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Purification and Partial Characterization of a *Nocardia brasiliensis* Extracellular Protease

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Nocardia brasiliensis possess proteolytic activities that can be readily detected in a variety of media. In a modified formulation of a growth medium originally used for *Streptomyces aureofaciens*, *N. brasiliensis* was found to secrete proteolytic enzymes, one of which was capable of hydrolyzing casein. This enzyme was purified to homogeneity from cell-free culture filtrates of *N. brasiliensis*. The purification procedure included ion-exchange chromatography on carboxymethyl-Sepharose, gel filtration on Sephadex G-100, and affinity chromatography, using a hemoglobin-Sepharose resin. The molecular weight of the *N. brasiliensis* protease was found to be 25,000 by gel filtration and 35,000 by sodium dodecyl sulfate-discontinuous gel electrophoresis. The enzyme is inhibited by *o*-phenanthroline and 8-hydroxyquinoline-5-sulfonic acid but is not affected by EDTA. Average values for its kinetic parameters were 0.288 μmol of hemoglobin solubilized per min per mg of enzyme for V_{max} and 0.76 mM for K_m, using hemoglobin as the substrate.

The ability of *Nocardia brasiliensis* to hydrolyze protein substrates constitutes a fundamental criterion for its differentiation from other *Nocardia* species (5). The enzyme or enzymes responsible for this phenomenon have never been characterized. The present study reports the purification of an extracellular protease from *N. brasiliensis* and describes some of its characteristics. The functions of this enzyme are unknown; however, the presence of an extracellular protease in a pathogenic organism suggests a role in the infective process.

MATERIALS AND METHODS

Bacterial strain. *N. brasiliensis* 27-78 was isolated from a human case of mycetoma and was given to us by P. Lavalle (Instituto Dermatológico Pascua, Mexico City, Mexico).

Cultures of *N. brasiliensis* 27-78 for enzyme purification. *N. brasiliensis* 27-78 was cultured by shaking at 30°C in a liquid medium containing (per liter): 6.796 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4$, 0.01 g of $MnSO_4$, 0.005 g of $CoSO_4$, 10 g of glucose, 2 g of neopeptone, and 0.5 g of yeast extract. For the initial studies on protease secretion versus growth of organisms, 125-ml Erlenmeyer flasks containing 25 ml of the above medium were inoculated with 10^6 organisms per ml. Every 8 to 12 h, the contents of one of the flasks were filtered through 0.22-μm preweighed GS filters (Millipore Corp., Bedford, Mass.); the samples were kept in a desiccator until they were completely dry and their weight remained constant for at least two readings (taken every 24 h). The filtrate from each sampling was aseptically placed in sterile disposable tubes and frozen until all samples had been taken. After the samples were thawed, they were assayed for proteolytic activity (see below), using a 1% casein solution as the substrate.

Purification of extracellular protease. The extracellular nature of the *N. brasiliensis* proteolytic activity was determined by testing the culture filtrates obtained after the removal of the organisms for the presence of a known intracellular marker (6-phosphogluconate dehydrogenase). The method used was that described by King (7), in which the activity of the enzyme is determined by the appearance of NADPH as indicated by an increase in absorption at 340 nm.

For purification of the extracellular protease produced by *N. brasiliensis*, organism cultivation was scaled up to 20 liters with a laboratory fermentor (model CFS-307; New Brunswick Scientific Co., Inc., Edison, N.J.). The culture filtrate was concentrated to ca. 800 ml by ultrafiltration, using the Pellicon system (Millipore), and extensively dialyzed for 48 h against 0.05 M imidazole buffer (pH 6.5) containing 0.1 mM EDTA and 0.1 mM dithiothreitol. The dialyzed material was passed through a carboxymethyl (CM)-Sepharose CL-6B column (Pharmacia Fine Chemicals, Piscataway, N.J.) that selectively adsorbed the protease. The enzyme was eluted by using a linear 0 to 0.3 M NaCl gradient. The peak of proteolytic activity was between 0.1 and 0.2 M ionic strength.

