Purification, Crystallization and Some Enzymatic Properties of Acid Protease of *Cladosporium* sp. No. 45-2†

By Sawao MURAO, Susumu FUNAKOSHI* and Kohei ODA

Department of Agricultural Chemistry, College of Agriculture,
University of Osaka Prefecture

Received November 29, 1971

An acid protease of *Cladosporium* sp. No. 45-2 was purified and crystallized by precipitation with ammonium sulfate, fractional precipitation with acetone, and pH adjustment. About 600 mg of third crystallized preparation was obtained from one liter of culture broth. The purified enzyme was chromatographically homogeneous and confirmed to be monodisperse by physicochemical criteria such as ultracentrifugal and electrophoretic analysis. The enzyme was most active at pH values between 2.5 and 2.7 toward both casein and hemoglobin and was stable at pH values from 2.5 to 7.0 on twenty hour incubation at 30°C.

Millimolar concentration of sodium lauryl sulfate markedly inhibited the enzyme, whereas diisopropyl phosphorofluoridate, sulfhydryl reagents, ethylenediaminetetraacetic acid, and divalent metal ion relatively little affected the activity. The enzyme was most resistant toward S-P1 among the acid proteases tested.

On the way of screening of yeast which produce extracellular acid protease, a potent yeast like strain was obtained. As the results of taxonomical studies, the isolated strain was identified to be a strain belonging to *Cladosporium*.¹⁾

Many investigators²⁻⁸⁾ have been studied on the acid protease produced from fungi, however, little is known on the acid protease of *Cladosporium*. So, we tried the purification and crystallization of the acid protease and studied the enzymatic and physicochemical properties on the crystalline preparation thus obtained.

The present paper describes the conditions of cultivation, purification procedures and some enzymatic properties of the acid protease of *Cladosporium* sp. No. 45-2.

† Studies on Acid Protease of *Cladosporium* sp. No. 45-2. Part I. The report was presented at the Annual Meeting of the Agricultural Chemical Society of Japan at Tokyo, April, 1971.

* Present address: Otsuka Pharmaceutical Co., Ltd., Tokushima.

MATERIALS AND METHODS

1. *Assay of protease activity.* The protease activity was measured by a modified Anson's method.⁹⁾ To 3 ml 4/3% milk casein solution containing 0.05 M lactate buffer, 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 Na₂CO₃ and then 1 ml of Folin-Ciocalteu reagent was added to it. The optical density of the color developed was measured at 660 mμ.

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 condition. The specific activity (unit/mg protein) was also measured according to the assay method by Fukumoto *et al.*⁷⁾

2. *Determination of Protein.* Protein content was assayed spectrophotometrically assuming that $E_{1\text{cm}}^{1\%}$ at 280 mμ of protein was 10.7, which was estimated from the dry weight of the purified enzyme preparation.

3. *Organism.* *Cladosporium* sp. No. 45-2 was isolated from the soil. The taxonomical study on *Cladosporium* sp. No. 45-2 will be reported in detail elsewhere.

4. *Ultracentrifugation.* Sedimentation analysis was carried out at 25°C with a Beckmann Spinco model E analytical ultracentrifuge.

5. *Moving-boundary electrophoresis.* The procedure was carried out with a Hitachi-Tiselius electrophoresis apparatus model HTB-2 at 5°C.

6. *Other proteolytic enzyme used.* *Rhizopus chinensis* acid protease,⁷⁾ *Mucor pusillus* rennin protease⁸⁾ and *Trametes sanguinea* acid protease¹⁰⁾ were kindly donated from Dr. Tsuru of Osaka City University, Dr. S. Iwasaki of Meitosangyo Co., Ltd., and Dr. K. Tomoda of Takeda Chemical Industries, respectively. Recrystallized pepsin was obtained from Washington Biochemical Co., Ltd. Partially purified preparation of *Aspergillus saitoi* acid protease⁵⁾ was purchased from Yashima Drug Co., Ltd., and used after further purification. *Rhodotulula glutinis* acid protease¹¹⁾ was purified as reported previously.

