J. Biochem. 93, 1119–1128 (1983)

Selective Cleavage of Peptide Bonds by Cathepsins L and B from Rat Liver[1]

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Received for publication, November 27, 1982

The selective cleavage of peptide bonds by cathepsin L from rat liver was examined with a hexapeptide, luteinizing hormone releasing hormone, neurotensin and oxidized insulin A chain as model substrates. The specificity of cathepsin L was compared with that of cathepsin B. Cathepsin L cleaved peptide bonds that have a hydrophobic amino acid, such as Phe, Leu, Val, and Trp or Tyr, in position P_2. A polar amino acid, such as Tyr, Ser, Gly, Glu, Asp, Gln, or Asn, in position P_1 enhanced the susceptibility of the peptide bond to cathespin L, though the importance of the amino acid residue in position P_1' was not as great as that of the amino acid in position P_2 for the action of cathepsin L. These results suggest that, in contrast to cathepsin B, cathepsin L shows very clear specificity.

Lysosomal thiol proteases are considered to be important in intracellular protein degradation, and limited proteolysis is thought to be an important initial step in protein degradation. Previously, we reported the purifications and some properties of two lysosomal thiol proteases, named cathepsin L [EC 3.4.22.-] (*1–3*) and cathepsin B [EC 3.4.22.1] (*4–6*). Our studies also showed that cathepsin L inactivated glucose-6-phosphate dehydrogenase and some other enzymes. Detailed information on the substrate specificities of lysosomal proteases is necessary for elucidating the mechanisms of degradation of proteins *in vitro* and *in vivo*. Therefore, in the present work, we investigated the proteolytic actions of cathepsin L from rat liver on the following polypeptide substrates: a hexapeptide, luteinizing hormone releasing hormone, neurotensin, and oxidized insulin A chain. The actions of cathepsin L on these substrates were compared with those of cathepsin B.

MATERIALS AND METHODS

Materials—Hexapeptide (Leu-Trp-Met-Arg-Phe-Ala) was purchased from Serva (Heidelberg). Oxidized insulin A chain, luteinizing hormone releasing hormone, neurotensin, and the danyl amino acids used as standards were purchased from Sigma Chemical Co. (St. Louis, Mo.). Dansyl chloride was obtained from Seikagaku Kogyo Co. (Tokyo). Dimethyl sulfoxide was obtained from Wako Pure Chemical Industries (Tokyo). Dowex 50W (×2) of 200–400 mesh was

[1] This work was supported by Grants-in-Aid for Scientific Research (Nos. 548370 and 56880028) from the Ministry of Education, Science and Culture of Japan.

purchased from Muromachi Kogyo Kaisha (Tokyo). All other chemicals were of reagent grade.

Methods—Cathepsin L and cathepsin B were purified from rat liver as described previously by Towatari *et al.* (*3*) and Towatari and Katunuma (*5*).

Digestion of polypeptide with cathepsin L: Hexapeptide, luteinizing hormone releasing hormone, neurotensin, and insulin A chain (oxidized form) were dissolved in distilled water at final concentrations of 10 μmol per ml. Total amounts of 1–4 μmol of each substrate were used and proteolytic hydrolysis was allowed to proceed at 25°C in 100 mM of acetate buffer, pH 5.0, containing a suitable amount of enzyme, 1.25 μmol of 2-mercaptoethanol, and 0.25 μmol of EDTA in a final volume of 0.25 ml. Reactions were stopped by addition of iodoacetic acid to a final concentration of 0.1 mM.

Digestion of polypeptides with cathepsin B: The reaction was carried out under the same conditions as for cathepsin L except that the reaction mixture contained 2.5 μmol of 2-mercaptoethanol.

