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PURIFICATION AND CHARACTERIZATION OF RAT LIVER LYSOSOMAL CATHEPSIN B2

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SUMMARY

1. Cathepsin B2 was purified 3900-fold over homogenate with respect to the hydrolysis of *N*-benzoyl-L-arginine amide (Bz-Arg-NH_2). Cathepsin B2 was shown to be a relatively nonspecific carboxypeptidase by virtue of its hydrolysis of N-blocked dipeptides. Only N-blocked dipeptides that contained proline, sarcosine, β-alanine, or D-amino acids were not hydrolyzed, which indicates a specificity for α-L-amino acids. The optimum hydrolysis of Bz-Arg-NH_2 and N-blocked dipeptides by cathepsin B2 occurred at pH 5.5–5.6, with K_m values in the range of 10–15 mM. Dipeptides were not hydrolyzed by cathepsin B2, but some tripeptides were hydrolyzed. A tetrapeptide (Leu–Trp–Met–Arg), a hexapeptide (Leu–Trp–Met–Arg–Phe–Ala), and glucagon were hydrolyzed by cathepsin B2 with the release of amino acids from the carboxyl terminus. Insulin A chain, insulin B chain, and bradykinin were not hydrolyzed by cathepsin B2, perhaps owing to the presence of cysteic acid or proline residues near the carboxyl terminus. No endopeptidase activity of cathepsin B2 was found. Cathepsin B2 was activated by sulfhydryl compounds and inhibited by *p*-hydroxymercuribenzoate.

INTRODUCTION

Cathepsin B (EC 3.4.22.1) has been defined classically by its amidase activity on α-*N*-benzoyl-L-arginine amide (Bz-Arg-NH_2), a model substrate for trypsin [1]. Cathepsin B also has been shown to hydrolyze other trypsin model substrates, including α-*N*-benzoyl-L,D-arginine-*p*-nitroanilide (Bz-DL-Arg–Nan) [2] and α-*N*-benzoyl-L, D-arginine-β-naphthylamide (Bz-DL-Arg–2-NNap) [3]. However, Otto [4] and Otto and Bhadki [5] isolated and characterized an enzyme from bovine spleen that inactivates glucokinase and hydrolyzes Bz-Arg-NH_2, Bz-DL-Arg–Nan, and Bz-DL-Arg–2-NNap. Otto [4] distinguished this enzyme, cathepsin B1, from another Bz-Arg-NH_2-hydrolyzing enzyme, cathepsin B2, which neither hydrolyzes Bz-DL-Arg–Nan nor Bz-DL-Arg–2-NNap and does not inactivate glucokinase. Recently, De Lumen and

Abbreviations: Bz-Arg-NH_2, α-*N*-benzoyl-L-arginine amide; Bz-DL-Arg–Nan, α-*N*-benzoyl-L-arginine-*p*-nitroanilide; Bz-DL-Arg–2-NNap, α-*N*-benzoyl-L, D-arginine-β-naphthylamide; Bz-Gly–Arg, hippuryl-L-arginine; Cbz-Gly–Glu, *N*-benzyloxycarbonylglycyl-α-L-glutamic acid.

Tappel [6] purified a cathepsin B1-like enzyme from rat liver that hydrolyzes Bz-DL-Arg–2-NNap specifically, and showed it to be distinct from cathepsin B2. Taylor and Tappel [7], while reporting the purification of rat liver lysosomal carboxypeptidases, pointed out the possibility that carboxypeptidase activity may be associated with cathepsin B2.

This study reports the purification of cathepsin B2 from rat liver lysosomes and presents results to support the carboxypeptidase nature of the enzyme.

MATERIALS AND METHODS

Enzyme source

Male Sprague–Dawley rats (400–500 g), fasted for 24 h, were killed by decapitation. The light-mitochondrial fraction obtained from a 10% liver homogenate prepared in 250 mM sucrose–1 mM EDTA by the procedure of De Duve et al. [8] was used as the source of the enzyme.

Column chromatography

The separation and purification of cathepsin B2 was carried out essentially as described by Taylor and Tappel [7]. The light-mitochondrial fractions were frozen and thawed 5 times, dialyzed against 50 mM sodium acetate buffer (pH 5.3) and centrifuged initially at 14 000 × *g* for 20 min and then at 100 000 × *g* for 60 min. The supernatant was subjected to $(NH_4)_2SO_4$ fractionation. The 30–70% $(NH_4)_2SO_4$ fraction was retained and dialyzed overnight at 4 °C against 4 changes of 5 mM sodium acetate buffer (pH 5.3) that contained 20 mM mercaptoethanol and 150 mM NaCl. The dialyzed fraction was applied to a Sephadex G-100 column, 94 cm × 3 cm, which had been equilibrated previously with the above buffer. The flow rate of the column was 28 ml/h, and 2.5-ml fractions were collected. The elution buffer was 5 mM sodium acetate buffer, (pH 5.3) that contained 20 mM mercaptoethanol and 150 mM NaCl. Protein was monitored by absorbance at 280 nm. The column fractions were analyzed for the activities of cathepsin B2 with Bz-Arg-NH_2, catheptic carboxypeptidases B and G with hippuryl-L-arginine (Bz-Gly–Arg) and *N*-benzyloxycarbonylglycyl-α-L-glutamic acid (Cbz-Gly–Glu), respectively, and cathepsin B1 with Bz-DL-Arg–2-NNap.

