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Secretory expression of *Lentinula edodes* intracellular laccase by yeast high-cell-density system: Sub-milligram production of difficult-to-express secretory protein

Takeshi Kurose,[1] Yuta Saito,[2] Koichi Kimata,[2] Yuko Nakagawa,[3,§] Akira Yano,[3] Keisuke Ito,[1,2] and Yasuaki Kawarasaki[1,2,*]

Biomolecular Engineering Lab, Graduate School of Integrated Pharmaceutical and Nutritional Sciences, University of Shizuoka, Yada 52-1, Suruga-ku, Shizuoka 422-8526, Japan,[1] School of Food and Nutritional Sciences, University of Shizuoka, Yada 52-1, Suruga-ku, Shizuoka 422-8526, Japan,[2] and Iwate Biotechnology Research Center, 22-174-4 Narita, Kitakami, Iwate 024-0003, Japan[3]

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While a number of heterologous expression systems have been reported for extracellular laccases, there are few for the intracellular counterparts. The *Lentinula edodes* intracellular laccase Lcc4 is an industrially potential enzyme with its unique substrate specificity. The heterologous production of the intracellular laccase, however, had been difficult because of its expression-dependent toxicity. We previously demonstrated that recombinant yeast cells synthesized and, interestingly, secreted Lcc4 only when they were suspended to an inducing medium in a high cell-density (J. Biosci. Bioeng., 113, 154–159, 2012). The high cell-density system was versatile and applicable to other difficult-to-express secretory proteins. Nevertheless, the system's great dependence on aeration, which was a practical obstacle to scale-up production of the enzyme and some other proteins, left the secretion pathway and enzymatic properties of the Lcc4 uncharacterized. In this report, we demonstrate a successful production of Lcc4 by applying a jar-fermentor to the high cell-density system. The elevated yield (0.6 mg L^{-1}) due to the sufficient aeration allowed us to prepare and purify the enzyme to homogeneity. The enzyme had been secreted as a hyper-glycosylated protein, resulting in smear band-formations in SDS-PAGE. The amino acid sequencing analysis suggested that the N-terminal 17 residues had been recognized as a secretion signal. The recombinant enzyme showed similar enzymatic properties to the naturally occurring Lcc4. The characteristics of the scale-upped expression system, which includes helpful information for the potential users, have also been described.

[**Key words:** *Lentinula edodes* laccase; High cell-density expression; Difficult-to-express protein; Yeast extracellular production; Recombinant protein]

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a widely occurring multi-copper enzyme capable of oxidizing a range of phenolic compounds with a simultaneous reduction of molecular oxygen (1). Fungal laccases possess a variety of enzymatic properties and play a primary role in degradation of wood components in ecosystem (2). Among various laccases, a limited species of extracellular enzymes such as those from *Trametes versicolor* and *Agaricus bisporus* have predominantly been used in a certain range of industrial processes (3,4). Further applications of other laccases are not fully progressing because of (i) difficulties in cultivation of their source organisms, (ii) their insufficient yields, (iii) high heterogeneity as well as impurity of the enzyme-containing fractions, and (iv) potential risks behind the use of enzymes from non-edible organisms (3–7). To circumvent those problems, many efforts have been made to establish heterologous expression systems. Nevertheless, the recombinant laccase productions are notoriously difficult in general as described (8,9).

Lentinula edodes, also known as shiitake mushroom, is an edible white-rot fungus and possesses paralogous genes for two distinct laccase isozymes. The two isozymes, Lcc1 (accession no. AB035409) (10) and Lcc4 (accession no. AB446445) (11), share a moderate (60%) identity in their amino acid sequences (12,13). These isozymes are synthesized in different organs and developmental stages in the *L. edodes* life cycle, and play different biological roles: Lcc1 is secreted from mycelia and capable of oxidizing lignin as well as other typical substrates such as 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (10), whereas the Lcc4 is synthesized in the mushroom's gill as an intracellular enzyme (11,13). Like Lcc1, Lcc4 is capable of oxidizing ABTS and possesses relatively higher activity to such phenolic compounds as catechols and catecholamines. Moreover, unlike Lcc1 and other fungal extracellular laccases, Lcc4 shows an expanding spectrum in substrate specificity to L-dihydroxyphenylalanine (L-DOPA), which is responsible for the melanin synthesis in the gill (11–13).