Samples of digests were divided in half and applied to silica gel TLC plates (20 cm × 20 cm) as 13-cm streaks. Peptide products were analyzed by ascending chromatography in *n*-butanol–acetic acid–water (30 : 10 : 10 or 40 : 10 : 10, by vol). The plates were dried and separated bands were located by staining guide-strips with ninhydrin solution (0.2 g of ninhydrin in 100 ml of acetone). Materials in the bands were eluted with 0.1 N HCl. In one experiment (with oxidized insulin A chain digest), the peptide products were initially separated by ion-exchange chromatography on Dowex 50W (×2) resin developed with a linear gradient of pyridine-acetate buffer (*7*). The peptides were located in the effluent with ninhydrin after alkaline hydrolysis (*8*). Fractions containing separated peptides were combined and dried by rotary evaporation under reduced pressure at 37°C. The concentrated fractions were then dissolved in 1.0 ml of distilled water and purified further by high voltage electrophoresis in pH 6.5 buffer (pyridine–acetic acid–water, 100 : 4 : 900). The peptides were then eluted from the paper with 0.1 N HCl and hydrolyzed for 24 h at 110°C with 6 N HCl containing 0.1% phenol under reduced pressure. The amino acid compositions of the hydrolysates were determined by the method of Moore and Stein (*9*) in Hitachi amino acid analyzers (types KLA5 and 835-50). On the basis of the known amino acid sequences of the substrate peptides, the sequence of each peptide product could be unequivocally deduced from its amino acid composition. Amino-terminal residues were identified by the dansyl chloride method, separating the dansyl amino acids on polyamide sheets (*10*). Other conditions for incubation of proteases with substrate polypeptides are given in the legends to figures and tables.

Protein determination: Protein concentrations were determined by the method of Lowry *et al.* (*11*) with crystalline bovine serum albumin as a standard.

RESULTS

To obtain more information on the specificity of cathepsin L from rat liver, we examined the hydrolyses of four polypeptides at various molar ratios of substrate to enzyme.

First, the hexapeptide Leu-Trp-Met-Arg-Phe-Ala was used as a model substrate. The progress of hexapeptide digestion was followed by ascending chromatography on a thin layer of silica gel of samples taken at various times during incubation for 2 h. Figure 1 shows that undigested hexapeptide still remained after 2 h of digestion at a molar ratio of hexapeptide to cathepsin L of 5,800 : 1; two major components were observed on silica gel thin layer chromatography. The peptide products separated by thin-layer chromatography were isolated by elution with 0.1 N HCl and subjected to amino-terminal amino acid analysis by dansylation and also to amino acid analysis. Amino acid analysis indicated that these fragments were generated by cleavage of the hexapeptide at Met(-3)-Arg(-4).

On digestion of luteinizing hormone releasing hormone at a molar substrate-enzyme ratio of 2,300 : 1 for 45 min and 4 h, five and six fragments, respectively, were isolated from the digests by silica gel thin layer chromatography and were identified by amino acid and amino-terminal analyses as shown in Table I. The products were peptides containing residues 8–10 (fragment I), 7–10 (fragment II-1), 1–4 (fragment III), 1–6 (fragment IV), and 5–6 (fragment V) and leucine (fragment VI). In addition to these products,

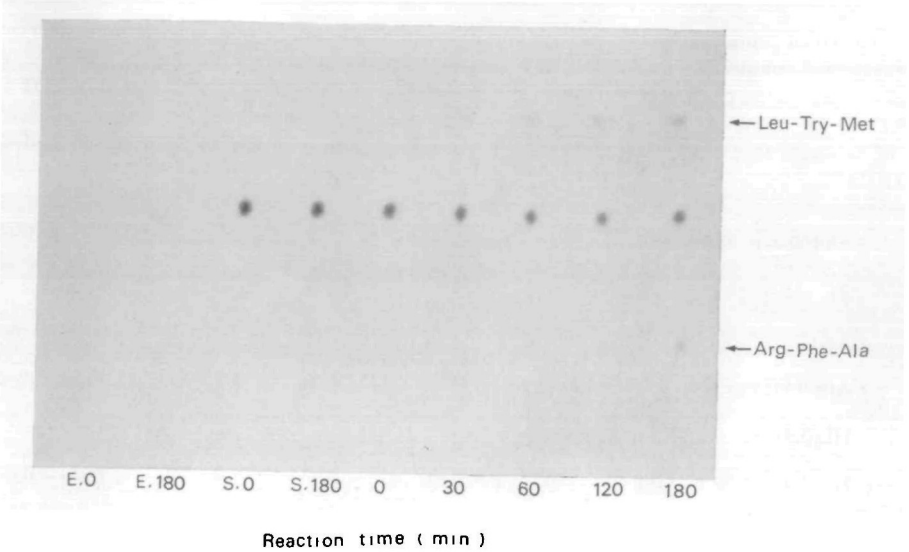

Fig. 1. Time course of hydrolysis of hexapeptide by cathepsin L from rat liver. The reaction mixture contained 1 μmol of hexapeptide and 4 μg of cathepsin L in 0.25 ml of 100 mM acetate buffer, pH 5.0, containing 1.25 μmol of 2-mercaptoethanol and 0.25 μmol of EDTA. Samples of 50 μl were removed after 0, 30, 60, 120, and 180 min and the reaction was stopped by addition of 0.05 μmol of iodoacetate. The products were analyzed as described in "MATERIALS AND METHODS." The lanes (left to right) show the patterns of enzyme controls (E, 0 min), (E, 180 min), the substrate controls (S, 0 min), (S, 180 min), and mixtures after reaction times of 0, 30, 60, 120, and 180 min.

