Hydrolysis of Water-Soluble and Water-Insoluble Cellulosic Substrates by 
Endo-\(\beta\)-1,4-Glucanase from *Acetobacter xylinum*

(Received April 30, 2004; Accepted June 9, 2004)


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Abstract: Morphology changes in bacterial cellulose produced by *Acetobacter xylinum* ATCC23769 were observed in the presence of \(\beta\)-glucodisaccharides such as gentiobiose and celllobiose. Endo-\(\beta\)-1,4-glucanase activity in culture broth was higher than that in the absence of those sugars. So we have investigated the properties of endo-\(\beta\)-1,4-glucanase (AEG) produced by this bacterium. This enzyme could hydrolyze water-soluble cellulose such as CMC, hydroxyethyl cellulose and celloextrin, and decreased the viscosity of the substrate solution. On the other hand, AEG could not produce any soluble sugars from water-insoluble cellulose such as Avicel and bacterial cellulose. These properties were completely different from endo-glucanase from fungi. AEG hydrolyzed cellohexaose and produced cellbiose, cellotriose and cellohexaose, but in the presence of bacterial cellulose, the soluble sugars produced from cellohexaose disappeared in the reaction mixture. It is suggested that AEG might have transglycosyl activity, though it belongs to glycosidase family 8. It is proposed that this activity is closely related to cellulose synthesis.

Key words: *Acetobacter xylinum*, endo-\(\beta\)-1,4-glucanase, *Irpex lacteus*

*A. xylinum* is a very simple and convenient system for studying the biochemical genetic aspects of cellulose biosynthesis. Why does *A. xylinum* synthesize cellulose? One possibility is that this bacterium would use cellulose as a form of glucose storage and its polymerizations cause a reduction in osmotic strength. If this is the case, *A. xylinum* must also encode a cellulase gene. Various reports have described the production of cellulolytic enzymes by *Acetobacter* strains. One of their genes encodes the endo-\(\beta\)-1,4-glucanase (CMCax) that can decompose cellulose. Because cellulose biosynthesis in *Acetobacter xylinum* was inhibited by the addition of the anti-CMCax serum to its growth medium, this enzyme is thought to be essential for cellulose biosynthesis. However, the relation between cellulose biosynthesis and cellulolytic enzyme has not yet been established.

From this point of view, it has been expected to make the properties of endo-glucanase clear, but there are few reports about the mode of action of *Acetobacter*-cellulase on various cellulosic materials. We have investigated the action pattern of this enzyme on water-soluble and -insoluble substrates.

**MATERIALS AND METHODS**

**Culture.** *A. xylinum* ATCC 23769 was used in this study by picking up “rough colonies” in 5-days-old agar plate. For seed culture, Hestrin and Schramm (SH) medium was used. The liquid medium (1000 mL) was sterilized at 121°C for 15 min, and inoculated and then incubated at 25°C for 15 days under the static condition.

**Enzymes.** An extracellular enzyme produced by *A. xylinum* (designated AEG) was obtained by the following. BC-free cells and the culture supernatant were recovered from the broth by filtration through a nylon membrane (13-XX100, SEFAR, Swiss). The filtrate was centrifuged at 30,000 \(\times\) g for 15 min at 4°C. The supernatant was treated with ammonium sulfate at 80% saturation, the precipitates formed were dissolved in 50 mL sodium acetate buffer (pH 5.0). The solution was dialyzed for 24 h at 4°C against distilled water and was used as the crude enzyme solution. The fungal endo-type cellulase (designated En-1) used in the present work was obtained from Driselase, a commercial product of *Irpex lacteus*, manufactured by Kyowa Hakko (Tokyo, Japan) according to procedures previously reported.

**Substrates.** Avicel, a microcrystalline cellulose powder (Art. 2331), was purchased from Merck (Darmstadt, Germany). Carboxymethyl cellulose (CMC) and ostazin brilliant red-hydroxyethyl cellulose (OBR-HEC) were purchased from Sigma Chemical (USA). Cotton was prepared as described by Hoshino et al. Phosphoric acid-swollen cotton (HC) and Avicel (HA) were prepared as described by Wood. Tamarind xylglucan was donated by T. Hayashi, Kyoto University. Cellohexaose (G₆) and \(p\)-nitrophenyl \(\beta\)-D-glucopyranoside (PNPG) were purchased from Seikagaku Kogyo (Tokyo, Japan).

**Protein measurements.** Enzyme protein was determined by the Lowry method using bovine serum albumin as the standard protein.

