The cellobiose-oxidizing enzymes CBQ and CbO as related to lignin and cellulose degradation – a review

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Abstract: In this review properties of cellobiose:quinone oxidoreductase (CBQ) and cellobiose oxidase (CbO) are presented and their possible involvement in lignin and cellulose degradation is discussed. Although these enzymes are produced by many different fungi, their importance for wood-degrading fungi is the topic here. CBQ is a FAD enzyme, while CbO also contains a heme group of the cytochrome b type. Protease activity is reported to convert CbO to CBQ. During oxidation of cellobiose (emanating from cellulose) to cellobiono-1,5-lactone, both enzymes reduce quinones produced by laccase and peroxidase during lignin degradation to the corresponding phenols. Many phenoxy and cation radicals are also reduced. Quinone reduction is more rapid than oxygen reduction, although oxygen is slowly reduced to superoxide and/or hydrogen peroxide. Thus, a more appropriate name for CbO is cellobiose dehydrogenase. CbO also reduces Fe(III) and together with hydrogen peroxide produced by the enzyme Fenton's reagent may be formed, resulting in hydroxyl radical production. This radical can degrade both lignin and cellulose, possibly indicating that cellobiose oxidase has a central role in degradation of wood by wood-degrading fungi.

Key words: Cellobiose:quinone oxidoreductase; Cellobiose oxidase; Cellobiose dehydrogenase; Lignin degradation; Cellulose degradation; Fenton's reagent; Hydroxyl radical; Peroxidases; Laccase

Introduction

The cellobiose-oxidizing enzymes cellobiose:quinone oxidoreductase (CBQ) and cellobiose oxidase (CbO) have gained an increased interest, due to new results related to their importance for both lignin and cellulose degradation. During the recent FEMS Symposium on Lignin Biodegradation and Transformation in Lisbon in April 1993 [1], one half session was devoted to recent findings about these enzymes. Other sessions dealt with genetics and physiology of white-rot fungi, with electron microscopy and with xylanase-assisted bleaching of paper pulp (see also other articles in this FEMS issue). In the present review, both old and new results regarding cellobiose-oxidizing enzymes will be discussed.

Cellobiose:quinone oxidoreductase (CBQ)

CBQ was discovered by Westermark and Eriksson in 1974 [2,64] during cultivation of Trametes versicolor on Kraft lignin-cellulose agar.
Fig. 1. Darkening and bleaching of kraft lignin-cellulose agar plates by *Trametes versicolor* [2].

plates to simulate natural conditions. After growth for 5 days, dark zones due to laccase activity (zone 1 in Fig. 1) and bleached zones (zone 2) due to quinone reduction were obtained. Little bleaching was obtained with glucose instead of cellulose. Cellobiose as carbon source gave an intermediary bleaching effect. From zone 2, having the strongest bleaching, an enzyme mixture was extracted and tested for guaiacol oxidation due to laccase activity. When cellobiose was added the colour disappeared, indicating also the presence of a quinone-reducing activity in this extract. The partially purified quinone-reducing enzyme, now called cellobiose : quinone oxidoreductase, was used for further studies.

Influence of CBQ plus cellobiose on consumption of guaiacol by laccase was followed as shown in Fig. 2A. With only laccase (a) almost all added guaiacol (25 µmol) disappeared due to polymerization. With laccase plus CBQ, less than 15% of the guaiacol polymerized (b). Oxygen consumption was measured as shown in Fig. 2. As expected, laccase alone consumed oxygen (a) and this consumption decreased as the guaiacol concentration decreased. With laccase plus CBQ, however, the oxygen consumption proceeded at the same rate for more than 60 min (b). This indicates that the phenoxy radicals produced by laccase from guaiacol are continuously reduced back to the phenol by active CBQ. So the laccase enzyme is active all the time trying to produce phenoxy radicals and is consuming oxygen.

