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Cellobiose:Quinone Oxidoreductase, a New Wood-degrading Enzyme from White-rot Fungi

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Production of an extracellular cellobiose: quinone oxidoreductase (cellobiose dehydrogenase) by *Polyporus versicolor* and *Chrysosporium lignorum** grown on cellulosic substrates was investigated. Highest yields of the enzyme occurred with powder cellulose as the carbon source. In *C. lignorum* development of oxidoreductase activity and cellulolytic enzymes were parallel.

The enzyme from *C. lignorum* was selected for further study. It was found to catalyze an oxidation – reduction reaction in which reduction of a quinone to the corresponding phenol was coupled to the conversion of cellobiose to cellobionic acid probably through cellobiono-δ-lactone. The enzyme has a high specificity for the oxidation of cellobiose. Lactose was oxidized at a slower rate but no other mono- or disaccharides tested served as substrate for the oxidoreductase. However, quinone requirement was less specific and the enzyme was able to reduce both *ortho* and *para* quinones. Optimum pH for oxidoreductase activity was approximately 4.5 – 5.0. An extracellular lactonase from *C. lignorum*, able to hydrolyze cellobiono-δ-lactone is also reported.

White-rot fungi utilize a number of extracellular hydrolytic enzymes for the degradation of cellulose.[1–3] In most studies of cellulose degradation, these enzymes are obtained by cultivation of fungi on pure cellulose. With cellulose and cellodextrins as substrates, the cellulolytic enzymes produce glucose and cellobiose as the final products of degradation. Results from these types of experiments are often extrapolated as description of the degradation of cellulose in wood. However, in this and in the accompanying paper,[4] we describe the occurrence of an extracellular enzyme which, in the presence of a suitable hydrogen acceptor, yields acidic sugars as breakdown products of cellulose. The enzyme can utilize reaction products of the phenol-oxidizing enzyme laccase and a suitable phenol, for example of lignin origin, as the hydrogen acceptor.

* Recent investigations have revealed that *C. lignorum* is identical with *Sporotrichum pulverulentum* Novobranova, and the latter name should be used.

EXPERIMENTAL

Organisms. *Chrysosporium lignorum* P 127-1 was obtained from Dr. T. Nilsson, Royal College of Forestry, Stockholm, Sweden. *Polyporus versicolor* L. ex Fr. isolate Madison, was obtained from Prof. E. B. Cowling, North Carolina State University, Raleigh, North Carolina, USA.

Cultivations. A basal salt medium [5] was used with 3 g of carbon source/liter.

To follow enzyme induction the fungi were grown in 250 ml Erlenmeyer flasks containing 80 ml culture solution on the following carbon sources: powder cellulose, wood meal, phosphoric acid-swollen cellulose, cellobiose, and sucrose. The flasks were incubated at 25°C on a reciprocal shaker at 135 rpm and two flasks were harvested every second day. The solid material was removed by filtration and the culture solution was concentrated 5-fold in a collodion tube before analysis.

For enzyme production, *C. lignorum* was cultivated in one-liter Erlenmeyer flasks on powder cellulose in 300 ml culture solution for 8 – 9 days at 25°C reciprocal shaking. The culture solution was filtered and then concentrated by ultrafiltration (membrane Amicon UM-10). The proteins were precipitated by addition of solid ammonium sulfate at room temperature to 90 % of saturation, redissolved and extensively dialyzed against distilled water before use.

Enzyme assays. Cellobiose dehydrogenase was assayed as follows: The reduction of 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2) was followed

spectrophotometrically at 360 nm at 25°C. The reaction mixture contained 300 μmol acetate buffer (pH 4.5), 1 μmol of the quinone, 2 μmol cellobiose and the enzyme in a total volume of 3 ml. Absorbancy was usually determined at one minute intervals. The decrease in absorbancy was linear with time until 90 % of the quinone was reduced. One enzyme unit was defined as the amount of enzyme that reduced 1 μmol quinone per min according to the Enzyme Commission.

