Cellobiose : quinone oxidoreductase does not prevent oxidative coupling of phenols or polymerisation of lignin by ligninase

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ABSTRACT

Phanerochaete chrysosporium ligninase/ H2O2 oxidized the lignin model compound acetosyringone to a phenoxy radical which was identified by ESR spectroscopy. Cellobiose : quinone oxidoreductase/cellobiose, previously suggested as a phenoxy radical scavenging system, was without effect on the radical. In accord with its oxidation of phenols to phenoxy radicals, ligninase/H2O2 polymerised guaiacol and it increased the molecular size of a synthetic lignin. The CBQase failed to prevent these polymerization reactions. Ligninase/H2O2 converted the lignin β-0-4 model dimer 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxy-[U-14C] phenoxy)-propane-1,3-diol (II) in part into higher molecular weight 14C-labelled products. CBQase/cellobiose did not affect the transformation of II by ligninase into polymeric material. It is concluded that CBQase is not involved in the prevention of phenol polymerization by ligninase. Since intact cultures of P. chrysosporium depolymerize synthetic lignin, an undescribed biochemical system must be responsible for the prevention of phenol polymerization during lignin biodegradation.

INTRODUCTION

A major degradative reaction following formation of aryl cation radicals catalysed by the ligninase (lignin peroxydase) from Phanerochaete chrysosporium in lignin model compounds degradation is C5=C6 cleavage in the propyl side-chain (Tien and Kirk, 1983). Studies with model representing the major substructure in lignin -- the arylglycerol-β-aryl ether type -- have shown that C5-C6 cleavage liberates a phenolic product from the ether-linked aryl moiety (Tien and Kirk, 1984). Ligninase has been shown to dimerize 4-t-butylguaiacol, indicating that it acts as a phenol-oxidizing enzyme as laccase or horseradish peroxidase (Tien and Kirk, 1984). Thus the phenolic degradation products would be expected to be repolymerized by
ligninase. In accord, Hammerli et al. (1986) showed that ligninase polymersizes lignin.

Intact cultures of P. chrysosporium, however, causes a rapid and extensive depolymerization of lignin (see Kirk and Rarrell, 1987). The fungus therefore possesses a biochemical mechanism to prevent repolymerization of phenols by ligninase. A candidate enzyme for this function is the extracellular flavoprotein cellobiose : quinone oxidoreductase (CBQase), discovered by Yestermark and Eriksson (1974) in Sporotrichum pulverulentum (now P. chrysosporium). This enzyme catalyses the oxidation of cellobiose to cellobiono-5-lactone with transfer of the electron non-specifically to quinones. Yestermark and Eriksson suggested that CBQase connects cellulose degradation with lignin degradation -- in the latter case by preventing polymerisation of suspected quinonoid products. They further suggested that CBQase might reduce phenoxy radicals as well as quinones but presented no evidence of phenoxy radical reduction. Nethertheless, cultures of P. chrysosporium in which CBQase is active do not polymerize lignin to the same extent as those without CBQase (grown with glucose) (see review Kirk and Farrell, 1987).

In this study, the effect of CBQase on ligninase polymerisation of guaiacol and lignin was investigated; the formation of a phenoxy radical from a phenol by ligninase was studied, and the action of CBQase on radical stability is determined.

RESULTS

Treatment of 14C guaiacol (I), with ligninase/H2O2 polymerized it, as determined by size exclusion chromatography (Fig. 1a). Repetition of the experiment in time gave the same results.

Incubation of a synthetic lignin with ligninase/H2O2 caused an increase in size, as shown by size exclusion chromatography; no depolymerization was observed (Fig. 1b). As with guaiacol, inclusion of CBQase + cellobiose was without effect. The experiment was repeated with the same results.

