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CHARACTERIZATION OF RAT LIVER LYSOSOMAL CATHEPSIN A1

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SUMMARY

1. Highly purified rat liver cathepsin A1 was shown to be a carboxypeptidase with a high degree of specificity for aromatic and hydrophobic amino acids.

2. In lysosomal soluble fractions, cathepsin A1 was partially responsible for the hydrolysis of the model substrates, *N*-benzyloxycarbonyl (Cbz)-Gly–Phe, Cbz-Glu–Phe, and Ac-Phe–Tyr. Dipeptides and tripeptides were not hydrolyzed, but N-blocked dipeptides that contain an aromatic or hydrophobic amino acid were hydrolyzed.

3. The optimum hydrolysis of N-blocked dipeptides by cathepsin A1 was at pH 5.0–5.8, with K_m values in the range of 1.2–54 mM.

4. The hexapeptide Leu–Trp–Met–Arg–Phe–Ala, glucagon, and insulin B chain were partially hydrolyzed by cathepsin A1 with the release of amino acids from the C-terminal end. These peptides all contain hydrophobic portions near the C-terminal end. Less hydrophobic peptides, such as insulin A chain and His–Ser–Gln–Gly–Thr–Phe, were not hydrolyzed. Very little hydrolysis of denatured hemoglobin was found.

5. Cathepsin A1 was activated by halide ions and inhibited by Ag^+ and Hg^{2+}.

INTRODUCTION

In the preceeding paper [1], a multiplicity of carboxypeptidases was shown to exist in rat liver lysosomes. Previous to this report, the only identified lysosomal carboxypeptidase was bovine spleen cathepsin A, which has similar specificity to that of pancreatic carboxypeptidase A [2, 3]. There are multiple forms of cathepsin A in rat liver lysosomes [1]. The major form of cathepsin A, cathepsin A1, was purified 1270-fold as measured by its activity toward Cbz–Gly–Phe. This study reports the characterization of highly purified cathepsin A1 from rat liver.

METHODS AND MATERIALS

Source and assay of cathepsin A1

Cathepsin A1 was purified from rat liver lysosomes, and was assayed with *N*-benzyloxycarbonyl (Cbz)-Gly–Phe, Cbz-Glu–Phe, or Ac–Phe-Tyr as previously described [1].

Electrophoretic identity of enzymes that hydrolyze Cbz-Gly–Phe, Cbz-Glu–Phe, and Ac-Phe–Tyr

Electrophoresis was carried out in 7% acrylamide gel polymerized with ammonium persulfate in the presence of 65 mM bis-Tris–HCl (pH 6.0). The reservoir buffer was 80 mM bis-Tris–maleate (pH 6.2). The purified DEAE-cellulose fraction of cathepsin A1 described previously [1] that contained 25 μg of protein in 100 μl of 250 mM sucrose was applied directly onto the gel. Migration was in the direction of the anode. Electrophoresis was done at 3.0 mA per tube for 90 min at 4 °C. The gel was removed and sliced into 3-mm pieces. The slices were homogenized in 1.0 ml of 100 mM citrate–phosphate buffer (pH 5.5) with a glass and Teflon tissue homogenizer. After centrifugation in a clinical centrifuge, 250-μl portions of the supernatants were removed for enzyme assay. A duplicate gel was stained with Coomassie Blue as described by Fishbein [4].

Measurement of the hydrolysis rates of N-blocked dipeptides, dipeptides, and tripeptides

The liberation of amino acids from N-blocked dipeptides, dipeptides, and tripeptides was followed with the fluorometric method of Taylor and Tappel [5].

Hydrolysis of polypeptides and proteins

For the polypeptides, a final concentration of 1 mg peptide per ml of 100 mM citrate–phosphate buffer (pH 5.5) was mixed with suitable amounts of purified cathepsin A1 to give a final volume of 0.5 ml. For denatured hemoglobin, a 0.5-ml reaction mixture contained 0.25 ml of 3% denatured hemoglobin in 50 mM citrate–phosphate buffer (pH 5.5), 0.15 ml deionized water, and 0.1 ml of enzyme solution. After incubation at 37 °C for 60 min, the reaction was stopped by addition of 0.5 ml of 10% trichloroacetic acid. After suitable dilution, the liberated amino acids were determined by the phthalaldehyde fluorometric method [5] with phenylalanine as a standard. The specific activity is expressed as nmoles phenylalanine equivalents released per min per mg of protein.

