Cellulose Dehydrogenase, an Active Agent in Cellulose Depolymerization

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The ability of cellobiose dehydrogenase purified from Phanerochaete chrysosporium to modify a Douglas fir kraft pulp was assessed. Although the addition of cellobiose dehydrogenase alone had little effect, supplementation with cellulose and iron resulted in a substantial reduction in the degree of polymerization of the pulp cellulose. When the reaction was monitored over time, a progressive depolymerization of the cellulose was apparent with the concomitant production of cellobiono-1,5-lactone. Analysis of the reaction filtrates indicated that glucose and arabinose were the only neutral sugars generated. These sugars are derived from the degradation of the cellobiose rather than resulting from modifications of the pulp. These results suggest that the action of cellobiose dehydrogenase results in the generation of hydroxyl radicals via Fenton's chemistry which subsequently results in the depolymerization of cellulose. This appears to be the mechanism whereby a substantial reduction in the degree of polymerization of the cellulose can be achieved without a significant release of sugar.

Microorganisms are known to degrade the cellulose, hemicellulose, and lignin components of wood through the action of extracellular enzymes. Hydrolytic enzymes such as cellulases, xylanases, and mannanases contribute to the degradation of the carbohydrate moieties, while oxidative enzymes such as laccases, lignin peroxidase, and manganese peroxidase, in combination with low-molecular-weight mediators, have been shown to be involved in lignin biodegradation (10).

Although many of the wood-degrading fungi can be grouped into categories such as white rot, brown rot, or soft rot fungi, this delineation does not readily carry over into the specific enzymes produced by these organisms involved in the degradation of the different wood components. While there has been a great deal of progress made in characterizing many of the enzymes involved in the degradation of the native wood constituents, there is still the apprehension that some enzymes may be multifunctional and cause modifications to more than one or all of the wood components.

One such group of enzymes are the extracellular cellobiose-oxidizing enzymes cellobiose dehydrogenase (CDH) and cellobiose:quinone oxidoreductase (CBQ), which have been shown to be produced by a number of basidiomycete fungi, including both white rot fungi (4, 22, 36, 41) and a brown rot fungus (37). This class of enzymes has also been isolated from the culture filtrates of nonligninolytic, cellulolytic microorganisms such as Monalsites stiphiola (12), Chaetomium cellulolyticum (15), Sporotrichium thermophilus (8), and bacterial strains (29).

CDH is an enzyme consisting of two prosthetic groups, a flavin and an adenine dinucleotide moiety (hemoflavinzyme). The latter domain is directly involved in both the oxidative and reductive half-reactions, while the former stimulates the reduction of one-electron acceptors such as cytochrome c and Fe\(^{3+}\) (19, 20). Recently, the size and shape of this enzyme have been determined by small-angle X-ray scattering (28), while the cDNA of Phanerochaete chrysosporium CDH has been successfully cloned and sequenced (34). It has also been shown that the proteolysis of CDH both in vitro (20) and by the action of isolated proteases (13, 16) results in the generation of a second active enzyme, CBQ (45). CBQ is a flavoenzyme which, in the presence of cellobiose, can reduce compounds such as quinones and phenoxy radicals (1).

Although these enzymes were originally isolated and characterized more than 20 years ago (41, 42), their role in wood decomposition has yet to be clearly defined. Several putative functional roles for this enzyme have been suggested: CDH acting as a vehicle to generate radicals as antibacterial agents (43); the reduction of quinones as a defense mechanism against toxins (32); CDH acting as a regulatory enzyme preventing the repolymerization of lignin radicals generated by other oxidative enzymes (1, 14); and the generation of highly active hydroxyl radicals which participate in Fenton’s reactions to degrade cellulose, xylan, and lignin (18, 46). However, as Morpeth so elegantly put it, “cellobiose oxidoreductases are enzymes in search of a function” (32).

