The Purification of the Extracellular Acid Protease of *Rhodotorula glutinis* K-24 and Its General Properties[†]

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The acid protease produced from *Rhodotorula glutinis* K-24 was purified and crystallized by precipitation with ammonium sulfate, fractional precipitation with acetone, gel-filtration with Sephadex G-100, and pH adjustment. The recovery of activity was about 40 per cent, and about 80~100 mg of third crystallized enzyme was obtained from one liter of broth. The purified enzyme was chromatographically homogeneous and confirmed to be monodispersive by physicochemical criteria. The enzyme was most active at pH values between 2.0 and 2.5 toward casein, and was stable at pH values from 2.5 to 6.5 on twenty hour incubation at 37°C. Comparing with several proteases obtained from various sources, the specific activity toward casein was potent and was 1.7 times that of pepsin. The enzyme was inactivated by pepsin inhibitor (S-PI) produced from *Streptomyces naniwaensis*, wheares it was not inactivated by SLS, divalent metal ions and sulfhydryl reagents.

Many kinds of molds have been known to produce a variety of acid proteases and some of these proteases were purified.¹⁻⁶⁾

Little is known, however, on the extracellular protease of yeasts, although *Candida albicans* of yeast was revealed to be producer of protease.⁷⁾ In the previous paper,⁸⁾ it was reported that during screening tests for extracellular protease of yeasts, a potent extracellular acid protease producing yeast was isolated from grape farm soil and was identified as *Rhodotorula glutinis* (Fres.) Harrison.

The enzyme was produced with high productivity on a synthetic medium. The present paper deals with studies on purification and general properties of the purified acid protease.

MATERIALS AND METHODS

1. *Microorganism and culture method.* *Rhodotorula glutinis* K-24 isolated from grape farm soil was used through out of this study. For the acid protease production, the following synthetic medium was employed: Glucose 7%, Polypeptone 2%, KH_2PO_4 0.6%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.08%, $\text{MnSO}_4 \cdot 6\text{H}_2\text{O}$ 4 ppm, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 4 ppm, Thiamine 2 ppm, pH 2.8.

R. glutinis K-24 was cultured under shaking by ordinary method at 25°C for 3 days.

2. *Assay of protease activity.* The protease activity was measured as follows. To 3 ml, 4/3% milk casein solution containing 0.05 M lactate buffer at pH 2.5 was added 1 ml of enzyme solution at 37°C. After incubation for ten minutes, the reaction was stopped by an addition of 4 ml of 0.44 M trichloroacetic acid, followed by filtration.

One ml of the filtrate was mixed with 5 ml of 0.44 M Na_2CO_3 and 1 ml of the Folin-Ciocalteu reagent was added to it. The optical density of the color developed was measured at 660 m μ . A unit of enzyme activity was defined as the enzyme quantity which liberate 1 μg of tyrosine per ml of the reaction mixture per minute under the conditions. The specific

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activity (units/mg protein) was also measured according to the assay method by Fukumoto *et al*.⁽⁴⁾

3. *Other proteolytic enzyme used.* For comparison, several proteolytic enzymes were used in the present experiment. Recrystallized pepsin was a product of Washington Biochemical Co., Ltd. Crystallized preparations of acid proteases of *Rhizopus chinensis* acid protease,⁽⁴⁾ *Mucos pusillus* rennin protease⁽⁵⁾ and *Trametes sanguinea* acid protease⁽³⁾ were kindly donated from Dr. D. Tsuru of Osaka City University, Dr. S. Iwasaki of Meitosangyo Co. Ltd., Dr. K. Tomoda of Takeda Chemical Industries, respectively.

Partially purified preparation of *Aspergillus saitoi* acid protease was purchased from Yashima drug Co., Ltd., and used after further purification by Ichishima's method.^(9,10) *Cladosporium* acid protease was purified as reported previously.⁽¹¹⁾

4. *Ultracentrifugal analysis.* Ultracentrifugal studies were performed with a Spinco Model E ultracentrifuge equipped with a schlieren cylindrical lens system.

