Note

Extracellular Dextran-induced p-Nitrophenyl-α-D-glucoside-hydrolyzing Enzyme of Bacillus circulans KA-304: A Producer of Schizophyllum commune-lytic Enzyme

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*p-NP-α-D-Glucoside-hydrolyzing activity in the culture filtrate of Bacillus circulans KA-304, a producer of Schizophyllum commune cell-wall lytic enzyme, increased remarkably when the bacterium was grown on dextran as a carbon source. It was suggested that the increase of the activity was caused by increases of two major species, *α*-d-glucosidase I and *α*-d-glucosidase II. *α*-D-Glucosidase I, which showed a certain activity toward dextran, was isolated from the filtrate (MW 70 kDa, 35-fold, 10% recovery). The enzyme was stable around pH 6.5-7.5 and showed its highest activity at pH 6.5. The enzyme preparation inactivated with *p*-chloromercuribenzoic acid recovered its activity by incubating with diithiothreitol. Its substrate specificity suggested that the enzyme was an exo-type enzyme with certain affinity toward α-1,6-glucosidic linkage.

Key words: Schizophyllum commune; *p-NP-α-D-glucosidase; Bacillus circulans KA-304

Bacillus circulans KA-304 showed an activity in its culture filtrate to release protoplasts from a wood-degrading basidiomycete, *Schizophyllum commune*, which was not observed in other bacterial strains. The filtrate also hydrolyzed *p*-nitrophenyl (*p-NP*)-α-D-glucoside, *p-NP*-β-D-glucoside, and *p-NP*-β-D-N-acetylglucosaminide. These hydrolyzing activities increased together with the protoplast-forming activity when *B. circulans* KA-304 was grown on a cell-wall preparation (CWP) of *S. commune* as a carbon source, and varied independently by replacing the CWP with various polysaccharide preparations. A result was also obtained, which indicated the occurrence of multiple enzyme species for each hydrolyzing activity. On the bases of these findings, we have worked to analyze the enzyme species participating in assimilation of the CWP, and eventually in protoplasm formation. During the course of the investigation, we found that *p-NP*-α-D-glucoside-hydrolyzing activity in the culture medium with dextran was about 40-fold higher than that in the medium with the CWP, and also that the analysis of each culture filtrate on the activity by gel-filtration gave all most the same result despite structural differences between dextran (α-1,6 linkage) and *S*. glucan (α-1,3 linkage) in *S. commune* cell-wall. This paper deals with variation and analysis of *p-NP*-α-D-glucoside-hydrolyzing activity, and some properties of a *p-NP*-α-D-glucoside-hydrolyzing enzyme showed activity toward dextran.

*B. circulans* KA-304 was grown on Medium B, and carbon source was changed to examine its effect on *p-NP*-α-D-glucoside-hydrolyzing activity. The activity was measured by the *p*-nitrophenol formed in the reaction mixture as described previously, and dextran-hydrolyzing activity by the reducing sugar formed in a mixture where *p-NP*-α-D-glucoside was replaced by 1% dextran. One unit of enzyme activity was defined as the amount that releases 1 nmole of *p*-nitrophenol or reducing sugar per min. Reducing sugar was estimated by the method of Somogyi and Nelson with glucose as a standard, and glucose with glucose oxidase (Glucose B-test Wako, Wako Pure Chemical Industries, Ltd.). Protein was measured by Lowry's method with egg albumin as a standard, and monitored by the absorbance at 280 nm during column chromatography. The CWP and S-glucan fraction were prepared from *S. commune* IFO 4928 as described previously. Trehalose, nigerose, panose, nigeran, and pullulan were supplied by Sigma Chemical Co. All other reagents were the highest grade of commercial products.

Table I indicates that *p-NP*-α-D-glucoside-hydrolyzing activity increased greatly in the medium with dextran (about 40-fold of that in the medium with the CWP) but

<table>
<thead>
<tr>
<th>Table I. Effect of Carbon Source on p-NP-α-D-Glucosidase Activity</th>
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<tbody>
<tr>
<td>Carbon source</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>Sucrose</td>
</tr>
<tr>
<td>Starch</td>
</tr>
<tr>
<td>Amylose</td>
</tr>
<tr>
<td>CWP of <em>S. commune</em></td>
</tr>
<tr>
<td>CWP of <em>S. commune</em> + sucrose</td>
</tr>
<tr>
<td>Dextran</td>
</tr>
<tr>
<td>Dextran + sucrose</td>
</tr>
</tbody>
</table>

*B. circulans* KA-304 was cultivated on 5 ml of Medium B containing 0.5% of glycerol as a carbon source in a test tube. After 24 h of cultivation at 30°C, the culture was transferred to 100 ml of Medium B containing each carbon source in a 500-ml Sakaguchi flask, and cultivated in a reciprocal shaker (100 rpm) at 30°C. A part of the culture was withdrawn periodically, and centrifuged (12,000 × g, 10 min, 4°C). *p-NP*-α-D-Glucoside-hydrolyzing activity in each supernatant was measured and expressed as the amount in 1 ml of it.