**Saccharification activity on water-soluble cellulose.** The reaction mixture contained 0.1 mL of 1.0% CMC solution, 0.1 mL of enzyme solution, and 0.2 mL of 0.05 M sodium acetate buffer (pH 5.0). The reaction mixture contained 0.1 mL of 1.0% CMC solution, 0.1 mL of enzyme solution, and 0.2 mL of 0.05 M sodium acetate buffer (pH 5.0).
m sodium acetate buffer (pH 5.0) in a total volume of 0.4 mL. After incubation at 30°C for 24 h, reducing sugars produced per mL of the reaction mixture were determined by the method of Somogyi-Nelson. One unit was defined as the amount of enzyme producing 1 μmol of glucose per min.

**Saccharification activity on water-insoluble cellulose.**

The reaction mixtures contained 0.1 mL of a substrate (1 wt% Avicel, 0.1 wt% HA, HC or 0.25 wt% BC), 0.1 mL of enzyme solution, and 0.2 mL of 0.05 M sodium acetate buffer, pH 5.0. Incubation was conducted at 30°C with mechanical shaking (100 strokes/min) for 24 h, and the mixtures were filtered through a glass filter. Reducing sugars produced were measured in the same way as the above. One unit was defined as the amount of enzyme producing 1 μmol of glucose per min.

**p-Nitrophenyl β-glucoside hydrolyzing activity.**

A reaction mixture contained 0.1 mL of 5 mM of pNPG, 0.1 mL of enzyme solution, and 0.2 mL of 0.05 M sodium acetate buffer, pH 5.0. After incubation at 30°C for 24 h, 1.0 mL of 1.0 wt% Na₂CO₃ and 2.0 mL of distilled water were added to the mixture. The amount of p-nitrophenol liberated was measured at 420 nm. One unit was defined as the amount of enzyme producing 1 μmol of p-nitrophenol per min.

**Hydrolyzing activity on OBR-HEC.**

The reaction mixture contained 0.35 mL of 1.0 wt% OBR-HEC solution, 0.35 mL of enzyme solution, and 0.7 mL of 0.05 M sodium acetate buffer, pH 5.0. After incubation at 30°C for 24 h, 0.4 mL of the reaction mixture was mixed with 1.2 mL of acetone. The precipitated substrate was removed by centrifugation and the absorbance of the supernatant was measured at 550 nm. One unit was defined as the amount of enzyme changing the absorbance (0.05/h).

**Viscometry of degradation of CMC and XG.**

The activity was assayed viscometrically in a viscometer at 30°C with 1 mL of enzyme solution and 4 mL of 0.05 M sodium acetate buffer (pH 5.0) and 1.0 mL of 1.0 wt% CMC or 0.5 wt% XG. One unit of activity was defined as the amount of enzyme required to cause a 10% decrease in relative viscosity for 24 h under these conditions.

**Molecular weight (Mw) distribution of CMC and XG.**

The molecular weight distributions of CMC and XG were determined using HPLC (801; JASCO, Tokyo, Japan). They were detected using a RI monitor (410; Wamer, Tokyo, Japan). The resolutions and absorptions of the supernatant were measured at 550 nm. One unit was defined as the amount of enzyme changing the absorbance (0.05/h).

**Thin-layer chromatography (TLC).**

The reaction mixture consisted of 0.1 mL of enzyme solution, 0.2 mL of 5 mg/mL cellohexaose or 5 mg/mL cellohexaose, to which was added 1 mg/mL bacterial cellulose and 0.1 mL of 0.05 M sodium acetate buffer at pH 5.0. After incubation for appropriate periods, 30 μL aliquots of reaction mixture and authentic sugars (cello-oligosaccharides) solution were spotted individually. Analytical TLC was performed with silica-gel 60 (0.5 mm thickness, Merck) in the solvent system of chloroform-methanol-water (90 : 65 : 15). The resolved sugars were detected by heating the plate at 120°C for 10 min after spraying with 30% sulfuric acid.

**RESULTS**

**Enzyme activity in crude enzyme preparation.**

The protein content of the crude enzyme preparation (AEG) was determined to be 1.43 mg/mL. Table 1 summarizes the hydrolyzing activity of AEG against water-soluble and insoluble substrates. AEG hydrolyzed water-soluble cellulose, but did not hydrolyze water-insoluble cellulose. AEG also containing β-glucosidase as pNPG was degraded. On the other hand, En-1 hydrolyzed water-soluble and insoluble cellulose as reported previously.

