Purification and Some Properties of $\beta$-Xylosidase from *Malbranchea pulchella* var. *sulfurea* No. 48†

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A $\beta$-xylosidase of a thermophilic fungus, *Malbranchea pulchella* var. *sulfurea* No. 48, was purified 99-fold from the culture filtrate after ammonium sulfate fractionation, DEAE-cellulose column chromatography, column electrophoresis and gel filtration on Sephadex G-200. The purified enzyme was found to be homogeneous upon ultracentrifugal analysis, disc electrophoresis and gel filtration. The molecular weight of the enzyme was estimated to be 26,000 by gel filtration, and the sedimentation coefficient was calculated to be 2.78S. $E_{1%}^{1cm}$ at 280 nm in phosphate buffer (pH 6.7) was 13.2. The optimum pH was found to be in the range of 6.2~6.8, and the optimum temperature was 50°C.

In a previous study,1 a thermophilic fungus (strain No. 48), which could produce a xylan hydrolysis activity, was isolated from soil and identified to be *Malbranchea pulchella* var. *sulfurea*. The xylanase of this strain was fairly thermostable, as compared with that of *Trichoderma viride*,2 and had its optimum pH in the neutral range. This optimum pH was not observed in most other fungal xylanases except for the one3 from *Pericaria oryzae*.

$\beta$-Xylosidases and/or saccharifying xylanases of most fungi, such as *Aspergillus niger*4–6 and *T. viride*,7 have been known to possess a high transglycosidase activity. The crude enzymes of several fungal strains, such as *M. pulchella* var. *sulfurea* No. 48, *Asp. niger*, *Chaetomium trilaterale*, *T. viride*, were tested to hydrolyze the hydrolyzate of xylan by *Streptomyces* xylanase, having an average degree of polymerization (D.P.) of 1.39, in a final concentration of 14.9% as xylose. The crude enzyme of *M. pulchella* var. *sulfurea* No. 48 alone was capable of hydrolyzing it and of lowering its D.P., but those of others were not.1

Two kinds of xylanase are distinguished by their modes of action on xylan: one is endo-type (a viscometric activity) and the other exotype (a $\beta$-xylosidase activity). These two kinds of xylanase have also been found in the *Malbranchea* crude enzyme. It is therefore reasonable to assume that the xylan hydrolysis was caused by the combined action of these two enzyme entities. The *Malbranchea* crude enzyme is thus considered most suitable for xylene production from xylan since it presumably possesses much less transferase activities than other fungal xylanases.

In this study, therefore, we purified xylanase to clarify the properties of xylanase system of this strain. The present paper describes the purification procedure and some properties of the $\beta$-xylosidase of *Malbranchea pulchella* var *sulfurea* No. 48.

**MATERIALS AND METHODS**

*Enzyme production. Malbranchea pulchella* var *sulfurea* No. 48 was cultured at 42°C for 40 hr with 20-liter jar-fermenter in the following medium: xylan (insoluble) 1.5%, polypeptone 1%, KH$_2$PO$_4$ 0.3%, (NH$_4$)$_2$PO$_4$ 0.3%, and corn steep liquor 0.4%.

*Assay of $\beta$-xylosidase activity. $\beta$-Xylosidase activity was assayed by measuring the amount of liberated phenol from phenyl-$\beta$-D-xyloside as described previously.1 The standard reaction mixture contained 3 ml of 0.02 M phenyl-$\beta$-D-xyloside, 2 ml of 0.1 M phosphat
buffer (pH 6.7) and 1 ml of enzyme solution. After incubation for 15 min at 45°C, 1 ml aliquots were withdrawn and colorimetrically analyzed for liberated phenol. A unit of β-xylosidase activity was defined as micromoles of phenol liberated per min.

**Determination of reducing sugar.** Reducing sugar concentration was determined by the method of Somogyi7) and Somogyi-Nelson8) and is expressed as xylose.

**Determination of protein concentration.** The protein concentration was determined by measuring the absorbancy at 280 nm with a Hitachi Spectrophotometer 139 and by the method of Lowry et al.9) with egg albumin as standard. The ultraviolet absorption spectrum of the *Malbranchea* β-xylosidase purified was measured with a Hitachi Spectrophotometer 124.

**Column electrophoresis.** Column electrophoresis was performed in a Mitsumi electrophoresis apparatus with Biogel P-100 as supporting medium.

**Disc electrophoresis.** The disc electrophoresis of the enzyme was carried out on a polyacrylamide gel at pH 8.3 by the method of Davis10) with an apparatus produced by Mitsumi Scientific Industry Co., Ltd.

**Ultracentrifugal analysis.** Ultracentrifugal analysis was carried out with a Hitachi UCA analytical centrifuge.

**Paper chromatography.** Paper chromatography was carried out as described previously1) with the solvent system of n-butanol: pyridine: water (6:4:3, v/v) by the ascending method, and the sugar component was detected with p-anisidine.

