Purification and Properties of β-Galactosidase from 
Aspergillus oryzae

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β-Galactosidase [EC 3.2.1.23] has been purified from a culture of Aspergillus oryzae by 2-propanol fractionation, column chromatography on DEAE-Sephadex A-50 and Sephadex G-200. The preparation was homogeneous on ultracentrifugation and disc electrophoresis.

The enzyme showed pH optima of 4.5 with ONPG1 as a substrate and 4.8 with lactose as a substrate. The stable pH range was from 4.0 to 9.0 and the optimum temperature was 46°.

The Michaelis constants were 7.2×10^{-4} M with ONPG and 1.8×10^{-2} M with lactose. Hg^{2+}, Cu^{2+}, N-bromosuccinimide, and sodium laurylsulfate caused marked inhibition.

The apparent molecular weight was calculated to be about 105,000 by Sephadex gel filtration and sucrose density gradient centrifugation.

β-Galactosidase [EC 3.2.1.23] is widely distributed in nature and many studies have been reported on this enzyme from bacteria (1-4), yeasts (5, 6), moulds (7), plants (8), and animal organs (9-13). The optimum pH of β-galactosidase from various microorganisms tends to be in the neutral range, e.g., 7.2 for E. coli (14), and that from mammals tends to be on the acidic side, e.g., 4.5 and 6.0 for human small intestine (11). Bahl and Agrawal isolated the enzyme from Aspergillus niger and found an optimum pH on the acidic side, pH 3.2-4.0. They described the pH stability and some kinetic properties (7). However, no detailed investigation of this enzyme has been carried out.

The present work describes a procedure for the purification of and also some properties of β-galactosidase from A. oryzae. This enzyme is stable and has an optimum pH on the acidic side. A. oryzae strain RT102 (FERM-P1680), which was used throughout this study, produced a large amount of the enzyme.

MATERIALS AND METHODS

Cultivation and Extraction Procedure—A culture medium containing 600 g of wheat bran suspended in 720 ml of distilled water
was sterilized, cooled, and inoculated with a spore suspension of A. oryzae strain RT102. Growth was allowed to take place at 30°C for 5 days. To 620 g of the cultured medium of A. oryzae strain RT102, 6.2 liters of water was added and the mixture was stirred overnight at room temperature, then filtered.

**Enzyme Assay with ONPG as a Substrate (Routine Assay Method)** — Hydrolysis of the chromogenic substrate ONPG was used as a measure of the enzyme activity. The reaction mixture contained 20 µmoles of ONPG and the enzyme in 4 ml of 0.1 M citrate-phosphate buffer (pH 4.5). After incubation for 10 min at 30°C, the reaction was stopped by adding 1 ml of 1 M Na₂CO₃. Then the absorbance at 420 nm was measured. One unit of the activity was defined as the amount of enzyme which liberated one µmole of o-nitrophenol per min at 30°C. ONPG was synthesized by the method of Seidman and Link (15).

**Enzyme Assay with Lactose as a Substrate** — The β-galactosidase activity was also assayed by measuring the liberation of D-glucose from lactose. The reaction mixture contained 200 µmoles of lactose and the enzyme in 5 ml of 0.1 M citrate-phosphate buffer (pH 4.8). The reaction mixture was incubated for 10 min at 30°C, and was then kept in boiling water for 2 min to stop the reaction. Then 0.5 ml of the reaction mixture and 3 ml of Glucostat reagent (Worthington Biochemical Co.) were incubated for 60 min at 37°C. The reaction was stopped by adding 0.5 ml of 5 N HCl and the absorbance at 400 nm was measured. One unit of the activity was defined as the amount of enzyme which liberated one µmole of D-glucose per min at 30°C.

**Determination of Protein Concentration** — The method of Lowry et al. (16) was used to determine the protein concentration in the enzyme preparation, with bovine serum albumin as a standard.

**Electrophoresis** — Disc electrophoresis was performed with 7.5% polyacrylamide gel (pH 8.2) at 5°C according to the method of Ornstein and Davis (17) with slight modifications. A current of 4 mA per tube was applied for 90 min. After electrophoresis, the gels were stained with 1% amido black, destained electrophoretically and stored in 7% acetic acid.