For the polypeptides, glucagon and oxidized insulin B chain, and the hexapeptide, Leu–Trp–Met–Arg–Phe–Ala, the products of hydrolysis were determined. The 2.2-ml reaction systems contained 1.2 ml of peptide (2 mg/ml) in 100 mM citrate–phosphate buffer (pH 5.5), 0.4 ml of 250 mM NaCl, and 0.8 ml of purified cathepsin A1. The mixtures were incubated at 37 °C and, at 0, 1, 5, 15, 30, and 60 min, 0.3 ml was removed and placed in a boiling water bath for 3 min to stop the reaction. Thin-layer chromatography was then carried out on Quantagram Q1 plates with an *n*-butanol–acetic acid–water (4:1:1, by vol.) developing system. Amino acid products were identified by spraying the plates with ninhydrin and comparing their R_F values with those of standards.

Sources of substrates and reagents

The sources of some of the substrates and reagents have been listed previously [1]. Other chemicals and sources include: α-L-aspartyl-L-phenylalanine and L-methionyl-L-phenylalanine from Fox Chemical Co., Los Angeles, Calif.; L-seryl-L-phenylalanine, L-histidyl-L-leucine and glycylglycyl-L-phenylalanine from Cyclo Chemical Corp., Los Angeles, Calif.; glycyl-D-leucine, D-leucylglycine, L-leucylglycine, L-methionyl-L-leucine, L-methionyl-L-phenylalanylglycine, glycyl-L-leucyl-L-

tyrosine, glycyl-L-phenylalanyl-L-phenylalanine, L-methionyl-L-leucylglycine, insulin A chain, insulin B chain, glucagon, and the N-terminal hexapeptide of glucagon from Schwarz–Mann, Orangeburg, N.Y.; L-valyl-L-leucine from Sigma Chemical Co., St. Louis, Mo.; the hexapeptide, L-leucyl-L-tryptophyl-L-methionyl-L-arginyl-L-phenylalanyl-L-alanine, from Research Plus Laboratories, Inc., Denville, N.J.; and hemoglobin from Worthington Biochemical Corp., Freehold, N.J.

RESULTS

Electrophoresis identity of Cbz-Gly–Phe, Cbz-Glu–Phe, and Ac-Phe–Tyr hydrolysis by cathepsin A1 preparation

Fig. 1 shows that the hydrolytic activity toward Cbz-Gly–Phe, Cbz-Glu–Phe, and Ac-Phe–Tyr was localized in one band after electrophoresis of the cathepsin A1