5. *Electrophoresis.* Electrophoretic experiment was carried out in a Hitachi HTB-2 Tiselius-Type electrophoresis apparatus. Disc electrophoresis was also conducted with a 7.5% polyacrylamide gel column at pH 9.5.

RESULTS AND DISCUSSION

I. Purification and crystallization of *R. glutinis* K-24 acid protease

The enzyme purification and crystallization were carried out as follows. After removing the cells of 5 liters cultured broth (pH 1.8) by the centrifugation at 10,000 rpm for 10 min, about 3.0 kg ammonium sulfate (0.8 saturation) were added to the culture solution, then stored at 3~5°C for 1~2 days.

The precipitate was separated from supernatant by centrifugation and dissolved immediately in about 100 ml 10⁻² M sodium citrate buffer, pH 4.0. After dissolved 2 g sodium chloride to the enzyme solution, cold acetone was added to the enzyme solution to a final concentration of 55 vol% and the mixture was allowed to stand overnight at 0~2°C.

The resulting precipitate was removed by centrifugation at 16,000 rpm for ten minutes in order to remove polysaccharides and other impurities. Cold acetone was added to the supernatant until about 75 vol%, then stored

FIG. 1. Column Chromatogram of Partially Purified Enzyme on Sephadex G-100.

Partially purified enzyme was loaded on a column (2.5×36 cm) which was equilibrated with 10⁻² M sodium citrate buffer, pH 4.0. Flow rate was 10 ml/hr. See the text for the experimental details.

○—○: Protein, O.D.₂₈₀,
●—●: Protease activity (PU/ml).

FIG. 2. Micrograph of Crystalline Acid Protease of *Rhodotorula glutinis* K-24.

TABLE I. PURIFICATION OF ACID PROTEASE OF *Rhodotorula glutinis* K-24

Procedure	Vol. (ml)	Activity (PU/ml) $\times 10^{-3}$	Specific activity (PU/O.D. 280 m μ)	Yield (%)
Culture broth	5000	0.32	56	100
(NH ₄) ₂ SO ₄ pptn				
Supernatant	100	14.7	136	92
Fractionation by acetone Chromatography on Sephadex				
Eluate	40	24.0	760	60
Acetone pptn				
Supernatant	10	88.0	800	55
pH adjustment (pH 4.5)				
Crystals			880	48
Recrystallization				
Freeze-dried preparation			1040	40

at 0°C for 2~3 hr. The precipitate was collected by a centrifugation, dissolved with 10 ml 10^{-2} M sodium citrate buffer, pH 4.0, and chromatographed on a column of Sephadex G-100 equilibrated with the buffer solution.

The representative chromatographic pattern is shown in Fig. 1.

The active fractions were combined and cold acetone was added to the solution until 75 vol%. The precipitate was collected by centrifugation at 16,000 rpm for 10 minutes, dissolved in a minimum amounts (about 7~10 ml) of 10^{-2} M sodium citrate buffer, pH 4.0, and then adjusted the pH to 4.5 with 1M sodium citrate solution. The enzyme solution was left at 2~5°C. Needle like crystals appeared within 2 hr and crystallization was completed after overnight storage at 2~5°C. The crystals were collected using a centrifuge and dissolved by 10^{-2} M sodium citrate buffer, pH 2.5~4.0, at room temperature.

After removing insoluble materials, if present, by centrifugation, the protease solution was adjusted to pH 4.5 with sodium citrate and stored in refrigerator. Needle like crystals were obtained in overnight storage. The crystallization method was repeated once more. In Fig. 2, microphotograph of crystalline en-

zyme preparation was presented. The purified enzyme was dissolved in distilled water and lyophilized. About 450 mg preparation was obtained from 5 liters cultured broth. The flow sheet of purification procedure was summarized in Table I.

II. Homogeneity of the enzyme preparation

Homogeneity of the enzyme was checked using the lyophilized preparation.

1. *SE(sulfoethyl)-Sephadex C-50 chromatography.* Homogeneity of the enzyme was checked by chromatography on a SE-Sephadex C-50 column equilibrated with 10^{-2} M sodium citrate buffer, pH 4.0, and a single symmetrical peak with specific activity 1,100 was found on a linear gradient elution system with sodium chloride as shown in Fig. 3.

