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PROPERTIES OF CATHEPSIN C FROM RAT LIVER

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SUMMARY

1. Rat liver cathepsin C (EC 3.4.4.9) was purified 1790-fold over the homogenate activity by a purification scheme that combined the techniques of centrifugation, acid precipitation, $(NH_4)_2SO_4$ precipitation, and Sephadex G-200, DEAE-cellulose, and CM-cellulose column fractionations.

2. The specific activity of the most purified CM- fraction with Gly–Tyr–NH_2 as substrate was 91.6 μmoles of hydroxamate produced per mg of protein per min, which is higher than previously reported values.

3. The K_m for Gly–Tyr–NH_2 was 6 mM when determined by hydroxamate formation, and that for Val–Leu–NH_2 was 2.5 mM.

4. The requirements for –SH compounds and for halide ions for activity were absolute. A dithioerythritol concentration of 25 mM and a Cl^- or Br^- concentration of 10 mM were required for full activation.

5. The enzyme catalyzed a hydrolysis reaction at pH 5, but the polymerization reaction catalyzed at pH 7–8 occurred at a greater rate.

6. The substrate specificity of cathepsin C was broader than that reported in older literature and the enzyme was confirmed to be a dipeptidyl aminopeptidase that acts upon a number of dipeptide amides and tripeptides. The most favorable substrates were tripeptides or dipeptide amides that have as the NH_2-terminal group a small residue and that have the aliphatic residue leucine at the penultimate position.

INTRODUCTION

Cathepsin C (EC 3.4.4.9), now known as a dipeptidyl aminopeptidase, was first described by Gutmann and Fruton[1] as the enzyme from hog kidney responsible for the deamination of Gly–Phe–NH_2. The best enzyme preparations have been obtained from beef spleen[2] and rat liver[3]. Since the preparation of the highly purified enzyme from beef spleen by Metrione *et al.*[2], the properties of cathepsin C have been increasingly explored. The enzyme has long been known to be activated by –SH compounds and to be completely inhibited by iodoacetamide or iodoacetic acid[4], yet many other specific properties of the enzyme have remained uncertain. Recently, the possible involvement of cathepsin C in the degradation of protein within lysosomes was reported[5,6]. This gives added importance to cathepsin C and to the need for

improved methods for purification of the enzyme for use in further investigations of its role in the hydrolysis of protein. This paper describes the general properties and substrate specificity of cathepsin C purified from rat liver.

MATERIALS

Gly–Tyr–NH_2 acetate was obtained from Mann Research Laboratories, Inc. All other dipeptides, dipeptide amides, and tripeptides were from either Cyclo Chemical Corp. or Fox Chemical Co.; these compounds showed single spots by thin-layer chromatography and the correct products upon hydrolysis. Sephadex G-200 was purchased from Pharmacia Fine Chemicals Inc. Reeve Angel was the source of the Whatman diethylaminoethyl cellulose and carboxymethyl cellulose. Eastman Chromagram sheets, No. 6061, were purchased from Eastman Kodak Co.

METHODS

Assay of cathepsin C

Cathepsin C activity was assayed either by the transamidation reaction described by Metrione *et al.*[2], or by the hydrolysis reaction with measurement of the release of NH_3 from dipeptide amides. For measurement of the transamidation reaction, the 0.5-ml reaction mixture consisted of 50 mM dipeptide amide, 25 mM dithioerythritol, 0.4 M hydroxylamine·HCl at pH 6.8, and diluted enzyme. After incubation for 20 min at 37 °C, the reaction was stopped by addition of 0.5 ml of 20% trichloroacetic acid. After centrifugation for 5 min, the formation of hydroxamate was measured in the decanted supernatant portion by addition of 0.5 ml of 5% $FeCl_3 \cdot 6\ H_2O$ in 0.1 M HCl and 1.0 ml of water. The absorbance at 510 nm was read within 10 min. The specific activity of the enzyme measured by this method is expressed as μmoles of hydroxamate produced per mg of protein per min. For measurement of NH_3, the 0.5-ml reaction mixture consisted of 50 mM dipeptide amide, 25 mM dithioerythritol, 10 mM NaCl, and 0.1 M sodium acetate buffer, pH 5. After incubation at 37 °C for 20 min in an NH_3 diffusion bottle (A. H. Thomas Company), the reaction was stopped by the addition of 1 ml of 0.2 M Na_2CO_3–$NaHCO_3$ buffer, pH 10. The released NH_3 was trapped by the micro-diffusion method of Seligson and Seligson[7], while the bottle was shaking at 37 °C, and was then determined colorimetrically by Nessler's method[8]. The specific activity of cathepsin C measured by this procedure is expressed as μmoles of NH_3 produced per mg of protein per min.

