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Enhanced archaeal laccase production in recombinant *Escherichia coli* by modification of N-terminal propeptide and twin arginine translocation motifs

Sivakumar Uthandi · Laurence Prunetti · Ian Mitchelle S. De Vera · Gail E. Fanucci · Alexander Angerhofer · Julie A. Maupin-Furlow

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Abstract Laccases are multicopper oxidases that couple the oxidation of phenolic polymers to the reduction of molecular oxygen. While an archaeal laccase has only recently been described (LccA from the culture broth of *Haloferax volcanii*), this enzyme appears promising for biotechnology applications based on its robust bilirubin oxidase and laccase activities as well as its ability to withstand prolonged exposure to extreme conditions. To further optimize LccA productivity and develop an option for LccA purification from whole cells, the encoding gene was modified through deletion of the twin-arginine translocation motif and N-terminal propeptide, and the modified genes were expressed in *Escherichia coli*. With this approach, LccA was readily purified (overall yield up to 54 %) from the soluble fraction of *E. coli* as a 74-kDa monomer with syringaldazine oxidizing activity as high as 33 U mg^{-1}. LccA proteins prepared from *H. volcanii* culture broth and the soluble fraction of *E. coli* cells were compared by ICP-AES, EPR, DSC, CD, and UV–Vis spectroscopy and found to have a similar folding pattern with T_m values and a rich β-sheet structure analogous to other multicopper oxidases. However, in contrast to the *H. volcanii*-purified LccA, which was loaded with copper, copper was not fully incorporated into the type-I Cu center of *E. coli* purified LccA, thus, providing insight into avenues for further optimization.

Keywords Multicopper oxidase · Haloarchaea · Recombinant protein · Phenol oxidation

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S. Uthandi · L. Prunetti · J. A. Maupin-Furlow (✉)
Department of Microbiology and Cell Science, University of Florida, PO Box 110700, Bldg. 981 Museum Rd., Gainesville, FL 32611-0700, USA
e-mail: jmaupin@ufl.edu

S. Uthandi
e-mail: usivakumartnau@gmail.com

L. Prunetti
e-mail: lprunetti@ufl.edu

I. M. S. De Vera · G. E. Fanucci · A. Angerhofer
Department of Chemistry, University of Florida, Gainesville, FL, USA
e-mail: idevera@ufl.edu

G. E. Fanucci
e-mail: fanucci@chem.ufl.edu

A. Angerhofer
e-mail: alex@chem.ufl.edu

J. A. Maupin-Furlow
Genetics Institute, University of Florida, Gainesville, FL 32611-7200, USA

Present Address:
S. Uthandi
Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, India

Introduction

Laccases (para-diphenol:dioxygen oxidoreductases, EC 1.10.3.2) are a diverse group of multicopper oxidases (MCOs) that catalyze the oxidation of a wide range of inorganic and aromatic compounds, particularly phenols such as lignin, while reducing molecular oxygen to water. Since laccases are implicated in numerous biological activities and oxidize a broad spectrum of substrates, these enzymes are of interest for use within different industrial fields, such as pulp delignification, textile dye bleaching, and bioremediation [27]. The search for new, efficient, and

environmentally safe processes for industries has increased interest in laccases, which work essentially as 'green' catalysts that use air and produce water as the only by-product, making them more generally available to the scientific community [27].

Laccases are produced by many filamentous fungi (e.g., *Coprinus cinereus* and *Neurospora crassa*), plants (e.g., *Rhus vernicifera*), a few insects (e.g., *Bombyx* species) and some bacteria (e.g., *Azospirillum lipoferum*) [20]. Among the increasing number of bacterial laccases reported, several with distinctive functions have been described, including laccases with roles in morphogenesis and sporulation, pigment production, and resistance to copper and phenolic compounds [4]. The bacterial CotA (a laccase from *Bacillus subtilis*) and McoA (a metallo-oxidase from *Aquifex aeolicus*) gained much importance owing to their potential for biotechnological application [10]. While laccases from eukaryotes and bacteria have been extensively studied, archaeal multicopper oxidase homologs are less understood. Only one archaeal laccase (LccA of the haloarchaeon *Haloferax volcanii*) that oxidizes phenolics has been characterized at the protein level so far [33]. A related multicopper oxidase (McoP) from the hyperthermophilic archaeon *Pyrobaculum aerophilum* has been shown to have nitrous oxide reductase activity [11]. Enzymes from extremophilic archaea, such as the *H. volcanii* LccA, are promising for industrial applications, as they have high intrinsic thermal and chemical stability [33].