**Hydrolysis of CMC by AEG and En-1.**

The time courses of hydrolysis of CMC were compared with typical endo-glucanase En-1 from *lacteus* (Fig. 1). En-1 hydrolyzed CMC rapidly at the initial stage, and decreased the viscosity of CMC following the typical curve of endo cellulase. On the other hand, AEG decreased the viscosity very slowly. The molecular weight distribution of CMC was determined using HPLC (Fig. 2). It was estimated at about 180,000 initially, and decreased to 300 after treatment with En-1 for 24 h. This suggests that the main products produced by En-1 were cello-oligosaccharides. On the other hand, the elution profile of CMC treated with AEG revealed that the original peak of CMC shifted to three clear peaks showing low molecular weights. Their molecular weights were estimated as 180,000, 50,000 and 5000 respectively. It is suggested this enzyme cleaved specific glycosidic bonds of CMC and then stopped. From these results, the mode of action of AEG was completely different from En-1.

**Table 1. Specific activities (SP) of AEG against various substrates.**

<table>
<thead>
<tr>
<th>Substrate (water soluble)</th>
<th>SP (×10⁻³ U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMC</td>
<td>0.66</td>
</tr>
<tr>
<td>XG</td>
<td>0.68</td>
</tr>
<tr>
<td>OBR-HEC</td>
<td>1.06</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate (water insoluble)</th>
<th>SP (×10⁻³ U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avicel</td>
<td>0.4</td>
</tr>
<tr>
<td>H₂PO₄-Avicel</td>
<td>0.4</td>
</tr>
<tr>
<td>H₂PO₄-cotton</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Fig. 1. Viscosity changes of CMC during incubation with AEG and En-1.

The activity was assayed viscometrically at 30°C with an enzyme solution and 1.0 wt% CMC. One unit of activity was defined as the amount of enzyme required to cause a 10% decrease in relative viscosity for 24 h under these conditions.
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AEG En-I

![Fig. 2. HPLC pattern of hydrolysis products from CMC by AEG and En-I.](image)

The molecular weight distributions of CMC were determined using HPLC. Numbers and arrows indicate molecular weight markers. (1), 180,000; (2), 50,000; (3), 5000; (4), 300.

Table 2. Molecular weight (Mw) changes of CMC during incubation with AEG and En-I.

<table>
<thead>
<tr>
<th>Mw</th>
<th>AEG 0 h</th>
<th>AEG 48 h</th>
<th>En-I 0 h</th>
<th>En-I 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>180,000</td>
<td>60%</td>
<td>55%</td>
<td>60%</td>
<td>—</td>
</tr>
<tr>
<td>50,000</td>
<td>40%</td>
<td>25%</td>
<td>40%</td>
<td>18%</td>
</tr>
<tr>
<td>5000</td>
<td>—</td>
<td>20%</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>300</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>82%</td>
</tr>
</tbody>
</table>

**Hydrolysis of XG by AEG and En-I.**

The time courses for the hydrolysis of the XG by endoglucanases were examined viscometrically (Fig. 3). Both AEG and En-I decreased the viscosity of the XG solution very slowly, but AEG decreased to a larger extent than En-I, and this result was the reverse of CMC degradation. The molecular weight of XG was estimated to be about 9,000,000 initially using HPLC (Fig. 4, Table 3). The molecular weight of XG after treatment with AEG or En-I shifted from 9,000,000 to 380,000, 120,000 and 60,000 after 48 h. From these results, the modes of action of both enzymes were almost similar to each other.

**Degradation of cellohexaose (G₆) with or without bacterial cellulose.**

The hydrolysis products from G₆ by AEG were analyzed by TLC (Fig. 5 (A)). The enzyme produced cellobiose and cellotetraose were also detected after 6 h incubation. This enzyme could not attack cellodextrin from cellobiose to cellopentaose and accumulated in the reaction mixture, though cellopentaose was degraded after prolonged incubation. This suggests that AEG required more than a hexaose unit of glucose for catalysis. The degradation pattern of G₆ in the presence of BC is shown in Fig. 5 (B). Although the hydrolysis products were similar to those from G₆ only, the amount of the hydrolysis products in the presence of bacterial cellulose obviously decreased compared with products from G₆ only. Interestingly, cello-oligosaccharides produced at the initial stage disappeared after prolonged incubation with AEG. It is suggested that AEG has a transglycosyl activity especially in the presence of insoluble cellulose.

**DISCUSSION**

Cellulase activity was detected in the culture broth of A. xylinum, as some researchers have reported. We investigated the properties of cellulase, especially the mode of action on various cellulosic substrates, as the role of...
Fig. 5. Thin-layer chromatograms of hydrolysis products from cellohexaose (A) and cellohexaose in the presence of bacterial cellulose (B) by ALG.