Determination of protein

Protein was determined by the method of Miller[9] or by a microassay based upon this method. Crystalline bovine serum albumin was used as a standard.

Preparation of mitochondrial–lysosomal fractions

Male Sprague–Dawley rats weighing 500–700 g were fasted for 24 h before decapitation. Livers were pooled from 20 to 24 rats and a 1:8 homogenate in 0.25 M sucrose was prepared and centrifuged at 1020 $\times$ g for 10 min to remove nuclear debris. The supernatant portion was centrifuged at 11700 $\times$ g for 35 min to sediment a broad mitochondrial fraction, which contained the lysosomes. This fraction was resuspended in deionized water with a Potter–Elvehjem homogenizer.

Purification of cathepsin C

After freezing and thawing 5 times, the mitochondrial–lysosomal fraction was adjusted to pH 3.5 with 1 M HCl. The precipitate was removed by centrifugation. The supernatant portion was brought to 40% saturation with solid $(NH_4)_2SO_4$. After centrifugation, the supernatant portion was increased to 70% saturation with solid $(NH_4)_2SO_4$. The final precipitate was dissolved in a minimal volume of water and dialyzed against 0.15 M NaCl to give the 40–70% $(NH_4)_2SO_4$ fraction. The dialyzed fraction, which contained 110 mg of protein, was chromatographed on a Sephadex G-200 column (2.5 cm × 100 cm) with 0.15 M NaCl. Fractions of 2.5 ml per 10 min were collected. The distribution of protein and of cathepsin C activity are shown in Fig. 1A. Fractions rich in cathepsin C activity were pooled and the protein was concentrated by $(NH_4)_2SO_4$ precipitation (80% of saturation). Salt was removed by dialysis against 5 mM sodium phosphate buffer, pH 6.8, that contained 2 mM β-mercaptoethanol. The dialyzed fraction was called the Sephadex G-200 fraction.

The Sephadex G-200 fraction (15 mg of protein) was chromatographed on a DEAE-cellulose column (1.5 cm × 30 cm) that had been treated according to the method of Sgarbieri *et al.*[10] and equilibrated with 5 mM sodium phosphate buffer, pH 6.8, that contained 2 mM β-mercaptoethanol. Protein was eluted with a linear 0–0.6 M NaCl gradient in the same buffer. Fractions of 2.8 ml per 15 min were collected. Fig. 1B shows the distribution of protein and of cathepsin C activity. The fractions rich in cathepsin C activity were pooled, concentrated by ultrafiltration (Diaflo, Amicon Corporation), and the protein was washed twice with 5 mM sodium acetate buffer, pH 5, that contained 2 mM β-mercaptoethanol (DEAE-fraction). The latter (8 mg of protein) was chromatographed on a CM-cellulose column (1.5 cm × 30 cm) that had been treated as described by Sgarbieri *et al.*[10]. Protein was eluted with a linear 0–0.6 M NaCl gradient in the same buffer. Fractions of 3 ml per 15 min were collected. The distribution of protein and of cathepsin C activity in the column eluate are shown in Fig. 1C. Protein in the fractions under the cathepsin C peak was concentrated by ultrafiltration, washed with 0.15 M NaCl, and stored at 3 °C (CM-fraction). For experiments in which the DEAE-fraction was used without further purification, the protein was first concentrated, washed, and stored in the same manner as was the CM-fraction protein.

