel-coated thin-layer plate. 125I-tryptic peptides were separated by electrophoresis in the first dimension, followed by ascending chromatography in the second dimension. Electrophoresis was carried out at 1000 V for 90 min at 4°C using a Pharmacia FBE 3000 flat-bed apparatus in the above acid mixture. Ascending chromatography was performed with normal butyl alcohol:pyridine:acetic acid:water (32:25:5:20, v/v/v). The dried plate was exposed to Fuji RX X-ray film for 7 days at −80°C.

Amino Acid Analysis. Amino acid analysis was carried out on acid hydrolysates (6 N HCl for 24 h and 48 h at 110°C) by a one-column procedure using a Nihondenshi (JOEL) Model JLC-6AH amino acid autoanalyzer.

RESULTS

Activities of Cathepsin D in Hepatoma and Normal Liver. The activity levels of the cathepsin D in hepatoma were significantly higher than those in normal human liver, although the activities of individual hepatoma tissues encompassed a somewhat wider range. The results of the statistical analysis are summarized in Table 1.

Purification of Cathepsin D from Hepatoma and Normal Liver. Cathepsin D could be easily purified with high yields mainly...

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Table 1. Statistical analysis of cathepsin D activity in human liver and hepatoma.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Normal Liver</th>
<th>Hepatoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity (units/mg protein)</td>
<td>0.0757 ± 0.0032</td>
<td>0.1459 ± 0.0210</td>
</tr>
</tbody>
</table>

* Mean ± SE.
* Numbers in parentheses, number of individual subjects.

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through the combination of acetone fractionation and affinity chromatography on pepstatin-AH-Sepharose. The enzyme was purified about 150-fold from human hepatoma tissues (specific activity, 32.3 units/mg protein), and the overall yield was about 30% (Table 2). The enzyme was also purified about 900-fold from normal human liver tissues (specific activity, 38.5 units/mg protein), and the overall yield was about 40% (Table 3).

Each enzyme preparation obtained exhibited two major bands: the heavy chain ($M_r$, 29,000) and the light chain ($M_r$, 14,000) of cathepsin D, on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 1). The same result was obtained when treatment with mercaptoethanol at 100°C for 5 min was omitted. The gel permeation method with Sephadex G-100 showed the enzyme to have a molecular weight of 42,000, and all the enzyme protein comigrated with cathepsin D activity (data not shown). From these results, the linkage between the heavy chain and the light chain appears to be noncovalent. The molecular weights of the heavy chain and the light chain were almost identical in cathepsin D of normal human liver and of hepatoma.

Immunological Analysis. The antigenicity of the enzyme was examined by double immunodiffusion. The enzyme from hepatoma exhibited the same antigenicity as that from normal liver (Fig. 2).

Charge Heterogeneity of Hepatoma Cathepsin D. Isoelectric focusing on polyacrylamide gel showed that normal liver cathepsin D was resolved into three major forms with isoelectric points (pis) of 5.6, 6.1, and 6.7 (Fig. 3, Lane 1). The precise pl values of the separated forms were determined by column isoelectric focusing using an LKB column (100 ml) and carrier ampholites (pH 3.5 to 10) as described by Vesterberg and Svensson (26). These three forms of cathepsin D in normal human liver were already observed by Barret (3) and were referred to as forms $\alpha$, $\beta$, and $\gamma$ in order of increasing pl. This was the case on cathepsin D purified from normal livers of six individuals. However, cathepsin D from hepatoma had another five to six more acidic forms ranging from pl 4.4 to 5.3, in addition to the forms in normal liver (Fig. 3, Lane 2). Although these acidic forms could be observed only slightly in cathepsin D from normal liver, they were detected apparently in the enzyme preparations from three hepatomas.

We purified cathepsin D from four individual hepatomas. Of these, one tumor had a prominent, and two tumors had moderate, charge heterogeneity. Only one tumor showed very little heterogeneity as was observed in normal human liver.

Effects of Exogenous Hydrolases on Cathepsin D from Hepatoma. In order to examine the cause of the appearance of the acidic forms, the cathepsin D from hepatoma was treated with exogenously added acid phosphatase, endoglycosidase H, or neuraminidase, followed by examination with isoelectric focusing (Fig. 4). Upon treatment with endoglycosidase H, which cleaves carbohydrate chains at the chitobiose unit of most glycopeptides with high mannose-type structures, acidic forms

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**Table 2** Purification of cathepsin D from hepatoma

