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ON THE SUBSTRATE SPECIFICITY OF CATHEPSINS B1 AND B2 INCLUDING A NEW FLUOROGENIC SUBSTRATE FOR CATHEPSIN B1

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Cathepsin B1 from bovine spleen exhibited its greatest rates of hydrolysis on peptide β-naphthylamide (βNA) derivatives containing paired basic residues, i.e., Cbz-Arg-Arg-βNA, t-Boc-Lys-Lys-βNA, and t-Boc-Lys-Arg-βNA. Internal peptide bonds were not attacked. At its pH 6.5 optimum, cathepsin B1 hydrolyzed Cbz-Arg-Arg-βNA (K_m 0.18 mM) 64 times faster than Bz-DL-Arg-βNA (K_m 3.3 mM or 1.6 mM for the L isomer) and was therefore chosen to replace the latter as a more soluble and sensitive substrate for the assay of cathepsin B1. Although cathepsin B2 had no action on the β-naphthylamide substrates, it did manifest carboxypeptidase activity by attacking COOH-terminal residues exposed by the action of cathepsin B1. At its pH 5.0 optimum, cathepsin B2 behaved as a SH-dependent, non-specific carboxypeptidase by releasing COOH-terminal amino acids from a variety of Cbz-Gly-X substrates and polypeptides such as glucagon, Val-Leu-Ser-Glu-Gly, and penta-lysine.

It was first deomonstrated by Otto (1) that bovine spleen cathepsin B (EC 3.4.22.1) prepared according to Greenbaum and Fruton (2) could be resolved into two distinct components (now termed cathepsins B1 and B2) by gel filtration. Both activities catalyzed a SH-dependent hydrolysis of α-N-benzoyl-L-arginine amide (Bz-Arg-NH_2), but only the smaller component (cathepsin B1) hydrolyzed the p-nitroanilide derivative. This finding was subsequently confirmed by us (3) whereupon it was shown that only cathepsin B1 hydrolyzed α-N-benzoyl-DL-arginine β-naphthylamide (Bz-Arg-βNA). In addition, it was demonstrated that the cathepsin B1 fraction contained a SH-activated, puromycin-sensitive aminopeptidase that was separable by gradient elution chromatography on DEAE-cellulose (3).

The present report describes the results of a substrate specificity study conducted with bovine spleen cathepsins B1 and B2 on a wide variety of synthetic peptide derivatives. A new, more soluble fluorogenic substrate is described that offers vastly improved sensitivity and specificity for the assay and histochemical detection of cathepsin B1--an endopeptidase. Beef spleen cathespin B2, on the other hand, was found to be a non-specific carboxypeptidase that now appears to have been responsible for the SH-dependent carboxypeptidase A and B activities previously observed in cathepsin B preparations by Greenbaum and Sherman (4). Similarly, Ninjoor et al. (5) recently reported that rat liver cathepsin B2 behaved as a non-specific carboxypeptidase.

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Methods and Materials

Preparation of Cathepsins B1 and B2 -- These enzymes were prepared from fresh bovine spleen using a procedure described earlier (3) that utilized a 22-hr acid extraction at pH 3.5 and 37° followed by recovery of the protein precipitated with ammonium sulfate between 40 and 70% saturation. These early steps are the same as those described by Metrione et al. (6) for the extraction of dipeptidyl aminopeptidase I (cathepsin C) from bovine spleen. The ammonium sulfate fraction was subjected to column chromatography on Sephadex G-100 as described earlier (3) to resolve cathepsin B into its B1 and B2 components. The SH-dependent aminopeptidase found to contaminate cathepsin B1 was then removed by gradient elution chromatography on DEAE-cellulose (3). This yielded a preparation of cathepsin B1 that hydrolyzed 0.6 μmole of Bz-Arg-βNA per min per mg protein under the conditions described for the fluorometric assay of cathepsin B1. This preparation, which was found to be free of other common lysosomal peptide hydrolases, was used in the studies described here.

