Purification and Some Properties of Crystalline Acid Protease from Acrocylindrium sp.

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A crystalline acid protease produced by a strain of Acrocylindrium in a submerged culture was prepared by treatment with acetone (60%), salting out with ammonium sulfate (saturated) and, after chromatography on Duolite CS-101 column, dialysis against distilled water. This preparation was homogeneous on sedimentation analysis, starch-gel electrophoresis and gel filtration with Sephadex G-75. The optimum pH was 2.0 for milk casein digestion and the pH stability was for 2.0–5.0 at 30°C for one day. The crystalline enzyme was completely stable below 50°C, but lost the activity at 70°C in ten minutes. The acid protease was almost equal to pepsin on specific activity when milk casein solution (pH 2.0) was used as substrate.

INTRODUCTION

Since all microorganisms which utilize proteins as nutrient materials produce proteolytic enzymes, there must be a large number of proteinase produced by various species of microorganisms. Among them, however, acid proteinases are relatively small in number, whose optimum pH values are below 3.0. Yoshida,\(^1\) obtained a crystalline acid proteinases from extract of solid culture of Aspergillus saitoi. His preparation was homogeneous on paper electrophoresis, most active at pH 2.5–3.0 and stable at pH 2.5–6.0. But, afterwards, Ichishima and Yoshida,\(^2\) found that this crystalline preparation was impure, and so they further purified it and obtained a homogeneous preparation on sedimentation analysis and boundary electrophoresis. An acid proteinase from Paecilomyces varioti Bainier TPR-220 was obtained in a crystalline form by Sawada.\(^5\) It was homogeneous on sedimentation analysis and electrophoresis, whose optimum pH was 2.5–3.0 and stable between pH 2.0–6.0. A crystalline acid proteinase obtained by Tomoda\(^6\) from a submerged culture of Trametes sanguinea, a wood-destroying fungus, was most active at pH 2.5 and stable over a pH range of 2.0–6.5. Koaze and his co-workers\(^7\) reported that Aspergillus niger var. macrosporus produced two crystalline acid proteinases which were fractionated by chromatography on hydroxyl apatite and S-E-cellulose. Their optimum pH ranges were 2.5–3.0 and 2.0, respectively. Besides these mentioned above, Aspergillus oryzae,\(^8\) Rhizopus javanicus,\(^9\) Mucor hiemalis,\(^10\) etc. were reported as fungi producing acid proteinases whose optimum pH were below 3.0.

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2) F. Yoshida and M. Nagasawa, ibid., 20, 257 (1956).
7) Y. Koaze et al., Seikagaku, 36, 637 (1964).
The present paper describes purification, crystallization and some enzymatic properties of an acid protease produced by a strain of *Acrocylindrium*, a plant pathogenic fungus. The acid protease was obtained in crystalline form on the way of purification of pectolytic enzymes from this microorganism.\(^{11)}\)

**MATERIALS AND METHODS**

**Microorganism.** A strain of *Acrocylindrium*, a plant pathogenic fungus, was used for producing an acid protease. This microorganism was selected by Kousaka\(^{12)}\) as the fungus producing pectolytic enzymes.

**Composition of the Medium.** The medium employed for experimental work is shown in Table 1.

<table>
<thead>
<tr>
<th>Components</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pectin*</td>
<td>5</td>
</tr>
<tr>
<td>NH(_4)NO(_3)</td>
<td>1</td>
</tr>
<tr>
<td>KH(_2)PO(_4)</td>
<td>0.5</td>
</tr>
<tr>
<td>MgSO(_4).7H(_2)O</td>
<td>0.05</td>
</tr>
<tr>
<td>CaCl(_2).2H(_2)O</td>
<td>0.01</td>
</tr>
<tr>
<td>Yeast extract**</td>
<td>0.2</td>
</tr>
<tr>
<td>pH</td>
<td>5.6</td>
</tr>
</tbody>
</table>

* Katayama Chemical Co.
** Oriental Yeast Co.

