The Specificity of Cathepsin D*

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SUMMARY

Bovine spleen cathepsin D has been partially purified for studies of its specificity. Isoelectric focusing of the purest preparation obtained indicated the presence of three molecular forms having approximately the same specific activity, but differing in their isoelectric points. Gel chromatography of this preparation on Sephadex G-100 gave an apparent molecular weight of about 42,000, and its sedimentation equilibrium behavior was found to be consistent with that of a dissociating system, with an apparent molecular weight of about 43,000 for an oligomer. Polycrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicated the presence of a subunit of molecular weight about 28,000.

A series of oligopeptides having a p-nitro-L-phenylalanyl-L-phenylalanyl (Phe(NO₂)-Phe) unit were tested as substrates for purified cathepsin D. It was found that for substrates of the type A-Phe(NO₂)-Phe-B, where the Phe(NO₂)-Phe bond is the only one cleaved by the enzyme, modification of the A and B groups markedly affected the kinetic parameters. The best substrate found was Phe-Gly-His-Phe-(NO₂)-Phe-Val-Leu-OMe. This substrate is cleaved by cathepsin D at pH 4 more slowly than by pepsin, but at about the same rate as by the acid proteinase of \textit{Rhizopus chinensis}. The data give further evidence of the similarity in the specificity of cathepsin D to that of other known acid proteinases and underline the importance of secondary enzyme-substrate interactions, at a distance from the site of catalytic action, in determining the apparent differences among these enzymes in their cleavage of oligopeptide substrates. The availability of a suitable synthetic substrate for cathepsin D has permitted the study of the competitive inhibition of this enzyme by peptides, including pepstatin.

Like other acid proteinases, cathepsin D is inhibited by active site-directed diazoketones, and it has been found that this enzyme is less susceptible to such inhibition than are pepsin and the \textit{Rhizopus} proteinase. This difference in behavior may be correlated with the apparent weaker binding of small oligopeptide substrates at the active site of cathepsin D.

The study of the cleavage, by aqueous extracts of animal tissues, of synthetic peptide substrates for crystalline pepsin, trypsin, and chymotrypsin showed the presence of at least three distinct proteolytic enzymes, denoted cathepsins A, B, and C, respectively (1); for reviews, see References 2 and 3. Subsequently, the presence of a proteinase having pepsin-like action near pH 3 on the B chain of insulin was found in bovine spleen by Press et al. (4). Since partially purified samples of this enzyme failed to cleave \textit{Ac-DL-Phe-Tyr}(I₄), the most sensitive synthetic substrate then available for porcine gastric pepsin (5), and also did not hydrolyze synthetic substrates for cathepsins A, B, and C, the spleen enzyme was named cathepsin D. In subsequent work, protease activity near pH 3 toward denatured hemoglobin, usually assigned to cathepsin D, has been identified in extracts of many animal tissues, and procedures have been described for the partial purification of the enzyme from bovine spleen (6, 7), from porcine thyroid (8, 9), from rabbit liver (10), from bovine uterus (11), from chicken muscle (12), and from rabbit ear and chick embryo cartilage (13). Also, the association of cathepsin D with the lysosomal fraction of liver homogenates was established (14).

The studies of Press et al. (4) on the action of their cathepsin D preparation on the B chain of oxidized insulin indicated a preferential attack at the Leu-15-Tyr-16, Phe-24-Phe-25, and Phe-25-Tyr-26 bonds, all of which are cleaved by pepsin. Other peptide bonds were also cleaved by both enzymes, with pepsin exhibiting a somewhat wider range of attack. These observations were largely confirmed in subsequent reports; Keilova and Keil (6) also showed that the heptapeptide corresponding to residues 23 to 29 of the B chain of insulin (Gly-Phe-Phe-Tyr-Thr-Pro-Lys) is cleaved by both cathepsin D and pepsin at the Phe-Phe and Phe-Tyr bonds.

There have been several reports on the action of cathepsin D preparations on synthetic peptides. For example, a bovine spleen preparation was found to effect the slow cleavage of Gly-Phe-Leu-Gly-Phe-Leu at the interior Phe-Leu bond, with a pH optimum near 4 (15). Woessner (16) has reported that the peptide Glu-Ala-Leu-Tyr-Leu-Val (corresponding to residues 13 to 18 of the B chain of insulin) is cleaved rapidly at the Leu-Tyr bond by a cathepsin D preparation from bovine spleen; no data on the kinetic parameters of this cleavage have come to our notice. The slow hydrolysis of Z-Gly-Phe-Phe-Tyr-OMe, Z-Phe-Tyr-Thr-OMe, and Phe-Tyr-Thr-n-Pro by this preparation has also been noted (16). Several oligopeptides were found to be hydrolyzed by a cathepsin D preparation from rat liver (17); the most sensitive of those tested appears to be Gly-Phe-

* The abbreviations used are: Ac, acetyl; Tyr(I₄), 3,5-diiodo-L-tyrosine; Z, benzoxycarbonyl; OMe, methoxy; Phe(NO₂), p-nitro-L-phenylalanyl; Boe, t-butyloxycarbonyl; OSu, oxysuccinimido.

