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Purification and Properties of *Mucor pusillus* Acid Protease[1]

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The protease produced by *Mucor pusillus* was recovered from a wheat bran medium by treatment with ammonium sulfate, ethyl alcohol, gel filtration and ion-exchange chromatography. The yield of the enzyme was 55%. The overall increase in the specific activity of the protease was 34-fold. The purified protease was most active at *p*H 3.8 and 5.6 against hemoglobin and casein, respectively. Optimal hydrolysis of casein was observed at 55 C. The enzyme was stable from *p*H 3.0 to 6.0. Enzyme inactivated by metal ions was reactivated by ethylenediaminetetraacetate and *o*-phenanthroline. Reducing agents and thiol poisons had no effect on the protease, suggesting that free sulfhydryl groups were not required for enzyme activity. Diisopropyl fluorophosphate did not inhibit the protease, indicating the probable absence of serine in the active center. The Michaelis-Menten constant for casein was 0.357%. Electrophoretic analysis of active protein recovered by ion-exchange chromatography showed that the protease preparation was homogeneous.

Recent literature abounds with information concerning the isolation and characterization of mold proteases having optimal activity at acidic *p*H. Studies on the properties of many mold proteases have been carried out with purified preparations (1, 6, 7, 8, 12, 18, 21, 23, 26). In some cases, however, only crude enzyme preparations have been used (5, 10, 11, 22, 25). Frequently, studies are mainly concerned with the milk-clotting activity of proteases (5, 9, 16, 17, 23, 24).

Mucor pusillus is a thermophilic mold (3) which synthesizes an acid protease under aerated conditions (K. Arima and S. Iwasaki, U.S. Patent 3,212,905,1965). Data have been published on the effect of *p*H, heat treatment, and calcium ions on the milk-clotting activity of the crude acid protease (9, 16). Factors influencing the synthesis of *M. pusillus* acid protease under submerged conditions have also been studied (20).

The aim of this investigation was the purification of the acid protease and the study of some of its properties.

MATERIALS AND METHODS

Organism. The culture of *M. pusillus* used throughout this study was obtained from the Purdue University Culture Collection. The culture was maintained on potato-malt (Difco)-agar slants at 37 C and transferred weekly.

Materials. Sephadex G-75 (medium) and diethylaminoethyl (DEAE) Sephadex A-50 (medium) were purchased from Pharmacia Fine Chemicals, Inc., New Market, N.J. Cellulose polyacetate strips (Sepraphore III) for electrophoresis were products of Gelman Instrument Co., Ann Arbor, Mich. Purified casein and ethylenediaminetetraacetic acid (EDTA) were products of Fisher Scientific Co., Pittsburgh, Pa. Cysteine and sodium thioglycolate were purchased from K & K Laboratories, Inc., Jamaica, N.Y., and Nutritional Biochemicals Corp., Cleveland, Ohio, respectively. Diisopropyl fluorophosphate (DIFP), hemoglobin (twice crystallized), and *N*-ethyl maleimide were obtained from Mann Research Laboratories, Inc., New York, N.Y. Monoiodoacetic acid and 2-mercaptoethanol were products of Matheson Coleman and Bell, East Rutherford, N.J. *o*-Phenanthroline and *p*-chloromercuribenzoate (pCMB) were products of J. T. Baker Chemical Co., North Phillipsburg, N.J., and Calbiochem, Los Angeles, Calif., respectively. All of the salts used were of reagent grade.

Buffers. Buffer solutions used in studying the properties of the acid protease were prepared according to Britton (2) and, Dawson and Elliott (4).

Isolation and purification of enzyme. A 5% wheat bran medium was adjusted to *p*H 5.0, dispensed into 2,000-ml Erlenmeyer flasks (350 ml/flask), sterilized, cooled, and inoculated with a spore suspension of *M. pusillus*. Flasks were agitated on a New Brunswick model 3-25 gyratory shaker operating at 240 cycles per min, for 5 days at 35 C. At the end of the incuba-

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tion period, the broth (*p*H 6.6) was first filtered through four layers of gauze and then through a 1-inch layer of Celite analytical filter aid. Solid ammonium sulfate was added in small amounts to the clear, dark yellow filtrate (1,000 ml), to bring the salt concentration to 85%. The mixture was allowed to stand in the cold (6 C) for 48 hr, and the precipitate was collected by centrifugation at 25,000 × *g* for 20 min in a model B-20 International centrifuge. The precipitate was dissolved in 0.05 M phosphate buffer (*p*H 6.0) and stirred for 30 min at room temperature; any insoluble matter was then removed by centrifugation, as described above.