**Method of Cultivation**

1) **Seed cultures.** Media were dispensed in amount of 100 ml to 500 ml shake flask and sterilized by autoclaving at 115°C for fifteen minutes. After inoculation from the stock-cultures which were maintained on potato agar slants, the cultures were incubated for three days at 30°C with reciprocal shaking (amplitude, 7 cm; 136 strokes per minute). Subsequently, the media inoculated by 2% of the cultures mentioned above were maintained under same conditions except for incubating time, one day.

2) **Large scale cultivation.** Ten l each of medium was poured into four 20-liter fermentors* and sterilized at 110°C for ten minutes. One hundred and fifty ml each of the seed cultures and 10 ml each of silicone oil (Shin-etsu Chem. Co.) as an anti-foaming agent were added into fermentors. During cultivation, the cultures were maintained at 30°C, aeraed at a flow rate of 10 liters per minute and agitated at about 375 r.p.m.

**Assay of Protease Activity.** Protease activity was assayed by Kunitz's method\(^{13)}\) with a slight modification. To 1 ml of an enzyme solution in a test tube, equilibrated at 37°C, was added 2 ml of 2.5% casein (Hammarsten, Merck Co.) in 0.1 M hydrochloric acid solution (pH 2.0) unless otherwise noted. After digestion for thirty minutes at 37°C, 2 ml of 0.4 M trichloroacetic acid (TCA) solution was added and incubation was subsequently continued for thirty minutes. The protein precipitated was removed by filtration and absorbancy of the filtrate was measured at 275 m\(_\mu\) against distilled water. On the other hand, the control test was performed by adding the casein solution after the TCA solution. One unit of protease activity (PU) was defined as the amount of enzyme which brought an increase of 0.001 in absorbancy at 275 m\(_\mu\), caused by net enzymatic digestion per minute at 37°C.

**Determination of Protein Concentration.** Protein concentration was estimated by Cu-Folin method\(^{14)}\) using ovalbumin as a standard protein.

**Starch-Gel Electrophoresis.** Starch-gel electrophoresis was carried out by Smithies' method\(^{15,16)}\) in order to ascertain the protein homogeneity. The size of starch gel was 8 x 15 x 0.3 cm and a current of 30 mA was passed through the gel in a cold room (4°C) for fifteen hours. Staining was carried out by dipping the gel into a saturated solution of amido black 10 B in methanol-water-glacial acetic acid (50 : 50 : 10).

**Ultracentrifugal Sedimentation.** The sedimentation analysis was carried out in a Spinco model E ultracentrifuge (Rotor, An-D, #1306; Angle, 60°) at 20°C and 59,780 r.p.m.

**Ultraviolet Absorption Spectrum.** The ultraviolet absorption spectrum of the crystalline acid protease was studied by Hitachi Recording Spectrophotometer (Model EPS-2).

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11) F. Uchino, Y. Kurono and S. Doi, This Journal, 30, 1066 (1966).
12) M. Kousaka, F. Uchino and S. Doi, Unpublished data.
RESULTS

Purification and Crystallization of the Enzyme

1) Precipitation with acetone. After cultivating for sixty hours, the culture was filtrated by suction for removing cells and then 541 of acetone (below -20°C) was added slowly to the clear filtrate (36 l) under stirring, cooling the mixture below -10°C with the coolant of dry ice-acetone. Standing two hours after addition, clear supernatant was sucked off and suspension of precipitate, remained in bottom, was centrifuged at 9,000 r.p.m. for ten minutes. The precipitate collected by centrifugation was dissolved in 21 of distilled water and insoluble precipitate was removed by filtration. The filtrate was subsequently dialyzed against running tap water for two days and then against distilled water for one day at 4°C.

2) Salting out with ammonium sulfate. The above dialyzed solution was saturated with solid ammonium sulfate at about 0°C. Standing overnight in a cold room (4°C), the precipitate was collected by centrifugation at 9,000 r.p.m. for ten minutes, dissolved in 500 ml of 0.05 M acetate buffer (pH 4.1) and dialyzed against six changes of 51 of the same buffer for three days at 4°C.

