Interaction of the \( \beta \)-Transglycosylase of *Trichoderma longibrachiatum* with Cellulose

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The effects of cellulose on the production and stimulation of \( \beta \)-transglycosylase were studied. The \( \beta \)-transglycosylase of *Trichoderma longibrachiatum* was produced specifically in the presence of cellulose in Czapek-Dox medium containing sucrose as a sole carbon source. The enzyme activity was stimulated by the addition of cellulose in the reaction mixture, where the transfer reaction product (a water-insoluble glucan) was apparently synthesized on the surface of the added cellulose fibers.

The hyphal wall fraction of the fungus had the same stimulatory effect on \( \beta \)-transglycosylase as the cellulose fibers. A cellulose-like material in this fraction was found by partial acid hydrolysis and gas chromatography. Cellotriose was the smallest substrate effective for the synthesis of a water-insoluble glucan in the presence of cellulose by the \( \beta \)-transglycosylase, though a significant amount of glucan could not be synthesized without the addition of cellulose.

The \( \beta \)-transglycosylase of *Trichoderma longibrachiatum* showed a typical disproportionating reaction using cellopentaose as the substrate, producing a linear \( \beta \)-1,4-glucan of DP 19 as the major transfer reaction product.\(^1\)\(^2\) The enzyme was originally isolated from a wheat bran Koji culture of the fungus, in which hydrolyses such as cellulase and \( \beta \)-glucosidase were also produced.\(^1\) As these hydrolytic enzymes should degrade such a \( \beta \)-1,4-glucan, the biological meaning of the \( \beta \)-transglycosylase seems complicated.

Several glucosyltransferases are known to synthesize cellulose using nucleotide sugars such as UDPglucose and GDPglucose as glucosyl donors and cellodextrin or cellulose molecules as glucosyl acceptors.\(^3\)\(^4\)\(^7\) The acceptor molecule should be essential for cellulose synthesis by these transferases. In a disproportionating reaction of the \( \beta \)-transglycosylase of *T. longibrachiatum*, all cellooligosaccharides or cellodextrins from cellopentaose upwards should also accept glycosyl moieties, leading to the \( \beta \)-1,4-glucan. In fact, the rate of the \( \beta \)-1,4-glucan synthesis was kept almost at the initial level or rather increased when cellopentaose had decreased significantly.\(^1\) Such an acceleration of the \( \beta \)-transglycosylase might depend on the \( \beta \)-1,4-glucan molecules accumulated in the reaction mixture.

We were interested in studying an interaction of the \( \beta \)-transglycosylase with a \( \beta \)-1,4-glucan or cellulose molecule to understand the transfer by the enzyme and also to find out its biological function. We investigated the effects of cellulose on production of the \( \beta \)-transglycosylase and stimulation of \( \beta \)-1,4-glucan synthesis by the enzyme. Also, interaction of the \( \beta \)-transglycosylase with the added cellulose fibers was studied with respect to a polysaccharide component in the hyphal wall fraction of *T. longibrachiatum*.

**MATERIALS AND METHODS**

*Assay of enzymes. \( \beta \)-Transglycosylase:* The enzyme we used was purified from a wheat bran Koji culture of *T. longibrachiatum*, and assayed in a 24-hr reaction with 1% cellopentaose at pH 6.0 and 30°C as previously reported.\(^1\) One unit of the activity was defined as the amount of enzyme that produced water-insoluble glucan (\( \beta \)-1,4-glucan) showing a turbidity of 1.0 per ml of the reaction mixture per 24 hr, when it was measured at 660 nm using
cells of 1-cm light-pass length. The purified enzyme (275.4 units/mg protein) gave a single protein band on polyacrylamide disc gel electrophoresis. Cellulase: Cellulase was assayed as described in our previous paper, and one unit was defined as the amount of enzyme that produced reducing sugars equivalent to 1 μmol of D-glucose per min. ⑧

Substrates. Celllobiose was purchased from Wako Pure Chemicals Ltd. Other cellooligosaccharides were prepared as described in our previous paper. ①⑧