Thin-layer chromatography

The solvent used for thin-layer chromatography was the upper phase of butanol–acetic acid–water (4:1:5, by vol.) and the spray used for location of products was 0.2% ninhydrin in acetone, unless otherwise specified.

RESULTS

Purification

Table I summarizes the purification scheme for rat liver cathepsin C. The specific activity in the DEAE-fraction with Gly–Tyr–NH_2 as substrate was 107.4 μmoles of hydroxamate produced per mg of protein per min; this was 1790-fold increased over the activity in the homogenate. When the DEAE-fraction was further chromatographed on a CM-cellulose column, despite the fact that some impurities were removed (Fig. 1C), the protein appeared in a greater number of fractions and

TABLE I

PURIFICATION OF CATHEPSIN C FROM RAT LIVER

Details of enzyme purification and assay are described in the text.

Fraction	*Total protein (mg)*	*Total activity**	*Specific activity**	*Yield (%)*	*Purification (-fold)*
Homogenate	60 294	3310	0.06	100	1
Mitochondrial–lysosomal fraction	9 937	3040	0.31	91.8	5.2
40–70% $(NH_4)_2SO_4$ fraction	110	765	6.9	23.1	115
Sephadex G-200 fraction	15	920	60.8	27.8	1013
DEAE-fraction	8	886	107.4	26.8	1790
CM-fraction	4	350	91.6	10.6	1527

* μmoles of hydroxamate produced per min.
** μmoles of hydroxamate produced per mg of protein per min.

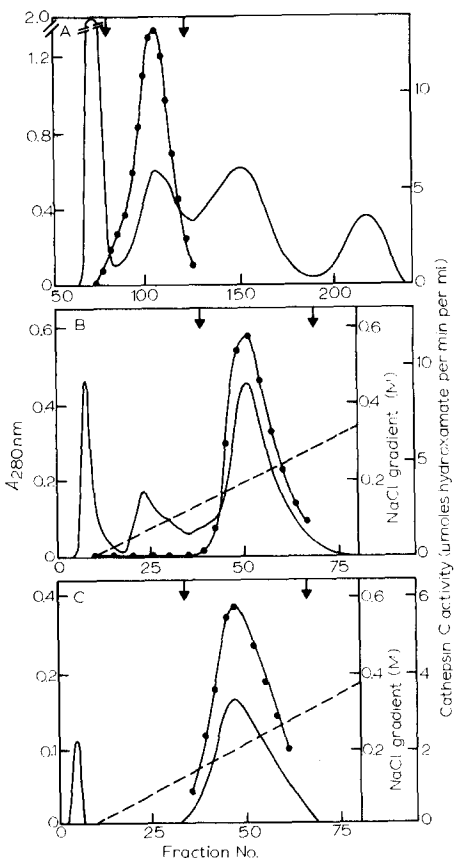

Fig. 1. Column fractionations of cathepsin C. Arrows indicate the pooled fractions. Chromatographic methods are described in the text. A. Sephadex G-200 column fractionation. B. DEAE-cellulose column fractionation. C. CM-cellulose column fractionation. ———, protein; ●—●, cathepsin C activity; — — —, NaCl.

the enzyme was partly inactivated, which resulted in a reduction of total and specific activity. In spite of loss of activity, the CM-fraction had a specific activity of 91.6 μmoles of hydroxamate produced per mg of protein per min, which is higher than previously reported values[2,3]. The separation of cathepsin C from other peptidases of lysosomes was noted. In Sephadex fractionations, cathepsin C eluted before cathepsins D and B. Both the DEAE- and the CM-fractions had negligible cathepsin D activity and no dipeptidase activity with either *N*-acetylphenylalanyltyrosine or cysteinyltyrosine as substrates.

