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ORIGINAL RESEARCH PAPER

Determination of a catalytic tyrosine in *Trametes cervina* lignin peroxidase with chemical modification techniques

Yuta Miki · Hirofumi Ichinose · Hiroyuki Wariishi

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Abstract *Trametes cervina* lignin peroxidase (LiP) lacks a catalytic tryptophan strictly conserved in other LiP and versatile peroxidases. It contains tyrosine^{181} at the potential catalytic site. This protein and the well-characterized *Phanerochaete chrysosporium* LiP with the catalytic tryptophan^{171} have been chemically modified: the tryptophan-specific modification with *N*-bromosuccinimide sufficiently disrupted oxidation of veratryl alcohol by *P. chrysosporium* LiP, whereas the activity of *T. cervina* LiP was not affected, suggesting no catalytic tryptophan in *T. cervina* LiP. On the other hand, the tyrosine-specific modification with tetranitromethane did not affect the activities of *P. chrysosporium* LiP lacking tyrosine but inactivated *T. cervina* LiP due to the nitration of tyrosine^{181}. These results strongly suggest that tyrosine^{181} is at the catalytic site in *T. cervina* LiP.

Keywords Lignin peroxidase · *Phanerochaete chrysosporium* · *Trametes cervina* · Tryptophan · Tyrosine

Y. Miki · H. Ichinose · H. Wariishi (✉)
Faculty of Agriculture, Kyushu University,
6-10-1, Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan
e-mail: hirowari@agr.kyushu-u.ac.jp

Present Address:
Y. Miki
CIB, CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain

Introduction

Lignin has a complex three-dimensional architecture containing different non-phenolic units (Gellerstedt and Henriksson 2008). Its degradation is a rate-limiting step for carbon recycling in ecosystem and a central issue for industrial uses of lignocellulose (Martínez et al. 2009). High redox-potential peroxidases from white-rot basidiomycetes, lignin peroxidase (LiP; EC: 1.11.1.14) and versatile peroxidase (VP; EC: 1.11.1.16), are responsible for initial oxidative depolymerization in lignin biodegradation (Kirk and Farrell 1987; Hammel and Cullen 2008). These enzymes can oxidize high redox-potential and large molecular substrates and are of interest as industrial biocatalysts (Ruiz-Dueñas and Martínez 2009).

LiP and VP oxidize 3,4-dimethoxybenzyl (veratryl) alcohol (VA), the physiological substrate of LiP, at the tryptophan residue located at the protein surface (Fig. 1a) via a long-range electron transfer pathway to heme (Doyle et al. 1998; Gelpke et al. 2002; Pérez-Boada et al. 2005). This catalytic tryptophan is conserved in almost all LiP and VP sequences (Martínez 2002).

Recently, a new type of LiP was discovered in the white-rot basidiomycete, *Trametes cervina*. Although this LiP exhibits the LiP-type catalytic properties (Miki et al. 2006), it lacks the catalytic tryptophan (Miki et al. 2010). Additionally, the homology molecular model of *T. cervina* LiP showed that a sole

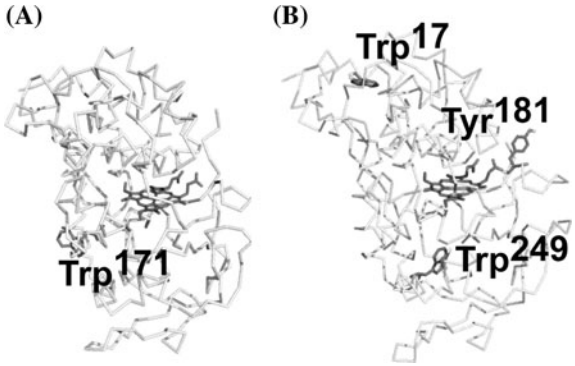

Fig. 1 The *P. chrysosporium* LiP structure (**a**) and the homology molecular model of *T. cervina* LiP (**b**). Overall protein structures are shown in *gray lines*, involving the heme cofactors (in the *center*) and the selected redox active residues (Trp^{171} in *P. chrysosporium* LiP, and Trp^{17}, Tyr^{181}, and Trp^{249} in *T. cervina* LiP, respectively) shown in *black sticks*. The *P. chrysosporium* LiP structure was obtained from PDB entry 1LLP. The structural model of *T. cervina* LiP was obtained by homology modeling with the Molecular Operating Environment program (Chemical Computing Group) (Miki et al. 2010)