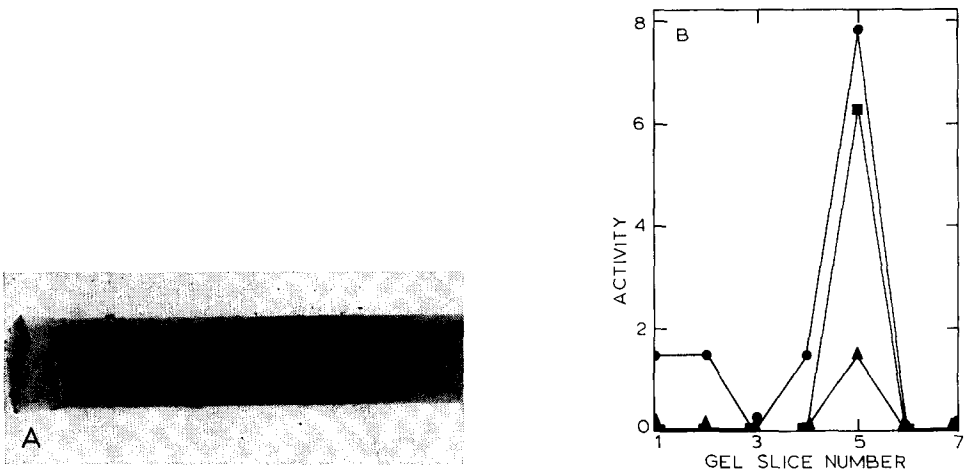

Fig. 1. (A) Protein pattern shown by Coomassie Blue stain after gel electrophoresis of purified cathepsin A1 fraction as described in Methods and Materials. (B) The localization of activities that hydrolyze Cbz-Gly–Phe (●—●), Cbz-Glu–Phe (▲—▲), and Ac-Phe–Tyr (■—■) after gel electrophoresis of purified cathepsin A1 fraction under identical conditions as in (A). No activity toward any of the substrates and no staining were noted beyond the seventh gel slice. Activity is expressed as nmoles amino acid liberated per min per ml gel slice homogenate.

preparation. This suggests that the highly purified cathepsin A1 preparation contains a single protein responsible for the hydrolysis of all three model substrates. The duplicate gel stained for protein is shown in Fig. 1A.

Hydrolysis of N-blocked dipeptides by cathepsin A1

Table I shows the hydrolysis rates of a series of N-blocked dipeptides by cathepsin A1. The most favorable rates of hydrolysis were obtained with substrates that contained hydrophobic amino acids, such as phenylalanine or leucine, in the C-terminal or penultimate positions. With a hydrophobic amino acid in the penultimate position, the hydrolysis rate was faster than with the same amino acid in the C-terminal position. Cathepsin A1 apparently recognizes features of the substrate beyond the penultimate amino acid, since Bz-Gly–Phe was hydrolyzed at a slower rate than

TABLE I

HYDROLYSIS OF N-BLOCKED DIPEPTIDES BY CATHEPSIN A1

Reaction mixtures contained 12.5 μmoles of N-blocked dipeptide and 1.25 μg of enzyme protein in 50 mM citrate–phosphate buffer (pH 5.5) in a 0.5-ml final volume. Reactions were carried out for 60 min at 37 °C. Other details are explained in Methods and Materials.

N-blocked dipeptide	Specific activity (nmoles amino acid/min per mg protein)	Cbz-Gly–Phe hydrolysis (percent)
Cbz-Gly–Phe	7 060	100
Cbz-Gly–Tyr	2 050	29
Cbz-Gly–Leu	7 010	99
Cbz-Gly–Met	5 090	72
Cbz-Gly–Trp	440	6
Cbz-Gly–His	0	0
Cbz-Gly–Arg	480	7
Cbz-Gly–Glu	47	0.7
Cbz-Gly–Ser	450	6
Cbz-Gly–Gly	560	8
Cbz-Gly–D-Phe	0	0
Bz-Gly–Phe	2 710	38
Cbz-Glu–Phe	920	13
Cbz-Glu–Tyr	10 900	155
Ac-Phe–Tyr	6 610	94
Cbz-Gly–Pro	60	0.8
Cbz-Gly–Sar	0	0
Cbz-Sar–Gly	220	3
Cbz-Pro–Gly	0	0
Cbz-Phe–Gly	29 500	420
Cbz-Tyr–Gly	19 900	280
Cbz-Leu–Gly	14 200	201
Cbz-Met–Gly	12 500	178
Cbz-Trp–Gly	9 630	136
Cbz-His–Gly	1 480	21
Cbz-Gln–Gly	910	13
Cbz-Glu–Gly	0	0
Cbz-Ser–Gly	220	3
Cbz-β-Ala–Gly	0	0
Ac-Gly–Leu–NH_2	150	2

Cbz-Gly–Phe. N-blocked dipeptides with histidine and tryptophan in the C-terminal position were hydrolyzed less readily than those with histidine or tryptophan in the penultimate position. Again, this indicates that the enzyme has specificity determinants for the penultimate position different from those for the C-terminal position. The carboxypeptidase nature of cathepsin A1 is indicated by the lack of hydrolysis of Ac-Gly–Leu–NH_2. Cathepsin A1 was specific for α-L-amino acids based on its lack of hydrolysis of Cbz-Gly–D-Phe, Cbz-Gly–Pro, Cbz-Pro–Gly, and Cbz-β-Ala–Gly.