The reaction mixture consisted of 0.1 mL of enzyme solution, 0.2 mL of 5 mg/mL cellohexaose or 5 mg/mL cellohexaose with the addition of 1 mg/mL bacterial cellulose and 0.1 mL of buffer. After incubation for appropriate periods, 30 µL aliquots of the reaction mixture and authentic sugar (cello-oligosaccharides) solution were spotted individually. Symbols: S, standard sugars; G, glucose; G, cellobiose; G, cellotriose; G, cellotetraose; G, cellopentaose; G, cellohexaose.

cellulase for cellulose synthesis of \textit{A. xylinum} was not established. Cellulase is defined as the enzyme that degrades cellulose and produces cello-oligosaccharides. However, \textit{Acetobacter} cellulase could not degrade cello-oligosaccharides less than DP 5, which is the smallest substrate as a completely soluble sugar. Furthermore, it could not produce soluble sugars from insoluble cellulosics, such as Avicel, bacterial cellulose and phosphoric acid-swollen cellulose. We have the questions of which substrates this cellulase reacts with and how it degrades cellulose. One possibility was derived from our results of cellohexaose degradation. Three products \(G_i > G_j \), \( G_k \), \( G_l \), were formed from \( G_6 \) that was partially solubilized in water, but it had a weak crystalline structure. From these results, \textit{Acetobacter} cellulase is an endo-glucanase, but is different from fungal endo-glucanases such as En-1 from \textit{Irpad lacticus}.

It is interesting that \textit{A. xylinum} produced enzymes that have transglycosyl activity, because cello-oligosaccharides were reused and disappeared in the reaction mixture of cellohexaose and bacterial cellulose. This phenomenon was observed only in the presence of bacterial cellulose that had the same conditions as the \textit{A. xylinum} culture. It is reported that the endo-glucanase from \textit{A. xylinum} belongs to glycosidase family 8, which is an inverting enzyme (http://afmb.crans-mrs.fr/CAZY/GH_8.html). As inverting enzymes merely catalyze the transglycosyl reaction, this reaction might be catalyzed by other enzymes because we did not purify this enzyme completely. Another possibility of the cellulase action in the culture is acetan degradation, as it degrades xylloglucan, which is a similar structure to acetan. Both substrates have the \( \beta \)-1,4-glucan backbone attached side chain composed of mixed oligosaccharides. Indeed, we detected some sugars composed of side chains such as rhamnose, mannose and gentiobiose (data not shown).

The cellulase from \textit{A. xylinum} did not attack purified bacterial cellulose produced by itself when we detected reducing sugars produced. However, it is reported that cellulose produced under the condition that it produced more cellulose than usual has a low degree of polymerization.\textsuperscript{15} We also measured the DP of bacterial cellulose when it was cultivated in the presence of \( \beta \)-glucodisaccharides such as gentiobiose and cellobiose. The DP of bacterial cellulose produced in the presence of \( \beta \)-glucodisaccharides was lower than that in the absence of those sugars. Furthermore, cellulase activity in the culture broth was also higher than usual (data not shown).

From these results, we propose that the cellulase is very important for cellulose production, and it might attack bacterial cellulose though it does not release soluble products.

Matthysse \textit{et al.}\textsuperscript{16,17} reported that an endoglucanase gene (designated \textit{celC}), homologous to \textit{A. xylinum} ATCC 23769, is contained in an operon of cellulose synthase in \textit{Agrobacterium tumefaciens}, and that there is a transposon insertion in \textit{celC} blocks cellulose synthesis, indicating that the production of CMC-hydrolyzing activity may be specifically associated with cellulose production. In higher plants, the activities of endo-\( \beta \)-1,4-glucanase are closely related to various physiological aspects of plant growth. Moreover, Tonouchi \textit{et al.}\textsuperscript{18} reported that cellulose production by strain BPR2001 was enhanced by the addition of a small amount of an endo-\( \beta \)-1,4-glucanase from \textit{Bacillus subtilis}. Cellulase, especially endo-\( \beta \)-1,4-glucanase, is somehow related to the biosynthesis of cellulose. The decrease in the DP of a polymer material is generally thought to influence its quality. We expect that AEG can contribute to the unique physical properties of BC.

This work was supposed by Grants-in-Aid for 21st Century COE Program by the Ministry of Education, Culture, Sports, Science, and Technology. We wish to thank Dr. T. Hayashi of the Wood...
Research Institute, Kyoto University, for supplying the xyloglucan of tamarind.

REFERENCES