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Phe-Tyr-Thr-Pro-Lys, with preferential cleavage at the Phe-Phe bond, but with significant hydrolysis at the Phe-Tyr and Tyr-Thr bonds as well. In another study, a cathepsin D preparation from bovine pituitary was found to act preferentially at the Phe-His and Leu-Leu bonds of Z-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-β-naphthylamide; omission of either the Pro-Phe-His or the Val-Tyr-Ser part of the peptide reduced the rate of cleavage greatly (18).

The available data on the action of cathepsin D on the B chain of insulin and on synthetic peptides thus appear to support the view that the specificity of this enzyme resembles that of porcine gastric pepsin A in respect of the nature of the amino acid residues flanking the sensitive peptide bond (19), but also suggest that additional secondary enzyme-substrate interactions may be decisive in conferring upon cathepsin D the ability to cleave rapidly such peptide linkages as the Phe-Phe bond in synthetic substrates of the type A-Phe-Phe-B. Attention has been drawn to the importance of secondary interactions, involving the A and B groups of such substrates, for pepsin (20-22) and for acid proteinases (e.g. rennin and mold proteinases) related to it (23). The purpose of the present investigation is to initiate a systematic study of the specificity and mechanism of action of cathepsin D from the standpoint developed in the case of pepsin (22, 24). In connection with this work, a method for the purification of cathepsin D from bovine spleen was devised; this procedure differs significantly from those described by other investigators, the details are reported here.

**EXPERIMENTAL PROCEDURE**

** Purification of Cathepsin D —** The course of the purification of cathepsin D was followed by means of a modification of the assay method of Anson (25) with 2% hemoglobin substrate powder (Worthington Biochemical Corporation) at pH 3.7 and 40°. After an incubation period of 30 min, 2 volumes of 5% trichloroacetic acid were added and the absorbance of the filtrate was determined at 280 nm. One cathepsin D unit is defined as the amount of enzyme that produces an increase in absorbance of 0.03 above the blank, using 1 cm cells in a Beckman DU spectrophotometer.

Within the range of cathepsin D activity used in the hemoglobin assays, the increase in absorbance was approximately proportional to the enzyme concentration. The specific activity (enzyme units per mg of protein) was determined by estimation of the protein content of the test enzyme solution, using the microbiuret reagent as described by Bailey (20), with bovine serum albumin as the standard.

In what follows, we give a typical laboratory scale preparation from three fresh bovine spleens. The outer membrane and major blood vessels were removed from the organs, yielding 1700 g of tissue. The tissue was minced with scissors and homogenized at 4° in a large Waring Blendor with 3400 ml of 1% NaCl solution containing 2% l-butanol. The resulting suspension at 5000 rpm for 10 min and was washed with 4 liters of neutralized 60% saturated ammonium sulfate. The washed precipitate could be stored in the freezer for several months without loss of activity. For the next step in the procedure, it was suspended in 450 ml of 0.01 M potassium phosphate buffer, pH 7.0, containing 0.03% (v/v) toluene, and the suspension was dialyzed extensively against this buffer. The dialysate was clarified by centrifugation; it is denoted in Table I as the 30 to 60 AS fraction.

