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Identification of an extracellular bacterial flavoenzyme that can prevent re-polymerisation of lignin fragments

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ABSTRACT

A significant problem in the oxidative breakdown of lignin is the tendency of phenolic radical fragments to re-polymerise to form higher molecular weight species. In this paper we identify an extracellular flavin-dependent dehydrolipoamide dehydrogenase from *Thermobifida fusca* that prevents oxidative dimerization of a dimeric lignin model compound, which could be used as an accessory enzyme for lignin depolymerisation.

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1. Introduction

The aromatic heteropolymer lignin is a major component of plant lignocellulose cell walls, and is liberated during cellulosic bioethanol production, and pulp/paper manufacture. The valorisation of lignin to produce aromatic chemicals is an important problem in the design of a future biorefinery for production of fuels and chemicals from renewable sources [1], and several new approaches involving either chemocatalysis [2–6] or biocatalysis [7–10] have been published in recent years.

One significant technical obstacle in the valorisation of lignin is the tendency of lignin fragments formed during oxidative cleavage to "repolymerise" or "condense" to form higher molecular weight species, via polymerisation of radical intermediates formed during lignin breakdown [11]. This problem has been encountered using fungal lignin peroxidase [12–14] and laccase enzymes [15,16] and with bacterial Dyp peroxidase [17] and laccase [18] enzymes, and is also encountered in chemocatalytic valorisation of lignin, where a high molecular weight char is observed alongside depolymerised products [1]. Kirk & Farrell commented on this problem in their review of microbial lignin oxidation in 1987, noting that polymerisation of lignin is not prominent *in vivo*, therefore they suggested that phenoxy radical intermediates "are reduced back to the phenols by an undiscovered enzyme and/or mechanism that prevents polymerisation" [19]. In basidiomycete *Pleurotus ostreatus*, a FAD-dependent aryl alcohol oxidase was shown in 1995 to reduce quinonoids and phenolic radicals, and to prevent polymerisation of laccase-generated phenolic compounds [20], but no further progress has been made to prevent lignin repolymerisation enzymatically, and no such enzyme has been identified in bacteria that can oxidise lignin.

In previous studies with bacterial *Rhodococcus jostii* peroxidase DypB, we found that addition of *C. kluyveri* diaphorase changed the distribution of products obtained upon conversion of a lignin model compound by DypB [17], consistent with an enzymatic single-electron reduction of phenolic radical intermediates by a reduced flavin coenzyme. We noted that in a proteomic study of thermophilic cellulose degrader *Thermobifida fusca* by Adav *et al* [21], the most abundant extracellular protein was found to be a dihydrolipoamide dehydrogenase (accession Q47R85), one of four dihydrolipoamide dehydrogenase sequences in the *T. fusca* genome (see Supplemental Fig. S1). We therefore hypothesised that this extracellular flavin-dependent enzyme might be responsible for one-electron reduction of phenolic radical intermediates during lignin breakdown. We report here evidence that this enzyme can prevent lignin repolymerisation.

2. Materials and methods

2.1. Cloning of T. fusca dihydrolipoamide dehydrogenase gene

The thermophile *Thermobifida fusca* YX (DSMZ) was grown in Luria-Bertani broth overnight at 45 °C. Genomic DNA was isolated using a Wizard Genomic DNA Purification Kit (Promega). Forward

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(5' CACC GTG AGT GAA AGC GGC GGC ACA TTC 3') and Reverse primers (5' TCA GTC GTG GAC GTG CAA CGG C 3') for the DLDH gene were designed with the intention of cloning into a pET151/D-TOPO vector. The gene was amplified from genomic DNA by polymerase chain reaction using Pfx Taq polymerase, using 34 cycles of: 30 s at 94 °C, 30 s at 58 °C; 90 s at 72 °C. The PCR product (1.4 kb) was excised from a 1% agarose gel and extracted using a GeneJET PCR Purification Kit (Thermo Scientific), and cloned into a pET151/D-TOPO vector (Invitrogen) using manufacturer's instructions, and transformed into One Shot TOP10 competent cells (Invitrogen). The DNA sequence was confirmed via DNA sequencing.

2.2. Overexpression of recombinant *T. fusca* dihydrolipoamide dehydrogenase

A 1L culture of *T. fusca* DHLDH pET151/TOP10 in Luria-Bertani broth containing 50 μg/ml ampicillin was grown at 37 °C for 2–3 h. At OD595 = 0.6, riboflavin (0.5 mM) and IPTG (0.5 mM) were added to the culture, which was then incubated in a shaker overnight at 15 °C at 200 rpm. After centrifugation at 6000*g*, the cell pellet was re-suspended in 10 ml of lysis buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10 mM imidazole). Cells were lysed using a cell disruptor at 21 kpsi. The cell lysate was then centrifuged at 13,000 g for 40 min. DLDH was then purified using nickel affinity chromatography. The supernatant was incubated with 2 ml of Ni^{2+} Sepharose beads at 4 °C for 60 min. This cell supernatant-Ni^{2+}-Sepharose mixture was then placed in disposable plastic column and the resin washed with 50 ml of wash buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 20 mM imidazole), then DLDH was eluted with 7 ml of elution buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl and 250 mM imidazole). Fractions were collected and analyzed by SDS-PAGE gel electrophoresis. Fractions containing DLDH (48 kDa band) were pooled and incubated overnight at room temperature with TEV protease (1 mg/ml) in order to remove the His tag. The DLDH-TEV mixture was centrifuged at 13,000 g for 15 min, and the supernatant was concentrated using a 10 kDa Millipore Centricon. Native DLDH was purified by passing the solution through a 5 ml HisTrap Ni^{2+} resin FPLC column, then desalted using a PD10 desalting column.