**Properties of CBQ**

Westemermark and Eriksson [3] purified CBQ from cellulose cultures of *Phanerochaete chrysosporium* and showed that CBQ is an FAD enzyme with a *M* value of 58 000 and a pH optimum of 4.5–5. It reduces a number of quinones, among them those shown in Fig. 3. Later, yet another quinone, 3,5-di-tert-butyl-o-benzoquinone, was used in CBQ assays [4]. CBQ oxidizes cellobiose to cellobiono-1,5-lactone (cellobiono-δ-lactone) during reduction of quinones. Morpeth and Jones [5] suggested that both superoxide radical and hydrogen peroxide are produced; the latter compound was shown as oxygen evolution using catalase and an oxygen electrode. Cellobiose with the apparent Michaelis constant *K*ₘ = 45 µM is a better substrate than lactose with a *K*ₘ of about 700 µM [5]. Cellopentaose was oxidized by the enzyme but not cellulose of DP 150 which is insoluble in water (see also below). The electron acceptors 2,6-dichlorophenolindophenol (DCIP)

![Diagram](https://example.com/diagram.png)

Fig. 2. (A) Influence of CBQ+cellobiose on consumption of guaiacol by laccase. (a) Control (laccase alone), (b) Laccase + CBQ. (B) Consumption of oxygen by laccase in the presence of CBQ. (a) Control (laccase alone), (b) Laccase + CBQ. Enzymes were from *T. versicolor* (laccase) and *P. chrysosporium* (CBQ) [2].
and butylbenzoquinone gave $K_m$ values of about 15 and 102–130, respectively. $V_{max}$ was, however, higher for benzoquinone than for DCIP.

CBQ (also called cellobiose dehydrogenase) was found in 25 white-rot fungi as detected by Ander and Eriksson [4] in 1977 during cultivation on cellulose in high nitrogen liquid media. Since the activity was measured using dibutylbenzoquinone, the assay does not distinguish between CBQ and cellobiose oxidase (see below). For all these fungi there was a stronger bleaching of kraft lignin agar (with 17 mM N) if cellulose was present. This bleaching is certainly due to both CBQ and cellobiose oxidase activity and to lignin degradation. The activities of these two enzymes together in the same cultures is almost totally unknown. Bleaching of lignin agar plates with low nitrogen media has not been tested.

A cellobiose dehydrogenase with a $M_r$ of 70,000 has also been isolated from the white-rot fungus *Fomes annosus* [65]. The enzyme tends to form aggregates with a $M_r$ of 300,000. Interestingly, the best activity was obtained with the combination cellobiose plus lignosulfonate. It is not known whether the enzyme preparation was a mixture of both CBQ and CbO. During many years there was controversy regarding the possible effects of CBQ on phenoxy radicals and whether the enzyme could inhibit peroxidase activity. Thus Odier et al. [6] in 1988 found that their CBQ had no influence on the phenoxy radical of acetosyringone or on polymerization of guaiacol and synthetic lignin by lignin peroxidase (ligninase). The reason for this is not known (see below). Samejima and Eriksson [7] reported in 1992 that CBQ could indeed reduce the phenoxy radical of acetosyringone.

In 1990, Ander et al. [8] found that active CBQ inhibited vanillic acid decarboxylation by laccase, ligninase, manganese peroxidase and horseradish peroxidase (Fig. 4). Veratryl alcohol oxidation by ligninase was also inhibited by CBQ – the inhibit-
ing effect being stronger at pH 4 than at pH 3.0 due to differences in pH optima of the enzymes (Fig. 5). Similarly, oxidation of 1,2,4,5-tetramethoxybenzene by ligninase as measured at 450 nm was stronger at pH 3 and less inhibited by active CBQ at this pH [8]. All these results indicate that CBQ is mainly interacting with quinones and radicals (other electron acceptors not considered now) produced by laccase and the peroxidases and not with the protein itself (see below). This idea is supported by results obtained with hemoglobin and vanillic acid decarboxylation reported by Ander and Pettersson in Kyoto in 1992 [9]. During investigations with hemoglobin (Hb) for use as a pulp bleaching agent it was found that Hb could act as an oxidase both in the presence of hydrogen peroxide or sodium chlorate as oxidant [10]. This oxidase activity, measured as vanillic acid decarboxylation, was again strongly inhibited by active CBQ (Table 1) [9]. The decarboxylation assay, described in [11] is not dependent on a stable quinone colour since evolved $^{12}$CO$_2$ is immediately trapped in NaOH.