Tyr^{181} occupies the position near the heme-propionate and is accessible to the exterior solvent (Fig. 1b), suggesting that this residue would be a catalytic site. On the other hand, the *T. cervina* LiP sequence contains other two tryptophans (Trp^{17} and Trp^{249}) at the positions different from the catalytic tryptophan in other LiPs and VPs (Fig. 1b). Their catalytic roles would also be possible. Thus, in this study, *T. cervina* LiP and *Phanerochaete chrysosporium* LiP (the latter molecule employed as a control molecule with the catalytic Trp^{171}) are modified by *N*-bromosuccinimide (NBS; tryptophan-specific modification reagent) and tetranitromethane (TNM; tyrosine-specific modification reagent), in order to determine the catalytic residue in *T. cervina* LiP.

Materials and methods

Peroxidases

Trametes cervina LiP and *P. chrysosporium* LiP were purified from the extracellular culture medium of *T. cervina* (WD550) and *P. chrysosporium* (ATCC 34541), respectively, as previously described (Gold et al. 1984; Miki et al. 2006). The concentrations of peroxidases were calculated from the absorbance at 407 nm ($\varepsilon_{407\ nm}$ 147 mM^{-1} cm^{-1}) for *T. cervina* LiP and 408 nm ($\varepsilon_{408\ nm}$ 133 mM^{-1} cm^{-1}) for *P. chrysosporium* LiP.

Chemical modification

N-Bromosuccinimide (NBS)-modification mixtures containing 3 μM peroxidases, 1 mM $CaCl_2$, and 0–50 μM NBS in 50 mM sodium acetate buffer (pH 4.0) were incubated for 30 min at 25°C. Tetranitromethane (TNM)-modification mixtures containing 3 μM peroxidases, 1 mM $CaCl_2$, and 0–60 mM TNM in 50 mM Tris–HCl buffer (pH 7.0) were incubated for 30 min at 25°C. After 30 min, the mixtures were applied into a 3 kDa Centricon (Millipore), and excess reagents and small byproducts were removed by buffer exchange using 20 mM sodium succinate buffer (pH 4.5) containing 1 mM $CaCl_2$. The resulting solutions containing the modified enzymes were used without further purification.

Enzyme activity assay

Oxidation activities were spectrophotometrically determined at 25°C. Initial oxidation velocities were estimated from the maximal absorbance changes at 310 nm for VA oxidation (veratrum aldehyde formation), 415 nm for ABTS oxidation (ABTS radical cation formation), 469 nm for 2,6-dimethoxyphenol oxidation (quinone dimer formation), and 550 nm for cytochrome c^{2+} oxidation (cytochrome c^{3+} formation). Cytochrome c^{2+} was prepared by reducing cytochrome c^{3+} with sodium dithionite, followed by removal of the excess dithionite with Sephadex G-25, immediately prior to use.

Results and discussion

Effects of NBS modification

T. cervina LiP and *P. chrysosporium* LiP were treated with NBS that selectively oxidizes tryptophan indole ring to oxindolamine. The activity for VA oxidation by *P. chrysosporium* LiP was decreased by the NBS pretreatment, in a linear fashion with increasing concentration of NBS used (Fig. 2); 80% of activity was lost with 50 μM NBS. This result agrees with previous studies that LiP and VP contain catalytic tryptophans using chemical modifications (Blodig

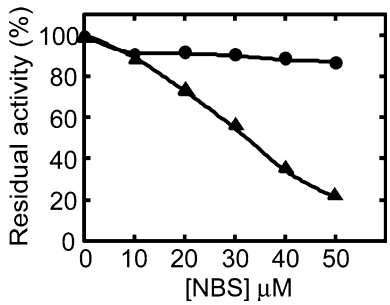

Fig. 2 The residual activities of *T. cervina* LiP (*closed circle*) and *P. chrysosporium* LiP (*closed triangle*) pretreated with NBS. VA oxidation rates were estimated in 20 mM sodium succinate buffer, pH 3.0, containing 0.1 mM H_2O_2, 0.5 mM VA, and 50 nM enzyme. The residual activities compared to the activity of untreated enzymes are shown

