Purification and Characterization of Cellobiose Dehydrogenase from White-Rot Basidiomycete *Trametes hirsuta*

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In order to save energy during the pulp making process, we tried to use white-rot basidiomycete, *Trametes hirsuta*, which degrades lignin efficiently. But a decrease in paper strength caused by cellulytic activity ruled this out for practical application. Since the cellulytic activity of the fungus must be decreased, we purified and characterized a cellobiose dehydrogenase (CDH) that was reported to damage pulp fiber. The CDH in the culture filtrate of *C. hirsutus* was purified by freeze-thawing and chromatographic methods. The pI of the enzyme was 4.2 and its molecular weight was 92 kDa. The optimal temperature and pH of the enzyme were 60–70 °C and 5.0 respectively. Since the purified CDH decreased the viscosity of pulp in the presence of Fe(III) and cellobiose, it was shown that the suppression of CDH should be an effective way to reduce cellulose damage.

Key words: *Trametes hirsuta*; basidiomycete; cellobiose dehydrogenase

In the 21st century, all industry, including the pulp and paper industry, is expected to achieve continuous growth by implementing environmentally friendly technologies that save energy and resources. During the pulp making process, lignin, one of the main components of wood, is removed or modified. Since white-rot fungi can degrade lignin, biological treatment of wood chips with white-rot fungi before the conventional pulp making process is a potential environmentally friendly technology. White-rot fungi produce multiple lignin-degrading enzymes, including lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac). These enzymes and low molecular weight components are involved in the degrading of lignin known as the laccase-mediator system and the lipid mediator system. Although the exact degradative mechanism of lignin is still unknown, utilization of white-rot fungi for biological treatment of wood chips has been reported. For example, *Phanerochaete chrysosporium* was shown to reduce the refining energy required in the mechanical pulping process. *Ceriporiopsis subvermispora* reduced energy in the mechanical and kraft pulping process. But problems arose in practice. The mutants of *P. chrysosporium* degraded less lignin than did wild-type strains when grown on wood, and did not result in energy savings during subsequent mechanical pulping. In the case of *C. subvermispora*, it was necessary to blow air from the bottom of the wood-chip pile because of the low tolerance of the microorganism to high temperatures. On the other hand, *Trametes hirsuta* (formerly *Coriolus hirsutus*) is a promising fungus for biological treatment because it efficiently degrades lignin at high temperatures and an efficient transformation system was developed. *T. hirsuta* secretes a series of lignin-degrading enzymes, phenoloxidase, LiP, and MnP (unpublished result). Two cDNAs and two genomic DNAs coding for allelic forms of phenoloxidase of *T. hirsuta* have been isolated and characterized. We cloned and characterized several LiP and MnP genes from *T. hirsuta* (unpublished result). Nonetheless, the wild type of *T. hirsuta* can’t be used for biological treatment, because it degrades cellulose, which causes losses in yield and paper strength. The degradative mechanism of cellulose by *T. hirsuta* appears to be the same as that of other white-rot fungi. White-rot fungi produce a series of cellulases that hydrolytically cleave cellulose chains by exo-wise fission (cellulohydrolases), endo-wise fission (endo-1,4-β-glucanases), and β-glucosidases. White-rot fungi also produce oxidoreductases such as cellobiose dehydrogenase (CDH). CDH has been isolated from white-rot fungi and characterized. It displays typical dehydrogenase properties, which mainly oxidize cellobiose and cello-oligosaccharides, while it reduces substrates, including quinones, transition metal ions, aromatic-based chromophores, and...
phenoxy radicals by sugar-derived electrons. Certain roles of CDH related to cellulose degradation have been reported. The CDH of *P. chrysosporium* with cellobiose and ferrous iron reduced the degree of polymerization (DP) of the cellulose by the Fenton reaction. The hydroxyl radical, which is generated in the Fenton reaction, is formed by the reaction of Fe(II) and hydrogen peroxide, which are reduced from Fe(III) and oxygen respectively by CDH. Hydrogen peroxide is also produced by autoxidation of Fe(II). The role of CDH has also been investigated by analyzing a CDH-deficient strain. The CDH-deficient strain from *T. versicolor* lacked the ability to invade wood without reducing any lignin-degrading activities. Hence CDH is considered to be the key enzyme in the degrading of cellulose. In the present study, we purified and characterized CDH from *T. hirsuta*.