Isolation of hyphal wall fraction from Trichoderma longibrachiatum. T. longibrachiatum was inoculated into a 5-l Erlenmeyer flask containing 700 ml of Czapeck-Dox medium: K₂HPO₄, 1 g; MgSO₄·7H₂O, 500 mg; NaNO₃, 2 g; Fe(NO₃)₃·9H₂O, 10 mg; KCl, 500 mg; sucrose, 30 g; and distilled water, 1000 ml, pH 6.8, and grown for 14 days at 30°C. The organism was harvested and washed several times with distilled water by repeated centrifugation. The mycelium (2 g wet weight) was homogenized in a Waring blender with 100 ml of distilled water for 2 min, and dried overnight at 60°C after washing several times with distilled water. The residue (1 g dry weight) was suspended in 10 ml of distilled water, and was sonicated using a 10 kc Branson Sonifier B-12 with 15.5 g of glass beads (150 μ average diameter). After removing the glass beads by decantation, the suspension was centrifuged for 5 min at 1,400 x g. The resulting precipitate was employed as a hyphal wall fraction.

The hyphal wall fraction was further fractionated by dissolving 400 mg of it, dry weight, in 10 ml of 17.5% KOH. The precipitate was washed twice with 10 ml of 17.5% KOH and several times with distilled water until the pH of the suspension became neutral. The residue (308 mg) obtained was called the 17.5% KOH-insoluble fraction of the hyphal wall.

Chemical analyses. The 17.5% KOH-insoluble fraction (100 mg) was refluxed with 10 ml of 1 N H₂SO₄. At times, portions (2 ml) were withdrawn and neutralized with BaCO₃. The amount of soluble carbohydrate in the supernatant, which was obtained after removing the precipitate by centrifugation, was measured by the anthrone-H₂SO₄ method using D-glucose as a standard. A 20 μl aliquot of the supernatant was analyzed by TLC using silica gel 60 (Merck) and 1-butanol, pyridine, and water (8 : 1 : 1, v/v) as the solvent system for 6 hr, and sugars were detected as described in our previous paper. ⑧ Disaccharide components in the supernatant were also analyzed by GLC as their trimethylsilyl-derivatives using a Shimadzu GC-6ATF gas chromatograph equipped with a column of Silicone OV-17 on Unipor HP (0.3 x 100 cm) at 250°C. The remaining aliquot (1.0 ml) of the supernatant was evaporated to dryness with sucrose (3.6 mg) as an internal standard. Trimethylsilylation of the residue was done with a mixture of pyridine, trimethylchlorosilane, and 1,1,1,3,3,3-hexamethyldisilazane (5:1:1, v/v) as reported by Bhatti et al. ⑨

Microscopy. Cellulose powder and the hyphal wall fraction of Trichoderma longibrachiatum were observed microscopically under an Olympus microscope FHT to see changes in their appearance.

Chemicals. Cellulose (Cellulose Powder A, 100~200 mesh) was purchased from Toyo Roshi Co., Ltd. Xylan (larch wood) was purchased from Aldrich Chemical Company, Inc. Chitosan (crab shells) was from Sigma Chemical Co. Chitin was from Wako Pure Chemical Industries, Ltd.

RESULTS
Effects of several polysaccharides on the production of β-transglycosylase
To find what conditions affect the production of the β-transglycosylase, T. longibrachiatum was grown in Czapeck-Dox medium containing 3% sucrose as a sole carbon source supplemented with each of several polysaccharides. The fungus was inoculated in 20 ml of the medium and grown at pH 6.8 and 30°C for 10 days. The β-transglycosylase activity of each culture fluid was assayed with 1% cellopentaose as the substrate in the presence of cycloheximide (1 mg/ml of the reaction mixture) to inhibit the growth of the fungus. As shown in Table I, the enzyme was not produced when the fungus was grown in Czapeck-Dox medium. Chitin, chitosan, and xylan were shown to have a small effect on the production of the enzyme. Significant production of the β-transglycosylase was observed in the medium