2.3. Dihydrolipoamide dehydrogenase assay

All assays were run in 50 mM sodium phosphate buffer pH 6.0 at 25 °C, in the presence of 0.2 mM NADH. Enzyme kinetics were measured through the decrease in the absorption spectrum of NADH at 340 nm over 2 min, as previously described by Youn et al. [22].

2.4. HPLC assays

Catalytic action of DHLDH toward oxidised lignin model compound was studied by reaction of model compound with *Pseudomonas fluorescens* Dyp1B enzyme in presence of *T. fusca* DLDH enzyme and NADH. The reaction was performed in 50 mM phosphate buffer pH 6.0 at 25 °C in presence of 0.4 mM model compound, 25–200 μM NADH, 560 nM Dyp1B, 210 nM DHLDH and 0.5 mM hydrogen peroxide. The following reactions were set up: 1) model compound + buffer + H_2O_2; 2) model compound + Dyp1B + H_2O_2; model compound + Dyp1B + H_2O_2 + NADH; 3) model compound + Dyp1B + H_2O_2 + NADH + DHLDH. The solutions were incubated at 25 °C for 30 min. Aliquots (500 μL) were taken and these fractions were analyzed by HPLC. Aliquots for HPLC were mixed with CCl_3COOH (100%, w/v, 50 μL), and the solution was then centrifuged for 10 min at 10,000 rpm. HPLC analysis was conducted using a Phenomenex Luna 5 μm C18 reverse phase column (100 Å, 50 mm, 4.6 mm) on a Hewlett-Packard Series 1100 analyzer, at a flow rate of 0.5 mL/min, with monitoring at 270 nm. The gradient was as follows: 50% MeOH/H_2O for 5 min; 50–80% MeOH/H_2O from 5 to 12 min; 80% MeOH/H_2O from 12 to 25 min; 80-30% MeOH/H_2O from 25 to 30 min. Peaks of interest were collected for further analysis by mass spectrometry and MS-MS fragmentation.

3. Results

T. fusca dihydrolipoamide dehydrogenase was cloned from genomic DNA, and was expressed as a His_6 fusion protein from a pET151 vector in *Escherichia coli*. The recombinant protein expressed well as a 48 kDa protein, was purified by metal affinity chromatography, and the fusion tag removed by digestion with TEV protease (see Supplemental Fig. S2). Addition of riboflavin to the growth media was found to yield recombinant enzyme with stoichiometric amounts of flavin cofactor (λ_{max} 460 nm, see Supplemental Fig. S3). The recombinant enzyme was catalytically active for NADH-dependent reduction of lipoic acid, with k_{cat} 0.14 s^{-1} and K_m 430 μM, and showed maximal activity at pH 6.0. The enzyme also showed NADH-dependent quinone reductase activity with *p*-benzoquinone (k_{cat} 0.42 s^{-1} K_m 540 μM, see Supplemental Figs. S4–S6), comparable with activity reported for dihydrolipoamide dehydrogenase from *Streptomyces seoulensis* [22].

We have previously reported that Dyp-type peroxidase DypB from *Rhodococcus jostii* RHA1 is able to oxidatively cleave β-aryl ether lignin model compound **1** [17]. Treatment of **1** with peroxidase Dyp1B from *Pseudomonas fluorescens* [23] or *Thermobifida fusca* Dyp peroxidase [24] leads to the formation of a new peak observed by C_{18} reverse phase HPLC at retention time 15.5 min (see Fig. 1, trace B). Analysis of this peak by electrospray mass spectrometry gave *m/z* 660.4, corresponding to an oxidative dimer of **1**, and further analysis of this species by MS-MS fragmentation has identified this species as dimer **2** arising from oxidative dimerization of **1** [24]. The formation of compound **2** therefore represented a convenient model for oxidative repolymerisation of lignin fragments.

Addition of 210 nM *T. fusca* dihydrolipoamide dehydrogenase (DHLDH) and 100 μM NADH to an incubation of 1.5 mM β-aryl ether **1** with *P. fluorescens* Dyp1B was found to completely prevent the formation of **2** (see Fig. 1 trace E, and Supplemental Fig. S7), whereas addition of NADH alone had no effect (trace B). Treatment of either the oxidised product **2** or lignin model compound **1** with *T. fusca* DHLDH gave no change as observed by HPLC, indicating that DHLDH acted upon an intermediate species formed during the oxidation of **1** to **2**. The disappearance of product **2** was found to be dependent upon the concentration of NADH, showing partial disappearance of **2** at 25 and 50 μM NADH (traces C,D). Since the dimer **2** is formed in approximately 10% yield from **1** (trace A), and prevention of dimerization occurs at 50–100 μM NADH, the stoichiometry of NADH required to prevent formation of **2** is in the range 1–2 nmoles NADH/nmole of dimer **2**.

The formation of **2** from **1** could be rationalised by dimerization of a phenoxy radical formed from **1**. In support of this mechanism, the conversion of **1** to **2** was also observed upon addition of nitroxyl radical reagent TEMPO to **1**. The catalytic mechanism of dihydrolipoamide dehydrogenase is known to proceed via reduction of an active site disulfide cysteine pair by reduced flavin [25,26]. We therefore propose the catalytic cycle shown in Fig. 2, where the reduced form of the active site disulfide reduces a phenoxy radical intermediate via a 1-electron transfer, to generate a cysteine radical intermediate. We have not observed any flavin semiquinone intermediate by pre-steady state kinetic analysis of *T. fusca* DHLDH, therefore we propose a further 1-electron transfer to a phenoxy radical, generating the oxidised active site disulfide, which is

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