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total enzyme activity (units)</th>
<th>Specific activity (units/mg protein)</th>
<th>Yield (%)</th>
<th>Fold (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Homogenate</td>
<td>33.670</td>
<td>7,160</td>
<td>0.213</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. 60% (NH$_4$)$_2$SO$_4$</td>
<td>8,640</td>
<td>2,520</td>
<td>0.292</td>
<td>35</td>
<td>1.37</td>
</tr>
<tr>
<td>3. 50% Acetone</td>
<td>3,340</td>
<td>2,920</td>
<td>0.872</td>
<td>41</td>
<td>4.09</td>
</tr>
<tr>
<td>4. Pepstatin-AH-Sepharose 4B</td>
<td>79.8</td>
<td>2,170</td>
<td>27.2</td>
<td>30</td>
<td>128</td>
</tr>
<tr>
<td>5. Sephadex G-100</td>
<td>61.5</td>
<td>1,989</td>
<td>32.3</td>
<td>28</td>
<td>152</td>
</tr>
</tbody>
</table>

**Table 3** Purification of cathepsin D from normal human liver

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total enzyme activity (units)</th>
<th>Specific activity (units/mg protein)</th>
<th>Yield (%)</th>
<th>Fold (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Homogenate</td>
<td>65,520</td>
<td>2,880</td>
<td>0.044</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. 60% (NH$_4$)$_2$SO$_4$</td>
<td>23,280</td>
<td>1,300</td>
<td>0.056</td>
<td>45</td>
<td>1.27</td>
</tr>
<tr>
<td>3. 50% Acetone</td>
<td>5,380</td>
<td>1,280</td>
<td>0.237</td>
<td>44</td>
<td>5.39</td>
</tr>
<tr>
<td>4. Pepstatin-AH-Sepharose 4B</td>
<td>35.8</td>
<td>1,450</td>
<td>40.6</td>
<td>50</td>
<td>923</td>
</tr>
<tr>
<td>5. Sephadex G-100</td>
<td>32.9</td>
<td>1,270</td>
<td>38.5</td>
<td>44</td>
<td>875</td>
</tr>
</tbody>
</table>

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Fig. 1. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Lane 1, molecular weight markers (Bio-Rad; 2 μl of 1:10 dilution): phosphorylase B ($M_r$, 92,500); bovine serum albumin ($M_r$, 66,200); ovalbumin ($M_r$, 45,000); carbonic anhydrase ($M_r$, 31,000); soybean trypsin inhibitor ($M_r$, 21,500); and lysozyme ($M_r$, 14,400). Lane 2, cathepsin D (10 μg) purified from normal human liver. Lane 3, cathepsin D (10 μg) purified from hepatoma. The gel was visualized with silver staining.

Fig. 2. Immunodiffusion of cathepsin D from normal human liver and hepatoma. Well A, rabbit anti-normal human liver cathepsin D IgG; Well B, normal liver enzyme; Well C, hepatoma enzyme.
processing including phosphorylation at nonreducing terminal mannose residues of the sugar chains bound to the enzymes (27), it seems likely that the appearance of acidic variant forms found in the hepatoma enzyme is attributable to an increased phosphorylation at the sugar chains of the enzyme. To examine this possibility, Man6-P content was enzymatically determined on cathepsin D from hepatoma and normal liver. The content of Man6-P in cathepsin D from normal liver was found to be 0.08 mol/mol of cathepsin D. This value was almost the same as that reported for porcine spleen cathepsin D (28). In contrast, the Man-6-P content in cathepsin D from hepatoma was found to be 0.17, twice as much as that in cathepsin D from normal liver.

It was examined whether GlcNAc of a phosphodiester present in carbohydrate moiety of cathepsin D is galactosylated by a milk β-galactosyltransferase. However, neither cathepsin D from normal liver nor that from hepatoma was galactosylated at all by milk β-galactosyltransferase under the conditions in which ovalbumin was fairly galactosylated.

Peptide Mapping. The autoradiograms of peptides from cathepsin D are shown in Fig. 5. Eight to ten major spots were detected in the maps of heavy chains of cathepsin D from normal liver and hepatoma, whereas only four to five spots were seen in the maps of light chains from the two sources. Although overall features of the maps of the two sources are very similar, additional spots (indicated by arrows) were seen on the heavy chain of hepatoma cathepsin D.

Amino Acid Composition. The results of amino acid analysis of the normal liver and hepatoma cathepsin D are shown in Table 4. Essentially, no difference was found between the enzymes in normal liver and hepatoma.

**DISCUSSION**

In the present study, we demonstrated significantly elevated activity of cathepsin D in human hepatoma tissues as compared to liver tissues free from pathological changes. This elevation of the activity is most probably due to increased amounts of enzyme protein in hepatoma, but not to more active enzyme, because the specific activity of the purified enzyme from normal liver and hepatoma was almost the same. Elevated activity of cathepsin D has been also demonstrated in human ovarian carcinoma (5).