3) Chromatography on Duolite CS-101. The dialyzed solution mentioned above (600 ml) was placed on a Duolite CS-101 column, 5.8 x 13 cm, which had been previously equilibrated with 0.05 M acetate buffer (pH 4.1), at a flow rate of 90 ml per hour. After the column was washed sufficiently with the same buffer, elution was carried out with 0.4 M acetate buffer (pH 6.0) at a flow rate of 150 ml per hour; fractions of 22 ml were collected. Fractions 28-132 were combined (2.41), whose absorbancy at 280 mμ was above 0.8.

4) Crystallization by dialysis. To the above eluate (2.41), solid ammonium sulfate was added very slowly in an ice bath under gentle stirring up to saturation. Standing overnight in the cold room, the precipitate was collected by centrifugation, dissolved in 150 ml of 0.05 M acetate buffer (pH 4.1) and dialyzed against six changes of 3 l of distilled water for three days. Crystal-like precipitate was found after two days in the dialyzing cellophane tube and dialysis was continued for additional one day. The dialyzed solution containing the precipitate was poured into a beaker and was incited mechanically for a few minutes by a glass stick. Standing two hours at a room temperature, crude crystals were developed. One day later the crystals were collected by centrifugation (Pectolytic enzymes remained in the supernatant), dissolved in 300 ml of 0.4 M acetate buffer (pH 4.1) and dialyzed against distilled water. In this case, crystallization was started after a four-days' dialysis in a cellophane tube without mechanical incitement and almost completed three days later. Recrystallization was carried out five times. Microscopic observations of the crystalline enzyme revealed two different forms of crystals, as shown in Fig. 1 and Fig. 2. The one shown in Fig. 1

![Fig. 1. Microphotograph of Crystalline Acid Protease (x250).](image1)

![Fig. 2. Microphotograph of Crystalline Acid Protease (x180).](image2)
Purification and Some Properties of Crystalline Acid Protease from Acrocylindrium sp.

TABLE II. OVER-ALL PURIFICATION OF THE ACID PROTEASE

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total activity (PU) $\times 10^{-6}$</th>
<th>Total protein mg</th>
<th>Specific activity (PU)/mg</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>4.7</td>
<td>58,700</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>1) Precipitated with acetone (60%) and dialyzed against distilled water</td>
<td>4.3</td>
<td>—</td>
<td>—</td>
<td>91</td>
</tr>
<tr>
<td>2) Precipitated with ammonium sulfate (saturated) and dialyzed against acetate buffer (0.05 M, pH 4.1)</td>
<td>4.2</td>
<td>25,800</td>
<td>163</td>
<td>89</td>
</tr>
<tr>
<td>3) Eluted from Duolite CS-101 column with acetate buffer (0.4 M, pH 6.0)</td>
<td>2.9</td>
<td>13,400</td>
<td>216</td>
<td>62</td>
</tr>
<tr>
<td>Crystals ($\times$5 recrystallized)</td>
<td>2.5</td>
<td>966</td>
<td>2,600</td>
<td>33</td>
</tr>
</tbody>
</table>

was obtained at a relatively high concentration of the enzyme protein and by fast crystallization. On the contrary, the other shown in Fig. 2 was obtained at a low concentration and by slow crystallization. The enzyme protein was so insoluble in distilled water or dilute buffer that crystals might be developed in 0.05 M acetate buffer having 0.02% of the enzyme protein by standing in the cold room (4°C).

The over-all procedure for preparing the crystalline acid protease from the culture filtrate is summarized in Table II, which shows that the enzyme was purified about 33-fold with 53% recovery of the original activity and that the specific activity was 2600(PU)/mg protein. The crystalline preparation was free from pectolytic activities.

