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Isolation of Alkaline Proteinase from *Aspergillus sojae* in Homogeneous Form*

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The alkaline proteinase of *Aspergillus sojae* was isolated in gram quantities as a homogeneous form. The purification procedures were, (1) batchwise-treatment with ion exchange resin Duolite CS 101, (2) fractional precipitation with ammonium sulfate, (3) precipitation with acetone, (4) column chromatography on DEAE-cellulose, and (5) gel filtration with Sephadex G-100. The recovery of the activity was about 12%. The purified enzyme preparation was found to be homogeneous by several criteria such as ultracentrifugation, paper and moving-boundary electrophoreses, etc. Any kinds of carbohydrate and phosphorus were not detected in this preparation, suggesting that this enzyme is a simple protein.

Aspergillus sojae,¹⁾ which is widely used in the microbial industry in Japan as is *Aspergillus oryzae*, is known to produce large amount of an extracellular alkaline proteinase. The purification of this enzyme has been tried by several investigators,^{2~4)} but it has not been isolated so far in the homogeneous form. As for an alkaline proteinase from *Aspergillus oryzae*, its purification has been tried by many workers.^{5~10)} However, any preparation which

was homogeneous both electrophoretically and ultracentrifugically was not obtained. Recently, the alkaline proteinase from *Aspergillus oryzae* has been isolated in homogeneous form¹¹⁾ and studied on some physical and chemical properties of the molecule.¹²⁾ However, little is known about the genetic relationship between the proteinases from different species of yellow aspergilli.

In the previous paper,¹³⁾ authors reported the outline of purification procedure and molecular characteristics on this enzyme. The present paper describes the purification procedure of *Aspergillus sojae* alkaline proteinase starting from wheat bran culture and some evidence as a simple protein in detail.

EXPERIMENTAL

Assay of proteinase activity. The proteinase activity was measured by a modification of the Anson's

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method.¹⁴⁾ To 1 ml 2% milk casein solution containing 0.2 M phosphate buffer at pH 7.5 was added 1 ml of enzyme solution at 30°C. After incubation for ten minutes, the reaction was stopped by an addition of 4 ml of 5% trichloroacetic acid, followed by filtrating. One ml of the resulted filtrate was mixed with 5 ml of 0.4 M Na₂CO₃ and then 1 ml of phenol reagent¹⁵⁾ was added to it. The optical density of the color developed was measured at 660 m μ . One proteinase unit (PU) was defined as 1 μ g of tyrosine liberated in the reaction mixture per one minute under the conditions described above.

Determination of protein. Protein content was assayed spectrophotometrically assuming that $E_{1\text{cm}}^{1\%}$ at 280 m μ of protein was 8.98, which was estimated by the dry weight measurement of the purified enzyme preparation.

Analysis of carbohydrates and phosphorus. Sugar content was determined by phenol-H₂SO₄ method¹⁶⁾ and orcin-H₂SO₄ method.¹⁷⁾ Pentose was determined by orcin-HCl-FeCl₃ method.¹⁸⁾ Hexosamine and uronic acid were analysed by Elson-Morgan's method¹⁹⁾ and naphthoresorcinol method,²⁰⁾ respectively. Allen-Nakamura's method²¹⁾ was applied for the determination of phosphorus.

Ultracentrifugation. Sedimentation analysis was carried out at 10°C with a Hitachi model UCA-1 analytical ultracentrifuge.

Moving-boundary electrophoresis. The procedure was carried out with a Hitachi-Tiselius electrophoresis apparatus model HTD-1 at 0°C.

Paper-electrophoresis. Paper-electrophoresis was performed on the Toyo filter paper No. 51, 2 $\times$ 40 cm, for sixteen hours at 500 V with currents of 4 to 6 mA. The zone of protein was detected by staining with Ponceau 3R.

RESULTS

1) Isolation of the Enzyme

This enzyme was first purified partially by the modification of the method reported by Mizumuma et al.,²⁾ followed by the application of chromatographical methods into the homogeneous state.

Preparation of crude enzyme extract. Six kilograms of wheat bran culture of *Aspergillus sojae* strain X816 was extracted with 20 l of water overnight at 4°C. Sixteen liters of the clear solution was obtained by filtration using hyfflosupercel.

Batchwise-treatment with Duolite CS 101. Duolite CS 101 resin (1.6 kg) treated with 0.05 M acetate buffer of pH 4.8 was added to the crude enzyme solution adjusted to pH 4.8 and stirred frequently. After four hours the proteinase-adsorbed resin was separated by the filtration, washed with 20 l of the same buffer described above, and then added into 4.5 l of 0.2 M phosphate buffer of pH 7.6. The resin suspension was adjusted to pH 5.3 with 10 N NaOH, stirred for two hours and filtrated. The volume of filtrate was 4 l.

Fractionation with ammonium sulfate. To the resin-treated extract was added, with constant stirring, solid ammonium sulphate to give 55% saturation. The resulting precipitate was removed by filtration. To the filtrate was added further solid ammonium sulphate to give 80% saturation. The resulting precipitate was separated by filtration, dissolved in 400 ml of cold water and dialized over night against water at 4°C.

Fractionation with acetone. Acetone was added to the dialized enzyme solution gradually at -10°C to the final concentration of 60%. The resulting precipitate was collected by the centrifugation at -5°C, dissolved in 150 ml of cold water, and dialized for two days at 4°C.