Although CDH has been shown to both enhance the action of cellulases on crystalline cellulose (7) and degrade model wood components such as carboxymethylcellulose, xylan, and synthetic lignin (18), there have been few studies to date on its direct role in cellulose modification in substrates such as pulp. In this report, we describe how we isolated CDH from the white rot fungus P. chrysosporium and assessed its ability to modify the degree of polymerization (DP) of a Douglas fir kraft pulp in the absence or presence of various cofactors.

MATERIALS AND METHODS

Growth conditions. P. chrysosporium BKM F-1767 was maintained on agar plates containing 5 g of glucose and 3.5 g of malt extract per liter. Plates were inoculated, and organisms were grown at 27°C. The growth medium was a modification of the medium described by Bao et al. (6), containing 2.28 g of (NH\(_4\))\(_2\)HPO\(_4\), 0.5 g of MgSO\(_4\) \(\cdot\) 7H\(_2\)O, 0.74 g of CaCl\(_2\), 0.0158 g of NaNO\(_3\), 0.01 mg of thiamine-HCl, 6.75 g of succinate disodium salt hexahydrate, and 10 g of unquilted cotton cosmetic pads (Safeway) per liter. P. chrysosporium was pregrown in 20 ml of medium containing 2% glucose and 0.5% yeast extract.
in 250 ml Erlenmeyer flasks at 27°C for 2 days at 150 rpm. The cultures were then homogenized in a Waring blender for 8 s and used to inoculate 250 ml of succinate medium in 500-ml Erlenmeyer flasks. The cultures were grown for 30 days at 27°C.

**Protein purification.** Culture filtrates were collected by vacuum filtration, and the protein content was determined in the sample using the method of Lowry et al. (28). Precipitated protein was collected by centrifugation at 8,000 rpm for 2 h. Between each purification step, the protein solution was washed with 10 mM ammonium acetate (pH 4.5) and concentrated with an Amicon system, using a Diaflo YM10 membrane with a 10-kDa cutoff. The concentrated protein was loaded on a DEAE-Sepharose CL-6B column (Pharmacia Biotech Inc.) equilibrated with 10 mM ammonium acetate (pH 4.5) and eluted with 1.5 liters of a linear gradient of 10 to 250 mM ammonium acetate (pH 4.5). Only fractions with an absorbance at 280 nm above 0.03 were pooled, washed, and concentrated. Subsequently, concentrated protein was loaded on a Phenyl-Superose HR 5/5 column (Pharmacia) equilibrated with 0.6 M (NH4)2SO4 in 50 mM ammonium acetate (pH 4.0) and eluted with 30 ml of a linear gradient of 0.6 to 0 M (NH4)2SO4 in 50 mM ammonium acetate (pH 4.0). Fractions with an A280/A260 ratio above 0.1 were pooled, washed, concentrated, and stored on a Superose 6 HR 10/30 column (Pharmacia) equilibrated with 50 mM ammonium acetate (pH 5.0). After gel filtration, CDH-containing fractions were pooled, concentrated, and stored at −80°C before use.

**Enzyme assays.** Cellobiose dehydrogenase activity was assessed by the reduction of cytochrome c at 550 nm (ε = 28 mM-1 cm-1). The assay mixture contained 3 mM cellobiose, 20 mM succinate (pH 4.5), 12.5 μM cytochrome c, and 2 mM sodium ascorbate with a total of 1 ml of 2.5-Dichlorophenolindophenol (DCPIP) was used to measure the combined CDH and CBQ activity at 515 nm (ε = 6.8 mM-1 cm-1). The DCPIP assay mixture contained 3 mM cellobiose, 20 mM succinate (pH 4.5), 7.5 μM DCPIP, and various amounts of enzymatic preparations to reach a total CDH unit. All assays were performed at 23°C. Enzyme activity, expressed in international units (IU), was equivalent to the reduction of 1 μmol of DCPIP per min. Cytochrome c activity of CDH was found to be equivalent to 5.4 U of the DCPIP activity.

**Pulp treatment conditions.** Unbleached kraft pulp derived from Douglas fir (Pseudotsuga menziesii) was obtained from the Crofton mill (Fletcher Challenge), British Columbia, Canada. The pulp was fractionated in a Bauer-McNett fiber length classifier to collect the 14R fraction (pulp fiber entrapped by the substrate for the enzymatic treatments. The longest fiber length fraction (14R) was used. This eliminated some of the variability that can arise in treating whole pulps.