Effect of substrate concentration and pH on hydrolysis of some N-blocked dipeptides by cathepsin A1

K_m values and pH optima for the hydrolysis of several N-blocked dipeptides by cathepsin A1 are presented in Table II. The K_m values lie between 10 and 50 mM

TABLE II

pH OPTIMA AND K_m VALUES FOR HYDROLYSIS OF N-BLOCKED DIPEPTIDES

Substrate	pH optimum*	V (nmoles/min per mg protein)	K_m** (mM)
Cbz-Gly–Phe	5.5	15 800	10.6
Ac-Phe–Tyr	5.5	38 900	33.3
Cbz-Phe–Gly	5.8	108 000	54.5
Cbz-Leu–Gly	5.5	22 000	16.0
Cbz-Glu–Phe	5.0	10 600	1.2

* pH-optima determinations were made in 0.5-ml reaction volumes that contained 12.5 μmoles of substrate, except for Cbz-Glu–Phe, 7.5 μmoles, and Ac-Phe–Tyr, 4.0 μmoles, and 2.5 μg of enzyme protein with 50 mM citrate–phosphate buffer for the pH range 3.0–5.8 and 50 mM potassium phosphate buffer for the pH range 6.0–8.0.

** K_m determinations were made at the pH optimum for each substrate with 2.5 μg of enzyme protein. Linear-regression analysis was applied to determine the K_m and V values. The methods for measurement of hydrolysis rate are detailed in Methods and Materials.

except for Cbz-Glu–Phe, which has a K_m value of 1.2 mM. Substrate-solubility limitations dictated that assays for hydrolysis of these substrates be run at less than saturating concentrations of substrate. However, under the conditions used for the substrates tested, the maximal hydrolysis within the time period utilized was 16% for Ac-Phe–Tyr. With most substrates, the hydrolysis within the time period utilized was less than 10%. The pH optimum for the hydrolysis of Cbz-Glu–Phe was the lowest of any substrate tested.

Effect of time and enzyme concentration on hydrolysis of Cbz-Gly–Phe by cathepsin A1

The hydrolysis of 25 mM Cbz-Gly–Phe at pH 5.5 was linear with time up to 60 min of hydrolysis and was proportional to enzyme concentration in the range of 0–3.8 μg of protein. Incubation times of 30–60 min and enzyme concentrations in the range of 0–3.8 μg of protein were used for hydrolysis of other substrates by cathepsin A1.

Hydrolysis of peptides and proteins by cathepsin A1

There was no detectable hydrolysis of dipeptides and tripeptides, including Gly–Phe, Asp–Phe, Arg–Phe, Met–Phe, Ser–Phe, Glu–Try, Leu–Try, Trp–Leu, Val–Leu, Gly–Leu, Met–Leu, Leu–Gly, D-Leu–Gly, Gly–D-Leu, Met–Phe–Gly, Gly–Leu–Tyr, Gly–Gly–Phe, Gly–Phe–Phe, and Met–Leu–Gly. As shown in Table III, hydrolysis of larger peptides was noted, particularly of glucagon, which yielded five amino acid products from its carboxyl end. The hydrolysis of the C-terminal peptide bond of glucagon, Asn–Thr, would not be predicted on the basis of the results of experiments on the hydrolysis of N-blocked dipeptides. However, the Asn–Thr bond was hydrolyzed rapidly with the four C-terminal amino acids, threonine, asparagine, methionine, and leucine, identifiable by thin-layer chromatography after 5 min of hydrolysis. A fifth spot corresponding to tryptophan appeared after 30 min of hydrolysis. The hydrophobic Trp–Leu–Met portion near the carboxyl end of glucagon may be important for the hydrolysis of this substrate. Likewise, hydrolysis of the C-terminal Lys–Ala

TABLE III

HYDROLYSIS OF POLYPEPTIDES AND PROTEINS BY CATHEPSIN A1

Details are given under Methods and Materials.