The active fractions from the CM-Sepharose column were pooled, concentrated by ultrafiltration with an Amicon cell, and fractionated on a Sephadex G-100 column (Pharmacia) equilibrated with the same buffer system used for the ion-exchange chromatography. fractions of 1.5 ml were collected. Active fractions were concentrated and chromatographed on a hemoglobin-Sepharose column prepared by the method of Chua and Bushuk (2). The column was washed after sample application, and then the enzyme was eluted as a single peak with 0.1 M acetate buffer (pH 4.0). Active fractions from that peak were pooled and concentrated by ultrafiltration to a final volume of 4 to 5 ml. The protein concentration of this preparation was determined by the Lowry assay (10) (see below) to be 0.1 to 0.12 mg/ml. The enzyme was stored at −20°C in samples of 100 μl each until future use.

Protein determination. Protein concentrations were determined by the Lowry assay (10). Bovine serum albumin or casein was used as the standard.

Determination of the enzymatic activity. Hydrolysis of casein (Gallard Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y.) was measured by the method described by Gallop et al. (4), by incubating 1 ml of a 1% (wt/vol)

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solution of casein in 0.05 M phosphate buffer (pH 7.2) with the enzyme preparation. The reaction was carried out at 37°C and was terminated after 30 min by adding 10% trichloroacetic acid (TCA). After filtration, samples of 75 μl each were removed for the Lowry assay. The optical density of the samples was measured at 700 nm in a Bausch & Lomb Spectronic 710 spectrophotometer. One unit of proteolytic activity was defined as the amount of enzyme that liberated 1 μg of TCA-soluble peptides from casein per min under the above conditions.

Electrophoresis. Homogeneity of the protease fraction was examined by the sodium dodecyl sulfate-disc gel electrophoresis method (SDS-PAGE) of Weber and Osborn (19) and by the native acid gel system developed by Reisfeld et al. (16). Both the acid and SDS gels were run at 15 mA per gel for the stacking gel and 30 mA per gel for the running gel. The migration markers used for the acid and SDS gels were Pyronine Y and bromphenol blue, respectively. Protein was stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Richmond, Calif.) and destained with a mixture of 40% methanol and 10% acetic acid in water.

Fibrinogen-containing SDS gels were used to determine the bands with proteolytic activity. These gels were prepared by the method of Lacks and Springhorn (8) and were electrophoresed with a current of 15 to 20 mA per gel for the stacking gel and 30 to 40 mA per gel for the running gel until the bromphenol blue dye had reached the bottom of the gels. The gels were then removed, rinsed in water, and washed by shaking in at least 10 times their volume of 0.04 M Tris (pH 7.6) for 3 h. The gels were incubated in 0.04 M Tris (pH 7.6)–2 mM $MgCl_2$–2 mM $CaCl_2$ overnight and stained with Coomassie brilliant blue R-250. The bands with proteolytic activity appeared as clear zones in a blue background.

Molecular weight determination. (i) Molecular-sieve chromatography. The molecular weight of the purified enzyme was estimated by the method of Andrews (1) with a Sephadex G-100 column (1.6 cm [inside diameter] by 59 cm). Chromatography was carried out at 4°C and run at 15 ml/h. Calibration standards (Pharmacia) included bovine serum albumin, ovalbumin, trypsinogen, and ribonuclease.

(ii) SDS-PAGE. SDS-PAGE was performed by the method of Weber and Osborn (19) with a vertical slab gel unit (SE 500; Hoefer Scientific Instruments, San Francisco, Calif). Electrophoresis was carried out at 15 mA per gel for the stacking gel and 30 mA per gel for the running gel with a 12% polyacrylamide gel containing 0.1% SDS (BDH, Poole, England). The protein was stained with Coomassie brilliant blue R-250 and destained with a mixture of 40% methanol and 10% acetic acid in water.