**Materials.** Insoluble xylan was prepared as described previously.1) Soluble xylan was prepared by the following procedure.11) Wood meal of Shirakambha Wood was treated with 2% aqueous ammonia at room temperature overnight, washed with water to remove ammonia, and extracted with 8% sodium hydroxide solution for 24 hr at room temperature. The yellow extract was filtered and acidified to pH 4.5 with acetic acid, and the xylan was precipitated with an equal volume of ethyl alcohol. The precipitate was collected on a filter cloth and dried under air at room temperature. Phenyl-β-D-xyloside was prepared as described previously. The molecular weight marker was purchased from Mann Research Laboratories and the DIAFLO ultrafiltration membrane, from Amicon Co., Ltd.

**RESULTS AND DISCUSSION**

**Purification of the enzyme**

All purification procedures were carried out at 4°C.

**Step 1. Culture filtrate.** The culture broth was filtered with filter paper, and the culture filtrate was adjusted to pH 6.7 with 1 N HCl.

**Step 2. Fractionation with ammonium sulfate.** The culture filtrate was saturated up to 80% with solid ammonium sulfate and adjusted to pH 6.7 with 1 N NaOH. After the filtrate was allowed to stand overnight, the resulting precipitate was collected by centrifugation and dialyzed against 0.01 M phosphate buffer at pH 6.7.

**Step 3. Column chromatography on DEAE-cellulose.** In a preliminary experiment for purification of β-xylosidase, it was found that the β-xylosidase activity could be adsorbed on DEAE-cellulose column but the liquefying xylanase activity (viscometric activity) could not. Therefore, DEAE-cellulose column chromatography was used to remove the liquefying xylanase activity from the culture filtrate.

After the removal of the insoluble materials, the dialyzed fraction was applied to a DEAE-cellulose column (5×30 cm) equilibrated with

--- protein; O--O, β-xylosidase activity; ----, molarity of NaCl.
0.05 M phosphate buffer at pH 6.7. The column was washed thoroughly with 5 liters of the same buffer. Elution was carried out by the application of a linear gradient of NaCl (0~0.3 M), as shown in Fig. 1.

**Step 4. Ultrafiltration.** The β-xylosidase-rich fractions (No. 27~44) were pooled, and were concentrated by ultrafiltration with a UM-10 membrane after filtration with an XM-100 membrane.

**Step 5. Column electrophoresis (I).** Column electrophoresis was performed with a column (4.5×58 cm) of Biogel P-100 as supporting medium. A voltage of 750 V (25 mA) was applied for 20 hr in 0.1 M phosphate buffer (pH 6.7) at 4°C. The zone volume was 15 ml, containing 990 mg of protein. After the electrophoresis was run for 20 hr, the column was eluted with the same buffer. After the elution, four protein peaks appeared as shown in Fig. 2.

**Step 6. Column electrophoresis (2).** The β-xylosidase-rich fraction in the first electrophoresis was pooled and subjected to the second electrophoresis under the same condition. One-fourth of the pooled fraction (Fraction I) was used in each electrophoretic run. The zone volume was 15 ml, containing 22 mg of protein. The β-xylosidase activity was found in a major peak (Fraction II) in the electrophoresis (Fig. 3).

**FIG. 2. Column Electrophoresis (I).**
Electrophoresis of desalted enzyme solution (990 mg, protein/15 ml) was carried out on a column (4.5×58 cm) of Biogel P-100 as supporting medium in 0.1 M phosphate buffer (pH 6.7) at 4°C. A voltage of 750 V (25 mA) was applied for 20 hr. The direction of migration is indicated in the figure by an arrow. The volume of each fraction collected was 10 ml.

---, protein; ○, β-xylosidase activity.

**FIG. 3. Column Electrophoresis (II).**
Electrophoresis of pooled Fraction I (22 mg, protein/15 ml) was carried out on a column (4.5×58 cm) of Biogel P-100 as supporting medium in 0.1 M phosphate buffer (pH 6.7) at 4°C. A voltage of 750 V (25 mA) was applied for 20 hr. The direction of migration is indicated in the figure by an arrow. The volume of each fraction collected was 5 ml.

---, protein; ○, β-xylosidase activity.

**FIG. 4. Gel-filtration of Malbranchea β-Xylosidase with Sephadex G-200.**
An aliquot of concentrated fraction II (10 mg, protein/3 ml) was passed through on Sephadex G-200 column (2.4×100 cm) which had been equilibrated with 0.1 M phosphate buffer (pH 6.7) at 4°C. The volume of each fraction collected was 5 ml.

