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Interaction of peroxidase systems with recombinant glyoxal oxidase from *Phanerochaete chrysosporium*

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SUMMARY

The modulation of recombinant glyoxal oxidase (rGLOX) activity from *Phanerochaete chrysosporium* was studied by using lignin peroxidase (LiP) or horseradish peroxidase (HRP) in coupled reactions. Peroxidase substrates included a phenol, a catechol, a series of methoxybenzene and spruce milled wood lignin (MWL). The crude rGLOX was shown to produce hydrogen peroxide in the absence of a coupled peroxidasic system. However, the activity is transient and the enzyme is partially and reversibly inactivated by the produced hydrogen peroxide. Results obtained with peroxidase substrates with varying redox potential strongly suggested that the oxidized intermediates produced by coupled peroxidases are the rGLOX activators. Notably rGLOX is activated when macromolecular spruce lignin is used in coupled reactions with LiP. In contrast, guaiacol and catechol are both inactivating, and such lignin degradation products are expected to have similar effects. Taken together, our results suggested that ligninolysis by peroxidase systems could be regulated by GLOX activity and *influenced* therefore by the presence of veratryl alcohol, lignin and lignin degradation products.

INTRODUCTION

Glyoxal oxidase (GLOX), an extracellular H_2O_2-generating enzyme produced by *Phanerochaete chrysosporium* (1), is thought to be an important partner in the ligninolysis catalyzed by the fungal lignin (Lip) and manganese peroxidases (MnP). Indeed, GLOX is a physiological source of H_2O_2 (1) and the enzyme has been shown to be reversibly activated during incubation with lignin peroxidase plus veratryl alcohol (VA) (2), a system known to partially depolymerize lignins (3, 4). In relation to both the latter point and the general characterization of the biophysical properties of purified GLOX (1) this paper reports some important features of the modulation of recombinant GLOX activity (5) by different coupled peroxidasic systems using LiP or horseradish peroxidase (HRP) in the presence of various methoxybenzene congeners or spruce milled wood lignins (MWL). The results presented here will be discussed relative to the physiological significance of such a regulated source of peroxide in ligninolysis.

MATERIALS AND METHODS

Enzymes. Lignin peroxidase (LiP) isozyme H1 was produced from *Phanerochaete chrysosporium* BKM F- 1767 (ATCC 24725) and purified according to (2) and (6). LiP

activity was assayed by veratryl alcohol oxidation at pH 3 (7). A small but non-significant residual GLOX activity was found in LiP H1 preparation. *Recombinant glyoxal oxidase* (rGLOX) corresponding to glx-lc of *P. chrysosporium* was produced with *Aspergillus nidulans* (5). The crude culture medum was filtered, concentrated and dialyzed and enzyme was used without further purification (8). GLOX activity was determined with a coupled peroxidasic system using HRP and phenol red (1). No peroxidase activity was found in the concentrated rGLOX preparation. *Horseradish peroxidase* (HRP) and other enzymes were commercial preparations and used as supplied. *Chemicals.* Methoxybenzenes were synthesized or purchased as described previously (9). Distilled veratryl alcohol was used throughout this study. *Oxygen and hydrogen peroxide measurements.* Dissolved oxygen consumption and hydrogen-peroxide formation rates in rGLOX-catalyzed reactions were determined with specific Pt-Ag electrodes in a capped thermostated chamber at 25°C (2). *Typical rGLOX/peroxidase coupled reactions.* Effects of peroxidase (LiP vs HRP) and peroxidase substrates in rGLOX/peroxidase coupled reactions were tested. rGLOX activity was monitored by dissolved O_2 consumption or peroxide formation rates. The components were used at the following concentrations: 0.02 M Na-dimethylsuccinate buffer (pH 4.5 or pH 6), 10 mM methylglyoxal, 4.5 nkat/ml rGLOX, 2 mM peroxidase substrate, and with either LiP or HRP at 12 nkat/ml (25 µg/ml) and 14 nkat/ml (10 µg/ml), respectively. Lignin was also used as a peroxidase substrate (1 mg/ml) and 5 to 10 % v/v dimethylformamide was also added to the reaction medium in order to obtain the desired colloidal state (4).