RESULTS AND DISCUSSION

Cultural condition for acid protease formation

The effect of various nitrogen, carbon source on the formation of acid protease was investigated. The maximal formation of acid protease was observed when the medium in Table I was used. Investigating the effect of initial pH on protease formation using some 230 strains of yeast and yeast-like fungi, the authors¹²⁾ found that initial pH is very important factor for extracellular protease formation. That is, there were many strains producing extracellular protease when initial pH was adjusted at 3.0, whereas there were a few strains when initial pH at 6.5.

We also tested the effect of initial pH on the acid protease formation of *Cladosporium* sp.

TABLE I. COMPOSITION OF CULTURE MEDIUM

Sucrose	7%
Polypeptone	2%
KH ₂ PO ₄	0.6%
MgSO ₄ ·7H ₂ O	0.05%
FeSO ₄ ·7H ₂ O	4 ppm
MnSO ₄ ·4-6H ₂ O	4 ppm
pH 3.0

FIG. 1. Effect of Initial pH on the Production of Acid Protease.

The *Cladosporium* sp. No. 45-2 was aerobically grown at 25°C for 5 days in the medium shown in Table I.

FIG. 2. Time Course of Acid Protease Production in Sucrose-Polypeptone Medium.

The *Cladosporium* sp. No. 45-2 was aerobically grown at 25°C in the medium indicated at Table I.

●—● Protease activity (PU/ml), ○—○ Growth (mg dry weight/ml broth), ○—○ Residual sugars (Glucose, mg/ml × 10⁻¹), ⊗—⊗ pH

No. 45-2. As shown in Fig. 1, this strain showed a similar inclination described previously.

For *Cladosporium* sp. No. 45-2, the initial pH, 3.0, was optimum. Figure 2 illustrates the time course of acid protease formation in the medium. The pH of broth was gradually

TABLE II. SUMMARY OF PURIFICATION PROCEDURE OF *Cladosporium* sp. No. 45-2 ACID PROTEASE

Procedure	pH	Volume (ml)	Total activity (units $\times 10^{-4}$)	Specific activity (unit/O.D. 280 $m\mu$)	Yield (%)
Culture filtrate	2.0	10,000	800	110	100
(NH ₄) ₂ SO ₄ precipitation and dialysis					
Dialyzate	3.0	600	760	500	95
active carbon treatment					
Filtrate	3.0	800	720	550	90
acetone precipitation and pH adjustment (pH 4.5)					
First crystals	—	—	640	680	80
recrystallization, twice and gel filtration					
Third crystals	—	—	520	760	65
dialysis					
Lyophilized preparation	—	—	480	740	60

lowered along with the consumption of sugar and retained at the range of 1.8~2.0. Protease production occurred in about 1 day after inoculation and reached the maximum yield at 4~5 days.

Purification and crystallization of enzyme

About 10 liters of culture broth was used. The protease was precipitated by the addition of solid ammonium sulfate to a final concentration of 0.8 saturation and allowed to stand overnight. The precipitate produced was collected by centrifugation (4×10^4 g, 10 min),

FIG. 3. Microscopic Photograph of *Cladosporium* sp. No. 45-2 Acid Protease.

FIG. 4. Column Chromatogram of Purified Enzyme on Sephadex G-100.

About 40 mg of purified enzyme was applied on a column (2.5 $\times$ 35 cm) which was equilibrated with 10^{-2} M citrate buffer, pH 3.0.

●—●: O.D. 280 $m\mu$, ○—○: Protease activity

dissolved in 10^{-2} M citrate buffer, pH 3.0, and dialyzed against the same buffer using a visking tube overnight. The dialyzed solution, 600 ml, was treated with active carbon (0.2% w/v), twice, and the colorless filtrate, 800 ml, was mixed with three volumes of cold acetone. The resulting precipitate was collected by centrifugation and dissolved in the same buffer

FIG. 5. Sedimentation Patterns of Acid Protease.

About one per cent enzyme solution in 10^{-2} M citrate buffer, pH 4.0 containing NaCl ($\mu=0.1$). Temperature was 25°C . The photographs were taken at indicated times reaching after full speed (56,000 rpm).

FIG. 6. Moving-boundary Electrophoretic Patterns of Acid Protease.