Lactonase was assayed by measuring the decrease in the amount of cellobiono-δ-lactone. The lactone was determined colorimetrically as its hydroxamic acid derivative.[6] After incubation at 25°C for different times, hydroxylamine solution (pH 5.0) was added and the reaction mixture was allowed to stand at room temperature for 15 min before the ferric chloride solution was added. All assays were compared with a blank incubation mixture lacking enzyme to correct for the spontaneous hydrolysis of the substrate. Aryl-β-glucosidase activity was measured colorimetrically.[7]

Cellulase activity was determined viscosimetrically with CMC as a substrate [8] and colorimetrically with Avicel as a substrate. For the Avicelase measurement, the enzyme solution was incubated in a reaction mixture containing 20 mg of Avicel (American Viscose Co., Marcus Hook, Pennsylvania, USA) and 300 μmol acetate buffer (pH 5.0) in a total reaction volume of 3 ml. The sample was incubated for 2 h at 30°C with continuous shaking, filtered, and analyzed for reducing sugars.[9] Reduction of viscosity of CMC gives only a measurement of randomly-hydrolyzing endo-β-1,4-glucanases, while production of reducing sugars from Avicel, a crystalline cellulose, gives a measurement of both endo- and exo-β-glucanases.

Chemical determinations. The concentrations of quinones were measured spectrophotometrically. 3-Methoxy-5-*tert*-butyl-benzoquinone-(1,2) λ_{max}(nm) = 360 (ε = 2180) in 0.1 M acetate buffer (pH 4.5); 2-methoxybenzoquinone-(1,4) λ_{max}(nm) = 368 (ε = 1238) in 0.1 M acetate buffer (pH 4.5); cerulignone λ_{max}(nm) = 472 (ε = 19 180) in 0.1 M acetate buffer (pH 4.5) containing 50 % dioxan.

Lactones were measured colorimetrically [6] at pH 5.0. The hydroxamic acid derivative of cellobionolactone has an ε = 239 at λ = 500 nm. If a quinoid hydrogen acceptor was present the reaction mixture was extracted with chloroform to remove phenols and quinones before analysis for lactones.

Paper chromatography. Identification of reaction products were made by ascending paper chromatography on Whatman No. 1 chromatography paper. The samples were extracted with chloroform to remove phenols, concentrated by evaporation under vaccum, adjusted to pH 8 with NaOH, boiled for 2 min and finally treated with a strong cation exchanger (Dowex 50WX8).

Cellobionic acid had the following $R_{glucose}$: 0.29 in ethylacetate – pyridine – water (4:2:1 v/v/v), 0.55 in butanol – ethanol – water (10:3:5), and 0.87 in ethylacetate – acetic acid – water (3:1:1).

Gas chromatography. Gas-liquid chromatography was carried out on the trimethylsilyl ethers of cellobionic acid and cellobiono-δ-lactone. Column: QF-1 11 % on Cromosorb W 100 – 120 mesh, 235°. The samples were extracted with chloroform, adjusted to pH 8 and placed on an anion exchange column (Dowex 1-X4) (acetate form). The column was carefully washed with distilled water to remove neutral sugars and the acidic reaction products were eluted with 1 M acetic acid. The acetic acid was then removed by repeated evaporation and the residue was silylated both directly and as the Na-salt. *N,O*-bis(trimethylsilyl)-trifluoroacetamide and trimethylchlorosilane were used in the preparation of the silyl ethers. Retention time for the trimethylsilylated cellobionolactone was 12.15 min and 13.4 min for the trimethylsilylated cellobionic acid.

Chemicals. Cellobiono-δ-lactone was synthesized by chlorite oxidation of cellobiose.[10] Cellobionolactone had a m.p. of 181.7 – 182.4°C. No gluconic acid could be detected in the preparation. Hydrolysis of the synthetic cellobionolactone (100°C, 1 M HCl, 4 h) yielded glucose and gluconic acid as identified by paper chromatography. Cellobionic acid was prepared by alkaline hydrolysis at pH 8 of cellobionolactone. The free acid was obtained by cation exchange (Dowex 50W-X8).