**Materials and Methods**

**Organism and culture conditions.** The fungal strain employed in this study, *Trametes hirsuta* IFO 4917, was a dikaryon. The no. 3 strain was a monokaryon derived from *T. hirsuta* IFO 4917 fruit-body. Stock cultures of *T. hirsuta* were maintained on potato-dextrose (PD) agar. One hundred ml of GP medium containing 3 g glucose, 1 g polypeptone, 0.1 g KH$_2$PO$_4$, 50 mg MgSO$_4$·7H$_2$O, and 0.2 mg thiamine HCl was inoculated with five agar plugs (0.5-cm diameter) taken from the periphery of a growing colony of *T. hirsuta*. The culture was incubated at 30 °C for 1 week with shaking at 105 rpm. For production of CDH, the culture were transferred to 100 ml of induction medium containing 1 g of oxygen-delignified hardwood kraft pulp (OKP) instead of glucose in the GP medium. The culture was sampled every 24 h. The cells and pulp were removed by centrifugation (13,000 rpm, 15 min), and cellulolytic activities in the supernatant were assayed.

**Assays.** CDH activity was assayed according to the method of Baminger, with a slight modification. This method follows the cellobiose-dependent decrease in absorbance of 2,6-dichlorophenolindophenol (DCPIP) spectrophotometrically at 520 nm. The assay mixture contained 0.6 mM cellobiose, 12 mM glucono-1,5-lactone, 4 mM NaF, and 0.5 mM DCPIP in 50 mM Na acetate buffer (pH 5.0) in a total volume of 500 μl. One unit of CDH was defined as the amount of the enzyme that reduced 1 μmol of DCPIP min$^{-1}$ ml$^{-1}$. Degradation of 4-methylumbelliferyl-β-D-glucoside (4-MUC) was determined with glucono-1,5-lactone at pH 5.0. β-Glucosidase activity was determined with 4-methylumbelliferyl-β-D-glucoside (4-MUG) at pH 5.0. The reaction mixtures for 4-MUC degrading activity and β-Glucosidase activity were incubated for 30 min at 40 °C, and stopped with glycine–NaOH (pH 10.5). The amounts of 4-methylumbelliferon (4-MU) were measured by fluorescence. One unit of 4-MUC degrading activity and β-Glucosidase was defined as the amount of the enzyme that released 1 μmol of 4-MU min$^{-1}$ ml$^{-1}$ from 4-MUC and 4-MUG respectively. CMCase activity was determined by release of reducing sugars from carboxymethylcellulose (CMC, 1.0% [w/v]) at pH 5.0 for 30 min at 40 °C. Avicelase activity was determined by release of reducing sugars from Avicel® SF (Asahi Kasei, Osaka, Japan, 1.0% [w/v]) at pH 5.0 for 2 h at 40 °C. One unit of Avicelase and CMCase was defined as the amount of the enzyme which released reducing sugars min$^{-1}$ ml$^{-1}$ from Avicel® SF and CMC respectively.

**Purification of CDH.** When maximum CDH activity was obtained (at 3 d), the culture supernatant was separated from the residual pulp, and a protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA). To remove polysaccharide produced by the fungi, the solution was frozen at −80 °C, thawed at 4 °C, and centrifuged. The clarified supernatant was concentrated by ultrafiltration with Centramate™ and Omega membrane (10 k) (Pall, East hills, NY, USA). The concentrated supernatant was applied to a POROS HP 2 column (PerSeptive Biosystems, Framingham, MA, USA; 16.0 mm by 30 mm) previously equilibrated with 20 mM Na phosphate (pH 6.0) and 1 mM ammonium sulfate. The column was washed with a linear salt gradient from 1 M to 0 M at a flow rate of 8.0 ml/min. Fractions containing CDH activity were pooled, dialyzed, and concentrated by ultrafiltration, and then applied to a Hi Load Superdex-200 column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA; 26 mm by 60 cm) previously equilibrated with 20 mM Na phosphate (pH 6.0) at a flow rate of 1.5 ml/min. Active fractions were pooled, dialyzed, and concentrated, and then applied to a mono Q HR5/5 column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) equilibrated with 20 mM Na phosphate (pH 6.0) and eluted with a linear salt gradient (0 to 400 mM) at a flow rate of 0.5 ml/min. Active fractions were pooled, dialyzed against water, concentrated, and stored at 4 °C.

**Protein concentration.** Protein concentrations were determined by the method of BCA, with bovine serum albumin as a standard.

**Enzyme kinetics.** The Eadie-Hofstee plot was used to determine the $K_m$ and $k_{cat}$ values of CDH. For the electron donor trials, DCPIP was used as an electron acceptor. For the electron acceptor trials, cellobiose was used as an electron donor. The reactions were done in 50 mM Na acetate buffer (pH 5.0).