The electrophoretic migration was carried out in acetate buffer over a pH range of 2.0 to 4.0, citrate buffer of pH 5.0, and McIlvaine buffer of pH 6.0. The ionic strength of the buffer solution was 0.1. The current was 10 mA at 5°C . The ascending and descending boundaries are shown 180 min after the start of electrophoresis.

described above as small quantity as possible. After removing the insoluble impurities by centrifugation, if any, the pH of the clear solution was adjusted to 4.5 with 10^{-1} M sodium citrate solution. The crystals appeared after about fifteen hours. The crude crystals were washed with 10^{-3} M citrate buffer, pH 4.5,

FIG. 7. Ultraviolet Absorption Spectrum of Acid Protease.

dissolved in 10^{-2} M citrate buffer, pH 3.0, as small quantity as possible and recrystallized twice. The crystal were dissolved in a small amount of 10^{-3} M citrate buffer solution, pH 3.0 and chromatographed on a column of Sephadex G-100 (2.5×40 cm) equilibrated with the same buffer. The active fractions were collected and crystallized as described above. The purified preparation was dissolved in 10^{-3} M acetate buffer, pH 3.0 and dialyzed against 10^{-4} M hydrochloric acid solution at 4°C and then lyophilized with little loss of activity. About 6g of the purified enzyme were obtained, amounting to about 65% recovery of the total proteolytic activity. The purification procedure is summarized in Table II, and the

photograph of crystalline enzyme is shown in Fig. 3.

Homogeneity of the lyophilized preparation was checked by chromatography on a Sephadex G-100 column equilibrated with 10^{-2} M citrate buffer, pH 3.0 and a single symmetrical peak with a constant specific activity of 760 (PU/O.D. at 280 m μ) at pH 2.5 was found as shown in Fig. 4.

Ultracentrifugal and moving boundary electrophoretic analysis showed that the preparation was monodisperse (Figs. 5 and 6).

The ultraviolet absorption spectrum of the enzyme is shown in Fig. 7.

Enzymatic properties

a) *Effect of pH on activity and stability of the enzyme.* Figure 8 illustrates the effect of pH on the protease activity. The pH of casein and hemoglobin substrate was adjusted by the following buffers: 10^{-1} M citrate buffer (pH 1.0~3.0), McIlvaine buffer (pH 2.0~6.0), and 10^{-1} M glycine-hydrochloric acid (pH 2.5~3.0). The protease was most active at pH values between 2.5 and 2.7 toward both substrates.

To check the pH stability relationship, the

FIG. 8. Effect of pH on Protease Activity.

Casein and hemoglobin were used as substrate. The reaction was carried at various pH values and 37°C for 10 min. See the text for the experimental details.

○—○: Casein, ●—●: Hemoglobin

FIG. 9. Effect of pH on the Stability of Acid Protease.

Enzyme solutions were incubated at each pH for 20 hr at 30°C and the remaining activities were assayed at pH 2.5.

enzyme was diluted with 10^{-1} M buffer of various pH values and incubated at 30°C for 20 hr. No loss of enzyme activity was observed at pH values between 2.5 and 6.5. (Fig. 9)

b) *Temperature optimum of the enzyme reaction and thermal stability of the enzyme.* The enzyme reaction was carried out at various temperature at pH 2.5 for ten minutes. The temperature optimum was at 55°C (Fig. 10). As shown in Fig. 11, the enzyme lost its activity after ten minutes incubation at 70°C at pH 2.5.

FIG. 10. Effect of Temperature on Enzyme Reaction.

The reaction was carried out for 10 min at pH 2.5 with casein at various temperatures.

FIG. 11. Thermal Stability of Acid Protease.

Enzyme solutions were incubated at each temperature for 10 min at pH 2.5 and the remaining activities were assayed at 37°C.

