Purification and Properties of *Mucor pusillus* Acid Protease

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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 pH 3.8 and 5.6 against hemoglobin and casein, respectively. Optimal hydrolysis of casein was observed at 55 C. The enzyme was stable from pH 3.0 to 6.0. Enzyme inactivated by metal ions was reactivated by ethylenediaminetetraacetic 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 pH. Studies on the properties of many mold proteases have been carried out with purified preparations (1, 6, 7, 8, 12, 18, 19, 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 pH, 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, Lafayette, Indiana 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 (Sephaphore 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 (DFIP), hemoglobin (twice crystallized), and N-ethyl maleimide were obtained from Mann Research Laboratories, Inc., New York, N.Y. Monothioacetic 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 (3) and Dawson and Elliott (4).

**Isolation and purification of enzyme.** A 5% wheat bran medium was adjusted to pH 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 incubation period the culture broth was centrifuged and the supernatant solution was used for purification of the enzyme.

**Chromatography.** Further purification of the crude enzyme was achieved by precipitation with ammonium sulfate. The precipitate obtained by adding 0.5 ml of saturated ammonium sulfate solution to 1 ml of enzyme solution was collected by centrifugation at 10,000 g for 10 min. The pellet was dissolved in a small volume of distilled water and dialyzed against 0.01 M acetic acid for 48 hr. The dialyzed solution was then subjected to chromatography on a column (2.5 x 50 cm) of Bio-Gel P-2 (50-200 mesh) in 0.01 M acetate buffer, pH 5.6. The fractions containing the protease were collected and dialyzed against 0.01 M potassium phosphate buffer, pH 8.0.

**Cation exchange chromatography.** The dialyzed solution was subjected to chromatography on a column (2.4 x 35 cm) of DEAE-Sephadex A-50 in 0.01 M potassium phosphate buffer, pH 8.0. The protease was eluted with a linear gradient of sodium chloride. Fractions containing the protease were collected and dialyzed against 0.01 M potassium phosphate buffer, pH 8.0.

**Gel filtration.** Gel filtration was carried out on a column (2.4 x 160 cm) of Sephadex G-75 in 0.01 M potassium phosphate buffer, pH 8.0. Fractions containing the protease were collected and dialyzed against 0.01 M potassium phosphate buffer, pH 8.0.

**Ion-exchange chromatography.** Further purification of the enzyme was achieved by chromatography on a column (2.2 x 110 cm) of DEAE-Sephadex G-75 in 0.01 M potassium phosphate buffer, pH 8.0. The protease was eluted with a linear gradient of sodium chloride. Fractions containing the protease were collected and dialyzed against 0.01 M potassium phosphate buffer, pH 8.0.

**Electrophoresis.** Electrophoretic analysis of the enzyme was carried out by using the sodium dodecyl sulfate (SDS)-gel method of Weber and Osborn (29) and by using the method of O'Farrell (26). The enzyme was dialyzed against 0.01 M potassium phosphate buffer, pH 7.0, prior to electrophoresis.

**Potentiometric pH measurement.** A pH meter (720C, Orion Research Inc., Beverly, Mass.) was used for the measurement of pH. The enzyme solution was titrated with 0.1 M sodium hydroxide solution using a microburet. The pH was recorded at 25 C.

**Gel diffusion.** Gel-diffusion analysis was carried out by using the method of Ouchterlony (24).

**Activity assay.** Casein (100 mg, Martin Dial) was dissolved in 0.01 M potassium phosphate buffer, pH 8.0, to give a concentration of 0.5 mg/ml. The enzyme solution was diluted to give an activity concentration of 0.01 units/ml. The assay mixture contained 0.01 M potassium phosphate buffer, pH 8.0, 0.5 mg/ml casein, and 0.01 units/ml of enzyme. The reaction mixture was incubated at 37 C for 30 min. The reaction was stopped by adding 10 ml of 6 N HCl. The precipitated casein was removed by centrifugation at 10,000 g for 10 min. The absorbance of the supernatant was measured at 280 nm. A unit of activity was defined as the amount of enzyme that hydrolyzes 1.0 mg of casein per min at 37 C.

**Optical rotatory dispersion.** Optical rotatory dispersion (ORD) and circular dichroism (CD) of the purified enzyme was determined using a Cary model 60 spectropolarimeter (30).

**Protein determination.** Protein was determined using the method of Lowry et al. (16).

**Enzyme molecular weight determination.** The molecular weight of the purified enzyme was determined by using the method of wild type (17).

**Enzyme stability.** The enzyme was stored frozen (-20 C) in 0.01 M potassium phosphate buffer, pH 8.0, containing 0.2 M glycerol. A unit sample was incubated at 37 C for 24 hr.

**Kinetic analysis.** The kinetic properties of the enzyme were determined by using the method of Hanes (13). The Michaelis-Menten constant was determined by using the method of Lineweaver and Burk (19). The double reciprocal plot was used to determine the Michaelis-Menten constant.

**Discussion.** 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 pH 3.8 and 5.6 against hemoglobin and casein, respectively. Optimal hydrolysis of casein was observed at 55 C. The enzyme was stable from pH 3.0 to 6.0. Enzyme inactivated by metal ions was reactivated by ethylenediaminetetraacetic 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.