undigested luteinizing hormone releasing hormone (fragment II-2) remained after 45 min of digestion. From these findings, it was deduced that cathepsin L mainly hydrolyzed the peptide bond between Gly(-6) and Leu(-7), though it also hydrolyzed the peptide bonds between Ser(-4) and Tyr(-5) and between Leu(-7) and Arg(-8) slightly, as shown in Fig. 2.

Neurotensin was digested similarly for 45 min and 4 h at 25°C at a molar substrate–enzyme ratio of 2,300 : 1 and the fragments were separated by silica gel thin layer chromatography. After incubation for 45 min, four main fragments were detected in addition to undigested neurotensin. As can be seen from the results of amino acid and amino-terminal amino acid analyses in Table II, the products were peptides containing residues 5–13 (fragment I), 4–13 (fragment II), 1–3 (fragment III), and 1–4 (fragment IV) of neurotensin, thus accounting for all the residues in neurotensin. No amino-terminal amino acid was detectable in fragment III or fragment IV, because the pyrrolidone carboxylyl residue is the original amino-terminal residue of neurotensin. As shown in Fig. 2, cathepsin L mainly hydrolyzed the bonds between Glu(-4) and Asn(-5) and between Tyr(-3) and Glu(-4) in neurotensin.

The peptide bonds cleaved by cathepsin L in the oxidized insulin A chain were also determined. The insulin A chain was digested for 45 min and 19 h at 25°C at a molar substrate–enzyme ratio of 2,300 : 1. The products were partially separated by cation-exchange chromatography, and the three ninhydrin positive components were then purified further by high-voltage electrophoresis. After digestion for 45 min and 4 h, four and eight peptides, respectively, were obtained and these were identified by amino acid and amino-terminal analyses, as shown in Table III. These peptides show that the enzyme mainly hydrolyzed the peptide bonds between Tyr(-14) and Gln(-15) and between Gln(-15) and Leu(-16), with slight actions on the peptide bonds between Glu(-4) and Gln(-5) and between Glu(-17) and Asn(-18) in the insulin A chain, as shown in Fig. 2. However, no peptides containing residues 1–14 and 5–14

TABLE I. Analysis of peptides obtained by digestion of luteinizing hormone releasing hormone with cathepsin L. Hydrolysis was carried out at 25°C with 1 μmol of luteinizing hormone releasing hormone and 10 μg of cathepsin L in 100 mM of acetate buffer, pH 5.0, containing 1.25 μmol of 2-mercaptoethanol and 0.25 μmol of EDTA in a total volume of 0.25 ml for 45 min or 4 h. The reaction was stopped by addition of 0.25 μmol of iodoacetate The molar ratio of substrate to enzyme was 2,300 : 1. The products were analyzed as described in "MATERIALS AND METHODS."

Peptide number	Amino acid analysis	N-Terminal residue	Recovery (nmol)	Peptide
	4 h digest (25°C)			
I	Arg(1.0); Pro(1.0), Gly(1)	Arg	22	$^{8}_{}$Arg--$^{10}_{}$Gly-amide
II-1	Arg(0.9); Pro(0.9); Gly(1); Leu(0.9)	Leu	359	$^{7}_{}$Leu--$^{10}_{}$Gly-amide
III	His(0.9); Ser(0.8); Glu(1)	—	387	$^{1}_{}$Pyr--$^{4}_{}$Ser
IV	His(0.9); Ser(0.8), Glu(1); Gly(0.9); Tyr(0.9)	—	40	$^{1}_{}$Pyr--$^{6}_{}$Gly
V	Gly(0.9); Tyr(1)	Tyr	439	$^{5}_{}$Tyr--$^{6}_{}$Gly
VI	Leu(1)	Leu	23	$^{7}_{}$Leu
	45 min digest (25°C)			
II-1	Arg(0.9); Pro(0.8); Gly(1); Leu(1.0)	Leu	330	$^{7}_{}$Leu--$^{10}_{}$Gly-amide
II-2	His(1.0); Arg(1.0); Ser(0.8), Glu(1.1); Pro(1.0); Gly(2), Leu(1.0); Tyr(0.9)	—	150	$^{1}_{}$Pyr--$^{10}_{}$Gly-amide
III	His(0.8); Ser(0.9); Glu(1)	—	97	$^{1}_{}$Pyr--$^{4}_{}$Ser
IV	His(0.8); Ser(0.8), Glu(1), Gly(1.0), Tyr(1.9)	—	205	$^{1}_{}$Pyr--$^{6}_{}$Gly
V	Gly(0.9), Tyr(1)	Tyr	118	$^{5}_{}$Tyr--$^{6}_{}$Gly