**Pulp treatment.** A time course experiment was conducted with CDH (0.1 IU/ml) in 50 mM sodium acetate buffer (pH 4.5) at 30°C, supplemented with 20 mM cellobiose-0.2 mM ferric chloride. The reactions were terminated at 1, 2, 6, 12, and 18 h, and the reaction mixtures were inactivated by boiling for 15 min. Reaction filtrates were then removed; a portion was used directly for monosaccharide and oligosaccharide determination, while the remaining portion was freeze-dried and acid hydrolysis (150 mM NaOH conditions) of the cellobionol-1-lactone during HPLC analysis, were separated on a CarboPac PA-1 column, using a Dionex DX-500 HPLC system (Dionex, Sunnyvale, Calif.) controlled by Peaknet 4.30 software. The carbohydrates were detected at 200 μm sodium acetate and regenerated with 500 mM NaOH. After injection of 20 μl of sample via a SpectraSYSTEM AS500 autoinjector (Spectra-Physics, Fremont, Calif.), the oligosaccharides were eluted with a 50 to 200 mM gradient of sodium acetate (over 20 min) at a flow rate of 1 ml/min. The oligosaccharides were monitored with a Waters 625 liquid chromatography system (Millipore Corp., Milford, Mass.). The cellulose triacetyl derivatives were purified on a Waters 486 spectrophotometer (Millipore) at a wavelength of 254 nm.

**RESULTS**

As we had previously used Douglas fir kraft pulp to assess the actions of various cellulases and xylanases (30, 31), we thought that this would be an effective substrate to use to evaluate what changes, if any, CDH caused to the carbohydrate constituents of the pulp. In an attempt to create a homogeneous substrate, the pulp was fractionated and only the longest fiber length fraction (14R) was used. This eliminated some of the variability that can arise in treating whole pulps. The 14R fiber length fraction accounted for 55% of the total pulp and was composed of approximately 75% glucose, 5.5% xylose, 6.3% mannose, 0.5% galactose, 0.4% arabinose, and 4.1% total lignin.

Previous work has shown that CDH initiates a two-electron oxidation of cellobextrins, generating the corresponding lactone (21). Cellobiose was found to be the substrate of choice, with cellobextrins of increasing DP demonstrating reduced activity. Meanwhile limited activity was observed when glucose was used as the substrate (19). The reductive half-reactions (see reaction scheme below) include a preferential single-electron reduction of Fe(III) to Fe(II) (9), while in systems of Fe(II) and CDH, CDH can reduce Fe(II) to generate H2O2 directly (40, 41) or via the production of superoxide, which subsequently react with Fe(II) (46). Furthermore, autooxidation of Fe(II) can result in the generation of hydrogen peroxide (46). Together hydrogen peroxide and reduced iron undergo Fenton’s chemistry, generating hydroxyl radicals, which then can

**Cellulose depolymerization by CDH**

Cellulose depolymerization by CDH was found to be equivalent to 3.4 U of the DCPIP activity. We also investigated controls of the above combinations without added iron. The GPC calibration curve was generated from the elution profile of polysaccharide standards with narrow molecular weight distributions. Using the Mark-Houwink coefficients previously reported for polysaccharide in THF, Kp = 1.18 × 10^{-3} and ν = 0.92, the molecular weight of the tricarbonylated cellulose was obtained (39). The DP of cellulose was obtained by dividing the molecular weight of the tricarbonylated polymer (MW) by the corresponding molecular weight of the tricarbonylated derivative of amyloglucoside (DP = MW/519).
CDH$_{ox}$ + cellobiose → CDH$_{red}$ + cellobiose-1,5-lactone (1)
2CDH$_{red}$ + 2Fe$_3$$^+$ → 2CDH$_{ox}$ + 2Fe$_2$$^+$ (2)
CDH$_{red}$ + O$_2$ → CDH$_{ox}$ + H$_2$O$_2$ (3)
2Fe$_2$$^+$ + O$_2$ + 2H$^+$ → 2Fe$_3$$^+$ + H$_2$O$_2$ (4)
Fe$_2$$^+$ + H$_2$O$_2$ → Fe$_3$$^+$ + OH$^-$ + OH$^-$ (5)