Heterologous expression systems for the Lcc1 and Lcc4 were initially constructed with tobacco BY-2 (14) and *Aspergillus oryzae* (15) as the host organisms, respectively. Other cost-efficient hosts including yeast *Saccharomyces cerevisiae*, however, have refused the expression of those isozymes, partly because of the expression-dependent cellular toxicity (16). Recently, Wong et al. reported

* Corresponding author at: Yada 52-1, Suruga-ku, Shizuoka 422-8526, Japan. Tel./fax: +81 54 264 5540.
E-mail address: kawarsky@u-shizuoka-ken.ac.jp (Y. Kawarasaki).
§ Present address: Department of Chemical Engineering, Ichinoseki National College of Technology, Hagisho, Ichinoseki, Iwate 021-8511, Japan.

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extracellular production of the two Lcc1 subtypes (i.e., Lcc1A [JN607250] and Lcc1B [JN607251]) by recombinant *Pichia pastoris* (9). Although their yields have not clearly been stated there, the relatively lower specific activity of the partially purified enzymes (3–5 U mg^{-1}) than the naturally occurring Lcc1 (348 U mg^{-1}) (10) and the heterogeneity that appears in their zymogram suggest that heterologous expression and purification of *L. edodes* laccases are yet challenging.

In parallel, we have developed a novel and versatile method, designated high cell-density system, for the functional expression of difficult-to-express secretory proteins (16). In this system, yeast cells harboring expression plasmid are suspended in a small aliquot of inducing medium to form a high cell-density culture (e.g., OD_{660} = 15). The system has been successfully applied to small-scale expressions of several difficult-to-express secretory proteins including extracellular catalytic domain of hDPPIV (17) and miracle fruit miraculin (18), as well as the two *L. edodes* laccases (16). Interestingly, the Lcc4, an intracellular enzyme, was synthesized as an extracellular enzyme like the Lcc1. As described, the yield of those products increased to 1000- to 6000-folds (16). A further analysis on the synthesized laccase was, however, not readily performed because of a practical difficulty; The high cell-density system is not amenable to simple scale-up, probably due to its increased oxygen-requirement that cannot be satisfied in a conventional flask-scale induction (16).

Here we show a preparative-scale production of Lcc4 by applying a bench-top jar-fermentor to the high cell-density expression system. To the best of our knowledge, this would be the first report that performed preparative-scale expression of fungal intracellular laccase with recombinant *S. cerevisiae*. The increase in the yield allowed us to purify the secreted Lcc4 to homogeneity by two-steps of column chromatography. Analyses of the purified enzyme revealed the path on which the intrinsically intracellular Lcc4 stepped to the culture supernatant in the recombined cell. Moreover, the results presented in the jar-fermentor-based system provide practically useful information to the researchers who struggle with his/her difficult-to-express secretory proteins.

MATERIALS AND METHODS

Materials and chemicals All chemicals used in this study were reagent-grade purity. Laccase substrates and amino acids were purchased from Wako Pure Chemical Industries (Osaka, Japan). Compounds for media were from Sigma–Aldrich (St. Louis, MO, USA) or Wako. The jar-fermentor MDL-200 was from B. E. Marubishi Co., Ltd (Tokyo, Japan). Resins (Toyopearl-Butyl and -SP) for the Lcc4 purification were from Tosoh (Tokyo, Japan). EndoH was purchased from New England Biolabs (Ipswich, MA, USA).

Yeast strain, culture media and cultivation condition The galactose-inducible expression vector pBG13 (19) was used for the construction of Lcc4 expression plasmid (pBGlcc4) as described previously (1). FGY217 (*MATa, pep4Δ, ura3-52, lys2Δ201*) was used as the host strain. The Lcc4-expressing transformant, FGY217 (pBGlcc4), was maintained and grown in a synthetic medium containing 2% glucose without uracil (SD-U). For the Lcc4 production, K-medium (SG supplemented with 0.5 mM $CuSO_4$, lacking uracil, cysteine and tyrosine) (16) or K2-medium [K-medium with enriched galactose (4%)] was used as the inducing medium. The high cell-density expression was performed as described: Briefly, FGY217 (pBGlcc4) cells precultured in SD-U at 30°C to OD_{660} = 3–4 were harvested by a centrifugation, then suspended in a small aliquot of K- or K2-medium to adjust the OD_{660} to 15. In the case of preparative-scale Lcc4 production, the cells cultured in totally 4-L of SD-U were suspended in K2-medium and poured into a jar-fermentor (model MDL-200 by B.E. Marubishi; vessel size: 2 L) equipped with a chilling-water circulation unit (Eyela CCA-1111, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The inducing cultivation was carried out at 20°C with a vigorous agitation (typically 2.0–2.2 vvm at 600 rpm).