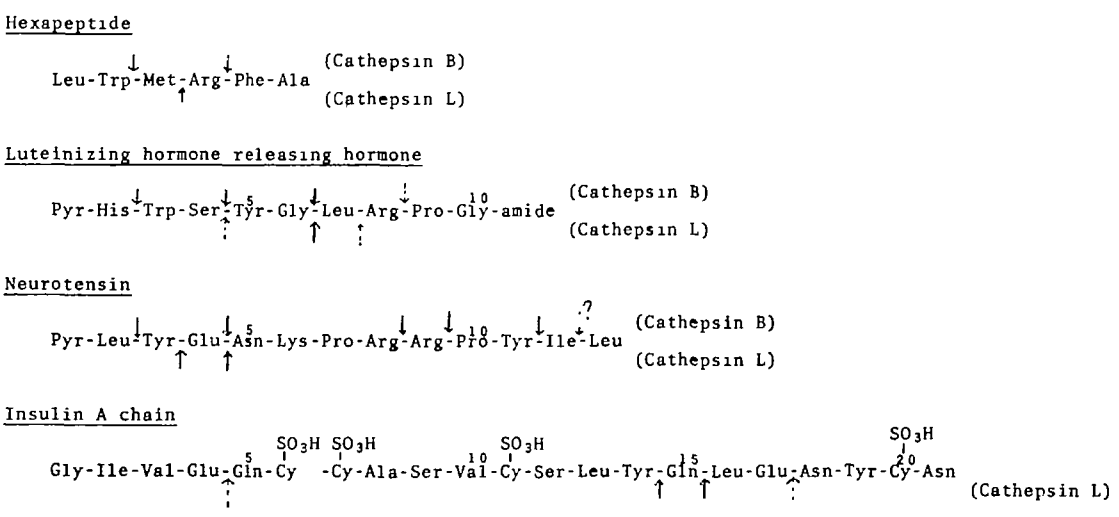

Fig. 2. Summary of the cleavage sites of cathepsin L and cathepsin B from rat liver on the hexapeptide, luteinizing hormone releasing hormone, neurotensin, and oxidized insulin A chain. Full arrows indicate major cleavage sites, while broken arrows indicate minor cleavage sites.

TABLE II. Analysis of peptides obtained by digestion of neurotensin with cathepsin L. Hydrolysis was carried out with 1 μmol of neurotensin and 10 μg of cathepsin L in a total volume of 0.25 ml for 45 min or 4 h. The molar ratio of substrate to enzyme was 2,300 : 1. Other experimental conditions were as for Table I.

Peptide number	Amino acid analysis	N-Terminal	Recovery (nmol)	Peptide
	4 h digest (25°C)			
I	Lys(1.2); Arg(2.3); Asp(1); Pro(2.2); Ile(1.2); Leu(1.3); Tyr(1.0)	Asn	243	$^{5}_{}$Asn--Leu^{13}
II	Lys(0.9); Arg(1.9); Asp(1); Glu(0.9); Pro(2.0); Ile(1.0); Leu(1.1); Tyr(0.9)	Glu	160	$^{4}_{}$Glu--Leu^{13}
IV	Glu(1); Leu(1.0); Tyr(0.8)	—	192	$^{1}_{}$Pyr--Tyr^{3}
V	Glu(1.8); Leu(1); Tyr(0.9)	—	384	$^{1}_{}$Pyr--Glu^{4}
	45 min digest (25°C)			
I	Lys(0.9); Arg(1.8); Asp(1); Pro(2.0); Ile(1.0); Leu(1.1); Tyr(0.9)	Asn	186	$^{5}_{}$Asn--Leu^{13}
II	Lys(0.7); Arg(1.3); Asp(1); Glu(1.0); Pro(1.9); Ile(0.9); Leu(1.0); Tyr(0.7)	Glu	89	$^{4}_{}$Glu--Leu^{13}
III	Lys(1.0); Arg(2.0); Asp(1); Glu(2.0); Pro(2.1); Ile(1.0); Leu(2.2); Tyr(1.9)	—	199	$^{1}_{}$Pyr--Leu^{13}
IV	Glu(1); Leu(0.9); Tyr(0.8)		99	$^{1}_{}$Pyr--Tyr^{3}
V	Glu(2); Leu(1.0); Tyr(0.9)		210	$^{1}_{}$Pyr--Glu^{4}