When cathepsin B2 was similarly chromatographed on DEAE-cellulose, contaminating cathepsin D emerged unadsorbed. The activity of cathepsin B2 measured on Bz-Arg-NH_2 did not elute until the salt gradient reached 0.23 M. Of particular note, the carboxypeptidase A and B activities (4) measured at pH 5.0 on Bz-Gly-Phe and Bz-Gly-Arg, respectively, were perfectly coincident with the cathepsin B2 peak. These activities exhibited a similar coincidence during prior gel filtration, and in separate electrofocusing experiments. Such parallel behavior strongly suggested that cathepsin B2 itself was responsible for the observed carboxypeptidase activities. Such a preparation of cathepsin B2 hydrolyzed 3.8 μmoles Bz-Arg-NH_2 per min per mg protein under the conditions described for the amidase assay of cathepsin B2, and was used to obtain the data described in this writing. Protein concentrations were determined by the Lowry procedure (7) with bovine serum albumin as the standard.

Fluorometric Assay of Cathepsin B1 -- To 3.8 ml of 10 mM dithiothreitol-10 mM EDTA-50 mM sodium phosphate buffer, pH 6.5, was added 0.1 ml of an appropriately diluted sample of cathepsin B1. The enzyme was preincubated 5 min at 37°, and the reaction was initiated by adding 0.1 ml of a 20 mM substrate solution. Substrates containing at least two basic residues were dissolved in water; the others were dissolved in dimethylformamide. Reaction rates were measured by a direct, recording technique using a fluorometer calibrated with free β-naphthylamine (8). A colorimetric method could also be employed (9).

Amidase Assay for Cathepsins B1 and B2 -- To 0.2 ml of diluted enzyme was added 0.2 ml of 20 mM dithiothreitol-40 mM EDTA-0.4 M sodium citrate buffer, pH 5.5. Following 5 min of preincubation at 37°, the reaction was initiated by adding 0.4 ml of 0.1 M Bz-Arg-NH_2. At intervals of 5 and 15 min, 0.2 ml aliquots were withdrawn for ammonia determination by diffusion and reaction with Nessler's reagent (10).

Other Peptide Hydrolases Assayed During Purification -- Cathepsin A was assayed on Cbz-Glu-Tyr at pH 5.5 (11), dipeptidyl aminopeptidase I (cathepsin C) on Gly-Phe-βNA at pH 6.0 (8), and cathepsin D on urea-denatured hemoglobin at pH 4.0 as described for the assay of proteinase I (12). The SH-dependent carboxypeptidase A and B activities were assayed at pH 5.0 on Bz-Gly-Phe and Bz-Gly-Arg, respectively (4). Catheptic carboxypeptidase C was assayed on Cbz-Pro-Phe at pH 5.5 (13), and lysosomal dipeptidase on Ser-Met at pH 5.5 (13).

Abbreviations: Cbz-, α-N-benzyloxycarbonyl-; Bz-, α-N-benzoyl-; t-Boc, tert-butyloxycarbonyl-; βNA, β-naphthylamide; 4MeOβNA, 4-methoxy-β-naphthylamide.

Assay Used to Survey Carboxypeptidase Specificity of Cathepsin B2 -- To 0.1 ml (2 µg) of cathepsin B2 was added 0.4 ml of 10 mM dithiothreitol-0.2 M sodium acetate buffer, pH 5.0. The mixture was preincubated 5 min at 37°, and the reaction was initiated by adding 0.5 ml of a 40 mM solution of N-blocked dipeptide. At intervals of 10, 20, and 30 min, 0.1 ml aliquots were combined with 2.9 ml of 0.15 M sodium borate buffer, pH 9.0. The concentration of free amino acid was determined fluorometrically (14) by adding 1.0 ml (0.2 mg) of fluorescamine (Roche, Nutley, N.J.) in acetone and reading the fluorescence in a fluorometer against a standard curve for the appropriate amino acid.