In isoelectric focusing experiments, cathepsin D purified from normal human liver exhibited three different forms with pl values of 5.6, 6.1, and 6.7. Similar observations were made by Barret (3), who named these forms α (pl 5.7), β (pl 6.0), and γ (pl 6.5). In contrast, the enzyme purified from hepatoma tissues was found to contain a number of acidic variant forms with pl values ranging from 4.4 to 5.3. As these acidic forms increase, the basic pl forms (5.6, 6.1, and 6.7) decrease, as compared to the more basic pl forms in normal liver.

Amino acid analysis did not show any apparent differences between cathepsin D from hepatoma and that from normal human liver. Therefore, it is conceivable that there are few, if any, tumor-associated changes in the protein moiety of the enzyme.

Although such charge heterogeneity in cathepsin D has not been noted in other human tissues, other lysosomal enzymes such as arylsulfatase B (6, 7), arylsulfatase A (8), and β-glucuronidase (10) have been shown to contain anionic variants in human neoplastic tissues.

Most lysosomal enzymes, including cathepsin D, are glycoproteins which contain asparagine-linked oligosaccharide
CATHEPSIN D IN HUMAN HEPATOMA

Fig. 5. Autoradiograms of tryptic peptide maps. The heavy chain and light chain of cathepsin D were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and cut from the gel. They were treated as described in "Materials and Methods." A, heavy chain from normal human liver enzyme; B, heavy chain from hepatoma enzyme; C, light chain from normal human liver enzyme; D, light chain from hepatoma enzyme. There are extra spots (arrows) on heavy chain from hepatoma.

Table 4: Amino acid composition of cathepsin D

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>Normal liver</th>
<th>Hepatoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>32.6</td>
<td>32.7</td>
</tr>
<tr>
<td>Thr</td>
<td>20.6</td>
<td>18.4</td>
</tr>
<tr>
<td>Ser</td>
<td>22.5</td>
<td>19.2</td>
</tr>
<tr>
<td>Glu</td>
<td>34.3</td>
<td>35.9</td>
</tr>
<tr>
<td>Gly</td>
<td>31.5</td>
<td>32.2</td>
</tr>
<tr>
<td>Ala</td>
<td>15.1</td>
<td>15.5</td>
</tr>
<tr>
<td>Val</td>
<td>28.0</td>
<td>30.6</td>
</tr>
<tr>
<td>Met</td>
<td>8.2</td>
<td>6.9</td>
</tr>
<tr>
<td>Ile</td>
<td>22.2</td>
<td>22.6</td>
</tr>
<tr>
<td>Leu</td>
<td>32.8</td>
<td>31.8</td>
</tr>
<tr>
<td>Tyr</td>
<td>17.2</td>
<td>18.5</td>
</tr>
<tr>
<td>Phe</td>
<td>14.6</td>
<td>14.7</td>
</tr>
<tr>
<td>Lys</td>
<td>21.1</td>
<td>20.8</td>
</tr>
<tr>
<td>His</td>
<td>4.7</td>
<td>4.2</td>
</tr>
<tr>
<td>Arg</td>
<td>9.9</td>
<td>10.2</td>
</tr>
</tbody>
</table>

phosphate has been indicated on the nonreducing terminal of the oligosaccharide chain, not only in the form of phosphomonoester (Man6-P), but also as phosphodiester (Man6-P-GlcNAc) (31).

Determinations of Man6-P revealed that cathepsin D from hepatoma contains more Man6-P than that from normal human liver. However, the contents were fairly low. This is supposed to be due to phosphate cleavage from cathepsin D during the purification, since phosphatase inhibitor was not included, and to the loss during the hydrolysis procedure with trifluoroacetic acid. The true values must be more than the values measured. The peptide mapping showed that the heavy chain of hepatoma cathepsin D afforded extra peptides, which may represent peptides containing phosphorylated oligosaccharides. According to the resistance to the acid phosphatase treatment, the Man6-P is presumed to be present in the form of phosphodiester, which is believed to be an intermediate form in the processing of cathepsin D. An oligosaccharide chain which contains an extra GlcNAc has been observed in cathepsin D from porcine spleen, although this chain was only 5% of the total content (29). An attempt was unsuccessful to demonstrate the presence of the phosphodiester by galactosylation of cathepsin D. Shanbacher et al. (32) observed that UDP-GlcNAc is a poor acceptor for β-galactosyltransferase. Since UDP-GlcNAc and Man6-P-GlcNAc are similar in having GlcNAc linked to phosphodiester, Man6-P-GlcNAc will be a poor acceptor. Therefore, the result of the galactosylation experiment does not mean to deny the presence of phosphodiester in hepatoma cathepsin D. It remains to study further whether an increase of the phosphodiester in hepatoma cathepsin D is due to decreased activity of α-N-acetylgalcosaminyl phosphodiesterase (33, 34) or elevated activity of N-acetylgalcosamine phosphotransferase (35). In any event, the increase of the acidic variant forms of cathepsin D may represent a tumor-associated impairment of the processing of the enzyme.
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REFERENCES

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