**Ultracentrifugation** — Sedimentation patterns were obtained on a Hitachi ultracentrifuge, model UCA-1. The run was carried out at 60,000 rpm at 9°C.

**Gel Filtration on Sephadex G-200** — A sample was loaded on a column of Sephadex G-200 (1.8×205 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 8.0) and eluted with the same buffer. Fractions of 1.2 ml were collected and assayed for enzyme activity. Horse liver alcohol dehydrogenase [EC 1.1.1.1] with an assigned molecular weight of 86,000 (18), bovine intestine alkaline phosphatase [EC 3.1.3.1] with an assigned molecular weight of 100,000–115,000 (19, 20), and beef heart lactate dehydrogenase [EC 1.1.1.27] with an assigned molecular weight of 136,000 (18) were used as standards.

**Sucrose Density Gradient Centrifugation** — A swinging bucket rotor (SW50L) was run at 38,000 rpm for 12 hr at 2°C in a Beckman-Spinco ultracentrifuge, model L2. A sample was layered on top of the gradient between 5 and 20% sucrose. After centrifugation, fractions were collected and assayed for enzyme activity. E. coli alkaline phosphatase with an assigned molecular weight of 80,000 (21) and yeast alcohol dehydrogenase with an assigned molecular weight of 150,000 (22) were used as standards.

**Amino Acid Analysis** — The preparations were hydrolyzed with glass-distilled 6 N HCl in sealed, evacuated tubes at 105°C for 24 and 66 hr. Amino acids in the hydrolysates were analyzed with a Yanagimoto amino acid analyzer, model LC-5. Tryptophan content was determined by the method of Goodwin and Morton (23).

**RESULTS AND DISCUSSION**

**Purification of β-Galactosidase** — β-Galactosidase of A. oryzae strain RT102 was readily solubilized in water, and about 95% of the enzyme was extracted without destruction of the cells. The purification procedure was performed at 0–5°C unless otherwise specified.

(1) **2-Propanol fractionation:** To the extract was added cold 2-propanol (4°C) up to 50%
(v/v) with stirring. The resulting precipitate was collected by centrifugation at 5,000 rpm for 15 min. One hundred grams (wet weight) of the precipitate thus obtained was suspended in 1,500 ml of 0.05 M phosphate buffer (pH 7.0) and stirred overnight. The insoluble residue was removed by centrifugation. The supernatant was brought to 30% (v/v) 2-propanol. After insoluble materials were removed, 2-propanol was further added to the supernatant to a final concentration of 45% (v/v). The precipitate collected by centrifugation was dissolved in 130 ml of 0.05 M phosphate buffer (pH 7.0) and dialyzed overnight against the same buffer.

(2) DEAE-Sephadex A-50 column chromatography: The dialyzate was applied to a column of DEAE-Sephadex A-50 (4.0 x 57 cm) equilibrated with 0.05 M phosphate buffer (pH 7.0). After the column had been washed with the same buffer, the adsorbed enzyme was eluted with a linear gradient from 0.05 M phosphate buffer, pH 7.0 (400 ml), to the same buffer containing 0.1 M NaCl (400 ml). The elution pattern is shown in Fig. 1. The fractions of β-galactosidase activity were pooled, concentrated and dialyzed against 0.05 M phosphate buffer, pH 7.0.

(3) Sephadex G-200 gel filtration: The dialyzate was loaded on a column of Sephadex G-200 (5.8 x 52 cm) equilibrated with 0.05 M phosphate buffer, pH 7.0. The column was eluted with the same buffer. The fractions containing β-galactosidase activity were pooled, concentrated and loaded again on a column of Sephadex G-200 (5.0 x 130 cm). The column was eluted with the same buffer. The elution pattern is shown in Fig. 2.