The fractions with high cathepsin B2 and carboxypeptidase activities were pooled and concentrated to a minimal volume in an Amicon–Diaflo apparatus with a PM-10 membrane. The resulting concentrate was dialyzed against 5 mM sodium acetate buffer (pH 5.3) that contained 20 mM mercaptoethanol and 30 mM NaCl, and applied to a DEAE-cellulose column (Whatman DE-32), 30 cm × 2 cm, previously equilibrated with the above buffer. Initial elution of unbound protein with the same buffer was followed by elution with a linear gradient of 30–400 mM NaCl. The buffer was pumped at a rate of 36.6 ml/h, and 4.7-ml fractions were collected. The fractions were analyzed for the activities of cathepsin B2, catheptic carboxypeptidases B and G, and cathepsin B1. Protein was monitored by absorbance at 280 nm.

Assay procedures

Lysosomal carboxypeptidase activities associated with cathepsin B2 were

measured essentially according to the procedure of Taylor and Tappel [7] in a 0.5-ml reaction mixture that contained 50–100 μl of enzyme fraction, 20 mM dithioerythritol, and 100 mM citrate–phosphate buffer. For catheptic carboxypeptidase B, 60 mM Bz-Gly–Arg was employed as the substrate at pH 5.5 or 5.6, and the incubation was for 30 min at 37 °C. Catheptic carboxypeptidase G was measured during an incubation period of 60 min at 37 °C at pH 5.5 or 5.6 with 25 mM Cbz-Gly–Glu as substrate. The reactions were stopped by the addition of 0.5 ml of 10% trichloroacetic acid. The precipitate, if any, was removed by centrifugation and a known aliquot of the supernatant was suitably diluted with deionized water. The concentration of the liberated amino acid was measured fluorometrically [9] with the appropriate amino acid standards.

Cathepsin B2 activity was measured with the reaction conditions described by De Lumen and Tappel [6] with Bz-Arg-NH_2 as substrate. The liberated ammonia was determined fluorometrically [10].

The procedure outlined by De Lumen and Tappel [11] was employed for the determination of cathepsin B1 activity. Protein was determined by the fluorescamine assay method [12] with bovine serum albumin as standard. For all determinations, interferring mercaptoethanol was removed by dialysis prior to the protein determination.

pH and thermal stability of the enzyme

Citrate–phosphate buffers of pH 3.0–6.5 and potassium phosphate buffers of pH 7.0 and 7.4 were used. The purified enzyme preparation that contained 2.3 μg of protein was suspended in 100 μl of 100 mM buffer at various pH levels and incubated for 10 min at 37, 50 and 60 °C. After addition of appropriate substrates, 20 mM dithioerythritol, and 100 mM citrate–phosphate buffer of pH 5.5 or pH 5.6, the activities of cathepsin B2 and the carboxypeptidases were determined.

Hydrolysis rates of N-blocked dipeptides

The 0.5-ml assay mixture contained 25 mM N-blocked dipeptide, 2.4 μg of enzyme protein in 70 mM citrate–phosphate buffer, pH 5.5, and 20 mM dithioerythritol. Incubation was at 37 °C for 60 min. The reaction was terminated by addition of 0.5 ml of 10% trichloroacetic acid, and the liberated amino acid was measured fluorometrically [9].

Hydrolysis of dipeptides, tripeptides, and polypeptides

The 0.5-ml reaction mixtures contained 25 mM dipeptides and tripeptides, 2.4 μg of enzyme protein, 70 mM citrate–phosphate buffer (pH 5.5) and 20 mM dithioerythritol. Incubation was at 37 °C for 60 min. The reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid. The liberated amino acids were determined fluorometrically and the extent of hydrolysis was calculated from the appropriate standard curve [9].

For the hydrolysis of polypeptides, an experiment was carried out to screen the extent of enzymic cleavage of various peptides, including bradykinin, insulin A and B chains, glucagon, a tetrapeptide (Leu–Trp–Met–Arg), and a hexapeptide (Leu–Trp–Met–Arg–Phe–Ala). The 0.5-ml reaction mixtures that contained 1 mg of the above substrates, 70 mM citrate–phosphate buffer (pH 5.5) and 20 mM dithioery-

thritol were incubated for 60 min at 37 °C. The reaction was terminated by the addition of 0.5 ml of 10% trichloroacetic acid. The liberated amino acids were measured both colorimetrically [13] and fluorometrically [9].

Thin-layer chromatography of the hydrolysates was 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 to those of standards.

Inhibition of cathepsin B2

The purified enzyme was dialyzed against 5 mM sodium acetate buffer (pH 5.3) to remove mercaptoethanol and NaCl. Enzyme protein, 1.2–2.0 μg, was incubated with and without the addition of *p*-hydroxymercuribenzoate at a concentration of 100 μM for 15 min at 37 °C. The enzyme reaction was initiated by the addition of substrate, dithioerythritol, and 100 mM citrate–phosphate buffer (pH 5.5).