Effects of incubation time and protein concentration

The CM-fraction was used to test the effects of incubation time and protein concentration on cathepsin C activity with Gly–Tyr–NH_2 and Val–Leu–NH_2 as substrates. Activity with Gly–Tyr–NH_2 was proportional to the enzyme concentration up to 191 ng of protein, and up to 38 ng of protein with Val–Leu–NH_2. The activity with both substrates increased linearly up to a period of 30 min. For subsequent analyses of activity using Gly–Tyr–NH_2, an incubation period of 20 min and 95 ng of protein were used. Under these conditions, activity fell within the linear range for measurement of initial velocity.

Effect of substrate concentration

The effect of concentration of both Gly–Tyr–NH_2 and Val–Leu–NH_2 on cathepsin C activity was studied with the CM-fraction. As calculated from double reciprocal curves, the K_m for Gly–Tyr–NH_2 was 6 mM and that for Val–Leu–NH_2 was 2.5 mM. At a high concentration of Val–Leu–NH_2 there was an inhibitory effect on cathepsin C activity. Gly–Tyr–NH_2 showed this effect only slightly. The substrate concentration used in the usual assay of cathepsin C was within the optimal range.

Requirement for –SH reagent

Cathepsin C is known to be an –SH enzyme. Compounds such as L-cysteine or β-mercaptoethanol have been used as enzyme activators[11,12], yet no kinetic studies on the –SH requirement of cathepsin C have been reported. The activation

TABLE II

RELATIVE EFFICIENCY OF –SH COMPOUNDS AS ACTIVATORS OF CATHEPSIN C

The activity of cathepsin C in the DEAE-fraction was determined by hydroxamate formation. The assay mixtures contained 1.2 μg of protein with 50 mM Gly–Tyr–NH_2, and 0.6 μg of protein with 25 mM Val–Leu–NH_2. Values are expressed as percent of activity obtained with dithioerythritol. Activator concentrations are 25 or 1 mM as indicated.

Activator	*Gly–Tyr–NH_2 (25 mM)*	*Val–Leu–NH_2 (25 mM)*	*Gly–Tyr–NH_2 (1 mM)*
Dithioerythritol	100	100	100
Mercaptoethanol	83	86	—
β-Mercaptoethylamine	114	111	—
L-Cysteine	140	146	107
DL-Homocysteine	101	116	72
Glutathione (reduced)	112	108	26
Ascorbic acid	—	—	22
Coenzyme A	—	—	72

of the DEAE-fraction cathepsin C activity by dithioerythritol was studied with both Gly–Tyr–NH_2 and Val–Leu–NH_2. The activation curves obtained were similar to the substrate saturation curves. Only 0.125 mM dithioerythritol was required for half-activation of cathepsin C when tested with both substrates, but maximum activity was obtained at a concentration of 25 mM dithioerythritol; this was the concentration used in the usual cathepsin C assay. Several other –SH activators were also tested to determine their relative effects on cathepsin C activity (Table II). At a concentration of 25 mM, all the compounds tested were efficient activators, and the values obtained with Gly–Tyr–NH_2 and Val–Leu–NH_2 were comparable. Some of the physiologically important reducing agents were also tested at a concentration of 1 mM, which is approximately the intracellular physiological concentration of –SH compounds. Among those compounds tested, L-cysteine was the most efficient activator.

Effect of pH

In the usual cathepsin C assay system at pH 6.8, hydroxylamine was included as acceptor for the activated dipeptidyl unit. The formation of hydroxamate provides a convenient way to determine the product. In the study of the effect of pH, the hydroxamate method was not appropriate; therefore, NH_3, the other reaction product, was measured. Sodium acetate, sodium phosphate, and Tris buffers were