Some Properties of the Crystalline Acid Protease

Ultraviolet absorption spectrum of the acid protease. As shown in Fig. 3, the ultraviolet absorption spectrum of the crystalline enzyme dissolved in 0.05 M acetate buffer of pH 4.1 (0.32 mg protein/ml) showed a maximum absorption peak at 280 m$\mu$, which is common for most proteins ($E_{1%}^{1cm}$ 280 m$\mu$ = 14.5).

Starch-Gel Electrophoresis

The crystalline enzyme gave a single sharply defined band at 0.9 cm on an anionic site from starting point on starch-gel electrophoresis in acetate buffer (ionic strength, 0.2; pH 4.1) and at 3.1 cm on a cationic site in phosphate buffer (ionic strength, 0.2; pH 7.0). Namely, this acid protease was homogeneous on starch-gel electrophoresis.

Ultracentrifugal Sedimentation Analysis

The sedimentation analysis on the crystalline acid protease was performed in order to investigate the homogeneity and the sedimentation coefficient. The protein concentrations were 1, 0.5 and 0.25% in 0.2 M acetate buffer of pH 4.0.

1) Homogeneity. All of the sedimentation patterns revealed that the peaks appeared very symmetrical and the pattern with 0.5% enzyme solution is shown in Fig. 4.

2) Sedimentation Coefficient. The sedimentation coefficients on protein concentrations of 1, 0.5 and 0.25% were calculated and the values of them were 3.37 S, 3.29 S, and 3.24 S, respectively.

When they were plotted against protein concentrations, it was found that they slightly depended on the concentrations and the extrapolated sedimentation coefficient for zero protein concentration was 3.20 S.

Fig. 3. Ultraviolet Absorption Spectrum of the Crystalline Enzyme.
Gel Filtration with Sephadex G-75

The gel filtration was carried out in order to examine whether the acid protease was homogeneous or not. The test solution (5.1 mg of crystals in 0.3 ml of 0.2 M acetate buffer, pH 4.0) was placed on a Sephadex G-75 column, 1.1 × 28 cm, which had been previously equilibrated with the same buffer, and the column was eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and diluted by five times with the same buffer. After that, the absorbancy at 280 m\(\mu\) and the protease activity of each fraction were measured.

As shown in Fig. 5, the protease activity coincided perfectly with the absorbancy at 280 m\(\mu\). That is to say, the specific activity was constant. In addition, the recovery of enzyme protein and protease activity was above 95%.

Therefore the crystalline enzyme was homogeneous on gel filtration with Sephadex G-75.

Effect of pH on the Activity

The enzyme activities were measured by incubating the enzyme solution with 2.5% casein solution at various pH values of from 0.7 to 8.0 at 37°C for thirty minutes. When casein solutions were prepared, above pH 5.0, casein was dissolved in 0.05 M phosphate buffer; between pH 2.0 and pH 5.0, in 0.05 M lactic acid solution; below pH 2.0, in 0.05 M hydrochloric acid solution, and adjusted to appropriate pH values by addition of 0.1 N hydrochloric acid or 0.1 N sodium hydroxide. As shown in Fig. 6, the acid protease was most active on casein at pH 2.0.

![Fig. 4. Sedimentation Pattern of Crystalline Acid Protease.](image)

Enzyme concentration, 0.5%; buffer solution, 0.2 M acetate buffer of pH 4.0; temperature, 20°C; revolution, 59,780 r.p.m. Photographs were taken at intervals of 16 minutes from right to left.