Since the crude enzyme preparation contained a membrane-weakening factor, elimination of excess salt by dialyzing in cellophane bags was impracticable. Consequently, the crude preparation was desalted by gel filtration on a Sephadex G-25 (fine) column (1.8 × 100 cm).

The next step in enzyme purification was treatment with alcohol. To the desalted active protein solution, 1.5 volumes of absolute ethyl alcohol were added dropwise. The precipitate which formed overnight in the cold (6 C) was centrifuged and discarded. To the supernatant fluid, another 1.5 volumes of absolute ethyl alcohol were added dropwise, and the mixture was allowed to stand in the cold. After 24 hr, the fine precipitate was recovered by centrifugation at 25,000 × *g* for 15 min and was dissolved in 0.05 M phosphate buffer (*p*H 6.0).

The active protein preparation was further fractionated by gel filtration. The protein was soaked into a column (2.4 × 55 cm) of Sephadex G-75 dextran gel equilibrated with 0.05 M phosphate buffer (*p*H 6.0). Elution of protein was carried out with the same buffer. Fractions (5 ml) were collected by means of an automatic fraction collector. The distribution of protein was established by determining the absorbance of each fraction at 280 mμ in a Beckman DU spectrophotometer.

The final step in enzyme purification was the ion-exchange chromatography of the protease obtained by gel filtration. The DEAE-Sephadex A-50 column (2.4 × 35 cm) was equilibrated with 0.02 M phosphate buffer containing 0.1 M NaCl. Elution of enzyme protein was achieved by a linear gradient from 0.1 to 0.48 M NaCl.

Electrophoresis. Active protein obtained by gradient ion-exchange chromatography was placed in dialysis bags and dialyzed against a large excess of distilled water, for 16 hr at 6 C. The protein solution was transferred into bottles and lyophilized. Approximately 500-μg amounts of the pale-yellow powder were loaded on Sepraphore III cellulose polyacetate strips, and electrophoresis was carried out in barbitone buffer (*p*H 8.6; ionic strength, 0.07, with 0.1 ma per strip) at room temperature, in a modified Beckman model R cell. The strips were stained with Ponceau S stain and were cleared by the acetic acid-ethyl alcohol clearing technique.

Protein determination. Protein concentration was measured by the method of Lowry et al. (14), with crystalline bovine albumin as the standard (Nutritional Biochemicals Corp.).

Protease assay. Proteolytic activity was measured by the method of McDonald and Chen (15). For routine analyses, the enzyme was incubated with 0.5% casein solution or 0.5% acid-denatured hemoglobin solution (19) as the substrate, in 0.1 M acetate buffer (*p*H 5.45) or 0.1 M phosphate buffer (*p*H 7.0). The amounts of enzyme and substrate, and the length of incubation, are specified for the individual experiments.

The degree of purification attained in each step of the purification procedure was checked by incubating various amounts of enzyme in 1-ml volume with 4 ml of 1% casein substrate in 0.1 M acetate buffer (*p*H 5.45), for 60 min at 30 C. Protein was precipitated by adding 5 ml of 5% trichloroacetic acid, and after 30 min the mixture was filtered through two layers of Whatman no. 42 filter paper. The amount of trichloroacetic acid-soluble matter was measured at 700 mμ. The increase in absorbance at 700 mμ was plotted against enzyme concentration, and, from the slope of the line, the activity was obtained in terms of micrograms of material absorbing at 700 mμ (calculated as tyrosine) solubilized per minute per milligram of enzyme. A unit of enzyme activity was defined as the amount of enzyme that solubilized 1 μg of material absorbing at 700 mμ (calculated as tyrosine) per minute at 30 C. Specific activity was defined as units of enzyme per milligram of protein.

RESULTS

Purification of protease. Table 1 summarizes the steps involved in the purification of the acid protease. After treatment with ammonium sulfate and ethyl alcohol, the active preparation was further fractionated on Sephadex G-75 (Fig. 1). Gel filtration effectively removed the considerable amounts of colored impurities (peak D) that were present in the crude enzyme preparation. Enzyme protein, as detected by protease assay, was concentrated under a distinct peak (peak B).

Ion-exchange chromatography of the enzyme on DEAE-Sephadex A-50 (Fig. 2) showed that all detectable activity was present under one symmetrical peak. Fractions under this active peak were pooled, lyophilized, and used for electrophoretic analysis. There was only a single band detectable on cellulose acetate strips stained with Ponceau S (Fig. 3).