<table>
<thead>
<tr>
<th>Addition</th>
<th>Conc. (%)</th>
<th>β-Transglycosylase activity (unit/ml of culture fluid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.0</td>
<td>0.00</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0.5</td>
<td>0.64</td>
</tr>
<tr>
<td>Chitin</td>
<td>0.5</td>
<td>0.07</td>
</tr>
<tr>
<td>Chitosan</td>
<td>0.5</td>
<td>0.02</td>
</tr>
<tr>
<td>Xylan</td>
<td>0.5</td>
<td>0.06</td>
</tr>
<tr>
<td>(Glucose)</td>
<td>1.0</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Table I. Production of β-Transglycosylase by Trichoderma longibrachiatum in the Presence of Several Polysaccharides
supplemented with cellulose, which also means that no significant amount of cellulase was produced. In fact, cellulase activity was not detected for the above culture fluid, and the amount of reducing sugars in the medium was at almost the same level (50 µg/ml) as that of the medium without cellulose during incubation.

Stimulation of β-transglycosylase activity by cellulose

The effects of cellulose on the transfer action of the β-transglycosylase were examined using a purified enzyme preparation. A reaction mixture (1.0 ml) containing 0.5% or 1.0% cellopentaose as the substrate and the β-transglycosylase (9 units) was incubated in the presence or absence of cellulose at pH 6.0 and 30°C. Figure 1 shows photographs of the reaction mixtures after 3 hr of incubation. Cellulose did not change its appearance after incubation with the β-transglycosylase, requiring more than 30 sec to sediment completely (Fig. 1 A-3, B-3). As shown in Fig. 1 A-1, the enzyme did not produce a water-insoluble glucan within 3 hr of incubation when 0.5% cellopentaose was used as the substrate. However, the cellulose powder distinctly changed its appearance after incubation with the β-transglycosylase, requiring not more than 20 sec to sediment (Fig. 1 A-2). As shown in Fig. 1 A-1, the enzyme did not produce a water-insoluble glucan within 3 hr of incubation when 0.5% cellopentaose was used as the substrate. However, the cellulose powder distinctly changed its appearance after incubation with 0.5% cellopentaose and the enzyme (Fig. 1 A-2). The cellulose powder, which looked swollen, required not more than 20 sec to sediment. When 1.0% cellopentaose was used as the substrate, the enzyme could synthesize a water-insoluble glucan within 3 hr of incubation when 0.5% cellopentaose was used as the substrate. However, the cellulose powder distinctly changed its appearance after incubation with 0.5% cellopentaose and the enzyme (Fig. 1 A-2). The cellulose powder, which looked swollen, required not more than 20 sec to sediment. When 1.0% cellopentaose was used as the substrate, the enzyme could synthesize a water-insoluble glucan within 3 hr of incubation (Fig. 1 B-1). However, the cellulose powder greatly increased the extent of swelling in the reaction mixture containing 1.0% cellopentaose as the substrate (Fig. 1 B-2).

An aliquot was taken from the reaction mixture containing 0.5% cellopentaose and cellulose before and after incubation with the β-transglycosylase (Fig. 1 A-2). It was observed that almost all of the cellulose fibers (particles) were surrounded by a water-insoluble glucan after 3 hr of incubation, as shown in Fig. 2. Some cellulose particles coagulated with water-insoluble glucan layers between them. The water-insoluble glucans should be synthesized adhering to the surface of the cellulose fiber. The β-transglycosylase activity was stimulated by the addition of cellulose, because the water-insoluble glucan could not be synthesized by the enzyme in the reaction mixture without cellulose (Fig. 1 A-1).

Effects of hyphal wall fraction on stimulation of the β-transglycosylase activity

A reaction mixture (1.0 ml) containing 0.5% cellopentaose and the β-transglycosylase (9 units) was incubated with the hyphal wall fraction (5 mg) at pH 6.0 and 30°C for 3 hr. The wall fraction (particles) changed in appearance as was observed for the cellulose powder. As shown in Fig. 3, the hyphal wall particles completely coagulated with water-insoluble glucan layers between them. A reaction mixture (1.0 ml) containing 0.5% cellopentaose and the β-transglycosylase (9 units) was incubated with chitin (10 mg) or chitosan (10 mg) at pH 6.0 and 30°C for 3 hr. However, they did not show any morphological change as described above by stimulating the production of a water-insoluble glucan.

