Purification of Alkaline Proteinase from *Aspergillus candidus*

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An alkaline proteinase of *Aspergillus candidus* was purified from wheat bran solid culture by batchwise treatment with Amberlite IRC-50 and sequential chromatography on DEAE-cellulose, hydroxylapatite and Sephadex G-100 gel. This purification results in a 18-fold increase of proteolytic activity and the enzyme preparation was homogeneous in sedimentation analysis of the ultracentrifuge and polyacrylamide gel disc electrophoresis. The molecular weight was estimated to be about 23,000 by gel filtration and 22,000 by calculation from the amino acid composition. The enzyme consisted of Lys₁₄, His₄, Arg₃, Asp₂₅, Thr₁₅, Ser₂₃, Glu₁₅, Pro₇, Gly₂₂, Ala₂₄, Met₂, Val₁₆, Ile₁₁, Leu₁₀, Tyr₆, Phe₇, Trp₂ and amide ammonia₁₄ and did not contain cysteine or cystine.

Serine proteases, generally identified by sensitivity to DFP (diisopropylphosphorofluoridate), were the proteolytic enzymes most widely distributed in the microbial world.¹⁾ Some of these enzymes are also referred to as alkaline proteinases characterized by their alkaline pH optima. Although a huge number of papers have been published on these enzymes from *Aspergillus* species, only a few enzymes were isolated in a homogeneous state and characterized in detail. For example, Subramanian and Kalnitsky²⁾ isolated aspergillopeptidase A from *A. oryzae*. Nordwig and Yahn³⁾ purified aspergillopeptidase C from the same species. Hayashi *et al.*⁴⁾ reported the isolation and characterization of alkaline proteinase from *A. sojae*. Similar work has been carried out by Turková *et al.*⁵⁾ with *A. flavus*, by Ito and Sugiura⁶⁾ with *A. melleus*, and by Danno and Yoshimura with *A. sydowii*⁷⁾ and *A. sulphureus*.⁸⁾

On the other hand, 20 out of 35 *Aspergillus* species were found in a previous paper⁹⁾ to produce alkaline proteinases most of which exhibit species-specific mobility in polyacrylamide gel electrophoresis. However, detailed

information of these enzymes is confined to the above several examples and enough data are not yet available to unravel the phylogenetic relationship of the proteinases from different species of the genus *Aspergillus*.

Therefore, it is of much interest to reveal the physico-chemical properties of those enzymes from viewpoints of comparative biochemistry and fungal classification. The present paper aims to describe the purification procedure of alkaline proteinase from *A. candidus* and to characterize the enzyme by analytical data. Enzymatic properties will be reported in the succeeding paper.

EXPERIMENTAL

Organism. *Aspergillus candidus* IAM 2015 was provided by the Culture Collection of the Institute of Applied Microbiology, University of Tokyo.

Chemicals. DEAE-cellulose was obtained from Seikagaku Kogyo Co. Ltd., Tokyo. Blue dextran and Sephadex G-100 were obtained from Pharmacia, Uppsala, Sweden. Amberlite IRC-50 and hydroxylapatite were purchased from Rohm and Haas Co., U.S.A. and Bio-Rad Laboratories, U.S.A., respectively. Pepsin and lysozyme from Nutritional Bio-chemicals

Corp., U.S.A., trypsin from Worthington Chemicals Co., U.S.A. and α -chymotrypsin from Wako Chemicals Co., Tokyo were used as standard proteins for molecular weight determination. Purified alkaline proteinases of *A. sojae* and *A. oryzae* were kindly supplied by Dr. K. Hayashi, Kikkoman Shoyu Co., Ltd. and Mr. T. Nakadai of this institute, respectively. Milk casein (Hammersten grade) used as the substrate in proteinase assay was purchased from Merck Co., Germany. All other chemicals were of guaranteed reagent grade.