TABLE III. EFFECT OF VARIOUS REAGENTS ON ENZYME ACTIVITY

The enzyme, 10 µg/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	93
PbCl ₂	10.0	100
MnCl ₂	10.0	100
CdCl ₂	10.0	100
FeCl ₂	10.0	100
NiCl ₂	10.0	90
ZnCl ₂	10.0	90
CuCl ₂	10.0	100
CoCl ₂	10.0	100
FeSO ₄	10.0	100
IAA	10.0	90
NaNO ₂	10.0	100
I ₂	10.0	100
KMnO ₄	1.0	80
SLS	1.0	0
PCMB	1.0	85
EDTA	10.0	100
DFP	1.0	100

c) *Effect of various protease inhibitors, surface active reagents and sulfhydryl reagents.* The enzyme was markedly inactivated by SLS but

slightly by divalent metal ions such as Hg²⁺, Ni²⁺, Zn²⁺ as shown in Table III.

Sulfhydryl reagent was also slightly inhibitory for the enzyme.

d) *Effect of Streptomyces-pepsin inhibitor (S-PI)*¹³¹ Effect of S-PI on the protease activity was tested, comparing with various acid proteases at optimal pH. The activity of pepsin

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

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

Protease	Concentration (µg)	Remaining activity (%)
<i>Cladosporium</i> sp. No. 45-2 acid	20	80
	40	65
	60	55
<i>Rhodotorula glutinis</i> K-24 acid	20	40
	40	25
	60	20
Pepsin	20	0
<i>Trametes sanguinea</i> acid	20	65
	40	50
	60	42
<i>Rhizopus chinensis</i> acid	20	42
	40	25
	60	22
<i>Aspergillus saitoi</i> acid ^{a)}	20	55

^{a)} Partially purified preparation.

TABLE V. PROTEOLYTIC ACTIVITY OF SEVERAL PROTEASE

Protease	Specific activity (units/mg protein) at optimum pH
<i>Cladosporium</i> sp. No. 45-2 acid	3,400
<i>Rhodotorula glutinis</i> K-24 acid	5,200
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 data was quoted from the paper of Fukumoto *et al.*⁷⁾

(2.9×10^{-8} M) was completely inactivated by $20 \mu\text{g}$ S-PI (3.1×10^{-8} M), whereas those of other protease were remained active. Among the acid protease tested, *Cladosporium* sp. No. 45-2 acid protease was most resistant toward S-PI. (Table IV)

e) *Comparative activity.* The specific activity of the acid protease obtained here was estimated to be 3,400 on casein substrates according the assay method by J. Fukumoto *et al.* As shown in Table V, the value indicates that the enzyme shows somewhat high activity than that of pepsin. The enzyme was more active than the acid proteases produced from *Rhizopus chinensis*, *Aspergillus saitoi*, *Trametes sanguinea* but less active than that produced from *Rhodotorula glutinis* K-24.

REFERENCES

- 1) S. Murao, M. Kamada, T. Nakase, S. Ogura and K. Oda, *Nippon Nogeikagaku Kaishi*, **46**, 167 (1972).
- 2) T. Hofmann and R. Shaw, *Biochim. Biophys. Acta*, **92**, 543 (1964).
- 3) W. E. Marshall, R. Manion and J. Porath, *ibid.*, **151**, 414 (1968).
- 4) K. Nakanishi, *J. Biochem. (Tokyo)*, **46**, 1263 (1959).
- 5) F. Yoshida and M. Nagasawa, *Bull. Agr. Chem. Soc. Japan*, **20**, 257 (1956).
- 6) J. Sawada, *Agr. Biol. Chem.*, **27**, 677 (1964).
- 7) J. Fukumoto, D. Tsuru and T. Yamamoto, *ibid.*, **31**, 710 (1967).
- 8) J. Yu, S. Iwasaki, G. Tamura and K. Arima, *ibid.*, **32**, 1051 (1968).
- 9) S. Akabori, "Method of Enzyme Research," Asakura Book Co., Ltd., 2, 1956, p. 240.
- 10) K. Tomoda and H. Shimazono, *Agr. Biol. Chem.*, **28**, 770 (1964).
- 11) M. Kamada, K. Oda and S. Murao, *ibid.*, **36**, 1095 (1972).
- 12) M. Kamada, S. Ogura, K. Oda and S. Murao, *Nippon Nogeikagaku Kaishi*, **46**, 171 (1972).
- 13) S. Murao and S. Sato, *Agr. Biol. Chem.*, **35**, 1477 (1971).