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starch or amylase was not as effective to increase the activity. Addition of sucrose, ineffective in itself, to the CWP or the dextran medium brought about a further increase of p-NP-α-D-glucoside-hydrolyzing activity.

p-NP-β-D-Glucoside- and p-NP-β-D-N-acetylglucosaminide-hydrolyzing activities were extremely low (3 and 2 units/ml, respectively, in the medium with dextran, data not shown), which were consistent with the previous observation\(^1\) that these hydrolyzing activities varied independently.

As shown in Fig. 1A, the culture filtrate with the CWP contained multiple α-D-glucoside-hydrolyzing enzymes with two major species, tentatively named α-D-glucosidase I (about 70% of the total activity) and α-D-glucosidase II (about 30%). The α-D-glucosidase I fractions showed certain dextran-hydrolyzing activity, while the α-D-glucosidase II fractions did not. The elution profile of the culture filtrate with dextran (Fig. 1B) was similar to Fig. 1A, and indicated that the fractions corresponding to α-D-glucosidase I also hydrolyzed dextran. These suggested that the great increase of p-NP-α-D-glucoside-hydrolyzing activity caused by dextran in the culture medium might be due to increase of α-D-glucosidases I and II. Characterization of α-D-glucosidase I was made as a part of enzymatic analysis of assimilation of the CWP by \textit{B. circulans} KA-304.

α-D-Glucosidase I was purified from a culture filtrate of \textit{B. circulans} KA-304 (Medium B\(^1\) containing 0.5% dextran and 0.5% sucrose, total 15 liters, 1 liter of medium in a 2-liter Sakaguchi flask, 8 days, 30°C) with ammonium sulfate fractionation (30–70% saturation), and column chromatographies on DEAE-cellulose, Sepharose CL-6B, butyl-Toyopearl 650M, and hydroxyapatite. Approximately 35-fold purification was achieved with an overall yield of 10%, and the final preparation was homogeneous on a criterion of polyacrylamide gel electrophoresis (PAGE). The relative molecular mass of p-NP-α-D-glucosidase I was estimated to be 70 kDa by a HPLC-gel filtration (Wako-sil 5-diole, 7.8 × 300 mm). Sodium dodecyl sulfate-PAGE gave a single band of 77 kDa, suggesting that the enzyme was a monomeric pro-

**Table II.** Substrate Specificity of α-D-Glucosidase I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Linkage</th>
<th>Concentration</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-NP-α-D-glucoside</td>
<td></td>
<td>10 mM</td>
<td>100</td>
</tr>
<tr>
<td>Trehalose</td>
<td>(Glc α 1→1Glc)</td>
<td>10 mM</td>
<td>N.D.</td>
</tr>
<tr>
<td>Nigerose</td>
<td>(Glc α 1→3Glc)</td>
<td>10 mM</td>
<td>N.D.</td>
</tr>
<tr>
<td>Mallose</td>
<td>(Glc α 1→4Glc)</td>
<td>10 mM</td>
<td>6</td>
</tr>
<tr>
<td>Isomaltose</td>
<td>(Glc α 1→6Glc)</td>
<td>10 mM</td>
<td>1</td>
</tr>
<tr>
<td>Isomaltohexose</td>
<td>(Glc α 1→6Glc)</td>
<td>10 mM</td>
<td>360</td>
</tr>
<tr>
<td>Panose</td>
<td>(Glc α 1→6Glc; Glc α 1→4Glc)</td>
<td>10 mM</td>
<td>220</td>
</tr>
<tr>
<td>Isomaltotriose</td>
<td>(Glc α 1→6Glc; Glc α 1→4Glc)</td>
<td>10 mM</td>
<td>200</td>
</tr>
<tr>
<td>Isomaltotriose</td>
<td>(Glc α 1→6Glc)</td>
<td>1 mM</td>
<td>220</td>
</tr>
<tr>
<td>Isomaltotriose</td>
<td>(Glc α 1→6Glc)</td>
<td>1 mM</td>
<td>173</td>
</tr>
<tr>
<td>Panose</td>
<td>(Glc α 1→6Glc; Glc α 1→4Glc)</td>
<td>1 mM</td>
<td>190</td>
</tr>
<tr>
<td>Isomaltotriose</td>
<td>(Glc α 1→6Glc)</td>
<td>1 mM</td>
<td>170</td>
</tr>
<tr>
<td>Isomaltotriose</td>
<td>(Glc α 1→6Glc)</td>
<td>1 mM</td>
<td>220</td>
</tr>
<tr>
<td>Isomaltotriose</td>
<td>(Glc α 1→6Glc)</td>
<td>1 mM</td>
<td>150</td>
</tr>
<tr>
<td>Dextran</td>
<td>(Glc α 1→6Glc)</td>
<td>1%</td>
<td>30</td>
</tr>
<tr>
<td>S-glucan fraction</td>
<td>(Glc α 1→3Glc)</td>
<td>1%</td>
<td>N.D.</td>
</tr>
<tr>
<td>Fine CWP powder of \textit{S. commune}</td>
<td>(S-glucan, R-glucan, and chitin)(^*)</td>
<td>1%</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