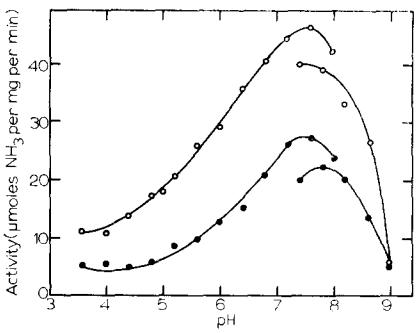

Fig. 2. Effect of pH on the release of NH_3 by cathepsin C. Protein from the DEAE-fraction used was 1.2 μg for conversion of 50 mM Gly–Tyr–NH_2 (○), and 0.6 μg for conversion of 25 mM Val–Leu–NH_2 (●). The remaining procedures are described in the text. Owing to alkaline lability of Gly–Tyr–NH_2 ,substrate blanks at each pH level were also determined. Buffers used were 0.1 M sodium acetate (pH 3.6–5.6), 0.1 M sodium phosphate (pH 6.0–8.0), and 0.1 M Tris (pH 7.4–9.0).

used at their suitable buffering ranges. Fig. 2 shows that the maximum range for NH_3 production was pH 7.2–7.8, in which range the polymerization reaction is thought to occur exclusively, and that hydrolysis at approximately pH 5 was much less than the polymerization reaction at pH 7.2–7.8. Also, the rate of NH_3 release from Val–Leu–NH_2 was one-half that from Gly–Tyr–NH_2. Utilization of Val–Leu–NH_2 was 5 times faster than that of Gly–Tyr–NH_2 when determined by hydroxamate production (Table III). The mechanisms basic to these differences are presently unknown. Tris, an amine, might have some effect on enzyme action as there was discontinuity in the pH curve when activity was determined in its presence.

TABLE III

RELATIVE RATES OF CONVERSION OF DIPEPTIDE AMIDES BY CATHEPSIN C

The standard assay procedure for cathepsin C was used with 1.2 μg of protein from the DEAE-fraction. The dipeptide amides, the tripeptide, and the tripeptide amide were used at a final concentration of 0.05 M, and the substituted NH_2-terminal dipeptide amides at a concentration of 12.5 mM.

Substrate	*Relative rate**
Aliphatic penultimate residue	
Gly–Ala–NH_2	0.6
Gly–Leu–NH_2	2.3
Pro–Leu–NH_2	2.3
Ser–Leu–NH_2	2.4
Thr–Leu–NH_2	3.2
Ala–Leu–NH_2	4.3
Val–Leu–NH_2	5.1
His–Leu–NH_2	0.6
Ile–Leu–NH_2	0.3
Try–Leu–NH_2	0
Aromatic penultimate residue	
Gly–Tyr–NH_2	1
Ser–Tyr–NH_2	1.12
Ala–Tyr–NH_2	1.18
Leu–Tyr–NH_2	0.4
His–Tyr–NH_2	0.23
Gly–Phe–NH_2	0.7
Pro–Phe–NH_2	0.6
Basic penultimate residue	
Ala–Arg–NH_2	1.4
Basic NH_2-terminal resideu	
Lys–Gly–NH_2	0.3
Lys–Leu–NH_2	0
Lys–Phe–NH_2	0
Substituted NH_2-terminal residue	
N–CBZ–Gly–Phe–NH_2**	0
Hippuryl–Phe–NH_2**	0
N-Acetyl–Gly–Leu–NH_2	0
Penultimate residue, proline	
Leu–Pro–NH_2	0
Gly–Pro–NH_2	0
Tripeptide	
Gly–Tyr–Gly	0.14
Tripeptide–NH_2	
Gly–Gly–Phe–NH_2	0.51

* Rates of conversion within 20 min are relative to the rate of conversion of Gly–Tyr–NH_2 within 20 min.

**N-CBZ-Gly–Phe–NH_2 and hippuryl–Phe–NH_2 were not completely soluble and were maintained in suspension by continuous agitation during incubation.