Identification of Split Products -- Products resulting from the hydrolysis of basic, fluorogenic substrates by cathepsins B1 and B2 were identified by thin layer chromatography on microcrystalline cellulose. The plates were developed with sec-butanol-3% NH_3 (75:30), dried, and sprayed with a 0.01% solution of phenanthrenequinone in 5% NaOH-80% ethanol. This reagent (15) converted all arginine-containing peptides to fluorescent derivatives that could be visualized and photographed under long-wave ultraviolet light. Free β-naphthylamine was detectable by its inherent UV fluorescence. The phenanthrenequinone spray was sometimes preceded by a ninhydrin spray when needed to detect fragments not containing arginine. A peptide spray (16) was used to detect N-substituted peptides. Peptide digests (other than of homologs of lysine) were also analyzed chromatographically, but with a solvent consisting of n-butanol-formic acid-water (70:15:15). Digests of penta-lysine were analyzed by high voltage paper electrophoresis using 2000 volts and 58 ma at pH 3.5 for 2 hr. All fragments were detected with 0.2% ninhydrin in acetone.

Sources of Substrates -- The peptide β-naphthylamide substrates were available from, or were custom synthesized by, Fox Chemical Company (Los Angeles, Calif.). Cbz-Arg-Arg-βNA was synthesized by Mr. Rao Makineni of Bachem, Inc. (Marina Del Ray, Calif.) according to a published procedure (17). Cbz-Arg-Arg-Arg-βNA was kindly provided by Dr. Robert E. Plapinger of the Sinai Hospital of Baltimore, Inc., and Cbz-Ala-Arg-Arg-4MeOβNA by Dr. Robert E. Smith of Lilly Research Laboratories (Indianapolis, Ind.). All other peptides were obtained in high purity from common commercial sources. The purity and identity of these substrates were established by thin layer chromatography and amino acid analysis. Except for Bz-DL-Arg-βNA, all the peptides were comprised of residues having the L configuration.

Results

Rates of Hydrolysis of Peptide β-Naphthylamides by Cathepsin B1 -- Table I compares the absolute and relative rates of cleavage, at pH 6.5, of the β-naphthylamide bond contained in a variety of peptide substrates. When these rates were compared with that obtained on the Bz-Arg-βNA, the traditional arylamide substrate for cathepsin B1, greatly enhanced rates were observed on a variety of substrates containing paired basic residues of arginine or lysine. For example, the rate on Cbz-Arg-Arg-βNA was 64 times greater than the rate on Bz-Arg-βNA. If the NH_2 terminus was unsubstituted, as in Arg-Arg-βNA, the rate of hydrolysis was reduced to about 1% of that on the NH_2-blocked substrate. Rates on other substrates included in Table I show that cathepsin B1 was also able to hydrolyze seryl and methionyl bonds at rates comparable to that observed on Bz-Arg-βNA. No hydrolysis was detected on the histidyl bond in pyroglutamyl-His-βNA or the phenylalanyl bond in glutaryl-Phe-βNA.

In contrast to the specificity of pancreatic trypsin, the action of cathepsin B1 was not enhanced by simply increasing the peptide chain length of the substrate, i.e., Cbz-Gly-Gly-Arg-βNA. Whereas trypsin is reported (18) to hydrolyze this substrate 220 times faster than Bz-Arg-βNA, cathepsin B1 actually showed a lower rate of hydrolysis on the larger substrate. Although

TABLE I
Rates of Hydrolysis of Peptide β-Naphthylamide Substrates
By Bovine Spleen Cathepsin B1 At pH 6.5 and 37°

Substrate	[S]	Hydrolysis Rate*	Relative Rates Cathepsin B1	Relative Rates Trypsin†
Bz-DL-Arg-βNA	0.5 mM	0.6	1.0	1.0
Cbz-Arg-Arg-Arg-βNA	"	45	75	113
Cbz-Arg-Arg-βNA	"	38	64	12
Cbz-Ala-Arg-Arg-4MeOβNA	"	38	64	
t-Boc-Lys-Arg-βNA	"	23	38	
t-Boc-Lys-Lys-βNA	"	14	23	
N-Acetyl-Ala-Met-βNA	0.25	2.2	3.6	
Cbz-His-Ser-βNA	"	1.5	2.5	
Arg-Arg-βNA	"	0.43	0.72	3.5
Cbz-Gly-Gly-Arg-4MeOβNA	"	0.24	0.40	
Cbz-Gly-Gly-Arg-βNA	"	0.18	0.30	220
Pro-Lys-Ala-4MeOβNA	"	0.18	0.30	
N-Acetyl-Gly-Lys-βNA	"	0.07	0.12	
Glutaryl-Phe-βNA	"	0	0	
Pyroglutamyl-His-βNA	"	0	0	

*Rates expressed as μmoles/min/mg protein.
†Rates reported by Nachlas et al. (Ref. 18)

Cbz-Arg-Arg-Arg-βNA was hydrolyzed most rapidly by cathepsin B1, Cbz-Arg-Arg-βNA was chosen as the preferred assay substrate because it was much less susceptible to attack by trypsin, and presumably other trypsin-like enzymes.

