Purification and Some Properties of β-Galactosidase from Penicillium multicolor

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β-Galactosidase of a strain of Penicillium multicolor was purified to homogeneity from culture broth. The enzyme was most active at pH 4.0 and at 60°C, and was stable in the pH range 3.5 to 7.5 and below 45°C. Only β-D-galactosides could be substrates. The activity to ONPG was highest among the galactosides tested and about 2-times higher than that to lactose. The apparent Km values were 0.6 and 8.9 mM for ONPG and lactose, respectively. Hg2⁺ and Cu2⁺ inhibited the activity while the other metal ions tested had no effect. p-Aminophenyl β-D-thiogalactopyranoside inhibited the activity competitively. The molecular weight of the enzyme was estimated to be 1.26 × 10⁵ and 1.3 × 10⁵ by sedimentation equilibrium and SDS-polyacrylamide gel electrophoresis, respectively. Leucine and glycine were the N- and C-terminal residues, respectively.

The β-galactosidases (β-D-galactoside galactohydrolase; EC 3.2.1.23) of microorganisms are one of the most extensively studied groups of enzymes and many reports have been published on the enzymes from bacteria,⁠¹⁻¹¹ yeasts,¹²⁻¹⁴ and molds.¹⁵⁻²⁷ Much interest has been focused not only on biochemical studies but also on food industry and pharmaceutical applications of the enzymes. β-Galactosidase from Aspergillus oryzae has been developed as a digestive.²¹

The enzyme from Penicillium citrinum was found to be produced extracellularly in a wheat-bran medium and to be active under nearly neutral conditions.²⁴ For pharmaceutical applications of the enzyme from the genus Penicillium, an extensive search has been done for an enzyme which is more stable under acidic and active under neutral conditions. The present paper describes the purification and some properties of β-galactosidase from Penicillium multicolor. The enzyme was found to be different from that from P. citrinum in some physical and enzymatic properties.

MATERIALS AND METHODS

Microorganisms. P. multicolor KU-0-132 (FERN-P-4375) was used in this study. The organism was maintained on malt extract-agar slants.

Medium and cultivation. The medium for preculture consisted of glucose (2.0%), rice-bran (2.0%), KH₂PO₄ (0.5%), malt extract (0.4%), and yeast extract (0.2%). The preculture was carried out in a 500-ml flask containing 200 ml of the medium at 29°C for 3 days on a rotary shaker. The medium for enzyme production consisted of rice-bran (4.0%), soybean meal (2.0%), (NH₄)₂SO₄ (0.5%), malt extract (0.4%), and yeast extract (0.2%). The preculture was carried out in a 500-ml flask containing 200 ml of the medium at 29°C for 3 days on a rotary shaker. The medium for enzyme production consisted of rice-bran (4.0%), soybean meal (2.0%), (NH₄)₂SO₄ (0.5%), and yeast extract (0.2%). The cultivation was started by transferring 1 liter of the preculture to 20 liters of the medium in a 30-liter jar fermentor and continued at 28°C for 140 hr.

Preparation of crude enzyme. Clear culture fluid was obtained by centrifugation, and was then dialyzed and concentrated by ultrafiltration (CL-100, Asahi Chemical). Cold acetone was added to the dialyzed solution to make a

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Abbreviations: ONPG, o-nitrophenyl β-D-galactopyranoside; EDTA, ethylenediaminetetraacetate; PCMB, p-chloromercuribenzoate; GSH, glutathione (reduced form); SDS, sodium dodecyl sulfate.
concentration of 50%. The resulting precipitates were collected by centrifugation and washed with cold acetone, which was then removed under reduced pressure. The dried powder thus obtained served as a crude enzyme preparation.