**Effect of pH on CDH stability.** The effect of pH on CDH stability was tested from pH 2 to 8. CDH was incubated in an appropriate buffer (50 mM) at 40 °C for 8 h. After incubation, CDH activity was measured at pH 5.0 using DCPIP as an electron acceptor. The buffers and pH ranges used in this experiment were as follows:
glycine–HCl buffer, pH 2–4; Na acetate buffer, pH 4–6; Na phosphate buffer, pH 6–8.

Thermostability of CDH. The stability of CDH against heat inactivation was determined by incubation at various temperatures for 30 min. An aliquot was withdrawn, and the remaining activity was measured by the DCPIP assay at 25°C.

Effect of CDH on cellulose depolymerization. To chelate metals, OKP was maintained with Na EDTA (38%) for 30 min at 50°C, and then thoroughly washed with distilled water. The OKP (500 mg) was treated as follows: (1) 10 U CDH, 0.2 mM ferric chloride (FeCl$_3$), 20 mM cellobiose; (2) 10 U CDH; (3) 0.2 mM FeCl$_3$, 20 mM cellobiose; in 50 mM Na acetate buffer (pH 5.0) at 30°C for 24 h. The reaction mixtures were filtrated with nylon membrane, and the degrees of viscosity (DP) of the residues were determined with Cannon-Fenske Viscometers. The DP of the OKP was also examined.

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with an ATTO PAGE $\text{G}$ system with an acrylamide gel (5 to 20% gradient). Isoelectric focusing (IEF) was performed with a Multiphor II Electrophoresis Unit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) with pH ranges of 3.5 to 9.5 and 4.0 to 6.5 in conjunction with an appropriate IEF isoelectric point (pl) standard (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Proteins were visualized by staining with Coomassie Blue.

Amino acid sequencing. The purified CDH (500 pmol) was treated with lysyl endopeptidase (1 pmol) for 24 h at 37°C. The reactant was concentrated and applied to a Wakopak Navi C-22-5 (Wako Pure Chemical Industries, Osaka, Japan; 2.0 mm by 250 mm) previously equilibrated with 0.05% of trifluoroacetic acid aqueous solution. The column was washed with a linear gradient with 0.05% of trifluoroacetic acid acetonitrile solution at a flow rate of 0.2 ml/min. Fractions were pooled, and then sequenced on an HP G1005A protein sequencing system (Hewlett-Packard, Palo Alto, CA, USA).

Spectroscopy. Absorption spectra were measured with a Hitachi spectrophotometer U-3300 in a cuvette with a 1-cm path length. Fluorescence was measured with a Fluoroskan Ascent FL (Thermo Labsystems, Helsinki, Finland).

Results

Time course of cellulolytic activities

Cellulolytic activities of the no. 3 strain were measured for 6 d in an inductive medium containing OKP (Fig. 1). The cellulolytic activities (CDH, Avicelase, CMCase, 4-MUC degrading activity, and β-Glucosidase) showed similar time course patterns. Until the second day, the activities were low. The activities were maximal on the third day, and then slightly decreased with longer incubation, except for 4-MUC degrading activity, which did not decrease with longer incubation. Since the measured cellulolytic activities including CDH maximized on the same day, it was shown that the CDH of T. hirsuta must be involved in the degradation of cellulose.

CDH purification

Since CDH was induced by cellulose, T. hirsuta was cultured in a broth containing OKP. CDH activity was the highest on the third day of cultivation. The culture was collected and filtrated to remove fungal cells. CDH was purified from culture filtrate by freeze-thawing, ultra filtration, a POROS HP 2 column, a HiLoad Superdex-200 column, and a Mono Q column, which yielded homogeneous enzyme as judged by SDS–PAGE (Fig. 2). This procedure provided approximately 204-fold purification with a specific activity of 16.3 U/mg for DCPIP reduction (Table 1). The relative molecular mass of the CDH was determined to be 91.7 kDa by SDS–PAGE analysis (Fig. 2). The pl of CDH was 4.2.

Optimal condition for CDH

CDH showed maximal activity on DCPIP at pH 5.0. The CDH activity on DCPIP was optimal at 60–70°C (Fig. 3).