Column chromatography on DEAE-cellulose. DEAE-cellulose (Brown) was equilibrated with 0.02 M phosphate buffer of pH 7.5. The treated cellulose suspension was poured into a column (3 $\times$ 90 cm) and allowed to settle

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FIG. 1. Elution Patterns of Alkaline Proteinase from *Aspergillus sojae* on DEAE-cellulose (top) and Sephadex G-100 (bottom). For experimental conditions see the text.

○—○ Protein ●—● Proteolytic activity

under gravity. The dialyzed enzyme solution described above was put onto this column. Gradient elution was performed introducing 0.3M sodium chloride solution containing the same buffer, through a 11 mixing chamber containing the same buffer. The rate of flow was adjusted to 100~120 ml/hr. and 10/ml fractions were collected. A typical elution pattern is shown in Fig. 1. The combined contents of tubes 40~72 from the DEAE-cellulose column were collected, desalted by dialysis, and then lyophilized.

Gel filtration on Sephadex G-100. Five hundred mg portion of lyophilized preparation was

applied to a Sephadex G-100 (Pharmacia, Uppsala, Sweden) column (2×90 cm) equilibrated with 0.02M ammonium acetate buffer of pH 6.0. Elution was performed with the same buffer and 10 ml fractions were collected. Fig. 1 showed a typical elution pattern. The combined contents of tubes 26~42 were collected and lyophilized.

The purification accomplished by these operations was summarized in Table I. The specific activity of the alkaline proteinase from *Aspergillus sojae* was shown to be 816 PU/mg, which was about 20% higher than that of the crystalline preparation by the previous method

TABLE I. PURIFICATION OF ALKALINE PROTEINASE OF *Aspergillus sojae*

Procedure	Vol. (ml)	Total Activity (PU $\times 10^{-4}$)	Specific Activity (PU/mg Protein)	Yield (%)
Crude enzyme solution	16000	712	8.52	100
Batchwise treatment with Doulite CS 101	4000	395	122	55.4
Fractionation with ammonium sulfate	500	292	309	44.1
Acetone precipitation	100	170	417	23.9
DEAE-cellulose chromatography	640	134	645	18.8
Gel filtration on Sephadex G-100	300	86	816	12.1

FIG. 2. Sedimentation Patterns of Alkaline Proteinase from *Aspergillus sojae*.

One decimal two per cent enzyme protein solution in phosphate buffer containing NaCl ($\mu=0.1$). Temperature was 10°C. The photographs were taken at indicated time reachings after full speed (55,430 r.p.m.).

FIG. 3. Moving-boundary Electrophoretic Patterns of Alkaline Proteinase from *Aspergillus sojae* at pH 7.7 and pH 3.4.

Left: 1.6% of enzyme protein in Veronal buffer (pH 7.7, $\mu=0.1$); Right: 1.4% of enzyme protein in glycine-HCl buffer (pH 3.4, $\mu=0.1$). 6 mA, 0°C.

Arrows show the direction of movement of boundary.

descending; $\bullet \leftarrow$; ascending $\circ \rightarrow$

FIG. 4. Paper-electrophoretic Patterns of Alkaline Proteinase from *Aspergillus sojae*.

Electrophoresis was carried out on Toyo filter paper No. 51, at 500 V, for 16 hr., 4°C. Applied enzyme was 200 μ g.

Upper: Acetate buffer containing NaCl (pH 4.0, $\mu=0.05$); Lower: Veronal-HCl buffer containing NaCl (pH 7.8, $\mu=0.05$).

Arrows show the line of application.

of Mizunuma et al.²¹ Six kilograms of the wheat bran culture of *Aspergillus sojae* strain X816 yields about 1.2 g of the purified enzyme, amounting to about 12% recovery of total proteolytic activity.

2) Homogeneity Studies

The purity of the final enzyme preparation, which seemed to be pure by criteria, such as chromatographical behavior, the absence of accompanying other enzyme activities, and a constant specific activity throughout the proteinase peak, was further confirmed by the following tests. Ultracentrifugal and moving-boundary electrophoretical analyses showed that the preparation was monodispersive. (Fig. 2 and 3) For the paper electrophoresis a large amount of the enzyme (200 μ g) was applied, but even a minor impurity could not be detected. (Fig. 4)

3) Some Evidence as Simple Protein

Subramanian et al. reported the presence of some carbohydrate (1~2%, equivalent of mannose) in their purified alkaline proteinase from *Aspergillus oryzae*. As shown in Table II, it was concluded that the purified enzyme from

TABLE II. ANALYSIS OF CARBOHYDRATES AND PHOSPHORUS IN ALKALINE PROTEINASE FROM *Aspergillus sojae*

Method	Molar ratio to the enzyme*	
Carbohydrates		
Phenol-H ₂ SO ₄ ¹⁶⁾	0.04	as <i>d</i> -glucose
Orcin-H ₂ SO ₄ ¹⁷⁾	0.06	as <i>d</i> -glucose
Orcin-HCl-FeCl ₃ ¹⁸⁾	0.03	as <i>d</i> -xylose
Elson-Morgan's ¹⁹⁾	0.05	as <i>d</i> -glucosamine-HCl
Naphthoresorcinol ²⁰⁾	0.00	as <i>d</i> -glucuronic acid
Phosphorus		
Allen-Nakamura's ²¹⁾	0.014	as KH ₂ PO ₄

* Calculated assuming the molecular weight of this enzyme to be 25,500.¹³⁾

Aspergillus sojae did not contain any carbohydrate and phosphorus, suggesting that this enzyme is a simple protein.

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