The results of linear gradient chromatography and electrophoretic analysis suggested that the protease activity found in the enzyme preparation was due to a single entity.

Effect of pH. The effect of hydrogen ion concentration on the activity of purified protease was determined with casein and acid-denatured hemoglobin as substrates, over the *p*H range 2.2 to 7.0. The buffer systems employed were Na_2HPO_4-citric acid from *p*H 2.2 to 7.0, sodium acetate-acetic acid from *p*H 3.6 to 5.6, and Na_2HPO_4-KH_2PO_4 from *p*H 5.8 to 7.0.

To determine the protease activity at various *p*H values, equal volumes of 1% substrate solution and appropriate buffer were mixed and equilibrated to 30 C. Then, 1 ml of enzyme solution (5 μg of protein) was mixed with 4 ml of substrate, and the assay mixture was incubated at 30 C for 30 min. The amount of liberated tyrosine was estimated by the McDonald-Chen method (15).

Figure 4 shows the effect of hydrogen ion concentration on the enzyme. The protease hydrolyzed hemoglobin most rapidly at about *p*H 3.8 to 4.0. Casein, which becomes less and less soluble as the isoelectric point is approached (*p*H 4.6), was not suitable for determining the *p*H optimum of the protease. However, it appeared that optimal casein hydrolysis was at about *p*H 5.6.

Stability of protease to pH. Samples (1 ml) of protease solution (184 μg of protein) were mixed with 12.5 ml of Britton-Robinson modified universal buffer of the appropriate *p*H (2). The protein solutions were allowed to stand in a 30 C water bath for 30 min. The remaining activity was assayed by mixing 1 ml of enzyme with 4 ml of 0.5% casein solution in 0.1 M acetate buffer (*p*H 5.45) and incubating the mixture at 30 C for 60 min.

TABLE 1. *Purification of Mucor pusillus acid protease*

Treatment	Total enzyme units	Total protein	Specific activity	Enzyme yield
		mg	*units/mg*	%
Crude filtrate.....	395 × 10^3	1,520	260	100
Ammonium sulfate.....	336 × 10^3	800	420	85
Ethyl alcohol.....	256 × 10^3	162	1,580	64
Sephadex.....	223 × 10^3	62	3,600	56
DEAE-Sephadex..	220 × 10^3	25	8,800	55

Figure 5 shows the *p*H stability relationship of the enzyme. Values plotted were averages of triplicate determinations. The enzyme was most stable between *p*H 3.0 and 6.0.

Effect of temperature. Protease activity at various temperatures was determined with casein used as the substrate. Samples of a 0.5% solution of casein in 0.1 M acetate buffer (*p*H 5.45) were held at various temperatures for 10 min. Then, 4 ml of substrate was added to 1 ml of enzyme (7.12 μg of protein), and the assay mixture was incubated at the selected temperature for 60 min. Figure 6 shows that the temperature optimum of the enzyme reaction was about 55 C.

To determine the heat stability of the enzyme, solutions of the active protein (8 μg/ml) in acetate buffer (*p*H 5.45) were allowed to stand in water baths set at various temperatures for 15 min. The heat-treated enzyme solutions were rapidly cooled, and the residual protease activity was determined at 30 C with casein as substrate. Figure 7 shows the heat stability of the enzyme. The protease lost about 90% of its activity after a 15-min exposure at 65 C.

Michaelis-Menten constant. A stock casein solution was diluted with 0.1 M acetate buffer (*p*H 5.45) to give different concentrations of substrate in the assay mixture. The enzyme concentration was held constant at 2.8 μg per ml.

The Michaelis-Menten constant (K_m) was determined graphically (Fig. 8), according to the