**Enzyme purification.** Seventy grams of the crude enzyme preparation were dissolved in 500 ml of 10mM acetate buffer (pH 4.5) and insoluble substances were removed by centrifugation. The supernatant solution was dialyzed against 10mM acetate buffer (pH 4.5). The dialyzed solution (610 ml) was applied to a column of SP-Sephadex C-50 (4.5 x 60 cm) equilibrated with 10mM acetate buffer (pH 4.5). The enzyme was eluted by a linear gradient of NaCl formed from 500 ml each of 0.6m NaCl, at a flow rate of 20 ml fractions. Active fractions were combined and dialyzed against 10mM phosphate buffer (pH 7.0). The dialyzed solution (280 ml) was applied to a column of DEAE-Sephadex A-50 (2.8 x 35 cm) equilibrated with 10mM phosphate buffer (pH 7.0). The enzyme was eluted by a linear gradient of NaCl formed from 500 ml each of 1.8m NaCl at a flow rate of 30 ml per hr. The eluate was collected in 10 ml fractions. Active fractions were combined (110 ml) and divided into 10 fractions. Each fraction was applied to a column of Ultrogel AcA 34 (2.8 x 135 cm) equilibrated with 0.1m acetate buffer (pH 5.0). The enzyme was eluted by the same buffer at a flow rate of 30 ml per hr. The eluate was collected in 10 ml fractions and active fractions were combined. Further purification was achieved by affinity chromatography using p-aminophenyl \( \beta \)-D-thiogalactopyranoside as the ligand.

**Preparation of affinity gel.** One gram of hydrated Affi-Gel 10 (24 g of wet cake) was added to 25 ml of 0.1m phosphate buffer (pH 7.0) containing 25 mg of p-aminophenyl \( \beta \)-D-thiogalactopyranoside and the mixture was agitated gently on a shaker at 25°C for 5 hr to couple the ligands with the carrier gel. Unreacted sites in the Affi-Gel 10 were blocked by adding 5 ml of 1m monoethanolamine. The gel thus prepared was packed into a column (1.5 x 10cm) and washed with 10mM acetate buffer (pH 4.5) until the washings become free from reactants as monitored at 257 nm.

**Affinity column chromatography.** To the affinity column was applied 150 ml of the eluate from the Ultrogel AcA 34 column containing about 4 mg of protein per ml. The column was washed with 10mM acetate buffer (pH 4.5) and the enzyme adsorbed was eluted by a linear gradient of buffer concentration formed from 150 ml each of 10mM and 200mM acetate buffer (pH 4.5), at a flow rate of 10 ml per hr. The eluate was collected in 5 ml fractions. Active fractions having a constant specific activity of about 150 units per mg of protein were combined and used as a purified enzyme preparation.

**Enzyme assay.** \( \beta \)-Galactosidase activity was determined using ONPG as the substrate. A reaction mixture (2 ml) containing 50 mM acetate buffer (pH 4.5), 5 mM ONPG, and the enzyme was incubated at 40°C for 15 min, and 0.2M Na2CO3 (2 ml) was added to stop the reaction. The o-nitrophenol released was determined at 410 nm. When lactose was used as the substrate, 10mM Lactose was substituted for ONPG and the glucose liberated was determined by Glucostat. One unit of the enzyme was defined as the amount of the enzyme which liberates 1 \( \mu \)mol of o-nitrophenol or glucose per min under the assay conditions.

**Protein determination.** Protein concentration was determined by the method of Lowry et al.\(^{28}\) using bovine serum albumin as a standard. In chromatographic procedures, the protein content was estimated by measuring the absorbance at 280 nm.

**Carbohydrate determination.** The carbohydrate in the enzyme was determined by the phenol-sulfuric acid method\(^{29}\) using mannose as a standard.

**Amino acid analysis.** For the determination of amino acid composition, the enzyme protein was hydrolyzed with 6N HCl at 110°C for 24 hr in evacuated sealed tubes. Analysis of amino acids in the hydrolysates was carried out according to the method described by Moore and Stein\(^{30}\) with a Hitachi amino acid analyzer KLA-5. Cysteine and half-cystine were estimated from the amount of cystic acid in performic acid-oxidized protein as described by Hirs.\(^{31}\) Tryptophan was determined spectrophotometrically as described by Goodwin and Morton.\(^{32}\)

**Terminal amino acid analysis.** The N-terminal amino acid of the enzyme was determined by the dansyl method according to Gray.\(^{33}\) To determine the C-terminal residue, the enzyme was hydrolyzed with carboxypeptidase A and the hydrolyzate was subjected to amino acid analysis.