Stability of CDH

CDH was very stable below 40°C in Na acetate buffer (50 mM, pH 5.0). At 40°C, CDH activity decreased by 25% after 30 min. Above 60°C, all activity was lost after 30 min. CDH was also stable in a pH range of 4–7 at 40°C for 8 h (Fig. 3).
Amino acid sequence

The N-terminal amino acid of the purified CDH could not be determined by blocking of the N-terminal of the amino acid. So, CDH was digested with lysyl endopeptidase to determine the internal amino acid sequences. Three amino acid sequences were as follows: ALVNPWLTN, ILALAG, KVLLLERGGPST. These sequences were found in the deduced amino acid sequence of the cdh gene from Trametes versicolor.32)

Specificities for substrates and electron acceptors

The ability of CDH to oxidize various sugar substrates was examined with DCPIP as an electron accepter. Among the tested monosaccarides, only glucose served as the substrate. Among the tested disaccarides, cellobiose, lactose, and maltose served as substrates. Cello-oligosaccarides such as cellotriose, cellotetraose, which

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtration</td>
<td>8.314</td>
<td>636</td>
<td>0.08</td>
<td>100</td>
<td>1.0</td>
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<tr>
<td>Ultra filtration</td>
<td>3.261</td>
<td>569</td>
<td>0.17</td>
<td>95</td>
<td>2.1</td>
</tr>
<tr>
<td>POROS HP 2</td>
<td>203</td>
<td>363</td>
<td>1.79</td>
<td>61</td>
<td>22.4</td>
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<tr>
<td>HiLoad Superdex-200</td>
<td>58.6</td>
<td>155</td>
<td>2.64</td>
<td>26</td>
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<tr>
<td>Mono Q</td>
<td>6.5</td>
<td>105</td>
<td>16.3</td>
<td>18</td>
<td>203.8</td>
</tr>
</tbody>
</table>

Protein was determined by BCA protein assay.
Activity was measured with DCPIP and cellobiose as substrates.

Table 1. Purification of T. hirsuta CDH

Fig. 2. SDS-Polyacrylamide Gel Electrophoresis of Purified CDH.
Lane 1, Molecular weight markers: myosin (200,000), β-galactosidase (116,000), bovine serum albumin (66,300), and aldolase (42,400). Lane 2, purified CDH.

Fig. 3. Effects of pH and Temperature on CDH Activity and Stability.
A, pH profiles of CDH; B, Temperature profiles of CDH; C, pH stability of CDH; D, Thermostability of CDH. pH profiles and pH stability were determined with 0.5 mM DCPIP in glycine–HCl buffer (○, pH 2–4), Na acetate buffer (•, pH 4–6), and Na phosphate buffer (△, pH 6–8). Temperature profiles and thermostability were determined with 0.5 mM DCPIP in Na acetate buffer (pH 5.0).
Table 2. Relative Activity of CDH with Various Sugar Substrates

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Relative Activity (%)</th>
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<tbody>
<tr>
<td>Cellobiase</td>
<td>100</td>
</tr>
<tr>
<td>Cellotriose</td>
<td>32</td>
</tr>
<tr>
<td>Cellotetraose</td>
<td>34</td>
</tr>
<tr>
<td>Glucose</td>
<td>9</td>
</tr>
<tr>
<td>Lactose</td>
<td>18</td>
</tr>
<tr>
<td>Maltose</td>
<td>25</td>
</tr>
<tr>
<td>Xylobiose</td>
<td>0</td>
</tr>
<tr>
<td>Xylose</td>
<td>0</td>
</tr>
<tr>
<td>Mannose</td>
<td>0</td>
</tr>
</tbody>
</table>

All experiments were performed in 50 mM Na-acetate buffer (pH 5.0). DCPIP (0.5 mM) was used as the electron acceptor.

Table 3. Specificity of CDH for Electron Donors and Acceptors

<table>
<thead>
<tr>
<th>Electron donors</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (mm⁻¹ s⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>Cellobiase</td>
<td>42</td>
<td>10.7</td>
<td>256</td>
</tr>
<tr>
<td>Cellotriose</td>
<td>216</td>
<td>3.5</td>
<td>16</td>
</tr>
<tr>
<td>Cellotetraose</td>
<td>1,396</td>
<td>9.5</td>
<td>6.8</td>
</tr>
<tr>
<td>Lactose</td>
<td>2,126</td>
<td>6.0</td>
<td>2.9</td>
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<table>
<thead>
<tr>
<th>Electron acceptors</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (mm⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCPIP</td>
<td>12.2</td>
<td>20.9</td>
<td>1,710</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>1.3</td>
<td>1.1</td>
<td>850</td>
</tr>
<tr>
<td>TBBQ</td>
<td>38.9</td>
<td>11.7</td>
<td>301</td>
</tr>
<tr>
<td>$K_3$Fe(CN)$_6$</td>
<td>214</td>
<td>7.0</td>
<td>33</td>
</tr>
</tbody>
</table>

Kinetic experiments were performed in 50 mM Na-acetate buffer (pH 5.0).