**FIG. 4.** Sedimentation Pattern of Crystalline Acid Protease.

**FIG. 5.** Gel Filtration of Acid Protease with Sephadex G-75.

- Column size: 1.1 × 28 cm
- Test solution: 0.3 ml
- Elution rate: 0.5 ml/min.
- Elution buffer: 0.2 M acetate buffer (pH 4.0)
- Fraction volume: 0.5 ml

- ○: Optical density
- ●: Protease activity

**FIG. 6.** Effect of pH on Protease Activity.

One ml of enzyme solution, containing 0.4 mg, and 2 ml of 2.5% casein solution of various pH values were incubated at 37°C for 30 min.
Effect of pH on the Stability
The enzyme solutions of various pH values of from 0.7 to 10.0 were incubated at 30°C for twenty-four hours to determine the effect of pH on the stability, and the remaining activities were measured. As shown in Fig. 7, almost all activity of the acid protease remained in the pH range of 2.5~5.0, but was lost below pH 0.7 and above pH 5.6, and it was found that pH values of the enzyme solutions incubated above pH 6.5 were lowered to about pH 5.7.

![Fig. 7. pH Stability of Acid Protease.](image)

Effect of Temperature on the Stability
The enzyme solution of pH 3.0 was incubated at various temperatures for ten minutes and the remaining activity was measured. As shown in Fig. 8, the acid protease was stable below 50°C and almost lost its activity above 70°C.

![Fig. 8. Heat Stability of Acid Protease.](image)

Comparison between Pepsin* and the Acid Protease
As the substrate, 2.5% casein solution (pH 2.0) was used to compare the acid protease with pepsin on the specific activity, and the measurement of their activities was performed simultaneously under the same condition; at 37°C for thirty minutes. The specific activity of the acid protease was 2,600 (PU)/mg protein and that of pepsin was 2,000 (PU)/mg protein.

DISCUSSION
As described above, the acid protease from Acrocylindrium sp. was found in crystalline form in the dialysis step in purification of pectolytic enzymes from this strain, and therefore, the cultural conditions for accumulation of large amount of the enzyme are not investigated yet.

In 1964, K. Tomoda and H. Shimazono reported that the acid proteinase from Trametes sanguinea was crystallized by dialysis against 0.02M hydrochloric acid-sodium acetate buffer (pH 3.5). However, in their case, the enzyme solution should be colorless and contain more than 5% of the enzyme protein, while our preparation was crystallized in only 0.02% enzyme solution by dialysis against distilled water.

Because the acid protease was insoluble in distilled water or in dilute buffer, crystals might have been found even in a few drops of the enzyme solution left in a beaker without being put on the Duolite CS-101 column. From this observation, it was examined whether crystals were obtained by dialysis of the cul-

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* Pepsin (Sigma Chem. Co., 2X Crystallized and Lyophilized) was used.
ture filtrate concentrated by the treatment of acetone-precipitation or not.

Expectedly, crystal-like precipitate was found, but the condition of this crystallization was not more exactly investigated.

On the basis of the data on the starch-gel electrophoresis, it was suggested that the isoelectric point of the present enzyme was between pH 4.0 and 7.0. This pH range may be higher than those obtained from the acid proteinases produced by Asp. saitoi\(^{17}\) (pH 3.65), Paecilomyces variotii Bainier TPR-220\(^{18}\) (pH 3.8) or Trametes sanguinea (pH 3.5), while the pH optimum of the protease from Acrocyindrium sp. was the lowest of them.

As the sedimentation coefficient of the present enzyme was 3.20 S, its molecular weight seemed to be larger than those of the acid proteinases from fungi mentioned above.

The optimum pH of the acid protease was pH 2.0, similar to that from Asp. niger var. macrosporus\(^{17}\) and the most acidic of all from fungi investigated so far.

As to the result in Table II, the yield of crystals was 53%, while the other acid proteinases described above were never obtained in crystalline forms with such a high value. The reason may be thought that the acid protease was very insoluble in dilute buffer or in distilled water.

Acknowledgement. We wish to express our appreciation to Dr. Y. Kaneko, Mr. M. Itoh and Mr. Y. Hasegawa of this laboratory, Nagoya University, for their valuable suggestions and encouragements throughout the work.

\(^{17}\) E. Ichishima and F. Yoshida, Seikagaku, 36, 637 (1964).
\(^{18}\) J. Sawada, This Journal, 30, 393 (1966).