The results of this purification experiment are summarized in Table I. From 620 g of the cultured medium of A. oryzae strain RT102, 326,000 units of β-galactosidase was extracted, and the specific activity was 3.0 units per mg of protein. The specific activity of the crude extract of β-galactosidase from A. niger is 0.42 units per mg of protein (7). From 50 g of the crude material of A. niger, 2,100 units of β-galactosidase was extracted (7) and from about 2.5 kg of wet cells of Saccharomyces fragilis, 94,700 units of β-galactosidase was extracted (6). Thus, A. oryzae strain RT102 produced a large quantity of enzyme. The specific activity always reached a constant value of about 85 units per mg of protein in several purification experiments, and this specific activity represented only about 30-fold purification. However, this purified preparation was homogeneous on ultracentrifugation and disc electrophoresis.

The purified β-galactosidase was stored at 2° and no significant loss of activity was observed after several months.

Test for Homogeneity—(1) Disc electrophoresis: Purity at various stages in the pur-
TABLE I. Purification of β-galactosidase from Aspergillus oryzae.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total activity (units)</th>
<th>Total protein (g)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery of activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction</td>
<td>326,000</td>
<td>108.7</td>
<td>3.0</td>
<td>(100)</td>
</tr>
<tr>
<td>2-Propanol fractionation</td>
<td>276,000</td>
<td>10.9</td>
<td>25.3</td>
<td>84.7</td>
</tr>
<tr>
<td>DEAE-Sephadex A-50 chromatography</td>
<td>179,000</td>
<td>4.4</td>
<td>40.7</td>
<td>54.9</td>
</tr>
<tr>
<td>Sephadex G-200 gel filtration</td>
<td>113,000</td>
<td>1.7</td>
<td>66.5</td>
<td>34.7</td>
</tr>
<tr>
<td>Sephadex G-200 gel filtration</td>
<td>70,000</td>
<td>0.8</td>
<td>87.5</td>
<td>21.5</td>
</tr>
</tbody>
</table>

Purification of β-galactosidase was monitored by disc electrophoresis. Figure 3 shows the electrophoretic patterns. The active fractions obtained by Sephadex G-200 chromatography gave a single protein band.

(2) Ultracentrifugation: The preparation of purified enzyme sedimented as a single homogeneous protein on sedimentation analysis (Fig. 4). The sedimentation coefficient estimated by extrapolation to zero concentration was 5.4S.

Effect of pH on the Enzyme Activity—The activity of the enzyme at various pH values was studied with ONPG and lactose as substrates. When ONPG was used as a substrate, the enzyme showed a pH optimum at 4.5, and when lactose was used as a substrate (Fig. 5), the optimum was 4.8.

Effect of pH on the Enzyme Stability—In order to examine the effect of pH on the enzyme stability, enzyme solutions (0.2%) in 0.05 M buffer of various pH values from 2.0 to 10.0 were allowed to stand at 30° for 1 hr. After these solutions had been adjusted to pH 4.5 by the addition of 0.1 M citrate-phosphate buffer, the remaining β-galactosidase activities were determined by the routine assay method. As shown in Fig. 6, the activity did not decrease in the pH range from 4.0 to 9.0.

Effect of Temperature on the Enzyme Activity—The activity of the enzyme at various temperatures was studied. Figure 7 shows the effect of incubation temperature on the enzyme activity. The maximum activity was observed at 46°.
Fig. 5. Effect of pH on the enzyme activity. The buffers used were HCl-NaCl buffer, pH 2.2, citrate-phosphate buffer from pH 2.6 to 7.0, and Tris-HCl buffer from pH 7.5 to 8.0. ■, ONPG as a substrate; ○, lactose as a substrate.

Fig. 6. Effect of pH on the enzyme stability. The buffers used were citrate-phosphate buffer from pH 2.5 to 7.0, Tris-HCl buffer from pH 8.0 to 9.0 and glycine-NaOH buffer from pH 9.0 to 10.0.

Effect of Temperature on the Enzyme Stability—The stability of β-galactosidase at various temperatures was examined with 0.05 M citrate-phosphate buffer, pH 3.5, 4.5, 6.0, and 9.0, for 10 min. These enzyme solutions were then chilled in ice-water, and, after adjustment to pH 4.5, the remaining enzyme activities were determined by the routine assay method. As shown in Fig. 8 at pH 3.5, more than 60% of the enzyme activity was lost after heating at 30° for 10 min but, at pH 4.5 and 6.0, the enzyme was reasonably stable at 40° for 10 min.