**Cellulose oxidase (CbO)**

In parallel with all these investigations, another sugar oxidase called cellulose oxidase had been isolated and purified from cellulose cultures of *P. chrysosporum* (earlier *Sporotrichum pulverulentum*) [12–14]. This enzyme and/or CBQ is also produced by non-ligninolytic cellulolytic fungi like *Monilia* [15] and *Sporotrichum (Chrysosporium) thermophile* [16,17]. From *Sclerotium rolfsii* an enzyme with catalytic properties rather similar to CBQ was purified [18]. FAD or heme was, however, not detected in that enzyme, making the results doubtful. CbO has also been found in the brown-rot fungus *Coniophora puteana* [19]. This brown-rot fungus is, however, not representative for the brown-rotters since it produces both endo- and exo-glucanases (exo-cellobiohydrolases). I did not find CBQ activity measured with butylbenzoquinone in six brown-rot fungi cultivated on wood meal, on cellulose, and on kraft lignin-cellulose agar plates [20]. If brown-rot fungi demethoxylate lignin there should, however, be a need for reducing the quinones thus formed. More research is needed here (see below).

Cellulose oxidase (CbO) and CBQ have many properties in common, the main one being the ability to reduce quinones and oxidize cellobiose and related oligosaccharides. Any oxygen atom is probably not incorporated in the cellobiose molecule but rather reduced directly to superoxide and/or hydrogen peroxide (Henriksson, personal communication). This is in contrast to a suggestion made by Ayers and Eriksson [21]. The oxidation of cellobiose as well as reduction of FAD, oxygen and other compounds are shown in Fig. 6.

CbO is a flavohemoprotein with $M_r$ 90,000 as determined by SDS/PAGE [12,13,22,23]. A molecular mass of 89,170 ± 440 was found using UV-laser-induced desorption mass spectrometry [24]. The heme group is of the cytochrome b type [13,14] with Soret bands of the oxidized form at 561, 530 and 419–421 nm. Reduced CbO has
Table 1
Influence of CBQ on decarboxylation of vanillic acid by hemoglobin (Hb) plus sodium chlorate

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Decarboxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin only</td>
<td>8.75 ± 0.20</td>
</tr>
<tr>
<td>Hb + cellobiose</td>
<td>9.86 ± 0.51</td>
</tr>
<tr>
<td>Hb + Na-chlorate</td>
<td>25.55 ± 0.31</td>
</tr>
<tr>
<td>Hb + Na-chlorate + Cb</td>
<td>24.21 ± 0.10</td>
</tr>
<tr>
<td>Hb + Na-chlorate + CBQ</td>
<td>17.45 ± 0.08</td>
</tr>
<tr>
<td>Hb + Na-chlorate + CBQ + Cb</td>
<td>0.19 ± 0.015</td>
</tr>
<tr>
<td>Hb + CBQ + Cb</td>
<td>0.14 ± 0.00</td>
</tr>
</tbody>
</table>

Hb 30 mg l⁻¹, 0.465 μM; Na-chlorate 2.82 mM; cellobiose (Cb) 0.40 mM. Acetate buffer 10 mM, pH 4.0-4.5. From ref. [9].

peaks at 562, 532 and 427–429 nm [12,13]. Cytochrome b’s are characterized by having the porphyrin ring non-covalently bound to the protein part, which indicates that it is easy to remove the heme group. This is also done by the fungus and it has been shown that protease activity (papain or V8 proteinase) can convert CbO to CBQ thus releasing a heme containing fragment [17,22,25]. Proteases are known to be produced by Phanerochaete in cellulose cultures [26]. The sum of the molecular masses for the heme fragment plus that of CBQ is very similar to that of CbO [22,25]. The heme group of CbO is probably used for storage and delivery of electrons from a two-electron donor such as cellobiose (via FADH₂) to one-electron acceptors such as radicals, cytochrome c, ferricyanide and oxygen (giving superoxide) (Fig. 6). How electrons are transferred between heme and flavin redox centres, perhaps in different CbO molecules, needs further investigation [14,27,28]. Cox et al. [29] found that the b-type heme ligation of CbO are histidine and methionine. This again suggests that the heme has an electron transfer function.

