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# PURIFICATION AND CHARACTERIZATION OF A NEW THIOL PROTEINASE FROM RAT KIDNEY

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## Summary

The levels of activity of a new proteinase, termed cathepsin T, in rat tissues were examined This enzyme had been previously reported to exist in rat liver on the basis of its catalysis of the conversion of multiple forms of tyrosine aminotransferase (L-tyrosine 2-oxoglutarate aminotransferase, EC 2.6.1.5). Kidney was found to be the richest source of cathepsin T activity, exhibiting about 10-times as great a specific activity as found in liver. The order of cathepsin T activity in tissues was kidney >> spleen > liver > small intestine > lung. The proteinase activity was not detectable in heart, skeletal muscle, brain and blood Kidney cathepsin T was purified about 1400-fold to homogeneity with a 20% yield by the purification procedure similar to that used for the liver enzyme (Gohda, E. and Pitot, H.C. (1980) J Biol. Chem 255, 7371—7379) Cathepsin T purified from rat kidney was found to be a glycoprotein, the molecular weight of which was between 33 500 and 35 000. Purified kidney cathepsin T converted Form I of tyrosine aminotransferase in the same way as the liver proteinase, with concomitant conversion of 52 500 dalton subunits of the aminotransferase to 48 000 dalton subunits. Kidney cathepsin T showed the same specific activities toward Form I of tyrosine aminotransferase, casein and acid-denatured hemoglobin as did the liver form of the enzyme. Many other characteristics common to the proteinases purified both from rat kidney and liver were found. We have concluded that kidney cathepsin T is the same enzyme as the liver proteinase

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Abbreviations SDS, sodium dodecyl sulfate, Mops, 3-(*N*-morpholino)propanesulfonic acid, Mes, 2-(*N*-morpholino)ethanesulfonic acid

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## Introduction

Previous work [1,2] from this laboratory has demonstrated that the purified convertase, which catalyzed the conversion of multiple forms of tyrosine aminotransferase (D-tyrosine: 2-oxoglutarate aminotransferase, EC 2.6.1.5), was a new endopeptidase in rat liver lysosomes. The convertase is a sulfhydryl enzyme having a molecular weight of 33 500—35 000. This proteinase showed potent azocaseinolytic activity 6-times higher than cathepsin L (EC 3.4.22.15), a thiol endopeptidase in rat liver lysosomes. This new endopeptidase has been named cathepsin T [2].

During examination of the tissue distribution of cathepsin T activity in rat tissues, we found that kidney contained a much higher specific activity of the proteinase than liver. Since only a small amount of the purified cathepsin T was obtained from rat liver [1], the presence of greater amounts of the enzyme in kidney provided an opportunity to obtain larger amounts of the enzyme for the further investigation of the properties and function of the protein. However, there was the possibility that the kidney convertase is an enzyme different from liver cathepsin T. This report describes purification and characterization of cathepsin T from rat kidney. No difference in the properties and characteristics of the proteinases from rat liver and kidney was observed.

## Materials and Methods

*Materials* Crystalline bovine serum albumin, bovine hemoglobin (type II), a set of molecular weight marker proteins for SDS-polyacrylamide gel electrophoresis, Mops and Mes were obtained from Sigma. Casein (Hammersten grade) was purchased from Fluka. Hydroxyapatite, SDS, acrylamide and other electrophoretic reagents were products of Bio-Rad. DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were purchased from Whatman; Sephadex G-75 (superfine) from Pharmacia. Other compounds were obtained from commercial sources.

*Animals* Albino male Holtzman rats (Madison, WI), weighing 200—250 g, were used in all experiments.

*Separation of multiple forms of tyrosine aminotransferase by hydroxyapatite chromatography.* Hydroxyapatite chromatography of the multiple forms of tyrosine aminotransferase using a linear phosphate gradient or a stepwise elution was carried out as described previously [1] at 4°C except that 0.5 M potassium phosphate buffer, pH 6.9, instead of 0.32 M buffer was used for the elution of Form III of the enzyme in the stepwise elution procedure.

