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A novel enzymatic decarboxylation of oxalic acid by the lignin peroxidase system of white-rot fungus *Phanerochaete chrysosporium**

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Oxidation of veratryl alcohol by lignin peroxidase (LiP) was potently inhibited by oxalic acid. The inhibition analysis with Lineweaver-Burk plots clearly showed that the type of inhibition is non-competitive. The enzymatic oxidation of veratryl alcohol in the presence of ^{14}C-oxalic acid yielded radioactive carbon dioxide. The results indicate that the apparent inhibition of LiP is caused by reduction of the veratryl alcohol cation radical intermediate back to the substrate level by oxalate, which is concomitantly oxidized to carbon dioxide.

Lignin peroxidase; Veratryl alcohol; Oxalic acid; Decarboxylation mechanism; Wood-rotting fungus; Veratryl alcohol peroxidase

1. INTRODUCTION

Lignin peroxidase (LiP) of the white-rot fungi, including *Phanerochaete chrysosporium*, is known to catalyze oxidations of not only lignin [1-3] but also a wide variety of other organic compounds [4-6] regardless of the lignin-related chemical structures. However, because the enzymatic systems do not depolymerize lignin completely [7], a new hypothesis has recently been put forward, proposing that LiP is not prerequisite for lignin degradation during white-rot wood decay processes [8]. Alternatively, it has been addressed that LiP plays primarily a key role in secondary metabolism of veratryl alcohol which is biosynthesized from L-phenylalanine in white-rot fungi, which might have successfully adapted veratryl alcohol peroxidase (VAP) or LiP to degradation of lignins and xenobiotics during their biochemical evolution [9].

Although the lignin-oxidizing systems such as LiP, Mn-peroxidase [10-11] and laccase [12] may play important roles in white-rot wood decay, their true physiological roles are still ambiguous. In this context, it is of great importance to further examine metabolic functions of the lignin degrading systems in connection with the physiological traits of woodrotting basidiomycetes; these fungi are known to commonly produce oxalic acid as a peculiar secondary metabolite. Importantly, the brown-rot fungi markedly accumulate it in their cultures, whereas the white-rot fungi do not [13-14]. Oxalate might be scavenged by the oxidative lignin degrading systems in extracellular sites of the latter fungi.

Quite recently, we have found out that only the oxalate buffer (50 mM, pH 4.5) potently inhibited the LiP-catalyzed oxidation of veratryl alcohol in contrast to the other organic acid buffers used for the reaction mixtures [15]. Interestingly, the enzymatic oxidation of veratryl alcohol decomposed ^{14}C-oxalate to radioactive carbon dioxide.

We report here the first example of decarboxylation of oxalic acid catalyzed by LiP of the white-rot fungus, *P. chrysosporium*. The reaction mechanism for the unprecedented enzymatic decarboxylation and a new role of the LiP system or veratryl alcohol peroxidase (VAP) are discussed in relation to oxalate metabolism of the white-rot fungi.

2. MATERIALS AND METHODS

2.1. *Chemicals*

^{14}C-Oxalic acid (0.13 GBq or 3.4 mCi/mmol) was purchased from New England Nuclear Inc. The radioactive sample was used after dilution with cold oxalic acid to the specific activity (2.45 MBq/μmol), which was determined by measurement of radioactivity of carbon dioxide produced after complete decomposition to carbon dioxide with $KMnO_4$ at 70°C. Veratryl alcohol and other chemicals were procured from Nakarai Tesque and Waken Chemicals, Kyoto.

2.2. *Preparation of LiP and assay conditions*

P. Chrysosporium was grown in the Kirk's medium which was modified by LC/MN medium with 0.2% glucose and 6 mM ammonium for carbon and nitrogen sources, respectively, in the presence of 0.1% Tween 80 under 21% oxygen as described previously [16]. The concentrate of the whole extracellular fluid was dialyzed against 50 mM tartrate buffer (pH 4.5) and used for oxidation of [^{14}C]oxalic acid. The LiP preparation (2.3 nkat/mg protein) thus obtained was

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*The new method for determination of oxalate in biological samples with LiP system is now applied for patent.

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further purified by Bio-gel A column chromatography as described previously [17]. The purified LiP preparation (50 nkats/mg protein) was used for the kinetic experiments to determine the inhibition type. The LiP activities were assayed by measurement of the increasing absorbance due to the formation of veratraldehyde ($\epsilon_{310}=9.3$ $mM^{-1}cm^{-1}$) at 310 nm in a volume of 3.0 ml at pH 4.5, as previously described [18].