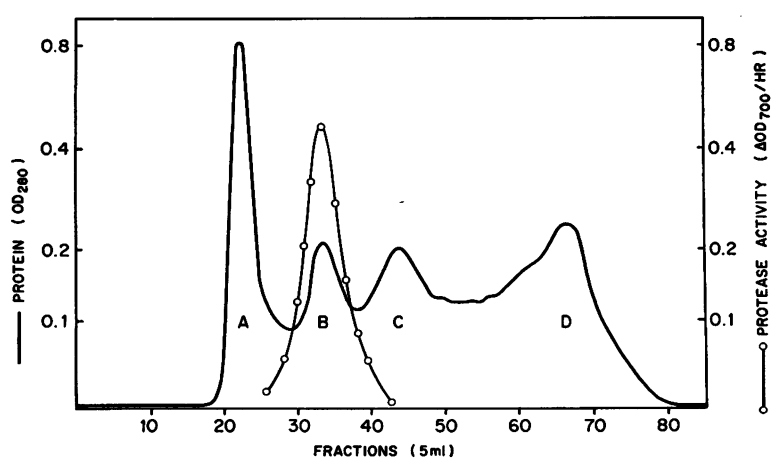

FIG. 1. *Gel filtration of ethyl alcohol-precipitated crude enzyme on Sephadex G-75. Column: 2.4 × 55 cm. Eluant: 0.05 M phosphate buffer (pH 6.0). Sample load: 32 mg. Flow rate: 0.25 ml* min^{-1} cm^{-2}, *at 6 C. Protease activity of each fraction was checked by mixing 0.5 ml with 4.5 ml of 0.5% casein (pH 5.45) and incubating the mixture at 30 C for 60 min.*

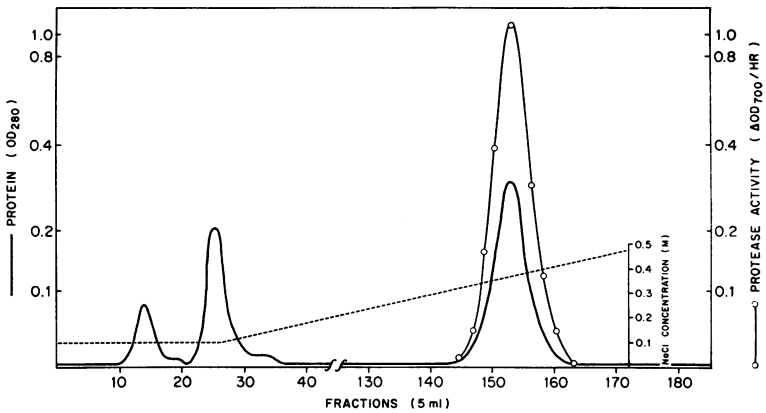

FIG. 2. *Chromatography of partially pure Mucor pusillus protease on DEAE-Sephadex A-50. Eluant: 0.02 M phosphate buffer, pH 6.7; a linear gradient of NaCl (from 0.1 to 0.48 M) was applied for the elution of protein. Protease assay: same as described in Fig. 1.*

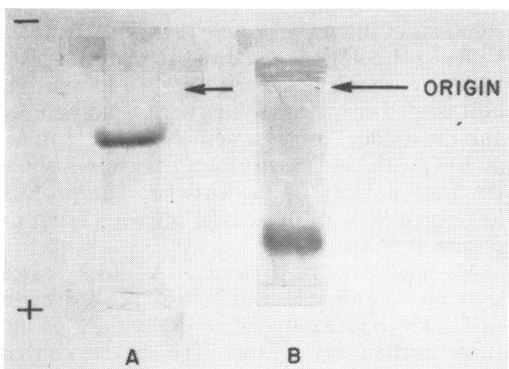

FIG. 3. *Electrophoresis of purified protease on cellulose acetate. Buffer: Sodium barbital, pH 8.6; ionic strength, 0.07. Current: 0.5 ma/strip. Stain: Ponceau S. A = migration of protein after 1 hr. B = migration of protein after 4 hr.*

method of Lineweaver and Burk (13). The value of K_m for casein was 0.357%.

Effect of chelating agents on enzyme activity. The effects of EDTA and *o*-phenanthroline were tested at 5×10^{-3} M and 10^{-3} M concentrations, in both acetate (*p*H 5.45) and phosphate (*p*H 7.0) buffer systems, with casein and hemoglobin as substrates. The enzyme was preincubated with the chelator for 30 min at 30 C before the addition of substrate. Protease assays were carried out under conditions described earlier. It was found that *o*-phenanthroline at a concentration of 5×10^{-3} M gave rise to cloudiness which interfered with the estimation of trichloroacetic acid-soluble matter at 700 mμ. However, the cloudiness could be effectively removed by filtration through Whatman no. 42 filter paper.