The 30 to 60 AS fraction was subjected to chromatography (in ~100 ml portions) on freshly prepared columns (5 x 25 cm) of DEAE-cellulose (bottom 15 cm; Bio-Rad Cellex-D, 0.75 meq per g) and CM-cellulose (top 10 cm; Bio-Rad Cellex-CM, 0.7 meq per g), the two layers being separated by Schleicher and Schuell filter paper No. 589; this combined cellulose column had been equilibrated previously with 0.01 M potassium phosphate buffer, pH 7.0, containing 0.03% (v/v) toluene. This buffer was used for elution at a rate of about 140 ml per hour, and 20-ml fractions were collected. The enzymic activity was located in the fractions from 450 to 700 ml of effluent. These fractions were pooled, concentrated by means of Aquacide II (Calbiochem), and dialyzed against the above phosphate buffer to yield DEAE-CM Fraction I (Table I). This enzyme solution was subjected to a second treatment on a fresh DEAE-CM-cellulose column, in the manner described above, to yield the dialyzed DEAE-CM Fraction II (Table I). The last named fraction was applied to a column (5 x 90 cm) of Sephadex G-75, previously equilibrated with the above phosphate buffer, and eluted with this buffer at a rate of ~100 ml per hour (30-cm pressure head). Fractions of 20 ml were collected; the active material appeared between 700 and 900 ml of the effluent solution. The active fractions were pooled, concentrated, and dialyzed in the manner described above to yield the dialyzed DEAE-CM Fraction II (Table I). The last named fraction was applied to a column (5 x 90 cm) of Sephadex G-75, previously equilibrated with the above phosphate buffer, and eluted with this buffer at a rate of ~100 ml per hour (30-cm pressure head). Fractions of 20 ml were collected; the active material appeared between 700 and 900 ml of the effluent solution. The active fractions were pooled, concentrated, and dialyzed in the manner described above to yield the G-75 Fraction I cited in Table I. A repetition of the above procedure on the same Sephadex G-75 column gave G-75 Fraction II (Table I); a typical elution pattern is shown in Fig. 1A. After being dialyzed against 0.014 M Tris buffer, pH 7.4, containing 0.03% toluene, the last named fraction was applied to a column (2.5 x 25 cm) of DEAE-cellulose equilibrated with this buffer. The enzyme was eluted by means of a linear NaCl gradient (1 liter of 0 to 0.25 M NaCl in Tris buffer), and 20-ml fractions were collected. After the appearance of a peak of inactive protein, the active material emerged under the peak at about 410 ml (Fig. 1B). The active fractions were pooled, concentrated, and dialyzed against 0.01 M potassium phosphate buffer.
The DEAE-Tris preparation was also examined in a sedimentation equilibrium study observing the meniscus depletion condition described by Yphantis (29). We are grateful to Dr. Robley C. Williams, Jr., Yale University, for his help in performing these measurements. A Beckman model E ultracentrifuge equipped with a helium-neon laser light source (30) was used in conjunction with Eastman type SO-410 film; the camera lens was focused at the two-thirds focal position in the cell. The temperature was set to 20° ± 0.05°, referred to a National Bureau of Standards thermometer. Three channels of an Yphantis-type six-channel centerpiece made of Kel-F with sapphire windows were filled with 0.12 ml of 0.1 M potassium phosphate buffer, pH 6.7, at 20°. The other three channels received appropriate quantities of the DEAE-Tris fraction, which had been extensively dialyzed against the pH 6.7 buffer, and appropriate quantities of buffer such that final protein concentrations in these channels approximated 0.92, 0.46, and 0.23 mg per ml. The centerpiece was tightened to a torque of 140 inch-pounds and inserted into a Beckman An-D rotor. The ultracentrifuge was brought to 34,000 rpm and maintained at speed for 24 hours before five photographs (2- to 6-s exposures) of the fringe patterns were taken. Similar photographs were taken later of a 34,000-rpm run with water in all six channels. X-Y coordinates of the fringe patterns for the 5-s exposures of both water blanks and protein solutions were obtained using a Nikon model 6C profile projector equipped with a Nikon mechanical stage and two IKL model 2-446BP digital micrometers. Data reduction was accomplished by the computer program of Roark and Yphantis (31). The density (ρ) of the pH 6.7 buffer was measured at 20° with a pyrometer; a partial specific volume of 0.74 was assumed (8).

The molecular size of the DEAE-Tris preparation was also estimated by means of gel chromatography with Sephadex G-100 (Pharmacia), with chymotrypsinogen A (Worthington) and bovine serum albumin (Pentex) run separately as standards. A column (1.2 cm internal diameter) with a total bed volume of 48 ml was used, and the flow rate (0.1 m potassium phosphate buffer, pH 7.0) was about 0.55 ml per min. The void volume (16 ml) was determined in the usual manner, using blue dextran 2000 (Pharmacia). After the protein samples (1 mg in 1 ml) were applied to the column, 1-ml fractions were collected, their absorbance at 280 nm was measured, and the enzymic activity of the cathepsin D fractions was also determined with hemoglobin as the substrate.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed in a manner similar to that of Weber and Osborn (32) using an EC Apparatus instrument (EC 470 vertical gel cell and EC 458 power supply). The buffer used to fill the tanks and to make up the gel mixture was a 0.1 M glycine-NaOH buffer, pH 9.0, 0.1% (w/v) in 99% sodium dodecyl sulfate (Pierce) and 0.1% (v/v) in β-mercaptoethanol. The gel mixture was 10% (w/v) in acrylamide, 0.3% (w/v) in bisacrylamide, 0.05% (v/v) in Temed, and 0.02% in ammonium persulfate. Solutions of the protein (~1 mg per ml) in glycine-NaOH buffer containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol were heated at 100° for 15 min and dialyzed against this buffer-detergent solution. After extensive further dialysis against the running buffer-detergent solution, 50-μl samples (1 μg per μl) were made 20% (w/v) in sucrose and 0.003% (w/v) in bromphenol blue. The voltage was maintained at 250 volts for 110 min. The gels were then washed and stained with 0.1% (w/v) Coomassie brilliant blue R (Sigma) and destained in the usual manner. The proteins used as standards were lysozyme (Miles), chymotrypsinogen A, alcohol de-
hydrogenase (Schwarz-Mann), ovalbumin (Schwarz-Mann), pyruvate kinase (Sigma), and bovine serum albumin.