Polyacrylamide-disc-gel electrophoresis

Disc-gel electrophoresis was carried out according to the procedure of Davis [14] with the exception that the sample (25–60 μg of protein) in 25% sucrose was layered directly onto the stacking gel. Electrophoresis was for 45 min at 4 °C with a current of 5 mA/tube. Gels were stained with Coomassie blue and destained by the method of Fishbein [15]. In other experiments, electrophoresis was carried out at pH 6.2 according to a procedure outlined elsewhere [7], and the gels were sliced into 1-mm sections. Each section was homogenized in 1 ml of 100 mM citrate–phosphate buffer (pH 5.5) with a glass and Teflon tissue homogenizer. Aliquots of these homogenates were tested for cathepsin B2 and carboxypeptidase activities as described above.

Sources of substrates and reagents

All N-blocked dipeptides, dipeptides, and tripeptides were products of one of the following companies: Fox Chemical Co., Los Angeles, Calif.; Cyclo Chemical Corp., Los Angeles, Calif.; Schwarz-Mann, Orangeburg, N.J.; or Sigma Chemical Corp., St. Louis, Mo. Bz-Arg-NH_2·HCl, Bz-Arg-NH_2·acetate, Bz-DL-Arg-2-NNap, and glucagon were obtained from Schwarz-Mann; ninhydrin, hydrindantin, bovine serum albumin, mercaptoethanol, *p*-hydroxymercuribenzoate, bovine insulin A and B chain, and dithioerythritol from Sigma Chemical Corp.; bradykinin from Cyclo Chemical Corp.; and Leu–Trp–Met–Arg and Leu–Trp–Met–Arg–Phe–Ala from Research Plus Laboratories, Denville, N.J. Sources of reagents for fluorometric amino acid analysis and disc-gel electrophoresis are given elsewhere [7, 9]. Fluorescamine was a generous gift from Dr S. Udenfriend of Hoffman-La Roche, Incorporated, Nutley, N.J. The chromatographic materials and blue dextran-2000 were products of Pharmacia Fine Chemicals, Uppsala, Sweden.

RESULTS

Chromatographic separation of cathepsin B2

Gel filtration of the 30–70% $(NH_4)_2SO_4$ fraction of rat liver light-mitochondria on Sephadex G-100 resulted in the separation of three major protein peaks (Fig. 1). The activity of catheptic carboxypeptidases B and G and the majority of cathepsin B2

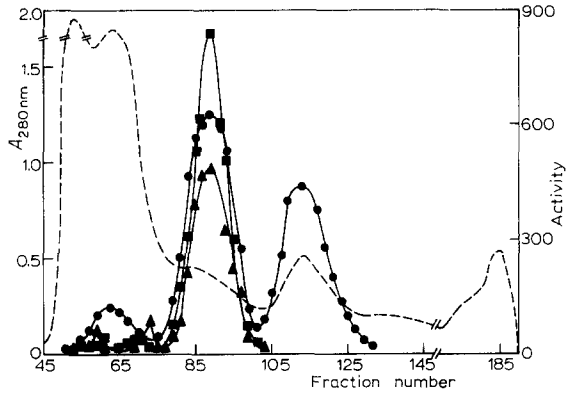

Fig. 1. Sephadex G-100 chromatogram of the 30–70% $(NH_4)_2SO_4$ fraction of soluble rat liver lysosomes. Chromatography conditions are detailed in Materials and Methods. The elution patterns for protein (– – –), Bz-Arg-NH_2-hydrolyzing activity (●—●), Bz-Gly–Arg-hydrolyzing activity (▲—▲) and Cbz-Gly–Glu-hydrolyzing activity (■—■) are shown. Activity is expressed in nmoles product liberated per min/ml.

activity eluted as a single, sharp peak following the first peak of protein. A second peak of Bz-Arg-NH_2 hydrolase activity eluted coincident with the second peak of protein and exhibited cathepsin B1 (Bz-DL-Arg–2-NNap amidohydrolase) activity. This result is in agreement with previous observations [7].

Fractions 80–97, which contained the major peak of cathepsin B2, catheptic carboxypeptidase B, and catheptic carboxypeptidase G were combined, concentrated, and chromatographed on a DEAE-cellulose column (Fig. 2). Hydrolytic activity against Bz-Arg-NH_2, Bz-Gly–Arg, and Cbz-Gly–Glu was eluted by 200 mM NaCl in a single peak of high specific activity. This peak was completely free from cathepsin

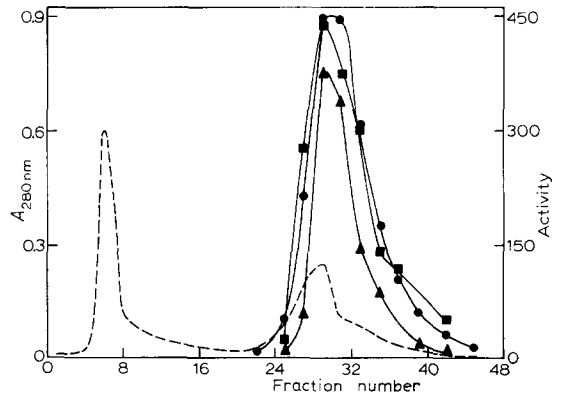

Fig. 2. DEAE-cellulose chromatogram of pooled, concentrated Fractions 80–97 from the Sephadex G-100 separation. Chromatography conditions are detailed in Materials and Methods. A linear gradient of 30–400 mM NaCl was run between Fractions 11 and 50. The elution patterns for protein (– – –), Bz-Arg-NH_2-hydrolyzing activity (●—●), Bz-Gly–Arg-hydrolyzing activity (▲—▲) and Cbz-Gly–Glu-hydrolyzing activity (■—■) are shown. Activity is expressed in nmoles product liberated per min/ml.