Kinetic Studies—The Michaelis constant (Km) and maximum velocity (Vmax) were determined by the method of Lineweaver and Burk
(24) for ONPG and lactose as substrates. The $K_m$ values were $7.2 \times 10^{-4}$ M with ONPG and $1.8 \times 10^{-2}$ M with lactose. The $V_{max}$ values were 86.7 $\mu$ mole/min/mg with ONPG and 121.9 $\mu$ mole/min/mg with lactose.

Effect of Metal Ions and Some Reagents on the Enzyme Activity—The hydrolysis of ONPG by $\beta$-galactosidase was inhibited by Cu$^{2+}$ and Ag$^+$ at $10^{-2}$ M concentration and Hg$^+$ at $10^{-4}$ M concentration. However, this enzyme was neither inhibited nor activated by various other metal ions at concentrations of $10^{-2}$ to $10^{-4}$ M. $\beta$-Galactosidase from E. coli was activated by Na$^+$ and K$^+$ (25), that from Saccharomyces lactis by Mg$^{2+}$ (5) and that from S. fragilis by K$^+$, Mn$^{2+}$, Mg$^{2+}$, and Co$^{2+}$ (6).

The enzyme activity was completely destroyed either by N-bromosuccinimide at $10^{-4}$ M concentration or by sodium lauryl sulfate at $10^{-2}$ M concentration. However, EDTA, mercaptoethanol, hydrogen peroxide, sodium thiosulfate, cysteine, and ascorbic acid at $10^{-2}$ M concentration had no effect on the enzyme activity.

TABLE II. Amino acid composition of $\beta$-galactosidase.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amino acid$^a$ (M/10$^9$ g protein)</th>
<th>Weight %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>58.1</td>
<td>5.2</td>
</tr>
<tr>
<td>Arginine</td>
<td>23.3</td>
<td>4.1</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>89.2</td>
<td>11.9</td>
</tr>
<tr>
<td>Cysteine</td>
<td>2.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>70.0</td>
<td>10.3</td>
</tr>
<tr>
<td>Glycine</td>
<td>82.2</td>
<td>6.2</td>
</tr>
<tr>
<td>Histidine</td>
<td>12.8</td>
<td>2.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>29.8</td>
<td>3.9</td>
</tr>
<tr>
<td>Leucine</td>
<td>69.7</td>
<td>9.1</td>
</tr>
<tr>
<td>Lysine</td>
<td>34.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Methionine</td>
<td>7.3</td>
<td>1.1</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>39.9</td>
<td>6.6</td>
</tr>
<tr>
<td>Proline</td>
<td>60.6</td>
<td>7.0</td>
</tr>
<tr>
<td>Serine</td>
<td>60.9</td>
<td>6.4</td>
</tr>
<tr>
<td>Threonine</td>
<td>53.9</td>
<td>6.4</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>10.5$^b$</td>
<td>2.1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>42.6</td>
<td>7.7</td>
</tr>
<tr>
<td>Valine</td>
<td>41.3</td>
<td>4.8</td>
</tr>
</tbody>
</table>

$^a$ Extrapolated to zero time of hydrolysis from the values of 24 and 66 hr hydrolysis. $^b$ By the ultraviolet absorption method (23).

Maltose, galactose, and lactose at $10^{-1}$ M concentration slightly inhibited the hydrolysis of ONPG by the enzyme but various other sugars at $10^{-4}$ M showed no inhibition.

Molecular Weight—The apparent molecular weight was determined by Sephadex G-200 gel filtration (26) and sucrose density gradient centrifugation. From Sephadex G-200, a molecular weight of 105,000 was obtained and from sucrose density gradient centrifugation, the apparent molecular weight was calculated to be 106,000 by the method of Martin and Ames (27). This value is small as compared with the molecular weight of $\beta$-galactosidase from E. coli, 540,000 (4) and from Bacillus megaterium, 150,000 (28).

Amino Acid Composition—The amino acid composition of $\beta$-galactosidase is shown in Table II. This $\beta$-galactosidase was rich in glycine and leucine, but poor in tryptophan, histidine, and sulfur-containing amino acids.

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REFERENCES


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