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A chimeric laccase with hybrid properties of the parental *Lentinula edodes* laccases

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Summary

We created a chimeric laccase from two different laccases, Lcc1 and Lcc4, from *Lentinula edodes*. Lcc1 is a secretory lignin-degrading enzyme produced in liquid cultures of *L. edodes*. Lcc4 is a tissue-accumulating-type enzyme, which is thought to be involved in melanin synthesis in fruiting body after harvesting. Lcc1 and Lcc4 differ in their *Km* values for some substrates, especially β-(3,4-dihydroxyphenyl) alanine (L-DOPA) and catechol. The novel chimeric laccase, Lcc4/1, has properties that are a hybrid of those of Lcc1 and Lcc4. Lcc4/1 acts upon both Lcc1 and Lcc4 substrates and most of its *Km* values are lower than those of Lcc1 and Lcc4. Homology modeling indicates that the deduced shape of the substrate-binding pocket of the chimeric laccase is larger than that of Lcc1 and similar to that of Lcc4. The other biochemical properties, such as temperature and pH dependency, are intermediate between those of Lcc1 and Lcc4.

Introduction

Laccases (EC 1.10.3.2) are blue multi-copper oxidoreductases that are widespread among bacteria, fungi, and plants. They are used in a wide range of industrial applications, including dye decolorization, wine clarification (Minussi et al. 2002), drug analysis, ethanol production (Larsson et al. 2001), and paper pulp bleaching (Couto and Toca-Herrera 2006; Mayer and Staples 2002; Sigoillot et al. 2004). Furthermore, laccases have

Abbreviations: ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; BPA, bisphenol A; CaMV35S promoter (35Sp), cauliflower mosaic virus 35S promoter; Lcc1, *L. edodes* laccase-1; Lcc4, *L. edodes* laccase-4; L-DOPA, β-(3,4-dihydroxyphenyl) alanine; ORF, open reading frame; PB, phosphate buffer; PAH, polycyclic aromatic hydrocarbon; PCB, polychlorinated biphenyl; PCP, pentachlorophenol; RBBR, Remazol Brilliant Blue R; RLcc1, recombinant laccase-1; RLcc4, recombinant laccase-4; RMSD, root mean square of difference; T1 Cu, Type I copper; TCP, 2,4,6-trichlorophenol; VA, violuric acid.

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potential uses for the biodegradation of phenolic compounds such as pentachlorophenol (PCP) (Ullah et al. 2000), pesticides (Baldrian 2006; Christian et al. 2005; Durán and Esposito 2000; Maruyama et al. 2006), and endocrine-disrupting chemicals such as bisphenol A (Fukuda et al. 2001; Tsutsumi et al. 2001), xenoestrogen nonylphenol (Junghanns et al. 2005), and 4-tert-octylphenol (Tamagawa et al. 2007). Remazol Brilliant Blue R (RBBR) is usually used as an indicator for detecting polychlorinated biphenyl (PCB) degradative activity, and the dye Poly R-478 is used as an indicator for detecting polycyclic aromatic hydrocarbon (PAH) degradative activity (Chroma et al. 2002; Novotný et al. 1997; Field et al. 1992). Nagai et al. (2002) have demonstrated the decolorization of RBBR and Poly R-478 by laccase1 (Lcc1) purified from *Lentinula edodes* and have suggested that it may be capable of degrading phenolic compounds. However, Lcc1 is not a versatile biodegrader. It cannot degrade β-(3,4-dihydroxyphenyl) alanine (L-DOPA) (Nagai et al. 2004), and phenolic pollutants were diverging in variety types. Therefore, the extension of Lcc1 reactivity to more compounds would be useful.