The determination of the NH₂-terminal amino acid residues was performed by the dansyl method as described by Weiner et al. (33).

**Peptide Substrates—**Except for Phe-Gly-His-Phe(NO₂)-Phe-Val-Leu-OMe, the synthesis of which is described below, the synthetic peptides tested as substrates in this study had been made previously in this laboratory (20, 34).

For the synthesis of Phe-Gly-His-Phe(NO₂)-Phe-Val-Leu-OMe, Z-His-Phe(NO₂)-Phe-N₉ (derived from 4 mmoles (2.6 g) of the hydrazide (19)) was coupled with Val-Leu-OMe hydrobromide (derived from 6 mmoles (2.3 g) of Z-Val-Leu-OMe (35) by treatment with HBr-acetic acid) in the manner described by Medzhibrodsy et al. (20) for the synthesis of Z-His-Phe(NO₂)-Phe-Ala-OMe. After recrystallization from isopropyl alcohol, the resulting Z-His-Phe(NO₂)-Phe-Val-Leu-OMe melted at 217–219°, with decomposition. Thin layer chromatography in 1-butanol-acetic acid-water, 4:1:1, on Eastman Chromagram 6061 sheets gave a single spot of RF 0.80 (iodine).

The Z-pentapeptide ester was treated with HBr-acetic acid in the usual manner to yield His-Phe(NO₂)-Phe-Val-Leu-OMe hydrobromide. This product (1.4 g, 1.6 mmoles) was used directly for coupling with Boc-Phe-Gly-OSu (0.7 g, 1.6 mmoles; prepared as described by Medzhibrodsy et al. (20) in the presence of triethylamine (3.2 mmoles), with dimethylformamide (90 ml) as the solvent. The reaction mixture was kept at room temperature for 20 hours, and water (30 ml) was added to yield 0.73 g (91%) of the crystalline Boc-heptapeptide ester; m.p. 208–210°, with decomposition. Thin layer chromatography in 1-butanol-acetic acid-water, 4:1:1, gave a single spot of RF 0.72 (iodine).

**C₄₆H₆₄N₁₈O₁₈** (mol wt 855.0)

Calculated: C 61.8, H 6.4, N 9.8
Found: C 61.5, H 6.6, N 10.0

The Z-pentapeptide ester was treated with HBr-acetic acid in the usual manner to yield His-Phe(NO₂)-Phe-Val-Leu-OMe hydrobromide. This product (1.4 g, 1.6 mmoles) was used directly for coupling with Boc-Phe-Gly-OSu (0.7 g, 1.6 mmoles; prepared as described by Medzhibrodsy et al. (20) in the presence of triethylamine (3.2 mmoles), with dimethylformamide (90 ml) as the solvent. The reaction mixture was kept at room temperature for 20 hours, and water (30 ml) was added to yield 0.73 g (91%) of the crystalline Boc-heptapeptide ester; m.p. 208–210°, with decomposition. Thin layer chromatography in 1-butanol-acetic acid-water, 4:1:1, gave a single spot of RF 0.72 (iodine).

**C₄₆H₆₄N₁₈O₁₈** (mol wt 1025.2)

Calculated: C 60.9, H 6.6, N 13.7
Found: C 60.7, H 6.7, N 13.0

This product (0.7 mmole) was treated with 20 ml of trifluoroacetic acid for 45 min at room temperature, the solution was evaporated in vacuo, and the residue was taken up in 5 ml of methanol. The addition of 50 ml of ether gave 0.73 g (91%) of the product, which was recrystallized from methanol-ether; m.p. 196–198°, with decomposition; [α]D = −14.1° (c 1, methanol). Thin layer chromatography in 1-butanol-acetic acid-water, 4:1:1, gave a major spot of RF 0.72 with slight evidence of impurity (iodine).