Stability of cathepsin C

Cathepsin C prepared by the procedure described here can be stored in the concentrated state under refrigeration for a period of at least 7 months with little loss of activity. Cathepsin C in crude enzyme preparations was relatively stable and it could be stored either at refrigerator (0–5 °C) or freezer temperature (−15 °C). Upon chromatographic purification, cathepsin C became cold-labile and frequently lost activity upon storage at −15 °C. When diluted, the enzyme lost approximately

one-third of its original activity upon standing at 0–4 °C for a few hours. Cold-lability and inactivation upon dilution have been interpreted as being caused by dissociation into subunits. The molecular weight of cathepsin C is 210 000 (see ref. 2) and the polymeric nature of the enzyme has been shown[13].

Effect of iodoacetamide and halide ions

It was found that cathepsin C was irreversibly inhibited by iodoacetamide and that there was an absolute requirement for halide ion. Cl^- and Br^- were most effective as activators of cathepsin C and maximum activity was obtained at a concentration of 10 mM. In this respect, cathepsin C prepared by the procedure described here behaved in the same manner as did the enzyme prepared by McDonald *et al.*[3,14].

Substrate specificity studies

Dipeptide amides, a tripeptide, and a tripeptide amide were used for testing the substrate specificity of cathepsin C. The relative rates of conversion of these substrates by cathepsin C in the DEAE-fraction were determined by hydroxamate production. The results of these studies are presented in Table III. Some of the generalities observed are as follows: (a) The dipeptide amides with the aliphatic residue leucine at the penultimate position were the most favorable substrates. If the dipeptide amide also had a small NH_2-terminal residue, it was even more susceptible to attack. Among the substrates tested, Val–Leu–NH_2 was the best. (b) When the penultimate residue was an aromatic amino acid and the NH_2-terminal amino acid was a small residue, such as glycine or serine or even proline, the amide bond was susceptible to attack at an intermediate relative rate. (c) Ala–Arg–NH_2, which has a basic penultimate residue, was favorable for attack; however, it was not many orders of magnitude higher than the rates exhibited on aromatic penultimate residues as has been reported for other substrates of this nature[3]. (d) There was little or no hydrolysis when the NH_2-terminal amino acid was a basic residue, and no hydrolysis when proline was at the penultimate position. (e) Substituted NH_2-terminal derivatives were not attacked. The products from hydrolysis of these substrates were assayed by hydroxamate formation, and those from 4-h incubation mixtures were analyzed by thin-layer chromatography. The chromatograms were developed with ninhydrin spray and with 0.5% Morin[15]. Only one spot appeared with the Morin spray and this corresponded with the intact substrate. (f) The tripeptide Gly–Tyr–Gly was hydrolyzed at a slow rate and the tripeptide amide Gly–Gly–Phe–NH_2 was hydrolyzed at an intermediate rate.

Some experiments were designed to determine which bond in the substrate was being cleaved and to show the gradual release of product. For analysis of the products, portions of each aliquot collected at various time intervals, together with corresponding authentic compounds, were spotted on thin-layer chromagram sheets for development and product identification. In Fig. 3 are shown the chromatograms traced from the original thin-layer sheets. When Gly–Tyr–NH_2 and Val–Leu–NH_2 were used as substrates, only the amide bond was cleaved. The dipeptides were gradually released and these were identified by comparison with standard dipeptides. NH_3, the other product of hydrolysis, should have appeared as ammonium acetate in the pH 5 acetate buffer that was used, but it apparently escaped from the mixture during the heating process. On chromatograms of samples incubated for longer time