Peptide or protein	Specific activity (nmoles phenylalanine equivalents/min per mg protein)	Product
Leu–Trp–Met–Arg–Phe–Ala	382	Ala, Phe
His–Ser–Gln–Gly–Thr–Phe	0	–*
Glucagon	4840	Thr, Asn, Met, Leu, Trp
Insulin A chain	120	–
Insulin B chain	500	Ala
Denatured hemoglobin	30	–

* Not identified.

peptide bond of insulin B chain would not be expected based on criteria developed from studies of the hydrolysis of N-blocked dipeptides. However, the hydrophobic Phe–Phe–Tyr portion near the C-terminal end may explain the hydrolysis of this bond. The less hydrophobic polypeptides, insulin A chain and His–Ser–Gln–Thr–Phe, were not hydrolyzed to any great extent. With the hexapeptide Leu–Trp–Met–Arg–Phe–Ala, the products identified were alanine and phenylalanine. No evidence was found for further hydrolysis of the remaining tetrapeptide despite the presence of the hydrophobic Leu–Trp–Met portion. This may reflect the chain-length specificity of cathepsin A1. This chain-length specificity of cathepsin A1 may be due to the close proximity of the positively charged amino end of the peptide to the carboxyl end of the peptide, which may inhibit binding or hydrolysis of the peptide. The only protein tested, denatured hemoglobin, was hydrolyzed at a slow rate. However, the hemoglobin concentration used was approx. 0.4 mM, which is lower than that used for the peptide substrates, so the rates may not be directly comparable. The concentration of C-terminal amino acids for hemoglobin is low. If the concentration of C-terminal residues had been constant for all substrates, the rate of hydrolysis of denatured hemoglobin by cathepsin A1 might have been appreciable.

Effect of inhibitors and activators on cathepsin A1

Percentage inhibition of Cbz-Gly–Phe hydrolysis by cathepsin A1 by metal ions at a final concentration of 200 μM were: Ag^+, 88%; Hg^{2+}, 94%; Zn^{2+}, 6%; and Cd^{2+}, 0%. Cathepsin A1 was inhibited only slightly by *p*-chloromercuribenzoate at a final concentration of 200 μM. The metal chelators, EDTA and *o*-phenanthroline, at a final concentration of 200 μM, activated the enzyme to 130% and 120% of control activity, respectively. Diisopropylfluorophosphate, at a final concentration of 200 μM, also activated the enzyme to 120% of control activity. Dithioerythritol, at 50 mM final concentration, had no effect on cathepsin A1 activity. A halide activation effect was noted for cathepsin A1. Maximal activation that occurred at a final concentration of 5 mM halide ion was 120% for F^- and 130% for Cl^-, Br^-, and I^-. At 50 mM final concentration, F^- and I^- inhibited cathepsin A1 9% and 13%, respectively. At 50 mM $NaNO_3$, 38% inhibition of cathepsin A1 was noted.

Stability of cathepsin A1

Purified cathepsin A1 was stable in frozen storage for at least 3 weeks. Since cathepsin A1 was unstable at neutral pH, storage was at pH 5.3.

DISCUSSION

Rat liver lysosomal cathepsin A1 appears to be a carboxypeptidase with specificity similar to that of pancreatic carboxypeptidase A and bovine pleen cathepsin [2, 3, 6]. Cathepsin A1 demonstrates a high degree of specificity for hydrophobic amino acids. However, it appears to hydrolyze peptide bonds that do not involve hydrophobic amino acids if a hydrophobic side-chain portion exists near the carboxyl end. Like pancreatic carboxypeptidase A [7], cathepsin A1 seems to have a definite chain-length requirement. Dipeptides and tripeptides are not hydrolyzed, and cathepsin A1 appears to recognize amino acid residues removed by three to five positions from the carboxyl end. Further work on chain-length specificity of cathepsin A1 is needed to determine the nature of the role that cathepsin A1 plays in lysosomal protein catabolism.