Both CBQ and CbO produce hydrogen peroxide and the inability of some researchers [6,12,17] to detect peroxide production is probably due to quinone reduction in coupled horseradish/o-dianisidine or horseradish/aminoantipyrine as-
Fig. 7. Comparison of cytochrome c-reducing ability of CbO and CBQ at various pH values [7].

This idea was put forward by the author already in January 1990 in an application to the Swedish Natural Science Research Council. Disturbance of peroxidase-based assays by active CbO has now been found by Henriksson et al. [27] using ABTS, and by Bao et al. [30,31] using o-dianisidine and guaiacol. However, the reduction of oxygen to produce hydrogen peroxide is a rather slow process [28,32] with a $K_m > 120 \mu M$ for oxygen. This makes $H_2O_2$ detection somewhat difficult; CbO may even degrade peroxide [27].

Reduction of quinones is much faster and both CBQ and CbO reduce a great number of quinones

Table 2
Quinones and radicals reduced by CBQ/CbO

<table>
<thead>
<tr>
<th>Quinones and radicals reduced by CBQ/CbO</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Methoxy-5-tert-butyl-benzoquinone (1.2)</td>
</tr>
<tr>
<td>2-Methoxy-benzoquinone (1.4)</td>
</tr>
<tr>
<td>Cerulignonone</td>
</tr>
<tr>
<td>2,6-Dichlorophenolindophenol (DCIP)</td>
</tr>
<tr>
<td>3,5-Di-tert-butyl-o-benzoquinone</td>
</tr>
<tr>
<td>Tetramethoxy-azo-p-methylenquinone (from syringaldazine)</td>
</tr>
<tr>
<td>Guaiacol phenoxy radical and the quinone</td>
</tr>
<tr>
<td>Vanillic acid phenoxy radical</td>
</tr>
<tr>
<td>Acetosyringone phenoxy radical</td>
</tr>
<tr>
<td>Veratryl alcohol cation radical</td>
</tr>
<tr>
<td>ABTS cation radical</td>
</tr>
<tr>
<td>1,2,4,5-Tetramethoxy benzene cation radical</td>
</tr>
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</table>

Fig. 8. Effect of cellobiose oxidase on syringaldazine (0.03 mM) oxidation by manganese peroxidase (MnP) in 20 mM Na-tartrate pH 4.5. (A) MnP (4050 nkat l$^{-1}$) + 0.1 mM Mn(II) + 1.0 mM cellobiose. The reaction was started by adding 50 μl H$_2$O$_2$ (final conc. 0.05 mM). All enzyme activities were the final. (B) 50 μl CbO (512 nkat l$^{-1}$) was added after 43 s, giving the break in the curve. (C) The same as in (B), but cellobiose not present (Ander and Sena-Martins, unpublished results).
and radicals. Most of these are shown in Table 2. The formulae of the first four of them were shown in Fig. 3. DCIP and 3,5-di-tert-butylbenzoquinone are now commonly used to assay the sum of CBQ and CbO activity. According to Samejima and Eriksson [7,33] cytochrome c could be used in a specific assay for CbO, although it was reported [5,13] that both CbO and CBQ reduced cytochrome c at pH 6.0. Fig. 7 shows that pH 4 is a very convenient pH to do this and the first report on simultaneous determination of CBQ and CbO in the same cultures was given by Costa-Ferreira et al. [34]. Since the superoxide radical is known to reduce cytochrome c [35], this could indicate that CbO is producing superoxide for this purpose. However, oxygen reduction is very slow and the 5000 or 9000 units of superoxide dismutase tested in this connection [5,13], can also be used as an electron acceptor. Bao et al. [31] did not observe any decrease in the rate of cytochrome c reduction by CbO in the presence of SOD. Thus cytochrome c is more probably reduced by FADH₂ in cooperation with the heme group of CbO.