The potential role of these various components encouraged us to assess the effect of different combinations of CDH, cellobiose, iron, and hydrogen peroxide on the molecular weight distribution of the 14R fraction of the Douglas fir kraft pulp (Fig. 1; Table 1). All control combinations without CDH (data not shown) gave profiles similar to that of the original sample buffer (DP$_N$ = 341; DP$_W$ = 2,337) with the exception of the reaction mixtures containing both hydrogen peroxide and ferric chloride (Fig. 1, line 2). This combination generates Fenton's reagent in the absence of cellobiose (line 5) caused a reduction in the DP of the cellulose even greater than that exhibited by the ferric chloride and hydrogen peroxide alone (line 2). This result confirmed that the CDH could modify the substrate without the addition of easily oxidizable substrates such as cellobiose (26). However, when the pulp samples were first subjected to chelation and then treated with CDH without the supplementation of iron, the ability of CDH to modify the DP of the pulp cellulose was substantially reduced (DP$_N$ = 331; DP$_W$ = 2,254). The greatest change in the DP of the cellulose occurred when the cellobiose and ferric chloride were added to the CDH. The mixture containing CDH, cellobiose, iron, and hydrogen peroxide demonstrated only slightly more depolymerization than the corresponding control lacking the enzyme. In all cases, the attack seems to be directed at the highercellulose morphology, the nature of the reaction was followed by monitoring the changes in DP through a time-controlled experiment (Fig. 2; Table 1). As the reaction proceeded, the enzyme complex continued to react with the cellulosic material of high DP (5,000), generating molecules of a lower DP and producing a new maximal peak at approximately a DP of 1,000 to 2,000 (Fig. 2; Table 1). A subsequent treatment with twice as much CDH enzyme resulted in a further reduction in the average DP (DP$_N$ = 193; DP$_W$ = 1,558), as indicated by the shift in the shoulder of material found between DPs of 200 and 800 (Fig. 2; Table 1). The supplementation of additional cellobiose, by a 20 mM cellobiose spike at hour 9 of an 18-h reaction, had no effect on the DP (data not shown).

Having established that the CDH supplemented with cellobiose and ferric chloride resulted in the greatest changes in cellulose morphology, the nature of the reaction was followed by monitoring the changes in DP through a time-controlled experiment (Fig. 2; Table 1). As the reaction proceeded, the enzyme complex continued to react with the cellulosic material of higher DP (5,000), generating molecules of a lower DP and producing a new maximal peak at approximately a DP of 1,000 to 2,000 (Fig. 2; Table 1). A subsequent treatment with twice as much CDH enzyme resulted in a further reduction in the average DP (DP$_N$ = 193; DP$_W$ = 1,558), as indicated by the shift in the shoulder of material found between DPs of 200 and 800 (Fig. 2; Table 1). The supplementation of additional cellobiose, by a 20 mM cellobiose spike at hour 9 of an 18-h reaction, had no effect on the DP (data not shown).

High-performance anion-exchange chromatography was subsequently used to determine if any oligosaccharides were...
TABLE 2. Polysaccharides liberated by CDH after 18 h of incubation

