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Role of Tyr residues on the protein surface of cationic cell-wall-peroxidase (CWPO-C) from poplar: Potential oxidation sites for oxidative polymerization of lignin

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Abstract

It was previously reported that an unique peroxidase isoenzyme, cationic cell-wall-bound peroxidase (CWPO-C), from poplar callus oxidizes sinapyl alcohol, ferrocytochrome *c* and synthetic lignin polymers, unlike other plant peroxidases. Here, the catalytic mechanism of CWPO-C was investigated using chemical modification and homology modeling. The simulated CWPO-C structure predicts that the entrance to the heme pocket of CWPO-C is the same size as those of other plant peroxidases, suggesting that ferrocytochrome *c* and synthetic lignin polymers cannot interact with the heme of CWPO-C. Since Trp and Tyr residues are redox-active, such residues located on the protein surface were predicted to be active sites for CWPO-C. Modification of CWPO-C Trp residues did not suppress its oxidation activities toward guaiacol and syringaldazine. On the other hand, modification of CWPO-C Tyr residues using tetranitromethane strongly suppressed its oxidation activities toward syringaldazine and 2,6-dimethoxyphenol by 90%, respectively, and also suppressed its guaiacol oxidation activity to a lesser extent. Ferrocytochrome *c* was not oxidized by Tyr-modified CWPO-C. These results indicate that the Tyr residues in CWPO-C mediate its oxidation of syringyl compounds and high-molecular-weight substrates. Homology modeling indicates that Tyr-177 and Tyr-74 are located near the heme and exposed on the protein surface of CWPO-C. These results suggest that Tyr residues on the protein surface are considered to be important for the oxidation activities of CWPO-C with a wide range of substrates, and potentially unique oxidation sites for the plant peroxidase family.
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1. Introduction

Lignin is a polymer of phenylpropanoid units with a variety of carbon–carbon and carbon–oxygen linkages, resulting in a complex structure. The final step of monolignol oxidation leading to lignin biosynthesis is catalyzed by plant peroxidases (Blee et al., 2003). Horseradish peroxidase (HRP) is a typical plant peroxidase that has been used to attempt to study the mechanism of monolignol dehydrogenative polymerization leading to lignins, even though HRP is not present in tissues that lignify to any extent (Syrjanen and Brunow, 1998, 2000). Angiosperm lignins consist of guaiacyl and syringyl units. HRP C and A2 oxidize coniferyl alcohol **1** efficiently, but not sinapyl alcohol **2** (Nielsen et al., 2001). *Arabidopsis thaliana* peroxidase A2 (ATP A2; 95% identity with HRP A2) was previously purified and cloned from a suspension culture of *A. thaliana* (Østergaard et al., 1996). Analysis of the substrate-binding mode of mature ATP A2 by docking simulation with three monolignols (*p*-coumaryl **3**, coniferyl **1** and sinapyl **2** alcohols) (Fig. 1) indicated that coniferyl **1** and *p*-coumaryl **3** alcohols bind well to the substrate-binding site of ATP A2 (Østergaard et al., 2000). On the other hand, sinapyl alcohol **2** did not bind to the substrate-binding site of ATP A2, since its 5-methoxy group overlapped with

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Pro-139 (Østergaard et al., 2000). This prediction was suggested to be generally applicable, since Pro-139 is highly conserved in the majority of plant peroxidases. For example, 69 of 73 peroxidases in *A. thaliana* contain Pro-139 (Nielsen et al., 2001; Welinder et al., 2002). Since HRP and ATP A2 share high identity including Pro-139, HRP can be used as a representative of plant peroxidases that cannot utilize sinapyl alcohol **2** as a preferred substrate.

An unique peroxidase isoenzyme from poplar callus, cationic cell-wall-bound peroxidase (CWPO-C), has broad substrate versatility and was reported to use sinapyl alcohol **2** and syringaldazine **6** as preferred substrates (Tsutsumi et al., 1994, 1998; Aoyama et al., 2002; Sasaki et al., 2004). Furthermore, CWPO-C catalyzes the oxidation of synthetic lignin polymers and ferrocytochrome *c*, unlike other plant peroxidases (Sasaki et al., 2004). These observations suggest that the oxidation mechanism of CWPO-C is quite different from those of other plant peroxidases and appears to be required for lignin formation in plant cell walls.

Ferrocytochrome *c* is a 13-kDa heme protein that is larger than the entrance of the substrate access channels of peroxidases. However, fungal lignin peroxidase (LiP) can directly oxidize ferrocytochrome *c* (Wariishi et al., 1994). The Trp-171 residue located on the LiP surface was determined to be an oxidation site for its substrates (Blodig et al., 1998; Doyle et al., 1998; Johjima et al., 1999). These findings allowed us to postulate that either the oxidation site of CWPO-C is located on the protein surface, or that the entrance of the substrate access channel of CWPO-C is larger than those of other plant peroxidases.