**Electrophoresis.** Polycrylamide gel electrophoresis was carried out according to the method of Davis\(^{34}\) with a column of 7.5% polacrylamide gel (0.5 x 6 cm) at pHs 4 and 9 at 5°C under a constant current of 3 mA per column. SDS-polycrylamide gel electrophoresis was carried out by the method of Weber and Osborn\(^{35}\) with a column of 5% polacrylamide gel (0.5 x 7 cm) containing 0.1% of SDS at room temperature under a constant current of 8 mA per column. Trypsin inhibitor (MW 21,500), bovine serum albumin (68,000), RNA polymerase \( \alpha \) (39,000), \( \beta \) (155,000), and \( \beta' \) (165,000) were used as the molecular weight markers.
**Isoelectric focusing.** The isoelectric point of the enzyme was determined by isoelectric focusing according to the method of Vesterberg and Svensson\(^3\) using carrier ampholites with a pH range of 3.5 to 10 at 5°C for 48 hr under a constant voltage of 800 V.

**Molecular weight determination.** Gel filtration was carried out according to the method of Andrews\(^4\) with a column of Sephadex G-200 (1.6 x 89 cm) equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 0.1 M NaCl. Ovalbumin (MW 43,000), bovine serum albumin (67,000), aldolase (158,000), and catalase (232,000) were used as the markers. Sedimentation equilibrium experiments were carried out as described by Van Hold and Baldwin\(^5\) using Hitachi analytical ultracentrifuge model 282 equipped with an absorption scanner, a multichannel cell and an RAM-18s rotor at 20°C for 24 hr.

**Materials.** The following materials were obtained from the sources indicated in parentheses: SP-Sephadex C-50, DEAE-Sephadex A-50, Sephadex G-200, and molecular weight marker proteins for gel filtration (Pharmacia Fine Chemicals); Ultrogel AcA 34 and carrier ampholites (LKB); molecular weight marker proteins for SDS-polyacrylamide gel electrophoresis (Böhringer Mannheim); reagents for polyacrylamide gel electrophoresis (Nakarai Chemicals); \(\alpha\)-aminophenyl \(\beta\)-d-thiogalactopyranoside (Calbiochem); Affi-Gel 10 (Bio-Rad). Other materials used were of the highest grade commercially available.

**RESULTS**

**Production of \(\beta\)-galactosidase and preparation of crude enzyme**

After 140 hr of cultivation, 17 liters of clear culture fluid was obtained, which contained 145 units of \(\beta\)-galactosidase per ml. From the culture fluid, 120 g of acetone dried crude enzyme preparation (18,500 units/g) was obtained.

**Purification of \(\beta\)-galactosidase**

Figures 1, 2, and 3 show elution profiles of the enzymes from SP-Sephadex C-50, DEAE-Sephadex A-50, and Ultrogel AcA 34 column, respectively. An elution pattern from the affinity column is shown in Fig. 4. In this affinity chromatography, the enzyme was not eluted by the buffer solution containing a substrate such as lactose or ONPG.

Results of the enzyme purification are summarized in Table I. The enzyme was purified about 3-fold at a recovery of 57%. The purified enzyme had a specific activity (ONPG as the substrate) of 145 units per mg of protein.
Table I. Purification of β-Galactosidase from P. multicolor.

<table>
<thead>
<tr>
<th></th>
<th>Total protein (g)</th>
<th>Total activity (unit)</th>
<th>Specific activity (u/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude preparation</td>
<td>22.7</td>
<td>1,230,000</td>
<td>54</td>
<td>100</td>
</tr>
<tr>
<td>SP-Sephadex</td>
<td>9.6</td>
<td>1,150,000</td>
<td>120</td>
<td>93</td>
</tr>
<tr>
<td>DEAE-Sephardex</td>
<td>6.6</td>
<td>863,000</td>
<td>130</td>
<td>70</td>
</tr>
<tr>
<td>Ultrogel AcA 34</td>
<td>5.6</td>
<td>777,000</td>
<td>140</td>
<td>63</td>
</tr>
<tr>
<td>Affinity column</td>
<td>4.8</td>
<td>699,000</td>
<td>145</td>
<td>57</td>
</tr>
</tbody>
</table>

Fig. 3. Gel Filtration Pattern on Ultrogel AcA 34 Column.
Experimental details are described in the text. Active fractions (Fractions 51 ~ 65) were combined.
---●---, β-galactosidase activity; ----, absorbance at 280 nm.

Homogeneity
The purified enzyme migrated as a single protein band in both polyacrylamide gel at different two pH values (pH 4 and 9) and in SDS gel electrophoresis as shown in Fig. 5. Terminal amino acid analysis, as described below, gave only leucine and glycine as the N- and C-terminal residues, respectively, also indicating the homogeneity of the purified preparation.