2. *Ultracentrifugal analysis.* Ultracentrifugal pattern of the enzyme was obtained as Fig. 4, the preparation was also homogeneous.

3. *Electrophoretic analysis.* The schlieren diagrams of the ascending and descending boundaries in an electrophoresis at pH 2.0, 4.0 and 6.0 are shown in Fig. 5. The photographs

FIG. 3. Column Chromatogram of Acid Protease on SE-Sephadex C-50.

Lyophilized preparation was applied on a column (1.5×97 cm) which was equilibrated with 10⁻² M sodium citrate buffer, pH 4.0. After washing the column with the same buffer described above, the protease was eluted with linear gradient increase in NaCl concentration at a flow rate of 9 ml/hr.

○—○: Protein, O.D.₂₈₀,
●—●: Protease activity (PU/ml).

were taken at an interval of 120 min with a current 7.5 mA. As shown in Fig. 5, the preparation was electrophoretically homogeneous at pH 2.0 and 4.0, but at pH 6.0, a few heterogeneity was observed. This heterogeneity probably arose mainly from autolysis during dialysis and electrophoresis.

To check this result, disc electrophoresis was further carried out at pH 9.5. As shown in Fig. 6, a single band appeared on acrylamide disc electrophoresis, which suggests that the preparation is homogeneous.

III. Properties of the purified enzyme

1. *Effect of pH on activity and stability of the enzyme.* The protease was most active at pH values between 2.0 and 2.5 toward casein, between 2.5 and 3.0 toward acid denatured

FIG. 4. Sedimentation Patterns of Acid Protease.

The enzyme concentration was about 1% in 10⁻¹ M sodium citrate buffer, pH 4.0, containing NaCl ($u=0.1$). Temperature was 25°C. The photographs were taken at indicated times reachings after full speed (56,000 rpm).

FIG. 5. Electrophoretic Pattern of Acid Protease.

The electrophoretic migration was carried out in 10⁻¹ M sodium citrate buffer of pH 2.0, 4.0 and in McIlvaine buffer of pH 6.0. The ionic strength of the buffer solution was 0.1. The current was 7.5 mA at 4°C. The ascending and descending boundaries are shown 120 min after the start of electrophoresis.

hemoglobin and was stable over the range 2.0 to 6.5 on a twenty hour incubation at 37°C as shown in Fig. 7 and Fig. 8.

FIG. 6. Polyacrylamide Disc Gel-electrophoresis of Purified Enzyme at pH 9.5.

Approximately 200 μ g of protein was applied on a column (0.5 $\times$ 5 cm) and electrophoresis was carried out at 2 mA per column at 5°C for 2 hr.

FIG. 7. Effect of pH on Protease Activity.

Casein and hemoglobin were dissolved in 10⁻¹ M sodium citrate buffer of various pH values.

○—○: Casein, ●—●: Hemoglobin.

FIG. 8. Effect of pH on the Stability of Acid Protease.

Enzymes were incubated at each pH for 20 hr at 37°C and the remaining activities were assayed at pH 2.5.

FIG. 9. Effect of Temperature on Enzyme Reaction.

The reaction was carried out for 10 min at various temperatures.

FIG. 10. Thermal Stability of Acid Protease.

Enzymes were incubated at each temperature for 10 min at pH 4.0 and the remaining activities were assayed at 37°C.

2. *Temperature optimum of the enzyme reaction and thermal stability.* The enzyme reaction was carried out at various temperature, at pH 2.5 for 10 min. The optimum temperature for casein was about 60°C as shown in Fig. 9. The enzyme dissolved in 10^{-2} M citrate buffer solution, pH 4.0 was incubated at various temperatures for 10 min and the remaining activity was assayed at 37°C. The results shown in Fig. 10 indicated that this enzyme was stable at temperature below 65°C and about 80% activity remained even at 70°C.