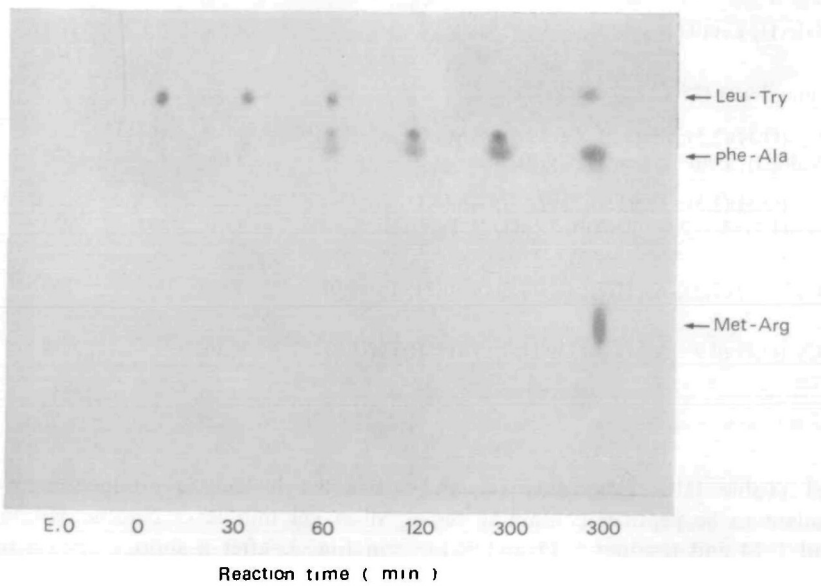

Fig. 3. Time course of hydrolysis of the hexapeptide by cathepsin B from rat liver. The reaction mixtures contained 1 μmol of hexapeptide and 5 μg or 200 μg of cathepsin B in 0.25 ml of 100 mM acetate buffer, pH 5.0, containing 2.5 μmol of 2-mercaptoethanol and 0.25 mol of EDTA. Samples of 50 μl were removed after 0, 30, 60, 120, and 300 min. The reaction was stopped by addition of 0.05 μmol of iodoacetate. Products were analyzed as described in "MATERIALS AND METHODS." The lanes (left to right) show results for the control (E, 0), and mixtures after reaction times of 0, 30, 60, 120, and 300 min with 5 μg of cathepsin B and 300 min with 200 μg of cathepsin B.

TABLE III. Analysis of peptides obtained by digestion of insulin A chain (oxidized form) with cathepsin L. Hydrolysis was carried out at 25°C with 4 μmol of oxidized insulin A chain and 40 μg of cathepsin L in 100 mM of acetate buffer, pH 5.0, containing 5 μmol of 2-mercaptoethanol and 1 μmol of EDTA in a total volume of 1.0 ml for 45 min or 19 h. The reaction was stopped by addition of 1 μmol of iodoacetate. The molar ratio of substrate to enzyme was 2,300 : 1. The products were analyzed as described in " MATERIALS AND METHODS."

Peptide number	Amino acid analysis	N-Terminal residue	Recovery (nmol)	Peptide
	19 h digest (25°C)			
I-1	Cys(O_3H)(3.0); Ser(2.0); Glu(3.0); Gly(1.0); Ala(1.0); Val(2.0); Ile(1.0); Leu(1); Tyr(1.0)	Gly	231	1 15 Gly--Gln
I-2	Cys(O_3H)(2.6); Ser(1.6); Glu(2.3); Ala(1); Val(1.0); Leu(1.2); Tyr(0.6)	Gln	405	5 15 Gln--Gln
II-1	Cys(O_3H)(0 8); Asp(2.2); Tyr(1)	Asn	240	18 21 Asn--Asn
II-1′	Glu(1.4); Leu(1)	Gln	88	15 17 Gln--Glu
II-2	Cys(O_3H)(0.7); Asp(2.0); Glu(1.1); Leu(1); Tyr(0.8)	Leu	833	16 21 Leu--Asn
II-3	Cys(O_3H)(0.8); Asp(2.3); Glu(1.8); Leu(1); Tyr(1.0)	Gln	160	15 21 Gln--Asn
III-1	Glu(1); Gly(1.0); Val(0.5); Ile(0.4)	Gly	352	1 4 Gly--Glu
III-2	Glu(1); Leu(0.8)	Leu	206	16 17 Leu--Glu
	45 min digest (25°C)			
I-1	Cys(O_3H)(2.5); Ser(1.2); Glu(3.6); Gly(1.0); Ala(1.2); Val(2.3); Ile(0.6); Leu(1); Tyr(0.7)	Gly	360	1 15 Gly--Gln
I-3	Cys(O_3H)(3.5); Asp(1.5); Ser(1.4); Glu(4.1); Gly(1); Ala(1.2); Val(2.0); Ile(0.6); Leu(1.8); Tyr(1.7)	Gly	924	1 21 Gly--Asn
II-2	Cys(O_3H)(0.9); Asp(1.7); Glu(1.2); Leu(1); Tyr(1.0)	Leu	256	16 21 Leu--Asn
II-3	Cys(O_3H)(0 9); Asp(1.6); Glu(1.8); Leu(1); Tyr(1.0)	Gln	158	15 21 Gln--Asn