CDH of Trametes hirsuta

Fig. 4. Cellulose Depolymerization by CDH.

The viscosity of each initial sample was defined as 100%. The viscosity of OKP after 24 h treatment of wood chips to save energy during the pulp making process, because it grows at higher temperatures than almost all other fungi and has high lignin-degrading ability, but the cellulolytic activities of T. hirsuta caused a reduction in the strength properties of paper. In order to reduce the cellulolytic activities of T. hirsuta effectively, we attempted to identify a key enzyme involved in cellulose degradation. But when T. hirsuta grew on OKP, various kinds of cellulolytic activities, such as cellobiohydrolase, endoglucanase, and cellulbiose dehydrogenase, were produced simultaneously. Since we could not identify the key enzyme, we focused on CDH, which is considered to be a key enzyme during cellulose degradation.

A monokaryon, strain no. 3, derived from a fruit-body of T. hirsuta IFO 4917, was used for production and purification of CDH, because IFO 4917, which might have an allele of the cdh gene, would produce more kinds of CDH than strain no. 3, which makes purification of the enzyme complicated. Strain no. 3 was cultivated in a medium containing OKP to induce CDH. Then the culture filtrate was concentrated and purified by chromatographic methods. As a result, CDH of T. hirsuta was purified to homogeneity. This procedure provided approximately 204-fold purification with a specific activity of 1710 mm⁻¹ s⁻¹.

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suta became acidic during the first 6 d (lower than 4.0), the electric charge of the CDH protein must be positive. Since OKP has a negative charge under these conditions, CDH and OKP interact with each other electrically. Thus the degradation of OKP in the culture proceeds efficiently. During biological treatment of wood chips by \textit{T. hirsuta}, CDH works effectively as well, because the condition of biological treatment is suitable for CDH. For example, the pH of wood chips is mild acidity and the temperature of wood chips is controlled below 40 °C, while CDH is very stable between pH 4 and 7 and below 40 °C.

When OKP was treated with a combination of purified CDH, Fe(III), and cellobiose, the viscosity of OKP decreased by 36.5% as compared with the initial viscosity, while the other reactions did not show a decrease in viscosity. The depolymerization mechanism of OKP caused by CDH, Fe(III), and cellobiose must be the Fenton reaction, which generates hydroxyl radicals, Fe(II) and hydrogen peroxide, which are needed for the Fenton reaction, are produced in the presence of CDH, Fe(III), and cellobiose, because CDH from \textit{T. hirsuta} reduced Fe(III) to Fe(II) in the presence of cellobiose, and hydrogen peroxide is generated by the reduction of oxygen and the autoxidation of Fe(II). The depolymerization of OKP and the condition of the biological treatment of wood chips clearly show that CDH of \textit{T. hirsuta} plays an important role in the depolymerization of cellulose.

Among the various carbohydrates examined, cellobiose, cellotriose, and lactose, which have the β-1,4 glucosidic bond, were oxidized by CDH, whereas xylobiose, which has the α-1,4 glucosidic bond was not oxidized. Maltose (α-1,4 glucosidic bond) and glucose were also oxidized. Determination of $k_{\text{cat}}/K_m$ for CDH-catalyzed sugar oxidations suggests that cellobiose is the most preferred substrate like other reported CDHs. Since many cellobiose are produced especially by cellulohydrolase, CDH acts efficiently on cellulose work to support the activity of CDH in vivo. A comparison of the $k_{\text{cat}}/K_m$ of the electron acceptors shows that DCPIP is the most preferred electron acceptor. Although DCPIP is not present in the wood, quinone derivatives are produced during the degradation of lignin by white-rot fungi. The quinone derivatives from lignin and cellobiose derived from cellulose work to support the activity of CDH in vivo.

Since the N-terminus of the purified CDH was blocked, CDH was treated with lysyl endopeptidase, and the sequences of certain resulting peptides were analyzed. The partial amino acid sequences of the CDH from \textit{T. hirsuta} were identical to the deduced amino acid sequences of CDH from \textit{T. versicolor}. This result supports the conclusion that the purified enzyme is similar to the CDH from \textit{T. versicolor} in constituent amino acids as well as activity.

In conclusion, although the other cellulolytic enzymes might also be involved in degrading cellulose, the fact that CDH from \textit{T. hirsuta} degrades wood-derived cellulose under the conditions of biological treatment indicates that CDH has a significant role in the degrading of cellulose during the biological treatment process. Judging from the role of CDH, suppression of CDH activity is necessary in applying \textit{T. hirsuta} to practical biological treatment of wood chips without negative effects on paper strength.

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References