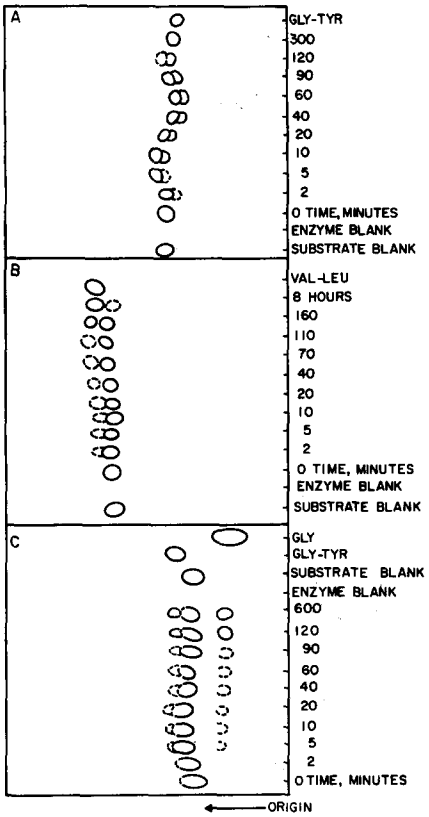

Fig. 3. Thin-layer chromatograms of reaction course analysis. The 0.5-ml reaction mixture consisted of 50 mM substrate, 25 mM dithioerythritol, 10 mM NaCl, and 50 mM sodium acetate buffer, pH 5. The reactions were initiated at 37 °C by addition of enzyme from the DEAE-fraction. Substrate and enzyme blanks were also determined. Aliquots of 50 μl were withdrawn at various time intervals, heated in boiling water and kept frozen until 5–10 μl portions were analyzed. A. Reaction course for the hydrolysis of Gly–Tyr–NH_2 by 3 μg of protein from the DEAE-fraction. Although the solvent did not progress evenly, the product was readily identified by its color difference from the substrate. B. Reaction course for the hydrolysis of Val–Leu–NH_2 by 1.2 μg of protein from the DEAE-fraction. C. Reaction course for the hydrolysis of Gly–Tyr–Gly by 3 μg of protein from the DEAE-fraction.

periods, ammonium acetate appeared as diffuse brown spots that corresponded with the authentic compound. When Gly–Tyr–Gly was used as substrate, the bond between –Tyr–Gly was cleaved but not the bond between Gly–Tyr–, and the products corresponded with standard Gly–Tyr and glycine. Dithioerythritol appeared as a faint spot near the solvent front, but it, as well as the ammonium acetate spot, was not traced from the original chromatogram.

Other tripeptides were also tested as substrates to determine the bond of cleavage and to show that cathepsin C acts as a dipeptidyl aminopeptidase, which cleaves dipeptides from the NH_2-terminal end, and not as an amidase, which cleaves NH_3 from peptide amides. The tripeptides used as substrates were Gly–Gly–Phe–NH_2, Leu–Gly–Gly, Gly–Tyr–Gly, Gly–Phe–Phe, Gly–Leu–Tyr, and Met–Leu–Gly. The products of hydrolysis were identified by thin-layer chromatography comparison

with standard compounds in a manner similar to that shown in Fig. 3. For the substrates Leu–Gly–Gly, Gly–Tyr–Gly, and Gly–Leu–Tyr, bond cleavage occurred between the penultimate amino acid and the COOH-terminal amino acid; Gly–Gly–Phe–NH_2 was cleaved between the second and third residues. These results clearly showed that cathepsin C acted as a dipeptidyl aminopeptidase, producing a dipeptide unit from the NH_2-terminal end during hydrolysis. Gly–Phe–Phe was shown also to have been cleaved slightly between the NH_2-terminal residue and the penultimate residue; however, judging from the equal color intensity of the glycine and the Phe–Phe, the tripeptide was not cleaved completely to amino acids. Met–Leu–Gly was shown to have been cleaved between –Leu–Gly; however, the dipeptide product, Met–Leu, was subsequently slightly cleaved to free amino acids. There was no hydrolysis of Glu–Ala–Ala, Arg–Gly–Gly, and Gly–Pro–Gly–Gly under the conditions studied, and the only spots that appeared on the chromatograms were those of the intact substrates.