**C₄₂H₄₈N₁₆O₁₄F₄** (mol wt 1152.1)

Calculated: C 53.2, H 5.5, N 12.2
Found: C 53.45, H 5.6, N 12.0

**Enzymic Cleavage of Synthetic Substrates—**In addition to the DEAE-Tris preparation of cathepsin D, preparations of twice crystallized porcine pepsin (Worthington, Lot P'M (933-7) and of three crystallized acid proteinase from Rhizopus chinensis (Miles Laboratories, Lot 8 U 06) were used. The specific activities of these two commercial preparations with hemoglobin as the substrate were 2,800 units per mg and 1,800 units per mg, respectively. The initial rate of cleavage (5 to 15%) of each synthetic substrate was determined at pH 4.0 (0.04 m sodium formate buffer) and 37.0° ± 0.1° by means of the spectrophotometric method described by Inouye and Fruton (34); this method depends on the increase in absorbance at 310 nm when the p-nitrophenylalanine residue is converted into its carboxylic acid form. In all cases, the data accorded with Michaelis-Menten kinetics over the range of substrate concentrations used. In the calculation of kcat, the molecular weights of the proteinases were assumed to be: cathepsin D, 42,000; pepsin, 34,200; and Rhizopus proteinase, 35,000. The precision (95% confidence limits) of kcat and Km were estimated by computer analysis (36). The position of enzymic cleavage of the peptide substrates was shown by thin layer chromatography (1-butanol-acetic acid-water, 4:1:1) to be restricted to the Phe(NO₂)-Phe bond; ninhydrin and the Pauly reagent were used to develop the chromatograms. Thus, in the case of the cleavage of Phe-Gly-His-Phe(NO₂)-Phe-Val-Leu-OMe by cathepsin D, pepsin, or the Rhizopus proteinase, samples were incubated for 24 hours, and it was found that a Pauly-positive, ninhydrin-positive component of RF 0.57 and a Pauly-negative, ninhydrin-positive component of RF 0.80 were present, and that the starting material (RF 0.72) was absent.

**Competitive Inhibition of Cathepsin D—**The inhibitors tested were Phe-OMe hydrobromide (34), Z-His-Phe-OMe (34), and pepstatin; the last was generously provided by Professor H. Umezawa, to whom we wish to express our gratitude. In all cases, the substrate was Phe-Gly-His-Phe(NO₂)-Phe-Val-Leu-OMe (pH 4.0, 37°) at three initial concentrations (0.1, 0.2, and 0.4 mM) and the inhibitor was tested at two or three concentrations; for each concentration of substrate and inhibitor, the initial velocity, v, was determined in triplicate by the spectrophotometric method for following the cleavage of the Phe(NO₂)-Phe bond of the substrate. The dissociation constant of the enzyme-inhibitor complex, Kᵢ, was estimated from plots of v₀ versus [I] (37), and the inhibition type was inferred from plots of the slopes of v₀ versus 1/S plots against inhibitor concentration (38, 39).

**Inhibition of Acid Proteinases by Diazoketones—**The synthesis of the two diazoketones, 1-diazoacetyl-2-phenylethane and 1,1'-bis(diazoacetyl)-2-phenylethane, used in this study has been described previously (40). The rate of inactivation of cathepsin D, pepsin, and Rhizopus proteinase by these reagents was determined at pH 5.0 (0.04 m sodium acetate buffer) and 30°, with denatured hemoglobin as the substrate, as described by Husain et al. (40). Appropriate controls in the absence of added diazoketone were run; no inhibition of enzymic activity was observed during the 30-min incubation period under the conditions of these studies.

**RESULTS**

**Purification of Cathepsin D—**The material obtained by the procedure summarized in Table I showed, on polyacrylamide gel electrophoresis in the two systems described under “Experimental Procedure,” a single protein band with a trace of a more slowly moving component. On isoelectric focusing, the DEAE-
Tris preparation was found to contain three components of isoelectric point 6.1, 6.3, and 6.7 (measured at 4°C); the specific activity of the three components was essentially the same (Fig. 2). An explanation of this appearance of multiple molecular forms of the enzyme requires further work to determine whether they represent genetically determined variants or chemically modified (e.g., partially degraded) active forms of the same protein. Multiple electrophoretic forms of cathepsin D have been observed by previous investigators; for a summary see Barrett (41).

Gel chromatography on Sephadex G-100, under conditions where chymotrypsinogen A emerged with a peak at 21 ml and bovine serum albumin with peaks at 21 ml (monomer) and 17 ml (dimer), gave an elution diagram (Fig. 3) with a single peak at 26 ml, indicating an approximate molecular weight of 42,000 for our cathepsin D preparation. Although the specific enzymic activity of the 1-ml fractions emerging between 21 ml and 36 ml was fairly constant (2,400 ± 200 units per mg), the elution curve shown in Fig. 3 exhibits asymmetry, with trailing at volumes corresponding to species of lower molecular weight. Sedimentation equilibrium analysis of our cathepsin D preparation gave data (Fig. 4) consistent with the behavior of a reversibly associating system involving an oligomeric protein of molecular weight about 43,000. Examination of the preparation by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicated the appearance of a component having an apparent molecular weight of about 28,000. Although the relative constancy of specific enzymic activity found upon isoelectric focusing and gel chromatography suggests the absence of gross inhomogeneity due to the presence of large amounts of inactive protein, it must be emphasized that the value of about 42,000 for our cathepsin D preparation is provisional, and that further work is needed on this problem. The values reported for other preparations of cathepsin D have ranged from 35,000 to 60,000 (41); in particular, Press et al. (4) reported a value of 58,000 for their bovine spleen preparation, based on a sedimentation velocity constant $\xi_{w2}$ of 3.3 at pH 6.75, and a similar molecular weight was found by Keilova et al. (42). On the other hand, Smith et al. (8) reported that their enzyme preparation from porcine thyroid undergoes a pH-dependent dimerization, involving monomers of molecular weight 21,000, the value found by sedimentation analysis at pH 3.7; near pH 7.5 the dimerization is maximal.