Lcc1 and laccase-4 (Lcc4) from *L. edodes* are expressed in different organs, at different developmental times, and play different physiological roles (Sakamoto et al. 2008, 2009). Lcc1 is the best-characterized laccase from *L. edodes* and is secreted into the culture medium from the vegetative mycelia (Nagai et al. 2003; Sakamoto et al. 2008). Lcc4 is known to accumulate in the gill tissue of *L. edodes* fruiting bodies (Nagai et al. 2003; Sakamoto et al. 2009; Yano et al. in press). The two enzymes are completely different in their biology, biochemistry, and molecular biology, with less than 60% homology in their cDNA sequences (Nagai et al. 2002, 2003; Sakamoto et al. 2008, 2009). Lcc1 oxidizing activity against 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) is greater than that of Lcc4 by comparison of *Kcat/Km* values, but the activity of Lcc4 against substrates such as catechol, pyrogallol, guaiacol, 2,6-dimethoxyphenol, and L-DOPA is higher than that of Lcc1 (Nagai et al. 2003). Therefore, modification of Lcc1 with Lcc4 is an attractive approach for extending Lcc1 reactivity.

Previously, we expressed recombinant Lcc1 (rLcc1) in tobacco BY-2 cell cultures (Sakamoto et al. 2008) and recombinant Lcc4 (rLcc4) in *Aspergillus oryzae* cultures (Yano et al. in press). There are two general methods for modifying gene sequences: random mutagenesis based on error-prone PCR techniques and DNA recombination or shuffling (Yuan et al. 2005). The DNA shuffling method is used to generate novel DNA fragments (Stemmer 1994). This can be used to combine DNA fragments independently of their genetic backgrounds and is dependent only on DNA sequence homologies. However, error-prone PCR and DNA shuffling both require the screening of large numbers of recombinants to obtain enzymes of desired characteristics. Unfortunately, our established laccase expression systems, tobacco cell culture and *A. oryzae* culture cannot be used for high-throughput expression for screening. Therefore, we have constructed a chimeric laccase cDNA, *Lcc4/1*, by conventional DNA recombination using the N-terminus of the *Lcc4* cDNA and the C-terminus of the *Lcc1* cDNA, expressed it in tobacco cell culture, and characterized the chimeric enzyme.

Materials and methods

Expression of chimeric laccases

The *L. edodes* dikaryotic cultivation strain H600 (obtained from Hokken Co., Ltd.) was used in all experiments. The deduced amino-acid sequences of Lcc1 and Lcc4 are shown in Figure 1A. The *Lcc4* (DNA Data Bank of Japan (DDBJ) accession no. AB446445) open reading frame (ORF) was amplified by PCR using cDNA isolated from the fully browned gills of *L. edodes* as a template. The PCR was carried out with KOD-Plus (Toyobo) using the following cycling parameters and primers: 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s, and 68 °C for 105 s; Lcc4-5′ (5′-ATGCGTCTACTCTTGACTTC-3′) and Lcc4-3′ (5′-TCAAAGCTGGTCGGGCTTGAG-3′). The resulting PCR fragment was cloned into pGEM-T (Promega) and sequenced, and the plasmid was designated pTLc4. The *Lcc1* (DDBJ accession no. AB035409) ORF was PCR amplified, using the same cycling parameters as above and the primers Lcc1-5′ (5′-ATGTTTTACTTCTCATCTTT-3′) and Lcc1-3′ (5′-TTAATTTCCACCAAGTTGTGC-3′), and cloned into pGEM-T. The resulting plasmid was designated pTLc1 (Sakamoto et al. 2008).

Chimeric laccases were constructed using pTLc4 as the PCR template. For amplification of the *Lcc4* N-terminus (ATG-849), primers Lcc4-5′ and Lcc4-852e-3′ (5′-ATTGGGAGAAGCCCGGATCC-3′) were used. For amplification of the *Lcc4* C-terminus (849-1584), primers Lcc4-852e-5′ (5′-GGATCCGGG-CTTCTCCCAAT-3′) and Lcc4-3′ were used. The PCR products and pTLc1 were digested with *Bam* HI and *Spe* I for *Lcc1/4*, and *Bam* HI and *Sph* I for *Lcc4/1*,