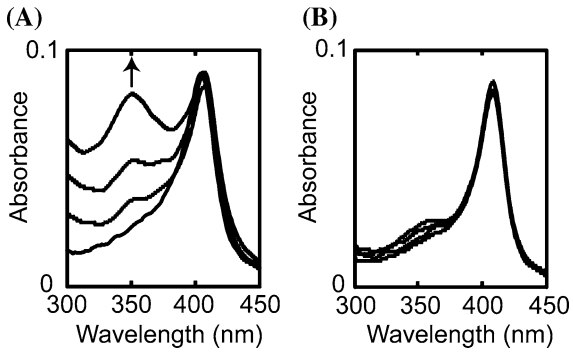

Fig. 3 Spectral changes of *T. cervina* LiP (**a**) and *P. chrysosporium* LiP (**b**) during TNM treatment. Spectra of peroxidases incubated for 30 min with 0, 20, 40 and 60 mM of TNM were recorded before buffer exchange. The absorbance increase at 350 nm due to generation of nitroformate anion, indicative of tyrosine nitration, is indicated by *arrow* in (**a**)

et al. 1998; Johjima et al. 2002; Kamitsuji et al. 2005). VA oxidation by *T. cervina* LiP, however, was unaffected by NBS (Fig. 2): more than 90% of the activity remained after pretreatment with 50 μM NBS. Thus, it is suggested that tryptophans in *T. cervina* LiP do not have a catalytic role.

Effects of TNM modification

T. cervina LiP and *P. chrysosporium* LiP were treated with TNM that selectively nitrates the aromatic ring of tyrosine and simultaneously generates nitroformate anion as a byproduct with a strong absorbance at 350 nm (Sokolovsky et al. 1967). The increase at 350 nm due to the formation of nitroformate anion, indicative of tyrosine nitration, was previously observed in the TNM treatment of plant cationic cell-wall-peroxidase with a catalytic tyrosine (Sasaki et al. 2008). Absorbance spectra of peroxidases modified by different concentrations of TNM are shown in Fig. 3. Spectra of *P. chrysosporium* LiP that lacks tyrosine was unaffected by TNM whereas TNM treatment of *T. cervina* LiP containing Tyr^{181} increased absorbance at 350 nm, suggesting the nitration of Tyr^{181}.

Consistent with the spectral results, VA oxidation by *P. chrysosporium* LiP was unaffected by TNM (Fig. 4a). More than 90% activity is observed after pretreatment with 60 mM TNM. Conversely, the activity for VA oxidation by *T. cervina* LiP is lowered by the TNM pretreatment, in a linear fashion with increasing concentrations of TNM used (Fig. 4a). Approx. 60% of VA oxidation activity was lost with

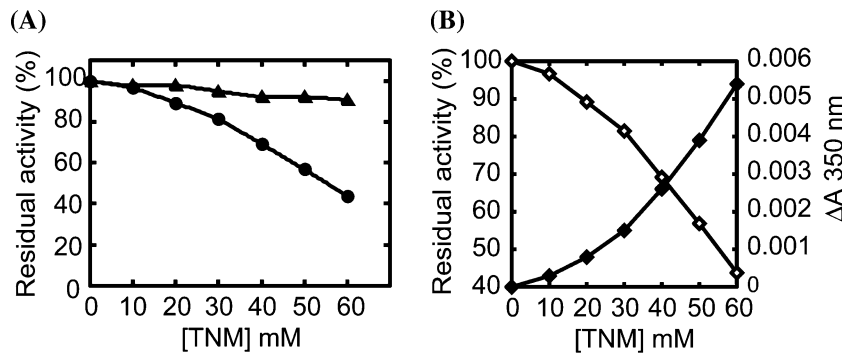

Fig. 4 Effects of the TNM pretreatment on the catalytic activities of peroxidases. **a** The residual activities of *T. cervina* LiP (*closed circle*) and *P. chrysosporium* LiP (*closed triangle*) pretreated with TNM. VA oxidation rates were estimated in 20 mM sodium succinate buffer, pH 3.0, containing 0.1 mM H_2O_2, 0.5 mM VA, and 50 nM enzymes. The residual activities compared to the activity of untreated enzymes are shown. **b** The correlation between the residual activities (*open diamond*) and the absorbance increases at 350 nm (*closed diamond*), the latter indicative of Tyr^{181}-nitration, in TNM-modified *T. cervina* LiP

60 mM TNM. As shown in Fig. 4b, the loss of the activity shows a good correlation with the absorbance increase at 350 nm, suggesting that the nitration of Tyr^{181} causes inactivation of *T. cervina* LiP.