Enzyme inhibition. Determination of enzyme inhibition in the presence of different amounts of inhibitors was carried out by incubating the protease with the inhibitor for 15 min at 37°C (17). One ml of a 1% (wt/vol) casein solution was immediately added, and the assay was carried out as described above. Results were recorded as the percent residual activity calculated with reference to activity of controls run in the absence of the inhibitor.

Initial velocity studies and calculation of the apparent K_m. Bovine hemoglobin (Sigma Chemical Co., St. Louis, Mo.) was chosen as the substrate for kinetic experiments with *N. brasiliensis* protease, since its amino acid sequence and molecular weight are more precisely defined than those of casein. Different concentrations of hemoglobin, ranging from 0.25 to 2.5 mM, were incubated with 10 μl (1.2 μg) of purified *N. brasiliensis* protease. Every five min the reaction was stopped by the addition of 10% TCA. The precipitate was removed by filtration, and the supernatants were tested for the presence of protein by the Lowry assay (10). Increases in absorbance at 700 nm for the different time points, determined versus absorbance of a control at time zero, were plotted against time. The linear part of the curve was used to estimate the values of the initial velocities. Their reciprocals, $1/v$, were plotted against 1/[S] values, and a line was fitted by the method of least squares. The apparent K_m and V_{max} were then obtained from the estimated intercept values. Initial reaction rates (velocity) were expressed as μmol of hemoglobin solubilized per min, and V_{max} was expressed as μmol of hemoglobin solubilized per min per mg of enzyme.

RESULTS

Preliminary studies on growth and enzyme formation. The growth curve of *N. brasiliensis* in the liquid medium used for enzyme purification is shown in Fig. 1. Data for total enzymatic activity in the supernatants indicate that proteolytic activity appears at ca. 40 h and peaks when the culture has reached early stationary phase.

Purification of the extracellular protease. The purification steps for the *N. brasiliensis* protease are summarized in Table 1. The total enzymatic activity present in the concentrated culture filtrate was recovered in a 75% yield after the CM-Sepharose step, producing a 29-fold enrichment in specific activity. The subsequent Sephadex G-100 run yielded a twofold increase in the specific activity of the protease, with a high yield of the total enzymatic activity. The final step of purification, chromatography on a hemoglobin-Sepharose column, produced the elution profile shown in Fig. 2, in which two peaks of proteolytic activity were present. Homogeneity of the peak eluting with 0.1 M acetate buffer (pH 4.0) was determined by SDS-PAGE, and a single band was observed (Fig. 3). This band exhibited proteolytic activity on the fibrinogen-containing SDS gel (Fig. 4) upon in situ

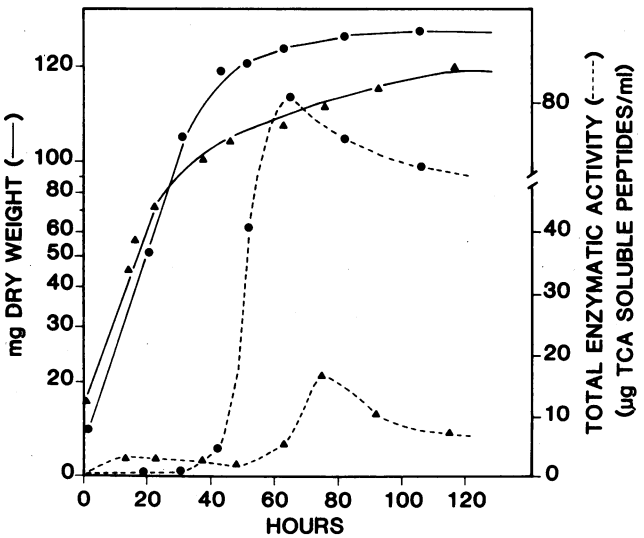

FIG. 1. Growth curves and total proteolytic activities for *N. brasiliensis* 27-78 in Sabouraud medium (▲) and in the medium (●) used for the enzyme purification. Growth curves were based on the determination of the dry weights of the organisms in 25-ml samples removed every 8 to 12 h. Total proteolytic activities were estimated by incubating 0.5-ml samples from the supernatant obtained after removing the organisms with 1 ml of 1% casein solution. After the addition of 10% TCA to stop the enzymatic reaction, the amount of TCA-soluble peptides was determined by the Lowry assay (10).