Molecular weight
The molecular weight of the enzyme was estimated to be $1.25 \times 10^5$ by gel filtration and $1.26 \times 10^5$ by sedimentation equilibrium. SDS-

Fig. 4. Elution Pattern from the Affinity Column.
Experimental details are described in the text.
---●---, β-galactosidase activity; ----, absorbance at 280 nm; ---, buffer concentration.

Fig. 5. Electrophoresis of Purified β-Galactosidase in Polyacrylamide Gel.
Thirty μg of protein was applied to gels A and B, and stained with Amido Black 10B. Two μg of protein was applied to gel C and stained with Coomassie Brilliant Blue R-250. Other experimental conditions are described in the text.
A, pH 4 gel; B, pH 9 gel; C, SDS-polyacrylamide gel.
Polyacrylamide gel electrophoresis gave a single protein band corresponding to a molecular weight of $1.3 \times 10^5$.

**Isoelectric point**

The isoelectric point of the enzyme was found to be 5.9 by isoelectric focusing (data not shown).

**Amino acid composition and terminal amino acid**

The amino acid composition of the enzyme is shown in Table II. It was found that the enzyme had relatively low contents of methionine and cysteine and/or cystine. Leucine and glycine was the N- and C-terminal residues, respectively.

**Carbohydrate content**

The carbohydrate content of the enzyme was determined to be 15% as mannose by the phenol–sulfuric acid method.

<table>
<thead>
<tr>
<th>Table II. Amino Acid Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid</td>
</tr>
<tr>
<td>Lys</td>
</tr>
<tr>
<td>His</td>
</tr>
<tr>
<td>Arg</td>
</tr>
<tr>
<td>Asp</td>
</tr>
<tr>
<td>Thr$^a$</td>
</tr>
<tr>
<td>Ser$^b$</td>
</tr>
<tr>
<td>Glu</td>
</tr>
<tr>
<td>Pro</td>
</tr>
<tr>
<td>Gly</td>
</tr>
<tr>
<td>Ala</td>
</tr>
<tr>
<td>Cys</td>
</tr>
<tr>
<td>Val</td>
</tr>
<tr>
<td>Met</td>
</tr>
<tr>
<td>Ile</td>
</tr>
<tr>
<td>Leu</td>
</tr>
<tr>
<td>Tyr</td>
</tr>
<tr>
<td>Phe</td>
</tr>
<tr>
<td>Trp</td>
</tr>
</tbody>
</table>

$^a$ Calculations were based upon molecular weight $1.26 \times 10^5$.

$^b$ Extrapolated to zero-time hydrolysis from 24- and 72-hr hydrolys.

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![Fig. 6](image-url)  
**Fig. 6.** Effects of pH on the Activity and Stability.  
(A) Effects of pH on the activity. The activity at various pH values was measured under the standard assay conditions using a McIlvaine type buffer system of constant ionic strength and expressed as percentages of the maximum activity. (●), ONPG; (○), lactose.  
(B) Effect of pH on the stability. The enzyme was kept at various pH values at 40°C for 3 hr using a McIlvaine type buffer system of constant ionic strength and then the solution was diluted with 50 mM acetate buffer (pH 4.5). The remaining activity was determined by the standard assay method and expressed as percentages of the original activity.
Effects of temperature on activity and stability

The optimum pH of the enzyme was shown to be 4.0 using lactose and ONPG as the substrate (Fig. 6A). The enzyme was stable in the pH range of 3.5 to 7.5 when incubated at 40°C for 3 hr. More than 80% of the original activity was observed to be retained at pH 2.5 after incubation at 40°C for 3 hr (Fig. 6B).

Effects of metal ions and chemicals

Effects of various metal ions on the enzyme activity are shown in Table III. Only Hg²⁺ and Cu²⁺ among the ions tested inhibited the activity. EDTA, PCMB, and GSH had no effect on the activity. p-Amiophenyl β-D-thiogalactopyranoside inhibited the activity competitively.