The presence of a cellulose-like material in the hyphal wall fraction was examined by analyzing its chemical composition. The wall fraction was further fractionated according to solubility in 17.5% KOH. Several sugar components were detected by TLC in partial acid hydrolysates of a 17.5% KOH-insoluble fraction with 1 N H₂SO₄ for 1 hr. Two of them had the Rf values corresponding to those of cellobiose and cellotriose, respectively. The component with the highest Rf value was identified as glucose. However, the other one, which was detected between glucose and cellobiose, could not be identified. Gas chromatographic analysis also revealed the presence of cellobiose in the acid hydrolysates described above, as shown in Table II. It is highly probable that the hyphal wall of T. longibrachiatum consists of a cellulose-like material as one of the carbohydrate components. The ratio of cellobiose to the total soluble carbohydrate was maximum at 1 hr of hydrolysis, suggesting
Fig. 1. Photographs Showing Morphological Changes of Cellulose Powder after Incubation with \(\beta\)-Transglycosylase.

A reaction mixture containing 0.5% (A-1) or 1.0% (B-1) cellopentaose and the \(\beta\)-transglycosylase (9 units) in 1.0 ml of 0.01 M phosphate buffer, pH 6.0 was incubated at 30°C for 3 hr. Reaction mixtures A-2 and B-2 were the same as A-1 and B-1, respectively, except that they were incubated with 10 mg of cellulose powder. Both A-3 and B-3 were incubated with the \(\beta\)-transglycosylase (9 units) and cellulose powder (10 mg). In both A-4 and B-4, cellulose powder (10 mg) was incubated without the \(\beta\)-transglycosylase and cellopentaose. Photographs were taken 20 sec after stirring each reaction mixture.

Fig. 2. Photomicrographs of Cellulose Powder before and after Incubation with \(\beta\)-Transglycosylase and Cellopentaose.

See the text for details. Arrows indicate the \(\beta\)-1,4-glucan layers.

Fig. 3. Photomicrographs of Hyphal Wall Particles before and after Incubation with \(\beta\)-Transglycosylase and Cellopentaose.

An aliquot was taken before and after 3 hr of incubation, and photographed. Arrows indicate the \(\beta\)-1,4-glucan layers.

Fig. 4. Photomicrographs of Cellulose Powders after Incubation with \(\beta\)-Transglycosylase and Cellotriose.

A reaction mixture (1.0 ml) containing 4% cellotriose and the \(\beta\)-transglycosylase (18 units) was incubated with 1 mg of cellulose powder at pH 6.0 and 30°C for 3 hr. An aliquot was taken after incubation, and photographed. Arrows indicate the \(\beta\)-1,4-glucan layers.

that the ratio of a cellulose-like glucan was more than 10.5%.
Table II. Liberation of Cellobiose from a 17.5% KOH-insoluble Fraction of the Hyphal Wall by Acid Hydrolysis

<table>
<thead>
<tr>
<th>Incubation time (hr)</th>
<th>Total sugar liberated (µg/mg dry weight)</th>
<th>Cellobiose liberated (µg/mg dry weight)</th>
</tr>
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<tbody>
<tr>
<td>0.5</td>
<td>140</td>
<td>18</td>
</tr>
<tr>
<td>1.0</td>
<td>270</td>
<td>105</td>
</tr>
<tr>
<td>4.0</td>
<td>680</td>
<td>44</td>
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</table>

Table III. Substrate Specificity of \( \beta \)-Transglycosylase in Water-insoluble Glucan Synthesis

<table>
<thead>
<tr>
<th>Substrate ( x )</th>
<th>Conc. (%)</th>
<th>Rate of water-insoluble glucan synthesis (turbidity at 660 nm/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( G_5 )</td>
<td>0.5</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.73</td>
</tr>
<tr>
<td>( G_4 )</td>
<td>1.0</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>0.12</td>
</tr>
<tr>
<td>( G_3 )</td>
<td>2.0</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>0.01</td>
</tr>
<tr>
<td>( G_2 )</td>
<td>7.0</td>
<td>0.00</td>
</tr>
</tbody>
</table>

\( x \) \( G_5 \), cellopentaose; \( G_4 \), cellotetraose; \( G_3 \), cellotriose; \( G_2 \), cellobiose.