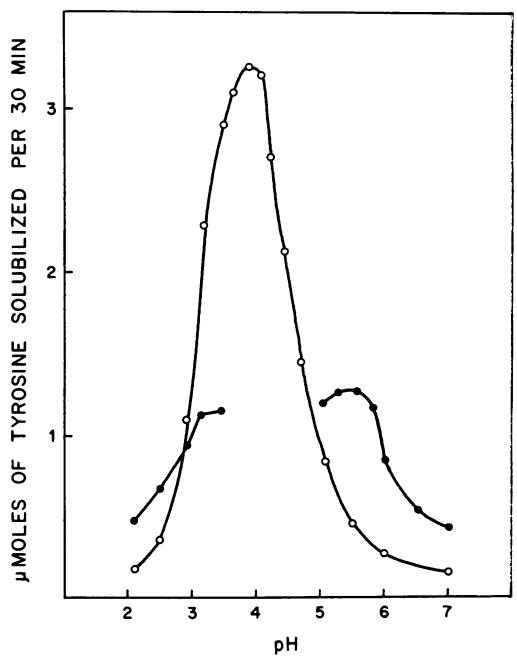

FIG. 4. *Effect of pH on protease activity. Reaction mixture contained 1 μg of enzyme per ml and 4 mg of substrate per ml, in McIlvaine (pH 2.2 to 7.0) and acetate (pH 5.0 to 5.8) buffers. Symbols: ○, hemoglobin digestion; ●, casein digestion.*

The results of protease assays indicated that neither EDTA nor *o*-phenanthroline influenced the hydrolysis of casein and hemoglobin.

Effect of metals on M. pusillus protease. The effects of a number of cations on the hydrolysis of casein by *M. pusillus* protease were tested. Mg^{++}, Ca^{++}, Fe^{++}, Cu^{++}, Hg^{++}, Co^{++}, Cd^{++},

Ni^{++}, and Fe^{+++} were tested as the chlorides, and Zn^{++} and Mn^{++}, as the sulfates.

Solutions of metallic salts were prepared in 0.1 M acetate buffer (*p*H 5.45). To 1 ml of enzyme solution (12 μg of protein), 1 ml of metallic salt solution was added, and the mixture was allowed to stand at 30 C for 30 min. Then, 3 ml of casein substrate was added to the enzyme-metal mixture, and protease activity was determined.

Table 2 shows the data on the effect of metal ions on enzyme activity. All of the cations tested except Mg^{++}, Ca^{++}, and Co^{++}, had an adverse effect on casein hydrolysis. At 10^{-3} M concentration, the order of inhibitory effectiveness was as follows: $Hg^{++} > Zn^{++} > Cu^{++} > Fe^{++} > Cd^{++} > Fe^{+++} > Ni^{++}$.

Reactivation of metal-inhibited protease by chelating agents. Although EDTA and *o*-phenanthroline did not influence the proteolysis of casein by *M. pusillus* protease, they were effective in reversing the inhibition of the enzyme by metal ions.

Enzyme was preincubated with inhibiting metal at 10^{-3} M for 60 min at 30 C. The chelator was added at 2×10^{-3} M to the enzyme-metal mixture, and this was immediately followed by the addition of casein substrate. Protease assays were carried out at 30 C for 60 min.

Table 2 shows the enzyme-reactivating effects of EDTA and *o*-phenanthroline on protease inhibited by various metal ions. EDTA was more effective than *o*-phenanthroline in recovering lost protease activity.

The effectiveness of EDTA as enzyme-reactivat-

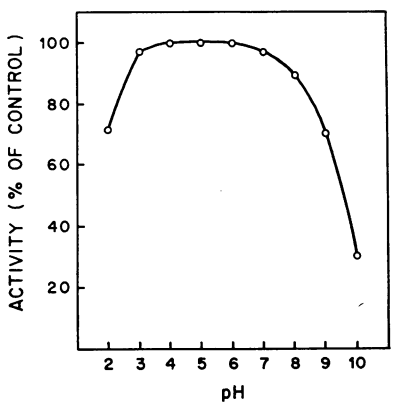

FIG. 5. *Stability of acid protease to pH. The enzyme (14 μg/ml) was incubated at various pH values for 30 min at 30 C before assay. Assay mixtures contained 2.8 μg of enzyme per ml and 4 mg of casein per ml. Incubation time: 60 min at 30 C.*

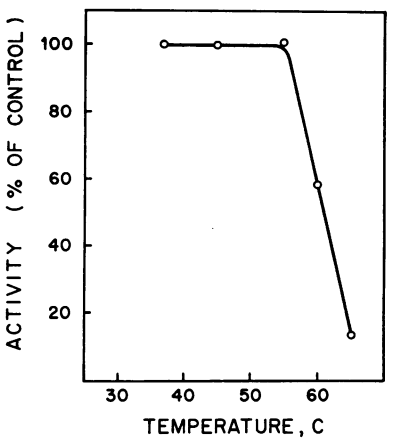

FIG. 7. *Heat stability of purified acid protease. Enzyme protein was exposed to temperatures indicated for 15 min. Residual activity was determined at 30 C. Incubation time: 60 min.*