RESULTS AND DISCUSSION

The experiments described here were designed to identify the interactions required for the reversible inactivation of GLOX and its reactivation in a coupled peroxidasic system. Thus, rGLOX activity was first studied in the absence of a peroxidase. Conditions for enzyme inactivation were determined and the possibility of end-product inhibition checked. The influence of peroxidases (LiP vs HRP) and their associated substrates were then investigated. Lignin as a natural substrate for LiP, was also tested for its effect on the coupled activity of rGLOX and to assess the physiological significance of such an enzymatic system.

rGLOX activity in the absence of a peroxidase. Whereas previous studies showed that purified GLOX is inactive without the presence of a peroxidase (1), rGLOX was shown to need just one of its substrates to be able to produce transiently H_2O_2 at a basal level. In this study, the substrate was methylglyoxal. However, a progressive inactivation of rGLOX was further obtained, related to some extent to the peroxide that accumulated during catalysis. Nevertheless, very large amounts of peroxide added to the reaction medium never succeeded in fully inhibiting the enzyme. This indicated unambiguously that some other unknown factors may participate in this reversible inactivation (data not shown).

Regulation of rGLOX activity by peroxidase-substrate couples. The (in)activation of rGLOX by peroxidase systems was characterized with three classes of substrates (non-phenolic monomers, phenolic monomers and polymeric lignins, table 1) and two peroxidases (LiP and HRP). Non-phenolic monomers were chosen to test whether the redox potential of these compounds could affect the rGLOX activation. The results obtained here strongly suggested that oxidized intermediates formed by coupled peroxidase system are the GLOX activators. Indeed, the best non-phenolic substrates for the peroxidases provided the best activation whereas poor peroxidase substrates yielded only moderate to low activation (figures 1 and 2, table 1). In contrast to the methoxybenzenes, guaiacol does not activate rGLOX in the presence of a peroxidase, even though it is oxidized by both LiP and HRP (figures 1 and 2). In fact, guaiacol strongly inhibited the oxidase activation in a coupled system with LiP and veratryl alcohol (figure 3), but had no effect on the progressive inactivation of rGLOX in the absence of a peroxidase. Another compound also found to readily inactivate rGLOX during catalysis was catechol (dihydroxybenzene). However, basal activity of rGLOX in the absence

of a peroxidase was also inhibited by catechol, suggesting that mechanisms involved in this process are different from those leading to rGLOX inactivation by guaiacol.

Mechanisms of rGLOX (in)activation. The multiple and divergent effects of peroxidase-substrate couples cannot be simply explained in terms of reactivity of the substrates with the peroxidases, even though oxidized intermediates may be privileged partners in the process. The specific components and their interaction in regulation (e.g. protein-protein interaction, electron transfer reactions, radical mediation etc...) must be determined before a mechanism for modulation of rGLOX activity can be established.

On *the physiological role of GLOX in ligninolysis.* The inhibition and activation of rGLOX was described in this study as was the prevention of activation by certain peroxidase substrates. Notably, rGLOX was activated during the oxidation of macromolecular spruce lignins by LiP, at least under initial conditions depicted in figure 1. Chemical characterization of lignins has also shown that catechol structures arising from demethylation reactions are formed during ligninolysis (4). This could be a source of potent rGLOX inhibitors during the lignin degradation process. A second set of reversible inhibitors could be the lignin degradation products related to the guaiacol series. Finally, lignin depolymerization reactions may also produce glycolaldehyde, a substrate for GLOX (10), suggesting that these components could interact together and provide full regulation of both LiP and GLOX activities during ligninolysis, as depicted in figure 4.

In conclusion, this study has demonstrated that rGLOX is regulated in the presence of various coupled peroxdase systems using both monomers and macromolecular lignins. Oxidized intermediates produced by peroxidases may be primarily involved in the control process of rGLOX activity. This strongly suggests that mutual regulation of peroxidase and rGLOX could lead to the controlled radical formation in lignin resulting in sustained activity for LiP and limitation of radical coupling reactions driving the oxidative process toward depolymerization.

REFERENCES

(1) Kersten, P.J. and Kirk, T.K. 1987 J. Bacteriol. 169: 2195

(2) Kersten, P.J. 1990 Proc. Natl. Acad. Sci. USA 87: 2936.

(3) Hammel, K.E., Jensen, K.A., Mozuch, M.D., Landucci, L.L., Tien, M. and Pease, E.A. 1993 J. Biol. Chem. 268(17): 12274.

(4) Kurek, B. and Monties, B. 1994 Enzyme Microb. Technol. 16(2): 125.

(5) Kersten, P.J. and Cullen, D. *submitted*

(6) Asther, M. Vilter, H., Kurek, B. and Meunier, J.C. 1992 Int. J. Biochem. 24(9): 1377.

(7) Tien, M. and Kirk, T.K. 1984 Proc. natl. Acad. Sci. USA 81: 2280.