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>Glucose (mg/ml)</th>
<th>Arabinose (mg/ml)</th>
<th>Cellobiose (mg/ml)</th>
<th>Cellobionic acid (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.022 (0.002)</td>
<td>0.000</td>
<td>6.799 (0.020)</td>
<td>0.000</td>
</tr>
<tr>
<td>C + Fe</td>
<td>0.023 (0.003)</td>
<td>0.000</td>
<td>6.811 (0.013)</td>
<td>0.000</td>
</tr>
<tr>
<td>C + F + H</td>
<td>0.114 (0.001)</td>
<td>0.006 (0.001)</td>
<td>6.772 (0.019)</td>
<td>0.000</td>
</tr>
<tr>
<td>C + CDH</td>
<td>0.157 (0.006)</td>
<td>0.008 (0.001)</td>
<td>6.046 (0.009)</td>
<td>1.285 (0.009)</td>
</tr>
<tr>
<td>C + H + CDH</td>
<td>0.225 (0.010)</td>
<td>0.013 (0.002)</td>
<td>6.188 (0.022)</td>
<td>0.893 (0.017)</td>
</tr>
<tr>
<td>C + F + CDH</td>
<td>0.171 (0.005)</td>
<td>0.008 (0.001)</td>
<td>5.252 (0.016)</td>
<td>1.999 (0.024)</td>
</tr>
<tr>
<td>C + F + H + CDH</td>
<td>0.175 (0.003)</td>
<td>0.010 (0.001)</td>
<td>6.389 (0.011)</td>
<td>0.463 (0.008)</td>
</tr>
<tr>
<td>CDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Reaction conditions included combinations of CDH (0.1 IU/ml), 20 mM cellobiose (C), 1.8 mM H₂O₂ (H), and 0.2 mM FeCl₃ (F).

DISCUSSION

To date, much of the work related to CDHs has focused on their interaction with ligninolytic enzymes and their action on lignin-related compounds (2, 36). It has been suggested that CDH may be one of the components of the fungal enzymatic machinery involved in the depolymerization-repolymerization of lignin-related compounds by a variety of fungi (1). This is most likely directly related to the oxidoreductive nature of this enzyme. However, other workers (7) have also shown that CDH enhanced the cellulosytic degradation of crystalline cellulose. These authors indicated that the supplementation of Trichoderma cellulases with CDH increased the substrate hydrolysis by approximately 20% over the non-CDH-supplemented reaction. More recently it has been demonstrated that CDH can degrade or modify cellulose and xylan derivatives, as well as synthetic lignin (18).

Previous studies using ¹H nuclear magnetic resonance spectroscopy indicated that CDH does selectively convert β-D-cellobiose to its cellobio-1,5-lactone derivative (21). Our present study using HPLC methods confirmed these findings, indicating the rapid production of cellobionolactone. Prolonged incubation times resulted in increased cellobionolactone generation, with the simultaneous generation of smaller amounts of cellobionic acid. Similarly, the addition of CDH and cellobiose alone to the pulp without either hydrogen peroxide or iron generated substantial amounts of the cellobionolactone, indicating that the iron content of the pulp (67 ppm) or other transition metals such as cobalt or copper (4 or 228 ppm, respectively) was high enough for the putative reaction to proceed. Furthermore, treating the pulp first with an EDTA chelation step effectively reduced the depolymerizing action of CDH. It is also possible that other reducible substrates such as quinones are present. In the situations when the reaction mixture was supplemented with hydrogen peroxide, reduced amounts of lactone were generated. These results are contrary to those found by Henrikkson et al. (18), who reported increased degradation of both xylan and cellulose when CDH was incubated in the presence of cellobiose, iron, and hydrogen peroxide. However, our reaction conditions differed not in iron concentration but in source. These previous workers (18) used ferric cyanide, while we used ferric acetate. It has been shown that ferric acetate participates much more readily in Fenton’s chemistry than does ferric cyanide (46), ultimately generating hydroxyl radicals. These radicals have a high and indiscriminate reactivity and a very short half-life. It has been postulated that enzyme damage can occur when the Fenton’s reaction takes place in close proximity to the enzyme (46). This is likely the case when additional hydrogen peroxide was added to this reaction mixture, while in the reaction mixtures not supplemented with hydrogen peroxide, this compound can readily be generated by the action of CDH and the supplemented iron acetate (see reaction scheme in Results). Our results clearly indicate that CDH can depolymerize wood-derived cellulose. In vivo, an even more efficient depolymerization may be possible, as an accumulation of hydrogen peroxide may result from the autoxidation of iron complexes such as Fe(II)
oxalate (46) and/or the slow continuous generation of small amounts of hydrogen peroxide by extracellular oxidases (10).