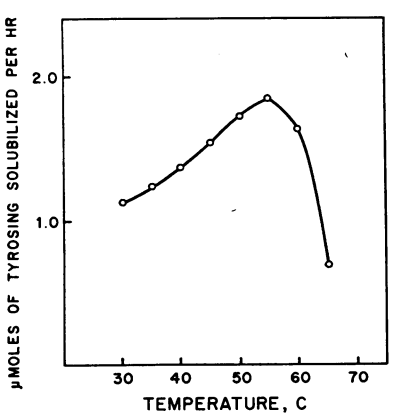

FIG. 6. *Effect of temperature on enzyme activity. Assay mixtures contained 1.42 μg of enzyme per ml and 4 mg of casein per ml. Incubation time: 60 min.*

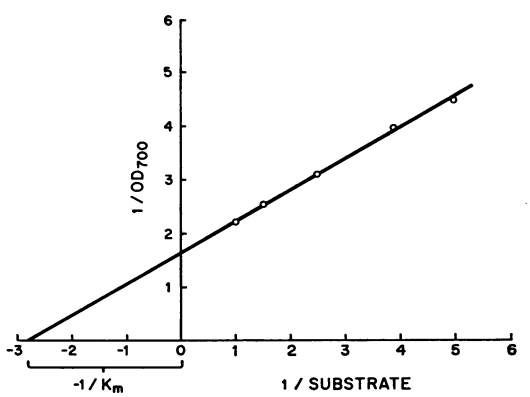

FIG. 8. *Lineweaver-Burk plot of reaction velocity (ΔOD_{700}) versus casein concentration (%). Reaction mixtures were incubated at 30 C for 60 min (pH 5.45 acetate buffer system).*

TABLE 2. *Inhibition of casein hydrolysis[a] by metal ions and enzyme reactivation by chelating agents*

Metal ion	Concn of metal ion[b]	
	2×10^{-3} M	10^{-3} M
Hg^{++}	27	38
$Hg^{++} + 2 \times 10^{-3}$ M EDTA	—	96
$Hg^{++} + 2 \times 10^{-3}$ M *o*-phenanthroline	—	62
Zn^{++}	25	42
$Zn^{++} + 2 \times 10^{-3}$ M EDTA	—	91
$Zn^{++} + 2 \times 10^{-3}$ M *o*-phenanthroline	—	67
Cu^{++}	37	52
$Cu^{++} + 2 \times 10^{-3}$ M EDTA	—	96
$Cu^{++} + 2 \times 10^{-3}$ M *o*-phenanthroline	—	83
Fe^{++}	66	76
Cd^{++}	72	81
Fe^{+++}	86	86
Ni^{++}	92	88
Mg^{++}	100	100
Ca^{++}	100	100
Co^{++}	100	100

[a] Expressed as percentage of control activity.

[b] Final concentrations in enzyme-metal incubation mixture before addition of substrate. Assay mixtures contained 2.4 μg of enzyme per ml and 3 mg of casein per ml. Appropriate blanks and controls were included for each experiment.

ing agent was studied with zinc-inhibited enzyme. The protease was incubated with zinc solutions containing various amounts of the metal (from 5×10^{-3} to 5×10^{-5} M), for 60 min before EDTA in equimolar amounts and casein substrate were added. The best recovery of enzyme activity was achieved with the lower concentrations of the metal inhibitor (up to 2.5×10^{-4} M). However, even at the highest inhibitor concentration tested (5×10^{-3} M), about 40% of lost protease activity was recovered by treatment with the chelator.

Effect of reducing agents, thiol poisons, DIFP, and other inhibitors on protease activity. The effects of cysteine, thioethanol, sodium thioglycolate, and sodium sulfite were tested at concentrations from 2×10^{-2} to 2×10^{-4} M. The effects of these compounds on the hydrolysis of casein were measured in both acetate (*p*H 5.45) and phosphate (*p*H 7.0) buffer systems. The enzyme was incubated with the reducing agents for 60 min before carrying out casein digestion for 60 min at 30 C. It was found that none of the reducing agents tested had any influence on the activity of the acid protease.