*Purification and assay of tyrosine aminotransferase* Purified and partially purified Form I of tyrosine aminotransferase was prepared from rat liver as described previously [1]. The specific activities of the preparations were 729 and 8.82 units/mg protein, respectively. Tyrosine aminotransferase activity was assayed by the modification of Diamondstone's method [3] described by Iwasaki and Pitot [4]. 1 unit of activity is defined as that amount of enzyme forming 1 $\mu$mol of *p*-hydroxyphenylpyruvate per min at 37°C. A value of 19 900 $M^{-1} \cdot cm^{-1}$ was used for the molar extinction coefficient of *p*-hydroxyphenylpyruvate [3].

*Purification of cathepsin T from rat kidney* Cathepsin T from rat kidney

was purified by essentially the same method as that described previously for liver enzyme [1] with the following modifications. All steps, unless otherwise stated, were carried out at 4°C. (a) The kidneys from 100 male rats which had been fasted overnight were rapidly removed, washed thoroughly with 0.25 M sucrose, and homogenized in 4 vol. of 50 mM potassium phosphate buffer, pH 6.5, containing 12.5% glycerol and 1 mM dithiothreitol (buffer A) by means of a Waring blendor for six 30 s periods. To the homogenate was added solid KCl to a final concentration of 0.3 M and the mixture was further stirred for 15 h. It was then centrifuged at 105 000 × *g* for 60 min, and the supernatant was saved for the next step of acetone fractionation. (b) The supernatant from the step of acid precipitation was diluted with 1.6 vol. of 10 mM potassium acetate/50 mM potassium phosphate buffer, pH 4.0, containing 12.5% glycerol and 1 mM dithiothreitol (buffer B). Diluted enzyme solution was applied to a CM-cellulose column (3.4 × 24 cm) equilibrated with buffer B containing 0.115 M KCl at a flow rate of 2.5 ml/min. After washing with 250 ml of the same buffer, the column was developed with a linear salt gradient (550 ml of buffer B containing 0.115 M KCl and 550 ml of buffer B containing 0.75 M KCl). The flow rate was 2.5 ml/min and 15-ml fractions were collected. A single peak of cathepsin T activity was eluted in the range of 0.16 M and 0.32 M KCl concentration. About 15 fractions with activity higher than 9.0 units/ml were pooled for the next step (c) The pooled active fractions (24 ml) from the step of Sephadex G-75 gel filtration were diluted with 0.5 vol. of 12.5% glycerol containing 1 mM dithiothreitol, and applied to a DEAE-cellulose column (1 × 18 cm) equilibrated with buffer A. The column was washed with 50 ml of the same buffer. The enzyme was then eluted with a linear gradient of 0—0.3 M KCl in buffer A (total gradient volume, 200 ml) at a flow rate of 0.4 ml/min and 2.5-ml fractions were collected The elution profile on a DEAE-cellulose column is shown in Fig. 1. Fractions 22 to 32 were collected, concentrated by ultrafiltration using a Diaflo PM10 membrane, and dialyzed for 3 h against buffer A containing 75 mM KCl. Dialyzed enzyme solution was stored at −70°C.

*Assay of cathepsin T activity* Cathepsin T activity was determined by mea-

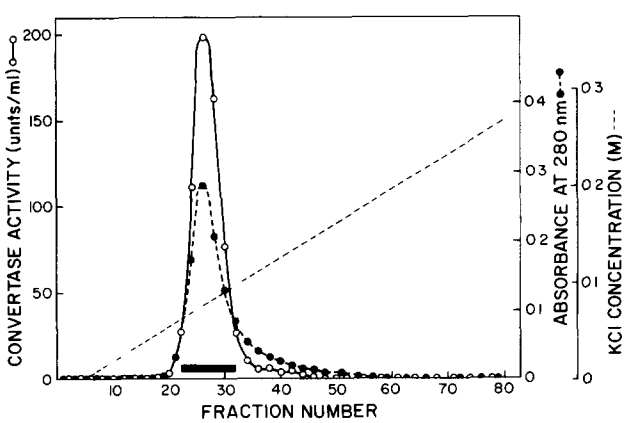


Fig 1 Chromatography of kidney cathepsin T on a DEAE-cellulose column The solid bar represents fractions pooled for further study

suring the formation of Form III from Form I of tyrosine aminotransferase as described previously [1]. For the assay of cathepsin T activity during purification or in crude preparations, partially purified Form I of tyrosine aminotransferase (1.27 mg, 11.2 units) was used. 1 unit of cathepsin T activity is defined as that amount of the enzyme forming 1 unit of Form III from partially purified Form I of tyrosine aminotransferase per min at 0°C. The activity of purified cathepsin T was also assayed using purified Form I of tyrosine aminotransferase (3.75 μg, 2.73 units). In both systems, no conversion of Form I of the aminotransferase to other forms was observed during incubation in the absence of a cathepsin T preparation. All assays were controlled by heating the reaction mixture for 10 min at 65°C immediately after adding a proteinase preparation and albumin solution.