Caution should be exercised when comparing the rates listed in Table 1 since K_m values were not determined for most of the substrates tested and substrate saturation may not have been achieved since solubility restrictions required that most rates be compared at a substrate concentration of 0.25 mM. In the case of Cbz-Arg-Arg-βNA, the substrate of primary interest, the assay concentration was limited to 0.5 mM (2.8 times K_m) since substrate inhibition was encountered at higher levels. Other substrates containing paired basic residues were similarly employed at 0.5 mM.

Cathepsin B2 had no action on any of the β-naphthylamide derivatives listed in Table 1.

Split Products and Mode of Attack Exhibited by Cathepsins B1 and B2 on Cbz-Arg-Arg-βNA -- Since the substrate specificity results in Table I revealed that cathepsin B1 had a strong preference for substrates containing paired basic residues, Cbz-Arg-Arg-βNA was therefore used in a time course analysis of split products to establish whether the internal -Arg-Arg- bond was cleaved by either cathepsin B1 or cathepsin B2. The thin layer chromatographic analysis shown in Fig. 1 revealed that the hydrolysis of the β-naphthylamide bond by cathepsin B1 was virtually complete within 10 min. The -Arg-Arg- bond was totally resistant as indicated by the fact that β-naphthylamine and Cbz-Arg-Arg were the only split products detected after 2 hr of digestion. An identical study conducted with Cbz-Ala-Arg-Arg-4MeOβNA revealed that the attack of cathepsin B1 was similarly limited to the arylamide bond. Cathepsin B2, on the other hand, showed no action on any of the bonds in Cbz-Arg-Arg-βNA. However, when cathepsin B2 was added following 2 hr of digestion by cathepsin B1 (Fig. 1), the former then behaved as a carboxypeptidase and cleaved the COOH-terminal arginine from Cbz-Arg-Arg. The pH was lowered to 4.5 to facilitate the carboxypeptidase activity of cathepsin B2.