Incubation of the labelled β-O-4 dimer II with ligninase/H2O2 resulted in partial conversion of the labeled moiety to both larger and smaller products than the starting material (Fig. 1c); some material excluded from the gel was produced. Again, repetition of the experiment gave the same results.
ESR spectroscopy showed that the lignin-related phenol acetosyringone is oxidized to a phenoxy radical by ligninase H8 (Fig. 2). The same spectrum was obtained (data not shown) and earlier by Caldwell and Steelink (1969) on incubation of acetosyringone with horseradish peroxidase (HRP), showing that ligninase acts as HRP and other phenol-oxidizing enzymes by oxidizing the substrate phenol by one electron.
Figure 2: ESR spectrum of the phenoxy radical produced on oxidation of acetosyringone by ligninase/H$_2$O$_2$; reaction mixture contained 0.1 M tartrate buffer pH 4.0, 0.38 nkat ligninase, 1.2 mM acetosyringone and 0.05 mM H$_2$O$_2$ in a total volume of 1 ml. Stability of the radical at pH 4.0 was readily demonstrated by monitoring the time course of phenoxy radical formation from acetosyringone (Fig. 3a). Inclusion of cellobiose + CBQase, in large activity excess, was without effect on the intensity of the signal (Fig. 3b).

Figure 3: Time course radical formation from acetosyringone in ligninase system. (a) acetosyringone + ligninase/H$_2$O$_2$; same as (a) but containing CBQase and (8.5 nkat) t cellobiose (7.3 µM); for conditions see legend of figure 2;
Evidence is presented here that oxidation of acetosyringone by ligninase/H$_2$O$_2$ proceeds primarily via the phenoxyl radical. Data not shown indicate that the phenoxy radical decays according to second-order kinetics.

Because the kinetic profile of the acetosyringone phenoxy radical obtained in the ligninase system is not affected by the addition of cellobiose and CBQase (Fig. 3a, 3b), it seems reasonable to conclude that the rate of formation of acetosyringone phenoxy radical is not sensitive to added CBQase. Thus our results show that CBQase does not reduce the phenoxy radical generated from acetosyringone, and similarly that it does not prevent the oxidative polymerization of guaiacol or lignin by ligninase. Both acetosyringone and guaiacol contain methoxyl substitution patterns found in lignin, so results with these substrates should be relevant to lignin.

Formation of higher molecular weight products from dimer II (Fig. 1c) is explained by the polymerisation of guaiacol released indirectly following C$_a$-C$_ß$ cleavage. Earlier results with non-phenolic $\beta$-O-4 dimers analogous to II showed that ligninase oxidation results in C$_a$-C$_ß$ cleavage (Tien and Kirk, 1984). It was proposed that an unstable hemiacetal is formed from the C$_ß$ moiety, followed by spontaneous decomposition which releases glycolaldehyde and a phenol. By analogy, a hemiketal would be formed from $\beta$-O-4 ether structures. In the case of model II, decomposition of the hemiacetal releases guaiacol which polymerizes.

Oxidation of model II by ligninase is expected to generate C- and O-centered radicals in addition to phenoxyl radicals (Hammel et al., 1985). Failure of CBQase to affect the molecular size distribution of labelled products generated from model II by ligninase action suggests that none of the radicals is affected. Further work is needed to elucidate the biomechanical mechanism for preventing phenol polymerization in vivo.

RESUME

La ligninase de Phanerochaete chrysosporium oxyde l'acetosyringone, modèle moléculaire de type lignine, en presence de H$_2$O$_2$ en radical phenoxy qui a été caractérisé par Spectroscopie de Résonance Magnétique Electronique (R.P.E.). La Cellobiose : quinone oxidoreductase en presence de cellobiose n'a pas d'action sur le radical forme. Le système ligninase/H$_2$O$_2$ polymérise le guaiacol et augmente la masse moléculaire de la lignine de synthèse via la formation de radicaux phénoxyl. A partir du
modèle moléculaire de type lignine 6-0-4 ether 1-(4-éthoxy-3-méthoxyphenyl)-2-(2-méthoxy-[U-14C]phényloxy)-propane-1,3-diol (II) des composes de masse moléculaire plus élevés sont également formées. La polyérisation du "C-guaiacol par couplage explique ce phénomène. La CBQase n'est pas capable d'inhiber ces réactions de polymérisation. On en déduit que la CBQase n'est pas impliquée dans la prevention de la polymérisation des phenols par action de la ligninase. Compte-tenu que des cultures entières de *P. chrysosporium* depolymérisent activement la lignine de synthase, un système biochimique non identifié doit intervenir pour empêcher la polymérisation des phénols formés au cours de la dégradation de la lignine par la ligninase.

**REFERENCES**


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