*Gel electrophoresis.* Polyacrylamide gel electrophoresis in the presence and absence of SDS was accomplished by the method of Weber et al. [5] and a modification [6] of the method of Davis [7], respectively, as described previously [1]. Gels were stained for protein with Coomassie brilliant blue and for carbohydrate by the periodic acid-Schiff procedure described by Zacharius et al. [8].

*Molecular weight determinations* Molecular weight of proteins was estimated by gel filtration [9] and SDS-polyacrylamide gel electrophoresis [5] as described previously [1].

*Assay of proteolysis of casein and acid-denatured hemoglobin by purified cathepsin T* Casein or acid-denatured hemoglobin [10] was incubated at a 1% (w/v) concentration with purified cathepsin T in 300 μl of 50 mM Mes buffer (pH 6.5 for casein and pH 5.8 for acid-denatured hemoglobin) for 10 min at 37°C After termination of the reaction with 0.9 ml of 6.67% trichloroacetic acid and filtration of the mixture at room temperature, the clear filtrate was used for determination of released peptides by the method of Lowry et al [11] with slight modifications [12]. The enzyme activity was expressed as mg tyrosine released during 10 min incubation.

*Protein determinations* Protein was measured by the method of Lowry et al. [11] using bovine serum albumin as standard. For protein concentrations lower than 200 μg/ml, the method of Bensadoun and Weinstein was used [13].

## Results

*Tissue distribution of cathepsin T activity in rats* Since only a small amount of purified cathepsin T was obtained from rat liver [1], the tissue distribution of the proteinase was examined in an attempt to identify richer sources of the enzyme. As shown in Table I, kidney contained a great amount of the enzyme activity. The specific activity in kidney was about 10 times higher than that in rat liver and the total activity of the enzyme in kidney was greater than that in liver. Spleen showed a slightly higher specific activity of cathepsin T than liver. The concentration of the enzyme's activity in small intestine and lung was the same as or lower than that in liver. Convertase activity was not detectable in heart, skeletal muscle, brain, and blood.

*Purification of cathepsin T from rat kidney.* Since kidney contained a much higher concentration of cathepsin T activity than liver, we attempted to purify

TABLE I

DISTRIBUTION OF CATHEPSIN T ACTIVITIES IN DIFFERENT RAT TISSUES

Rats were fed ad libitum on laboratory chow before all experiments Rat tissues were homogenized in 7 vol of 63 5 mM potassium phosphate buffer (pH 6 0), containing 15 9% glycerol and 1 27 mM dithiothreitol with Polytron for 1 min at maximum intensity and 0°C After addition of 0 1 vol of 5% Triton X-100 containing 3 M KCl, homogenates were kept at 0°C for 3—5 h with occasional shaking Aliquots (less than 0 30 mg of protein) were used for the assay of cathepsin T activity employing partially purified Form I of tyrosine aminotransferase as substrate Values are expressed as mean ± S D for six rats Activity was not detected in heart, skeletal muscle, brain or blood samples

| Tissue | Cathepsin T activity | |
|---|---|---|
| | (units/g tissue) | (munits/mg protein) |
| Liver | 7 94 ± 0 44 | 49 1 ± 1 6 |
| Kidney | 77 2 ± 10 9 | 526 ± 76 |
| Spleen | 9 95 ± 1 75 | 68 8 ± 11 1 |
| Lung | 2 46 ± 0 31 | 21 0 ± 3 0 |
| Small intestine | 3 96 ± 0 16 | 50 0 ± 2 7 |

the enzyme from rat kidney according to the method described in Materials and Methods. Table II shows a typical result of purification. Starting with 166 g of kidney, we obtained 3.4 mg of the enzyme, representing a 1420-fold purification and a 19.9% yield.