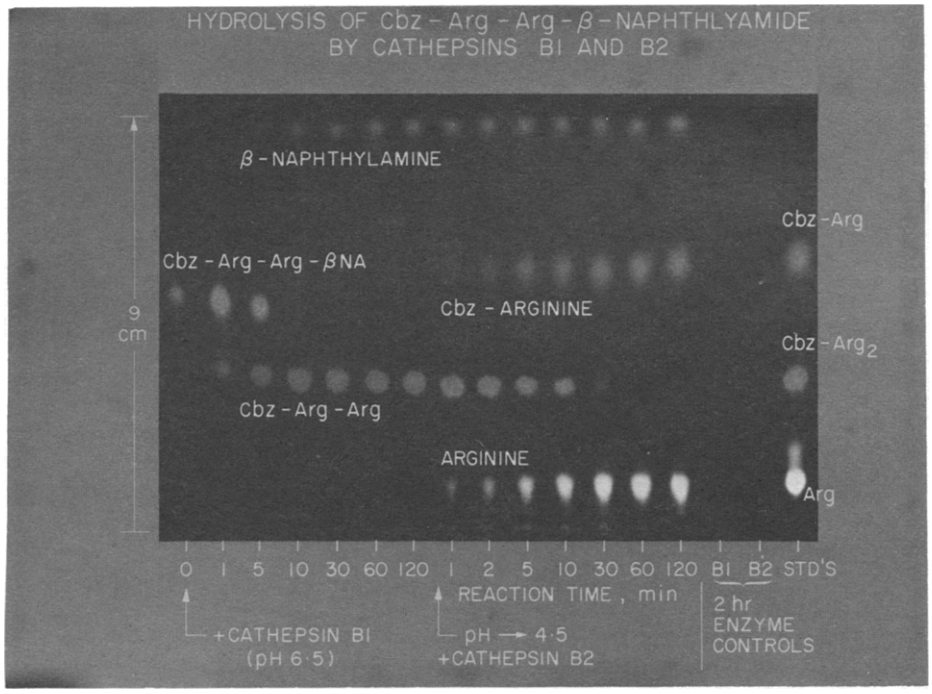

FIG. 1

Chromatographic identification of the products of Cbz-Arg-Arg-β-naphthylamide hydrolysis by cathepsins B1 and B2. Cathepsin B1 (18 µg) was preincubated for 5 min at 37° in 0.5 ml of 10 mM dithiothreitol-10 mM EDTA-0.1 M sodium phosphate buffer, pH 6.5. To this was added 0.5 ml of 6 mM Cbz-Arg-Arg-β-naphthylamide in water. At the designated time intervals, 0.5 µl aliquots were spotted on thin layer cellulose. For the cathepsin B2 reaction, a 50 µl aliquot of the 2-hr reaction mixture was combined with 20 µl of 0.1 M acetic acid to give pH 4.5, followed by 25 µl of 40 mM dithiothreitol-40 mM EDTA, and finally 5 µl (23 µg) of cathepsin B2 (or water for the substrate control). Aliquots (1 µl) were spotted at the designated time intervals. Enzyme controls (B1 and B2) and standards were included. The chromatogram was developed and visualized as described under Methods.

Controls subjected to this pH shift showed no liberation of arginine without the addition of cathepsin B2.

Effect of Activators, pH, and Substrate Concentration on the Hydrolysis of Cbz-Arg-Arg-βNA by Cathepsin B1 -- Cathepsin B1 exhibited a pH optimum of 6.5 in 50 mM sodium phosphate buffer for the hydrolysis of Cbz-Arg-Arg-βNA. About 50% of the maximum rate was manifested at pH 7.2. The rate response to substrate concentration, which was rapid compared to that for Bz-Arg-βNA, reached a maximum at 0.5 mM. Substrate inhibition occurred at higher levels. The K_m values at pH 6.5 and 37° were found to be 0.18 mM for Cbz-Arg-Arg-βNA and 3.3 mM for Bz-DL-Arg-βNA (or 1.6 mM for the L isomer). V_{max} was about 33 times higher for Cbz-Arg-Arg-βNA. Sulfhydryl activation was achieved with dithiothreitol. The activation was maximal at about 10 mM, and 80% maximal at

1 mM. At all levels of dithiothreitol the hydrolysis rates could be enhanced 2 to 3 fold by incorporated EDTA at 10 mM. EDTA alone was relatively ineffective.

Carboxypeptidase Specificity of Cathepsin B2 -- Pursuant to the observation (Fig. 1) that cathepsin B2 catalyzed a carboxypeptidase attack on Cbz-Arg-Arg, a more thorough assessment of its carboxypeptidase activity was carried out using a range of Cbz-Gly-X substrates. The survey was conducted at pH 5.0 since this was the optimum found on Bz-Gly-Arg and Bz-Gly-Phe. The rates listed in Table II show that cathepsin B2 acted with relatively little specificity when attacking COOH-terminal residues. The same appeared to be true when polypeptides were tested as substrates. Glycine, glutamic acid, and serine were liberated in that order from Val-Leu-Ser-Glu-Gly. Only traces of free valine and leucine were detected. Digests of glucagon gave rise to at least six amino acids known to reside at the COOH terminus, e.g., Thr, Asn, Met Leu, Trp, Gln. Penta-lysine was degraded to a mixture comprised of free lysine and tri-lysine. Tetra-lysine was seen as a transient intermediate during the time course.

Although the substrate concentration (20 mM) employed in Table II probably exceeded the K_m values for the substrates tested, some caution should be exercised when interpreting relative rates.