The rate of water-insoluble glucan synthesis was expressed as an increase of the turbidity per hr of the reaction.

Substrate specificity of the \( \beta \)-transglycosylase

Substrate specificity of the \( \beta \)-transglycosylase was examined again using the enzyme (18 units/ml) and more than 1% of several cellooligosaccharides as the substrate. The rate of water-insoluble glucan synthesis with 0.5% cellopentaose was lower than that determined with 1.0% cellopentaose, as shown in Table III. Cellotetraose was also shown to be effective as the substrate for a water-insoluble glucan synthesis, when it was used at the concentration of more than 2%. However, the rate of the glucan synthesis with cellotriose was negligible even when it was used at the concentration of 4%. Cellobiose was not effective for the reaction.

Effects of cellulose on water-insoluble glucan synthesis were examined using 4% cellotriose or cellobiose as the substrate. The cellulose powder swelled after 3 hr of incubation with the \( \beta \)-transglycosylase and 4% cellotriose, though it did not show any change in the reaction mixture with 4% cellobiose even after 24 hr of incubation. A water-insoluble glucan was synthesized on the surface of the cellulose powder, indicating the stimulation of the \( \beta \)-transglycosylase activity, as shown in Fig. 4.

**DISCUSSION**

*Trichoderma longibrachiatum* is a cellulytic fungus found in cellulosic materials in the soil. The \( \beta \)-transglycosylase of *T. longibrachiatum* is not a constitutive enzyme because it was not produced when the fungus was grown on a synthetic medium. The addition of cellulose induced the production of the enzyme in the medium; the fungus grew adhesively on the cellulose powder, in a film-like mycelium. The addition of glucose had no effect on production of the \( \beta \)-transglycosylase, though much better growth of the fungus was observed. Probably, cellulose directly affected the production of the enzyme by itself.

The synthesis of a transfer reaction product (a water-insoluble glucan) on the surface of the cellulose powder was found to result in its swelling after incubation with the \( \beta \)-transglycosylase and cellopentaose. In our previous paper, we found that the \( \beta \)-transglycosylase was strictly specific and elongated \( \beta \)-1,4-glucosidic linkages.\(^1\)\(^2\) The water-insoluble glucan that was synthesized on the surface of the cellulose powder must be a linear \( \beta \)-1,4-glucan. While, such a complex of \( \beta \)-1,4-glucan and cellulose as described above was not produced when the glucan synthesized in a reaction mixture with 1% cellopentaose was mixed with cellulose powder. Therefore, it seems necessary for production of the complex that the \( \beta \)-transglycosylase acts on the surface of the cellulose fiber. However, it is still unknown how the \( \beta \)-1,4-glucan molecule was bound to the cellulose in the complex. The cellulose
molecule might serve as an acceptor of glycosyl moieties as was expected from the transfer action mediated by several cellulose synthases.

Chitin is one of the polysaccharide components of the hyphal wall in many fungi and cellulose was found in the hyphal wall of *Oomycetes* such as *Phytophthora cinnamomi* and *P. parasitica*. In this study, a cellulose-like glucan having cellobiose as an unit was demonstrated in the hyphal wall of *T. longibrachiatum*. Stimulation of the β-transglycosylase activity by the fraction might depend on the glucan, resulting in coagulation of the fraction.

The β-transglycosylase did not show any hydrolytic action on cellulose producing reducing sugars. The enzyme seems to interact with cellulose only in the presence of an effective substrate. In that sense, cellotriose was regarded as the smallest substrate. However, it is not apparent whether the enzyme acts on cellulose directly by transferring glycosyl moieties to another cellulose molecule, a disproportionation reaction. Such a change seems not to affect the chemical properties of cellulose or its appearance. Also, it is quite uncertain whether the transfer action of the β-transglycosylase is involved in the synthesis of a cellulose-like glucan of the hyphal wall. Further investigation on this is necessary.

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REFERENCES