Phosphoric acid-swollen cellulose was prepared from Munktell's Cellulose Powder No. 400.[11] The 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2) and cerulignone were kindly supplied by Dr. Augustine E. Opara of this department.

RESULTS

Induction of cellobiose dehydrogenase on different carbon sources

The production of cellobiose dehydrogenase from *C. lignorum* and *P. versicolor* was followed during a twenty day cultivation. *C. lignorum* was cultivated on wood meal, cellulose, phosphoric acid-swollen cellulose, cellobiose, and sucrose. *P. versicolor* was cultivated on cellulose and swollen cellulose. Fig. 1 shows the development of enzyme in the culture solution of *C. lignorum*. Cellulose induced the highest cellobiose dehydrogenase activity in the culture solution. The other cellulosic materials, wood meal and swollen cellulose, also induced enzyme activity. Very small amounts of enzyme were

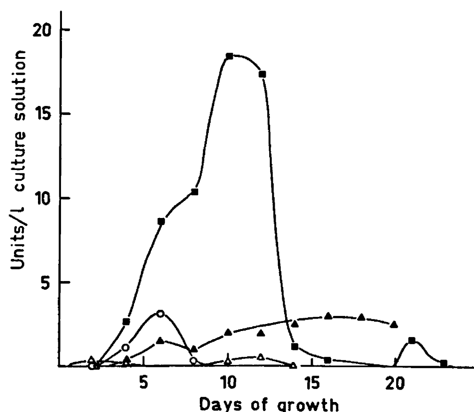


Fig. 1. Production of cellobiose dehydrogenase by *C. lignorum* grown on different carbon sources. ■ Cellulose powder. ○ Phosphoric acid swollen cellulose. ▲ Wood meal. △ Cellobiose.

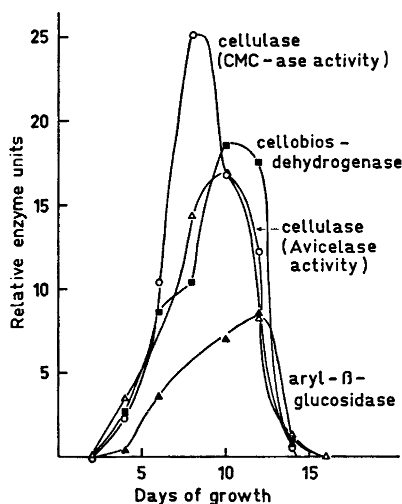


Fig. 2. Development of cellulose-degrading enzymes in a culture solution of *C. lignorum* grown on powder cellulose.

produced with cellobiose and no activity was detected with sucrose. Cultivations of *P. versicolor* gave similar results but the maximal production was about 13 units/l culture solution compared with 18 units/l culture solution for *C. lignorum*. Therefore, the latter fungus was selected for further study.

Fig. 2 shows the development of cellulose-degrading enzymes in a culture solution of *C. lignorum* grown on powder cellulose. The development of cellobiose dehydrogenase was parallel to the cellulose-degrading enzymes. Cellobiose dehydrogenase, aryl-β-glucosidase, and avicelase activity all reached maximum after about ten days. CMC-ase activity reached maximum after about eight days. Without added cellobiose the quinone-reducing activity of the culture solution was very low during the whole cultivation period.

Properties of cellobiose dehydrogenase

(a) Isolation and identification of the reaction products. Quinone reduction. Treatment of 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2) with the enzyme in the presence of cellobiose yielded one single aromatic compound. It was identified as the corresponding reduced quinone, 3-methoxy-5-*tert*-butyl-catechol-(1,2). The ultraviolet spectrum of the product of the enzymic reduction was in agreement with the spectrum of the authentic catechol. The identity of the product was further confirmed by thin-layer chromatography using authentic material as a reference compound.