The thiol poisons pCMB (8.3×10^{-4} M), monoiodoacetate (5×10^{-2} and 1×10^{-4} M), and *N*-ethyl maleimide (5×10^{-2} and 1×10^{-4} M) were also tested in both acetate (*p*H 5.45) and phosphate (*p*H 7.0) buffer systems. As with reducing compounds, the enzyme was incubated with the sulfhydryl inhibitor for 60 min at 30 C before the addition of casein substrate. The results indicated that the protease was not affected by the presence of sulfhydryl reagents in the enzyme assay mixture.

Experiments that were carried out with DIFP (from 5×10^{-3} to 1×10^{-4} M) showed that the protease was not susceptible to organofluoride inhibition.

The effects of potassium cyanide and sodium fluoride were tested between 5×10^{-2} and 1×10^{-4} M concentrations. Protease assays showed no inhibition by these compounds, either at *p*H 5.45 (acetate system) or at *p*H 7.0 (phosphate system).

Effect of phosphate ion. Phosphate ion, supplied as KH_2PO_4, was tested from 5×10^{-3} to 0.2 M concentration. Protease assays showed no detectable change in the amount of liberated trichloroacetic acid-soluble matter at any of the phosphate concentrations tested.

DISCUSSION

It is generally recognized that many microorganisms elaborate exoenzymes that are concerned with the degradation of macromolecules whose transport into the cell is restricted because of their size. When *M. pusillus* is grown in a complex medium (wheat bran), proteolytic activity in the medium can be detected very early during the incubation period (20). Thus, it appears safe to assume that the protease is an extracellular enzyme.

Purification of the crude enzyme yielded a preparation which, upon ion-exchange chromatography and electrophoretic analysis, appeared to be homogeneous. The data suggested that *M. pusillus*, under the conditions of cultivation used, secreted only one type of protease into the environment. This protease was optimally active at about *p*H 3.8, when acid-denatured hemoglobin was used as the substrate, and at about *p*H 5.4 to 5.6 with casein substrate. The discrepancy between the *p*H optima for casein and hemoglobin degradation probably resulted from the decreasing solubility of casein near its isoelectric point (*p*H 4.6). The protease retained all of its activity after exposure to different hydrogen ion concentrations, from *p*H 3.0 to 6.0. Casein substrate was optimally hydrolyzed at about 55 C, and the enzyme was irreversibly destroyed at 65 C, showing a 90% loss of activity.

The enzyme was sensitive to most metal ions tested. The inhibition of protease by metals could be removed by treatment with EDTA or *o*-phenanthroline, the latter being somewhat less effective. The effectiveness of EDTA as enzyme reactivator depended on the concentration of the metal inhibitor. The findings suggested that, at high concentrations, the metal inhibitor brought about irreversible changes in the enzyme protein, because activity could not be fully recovered after the removal of inhibitor by the chelating agent.

The observation that chelating agents did not inhibit protease activity suggested that the protease was not a metalloenzyme. In this respect, the enzyme of *M. pusillus* resembles the acid proteases of *Aspergillus oryzae* (1), *Paecilomyces varioti* (18), and *Rhizopus chinensis* (6).

Reducing compounds and sulfhydryl reagents failed to influence casein hydrolysis, indicating that free sulfhydryl group(s) was not required for enzyme activity. This property of the enzyme was similar to that of *A. oryzae* (1) and *R. chinensis* (6).

Protease activity was not affected by the presence of DIFP in the reaction mixture. This finding suggested the probable absence of a serine residue at the active center of the enzyme. In this respect, the acid protease of *M. pusillus* resembles the enzymes synthesized by *P. varioti* (18) and *A. saitoi* (26).