DISCUSSION

The cathepsin C purification scheme described is well adapted for a preparative scale. Mitochondrial–lysosomal fractions prepared from separate batches of rat liver homogenate can be combined for subsequent fractionation. A large quantity of starting material is required so that the enzyme protein is not too diluted in the final stage of purification. The CM-fraction obtained by this procedure had a cathepsin C specific activity of 91.6 μmoles hydroxamate produced per mg of protein per min; this is a higher specific activity than that obtained with the highly purified enzyme from beef spleen[2] or that reported for rat liver cathepsin C[3]. Purified lysosomes can also be used as starting material for purification of cathepsin C by this method. Lysosome preparations contain less contaminating protein and yield a product with a higher specific activity than can be obtained from the mitochondrial–lysosomal fractions, but they give a lower yield of protein and of total activity than the latter.

The activation of cathepsin C by –SH compounds is necessary for enzyme activity. Why a concentration of 25 mM –SH activator was required for full activation is unknown. Such a high concentration of –SH activator inside the cell would be difficult to maintain, but when the intracellular reducing atmosphere is considered, the *in vivo* requirement for –SH protector may be quite different from the *in vitro* requirement. *In vivo* the protection of –SH enzymes by reduced glutathione might be afforded through a thiol–disulfide exchange. Recently, a thiol:protein disulfide oxidoreductase from rat liver was characterized[16], and glutathione and cysteine were shown to be substrates for the enzyme. The subcellular localization of this enzyme has not been examined, but it could be involved in the activation of cathepsin C by thiols.

Cathepsin C catalyzes the transfer of a dipeptidyl group of a suitable substrate to nucleophilic amines, such as hydroxylamine, free amino acids, and excess dipeptide amide substrate (to form polymers), or even water (to cause hydrolysis)[17,18]. The relative extent to which the activated dipeptidyl unit reacts with water or with the amino group of another substrate depends upon the pK_a of the corresponding ammonium group and upon the pH of the solution[19]. At pH values at which the NH_2-

terminal group of the substrate is largely protonated (pH < 6), the partition between hydrolysis and polymerization is strongly in favor of the former process. In the range of pH 7–8, where the conjugate acids of the substrate have their pK_a values, the predominant reaction is polymerization. For these reasons, the study of the specificity cathepsin C as a hydrolase was done at pH 5. From the pH activity profile, the catalysis of the polymerization reaction at pH 7–8 by cathepsin C was seen to be more active than the hydrolytic action at approximately pH 5. Whether the polymerization reaction is physiologically important is not known, although this reaction has been intensively studied *in vitro*[20]. Cathepsin C is a lysosomal enzyme, and upon consideration of the assumed acidic pH inside the lysosome, it seems improbable that polymerization reactions would be significant under physiological conditions.

The substrate specificity study confirmed cathepsin C to be a dipeptidyl aminopeptidase[3]. The specificity appeared to be broader than that reported in the older literature[12]. The enzyme was active against a variety of dipeptide amides when examined by its transfer action. Among the dipeptide amides tested, those having leucine as the aliphatic residue in the penultimate position and at the same time having a small NH_2-terminal residue, were most susceptible to attack. The preferential attack on peptides with hydrophobic[19] and aromatic[21] residues in the penultimate position has been reported previously. These results differed from the general conclusion of McDonald *et al.*[3] that dipeptide derivatives having basic residues at the penultimate position were the best substrates for cathepsin C; however, the same substrates were not used in this study as were used by McDonald *et al.*[3]. There are limitations to the types of bonds that can be hydrolyzed by cathepsin C. As reported in the literature, those dipeptide derivatives with a substituted, basic, or acidic residue at the NH_2-terminal position, or having proline at the penultimate position were inert to the action of cathepsin C[3,11]. These limitations were found for dipeptide amide hydrolysis.

The broader substrate specificity of cathepsin C observed in these studies and by McDonald *et al.*[3] certainly adds more possibility to a role for cathepsin C in protein hydrolysis within lysosomes. Purified cathepsin C also has been shown to be active on certain polypeptide hormones[3,14], and has been considered to be the specific enzyme for glucagon degradation[14]. As more data develop, the evidence indicates that cathepsin C, acting in concert with the other lysosomal proteolytic enzymes, must be important in the process of protein and peptide hydrolysis and turnover.

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