To identify the products of the enzymic oxidation of cellobiose, the following two mixtures were used: (I) 1 mmol cellobiose, 3 mmol acetate buffer (pH 5.0), 25 μmol guaiacol, the phenol-oxidizing enzyme laccase and the cellobiose dehydrogenase in a reaction mixture of 18 ml. The reaction proceeded for 5 h at 25°C with shaking to supply the laccase with oxygen. (II) 50 μmol cellobiose, 50 μmol 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2), 5 mmol acetate buffer (pH 5.0), and cellobiose dehydrogenase in a total volume of 50 ml. The reaction proceeded at 25°C until all the quinone was reduced.

The reaction products were indentified by paper chromatography with glucose, cellobiose gluconic acid, and cellobionic acid as reference compounds. Spots running the same as glucose, cellobiose, and cellobionic acid were identified from the reaction mixture but no gluconic acid was found. Part of the reaction mixture (I) was also analyzed by gas-liquid chromatography. The silylated samples from the reaction mixture gave peaks with the same retention time as the synthetic cellobiono-δ-lactone and cellobionic acid. The aldonic acids are in equilibrium with

Table 1. Stoichiometry of reaction. Reduction of 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2) by cellobiose dehydrogenase. The reaction mixture contained 300 μmol acetate buffer (pH 4.5), quinone and cellobiose according to the table, and the enzyme in a total volume of 3 ml.

Quinone μmol	Cellobiose μmol	Quinone reduced μmol
2.0	2.0	2.0
2.0	1.0	1.0
2.0	0.2	0.2
2.0	0	0
2.0	200	2.0

Table 2. Stoichiometry of reaction. Acid formation from the oxidation of cellobiose by cellobiose dehydrogenase. The reaction mixture contained cellobiose and 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2) according to the table together with enzyme in 18 ml distilled water. The initial pH was adjusted to 5 with nitric acid and the acid-formation was followed at pH 5 with a pH-stat.

Quinone μmol	Cellobiose μmol	Acid formed μmol
37	75	31
18	75	16
11	75	10
0	75	0
18	0	0

their lactones at acidic pH and the working up procedure did not ascertain whether cellobionic acid or cellobiono-δ-lactone was the primary product of the reaction. Direct analysis of the assay mixture (I) during the reaction failed to detect the presence of lactones. However, the enzyme preparations used contained high lactonase activities (see later).

(b) Stoichiometry of reaction. To evaluate the stoichiometry of the reaction, two series of experiments were run, one with cellobiose and one with quinone as the limiting substrate. Table 1 shows the reduction of the quinone when cellobiose was limiting and Table 2 the production of acid from cellobiose with limiting quinone.

The acid production was followed in a pH-stat at pH 5.0. Both Table 1 and Table 2 suggest that one mol of quinone is reduced when one mol of cellobiose is oxidized.

Table 3. Oxidation of different sugars. The reaction mixture contained 300 μmol acetate buffer (pH 4.5), 1 μmol 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2), 2 μmol of sugar and the enzyme in a total reaction volume of 3 ml. The reaction was measured by following the reduction of the quinone.

Substrate	Relative rate of oxidation
Cellobiose	100
Lactose	44
Maltose	0
Sucrose	0
Xylobiose	0
Glucose	0
Galactose	0
Mannose	0
Gluconolactone	0
Arabinose	0
Xylose	0

(c) Substrate specificity. (a) Carbohydrate oxidation. The relative rates of oxidation for a number of mono- and disaccharides were investigated and the results are presented in Table 3. The enzyme appears to have high specificity for the oxidation of cellobiose. Glucose was not oxidized at all by the enzyme and oligosaccharides could not be tested, since they would rapidly be degraded to cellobiose by contaminating enzymes.