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LITERATURE CITED

1. BERGVIST, R. 1963. The proteolytic enzymes of *Aspergillus oryzae*. II. Properties of the proteolytic enzymes. Acta Chem. Scand. **17**:1541–1551.
2. BRITTON, H. T. S. 1943. Hydrogen ions, p. 300–324. D. Van Nostrand Co., Inc., New York.
3. COONEY, D. G., AND R. EMERSON. 1964. Thermophilic fungi, p.17–27. W. H. Freeman and Co., San Francisco.
4. DAWSON, R. M. C., AND W. H. ELLIOTT. 1959. Buffers and physiological media, p. 192–209. *In* R. M. C. Dawson, D. C. Elliott, W. H. Elliott, and K. M. Jones [ed.], Data for biochemical research, Oxford University Press, Oxford.
5. DYACHENKO, P. F., AND V. V. SLAVYANOVA. 1962. Studies of rennet activity of vegetable proteolytic enzyme preparations. Intern. Dairy Congr. Proc., 16th, Copenhagen **B**:349–352.
6. FUKUMOTO, J., D. TSURU, AND T. YAMAMOTO. 1967. Studies on mold protease. I. Purification, crystallization and some enzymatic properties of acid protease of *Rhizopus chinensis*. Agr. Biol. Chem. (Tokyo) **31**:710–717.
7. HOFMAN, T., AND R. SHAW. 1964. Proteolytic enzymes of *Penicillium janthinellum*. I. Purification and properties of a trypsinogen activating enzyme (peptidase A). Biochim. Biophys. Acta **92**:543–557.
8. ICHISHIMA, E., AND F. YOSIDA. 1965. Chromatographic purification and physical homogeneity of acid protease of *Aspergillus saitoi*. Biochim. Biophys. Acta **99**:360–366.
9. IWASAKI, S., G. TAMURA, AND K. ARIMA. 1967. Milk clotting enzyme from microorganisms. II. The enzyme production and the properties of crude enzyme. Agr. Biol. Chem. (Tokyo) **31**:546–551.
10. JONSSON, A. G., AND S. M. MARTIN. 1964. Protease production by *Aspergillus fumigatus*. Agr. Biol. Chem. (Tokyo) **28**:734–739.
11. JONSSON, A. G., AND S. M. MARTIN. 1965. Protease production by *Alternaria tenuissima*. Agr. Biol. Chem. (Tokyo) **29**:787–791.
12. KOAZE, Y., G. HITOSHI, E. KAZUMI, Y. YUJIRO, AND H. TAKESHI. 1964. Fungal proteolytic enzymes. I. Isolation of two kinds of acid proteases excreted by *Aspergillus niger* var. *macrosporus*. Agr. Biol. Chem. (Tokyo) **28**:216–223.
13. LINEWEAVER, H., AND D. BURK. 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. **56**:658–666.
14. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. MCDONALD, C. E., AND L. L. CHEN. 1965. The Lowry modification of the Folin reagent for determination of proteinase activity. Anal. Biochem. **10**:175–177.
16. RICHARDSON, G. H., J. H. NELSON, R. E. LUBNOW, AND R. L. SCHWARBERG. 1967. Renin-like enzyme from *Mucor pusillus* for cheese manufacture. J. Dairy Sci. **50**:1066–1072.
17. SARDINAS, J. L. 1968. Renin enzyme of *Endothia parasitica*. Appl. Microbiol. **16**:248–255.
18. SAWADA, J. 1964. Studies on the acid protease of *Paecilomyces varioti* Bainier TPR-220. II. Some enzymic properties of the crystalline protease. Agr. Biol. Chem. (Tokyo). **28**:348–355.
19. SCHLAMOWITZ, M., AND L. U. PETERSON. 1959. Studies on the optimum pH for the action of pepsin on native and denatured bovine serum albumin and bovine hemoglobin. J. Biol. Chem. **234**:3137–3145.
20. SOMKUTI, G. A., AND F. J. BABEL. 1967. Conditions influencing the synthesis of acid protease by *Mucor pusillus* Lindt. Appl. Microbiol. **15**: 1309–1312.
21. TOMODA, K., AND H. SHIMAZONO. 1964. Acid protease produced by *Trametes sanguinea*, a wood-destroying fungus. I. Purification and

crystallization of the enzyme. Agr. Biol. Chem. (Tokyo) **28**:770–773.

22. TSUGO, T., AND K. YAMAUCHI. 1960. Comparison of clotting action of various milk coagulating enzymes. I. Comparison of factors affecting clotting time of milk. Intern. Dairy Congr. Proc., 15th London, 1959 **2**:636–642.
23. UCHINO, F., Y. KURONO, AND S. DOI. 1967. Purification and some properties of crystalline acid protease from *Acrocylindrium* sp. Agr. Biol. Chem. (Tokyo). **31**:428–434.
24. VESELOV, I. Y., D. Y. TIPOGRAF, AND T. A. PETINA. 1965. *Aspergillus candidus* as a renin producer. Prikl. Biokim. Mikrobiol. **1**:52–56.
25. WANG, H. L. 1967. Release of proteinase from mycelium of *Mucor hiemalis*. J. Bacteriol. **93**: 1794–1799.
26. YOSHIDA, F., AND M. NAGASAWA. 1958. Studies on the proteolytic enzymes of black *Aspergilli*. Proc. Intern. Symp. Enzyme Chem. Tokyo-Kyoto 1957, **2**:504–510.