Reduction of Fe(III) is more rapid than oxygen reduction. Thus CbO can be called a Fe(III) reductase [32], while Eriksson prefers to call it a cytochrome c reductase [7]. The $K_m$ for Fe(III) acetate was 34 ± 12 µM while that for cytochrome c was 13 µM, indicating that cytochrome c is slightly easier to reduce than Fe(III). Even Mn(III) is reduced by CbO as reported by Bao et al. [30,31]. Here the possible connection with Mn-peroxidase is an interesting question. Since CbO is transferring electrons much more slowly to O₂ than to other electron acceptors, CbO should be called cellobiose dehydrogenase rather than cellobiose oxidase [31]. In Bao’s hands, the $K_m$ for cytochrome c was as low as 1.2 µM followed by DCIP 3.6 µM, ferricyanide 5.2 µM, dibutylybenzoquinone 12 µM and Mn(III)-malonate 150 µM.

The quinone formed from syringaldazine by Mn-peroxidase is also reduced by CbO as shown in Fig. 8. A strong effect was obtained with active CbO, while inactive CbO (without cellobiose) had no effect (Ander and Sena-Martins, unpublished results). Reduction of guaiacol quinones and vanillic acid phenoxy radicals have been reported [2,8,30,31], like reduction of the 1,2,4,5-tetramethoxybenzene cation radical by both CBQ and CbO [7,8,27,36]. CbO reduced the cation radical better than CBQ [7,27].

**Interaction with peroxidases**

Inhibition of ligninase-catalyzed oxidation of veratryl alcohol by CBQ has been shown [8] and the redox interaction between ligninase and CBQ/CbO was further studied by Eriksson’s group in Georgia [7,36]. By measuring the FAD absorbance at 457 nm they found that CBQ-FADH₂ was oxidized by ligninase (LiP) + H₂O₂ + veratryl alcohol, and that this oxidation was stronger than by only ligninase + H₂O₂. The inhibition of veratryl alcohol oxidation by the cellobiose oxidases is non-competitive and the redox cycles of LiP and CBQ are connected through veratryl alcohol as shown in Fig. 9. Thus veratryl alcohol can be considered to be a mediator in oxidation of reduced CBQ.

In what way the above results are connected with results obtained by Ander et al. [37] is not known at present. We found (Fig. 10) that ligninase compound II (LiP II) obtained by adding about six equivalents of H₂O₂, was reduced back to native LiP much more rapidly if active cellobiose oxidase was also present. When only peroxide was present, return to native LiP started 11 min after peroxide addition as shown by the decrease in absorbance at 420 nm. With the three different CbO concentrations tested, reversion started already after 2–4 min in a CbO concentration-dependent manner (Fig. 10). If, however, veratryl alcohol was added to LiP II instead of...
CbO, reduction back to native LiP took place immediately.

Initially, these studies were started to investigate if the cellobiose-oxidizing enzymes could act not only on quinones and radicals but also directly on the peroxidase protein itself. A similar idea has been mentioned earlier [38].

Reduction of MnP compound II (MnP II) with and without the presence of active CbO gave similar results as with LiP II [37]. Fig. 11A shows the change of MnP II absorbance at 420 nm, 19 min after addition of 11 equivalents of peroxide. During these first 19 min there was no change in the spectrum but a slow decrease in absorbance appeared 50 s after start of the time scan giving a total time of 19 min 50 s before conversion to native MnP started. The change was finished 400 s later. In the presence of CbO + cellobiose, however, the disappearance of MnP II measured at 420 nm started already after 150 s and was completed after another 50 s (B). The increase in absorbance at 407 nm, indicative of formation of native MnP is shown in (C). Again the presence of active CbO gave a rapid reduction of MnP II to native MnP after 150 s. MnP II-reducing activities were 0.15, 2.06 and 5.2 nkat l⁻¹, respectively.

Time scans like those in Fig. 11 were used to calculate MnP II-reducing activity of cellobiose oxidase. The result is shown in Fig. 12. A good correlation with CbO activity was obtained. Addition of Mn²⁺ instead of CbO resulted in an immediate reduction of MnP II.