Preparation of crude enzyme. *A. candidus* was grown at 30°C for 3 days on 1.2 kg of wheat bran moistened with 960 ml of tap water. Extraction of the enzyme was performed at 4°C overnight with 5 liters of 1% sodium chloride solution. To the extract about two volumes of cold acetone precooled at -20°C was added to precipitate the enzyme. The precipitates were collected by filtration and dissolved in 1 liter of distilled water. Insoluble materials were removed by centrifuge at 17,000 $\times g$ for 30 min. The supernatant solution was dialyzed overnight against three change (each 10 liters) of 5 mM acetate buffer at pH 4.6. Unless stated otherwise, further purification was carried out in a cold room at 4°C.

Enzyme assay. Alkaline proteinase activity was determined by a modification of the Anson's method¹⁰⁾ as described in a previous paper¹¹⁾ and expressed as (PU) units or optical density at 660 nm in some cases. The concentration of the enzyme protein was spectrophotometrically determined on the basis that the $E_{1\text{cm}}^{1\%}$ at 280 nm of the enzyme protein was 7.1 which was estimated by dry weight measurement of the purified preparation.

Ultracentrifugal analysis. Sedimentation analysis was performed with a Hitachi model UCA-1 analytical centrifuge at 20°C in a single sector cell. The sedimentation coefficient was calculated as described by Schachman¹²⁾ and was corrected at 20°C in water.

Electrophoresis. Polyacrylamide gel electrophoresis was carried out by the standard method of Davis.¹³⁾ For each run 100 μg of the protein sample was employed and electrophoresis at 5 mA per gel column was performed at 4°C for 60 min. Gels were stained with 1% amido schwarz solution in 7% acetic acid and destained by repeated washing with 7% acetic acid.

Determination of amino acid composition. The enzyme protein was hydrolyzed with 6 N hydrochloric acid at 110°C for 24, 48, 72 and 100 hr in evacuated

sealed tubes. Amino acid analysis was performed with a Hitachi KLA-2 automatic amino acid analyzer. Since serine, threonine and tyrosine were gradually decomposed with increased time of hydrolysis, the analytical values for these amino acids were obtained by extrapolation of the data at the above periods to zero hr of hydrolysis. Tryptophan content was calculated from the ultraviolet absorption of the enzyme dissolved in 0.1 N sodium hydroxide solution.¹⁴⁾ The determination of amide ammonia was performed by the Conway's micro-diffusion method¹⁵⁾ after the treatment according to the Bailey's method.¹⁶⁾

Estimation of molecular weight. The molecular weight of the alkaline proteinase of *A. candidus* was estimated by Whitaker's method¹⁷⁾ using Sephadex G-100 column equilibrated with 0.1 M sodium acetate buffer at pH 6.0. The void volume of the column was measured with blue dextran. Pepsin (M. W. 35,000), α -chymotrypsin (M. W. 25,200), trypsin (M. W. 23,300) and lysozyme (M. W. 14,307) were used as marker proteins.

RESULTS

Purification of alkaline proteinase

Batchwise treatment with Amberlite IRC-50. To crude enzyme solution adjusted to pH 4.8 about 300 ml of Amberlite IRC-50 equilibrated with 0.01 M acetate buffer at pH 4.8 was added and frequently agitated for 3 hr. Then the enzyme protein was eluted with 500 ml of 0.2 M phosphate buffer at pH 7.5 and dialyzed against five changes (each 2 liters) of 0.01 M phosphate buffer at pH 7.5 for 36 hr.

Fractionation on DEAE cellulose. The dialyzed enzyme preparation was applied to a column of DEAE-cellulose equilibrated with the same buffer as dialyzing solution. The column was washed with 1.7 liter of the same buffer and subsequently eluted with a linear gradient of sodium chloride increasing from zero to 0.2 M in 1.4 liter of the same buffer. This procedure separated alkaline proteinase from most of non-enzymatic proteins and colored substances. The peak of the enzyme protein was found at 0.07 M of sodium chloride

concentration (Fig. 1). The fractions indicated by the bracket in Fig. 1 were pooled and dialyzed overnight against 0.01 M phosphate buffer at pH 7.0.

FIG. 1. Separation of *Aspergillus candidus* Alkaline Proteinase on a DEAE-cellulose Column.