*Properties and characteristics of cathepsin T purified from rat kidney*

*(a) Polyacrylamide gel electrophoresis.* The final enzyme preparation gave a single protein band stained with Coomassie blue after electrophoresis of the denatured enzyme in polyacrylamide gels containing 0.1% SDS (Fig. 2A). This band was stained by the periodic acid-Schiff procedure (Fig. 2B), indicating the presence of carbohydrates. A single major protein band was also obtained after electrophoresis in polyacrylamide gels at pH 8.3; it also gave a positive periodic acid-Schiff stain (Fig. 2C and D). In this case, however, care had to be taken to prepare the samples, otherwise, the original band was preceded by at least one

TABLE II

PURIFICATION OF CATHEPSIN T FROM RAT KIDNEY

The data presented are the results of a typical experiment starting with 166 g of rat kidney

| Fraction | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Yield (%) | Purification (-fold) |
|---|---|---|---|---|---|
| Homogenate | 24 300 | 11 700 | 0 481 | 100 | 1 00 |
| Kidney extract | 11 800 | 10 300 | 0.873 | 88 0 | 1 81 |
| Acetone, 45% precipitate | 3320 | 8500 | 2 56 | 72 6 | 5 32 |
| Acid supernatant | 1280 | 6430 | 5 02 | 55 0 | 10 4 |
| CM-cellulose | 151 | 4250 | 28 1 | 36 3 | 58 4 |
| Hydroxyapatite | 12 4 | 3300 | 266 | 28 2 | 553 |
| Sephadex G-75 | 4 00 | 2880 | 720 | 24 6 | 1497 |
| DEAE-cellulose | 3.40 | 2330 | 685 | 19 9 | 1420 |

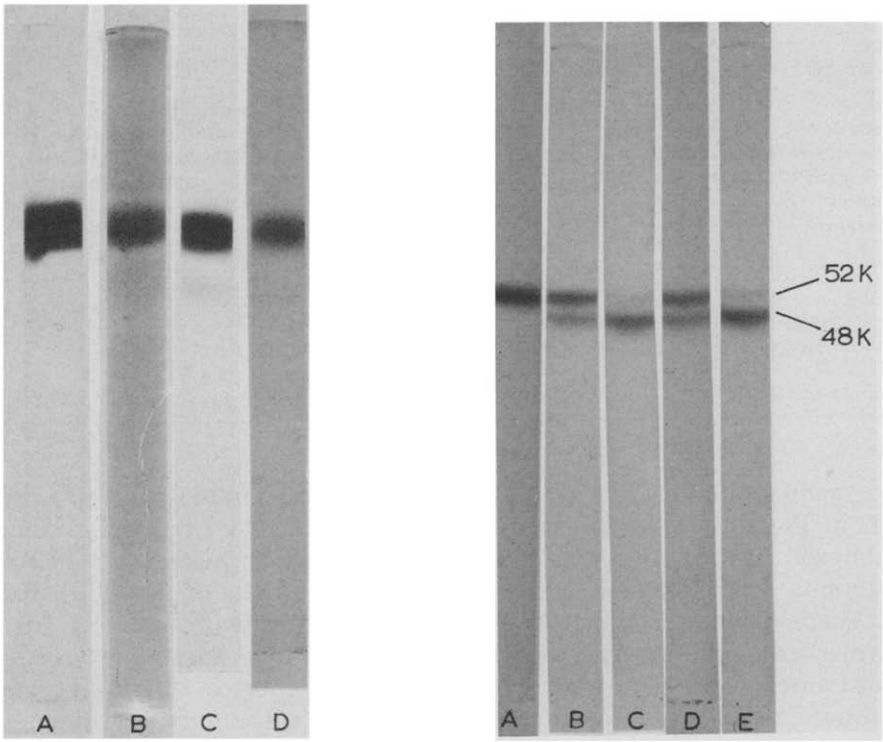


Fig 2 Polyacrylamide gel electrophoresis of cathepsin T purified from rat kidney In gels A and B, purified cathepsin T (30 μg of protein) was electrophoresed on a 12 5% polyacrylamide gel in the presence of 0 1% SDS In gels C and D, purified cathepsin T (20 μg of protein) was electrophoresed at pH 8 3 and 0°C on a polyacrylamide gel Migration was from the cathode at the top of the gels to the anode at the bottom. Gels were stained for protein with Coomassie blue (gels A and C) and for carbohydrate by the periodic acid-Schiff procedure (gels B and D)