were detected (Table III). Fragments I-1 and II-2 were thought to be peptides containing residues 1–15 and 1–14 and residues 5–15 and 5–14, respectively, but these peptides were not separated completely by high-voltage electrophoresis.

For comparison, parallel experiments were carried out on the hydrolyses of the hexapeptide, luteinizing hormone releasing hormone, and neurotensin by cathepsin B. The hexapeptide was digested at a molar substrate–enzyme ratio of 5,800 : 1 for various times of up to 5 h at 25°C, and the hydrolytic products were separated by silica gel thin layer chromatography. As shown in Fig. 3, after a short digestion time, two components were detected in addition to uncleaved hexapeptide. One of them disappeared with the appearance of two new components on digestion for 5 h at a molar substrate–enzyme ratio of 150 : 1. These components were eluted with 0.1 N HCl and subjected to amino-terminal amino acid analysis by dansylation and amino acid analysis. The final products were peptides containing resi-

dues 1–2 (fragment I), 3–4 (fragment II), and 5–6 (fragment III). Thus it was deduced that cathepsin B hydrolyzed the peptide bonds between Arg(-4) and Phe(-5) and between Trp(-2) and Met(-3). Dipeptides originating from the carboxyl-terminal portion of the hexapeptide were released successively.

The luteinizing hormone releasing hormone was digested for 45 min and 9 h at 25°C at a molar substrate-enzyme ratio of 630 : 1. Five and nine peptides were detected after digestion for 45 min and 9 h, respectively. As can be seen from the results of amino acid analysis and dansylation of amino-terminal amino acids (shown in Table IV), cathepsin B attacked the peptide bonds between Gly(-6) and Leu(-7), Ser(-4) and Tyr(-5), and His(-2) and Trp(-3) successively from the carboxyl-terminal end and caused slight cleavage of the

TABLE IV. Analysis of peptides obtained by digestion of luteinizing hormone releasing hormone with cathepsin B. Hydrolysis was carried out at 25°C with 1 μmol of luteinizing hormone releasing hormone and 50 μg of cathepsin B in 100 mM of acetate buffer, pH 5.0, containing 2.5 μmol of 2-mercaptoethanol and 0.25 μmol of EDTA in a total volume of 0.25 ml for 45 min or 9 h. The reaction was stopped by addition of 0.25 μmol of iodoacetate. The molar ratio of substrate to enzyme was 630 : 1. The products were analyzed as described in " MATERIALS AND METHODS."

Peptide number	Amino acid analysis	N-Terminal residue	Recovery (nmol)	Peptide
	9 h digest (25°C)			
I-1	Arg(0.9); Pro(1.0); Gly(1.0); Leu(1)	Leu	327	$^{7}_{10}$ Leu--Gly-amide
I-2	Pro(0.5); Gly(1)	Pro	120	$^{9}_{10}$ Pro--Gly-amide
I-3	His(0.9); Glu(1)	—	113	$^{1}_{2}$ Pyr--His
II	His(0.9); Ser(0 9); Glu(1)	—	102	$^{1}_{4}$ Pyr--Ser
III	Arg(1.1); Leu(1)	Leu	104	$^{7}_{8}$ Leu--Arg
IV-1	His(0.8); Glu(1); Gly(1.2); Tyr(1.0)	—	76	$^{1}_{6}$ Pyr--Gly
IV-2	Gly(1); Tyr(0.8)	Tyr	299	$^{5}_{6}$ Tyr--Gly
V	Ser(1)	—	85	$^{3}_{4}$ Trp--Ser
	45 min digest (25°C)			
I-1	Arg(0.9); Pro(0.8); Gly(1 0); Leu(1)	Leu	203	$^{7}_{10}$ Leu--Gly-amide
II	His(0.8); Ser(0.8); Glu(1)	—	117	$^{1}_{4}$ Pyr--Ser
IV-1	His(0.8); Ser(0.8); Glu(1); Gly(1.0); Tyr(1.0)	—	68	$^{1}_{6}$ Pyr--Gly
IV-2	Gly(1); Tyr(0.8)	Tyr	114	$^{5}_{6}$ Tyr--Gly
VI	His(1); Arg(1.0): Ser(0 8); Glu(1.1); Pro(1.0); Gly(2.0); Leu(1); Tyr(0.9)		274	$^{1}_{10}$ Pyr--Gly-amide