B1 activity. Highly active catheptic carboxypeptidase B and G emerged along with cathepsin B2 activity after Sephadex G-100 and DEAE-cellulose chromatography, therefore, it may be inferred that the three hydrolytic activities are exhibited by a single enzyme.

The cathepsin B2 peak from DEAE-cellulose chromatography showed two protein bands when subjected to disc-gel electrophoresis at pH 8.9. However, enzyme activity could not be demonstrated in the gel, owing to its instability at pH 8.9. Also, the enzyme activity could not be located following electrophoresis at pH 6.2, owing to the lack of mobility of the enzyme in acidic gels.

The stepwise purification of cathepsin B2 with respect to Bz-Arg-NH_2, Bz-Gly–Arg, and Cbz-Gly–Glu hydrolysis is shown in Table I. Sephadex G-100 chromatography resulted in the following purification of cathepsin B2 over the

TABLE I

PURIFICATION OF CATHEPSIN B2

Details are described under Materials and Methods.

Substrate and fraction	Total protein (mg)	Total activity (nmoles product/min)	Spec. act. (nmoles product/min/mg protein)	Yield (%)	Purification (-fold)
Bz-Arg-NH_2					
Homogenate	146 000	701 000	4.8	100	1
Light-mitochondrial supernatant	367	83 000	227	12	47
30–70% $(NH_4)_2SO_4$ fraction	125	35 000	277	5.0	58
Sephadex G-100 Fractions 80–97	5.6	18 000	3100	2.5	646
DEAE-cellulose Fractions 27–31	0.55	10 000	18 800	1.5	3900
Bz-Gly–Arg					
Homogenate	146 000	940 000	6.4	100	1
Light-mitochondrial supernatant	367	10 000	29.2	1.1	4.5
30–70% $(NH_4)_2SO_4$ fraction	125	25 000	198	2.6	31
Sephadex G-100 Fractions 80–97	5.6	14 000	2550	1.5	395
DEAE-cellulose Fractions 27–31	0.55	8 800	15 700	0.9	2430
Cbz-Gly–Glu					
Homogenate	146 000	830 000	5.7	100	1
Light-mitochondrial supernatant	367	27 000	73.5	3.2	13
30–70% $(NH_4)_2SO_4$ fraction	125	35 000	280	4.2	49
Sephadex G-100 Fractions 80–97	5.6	20 000	3620	2.4	631
DEAE-cellulose Fractions 27–31	0.55	12 000	18 300	1.2	3190

activity in homogenate: Bz-Arg-NH_2 hydrolase activity, 646-fold; Bz-Gly–Arg hydrolase activity, 395-fold; and Cbz-Gly–Glu hydrolase activity, 631-fold. After DEAE-cellulose chromatography, there was 3900-fold, 2420-fold, and 3190-fold purification over homogenate with Bz-Arg-NH_2, Bz-Gly–Arg and Cbz-Gly–Glu as substrates, respectively. Thus, the same ratio of purification factors as obtained with Sephadex G-100 gel filtration was retained. This constant purification factor ratio, 1.6:1.0:1.6, among the three hydrolytic activities again suggests that these activities are exhibited by a single enzyme. The dissimilarity of purification factor ratios in stages before Sephadex G-100 chromatography may be due to the presence of multiple forms of hydrolytic activity toward Bz-Arg-NH_2, Bz-Gly–Arg, and Cbz-Gly–Glu. For example, the cathepsin B1 peak from Sephadex G-100 possesses some Bz-Arg-NH_2 hydrolase activity but no measureable activity toward Bz-Gly–Arg or Cbz-Gly–Glu.

Effect of pH and substrate concentration on cathepsin B2

K_m and V values and pH optima for the hydrolysis of Bz-Arg-NH_2, Bz-Gly–Arg, and Cbz-Gly–Glu are presented in Table II. Cathepsin B2 had maximum activity at pH 5.6 with Bz-Arg-NH_2 as substrate and at pH 5.0–5.5 with Bz-Gly–Arg and Cbz-Gly–Glu as substrates. The K_m values for all three substrates were in the concentration range of 10–15 mM.

TABLE II

pH OPTIMA, K_m VALUES AND V OF CATHEPSIN B2

For pH optima studies, the 0.5-ml reaction mixtures contained 2.4 μg of enzyme protein, 50 μmoles citrate–phosphate buffers at different pH values, 20 μmoles dithioerythritol, and either 25 μmoles of Cbz-Gly–Glu or 50 μmoles of Bz-Gly–Arg or Bz-Arg-NH_2. The incubation period ranged from 30 to 60 min. The reaction was terminated by addition of 0.5 ml of 10% trichloroacetic acid, and the released amino acids were measured fluorometrically as described in Materials and Methods. K_m determinations were made at the pH optimum for each substrate with 2.4 μg of enzyme protein. Linear regression analysis was applied to determine the K_m and V values.