During these experiments also reduction of cytochrome c was measured at pH 4.5. Again a linear dependence was obtained for the different CbO concentrations (Fig. 13). Horseradish peroxidase Cpd II was also reduced by active CbO as found by us [37] and by others [27]. Our results are summarized in Table 3. It is seen that cytochrome c as well as compound II of MnP, LiP and HRP were all reduced by active CbO, that is they were all electron acceptors. Cytochrome c was much more easily reduced than the peroxidases; for MnP II the difference was 40 times.

It seems as if very high CbO activity could give so much hydrogen peroxide that a decreased rate of compound II reduction was obtained (see MnP and LiP). Peroxide was added to test this possibility. If compound II is present, the peroxidase will still be active producing phenoxy radicals and CbO will be less effective. This could explain the results of Odier et al. [6]. Formation of more than 0.59 mM cellobionolactone would also inhibit CbO activity [31].

In summary, we found [37] that active CbO did not repress formation or reduction of compound 430

![Graph](https://example.com/graph.png)

**Fig. 10.** Influence of CbO + cellobiose on reduction of H₂O₂-treated ligninase, measured as change in wavelength of LiP compound II (420 nm) to native LiP (409 nm). 0 µl CbO means spontaneous reversion of LiP II to native LiP (Ander and Sena-Martins, unpublished results).
Fig. 11. Time scan for reduction of MnP compound II (420 nm) obtained by adding eight equivalents of H₂O₂ to native MnP (0.5 μM). (A) No CbO, time scan started 19 min after peroxide addition. The arrows show start and end of reduction. (B) 75 μl CbO (2288 nkat l⁻¹). Time scan started directly after H₂O₂ addition. (C) Reduction of MnP II measured as increase in absorbance at 407 nm with 75 μl CbO as in (B). Conditions: Na-tartrate 20 mM pH 4.5 containing 0.5 mM cellobiose (Ander and Sena-Martins, unpublished results).

I. Thus, compound I did not accept electrons from CbO/FADH₂ and Cpd II formation was unaffected by CbO. Mn²⁺/veratryl alcohol reduced compound II of the peroxidases much more rapidly than active CbO. This indicates that in the presence of peroxidase substrates, MnP and LiP can complete their catalytic cycles and function normally without interference from cellobiose oxidase. The inhibition of peroxidase ac-

Fig. 12. MnP II-reducing activity of CbO plus cellobiose measured as increase in absorbance at 407 nm. The reaction mixture contained 0.4 μM MnP, 0.25 mM cellobiose and CbO in 20 mM Na-tartrate pH 4.5 and was initiated by adding 2.5 equivalents of H₂O₂ (Ander and Sena-Martins, unpublished results).

Fig. 13. Reduction of cytochrome c by CbO plus cellobiose measured as increase in absorbance at 550 nm. The reaction mixtures contained 1.08 μM (25 μl) cytochrome c, 0.25 mM cellobiose and CbO in 20 mM Na-tartrate pH 4.5 and was initiated by adding 55–666 nkat l⁻¹ of CbO (Ander and Sena-Martins, unpublished results).
Degradation of lignin

Cellobiose-oxidizing enzymes may be necessary to reduce toxic quinones like methoxyquinones (formed by peroxidases and laccase) to the corresponding phenols. During this reduction also phenoxy radicals are reduced thereby decreasing repolymerization which is an unwanted effect of peroxidases and laccase. Although not proven, this is supposed to increase lignin degradation [39]. Indeed, Fig. 14 shows the first indication of increased lignin degradation in the presence of LiP and CBQ [8]. It shows HPLC of kraft lignin and Fig. 14A is untreated lignin, Fig. 14B is kraft lignin + crude LiP + H₂O₂ giving polymerization. Fig. 14C shows LiP + H₂O₂ + CBQ + cellobiose giving smaller peaks and a new small peak eluting at about 12 min. Since the relative peak areas in A, B and C is 100 : 99 : 68, a substantial (32%) loss of 280 nm absorbing material was obtained in the presence of active LiP and CBQ as in C. This could be due to aromatic ring-cleavage or other changes in the aromatic compounds. Confirmation of this interesting result, however, is needed.