TABLE II

Rates of Hydrolysis of Various COOH-Terminal Residues By Beef Spleen Cathepsin B2 At pH 5.0 and 37°

Substrate (20 mM)	Rates of Hydrolysis Absolute*	Relative (%)
Cbz-Gly-Met	4.0	100
Cbz-Gly-Phe	3.7	93
Cbz-Gly-Ser	3.4	85
Cbz-Gly-Asp	2.8	70
Cbz-Gly-Arg	2.3	58
Cbz-Gly-Leu	1.9	47
Cbz-Gly-Gly	0.9	22

*Expressed as μmoles/min/mg protein.

Discussion

Most notable was the finding that bovine spleen cathepsin B1 exhibited greatly enhanced rates of hydrolysis on several peptide β-naphthylamide substrates containing paired basic residues. In sharp contrast with the reported specificity of trypsin (18), Cbz-Gly-Gly-Arg-βNA, with a single basic residue, was found to be a poor substrate for cathepsin B1. These studies led to the identification of Cbz-Arg-Arg-βNA as an improved substrate for the assay and localization of cathepsin B1. If necessary, the action of trypsin-like enzymes could probably be blocked by adding pancreatic trypsin inhibitor to the assay system. This inhibitor, when incorporated at a concentration as high as 150 μg per ml, had no affect on the rate of hydrolysis of Cbz-Arg-Arg-βNA by cathepsin B1.

The carboxypeptidase specificity exhibited by bovine spleen cathepsin B2 has also been observed for cathepsin B2 purified from rat liver lysosomes (5). Both enzymes were relatively non-specific in their action on NH_2-blocked dipeptides and polypeptides. Although the enzymes from both sources exhibited little or no action on tri-lysine, the bovine spleen enzyme rapidly degraded

penta-lysine to free lysine and tri-lysine. In view of the reported histone hydrolase activity of rat liver cathepsin B2 (19), it seems likely that this enzyme is capable of carrying out a carboxypeptidase attack on large, basic polypeptides. In all probability cathepsin B2 has also been responsible for the thiol-dependent activities previously referred to as catheptic carboxypeptidases A and B (4).

The one apparent substrate specificity anomaly exhibited by cathepsin B2 was its ability to hydrolyze Bz-Arg-NH_2 (and Bz-Arg-OEt) (1). However, this anomaly is shared with pancreatic carboxypeptidase B where it is believed that peptide and ester substrates occupy different loci on the enzyme (20). We suspect that cathepsin B2 may also have more than one active center since it was not possible to demonstrate competitive kinetics for Bz-Arg-NH_2 and Bz-Gly-Arg.

The observed specificity of cathepsin B1 was of special interest in view of recent observations that several peptide hormones appear to arise from prohormone precursors as a result of limited proteolysis involving the cleavage of bonds adjacent to paired basic residues (21). In studies concerned with this possibility (22), supporting evidence was obtained when cathespsin B1 was reacted with proinsulin at pH 6.5. The major product of this reaction had the same electrophoretic mobility as a reference sample of diarginyl insulin (comprised of insulin with two additional arginine residues attached at the COOH terminus of the B chain). Histochemical evidence for the presence of a cathepsin B1-like enzyme in the β-cells of the pancreas has been described by Smith et al. (23) using Cbz-Ala-Arg-Arg-4MeOβNA. Cathepsin B1 itself might possibly have such a function since the Golgi apparatus of these secretory cells may have the capability of incorporating this enzyme into newly-formed secretory granules as well as into lysosomes. The carboxypeptidase activity of a cathepsin B2-like enzyme could conceivably serve to complete the conversion to insulin by removing the arginine residues from diarginyl insulin.

It is now quite clear that the lysosomes of bovine spleen and rat liver contain three distinct carboxypeptidases, all of which carry the vestiges of early cathepsin nomenclature. Consistent with published studies, it is suggested that cathepsin A (11) be termed lysosomal carboxypeptidase A, cathepsin B2 be termed lysosomal carboxypeptidase B, and catheptic carboxypeptidase C (13) be termed lysosomal carboxypeptidase C. It follows that cathepsin B1 need only be referred to as cathepsin B. These lysosomal carboxypeptidases, together with two dipeptidyl aminopeptidases (24) and one dipeptidase (13) constitute the recognized exopeptidases undoubtedly responsible for the high yield of amino acids and dipeptides shown to result from the breakdown of complex proteins by lysosomal extracts (25).

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