Substrate specificity

Activities of the enzyme on various glycosides are shown in Table IV. The enzyme was active only on β-D-galactopyranosides. The activity was highest towards ONPG among the β-galactosides examined and was about 2-

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Table III. Effects of Metal Ions on the Activity

<table>
<thead>
<tr>
<th>Ion</th>
<th>Concentration (mm)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>No addition</td>
<td>100.0</td>
</tr>
<tr>
<td>Na⁺</td>
<td>1</td>
<td>100.0</td>
</tr>
<tr>
<td>K⁺</td>
<td>1</td>
<td>99.8</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>1</td>
<td>99.0</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>1</td>
<td>98.5</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>1</td>
<td>47.6</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1</td>
<td>97.8</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

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Fig. 7. Effects of Temperature on the Activity and Stability.

(A) Effect of temperature on the activity. The activity at temperatures indicated was measured by the standard assay method and expressed as percentages of the maximum activity.

(B) Effect of temperature on the stability. The enzyme was kept at the indicated temperatures for 15 min in 20 mM acetate buffer (pH 5.6), and then in an ice bath for 30 min. The remaining activity was determined by the standard assay method and expressed as percentages of the original activity.
Table IV. Substrate Specificity

The activity was measured under the standard assay conditions and expressed as percentages of that to lactose.

The concentration of substrates was 5mM except for lactose (10mM).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>100</td>
</tr>
<tr>
<td>β-ONPG</td>
<td>225</td>
</tr>
<tr>
<td>β-PNPG</td>
<td>210</td>
</tr>
<tr>
<td>Phenyl β-galactoside</td>
<td>131</td>
</tr>
<tr>
<td>α-PNPG</td>
<td>0</td>
</tr>
<tr>
<td>Phenyl α-mannoside</td>
<td>0</td>
</tr>
<tr>
<td>Phenyl β-arabinoside</td>
<td>0</td>
</tr>
<tr>
<td>Phenyl β-xyloside</td>
<td>0</td>
</tr>
<tr>
<td>Phenyl β-glucoside</td>
<td>0</td>
</tr>
<tr>
<td>Phenyl α-glucoside</td>
<td>0</td>
</tr>
</tbody>
</table>

times higher than that towards lactose. Soybean β-1,4-galactan could not act as substrate for the enzyme. Apparent Km values for lactose and ONPG were 8.9 and 0.6mM, respectively.

DISCUSSION

In the utilization of enzymes, economical production and suitable properties of the enzymes are indispensable.

β-Galactosidase of P. multicolor was produced in culture fluid when the mold was grown in a liquid medium consisting of rice-bran, soybean meal, and inorganic salts. A crude enzyme preparation which appeared to be already about one-third pure, because the enzyme could be purified to homogeneity after only a 3 fold-increase in specific activity, could be obtained from the culture fluid by acetone precipitation alone. From this crude preparation, the enzyme was purified to the homogeneous state with a high recovery by relatively simple procedures. The enzyme was stable during the purification and storage. No requirement of metal ions was observed for the activity or stability of the enzyme.

β-Galactosidase from P. multicolor was highly specific to β-D-galactopyranoside. The Km values of the enzyme for lactose and ONPG were similar to or lower than those of β-galactosidases from other sources. The activity of various β-galactosidases to ONPG, a synthetic substrate for activity determination, is generally 3 to 5 times higher than to lactose, the substrate in practical use, while that of the enzyme from P. multicolor was about 2 times.

In other words, the activity to lactose of the enzyme is higher than that of other β-galactosidases, assuming the activity level to ONPG to be equal.

For the pharmaceutical application of enzymes as digestives, properties such as stability in an acidic and activity in a neutral pH range are required of the enzymes. β-Galactosidase from P. multicolor had the highest activity at pH 4.0 and still showed about a half of its maximum activity at pH 6.0. Moreover, the enzyme exhibited remarkable stability at pHs as low as 2.5. As reported previously, β-galactosidase from P. citrinum had considerable activity at neutral pH values but was unstable under acidic conditions. Contrarily, β-galactosidases from some acidophilic molds showed outstanding stability at low pH values but had little activity under neutral conditions.

β-Galactosidase from P. multicolor was found to differ in some properties from that from P. citrinum. The enzyme from P. multicolor had a monomeric structure with a molecular weight of about 1.3 x 10^5, gave leucine as the N-terminal residue and was inhibited competitively by p-aminophenyl β-D-thiogalactopyranoside, while the enzyme from P. citrinum was a dimer with a molecular weight of about 1.1 x 10^5, had histidine as the N-terminal residue and was not inhibited by the thiogalactoside.

REFERENCES

4) G. R. Craven, E. Steers, Jr. and C. B. Anfinsen, J.