Table 3
Reduction rate of cytochrome c and peroxide-treated MnP, LiP and HRP by CbO plus cellobiose in 20 mM sodium tartrate, pH 4.5

<table>
<thead>
<tr>
<th></th>
<th>CbO Reducing activity (nkat l⁻¹)</th>
<th>Reducing activity (nkat l⁻¹)</th>
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<tbody>
<tr>
<td>Cytochrome c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>55</td>
<td>14.7</td>
</tr>
<tr>
<td>111</td>
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<td>21.4</td>
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<td>222</td>
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<td>76.9</td>
</tr>
<tr>
<td>666</td>
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<td>111</td>
</tr>
<tr>
<td>Manganese peroxidase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>663</td>
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<td>1024</td>
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<tr>
<td>3315</td>
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<td>11.9</td>
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<td>Horseradish peroxidase</td>
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<td>25</td>
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<td>5.10</td>
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</tbody>
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From ref. [37].

Fig. 14. Treatment of Indulin AT kraft lignin with ligninase with and without the presence of CBQ + cellobiose followed by gel chromatography on TSK 3000 PW in acetate buffer. (A) Lignin alone. (B) Lignin plus fungal extract containing ligninase (H₂O₂; 0.24 mM). (C) Lignin plus ligninase, H₂O₂, CBQ and cellobiose. (D) Fungal crude extract alone. (E) CBQ alone [8].

Similar results were obtained in a related HPLC system [8].

In the presence of the growing fungus it may be that oxidation and reduction takes place sequentially: (i) oxidation by LiP, MnP, laccase; (ii) reduction with quinone reductases (other reductases produced in glucose cultures); (iii) oxidation; and (iv) reduction.
Schoemaker [40] said: “Rapid metabolism of quinone type intermediates represents one possible way of shifting the polymerization–depolymerization equilibrium, induced by lignin peroxidase and phenoloxidases, towards degradation”.

The inhibition of MnP- or LiP-catalyzed polymerization of phenols or lignin degradation products via phenoxy radical reduction may be of importance both for cellulose and lignin biodegradation. Both CBQ and CbO binds strongly to cellulose [22,41,42] and their radical-reducing activity may decrease repolymerization and precipitation of lignin-like polymers on the cellulose surface, thereby facilitating cellulose degradation [27].

Degradation of cellulose – recent hydroxyl radical studies

Cellulbiose oxidase is very interesting for cellulose degradation studies and it has been recently shown by Kremer and Wood [32,43] that CbO can reduce Fe(III) to Fe(II), as was already indicated for CBQ by Coudray et al. in 1982 [16]. Together with hydrogen peroxide also produced by CbO, Fenton’s reagent will be formed to get hydroxyl radicals which can attack both lignin and cellulose [44–48]:

\[
\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \cdot \text{OH} + \text{OH}^-
\]

Earlier studies were often done in acetate buffers and Fe(III)acetate plus CbO/Cb was found by Kremer and Wood [32,43,49] to produce ·OH under certain conditions. As a test for hydroxyl radical production, they used hydroxylation of salicylic acid to 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid. Another test for this radical was conversion of deoxynibose to malonodialdehyde. Wood [49] suggested that an autoxidizable compound such as oxalic acid could be used in CbO experiments. This would produce hydrogen peroxide separated from Fe(III) reduction, in order not to damage CbO by the hydroxyl radical.

A Fenton type system for degradation of carboxymethyl cellulose (CMC) was also tested by Henriksson et al. [41]. They found that a redox system containing CbO, cellulbiose, ferricyanide and hydrogen peroxide degraded CMC as measured by viscometry. The two-electron acceptor 3,5-di-tert-butylbenzoquinone and the radical scavenger dimethylsulfoxide inhibited this degradation, suggesting that hydroxyl radicals are responsible for degradation. Since the FAD fragment from CbO had less activity than the original CbO it was proposed that the heme group stimulated one-electron reduction [27,41].

In our laboratory, we have started to investigate ·OH production by CbO using benzoic acid decarboxylation [50]. We will continue to study degradation of radioactive synthetic lignin (DHP) and cellulbiose by CbO (Henriksson et al., submitted to Appl. Microbiol. Biotechnol.).