peptide bond between Arg(-8) and Pro(-9) in the peptide containing residues 7–10 (fragment I).

Neurotensin was digested for 19 h at 25°C at a molar substrate-enzyme ratio of 290 : 1 or 2,900 : 1 and the products were separated by high-voltage electrophoresis and purified further by thin layer chromatography. As shown in Table V, after mild digestion, a peptide containing residues 12–13 (fragment II-4) was released from the carboxyl-terminal, followed by a peptide containing residues 10–11 (fragment II-2). On more extensive digestion at a molar ratio of 290 : 1, nine fragments were detected, from which it was deduced that cathepsin B hydrolyzed the peptide bonds between Tyr(-11) and Ile(-12), Arg(-9) and Pro(-10), Arg(-8) and Arg(-9), Glu(-4) and Asn(-5), and Leu(-2) and Tyr(-3).

Thus, cathepsin B hydrolyzed luteinizing hormone releasing hormone and neurotensin successively from the carboxyl-terminal. Its action was not necessarily the same as that on glucagon, although it hydrolyzed the hexapeptide by se-

TABLE V. Analysis of peptides obtained by digestion of neurotensin with cathepsin B. Hydrolysis was carried out at 25°C with 1.5 μmol of neurotensin and 15 μg or 150 μg of cathepsin B from rat liver. Other experimental conditions were as for Table IV. The molar ratio of substrate to enzyme was 2,900 · 1 or 290 : 1. The products were analyzed as described in " MATERIALS AND METHODS."

Peptide number	Amino acid analysis	N-Terminal residue	Recovery (nmol)	Peptide
	19 h digest (25°C) at molar ratio of 290 . 1			
I-1	Glu(1); Leu(1.0)	—	25.3	1 2 Pyr--Leu
I-2	Glu(1); Tyr(1.2)	Tyr	37.5	3 4 Tyr--Glu
II-1	Lys(1.0); Arg(0.9); Asp(1); Glu(1.8); Pro(0.9); Leu(1.0); Tyr(0.7)	—	96.7	1 8 Pyr--Arg
II-2	Pro(1); Tyr(0.9)	Pro	610.8	10 11 Pro--Tyr
II-3	Leu(1)	Leu	99.4	Leu
II-4	Ile(1.1); Leu(1)	Ile	539.4	12 13 Ile--Leu
III	Lys(0.8); Arg(1.4); Asp(1); Glu(1.9); Pro(1.0); Leu(1.0); Tyr(0.9)	—	461.1	1 9 Pyr--Arg
IV	Lys(1.0); Arg(1.1); Asp(1); Pro(1.4)	Asn	41.7	5 8 Asn--Arg
V	Arg(1)	Arg	99.7	9 Arg
	19 h digest (25°C at molar ratio of 2,900 · 1			
II-2	Pro(1); Tyr(0.8)	Pro	409.5	10 11 Pro--Tyr
II-4	Ile(1.0); Leu(1)	Ile	492	12 13 Ile--Leu
III	Lys(1.2); Arg(2.3); Asp(1); Glu(2.1), Pro(1.1); Leu(1.3); Tyr(1.3)	—	543	1 9 Pyr--Arg
VI	Lys(1.2); Arg(2.2); Asp(1); Glu(2.0); Pro(2.0), Leu(1.3); Tyr(1.0)	—	58.5	1 11 Pyr--Tyr

quential cleavage of dipeptides from the carboxyl-terminal, as suggested by Aronson and Barrett (*12*).

DISCUSSION

Studies on the substrate specificities of cathepsin L and cathepsin B from rat liver are useful in elucidating the reasons for limited proteolysis of macromolecular proteins.