---, protein; ○, β-xylosidase activity.
TABLE I. SUMMARY OF THE PURIFICATION PROCEDURE OF Malbranchea β-Xylosidase

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>4000</td>
<td>23080</td>
<td>2440</td>
<td>0.106</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄-precipitation</td>
<td>360</td>
<td>7470</td>
<td>1224</td>
<td>0.164</td>
<td>50.1</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>324</td>
<td>1315</td>
<td>1105</td>
<td>0.840</td>
<td>45.3</td>
</tr>
<tr>
<td>Ultrafiltration (1)</td>
<td>16</td>
<td>990</td>
<td>905</td>
<td>0.914</td>
<td>37.1</td>
</tr>
<tr>
<td>Electrophoresis (1)</td>
<td>60</td>
<td>88</td>
<td>804</td>
<td>9.14</td>
<td>32.9</td>
</tr>
<tr>
<td>Electrophoresis (2)*</td>
<td>400</td>
<td>54</td>
<td>512</td>
<td>9.48</td>
<td>21.0</td>
</tr>
<tr>
<td>Ultrafiltration (2)</td>
<td>16</td>
<td>51</td>
<td>506</td>
<td>9.92</td>
<td>20.7</td>
</tr>
<tr>
<td>Sephadex G-200b)</td>
<td>50</td>
<td>9</td>
<td>94</td>
<td>10.5</td>
<td></td>
</tr>
</tbody>
</table>

a) The value indicated is the sum of each of the second electrophoretic runs.
b) Three ml of step 7 (ultrafiltration (2))-fraction was loaded onto a Sephadex G-200 column.

Step 7. Ultrafiltration. The β-xylosidase-rich fractions from each of the second electrophoretic runs were collected and concentrated by ultrafiltration with a UM-10 membrane (Ultrafiltration (2)).

Step 8. Gel filtration on Sephadex G-200. Three ml of the concentrated β-xylosidase-rich solution (Ultrafiltration (2)) was applied to a column of Sephadex G-200 which had previously been equilibrated with 0.01 M phosphate buffer (pH 6.7) and eluted with the same buffer. The gel filtration gave a symmetrical elution pattern with respect to the protein concentration and the enzyme activity as shown in Fig. 4. The results of purification are summarized in Table I.

**Homogeneity of the enzyme**

Homogeneity of the Malbranchea β-xylosidase in various preparations were examined by disc electrophoresis on a polyacrylamide gel at pH 8.3. As shown in Fig. 5, electrophoresis gave three bands in Fraction I but only a clean single band in the purified enzyme. This result indicated that the purified enzyme was electrophoretically homogeneous.

![Fig. 5. Purity at Various Stages in the Purification of Malbranchea β-Xylosidase as Monitored by Polyacrylamide Gel Disc Electrophoresis.](image)

**Enzyme Samples:**

A, Precipitate by the ammonium sulfate (0~0.8 saturation).
B, After 1st column electrophoresis.
C, After gel filtration on Sephadex G-200.

**Electrophoretic Conditions:** Run at pH 8.3 and 5°C for 1 hr.

![Fig. 6. Sedimentation Patterns of Malbranchea β-Xylosidase.](image)

The rotor speed was 60,000 rpm. A 0.7% β-xylosidase solution in 0.1 M phosphate buffer (pH 6.7) was used. Photographs were taken at indicated times after the maximum speed was reached at 20°C. Synthetic boundary cell was used.
Homogeneity of the purified enzyme was also examined by ultracentrifugation. The purified enzyme was dissolved in 0.1 M phosphate buffer at pH 6.7 to give a protein concentration of 0.7%. A discrete single sedimentation pattern was observed as shown in Fig. 6. These results, along with other observations cited above, can be considered strong evidence for homogeneity of the purified enzyme.

Some physicochemical properties of the purified enzyme

The ultraviolet absorption spectrum was determined at an enzyme concentration of 0.43 mg per ml in phosphate buffer at pH 6.7. The resulting spectrum is shown in Fig. 7, and $E_{280}^{1\%}$ was equal to 13.2.

From the data in Fig. 6, the sedimentation coefficient, $s_{20,W}$, of the Malbranchea $\beta$-xylosidase was estimated to be 2.78S by the method of Shachman.

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Effect of pH and temperature on the enzyme activity. The effect of pH on the enzyme activity is shown in Fig. 9. The enzyme was

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Some enzymatic properties of the purified enzyme

Effect of pH and temperature on the enzyme activity. The effect of pH on the enzyme activity is shown in Fig. 9. The enzyme was
most active in the range of pH 6.2 to 6.8. Generally, the optimum pH of fungal β-xylanase has been known to be acidic and that of bacterial β-xylanase, neutral. It is of particular interest that *Malbranchea* β-xylanase was shown to differ from other fungal β-xylanase with respect to pH optimum. The effect of temperature on the enzyme activity at pH 6.7 is shown in Fig. 10. The optimum temperature for the enzyme reaction was about 50°C, and 50% of maximal activity was found at 40°C and 58°C.

**Effect of pH and temperature on the purified enzyme stability.**

Stability tests of the enzyme at various pH values were made. As shown in Fig. 11, this enzyme was stable in the range of pH 6.3 to 6.7 at 50°C. Thermal stability tests were carried out at various temperatures, and the results are shown in Fig. 12. The enzyme retained about 80% of its activity by incubation at 50°C for 15 min without substrate.

**REFERENCES**

2) T. Yasui, M. Sudo and T. Kobayashi, Abstracts of Papers, 22th Annual Meeting of the Society of Fermentation Technology, Japan, Osaka, Novem-
Purification and Some Properties of *Malbranchea* β-Xylosidase