(b) Quinone reduction. The specificity of the quinone-reducing ability of the enzyme appears to be less restricted and quinones used as sub-

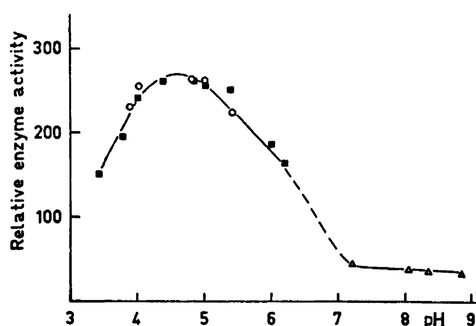


Fig. 3. Effect of pH on the activity of cellobiose dehydrogenase. The reaction mixture contained 300 μmol buffer, 1 μmol 3-methoxy-5-*tert*-butyl-benzoquinone-(1,2), 2 μmol cellobiose and the enzyme in a total volume of 3 ml. ■ Citrate buffer. ○ Acetate buffer. △ Tris buffer.

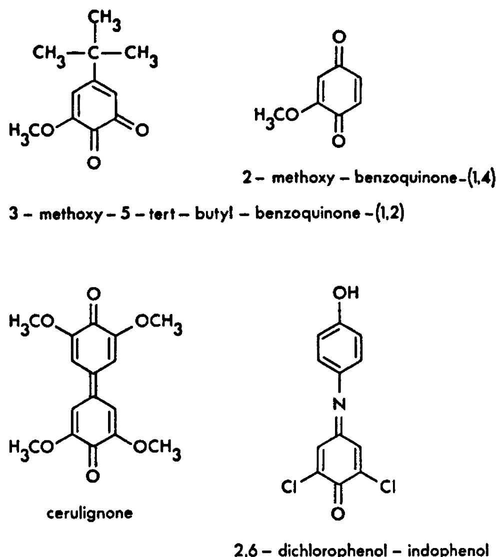


Fig. 4. Quinones which can be reduced by cellobiose dehydrogenase.

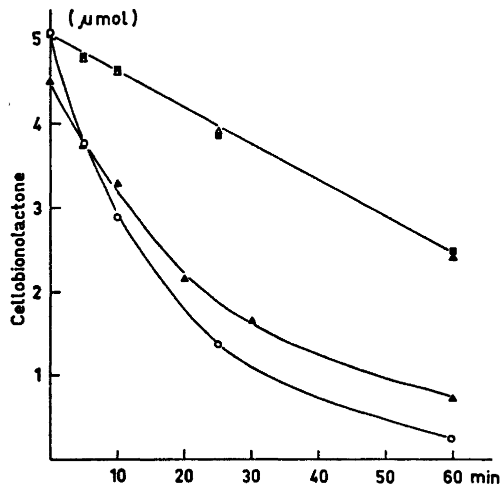


Fig. 5. Hydrolysis of cellobiono-δ-lactone. The reaction mixture contained 100 μmol of acetate buffer (pH 5.0), 5 μmol cellobiono-δ-lactone and the enzyme in a total volume of 1 ml. ○ 0.15 ml enzyme. ▲ 0.1 ml enzyme. △ Boiled enzyme control. ■ No enzyme (spontaneous hydrolysis).

strates for the enzyme are summarized in Fig. 4. The oxidoreductase can reduce both *ortho* and *para* quinones and also the artificial electron acceptor 2,6-dichlorophenolindophenol, but it cannot use molecular oxygen as hydrogen acceptor. What is more interesting from the point of view of wood degradation is that the enzyme can use reaction products of the phenol-oxidizing enzyme laccase and a suitable phenolic compound, for example lignin or lignin-degradation products, as hydrogen acceptor. As discussed in Ref. 4 the phenoxy radical formed from laccase oxidation of phenols is probably the substrate for the enzyme.

(d) pH-Optimum for the enzyme. The effect of pH on the enzymic activity was measured in citrate, acetate, and tris-buffers. The results given in Fig. 3 show that the enzyme has its optimal activity around 4.5 – 5. Phosphate buffer was somewhat inhibitory to the enzyme.