Fig 3 The conversion of 52 000 dalton subunits of tyrosine aminotransferase to 48 000 dalton subunits by cathepsin T purified from rat liver or kidney Purified Form I of tyrosine aminotransferase (15 0 μg, 10 9 units) was incubated with cathepsin T (16 7 ng) purified from rat liver or kidney in 100 μl of 50 mM Mops buffer, pH 7 0, containing 2 mM dithiothreitol, at 0°C At 5 and 30 min of incubation, 20 μl of the incubation mixture was taken, processed, and electrophoresed on a 7 5% polyacrylamide gel in the presence of 0.1% SDS A, no cathepsin T, B, liver cathepsin T, 5 min, C, liver cathepsin T, 30 min, D, kidney cathepsin T, 5 min, E, kidney cathepsin T, 30 min

more band, as described previously in enzyme preparations from rat liver [1]. Purified kidney cathepsin T showed the same mobility as the liver enzyme on these polyacrylamide gels in the presence and absence of SDS.

*(b) Molecular weight.* The molecular weight of the proteinase was estimated by SDS-polyacrylamide gel electrophoresis and by Sephadex G-75 gel filtration On 12.5% polyacrylamide gels in SDS, the molecular weight of kidney cathepsin T was calculated as 35 000. The proteinase purified from rat kidney emerged at the same position as the enzyme from rat liver on Sephadex G-75 gel filtration, corresponding to a molecular weight of 33 500.

*(c) Comparison of properties of cathepsin T purified from rat liver and kidney* Kidney cathepsin T converted Form I of tyrosine aminotransferase to Forms II and III as the liver enzyme [1], when judged by hydroxyapatite

TABLE III

COMPARISON BETWEEN SPECIFIC ACTIVITIES OF PURIFIED CATHEPSIN T FROM RAT LIVER AND KIDNEY AGAINST VARIOUS PROTEIN SUBSTRATES

DEAE-cellulose fraction of cathepsin T purified from rat liver or kidney was incubated with the substrate indicated The specific activity is expressed in units/mg protein for Form I of tyrosine aminotransferase and mg tyrosine released/10 min per mg protein for the other two substrates The values are the mean ± S D of three purified enzyme preparations Partially purified Form I of tyrosine aminotransferase from rat liver was used

| Substrate | Specific activity of purified cathepsin T | |
|---|---|---|
| | Liver | Kidney |
| Tyrosine aminotransferase, Form I | 686 ± 35 | 680 ± 56 |
| Casein | 23 2 ± 1 7 | 22 4 ± 2 4 |
| Acid-denatured hemoglobin | 6 87 ± 0 62 | 6 64 ± 0 25 |

chromatography employing linear phosphate gradient elution (data not shown) As shown in Fig 3, this conversion of the multiple forms of the aminotransferase catalyzed by both enzymes was accompanied by an increase of 48 000 dalton subunits of the enzyme with a concomitant decrease of 52 000 dalton subunits. There was no difference between the specific activities of both purified enzymes as measured by the conversion of multiple forms of tyrosine aminotransferase (Table III). Liver cathepsin T was reported to have potent azocaseinolytic activity [1]. As presented in Table III, the kidney proteinase demonstrated the same specific activity as the liver enzyme in the hydrolysis of casein and acid-denatured hemoglobin. Conversion of multiple forms of tyrosine aminotransferase by both enzymes was strongly inhibited by the presence of oxidized ribonuclease A (data not shown), which had been shown to serve as substrate of the proteinase from rat liver [2] Activity of the kidney enzyme was enhanced by sulfhydryl compounds and inhibited by sulfhydryl reactive reagents (not shown) as with the liver proteinase [2].

## Discussion

The purification procedure of kidney cathepsin T described in this report was less complicated than that of the liver enzyme described previously [1] The step of separation of a mitochondrial-lysosomal fraction was not necessary. This step lost 40% of the convertase activity during the purification of cathepsin T from rat liver. The recovery of the proteinase from rat kidney was increased compared with that of the liver enzyme [1].