TABLE 1. Purification of the *N. brasiliensis* protease

Purification step	Total protein (mg)	Total vol (ml)	Total activity (U)[a]	sp act (U/mg of protein)	% Yield	Purification factor
Concentrated culture filtrate	1,259.8	810	2,920	2.3	100	1
CM-Sepharose eluate	33.4	5.5	2,200	65.8	75	29
Sephadex G-100	13.2	11.0	1,818	137.7	62	60
Hemoglobin-Sepharose pool	0.472	4.0	765	1,620.0	26	705

[a] One unit = the amount of enzyme that liberates 1 μg of TCA-soluble peptides from casein per min, under the conditions of the assay.

renaturation of the enzyme. The zone of clearing in Fig. 4, lane 6, is partially obscured by the contaminating protein. The cleared zone in lanes 2 through 5 is clearly contaminating protease from lane 1 (Fig. 4).

Molecular weight determination. The molecular weight of the purified protease was estimated by SDS-PAGE and by gel filtration on Sephadex G-150. The values obtained were 35,000 and 25,000, respectively. The apparent discrepancy between the values obtained is not unexpected, since the methodologies used allow the estimation of molecular weights on the basis of different properties of a molecule, namely, its electrophoretic mobility under denaturing conditions (SDS-PAGE) and its effective Stokes radius under nondenaturing conditions (gel filtration).

Effect of different inhibitors on the *N. brasiliensis* proteolytic activity. Metal-chelating agents such as *o*-phenanthroline and 8-hydroxyquinoline-5-sulfonic acid caused considerable inhibition of the enzyme (Table 2). Sulfhydryl inhibitors such as iodoacetic acid and *p*-chloromercuribenzoic acid did not affect the protease. The purified enzyme was also not inactivated by phenylmethylsulfonyl fluoride or soybean trypsin inhibitor; EDTA, 2,2'-dipyridyl, and 2,9-dimethyl-1,10-phenanthroline had little effect on *N. brasiliensis* proteolytic activity.

Effect of metal ions on *o*-phenanthroline-inhibited enzyme. *o*-Phenanthroline-inhibited enzyme was incubated with various concentrations of either zinc or calcium chloride for 15 min, and the activity was determined as described above. The residual activities of the enzyme indicated that $ZnCl_2$ restored the activity of the enzyme to almost 90%. $CaCl_2$, however, had no significant effect (data not shown).

V_{max} and apparent K_m for the *N. brasiliensis* protease, using hemoglobin as the substrate. The progress of the enzymatic reaction versus time showed an initial reaction that was not linear, probably due to the ability of the enzyme to hydrolyze certain bonds in the hemoglobin molecule very rapidly. After 5 min, the reaction progressed linearly, and this part of the curve was used for the calculations of the initial reaction rate. The amount of the total substrate cleaved during the course of the reaction was <1%. The values obtained for V_{max} and K_m from the Lineweaver-Burk double-reciprocal plots were 0.288 μmol/min per mg of enzyme and 0.76 mM, respectively.

DISCUSSION

Among the enzymes produced by the actinomycetes, the proteases from *Streptomyces* species have been the subject of much research (12, 15, 18). Proteolytic enzymes from other *Actinomycetales*, however, have not been well studied, probably because of the difficulties associated with growth of the organisms.