Other cellulose degradation studies

Degradation of cotton and views on cellulose degradation by brown-rot and white-rot fungi

Already in 1965, Halliwell [44] reported that cotton fibres were degraded by a mixture of hydrogen peroxide and ferrous salts. However, up to 0.4% of \( \text{H}_2\text{O}_2 \) and a long incubation time, 7 days, was used. These studies were repeated and confirmed by Koenigs [45] using less peroxide and shorter incubation times. It was also mentioned by Koenigs, taking data from Cowling [51], that during brown-rot decay of cellulose in sweetgum wood, there was a very rapid decrease in the degree of polymerization of residual cellulose (Fig. 15). This is in opposite to white-rot degraded sweetgum, where the residual cellulose had a relatively small change in the DP-value. Hydroxyl radicals are supposed to be involved in cellulose degradation by brown-rot fungi [45,51,52], but whether CbO is involved is not known. White-rot fungi do not appear to use the same mechanism as brown-rot fungi, if only DP of the cellulose is considered. Oxalic acid is produced by both brown-rot and white-rot fungi but its role in wood biodegradation is not clear [53].

The first indication for an enzyme activity important for oxidative cellulose degradation was reported by Eriksson et al. [54] in 1974. They found that in 68 h, a concentrated but unfrac-
White rot caused by *Poria placenta* (earlier *Poria monticola*) and by the white-rot fungus *Trametes* (*Coriolus*) *versicolor*. From refs. [51,52].

Fig. 15. Depolymerization of cellulose in wood by the brown fungus *Poria placenta* (earlier *Poria monticola*) and by the white-rot fungus *Trametes* (*Coriolus*) *versicolor*. From refs. [51,52].

Continuous monitoring of cellulase activity

Continuous monitoring of cellulase activity against microcrystalline cellulose (Avicel) was studied by Kremer and Wood [55,56] using cellobiose oxidase and by Kelleher et al. [57] using CBQ to monitor saccharification. With Avicel, CBQ caused oxygen consumption, suggesting that this insoluble cellulose is a substrate for CBQ [55]. Canevascini [58] in 1985 used CBQ in a cellulase assay. He used ferricyanide as an electron acceptor and different celluloses (amorphous cellulose, filter paper, Avicel, cotton and CMC). Bao et al. [59] recently reported that CBQ enhanced degradation of crystalline cellulose (SigmaCell type 50) by *Trichoderma* cellulase. It was suggested that low concentrations of CBQ was better than high concentrations, giving inhibition of cellulase activity due to hydrogen peroxide.

Use of cellobiose oxidase as a biosensor

Research is conducted at the University of Uppsala to use CBQ cross-linked in a redox polymer matrix on a rotating disk electrode as a biosensor [23]. Under certain conditions, cellobiose, lactose, maltose, cellobiose up to cellulose can be determined by this biosensor.

Fig. 16. Interaction of CBQ and CBQ with quinones and radicals produced by Mn-peroxidase, ligninase and laccase during cellulose and lignin biodegradation. CBQ and CBQ slowly form hydrogen peroxide and reduces Fe(III) to Fe(II); this combination can lead to hydroxyl radical formation if present simultaneously. Peroxide may also be formed by other sugar-oxidizing enzymes or by certain autoxidizable compounds.
In combination with glucose oxidase it is also possible to determine glucose [60].

**Final statement**

Although under laboratory conditions, cellulose and lignin biodegradation usually takes place during different culture conditions [20,34,39,61], it is common in Nature that cellulose and lignin are degraded simultaneously [62,63]. This suggests that cellulases, hemicellulases, CbO, CBQ and the different peroxidases (and laccase) may be produced relatively close to each other during wood degradation. Sugar oxidases like glucose oxidases will also be present partly to produce hydrogen peroxide. However, the combination and spatial distribution of the fungal enzyme machinery produced during wood degradation is very little known at present. Studies of the interaction of the above-mentioned oxidases, as well as electron microscopy of white-rotted wood, would certainly increase the knowledge on how celllobiose-oxidizing enzymes can act as a connection between lignin and cellulose degradation in Nature.

Finally, an updated version of the old CBQ/laccase cycle is shown (Fig. 16). Here most of the new results, including CbO and hydroxyl radical formation by Fe(II) + H₂O₂, are incorporated.

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