**References.**

1. Presented in part at Annual Meeting of the American Society for Microbiology, New York, New York, 30 April-4 May 1967. Published with the approval of the Director of the Purdue University Agricultural Experiment Station as Journal Series Paper No. 3283.
tion period, the broth (pH 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 (pH 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 (pH 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 (pH 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 300-μg amounts of the pale-yellow powder were loaded onto Sepaphore III cellulose polyacetate strips, and electrophoresis was carried out in barbrite buffer (pH 8.6; ionic strength, 0.07, with 0.1 mα 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 (pH 5.45) or 0.1 M phosphate buffer (pH 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 (pH 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 μm. The increase in absorbance at 700 μm 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 μm (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 μm (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 pH range 2.2 to 7.0. The buffer systems employed were Na2HPO4-citric acid from pH 2.2 to 7.0, sodium acetate-acetic acid from pH 3.6 to 5.6, and Na2HPO4-KH2PO4 from pH 5.8 to 7.0.
To determine the protease activity at various pH 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 pH 3.8 to 4.0. Casein, which becomes less and less soluble as the isoelectric point is approached (pH 4.6), was not suitable for determining the pH optimum of the protease. However, it appeared that optimal casein hydrolysis was at about pH 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 pH (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 (pH 5.45) and incubating the mixture at 30°C for 60 min.

Figure 5 shows the pH stability relationship of the enzyme. Values plotted were averages of triplicate determinations. The enzyme was most stable between pH 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 (pH 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 (pH 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 (pH 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 (Km) was determined graphically (Fig. 8), according to the

### Table 1. Purification of Mucor pusillus acid protease

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total enzyme units</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Enzyme yield mg units/mg %</th>
</tr>
</thead>
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<tr>
<td>Crude filtrate</td>
<td>395 × 10⁶</td>
<td>1,520</td>
<td>260</td>
<td>100</td>
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<tr>
<td>Ammonium sulfate</td>
<td>336 × 10⁶</td>
<td>800</td>
<td>240</td>
<td>85</td>
</tr>
<tr>
<td>Ethyl alcohol</td>
<td>256 × 10⁶</td>
<td>162</td>
<td>1,580</td>
<td>64</td>
</tr>
<tr>
<td>Sephadex</td>
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<td>62</td>
<td>3,600</td>
<td>56</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>220 × 10⁶</td>
<td>25</td>
<td>8,800</td>
<td>55</td>
</tr>
</tbody>
</table>

**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⁻³, 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.
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 $5 \times 10^{-3}$ M and $10^{-4}$ M concentrations, in both acetate (pH 5.45) and phosphate (pH 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 $5 \times 10^{-3}$ M gave rise to cloudiness which interfered with the estimation of trichloroacetic acid-soluble matter at 700 nm. However, the cloudiness could be effectively removed by filtration through Whatman no. 42 filter paper.

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 (pH 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 x 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-

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**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. 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. 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. 8.** Lineweaver-Burk plot of reaction velocity (ΔOD750) 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 by metal ions and enzyme reactivation by chelating agents

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Conc of metal ion</th>
<th>Conc of metal ion</th>
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<tbody>
<tr>
<td>Hg++</td>
<td>2 × 10^-3 M</td>
<td>10^-1 M</td>
</tr>
<tr>
<td>Hg++ + 2 × 10^-3 M EDTA</td>
<td>—</td>
<td>96</td>
</tr>
<tr>
<td>Hg++ + 2 × 10^-4 M α-phenanthroline</td>
<td>—</td>
<td>62</td>
</tr>
<tr>
<td>Zn++</td>
<td>25</td>
<td>42</td>
</tr>
<tr>
<td>Zn++ + 2 × 10^-3 M EDTA</td>
<td>—</td>
<td>91</td>
</tr>
<tr>
<td>Zn++ + 2 × 10^-2 M α-phenanthroline</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cu++</td>
<td>37</td>
<td>52</td>
</tr>
<tr>
<td>Cu++ + 2 × 10^-2 M EDTA</td>
<td>—</td>
<td>96</td>
</tr>
<tr>
<td>Cu++ + 2 × 10^-3 M α-phenanthroline</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Fe+++</td>
<td>66</td>
<td>76</td>
</tr>
<tr>
<td>Cd++</td>
<td>72</td>
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<td>Fe++/++</td>
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</tr>
<tr>
<td>Ca++</td>
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<td>100</td>
</tr>
<tr>
<td>Co++</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* Expressed as percentage of control activity.
† 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.

The thiol agent was studied with zinc-inhibited enzyme. The protease was incubated with zinc solutions containing various amounts of the metal (from 5 × 10^-2 to 5 × 10^-4 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 (pH 5.45) and phosphate (pH 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 (pH 5.45) and phosphate (pH 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^-2 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 pH 5.45 (acetate system) or at pH 7.0 (phosphate system).

**Effect of phosphate ion.** Phosphate ion, supplied as K2HPO4, was tested from 5 × 10^-2 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 one type of protease into the environment. This protease was optimally active at about pH 3.8, when acid-denatured hemoglobin was used as the substrate, and at about pH 5.4 to 5.6 with casein substrate. The discrepancy between the pH optima for casein and hemoglobin degradation probably resulted from the decreasing solubility of casein near its isoelectric point (pH 4.6). The protease retained all of its activity after exposure to different hydrogen ion concentrations, from pH 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 p-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