2.3. *Inhibition of LiP by oxalate*

The initial rates for the LiP activity were determined at the various concentrations of the substrate in the presence of 0.1 mM, 0.5 mM and 1 mM oxalic acid and the absence of oxalate. The Lineweaver-Burk plots were obtained by plotting reciprocals of the initial rates (absorbance at 310 nm/min) against those of the various substrate concentrations (mM). The same amount (0.5 nkat) of the purified LPO was used for each assay.

2.4. *Enzymatic decomposition of ^{14}C-oxalate*

The complete reaction mixture (3.0 ml) placed in 100 ml Erlenmyer flask with tubes for air-flushing contained 1.0 nkat of the concentrated crude LiP preparation, veratryl alcohol substrate (10 μmol), ^{14}C-oxalate (4 μmol) and H_2O_2 (1.5 μmol) and 2.78 ml of 50 mM tartrate buffer (pH 4.5) was incubated at 30°C for 1 h. At 20 min intervals during the incubation period, the radioactive carbon dioxide produced was purged out by air into the scintillation cocktail with ethanol amine as previously reported by Kirk et al. [19]. The sum of three measurements of the radioactivities was recorded for each incubation mixture and the amounts of carbon dioxide evolved were calculated in consideration of counting efficiency (85%). For the control experiments, either LiP, veratryl alcohol, hydrogen peroxide, or ^{14}C-oxalate was singly omitted from the complete reaction system.

2.5. *Instrumentation*

Beckman LS-1800 scintillation counter and Hitachi UV-Spectrophotometer Model 200-20 were used for measurement of radioactivities and enzyme activities, respectively.

3. RESULTS

3.1. *Inhibition of LiP by oxalic acid*

In order to determine the type of inhibition by oxalate, the Lineweaver-Burk plots were obtained as shown in Fig. 1. The results clearly show that all the four lines have different slopes but the same intercept (-1 K_m) on the abscissa, indicating that the type of inhibition is noncompetitive; K_m value (0.2 mM determined graphically) for veratryl alcohol does not change, whereas the maximal velocity (V_{max}) decreased, by increasing the concentrations of oxalate. This means that oxalate inhibitor does not compete with the substrate for the same binding site of the enzyme molecule. On the assumption that this type of inhibition is expressed by an allosteric effect of oxalate, the apparent K_i constant is determined to be 0.4 mM, which is the concentration to bring about 50% inhibition of V_{max}.

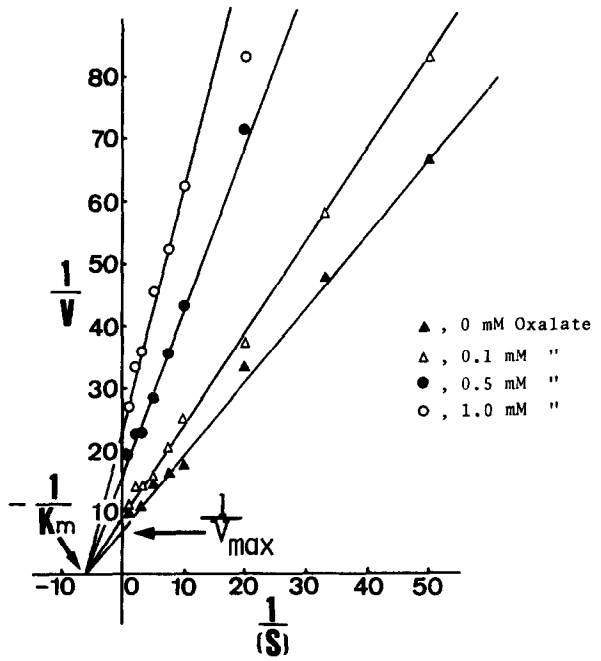

Fig. 1. Lineweaver-Burk plots for LiP activities in the presence and absence of different concentrations of oxalate. $1/V$, reciprocals of the reaction rates, $1/S$, reciprocals of the substrate (mM).

Table I

The LiP-System-dependent decarboxylation of ^{14}C-oxalate

Enzyme System	CO_2 produced (nmoles)*
Complete	390
" - enzyme	0
" - veratryl alcohol	4
" - H_2O_2	0
" - ^{14}C-oxalate	0

* The reaction conditions are described in the text. The net amounts of CO_2 produced were determined in consideration of the specific radioactivities of [^{14}C]oxalate after background (20 kBq) corrections were made.