Substrate	pH optimum	K_m (mM)	V (nmoles/min/ml)
Bz-Arg-NH_2	5.6	11.8	6950
Bz-Gly–Arg	5.0–5.5	10.2	208
Cbz-Gly–Glu	5.0–5.5	14.8	185

Effect of sulfhydryl activators and Cl^-

The purified enzyme preparation, extensively dialyzed to remove mercaptoethanol and NaCl, was used to study the effect of sulfhydryl compounds and Cl^- on hydrolytic activity. With Bz-Arg-NH_2·acetate, Cbz-Gly–Glu, and Bz-Gly–Arg as substrates, no effect of NaCl on the activity of the enzyme was noted. However, with all three substrates, maximal activity of the enzyme was obtained with 6 mM dithioerythritol, which suggests a requirement for sulfhydryl compounds. Other sulfhydryl compounds tested include mercaptoethanol and glutathione. Dithioerythritol exerted the maximum activation effect on the enzyme, while a larger amount of mercaptoethanol was required to obtain a similar effect. The effect of glutathione could be

tested only on Bz-Arg-NH_2 hydrolase, since this compound interfered with fluorometric measurement of amino acids released from Bz-Gly–Arg and Cbz-Gly–Glu. The activation effect of glutathione was considerably less than the activation effects found with dithioerythritol and mercaptoethanol.

pH and heat stability

The response of cathepsin B2 to different pH and temperature conditions as measured by the hydrolysis of Bz-Arg-NH_2, Bz-Gly–Arg, and Cbz-Gly–Glu is depicted in Fig. 3. The three hydrolytic activities of cathepsin B2 were not stable above pH 6.5 for all temperature conditions tested. The hydrolytic activities were

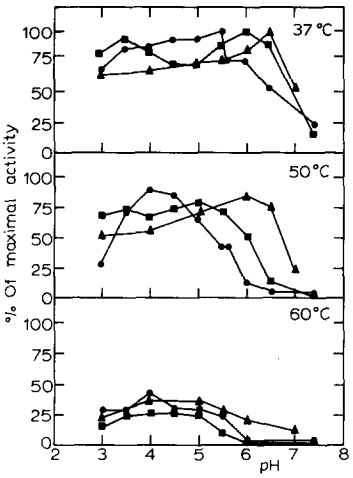

Fig. 3. pH and heat stability of cathepsin B2 activity toward Bz-Arg-NH_2 (●—●), Bz-Gly–Arg (▲—▲) and Cbz-Gly–Glu (■—■). Results are presented as a percentage of the activity of an unincubated control. Experimental details are outlined under Materials and Methods.

stable between pH 4.0 and 6.0 at 37, 50 and 60 °C, although the activities were diminished at higher temperatures. Some minor variations occur in the pH–stability curves at 37, 50 and 60 °C for the three hydrolytic activities. Some of this variation is due to experimental error, and these curves represent the mean values for one experiment. At 50 °C, the discrepancies seem to be greater than at other temperatures, and this is difficult to rationalize. In general, the pH–stability relationships for the three hydrolytic activities are reasonably similar, which suggests that these activities may originate from a single enzyme.

Hydrolysis of N-blocked dipeptides by cathepsin B2

Table III shows the hydrolysis rate of a series of N-blocked dipeptides by cathepsin B2. The highest rates of hydrolysis were obtained with substrates that contained the amino acids tryptophan, tyrosine, phenylalanine, glutamic acid, or serine in the C-terminal position. However, the hydrolysis rates were appreciable for all the C-terminal amino acids tested. This suggests that cathepsin B2 is relatively nonspecific with regard to the C-terminal amino acid. The hydrolysis rates were decreased

TABLE III

HYDROLYSIS OF N-BLOCKED DIPEPTIDES BY CATHEPSIN B2

The details are given under Materials and Methods. Spec. act. is expressed as μmoles of amino acid released per min per mg protein. Hydrolysis of each substrate is compared to that of Cbz-Gly–Glu.

N-blocked dipeptide	Spec. act.	Hydrolysis (%)
Cbz-Gly–Glu	25.4	100
Cbz-Glu–Phe	24.4	96
Cbz-Glu–Tyr	22.3	88
Cbz-Gly–Gly	12.7	49
Cbz-Gly–Met	17.8	70
Cbz-Gly–Ser	20.8	82
Cbz-Gly–Leu	11.8	46
Cbz-Gly–Tyr	16.3	64
Cbz-Gly–Arg	11.3	44
Cbz-Gly–Asp	14.4	56
Cbz-Gly–Trp	25.8	102
Cbz-Gly–Phe	15.8	62
Cbz-Gly–D-Phe	1.6	6
Cbz-Gly–His	16.9	67
Cbz-Glu–Gly	15.5	61
Cbz-Leu–Gly	12.0	47
Cbz-Trp–Gly	8.9	35
Cbz-Phe–Gly	4.8	19
Cbz-Tyr–Gly	3.8	15
Cbz-His–Gly	4.4	17
Cbz-Ser–Gly	7.7	30
Cbz-Met–Gly	13.6	53
Cbz-Gln–Gly	8.4	33
Bz-Gly–Arg	8.9	35
Cbz-Sar–Gly	0.7	3
Cbz-Pro–Gly	1.0	4
Cbz-β-Ala–Gly	1.0	4
Bz-Arg	0.4	1
N-Ac-Gly–Leu amide	0	0