Enzymic hydrolysis of sugar lactones. It was expected that the primary product of cellobiose oxidation should be the cellobiono-δ-lactone if the enzyme reacted with the pyranoside form of cellobiose. However, all experiments to show the presence of the lactone failed. The cellobionolactone is spontaneously hydrolyzed at pH 5.0 (half life of about 58 min) but this could not explain the fact that no lactone could be detected in the reaction mixture after the oxidation of cellobiose.

To determine if enzymatic hydrolysis of lactones occurred, concentrated culture filtrates were assayed for lactonizing activity as described under methods. Fig. 5 shows the rate of hydrolysis of cellobionolactone by the culture solution. The concentrated culture solution also contained activity against glucono-δ-lactone but arabino-γ-lactone was not hydrolyzed by the enzyme solution. Thus, the first product of the cellobiose oxidation may be the cellobiono-δ-lactone which is immediately hydrolyzed by the lactonase, also secreted into the medium by the fungus.

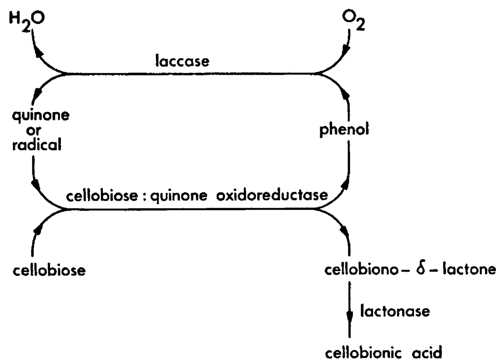
DISCUSSION

Certain bacteria, especially from the genus *Pseudomonas*,[12] can oxidize the disaccharides lactose and maltose to the corresponding aldobionic acids in the presence of a suitable hydrogen acceptor, without prior hydrolysis to monosaccharides. The enzyme responsible for the oxidation of lactose in *Pseudomonas graveolens* has been studied [13] and seems to have a similar mechanism of action as the *C. lignorum*

enzyme. However, the *P. graveolens* enzyme is intracellular, localized in the particulate fraction, while the *C. lignorum* is extracellular. Furthermore, the *P. graveolens* enzyme can oxidize several disaccharides including cellobiose, and also pentoses and hexoses, while the *C. lignorum* enzyme seems to be quite specific for cellobiose and fails to oxidize monosaccharides.

Cellobiose dehydrogenase may be involved in the enzymic degradation of both cellulose and lignin in wood. Several points suggest that carbohydrate oxidation is the important function of the enzyme. First, cellobiose dehydrogenase is induced by cellulose and cellulosic materials and is produced simultaneously with the cellulolytic enzymes in *C. lignorum*. The pH-optimum of the enzyme is in accordance with that of the cellulases from the same fungus.[14] Also, cellobiose dehydrogenase seems to have a high specificity for cellobiose which is an abundant product of cellulose degradation. It was impossible to determine if higher oligosaccharides and cellulose could also be oxidized by the enzyme, since the preparation contained cellulases. Finally, preliminary experiments indicate that cultivation of the fungus on xylan produces a similar enzyme specific for oxidation of xylan degradation products.

The function of laccase in white-rot fungi during wood degradation has been a topic of much discussion. It has been observed that some aromatic compounds stimulate the growth of white-rot fungi[15] and it was suggested that laccase participates in their electron-transport chain. The results in this paper indicate that laccase may, in fact, function as a link in an extracellular "electron-transport chain." A proposed mechanism for cellobiose dehydrogenase is given in Scheme 1.



Scheme 1.

Cellobionolactone could not be detected in the reaction but the presence of an extracellular lactonase which hydrolyzes cellobionolactone to cellobionic acid suggests that the primary product of the oxidation is the lactone. It is known that sugar lactones, especially glucono-1,5-lactone, inhibit cellulases and β-glucosidases[16] so it is probably important for the fungus to hydrolyze immediately any lactones formed. In a study of the enzymic cleavage of cellobionic acid[17] it was found that enzymes in a commercial cellulase preparation and also an enzyme extract from *Aspergillus niger* could cleave cellobionic acid to glucose and gluconic acid.

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