T. cervina LiP pretreated with 60 mM TNM also had lower activities for oxidation of ABTS (37%), 2,6-dimethoxyphenol (41%), and cytochrome c^{2+} (50%) than untreated enzyme (Fig. 5). These results suggest that Tyr^{181} is involved not only in oxidation of high-redox potential non-phenolic substrate (VA) but also in oxidation of low redox-potential anionic substrate (ABTS) and a phenolic substrate (2,6-dimethoxyphenol), though other LiPs and VPs are suggested to possess different catalytic sites for these substrates (Johjima et al. 2002; Ruiz-Dueñas et al. 2009a). In addition, it is confirmed that Tyr^{181} also contributes to the ability to oxidize large molecule (cytochrome *c*), which is the requisite property for direct oxidation of polymeric lignin.

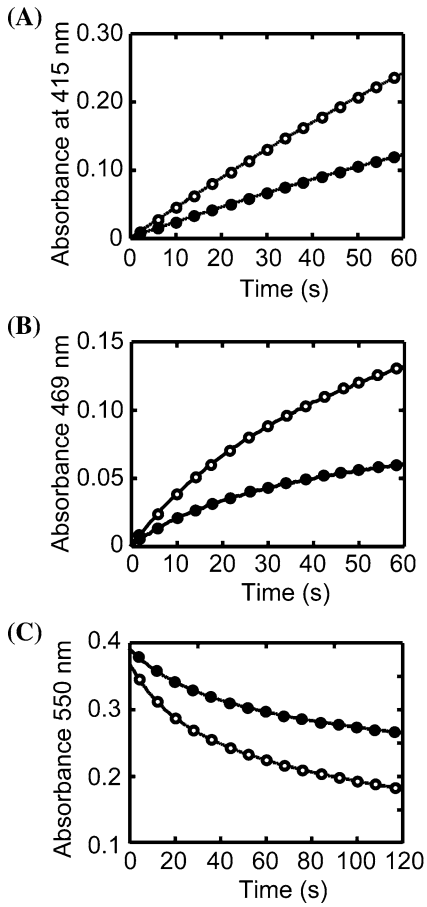

Fig. 5 Kinetic traces for oxidation of ABTS (**a**), 2,6-dimethoxyphenol (**b**) and cytochrome c^{2+}(**c**) by *T. cervina* LiP pretreated with 60 mM TNM (*closed circle*) and unmodified enzyme (*open circle*). Oxidation of ABTS and 2,6-dimethoxyphenol were measured in 20 mM sodium succinate buffer, pH 3.0, containing 0.5 mM substrate, 5 nM enzyme, and 0.1 mM H_2O_2. Cytochrome c^{2+} oxidation was measured in 20 mM sodium succinate buffer, pH 4.0, containing 14 μM cytochrome c^{2+}, 15 nM enzyme, and 0.1 mM H_2O_2

Conclusions

Several structural features contribute to the LiP-type catalytic properties for oxidation of high redox potential and large-molecular substrate such as lignin. One of the structural features is the catalytic site exposing to the exterior solvent (Ruiz-Dueñas and Martínez 2009). Although all other LiP and VP conserve the exposed catalytic tryptophan (Martínez 2002), this present study shows for the first time that Tyr^{181} is a catalytic site in *T. cervina* LiP. Though a catalytic tyrosine had never been found in other LiP and VP, different redox enzymes use this aromatic residue as a catalytic site (Stubbe and van der Donk 1998). Additionally, a VP mutant, W164Y, in which the catalytic Trp^{164} was substituted by tyrosine, contained a tyrosyl radical (Ruiz-Dueñas et al. 2009b) that would function as the catalytic radical like the tryptophanyl radical in native LiP and VP (Blodig et al. 1999; Pogni et al. 2006; Smith et al. 2009). These observations strongly support the idea that Tyr^{181} in *T. cervina* LiP would oxidize substrates via tyrosyl radical. Since the nature and length of the electron transfer pathway in *T. cervina* LiP are different from others and novel, the structure–function characterization of this enzyme is of high interest. Recently, production of recombinant *T. cervina* LiP by *E. coli*, followed by in vitro refolding, was optimized (Miki et al. 2009). Thus, further detailed mechanistic studies of this enzyme would be performed in the near future.

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