Enzyme assay The amount of extracellularly produced laccase was measured by quantifying the enzyme activity in the culture supernatant. The assay was basically performed at pH 4.0, according to the method described by Nagai et al., with ABTS as a substrate (10,11). One unit of laccase activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of ABTS in 1 min at 30°C (11). Characterization of the purified Lcc4 was also performed according to the Nagai's publication (11).

Enzyme purification Purification of Lcc4 from the culture supernatant was carried out by two-steps of column chromatography. First, ammonium sulfate and Triton X-100 were added to the culture supernatant to give final concentrations of 30 saturation% and 0.03 w/v%, respectively. A small aliquot of 1M acetic acid was then added to adjust the pH to 4.5. After a glasswool-clarification, proteins in the culture supernatant were adsorbed on a Toyopearl-butyl column (Φ = 15, L = 30 mm), and eluted by a linear gradient of ammonium sulfate (30-0 sat%; buffered with 20 mM sodium acetate (pH 4.5) containing 0.03 w/v% Triton X-100). The fractions with laccase activity were pooled, then subjected to Toyopearl-SP column chromatography (Φ = 15, L = 30 mm). The adsorbed Lcc4 was eluted by a linear gradient of sodium chloride [0–200 mM; buffered with 20 mM sodium acetate (pH 4.5)].

RESULTS AND DISCUSSION

Preparative-scale production of Lcc4 using bench-top fermentor The naturally occurring Lcc4 is synthesized in shiitake fruiting body as an intracellular enzyme, whereas the recombinant enzyme was somehow secreted outside of the yeast host cells when expressed in the high cell-density system. This is, however, a practically preferable nature, since extracellular productions of enzymes simplify the downstream purification in general. Nevertheless, understanding the secretion mechanism as well as clarifying the differences between the recombinant Lcc4 and its native version are necessary for the future application of the recombinant enzyme.

To obtain sufficient amount of recombinant Lcc4 for the purification, we first optimized the expression condition in test-tube- or flask-scale cultivation, where the aeration was on the critical or less sufficient for the laccase production (16). Briefly, high cell-density expression (i) at lower temperature (18–20°C, Supplementary Fig. S1), (ii) with an increased amount of the inducer (i.e., galactose up to 4%, Supplementary Fig. S2) enabled the densely suspended host cells to secrete the elevated amount of Lcc4 in a prolonged induction period in a test-tube scale. Yet, Lcc4 production was diminished in the flask-scale cultivation (Fig. 1). Since the synthesized laccase was stable in the supernatant for a week (data not shown), the diminished laccase yield in the scale-up production was unlikely caused either by the increased instability of the enzyme or its susceptibility to proteases.

We next tested a bench-top jar-fermentor system for the high cell-density expression, to supply a sufficient amount of air to the cell suspension. The yeast cells precultured in non-inducing medium (4 L in total) were harvested, and suspended to inducing K2-medium to give a dense cell suspension (i.e., 15 OD_{660}), then poured into the Marubishi fermentor vessel (2 L in volume). The cell suspension was agitated at 600 rpm at 20°C, with a vigorous (2 L

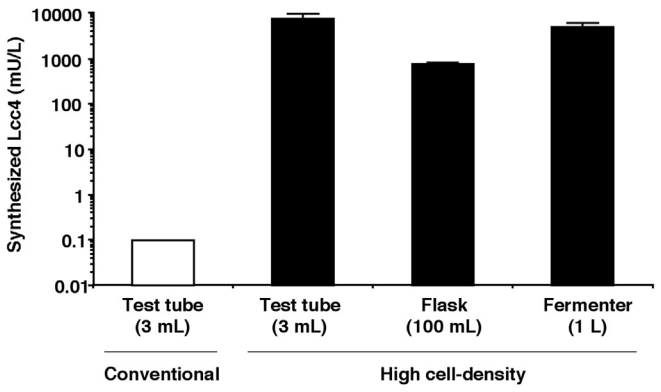

FIG. 1. Yield comparison. The amounts of extracellularly synthesized Lcc4 yielded in various production systems are illustrated in bars. The open bar on the left represents the amount of Lcc4 synthesized in a conventional growth-associated manner (test-tube scale, in 3 days). The high cell-density expression was performed in various scales with different cultivation vessels, with the same induction condition (20°C for 3 days with vigorous agitation). The volumes of the cell suspension have also been shown. Note that bars were depicted in logarithmic scale.