for N-blocked dipeptides that contained glycine in the C-terminal position. Cathepsin B2 had somewhat different specificity for the amino acid in the penultimate position. Glutamic acid, serine, and tryptophan were among the favorable amino acids for the penultimate position, but Cbz-Phe–Gly and Cbz-Tyr–Gly were not particularly good substrates. Overall, cathepsin B2 seems to be rather nonspecific with regard to the penultimate amino acid, except proline, which is not hydrolyzed. The carboxypeptidase nature of cathepsin B2 is evident from the lack of hydrolysis of Ac-Gly–Leu-NH_2. However, since cathepsin B2 hydrolyzes Bz-Arg-NH_2, it must have a certain amount of amidase activity. Cathepsin B2 also had specificity for L-α-amino acids, based on the lack of hydrolysis of Cbz-Gly–D-Phe, Cbz-β-Ala–Gly, Cbz-Sar–Gly, and Cbz-Pro–Gly.

Hydrolysis of dipeptides, tripeptides, and polypeptides

The results on the hydrolysis of dipeptides and tripeptides by purified cathepsin B2 are presented in Table IV. Dipeptides were not hydrolyzed by cathepsin B2, but

TABLE IV

HYDROLYSIS OF DIPEPTIDES AND TRIPEPTIDES BY CATHEPSIN B2

Details are given under Materials and Methods.

Substrate	Total activity (nmoles product/min/ml)	Products identified
Glu–Tyr, Gly–Glu, Trp–Glu, His–Ser, Trp–Leu, Gly–Phe, Arg–Phe, Arg–Gly, Gly–Lys, Gln–Gly	0	–
Glu–Gly–Phe	308	Phe, Glu–Gly
Arg–Gly–Gly	27	–
Gly–Gly–Glu	15	–
Lys–Lys–Lys	0	–

hydrolysis was noted of the tripeptide substrates tested, with the exception of trilysine. Highest activity was obtained with Glu–Gly–Phe, and thin-layer chromatography of the hydrolytic products yielded distinct spots for phenylalanine and Glu–Gly. This again pointed out the inability of the enzyme to hydrolyze dipeptides.

The hydrolysis of longer chain-length peptides by cathepsin B2 is shown in Table V. Comparatively low total activity values for released products were obtained by the phthalaldehyde detection method when compared to those obtained by the ninhydrin detection method. This may be explained by the fact that ninhydrin reacts with unreacted peptides, particularly dipeptides, to a greater extent that does phthalaldehyde. The most extensive hydrolysis occurred with short-chain peptide substrates, perhaps because the concentration of C-terminal amino acids in the reaction mixtures was greater. When the tetra- and hexapeptides were tested, all the amino acid products were identified with the exception of leucine and tryptophan. The N-terminal dipeptide, Leu–Trp, seemed to be the limiting factor to further hydrolysis because of the inability of the enzyme to hydrolyze dipeptides. Among the polypeptides tested, glucagon was hydrolyzed to the greatest extent. The hydrolysis of glucagon yielded six amino acid products from its C-terminal end. Further hydrolysis of glucagon might be expected based upon the results obtained with the N-blocked dipeptides

TABLE V

HYDROLYSIS OF POLYPEPTIDES BY CATHEPSIN B2

The details of the experiment are described in Materials and Methods.

Peptide	Total activity (nmoles amino acid/min/ml)		Products
	Ninhydrin method	Fluorometric method	
Leu–Trp–Met–Arg	655	528	Met, Arg, Leu–Trp
Leu–Trp–Met–Arg–Phe–Ala	417	288	Ala, Phe, Arg, Met, Leu–Trp
Glucagon	202	123	Thr, Asn, Met, Leu, Gln, Trp
Insulin A chain	33	5	–
Insulin B chain	42	15	–
Bradykinin	6	27	–

(Table III). Hydrolysis for longer periods of time may be required to release further amino acids. Insulin A and B chains and bradykinin were not hydrolyzed to any great extent, even though they possessed C-terminal amino acids that should have been released (Table III). This may be explained by the presence of proline near the C-terminus of insulin B chain and bradykinin. The lack of hydrolysis of insulin A chain might be due to the presence of a cysteic acid residue in the penultimate position. No endopeptidase activity by cathepsin B2 was noted for any of the polypeptide substrates.

Effect of p-hydroxymercuribenzoate on cathepsin B2 activity

p-Hydroxymercuribenzoate at a concentration of 100 μM inhibited the various hydrolytic activities of cathepsin B2 to the following extent: Bz-Arg-NH_2 hydrolase, 95%; Bz-Gly–Arg hydrolase, 76%; and Cbz-Gly–Glu hydrolase, 100%.