The column (4 × 40 cm) was washed and eluted at a flow rate of 60 ml/hr in 7 ml fractions. ○—○ Proteins, ●—● Enzyme activity, △—△ NaCl concentration.

Fractionation on hydroxylapatite. The above dialyzed sample was applied to a column of hydroxylapatite. The column was fractionated stepwisely with 0.01, 0.04 and 0.06 M phosphate buffer at pH 7.0. Alkaline proteinase activity was eluted with 0.04 M buffer (Fig. 2). The enzyme fractions were collected and concentrated in a collodion bag.

Gel filtration through Sephadex G-100. The concentrated sample was fractionated on a column of Sephadex G-100 equilibrated with 0.01 M ammonium acetate buffer at pH 7.0 containing 1 mM calcium acetate. Figure 3 shows the good correspondence between enzyme activity and the protein profile. After dialysis against water, the peak fractions were lyophilized and used as the purified enzyme for further experiments. Table I summarizes the purification procedure and shows about 18-fold increase of specific activity.

Characterization of alkaline proteinase

Sedimentation coefficient. The purity of

FIG. 2. Chromatography of *A. candidus* Alkaline Proteinase on Hydroxylapatite.

The column (3 × 15 cm) was eluted stepwisely at a flow rate of 12 ml/hr in 10 ml fractions. ○—○ Protein, ●—● Enzyme activity.

FIG. 3. Sephadex G-100 Elution Pattern of *A. candidus* Alkaline Proteinase.

The column (2 × 120 cm) was eluted at a flow rate of 7 ml/hr in 10 ml fractions. ○—○ Protein, ●—● Enzyme activity.

the final enzyme preparation was examined by ultracentrifugal analysis. A single protein peak was found during the ultracentrifugal run as shown in Fig. 4. The sedimentation coefficient, s_{20}^w , of *A. candidus* alkaline proteinase (1% solution) was calculated to be

FIG. 4. Sedimentation Patterns of *A. candidus* Alkaline Proteinase.

The sample was 1% solution of the enzyme in 0.01 M phosphate buffer (pH 7.0) containing 0.1 M NaCl. The photographs were taken at the indicated time after reaching the full speed (55,430 rpm).

TABLE I. PURIFICATION OF *A. candidus* ALKALINE PROTEINASE

Procedure	Volume (ml)	Total activity (PU $\times 10^3$)	Total protein (mg)	Specific activity (PU/mg protein)	Recovery (%)
Crude enzyme	1,100	1,172	33,011	35.5	100
Amberlite IRC-50 treatment	580	410	7,694	51.5	35
DEAE-cellulose chromatography	350	246	579	450	21
Hydroxylapatite chromatography	380	141	251	560	12
Sephadex G-100 chromatography	65	94	150	625	8

3.0 S from sedimentation velocity at 55,430 rpm and the partial specific volume (0.70) of the enzyme molecule.

Electrophoresis. The purity of the enzyme preparation was also investigated by disc electrophoresis. A single protein band was observed in polyacrylamide gel electrophoresis at pH 9.4 as photographed in Fig. 5-C. The electrophoretic mobility of the alkaline proteinase from *A. candidus* was distinctively different from that from *A. sojae* or *A. oryzae* (Fig. 5).

Estimation of molecular weight. The determination of the molecular weight was performed by gel filtration though Sephadex

G-100 column. Figure 6 shows the plots of the elution volumes versus the logarithms of the known molecular weights of standard proteins. The molecular weight of *A. candidus* alkaline proteinase was estimated to be 23,000 by this method.

Amino acid composition. The results of amino acid analysis of *A. candidus* enzyme were shown in Table II with those of some other *Aspergillus* alkaline proteinases. Based on amino acid analysis, the molecular weight of the alkaline proteinase of *A. candidus* was calculated to be about 22,000. Both cysteine and cystine were absent in its amino acid pattern. On the other hand, a content of aspartic acid, alanine, serine and glycine was