In this report, purification of an extracellular protease from *N. brasiliensis* was accomplished by conventional methods and by a final affinity chromatography step that made use of a hemoglobin-Sepharose column originally devised for the purification of wheat proteases (2). The final

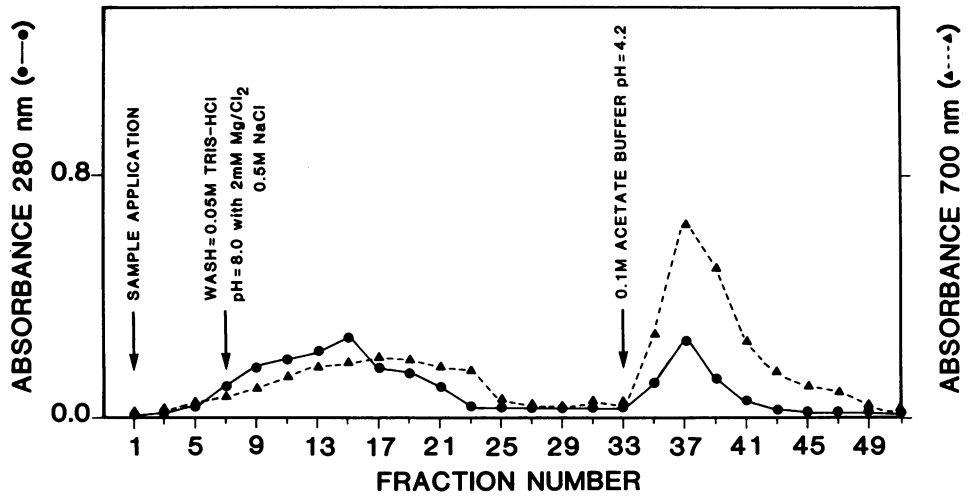

FIG. 2. Elution profile for the chromatography of the G-100 eluate on a hemoglobin-Sepharose column. A 4-ml sample volume was applied to the column. This was then washed with 0.05 M Tris-hydrochloride (pH 8.0) containing 2 mM $MgCl_2$ and 0.5 M NaCl. The major peak with proteolytic activity was subsequently eluted with 0.1 M acetate buffer (pH 4.2). Protein in the diagram is indicated as absorbance at 280 nm (●), and enzyme activity is indicated as absorbance at 700 nm (▲). The incubation mixture for the enzymatic assay was composed of 25 μl of eluate and 250 μl of a 1% casein solution in 0.05 M phosphate buffer (pH 7.0).

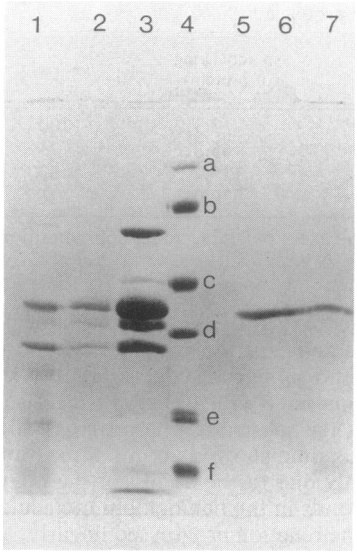

FIG. 3. Twelve percent SDS-PAGE of hemoglobin-Sepharose fractions. Samples in lanes 1, 2, and 3 of the gel correspond to fractions 9, 13, and 17, respectively, from the first protein peak obtained when the hemoglobin-Sepharose column was washed with 0.05 M Tris-hydrochloride (pH 8.0) containing 2 mM $MgCl_2$ and 0.5 M NaCl. Lane 4 has the molecular weight standards: (a) phosphorylase b, (b) bovine serum albumin, (c) ovalbumin, (d) carbonic anhydrase, (e) soybean trypsin, and (f) α-lactalbumin. Lane 5 was empty. Lanes 6 and 7 were loaded with fractions from the late protein peak of the hemoglobin-Sepharose column, eluting with 0.1 M acetate buffer (pH 4.2).

yield of the enzyme was 26%, and the purification factor was 705-fold compared with that of the initial concentrated culture filtrate. This yield is good, considering that the initial extracts contained other proteolytic activities which were removed by the purification procedure.