The present study with various polypeptides as substrates showed that cathepsin L has a unique specificity. It hydrolyzed the synthetic hexapeptide Leu-Trp-Met-Arg-Phe-Ala to peptides containing residues 1–3 and residues 4–6, whereas cathepsin B hydrolyzed the hexapeptide to three peptides containing residues 1–2, residues 3–4, and residues 5–6 by sequential cleavage of dipeptides from the carboxyl-terminal. In contrast, trypsin cleaves the Arg-Phe bond and chymotrypsin first hydrolyzes the Trp-Met bond of the hexapeptide and later the Phe-Ala bond. Thus, cathepsin L does not have the same action as either chymotrypsin or trypsin. Figure 2 summarizes the peptide bonds in the four polypeptides cleaved by cathepsin L and cathepsin B. The scheme includes the amino acid residues adjacent to peptide bonds cleaved preferentially by cathepsin L. Kärgel *et al.* examined the action of cathepsin L on the oxidized insulin B chain (*13*), finding that the peptide bonds cleaved by cathepsin L show a clear specificity in that positions P_2 and P_3 (*14*) at least must be occupied by particular hydrophobic amino acids. Our results, summarized in Figs. 2 and 4, suggest that cathepsin L cleaves peptide bonds containing a hydrophobic amino acid, such as Phe, Leu, Val, Trp, or Tyr, or Gly in position P_2 of the substrate molecule. This is consistent with the finding of Kärgel *et al.* (*13*). The importance of the amino acid residue in position P_3 on the action of cathepsin L is not so clear as that of the amino acid in position P_2, since many peptide bonds that do not contain a hydrophobic amino acid in position P_3 are cleaved by cathepsin L.

Our results also indicate that in addition to a hydrophobic amino acid in position P_2, a polar amino acid, such as Tyr, Ser, Gly, Glu, Asp, Gln, or Asn, in position P_1 may increase the susceptibility of the peptide bond to cathepsin L, although peptide bonds containing Met or Leu in position P_1 were also hydrolyzed and some peptides containing Arg, Tyr, Thr, or Cys(O_3H) in position P_1 were not hydrolyzed. No requirement for a special amino acid in the P_1' or P_3 position was observed. As cathepsin L cleaved the insulin A chain at Tyr(-14)-Gln(-15) with Leu in position P_2 but did not cleave the insulin B chain at Tyr (-16)-Leu(-17) with Leu in position P_2, it is uncertain whether the amino acid residue in position P_1' or position P_3 is important.

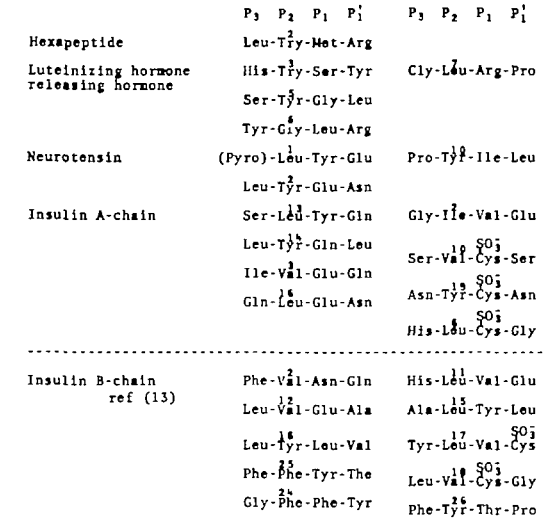

Fig. 4. Peptide bonds cleaved and not cleaved by cathepsin L. The peptide bonds have a hydrophobic amino acid at position P_2. The peptide bonds on the left are cleaved preferentially by cathepsin L, while those on the right are not cleaved.

Aronson and Barrett found that cathepsin B degraded glucagon and inactivated rabbit muscle aldolase by sequential cleavage of dipeptides from the carboxyl-terminal end of the molecule (*12*, *15*), but its action on the insulin B chain was quite different (*16*, *17*). Our results also indicate that cathepsin B exhibits the same behavior on the hexapeptide as on glucagon, but cleaves luteinizing hormone releasing hormone and neurotensin in a quite different manner. Thus, comparison of the numerous peptide bonds cleaved by cathepsin B does not reveal any clear specificity of the enzyme.

We would like to thank Mr. H. Miyai and Dr. T. Watanabe for assistance in amino acid analysis and Mrs. E. Inai for help in the preparation of this manuscript.

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