In the present study, *in vitro* chemical modification techniques with *N*-bromosuccinimide (NBS) and tetranitromethane (TNM) were used to determine the key catalytic amino acid residues of CWPO-C. These were used since NBS can be employed for selective oxidation of exposed Trp residues to oxindolealanine (Blodig et al., 1998; Spande and Witkop, 1967), while TNM converts Tyr residues to 3-nitrotyrosine and has been used to assess the roles of Tyr residues in protein functions (Landfear et al., 1978; Miki and Wariishi, 2005). In addition to *in vitro* experiments, *in silico* homology modeling was employed to investigate the entrance size of the substrate access channel and determine the oxidation sites of CWPO-C.

2. Results and discussion

2.1. No definitive differences between the heme environment of CWPO-C and those of other plant peroxidases

A full-length CWPO-C cDNA was cloned using RT-PCR and RACE-PCR techniques. The predicted CWPO-C amino acid sequence contains a putative signal peptide (22 amino acids) at its *N*-terminus (Sasaki et al., 2006). The M_w and *p*I of the deduced amino acid sequence of the mature CWPO-C were calculated to be 32.3 kDa and 8.06, respectively. The molecular weight of the purified CWPO-C was previously determined to be approximately 32 kDa by SDS-PAGE (Tsutsumi et al., 1994).

An alignment analysis indicated that the catalytic sites of CWPO-C, including Arg-39, His-43, Pro-135 and His-165, were identical to those of ATP A2, HRP C and other plant peroxidases (Fig. 2) (Welinder, 1992). CWPO-C showed absorption maxima at 402 (Soret band), 496 and 635 nm, suggesting that it is a high-spin ferric peroxidase (Fig. 3a) (Tamura et al., 1972). The conserved functionally important amino acid residues and absorption spectrum of the CWPO-C also indicated that the heme environment of CWPO-C was identical to those of other plant peroxidases.

The simulated CWPO-C structure constructed by the Molecular Operating Environment (MOE) program predicts that the entrance to the heme pocket of CWPO-C is of the same size as those of the plant peroxidases HRP C and ATP A2 (Supplementary Figure). It was reported that binding of sinapyl alcohol **2** is sterically hindered in the ATP A2 peroxidase and other plant peroxidases due to overlap of the methoxyl group on the syringyl aromatic ring and Pro-139 (Østergaard et al., 2000). Our alignment analysis indicated that CWPO-C has a conserved Pro-135, which corresponds to Pro-139 in the other plant peroxidases (Fig. 2). Since ferrocytochrome *c* and syringyl

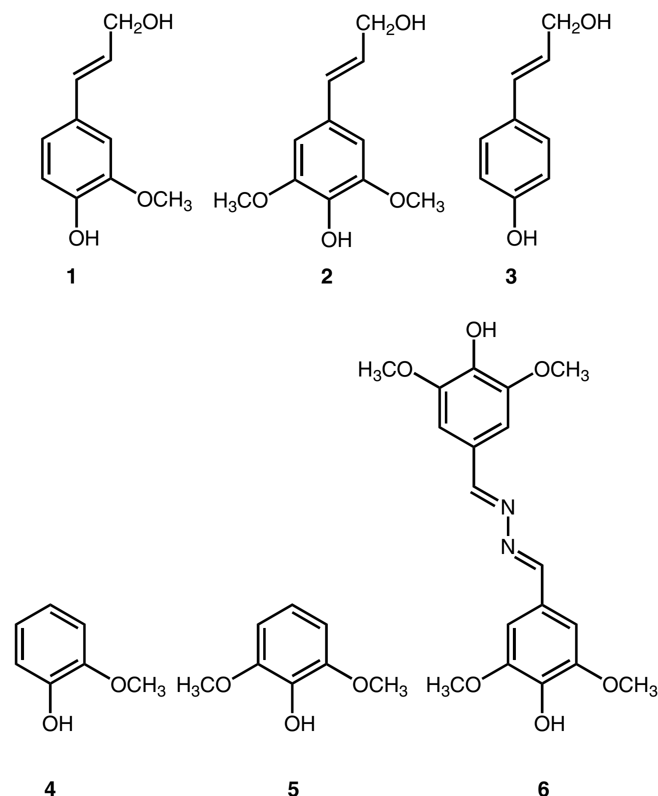

Fig. 1. Chemical structures of monolignols and substrates used for peroxidase assay. Monolignols, **1**: coniferyl alcohol; **2**: sinapyl alcohol; **3**: *p*-coumaryl alcohol. Substrates for peroxidase assay, **4**: guaiacol; **5**: 2,6-dimethoxyphenol; **6**: syringaldazine.

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