An important early purification step was the ion-exchange chromatography on CM-Sepharose. This step was found to adsorb the protease, while allowing the removal of most of the protein and peptide contaminants present in the culture filtrate. The G-100 Sephadex step yielded a preparation that was not homogenous, although its specific activity increased twofold. The contaminants present in this preparation were removed by chromatography on the hemoglobin-Sepharose resin. The recovery of active material from this last step was somewhat lower than expected, due to the relatively low affinity of the enzyme for hemoglobin (see below).

The original medium for the growth of *Streptomyces aureofaciens* and induction of its caseinolytic activity contained leucine and lysine as sole nitrogen sources (9). This medium did not induce the secretion of *N. brasiliensis* proteolytic activity. Substitution of the two amino acids by the more complex nitrogenous source from neopeptone was sufficient to allow secretion of the enzyme. Induction did not occur until stationary phase of growth; however, once induced, activity remained in the culture filtrates for several hours.

The molecular weights for proteolytic enzymes from microbial sources have been considered by Morihara (12) and Nakadai et al. (14) to fall within the range of 22,000 to 77,000. The molecular weight for the *N. brasiliensis* protease as estimated by SDS-PAGE and by gel filtration on Sephadex G-100 is in agreement with these figures.

The kinetic parameters (V_{max} and K_m) for *N. brasiliensis* proteolytic activity were estimated, using hemoglobin as the substrate. The average K_m value obtained, 0.76 mM, indicates that the affinity of the enzyme for the substrate is very low. Although the apparent V_{max} of 0.288 μmol of hemoglobin solubilized per min per mg of protein appears low, a minimum of 13 proteolytic cleavages must be made per polypeptide chain, since the assay recognizes TCA-soluble peptides. In general, peptides must be below a molecular weight of 5,000 to remain soluble under these conditions (3). Thus, in terms of peptide bond cleavage the enzyme catalyzes approximately two events per s based on an enzyme molecular weight of 30,000. This value is in agreement with those values reported for other microbial proteases with different substrates, for example, the *Bacillus subtilis* neutral protease (13) and the α-lytic protease from *Myxobacterium* (20).

The *N. brasiliensis* protease was not inhibited by the serine protease inhibitors phenylmethylsulfonyl fluoride and soybean trypsin inhibitor (Table 2). Among the sulfhydryl inhibitors only iodoacetic acid exerted a slight inhibition; others such as *p*-chloromercuribenzoate did not affect the enzymatic activity. These observations indicate that the enzyme is neither a serine nor a thiol protease. The enzymatic activity was inhibited 86% by 0.1 mM *o*-phenanthroline and 77% by 1.3 mM 8-hydroxyquinoline-5-sulfonic acid. However, other metal-chelating reagents such as EDTA and 2,2'-dipyridyl did not considerably affect the enzymatic activity at the concentration used. The inhibitory effects of