A determination of the NH$_2$-terminal residues by the dansyl method (33) indicated that the DEAE-Tris preparation contained glycine as the NH$_2$-terminal amino acid, in agreement with earlier reports for preparations of bovine spleen cathepsin D (4, 42).

Action of Cathepsin D on Synthetic Peptides—A series of peptides of the type A-Phe(NO$_2$Phe-B, previously found to be readily cleaved by pepsin at the Phe(NO$_2$Phe bond (20), were tested as substrates for the DEAE-Tris preparation of cathepsin D. At an enzyme concentration of 1.8 mg/ml, the peptides having A = Z-His, Z-Gly-His, or Phe-Gly-His and B = OMe or His-OMe were not cleaved significantly during 30 min by cathepsin D at pH 4.0 and 37°C. This behavior may be contrasted to the susceptibility of these peptides to pepsin; the $k_{cat}/K_m$ values (in seconds$^{-1}$ mM$^{-1}$) found previously were 0.63 for Z-His-Phe(NO$_2$Phe-OMe, 0.38 for Z-Gly-His-Phe(NO$_2$Phe-OMe, 0.25 for Phe-Gly-His-Phe(NO$_2$Phe-OMe, and 0.5 for Z-His-Phe(NO$_2$Phe-OMe (20). The peptides Z-His-Phe(NO$_2$Phe-
Ala-OMe and Phe-Gly-His-Phe(NO₂)-Phe-Ala, which are hydrolyzed by pepsin with $k_{cat}/K_m = 8.3$ and 1.4, respectively (20), were cleaved very slowly by 1.8 $\mu M$ cathepsin D, and the rates were unsuitable for determination of the kinetic parameters. One of the synthetic substrates that was found to be completely cleaved by cathepsin D in 30 min, namely Z-His-Phe(NO₂)-Phe-Ala-OMe, is sparingly soluble at pH 4.0, and initial concentrations above 0.15 mM could not be maintained. Preliminary experiments showed the $K_m$ value for the cleavage of this substrate by cathepsin D to be well above the limit of solubility, in contrast to the situation with pepsin, where $K_m = 0.13$ mM (20). It should be added that the peptides listed above, including Z-His-Phe(NO₂)-Phe-OMe, were readily hydrolyzed by the Rhizopus proteinase at pH 4 (23).

In Table II are given data for those cathepsin D substrates tested whose solubility and susceptibility permitted determination of the kinetic parameters for the cleavage of the Phe(NO₂)-Phe bond in substrates of the type Phe-Gly-His-Phe(NO₂)-Phe-B. Three of these, with $B = \text{Ala-Ala, Ala-OMe, and Ala-Phe-OMe}$, had previously been found to be cleaved by pepsin with $k_{cat}$ values ranging between 19 and 28 s⁻¹ and $K_m$ between 0.04 and 0.27 mM (20). In addition, the Rhizopus proteinase had been found to cleave the peptide with $B = \text{Ala-Phe-OMe}$ with $k_{cat} = 0.45$ s⁻¹ and $K_m = 0.04$ mM (23). It will be seen in Table II that, with cathepsin D, the values of $k_{cat}$ for these three substrates ($>0.1$ s⁻¹) are considerably below those for pepsin, and that the $K_m$ values (0.24 to 0.51 mM) are somewhat higher.

These results on the relative specificity of cathepsin D and pepsin support the view that, as in the case of pepsin, the addition of hydrophobic amino acid residues to the $B$ group of substrates of the type $\text{A-Phe(NO₃)-Phe-B}$ greatly increases the sensitivity of the Phe(NO₂)-Phe bond. It is also clear, however, that both the binding of substrate to enzyme (assumed to be reflected in $K_m$) and the catalytic efficiency ($k_{cat}$) are much less favorable for enzymic action in the case of cathepsin D than with pepsin. In view of these findings, a substrate was designed in which the $B$ group is Val-Leu-OMe, so chosen because of the favorable effect of the Leu-17-Val-18 sequence in the $B$ chain of insulin on the cleavage of the Leu-15-Tyr-16 bond. The Val-Leu sequence was preferred to that present in insulin to prevent the possible cleavage of an additional peptide bond in the substrate; previous work had shown that such substrates as Z-His-Val-Phe-OMe are resistant to pepsin action (10). It will be noted in Table II that Phe-Gly-His-Phe(NO₂)-Phe-Val-Leu-OMe is indeed hydrolyzed rapidly by cathepsin D; although its $k_{cat}$ value is only about one-seventh of that for pepsin, it is near that for the Rhizopus proteinase, and the $K_m$ values for the actions of the three acid proteinases on this substrate are similar (0.02 to 0.09 mM). Since only the Phe(NO₂)-Phe peptide bond is broken during the cleavage of Phe-Gly-His-Phe(NO₂)-Phe-Val-Leu-OMe by cathepsin D and the rate can be followed spectrophotometrically, this peptide represents a useful material for further studies of the enzyme. No data are available at present on the effect of structural modification of the $A$ group in A-Phe(NO₂)-Phe-Val-Leu-OMe, but earlier results on the specificity of pepsin (21) suggest that it may perhaps be possible to synthesize peptide substrates of greater sensitivity to cathepsin D by modification of the $A$ group.