3.2. *Decomposition of oxalic acid by LiP system*

The amounts of carbon dioxide produced from ^{14}C-oxalic acid in the complete and the control systems are given in Table I. The results demonstrate that the decomposition of oxalic acid to carbon dioxide depends on the presence of the complete LiP system, since little radioactive carbon dioxide is produced in the control systems. The system with the heat-denatured enzyme is not active, either. The yield of CO_2 produced was found to be about 26% based on hydrogen peroxide used but higher yield (68%) was obtained when the reaction was carried out at pH 3.0 which is close to the optimal pH of LiP [18].

The use of purified LiP together with the glucose oxidase/glucose system which was employed as a source of H_2O_2 attained 91% conversion of oxalate to carbon dioxide, which will be reported elsewhere. Interestingly, replacement of veratryl alcohol with vanillic acid did not produce CO_2 from oxalic acid.

4. DISCUSSION

4.1. *The mechanism for decarboxylation*

This investigation provides important evidence for a mechanism of the oxalate decarboxylation by the LiP system (Fig. 2). First, the decarboxylation of oxalate thoroughly depends on the complete LiP system containing the enzyme, veratryl alcohol, and H_2O_2 (Table I). Second, the inhibition analysis (Fig. 1) revealed that

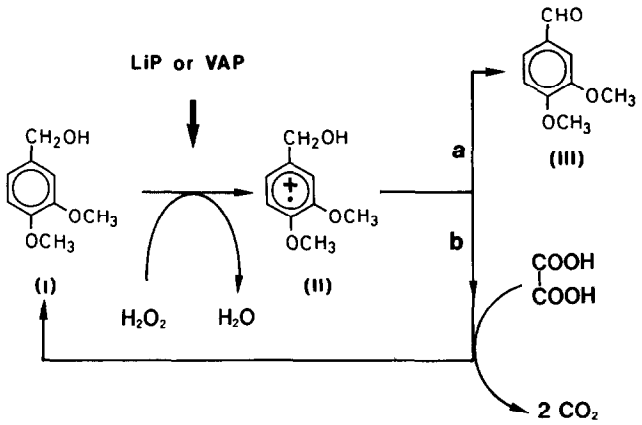

Fig. 2. Mechanism proposed for decarboxylation of oxalate (route b) coupling with the LiP or VAP system during oxidation of veratryl alcohol (I) to veratraldehyde (III) (route a).

oxalate does not compete with veratryl alcohol for the same active site of the enzyme.

In the LiP system, yielding veratraldehyde product (III) from veratryl alcohol substrate (I) (route a in Fig. 2), the cation radical intermediate (II) is regarded as an active oxidizing species, because formation of similar species of alkoxybenzene substrates have been established in the LiP-catalyzed reaction [20]. Therefore, it is proposed that the radical intermediate (II) is reduced back to the substrate (I) (route b) by oxalate, which is concomitantly decomposed to CO_2; the reduction of the intermediate (II) by oxalate prevents formation of the product (III) (route a). Accordingly, the noncompetitive inhibition observed (Fig. 1) is explained reasonably in terms of the coupling of the LiP system with oxalate oxidation rather than the allosteric binding of oxalate to the enzyme molecule. Conversely, veratryl alcohol (I) acts as a mediator for oxidation of oxalic acid in this system, which is in good accord with the previous report on the role of veratryl alcohol as a mediator in lignin degradation [21].

4.2. *Roles of LiP in oxalate metabolism*

From medicinal viewpoints, oxalic acid has long been receiving considerable attention as a toxic metabolite occurring in plants such as spinach and wood sorrel, cultures of molds and basidiomycetous fungi, and even human urine [22]. However, functions of oxalate metabolism in those biological systems, including the wood-rotting fungi are little understood.

It is noteworthy that, in contrast to the brown-rot fungi, most of white-rot fungi have intracellular oxalate decarboxylase which catalyzes decarboxylation of oxalate to formate and CO_2 [23]. Although it is not clear yet whether *P. chrysosporium* also contains such a decarboxylase, this investigation demonstrates that this fungus decarboxylates oxalate with the LiP system, probably, in the extracellular site. Furthermore, the oxalate inhibition (Fig. 1) indicates that lignin degradation by LiP is greatly hampered as long as both veratryl alcohol and oxalic acid are accumulating in the same reaction site, because H_2O_2 is also depleted by this system. Therefore, lignin degradation or veratryl alcohol oxidation is controlled by the level of oxalate on one side and detoxication of the metabolite is affected by VAP or LiP activity on the other.

In conclusion, VAP or 'LiP' plays an important physiological role in scavenging toxic oxalate derived originally from wood carbohydrates during the white-rot decay process. The reason for little accumulation of oxalate in the cultures of white-rot fungi is also rationally explained by such a unique role of VAP.

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