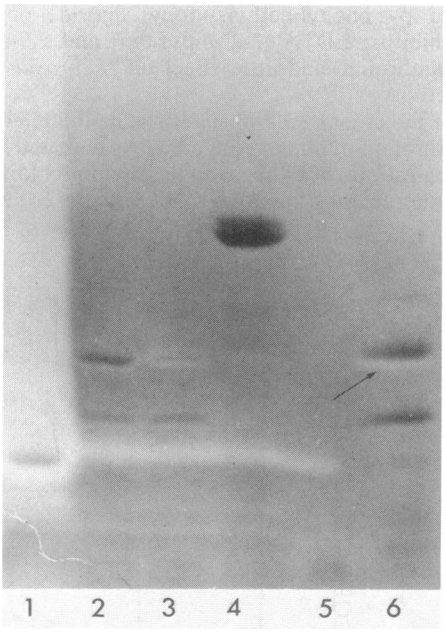

FIG. 4. In situ renaturation of the *N. brasiliensis* protease after SDS-PAGE. The gel contained fibrinogen at 170 μg/ml and was run under the conditions described in the text. Lanes were loaded with: (1) 1 μg of chymotrypsin; (2 and 3) the pool with proteolytic activity from the G-100 column, not treated with 2-mercaptoethanol; (4) fraction 37 from the CM-Sepharose column; (5) nothing; (6) the pool with proteolytic activity from the G-100 column, treated with 2-mercaptoethanol. The arrow indicates the band with proteolytic activity. Proteins with proteolytic activity under these conditions are detected by the halo of gel which has been depleted of fibrinogen.

TABLE 2. Effect of inhibitors on *N. brasiliensis* proteolytic activity

Inhibitor[a]	Final concn (M)	% Residual activity
Soybean trypsin	10×10^{-6}	102.0
Phenylmethylsulfonyl fluoride	1.0×10^{-3}	97.7
	2.0×10^{-3}	96.6
	4.0×10^{-3}	96.7
p-Chloromercuribenzoic acid	10.0×10^{-6}	80.8
Iodoacetic acid	0.2×10^{-3}	82.3
	0.4×10^{-3}	60.3
EDTA	3.0×10^{-3}	75.2
o-Phenanthroline	1×10^{-5}	96.8
	4×10^{-5}	27.7
	1×10^{-4}	13.9
2,9-Dimethyl-1,10-phenanthroline	4×10^{-5}	88.0
	1×10^{-4}	106.0
8-Hydroxyquinoline-5-sulfonic acid	3.2×10^{-4}	89.4
	13×10^{-4}	22.9
2,2'-Dipyridyl	1.3×10^{-4}	92.4
	2.6×10^{-4}	88.3

[a] The inhibitors were added to either 0.01 M Tris-hydrochloride (pH 8.0) containing 1.5 mM $CaCl_2$ (soybean trypsin and phenylmethylsulfonyl fluoride), 0.05 M Tris-hydrochloride (pH 7.5) containing 5 mM cystein and 2 mM EDTA (*p*-Chloromercuribenzoic acid and iodoacetic acid), or 0.1 M phosphate buffer (pH 7.0) (EDTA, *o*-phenanthroline, 2,9-dimethyl-1,10-phenanthroline, 8-hydroxyquinoline-5-sulfonic acid, and 2,2'-dipyridyl), in amounts ranging from 0.1 to 0.4 ml of stock solutions to yield the final concentration given above. At timed intervals, 0.1 ml of enzyme solution (10 μg protein) was added to each tube. Incubation was allowed to proceed at 37°C for 15 min. The remaining enzymatic activity was then assayed by the method described in the text.

o-phenanthroline and 8-hydroxyquinoline-5-sulfonic acid could be explained by the chelation of some divalent metal ion necessary for enzymatic activity. The protective effects of zinc, as evidenced by the fact that its addition to *o*-phenanthroline-inhibited enzyme restores its activity, suggest that the metal ion is responsible for enzymatic activity. In addition, the slight inhibition by 2,9-dimethyl-1,10-phenanthroline, an analog of *o*-phenanthroline and a weaker chelator of zinc than is *o*-phenanthroline (6), is also consistent with a catalytic role for zinc. However, further experimentation is required to show beyond doubt that the *N. brasiliensis* protease is a zinc metalloenzyme.

The characteristics of the *N. brasiliensis* protease that have been discussed are consistent with those established for other metal chelator-sensitive proteases from organisms such as *Streptomyces griseus* (15, 17) and *Pseudomonas aeruginosa* (11). The metabolic role of the *N. brasiliensis* enzyme is presently unknown. However, the availability of techniques to express and purify the enzyme should prove useful toward the study of its possible role in the pathogenicity of the microorganism.

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