The incubation period was varied to estimate initial velocity of the reaction according to the enzyme activity toward each substrate. Glucose formed was measured with glucose oxidase, and the activities are expressed relative to the initial velocity of p-NP-α-D-glucoside-hydrolyzing activity.

N.D.: No glucose was detected even after 24 h of reaction at 30°C.

\(^*\) See reference 10.
tein. The molecular mass was heavier than those (55-64 kDa) of similar enzymes from other Bacilli.\(^5,^9\)

\(\alpha\)-D-Glucosidase I was stable from pH 6.5 to 7.5, and retained the full activity after 10 min of incubation at 45°C in 100 mM potassium phosphate buffer (pH 7.0). The reaction with \(p\)-NP-\(\alpha\)-D-glucoside proceeded at pH 6-7 with an optimum of 6.5. The enzyme was activated by SH-reducing reagents, and inhibited by several SH-modifying oxidizing reagents. The enzyme preparation inactivated by 0.2 mM of \(p\)-chloromercuribenzoic acid (30°C, 1 h) recovered its activity (60% of the initial amount) by incubating with 0.2 mM dithiothreitol at 30°C for 1 h. These indicated that SH-group(s) might play some roles in the reaction. The effects of metal ions were not obvious.

\(\alpha\)-D-Glucosidase I hydrolyzed only \(p\)-NP-\(\alpha\)-D-gluco-
side (\(K_m=5.2 \text{ mM, data not shown}\)) among various \(p\)-
glycosides. As summarized in Table II, the enzyme
showed high reactivity toward isomalto (\(\alpha\)-1,6
linkage), and certain to nigerose (\(\alpha\)-1,3 linkage). The en-
zyme hydrolyzed isomaltooltriose and panose with almost
the same reactivity at the concentration of 10 mM. A
qualitative analysis by TLC of the reaction mixture indi-
cated that glucose and maltose were formed from
panose with scarce formation of isomalto. A series of
isomalto-oligosaccharides were also hydrolyzed by the
enzyme, though their reactivities were variable by their
concentration and some oligosaccharides inhibited the
enzyme at higher concentration. Table II comparing
reactivity at the concentration of 1 mM indicated that
reactivity of isomaltooligosaccharides did not so
decreased with their degree of polymerization. Dextran
was hydrolyzed with 30% reactivity to liberate glucose,
but neither formation of glucose nor reducing sugar was
observed in the mixture with S-glucan, fine CWP of \(S.
commune\), pullulan, nigeran, or such \(\alpha\)-1,4 glucans as
amylose, amylopectin, glycogen, and limit dextrin,
some of which branched at various position with \(\alpha\)-1,6
glucosidic linkages.

The substrate specificity as well as several properties
was close to those of the enzymes from other Bacilli,\(^6,^8\)
and indicated that \(\alpha\)-D-glucosidase I might be an exo-
type enzyme releasing the terminal glucose from isomal-
tooligosaccharides or dextran. The result suggested as if
the enzyme plays no role in assimilation of the CWP,
though inducible formation of the enzyme by the CWP
and its certain reactivity toward \(\alpha\)-1,3 glucosidic linkage
were not exclusion.

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