Storage stability of cathepsin B2

When the purified enzyme was dialyzed against water, 30% of the activity was lost within 24 h, irrespective of storage conditions. However, when the enzyme was dialyzed against buffer at pH 5.3 in the presence of mercaptoethanol, it could be stored for a week at 0–4 °C without loss of activity.

DISCUSSION

Rat liver cathepsin B2 has been shown to be a rather nonspecific sulfhydryl-activated carboxypeptidase. The similar elution properties of the carboxypeptidase activity and the Bz-Arg-NH_2 hydrolyzing activity on both gel filtration and ion-exchange chromatography, coupled with the similar pH and temperature stabilities and the similar inhibition by *p*-hydroxymercuribenzoate support this conclusion. Although most of the evidence found in this study supports the concept of a single enzyme that hydrolyzes Bz-Arg-NH_2, Bz-Gly–Arg, and Cbz-Gly–Glu, it is difficult to envision a carboxypeptidase with amidase activity. More work is needed to firmly establish this identity, such as further purification to homogeneity, studies with a greater variety of inhibitors, and K_i determinations for these inhibitors. Cathepsin B2 had no endopeptidase activity toward glucagon, bradykinin, insulin A chain, or insulin B chain. Previous work [16] has shown extensive hydrolysis of histones by cathepsin B2. However, this may be the result of extensive carboxypeptidase activity. Results obtained with peptide hydrolysis suggest that proline and cysteic acid are the limiting factors for the carboxypeptidase activity of cathepsin B2. Histones contain relatively few proline residues. Additional support for this possibility is the observation that other basic proteins such as lysozyme, cytochrome *c*, and protamine sulfate are not extensively hydrolyzed by cathepsin B2 [16]. Although evidence for lysosomal dipeptidase activity exists [17], cathepsin B2 did not hydrolyze dipeptides, but tripeptides and larger peptides were readily hydrolyzed.

Several carboxypeptidases that are active in the acid pH region have been reported in the literature, including cathepsin A [18, 19], catheptic carboxypeptidase A [20], catheptic carboxypeptidase B [21], catheptic carboxypeptidase C [22] or prolyl carboxypeptidase [23], catheptic carboxypeptidase G [7], Ac-Phe–Tyr hydrolase [7, 24], a tripeptide carboxypeptidase [25], angiotensinase [26, 27], and brady-

kininase [21]. Many of these acid carboxypeptidases are likely to be lysosomal, although this had not been critically determined in most instances. Taylor and Tappel [18] have shown that rat liver cathepsin A is a carboxypeptidase with specificity for hydrophobic amino acids. Cathepsin A and Ac-Phe–Tyr hydrolase were shown to be identical in rat liver [18]. Cathepsin B2 has been found to be responsible for the catheptic carboxypeptidase B and catheptic carboxypeptidase G activity reported earlier [7]. A third, distinct carboxypeptidase, prolyl carboxypeptidase, exists in rat liver lysosomes [22, 23], and is probably responsible for the angiotensinase [26, 27] activity of lysosomes. Lysosomes seem to have a full complement of the requisite carboxypeptidases for complete hydrolysis of peptides and proteins from the C-terminus.

Several partial purifications of cathepsin B2 have been reported earlier [4, 28]. Otto [4] described a preparation of cathepsin B2 from bovine spleen that was almost free from the endopeptidase activity of cathepsin D. Taylor and Tappel [7] showed that gel filtration of a lysosomal $(NH_4)_2SO_4$ fraction on Sephadex G-100 led to a cathepsin B2 preparation completely free from cathepsins A, B1, C and D. This work was expanded here by use of an ion-exchange column after the gel-filtration step. The 3900-fold purification of cathepsin B2 for the Bz-Arg-NH_2-hydrolyzing activity is the highest purification and the specific activity was the highest ever achieved for this enzyme. Although electrophoresis showed that this preparation was not completely pure, contaminating protease activity was not found. Attempts to purify cathepsin B2 further were unsuccessful, perhaps owing to instability of cathepsin B2 upon excessive dilution.

The sulfhydryl dependency of cathepsin B2 coupled with the inhibition by *p*-hydroxymercuribenzoate suggests the presence of critical cysteine residues in the enzyme. The sulfhydryl dependence of cathepsin B2 has been reported in several previous studies [4, 16]. De Lumen and Tappel [16] reported halide activation of the histone hydrolase and Bz-Arg-NH_2 hydrolase activities of cathepsin B2. However, no halide activation was observed for the Bz-Arg-NH_2-hydrolyzing or carboxypeptidase activities of this cathepsin B2 preparation. Halide ions may serve to neutralize the positive charge of histones at pH 5.5 to cause an activation effect, but no satisfactory explanation of the differences in halide activation of Bz-Arg-NH_2 hydrolase activity is readily apparent. Further work is in progress to elucidate the active site components of cathepsin B2.