Rat liver cathepsin A1 is somewhat different from other cathepsin A preparations that have been reported. Cathepsin A was originally defined by its hydrolysis of Cbz-Glu–Tyr, a synthetic substrate for pepsin [8]. The first purified cathepsin A preparation from chicken breast muscle showed higher activity toward Cbz-Glu–Phe than toward Cbz-Glu–Tyr [9]. Rat liver cathepsin A1 hydrolyzes Cbz-Glu–Phe at a much lower maximal rate than it hydrolyses Cbz-Glu–Tyr, and the best substrates with respect to V appear to be Cbz-Phe–Gly and Ac-Phe–Tyr. However, while a lower maximal hydrolysis rate was demonstrated toward Cbz-Glu–Phe, a lower K_m for this substrate was also found. Since less than saturating levels of the substrates were used in these assays, the velocity of the reaction can be approximated by V/K_m. The ratio, V/K_m is highest for Cbz-Glu–Phe, which indicates that this substrate is hydrolyzed at the fastest rate by cathepsin A1. Iodice [2] found that purified bovine spleen cathepsin A could hydrolyze the five C-terminal amino acids from glucagon. Logunov and Orekovich [3] also purified cathepsin A from bovine spleen but they found it had both carboxypeptidase and endopeptidase activity. While rat liver cathepsin A1 hydrolyzes the five C-terminal amino acids from glucagon, it has no endopeptidase activity. This finding is in agreement with further work by Iodice [6] that indicated no endopeptidase activity by the bovine spleen enzyme. Logunov and Orekovich [10] reported that their bovine spleen cathepsin A preparation was inhibited by diisopropylfluorophosphate, Ag^+, Hg^{2+}, and Cu^{2+}. Rat liver cathepsin A1 was not inhibited by diisopropylfluorophosphate but was inhibited by Ag^+ and Hg^{2+}. The bovine spleen preparation of Logunov and Orekovich [3] appears to be either an endopeptidase with specificity for hydrophobic amino acids or a cathepsin A fraction contaminated with cathepsin D, which could explain the hydrolysis of the Val–Tyr bond in angiotensin II [11, 12]. The rat liver cathepsin A1 and the bovine spleen cathepsin A preparation of Logunov and Orekovich [3] appear to be very different.

The inhibition of cathepsin A1 by Ag^+ and Hg^{2+} is not necessarily indicative that cysteine is at the active site, especially since cathepsin A1 was inhibited only slightly by *p*-chloromercuribenzoate and was not activated by dithioerythritol. The

Ag^+ and Hg^{2+} may be reacting with some functional group in cathepsin A1 other than cysteine, such as histidine, but this possibility was not tested further.

Another interesting feature of rat liver cathepsin A1 is the halide activation effect. Halide activation of several lysosomal proteases, namely cathepsin C [13] and cathepsin B2 [14], has been described. Cathepsin C is a known exopeptidase [15] and, based on the results presented in the preceeding paper [1], cathepsin B2 may be an exopeptidase also. Halide activation in vivo, particularly by Cl^-, could play a role in increasing lysosomal exopeptidase activity. F^- does not activate either rat liver cathepsin A1 or cathepsin B2 [14] to the same extent as do the other halide ions. However, it should be noted that F^- has a larger hydrated radius than the other halide ions, which could explain this disparity [16].

Rat liver cathepsin A1 had little activity toward denatured hemoglobin, although this observation is perhaps due to the low concentration of C-terminal amino acids present. Iodice et al. [9] found similar results, but noted that chicken breast-muscle cathepsin A could act synergistically with cathepsin D in the hydrolysis of hemoglobin. Synergistic effects between partially purified rat liver cathepsin A and cathepsin D have been noted in the hydrolysis of hemoglobin and insulin B chain [17]. These synergistic effects are likely owing to the increase in C-terminal amino acid concentration. Through this synergistic mechanism, exopeptidases such as cathepsin A1 may play a vital role in determination of the rate and extent of protein catabolism in lysosomes.

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