Inhibition of Cathepsin D by Peptides—The availability of a suitable synthetic substrate for cathepsin D permitted examination of the ability of several peptides known to be inhibitors (or substrates) of pepsin to inhibit the action of the DEAE-Tris preparation of cathepsin D. With Phe-Gly-His-Phe(NO₂)-Phe-Val-Leu-OMe as the substrate, Phe-OMe and Z-His-Phe-OMe (which is resistant to cathepsin D) were found to be linear competitive inhibitors of cathepsin D at pH 4.0 and 37°, with $K_i$ values of 1.0 mM and 0.2 mM, respectively. With pepsin, the $K_i$ value found for Phe-OMe was 0.25 mM (18) and the $K_i$ value for Z-His-Phe-OMe was 0.37 mM (34). The naturally occurring isovaleryl pentapeptide pepstatin (44) was found to inhibit the action of cathepsin D on the heptapeptide substrate with a $K_i$ of approximately $1 \times 10^{-5}$ mM, close to the $K_i$ value reported for the inhibition of the cleavage of hemoglobin by pepsin at pH 4 (45). It should be added that whereas the plots of $1/v$ versus $1/S$ for the inhibition of cathepsin D by Phe-OMe and Z-His-Phe-OMe gave sets of lines that clearly intersected on the 1/s axis, the corresponding lines for pepstatin failed to do so. This may be attributed to the wide disparity in the binding of the substrate and inhibitor, which made it difficult to determine the position of the intercept accurately. A similar result was obtained for the inhibition of pepsin by pepstatin, using Z-His-Phe(NO₂)-Phe-OMe as the substrate. Barrett and Dingle (46) have recently reported the inhibition, by pepstatin, of the cleavage of hemoglobin by preparations of liver cathepsin D.

Inhibition of Cathepsin D by Diazocompounds—Previous work in this laboratory (40, 47) and by other investigators (48-50) has shown that pepsin is stoichiometrically inactivated by some diazo compounds, such as tosyl-L-phenylalanyldiazomethane (47), diazocetyl-L-phenylalanine methyl ester (47, 50), diazocetyl N-norleucine methyl ester (48, 51), 1-diazocetyl-2-phenylethene (40, 49), and 1,1'-bis(diazocetyl)-2-phenylethene (40). The inhibition of partially purified cathepsin D preparations by diazocetyl-N-norleucine methyl ester has been reported (8, 32).

In previous work (40) it was found that the

### Table II

**Kinetics of cleavage of synthetic substrates by acid proteinases**

Experiments were carried out at pH 4.0 (0.04 M formate buffer) and 57°.

| Enzyme     | Substrate                         | $k_{cat}/K_m (s^{1/2})$ | $K_m$ (mM) | $k_{cat}/K_m$ |
|------------|-----------------------------------|--------------------------|------------|---------------|
| Cathepsin D| Phe-Gly-His-Phe(NO₂)-Phe-Ala-Ala   | 0.15-0.55 (15)           | 0.09 ± 0.01| 0.47 ± 0.10   |
| Cathepsin D| Phe-Gly-His-Phe(NO₂)-Phe-Ala-OMe  | 0.15-0.55 (14)           | 0.07 ± 0.02| 0.5 ± 0.2     |
| Cathepsin D| Phe-Gly-His-Phe(NO₂)-Phe-Phe-OMe  | 0.15-0.55 (15)           | 0.12 ± 0.02| 0.27 ± 0.09   |
| Cathepsin D| Phe-Gly-His-Phe(NO₂)-Phe-Val-Leu-OMe| 0.10-0.55 (18)           | 0.08       | 0.87 ± 0.13   |
| Pepsin     | Phe-Gly-His-Phe(NO₂)-Phe-Val-Leu-OMe| 0.025-0.125 (18)         | 0.01       | 0.22 ± 0.02   |
| Rhizopus proteinase | Phe-Gly-His-Phe(NO₂)-Phe-Val-Leu-OMe | 0.025-0.2 (20)           | 0.08       | 1.3 ± 0.2     |