The existence of a relatively nonspecific carboxypeptidase, such as cathepsin B2, in rat liver lysosomes is very significant in the determination of the rate and extent of lysosomal protein hydrolysis. Exhaustive hydrolysis of proteins by lysosomal protease–peptidase mixtures has been shown to yield dipeptide and amino acid products [29]. Sulfhydryl activation of this overall hydrolysis has been noted, also [30, 31]. The concerted action of endopeptidases such as cathepsin D and cathepsin B1 and exopeptidases such as cathepsin A, cathepsin B2, and cathepsin C could account for the hydrolysis of proteins to dipeptides and amino acids. Kussendrager et al. [32] have identified the predominant products of extensive insulin B chain hydrolysis as amino acids, dipeptides, and three cysteic acid-containing tetrapeptides. From the known specificity of rat liver lysosomal cathepsin C [33] and cathepsin D [2] on insulin B chain, coupled with the specificity of cathepsin B2 shown here, these products might be predicted with the exception that the Pro^{28}–Lys^{29} bond

was split. The presence of other endopeptidases and exopeptidases, such as cathepsin B1 and cathepsin A, would affect only the rate and not the extent of hydrolysis of insulin B chain. However, cathepsin B1 and cathepsin A may play important roles in the hydrolysis of other peptides and proteins, such as the collagenase activity recently ascribed to cathepsin B1 [34]. One relatively important point is that cathepsins B1 and D tend to expose peptide fragments with hydrophobic amino acids in the C-terminal positions, and the lysosomal carboxypeptidases, cathepsin B2 and cathepsin A, favor hydrophobic amino acids in the C-terminus. Therefore, the synergistic effect of exopeptidases, especially the relatively nonspecific exopeptidases such as cathepsin B2, on the hydrolysis rates of proteins by endopeptidases could be particularly dramatic.

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REFERENCES

1 Tallan, H. H., Jones, M. E. and Fruton, J. S. (1952) J. Biol. Chem. 194, 793–805
2 Kielova, H. (1971) in Tissue Proteinases (Barrett, A. J. and Dingle, J. T. ,eds) pp. 45–68, American Elsevier, New York
3 Blackwood, C. and Mandl, I. (1961) Anal. Biochem. 2, 370–379
4 Otto, K. (1971) in Tissue Proteinases (Barrett, A. J. and Dingle, J. T., eds) pp. 1–28, American Elsevier, New York
5 Otto, K. and Bhakdi, S. (1969) Hoppe-Seyler's Z. Physiol. Chem. 350, 1577–1588
6 de Lumen, B. O. and Tappel, A. L. (1972) J. Biol. Chem. 247, 3552–3557
7 Taylor, S. L. and Tappel, A. L. (1974) Biochim. Biophys. Acta 341, 99–111
8 De Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Biochem. J. 60, 604–617
9 Taylor, S. L. and Tappel, A. L. (1973) Anal. Biochem. 56, 140–148
10 Taylor, S. L., Ninjoor, V., Dowd, D. M. and Tappel, A. L. (1974) Anal. Biochem., 60, 153–162
11 De Lumen, B. O. and Tappel, A. L. (1972) Anal. Biochem. 48, 378–385
12 Bohlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155. 213–220
13 Moore, S. and Stein, W. H. (1954) J. Biol. Chem. 211, 907–913
14 Davis, B. J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427
15 Fishbein, W. N. (1972) Anal. Biochem. 46, 388–401
16 De Lumen, B. O. and Tappel, A. L. (1973) Biochim. Biophys. Acta 293, 217–225
17 Goldman, R. (1973) FEBS Lett. 33, 208–212
18 Taylor, S. L. and Tappel, A. L. (1974) Biochim. Biophys. Acta 341, 112–119
19 Iodice, A. A. (1967) Arch. Biochem. Biophys. 121, 241–242
20 Mellors, A. (1971) Arch. Biochem. Biophys. 144, 281–285
21 Greenbaum, L. M. and Yamafuji, K. (1965) Life Sci. 4, 657–663
22 McDonald, J. K., Zeitman, B. B. and Ellis, S. (1972) Biochem. Biophys. Res. Commun. 46, 62–70
23 Kakimoto, T., Oshima, G., Yeh, H. S. J. and Erdos, E. G. (1973) Biochim. Biophys. Acta 302, 178–182
24 Menzies, C. A. and McQuillan, M. T. (1967) Biochim. Biophys. Acta 132, 444–453
25 Stein, U., Weber, U. and Buddecke, E. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 472–484
26 Matsunaga, M. (1971) Jap. Circ. J. 35, 333–338

27 Yang, H. Y. T., Erdos, E. G., Chiang, T. S., Jenssen, T. A. and Rodgers, J. G. (1970) Biochem Pharm. 19, 1201–1211
28 Franklin, S. G. and Metrione, R. M. (1972) Biochem. J. 127, 207–213
29 Coffey, J. W. and De Duve, C. (1968) J. Biol. Chem. 243, 3255–3263
30 Huang, F. L. and Tappel, A. L. (1971) Biochim. Biophys. Acta 236, 739–748
31 Huisman, W., Bouma, J. M. W. and Gruber, M. (1973) Biochim. Biophys. Acta 297, 98–109
32 Kussendrager, K. D., de Jong, Y., Bouma, J. M. W. and Gruber, M. (1972) Biochim. Biophys Acta 279, 75–86
33 Callahan, P. X., McDonald, J. K. and Ellis, S. (1972) Fed. Proc. 31, 1105–1113
34 Burleigh, M. C., Barrett, A. J. and Lazarus, G. S. (1974) Biochem. J. 137, 387–398