The attack on the natural woody substrate seems to be directed at the cellulose of higher DP, generating molecules of cellulosic material of smaller size. This is indicated by the appearance of the shoulders in the lower DP ranges of the chromatographs. Recently, Ander et al. (3) found, through the use of polarized light micrographs, that the attack by CDH on pine holocellulose fiber seemed to be directed at specific sites of compression. These “nodes,” whose origin are still under investigation, appear approximately every 100 to 200 μm. It would be interesting to see whether these nodes are enriched in iron or other transition metals, as concentrated deposits in these regions would support the localized attack previously observed (3).

The appearance of arabinose in the acid hydrolysates of the reaction filtrates was rather surprising, as no xylose was detected. Since the majority of the arabinose found in pulp is directly associated with the xylan backbone, and no xylose was liberated, this observation suggested that the arabinose did not result from the selective liberation of a neutral wood sugar from the pulp but rather was a degradation product of the supplemented cellbiose. This assumption was confirmed when arabinose was detected in reaction filtrates which mimicked the pulp treatments but lacked pulp as a substrate. Maximal arabinose was detected in samples which had been supplemented with hydrogen peroxide, suggesting that Fenton’s chemistry was directly involved in arabinose generation. Previously it had been reported that the products of celllobiose degradation under Fenton’s chemistry include both glucose and arabinose as well as other organic acids (23). Thus, it is highly likely that the observed cellulose depolymerization was directly related to the action of hydroxyl radicals generated by this enzyme. The breakdown products of cellbiose were also observed when only cellbiose and CDH were added to pulp, suggesting that the concentration of transition metals within the pulp was enough to initiate Fenton-type reactions. However, this did not occur to the extent that was exhibited when iron was added to the reaction mixture and was reduced substantially when the pulps were first subjected to a chelation step. This observation was further supported by the fact that CDH and cellbiose alone could depolymerize cellulose to a greater extent than was observed with just the addition of iron and hydrogen peroxide.

Most of the past work on CDH has primarily investigated its action on lignin or lignin-related compounds, implying that this enzyme may have a primary role in lignin degradation. However, the fact that this enzyme contains a cellulose binding domain (35), is produced during primary metabolism with cellulose as a substrate (33), and demonstrates the ability to degrade both native cellulose and cellulose and xylan derivatives suggests that this enzyme may actually be more closely associated with the cellwallases than the ligninases, while it functions as a dehydrogenase. Recent work by Ander et al. (3) supports this suggestion, as these workers found that the attack by CDH on pine holocellulose was more pronounced when the reaction mixture was supplemented with exoglucanases compared to endoglucanases. As the natural iron content of the pulp (20 to 100 ppm) (27) seems to support enzyme activity and the generation of cellbiose occurs by the action of accompanying cellwallases (exoglucanases), it is probable that all of the required cofactors will be present in the natural environment. Further evidence for its close association with the cellwallases is that CDH has been found in nonligninolytic fungi such as Monilia sp. (12), Sporotrichum thermophilum (8), and Chaetomium cellulolyticum (15) and in bacteria (29), which all possess a complete cellwallase system and no ligninolytic enzymes. It is also known that celllobioalactone, which is generated by CDH, can act as an inducer of celllobioalzymes while inhibiting β-glucosidases. By actively competing for the available cellbiose with the β-glucosidases, this may limit the actual amount of degradation that occurs naturally.

It is worth noting that all of the white rot fungi and the single strain of brown rot fungus (Coniophora puteana) from which CDH has been purified to date contain a complete cellwallase system. Other brown rot fungi, which are not members of the family Coniophoraceae, do not possess a complete cellwallase system, as they lack exoglucanases, and these fungi have yet to demonstrate the existence of CDH as part of their enzymatic machinery. Therefore, the generation of cellbiose by exoglucanases and the natural iron content in woody material complete the requirements for active CDH enzyme, which could then act in concert with the celllobioalzymes to degrade the carbohydrate moieties while also causing modifications to the lignin.

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