*The numbers in parentheses denote the number of runs.*

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rate of inactivation of pepsin by 1,1'-bis(diazoacetyl)-2-phenylethane under comparable conditions, and it was of interest to examine the action of these two diazoketones on the DEAE-Tris preparation of cathepsin D. It will be seen in Fig. 5 that, under comparable conditions, and it was of interest to examine the action of these two diazoketones on the DEAE-Tris prepara-

The available data on the specificity of cathepsin D indicate that the reason for the difference in its apparent specificity as compared with pepsin must be sought in the role of secondary enzyme-substrate interactions, at a distance from the Phe-Phe unit, in determining the rate of cleavage of the sensitive bond. It is evident from the data in Table II that modification of the nature of the B group of substrates of the type Phe-Gly-His-Phe-Phe-B can have greater effects on the kinetics of cathepsin D than on the action of pepsin. Although further work is needed to examine the role of secondary enzyme-substrate interactions in the case of cathepsin D, there can be little doubt that as for other pepsin-like acid proteinases (rennin, Rhizopus proteinase, etc.) the active site of the enzyme represents an extended region, and that specific interactions at a distance from the catalytic groups can modify greatly the catalytic efficiency of cathepsin D action. This has been shown by Raymond et al. (54) for the cleavage of the Phe-Met bond in oligopeptide substrates of rennin.

Comparison of the kinetic parameters reported in Table II with earlier data in the literature is difficult. Keilova et al. (15) reported a $K_m$ of 0.33 mM (pH 4.3, 37°C) for Gly-Lys-Leu-Gly-Phe-Leu, which was cleaved by bovine spleen cathepsin D only at the Phe-Leu bond, but no $k_{cat}$ value was given. They also found that in the COOH-terminal leucyl residue by its enantiomer made the peptide more resistant to enzymic cleavage; the diastereoisomeric peptide acted as a competitive inhibitor with $K_i$ of 0.07 mM. Kazakova et al. (17) reported both $K_m$ and $k_{cat}$ values for the cleavage by rat liver cathepsin D of several peptides made by the solid phase method of synthesis, the purity of which was documented only by thin layer chromatography and amino acid composition. The peptides selected for kinetic study were cleaved at multiple peptide bonds, and $K_m$ and $k_{cat}$ values were given for the cleavage of the bond apparently hydrolyzed most rapidly. Thus, for the cleavage of Ala-Phe-Gly-Leu-Phe-Val at the Leu-Phe bond, the values (at pH 3.0 and 37°C) were stated to be $1.56 \times 10^{-4}$ M and $1 \times 10^6$ min$^{-1}$, respectively. Similar kinetic parameters were reported for the cleavage of the Gly-Leu bond in Ala-Phe-Gly-Leu-Asp-Val and in Ala-Asp-Gly-Leu-Phe-Val. In view of the fact that, in separate experiments, only 25 to 40% of the most sensitive bond was reported to have been cleaved in 17 hours when the ratio of peptide to enzyme was 50:1, the reported high $k_{cat}$ values, corresponding to 20 to 70 s$^{-1}$, are difficult to understand. We believe that the data reported by Kazakova et al. (17) need careful scrutiny.

Like pepsin, cathepsin D and other acid proteinases are in-

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**FIG. 5.** Inhibition of cathepsin D, pepsin, and Rhizopus proteinase by 1-diazoacetyl-2-phenylethane (mono) and 1,1'-bis(diazoacetyl)-2-phenylethane (bis). A and B ( ), 0.65 µM cathepsin D, 105 µM diazoketone (A, mono; B, bis), and 780 µM CuSO$_4$. C and D ( ), 0.65 µM pepsin, 32.5 µM diazoketone (C, bis; D, mono), and 130 µM CuSO$_4$. E and F ( ), 0.65 µM Rhizopus proteinase, 32.5 µM diazoketone (E, mono; F, bis), and 130 µM CuSO$_4$. All experiments were conducted at pH 5.0 (8 mM sodium acetate buffer) at 30°C, in the presence of 170 µl ethanol. The ordinate denotes the per cent original activity, with hemoglobin as the substrate.
activated by suitable diazo compounds, and the data in Fig. 5 provide a comparison of the susceptibility of cathepsin D, pepsin, and Rhizopus proteinase to two such inhibitors. The available data do not permit speculation about the reason for the strikingly lower sensitivity of cathepsin D, but the possibility must be considered that the benzyl group of the diazoketones is bound less strongly at the active site in the case of cathepsin D than with the other two enzymes. Such apparently lower affinity of the reagent for the active site is consistent with the weaker binding of comparable small oligopeptides.

Note Added in Proof—After the acceptance of this paper for publication, we learned of the report by McAdoo et al. (55) that a partially purified cathepsin D preparation from bovine lung, when tested with hemoglobin as the substrate, is inhibited by pepstatin and by 1-diazoacetyl-2-phenylethylamine.

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