3. *Influence of metal ions and other chemicals.* Effects of several metal ions and other chemical reagents on the protease activity were examined. Various metal ions, EDTA(ethylenediaminetetra acetic acid sodium salt) and sulfhydryl reagents such as *p*-chloromercuribenzoate and moniodo acetic acid did not inhibit the acid protease produced from *R. glutinis* K-24. Even SLS (sodium lauryl sulfate) which completely inactivate the mold acid proteases, did not inhibit (Table II).

TABLE II. EFFECT OF VARIOUS REAGENTS ON ENZYME ACTIVITY

The enzyme, 10 μ g/ml, was incubated at 37°C for 15 min with various chemicals at pH 4.0 or 6.0, and then the remaining activity was assayed at pH 2.5.

Chemicals	Concentration (mM)	Remaining activity (%)
None	—	100
HgCl ₂	1.0	100
PbCl ₂	10.0	100
ZnCl ₂	10.0	100
MgCl ₂	10.0	100
CuCl ₂	10.0	100
FeCl ₂	1.0	97
NiCl ₂	10.0	100
CoCl ₂	10.0	100
IAA	10.0	100
EDTA (pH 6)	10.0	100
PCMB (pH 6)	1.0	100
SLS	1.0	100

IAA: Moniodo acetic acid, EDTA: Ethylenediaminetetraacetic acid sodium salt. PCMB: *p*-Chloromercuribenzoate, SLS: Sodium lauryl sulfate.

E. Ichishima and F. Yoshida¹²⁾ reported that conformation of Aspergillopeptidase A was apparently converted by sodium lauryl sulfate, into a partially α -helical conformation and the residual activity failed to zero ($1 \sim 5 \times 10^{-3}$ M).

4. *Effect of pepsin inhibitor (S-PI) produced from Streptomyces naniwaensis.*¹³⁾ Effect of S-PI on *R. glutinis* acid protease was compared with other acid protease at its optimum pH. One mg of *R. glutinis* acid protease (3.3×10^{-8} M) was inhibited by 60% of original activity at 20 μ g of S-PI (3.1×10^{-8} M). At this concentration of S-PI, one mg of pepsin (2.9×10^{-8} M) was completely inhibited.

The effect of S-PI was specific on acid protease and the rate of inhibition was different on each acid proteases as shown in Table III.

Among the acid protease tested, *Cladosporium* sp. No. 45-2 acid protease was most resistant toward S-PI.

5. *Comparative proteolytic activity.* The proteolytic activity on casein of *R. glutinis* K-24 acid protease was compared with several acid

TABLE III. EFFECT OF *Streptomyces*-PEPSIN INHIBITOR (S-PI) ON VARIOUS ACID PROTEASES

The enzyme, 1 mg/ml, was incubated at 37°C for 10min with equal volume of S-PI solution at optimum pH and then the remaining activity was assayed.

Protease	Concentration (μ g)	Remaining activity (%)
<i>Rhodotorula glutinis</i>	20	40
K-24 acid	10,000	0
<i>Cladosporium</i> sp.	20	80
No. 45-2 acid	1,000	0
Pepsin	20	0
	10,000	0
<i>Trametes sanguinea</i> acid	20	65
	10,000	0
<i>Rhizopus chinensis</i> acid	20	42
	10,000	0
<i>Aspergillus saitoi</i> acid ^{a1)}	20	55
	10,000	0

^{a1)} Partially purified preparation.

proteases obtained from various sources. The proteolytic activity of *R. glutinis* K-24 acid protease was shown to be most potent enzyme among these of acid proteases (Table IV).

TABLE IV. PROTEOLYTIC ACTIVITY OF SEVERAL
PROTEASES

Protease	Specific activity (PU/mg protein) at optimal pH
<i>Rhodotorula glutinis</i> K-24 acid	5,200
<i>Cladosporium</i> sp. No. 45-2 acid	3,400
Pepsin	3,000
<i>Rhizopus chinensis</i> acid	2,200
<i>Aspergillus saitoi</i> acid ^{a)}	2,000
<i>Trametes sanguinea</i> acid	2,200

^{a)} This datum was quoted from the paper of J. Fukumoto, D. Tsuru and T. Yamamoto.⁴⁾

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