Lysosomal proteinases in rat kidney have not been well characterized. Recently, Strewler and Manganiello [14] have purified and characterized a thiol proteinase from the lysosomal fraction of rat kidney cortex, which activated cyclic nucleotide phosphodiesterase. The properties of this proteinase closely resembled those of cathepsin L from rat liver. They have also reported that rat kidney cortex contained lysosomal proteinases analogous to cathepsins $B_1$ and D of other rat tissues in addition to the cathepsin L-like proteinase. In this report, we demonstrated that rat kidney also contained large amounts of the proteinase which catalyzed the conversion of multiple forms of tyrosine

aminotransferase. Several properties and characteristics of this proteinase purified from rat kidney are consistent with the interpretation that the proteinase is the same enzyme as cathepsin T purified from rat liver [1]. (1) Both enzymes were purified to homogeneity by almost the same purification procedures. (2) Both proteinases were not able to be separated from each other on polyacrylamide gel electrophoresis in the presence or absence of SDS. (3) The molecular weights of both proteinases were 33 500—35 000. (4) Both proteinases converted Form I of tyrosine aminotransferase to Forms II and III as judged by hydroxyapatite chromatography. Both enzymes catalyzed the conversion of 52 000 dalton subunits to 48 000 dalton subunits of tyrosine aminotransferase (Fig 3). (5) No differences were observed between the specific activities of both proteinases toward various protein substrates, including Form I of tyrosine aminotransferase (Table III). (6) The conversion of multiple forms of tyrosine aminotransferase by both enzymes was strongly inhibited by oxidized ribonuclease A. (7) Activities of both enzymes were enhanced by sulfhydryl compounds and inhibited by sulfhydryl reactive reagents. Typical of many other lysosomal proteins and enzymes, cathepsin T was a glycoprotein, as judged by periodic acid-Schiff staining for carbohydrate (Fig. 2).

The specific activity of cathepsin T in rat kidney was 10 times higher than that in rat liver (Table I). Since the assay of cathepsin T activity in rat tissues was performed using a tissue homogenate as a proteinase preparation, the possibility is not ruled out that proteinases other than cathepsin T are also responsible for the converting activity of tyrosine aminotransferase forms. In fact, as discussed previously [1], limited proteolysis of Form I of tyrosine aminotransferase by trypsin or chymotrypsin partially resembled the action of cathepsin T. However, any peaks of converting activity other than that of cathepsin T were not observed, using several methods of chromatography during purification of the enzymes from rat kidney and liver, and yield of kidney cathepsin T was relatively high. Therefore, the assay method used in this study employing Form I of tyrosine aminotransferase as substrate is likely to be specific for cathepsin T at least in rat liver and kidney.

Our results on the tissue distribution of cathepsin T activity were somewhat different from those of Boctor and Grossman [15]. They observed converting factor activity, equivalent to that in liver, in particulate fractions from rat kidney and no detectable activity in small intestine and lung They used particulate fractions from tissues as a preparation of converting factor, whereas we used tissue homogenates. In addition, the methods of assay for converting factor activity employed were different in their study from those reported herein. CM-Sephadex was used for the separation of the multiple forms of tyrosine aminotransferase in their study [16]. The activity of the converting factor was expressed as a decrease of substrate concentration, Form I of tyrosine aminotransferase [16], thus reducing the sensitivity of the assay method.

Lenney et al. [17] have demonstrated that among rat tissues, kidney, liver and spleen were by far the richest source of proteolytic activity with $\alpha$-*N*-benzoyl-DL-arginine-$\beta$-napthylamide as substrate. The kidney contained about three times as much activity per g as did liver or spleen, when rat tissues were autolyzed at pH 3.8. Our determinations showed that the specific activity of $\alpha$-*N*-benzoyl-DL-arginine-$\beta$-napthylamide hydrolase in rat kidney homogenate

was about 3 times higher than that in rat liver homogenate, whereas rat kidney contained 1.5—2 times as much acid hemoglobin hydrolyzing activity per mg protein as rat liver (Gohda, E. and Pitot, H.C., unpublished data). Thus the ratio of cathepsin T activity in kidney to that in liver was higher than that of other cathepsins common to these two tissues. The high yield of cathepsin T from rat kidney, reported in this study, should be helpful in the determination of the specificity of the peptide bond cleaved and in structural studies of this new proteinase.

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