TABLE II. AMINO ACID COMPOSITION OF ALKALINE PROTEINASE FROM SOME *Aspergillus* SPECIES

Amino acid	<i>A. candidus</i>	<i>A. sojae</i> ⁽⁴⁾	<i>A. oryzae</i> ⁽²⁾	<i>A. flavus</i> ⁽⁵⁾	<i>A. sulphureus</i> ⁽⁸⁾
Lysine	14	12	11~12	11	12
Histidine	4	4	4	3~4	5
Arginine	3	3	2	2	4
Aspartic acid	25	27	21	21	27
Threonine	15	16	11	11	19
Serine	23	25	19	20	20
Glutamic acid	15	16	12	12~13	12
Proline	7	6	4	4~5	5
Glycine	22	24	19	20	31
Alanine	24	28	23	23	31
Half cystine	0	0	0	0	0
Methionine	2	2	0	1	0
Valine	16	16	15	15	21
Isoleucine	11	12	9~10	9~10	10
Tyrosine	6	7	5	5	7
Phenylalanine	7	6	5	5	5
Tryptophan	2	2	2	2	2
Amide ammonia	14	18	15	17	18

FIG. 5. Polyacrylamide Gel Electrophoresis of Alkaline Proteinases of *A. oryzae* (A), *A. sojae* (B) and *A. candidus* (C).

relatively high.

DISCUSSION

Aspergillus candidus produces a large amount of alkaline proteinase in wheat bran solid culture. This enzyme migrates 3 to 4 times as fast as alkaline proteinase of *A. oryzae* does in polyacrylamide gel electrophoresis and shows the fastest electrophoretic mobility of all other alkaline proteinases of 20 different

FIG. 6. Estimation of Molecular Weight by Sephadex G-100 Gel Filtration.

L: Lysozyme, S: *A. sojae* alkaline proteinase, T: Trypsin, C: *A. candidus* alkaline proteinase, CHT: α -Chymotrypsin, P: Pepsin.

Aspergillus species.⁽⁹⁾

Such a character suggests that the *A. candidus* enzyme may be different from the other *Aspergillus* proteinases, since the rate of migration in polyacrylamide gel electrophoresis is con-

sidered to reflect the net charge, shape and size of an enzyme protein molecule.

Alkaline proteinase of *A. candidus* was purified to be homogeneous in ultracentrifugal and electrophoretic analyses by treatment of Amberlite IRC-50, chromatography on DEAE-cellulose and hydroxylapatite columns and gel filtration through Sephadex G-100 column. The behavior of the enzyme protein during the above purification procedure is very similar to that of the *A. sojae* enzyme.⁴⁾

The sedimentation coefficient, s_{20}^w (3.0 S) of the *A. candidus* enzyme, is consistent with that of *A. sojae* alkaline proteinase.¹⁰⁾ Moreover, the molecular weight (23,000) of alkaline proteinase from *A. candidus* determined by gel filtration method is also in good agreement with those from *A. sulphureus* (M. W. 23,000)⁸⁾ *A. sojae* (M. W. 23,000~24,000)¹⁸⁾ and *A. flavus* (M. W. 19,500~22,000)⁵⁾ but somewhat smaller than that from *A. melleus* (M. W. 27,900).⁶⁾ In spite of big difference in electrophoretic mobility, therefore, the molecular sizes of alkaline proteinases from *Aspergillus* species seem to be much alike.

The absence of both cysteine and cystine is noteworthy aspect in amino acid composition of the enzyme. This character is universal, so far studied,^{3, 5, 6, 18, 19)} in all *Aspergillus* alkaline proteinases, since the presence of one cysteine residue in the *A. sojae* enzyme²⁰⁾ has been corrected by further investigation.¹⁸⁾ On the other hand, two methionine residues are found in the *A. candidus* enzyme as in the *A. sojae* proteinase, although only one methionine residue is detected in the *A. flavus* enzyme⁵⁾ and *A. oryzae* aspergillopeptidase C.³⁾ To this contrary, the absence of methionine is reported in the *A. sulphureus* enzyme⁸⁾ and *A. oryzae* aspergillopeptidase B.¹⁹⁾ The content of the other amino acids is not so variable each other among the above enzymes.

Similarity of the enzymatic properties of

fungal alkaline proteinases referred above may be expected by the close resemblance of the data of their molecular weights and amino acid patterns.

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