(8) Kurek, B. and Kersten, P.J. 1995 Enzyme. Microb. Technol. *in press*

(9) Kersten, P.J., Kalyanaraman, B., Hammel, K.E., Reinhammar, B. and Kirk, T.K. 1990 Biochem. J. 268: 475.

(10) Hammel, K., Mozuch, M.D., Jensen, K.A. and Kersten, P.J. 1994 Biochemistry 33: 13349.

table 1: oxidation of methoxybenzenes, model compounds and lignin by LiP and HRP[a].

compound	$E_{1/2}$	oxidation by	
		LiP	HRP
anisole[b]	1.76	-	-
1,2,3,4 TMB[c]	1.25	+	-
1,2,3,5 TMB	1.09	+	w
1,2,4,5 TMB	0.81	+	+
guaiacol	0.89	w	+
catechol[d]	0.71	nd	nd
veratryl alcohol	na	+	-
lignin	na	+	+

[a] adapted from ref 9; [b] =methoxybenzene; [c] =tetramethoxybenzene; [d] =1,2 dihydroxybenzene; na: not available; nd: not determined; « + » and « - » indicate that the compound was or was not oxidized, respectively; « w » indicates that weak oxidation occurred

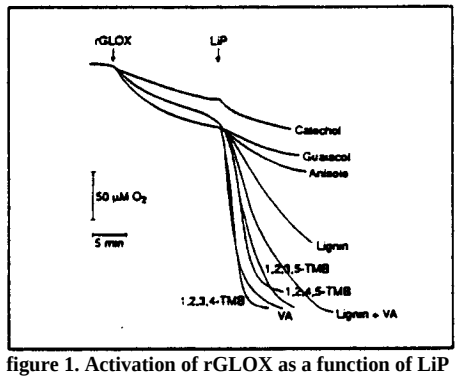

figure 1. Activation of rGLOX as a function of LiP substrate. Reactions were initiated with rGLOX (arrow) and oxygen consumption was measured; after inactivation of oxidase, LiP was added (arrow); when lignin was used as peroxidase substrate. 5% dimethylformamide was also included in the reaction medium

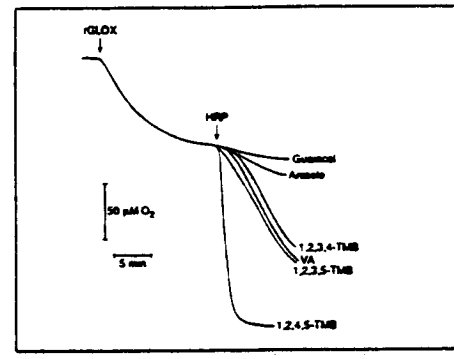

figure 2. Activation of rGLOX as a function of HRP substrate. Reactions were as in figure 1.

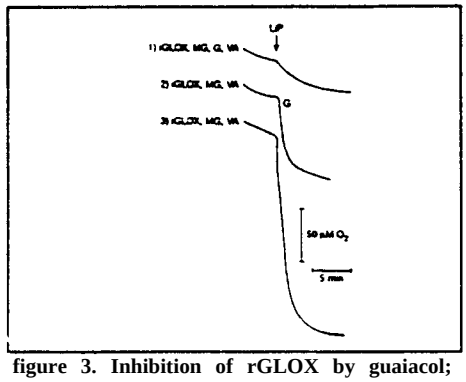

figure 3. Inhibition of rGLOX by guaiacol; reactions were first run with methylglyoxal (MG) plus guaiacol (G) and veratryl alcohol (VA, trace 1) or MG plus VA (traces 2 and 3). For clarity, O_2 consumption traces are shown only after almost total inactivation of rGLOX. The reactions were further continued with LiP (traces 1 and 3) or with LiP plus immediate addition of G (trace 2).

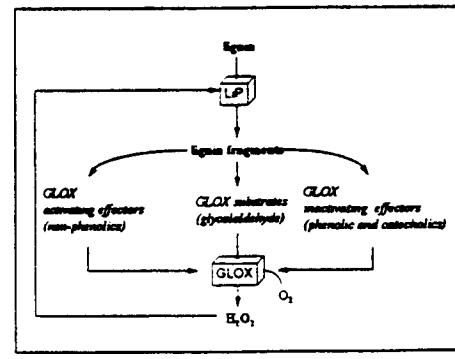

figure 4. Possible interactions between Lip, GLOX and lignin fragments during ligninolysis. Further control maybe influenced by the intracellular metabolism of the lignin-derived GLOX effectors.

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