To cater to the needs of industries for various applications, it is desirable to produce biocatalysts in large quantities and at low cost. Heterologous laccase production may enhance enzyme yield and permit the synthesis of modified forms of the enzyme that are optimized for industrial applications. Conversely, laccases purified from native sources are often associated with low yield and high input cost, which is unsuitable for large-scale enzyme production. In the past, laccases have been expressed in different recombinant host systems such as *Saccharomyces cerevisiae* [18], *Pichia pastoris* [15, 26], *Trichoderma reesei* [29], *Aspergillus oryzae* [2], *Aspergillus niger* [28], and *E. coli* [11, 22, 32]. We recently reported purification of a highly active laccase (LccA) from the extracellular fractions of the archaeon *H. volcanii* [33]. LccA catalyzes the oxidation of bilirubin, syringaldazine, and other phenolics [33]. Consistent with its discovery in an extremophilic archaeon, LccA is stable at high temperature (50 °C) and high concentrations of salt (2M NaCl) and solvent (25 % [v/v] methanol, ethanol, DMSO or DMF) with half-lives of inactivation greater than 1 day [33].

The present work is focused on the development of an expression system to produce the archaeal LccA in a host cell that is commonly used in the biotechnology industry (i.e., the bacterium *Escherichia coli*). Our previous work failed to yield high levels of LccA protein in recombinant *E. coli* when the full-length *H. volcanii lccA* gene was used for the expression system [33]. Here we report that modification of the twin-arginine translocation (TAT) motif and the N-terminal propeptide sequence of *H. volcanii* LccA results in successful production of LccA in a soluble and active form in recombinant *E. coli*. This study provides one of the few examples of an *E. coli*-expressed laccase that is not produced as an insoluble aggregate in the cell.

Materials and methods

Materials

Biochemicals were from Sigma-Aldrich (St. Louis, MO). Other organic and inorganic analytical-grade chemicals were from Fisher Scientific (Atlanta, GA). Restriction endonucleases, T4 DNA ligase and Vent DNA polymerase were from New England Biolabs (Ipswich, MA). Phusion DNA polymerase was from New England Biolabs (Ipswich, MA). Desalted oligonucleotides were from Integrated DNA Technologies (Coralville, IA). Molecular biology grade agarose, prestained Kaleidoscope protein standards and Precision Plus unstained protein standards were from Bio-Rad Laboratories (Hercules, CA). SeaKem GTG agarose used for separation and isolation of DNA fragments prior to ligation was from FMC Bioproducts (Rockland, ME).

Microbial strains and media

Bacterial and archaeal strains used in this study are summarized in Table 1. *E. coli* DH5α was used for routine recombinant DNA experiments. *E. coli* Rosetta (DE3) was used for expression of *lccA* and its site-directed variants. *E. coli* strains were grown at 37 °C in Luria–Bertani medium unless otherwise indicated. *Haloferax volcanii* strains were grown at 42 °C in complex (YPC) medium as previously described [8]. Ampicillin (Ap, 100 $\mu g\ ml^{-1}$), kanamycin (Km, 50 $\mu g{\cdot}ml^{-1}$), chloramphenicol (Cm, 30 $\mu g\ ml^{-1}$), novobiocin (Nv, 0.1 $\mu g\ ml^{-1}$) and $CuSO_4$ (250 μM) were included as needed. Cultures were grown in liquid (with rotary shaking at 150–200 rpm) and solid media (15 % [w/v] agar plates).

Site-directed mutagenesis and strain construction

Plasmids and primers used in this study are summarized in Table 1. Plasmid pJAM823 [33], carrying *lccA* (Hvo_B0205), was prepared from *E. coli* DH5α and used as a template in a polymerase chain reaction (PCR) with

Table 1 List of strains, plasmids, and oligonucleotide primers used in this study

Strain, plasmid, or primer	Description	Source or reference
Strains		
E. coli		
DH5α	F^- *recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA relA1*	Life Technologies
Rosetta(DE3)	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pRARE (Cm^R)	Novagen
X-L1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* F′*proAB lac*IqZΔM15 Tn10 Tet^r	Stratagene
US02E	Rosetta (DE3)-pJAM823 for synthesis of LccA	[33]
US04	Rosetta (DE3)-pJAM831 for synthesis of LccA R6K R7K R8K	This study
US05	Rosetta (DE3)-pJAM835 for synthesis of LccA Δpro	This study
H. volcanii		
DS70	Wild-type isolate DS2 cured of plasmid pHV2	[34]
H26	DS70 *pyrE2*	[1]
US02	H26-pJAM824 for synthesis of LccA	[33]
Plasmids		
pET24b	Km^r; *E. coli* expression vector	Novagen
pJAM823	Km^r; pET24b with *lccA* for expression in Rosetta (DE3)	[33]
pJAM824	Ap^r; Nv^r; pJAM202 with P2$_{rrn}$-*lccA* for expression in *H. volcanii*	[33]
pJAM830	Ap^r; Nv^r; pJAM202 with P2$_{rrn}$-*lccA* with R6K R7K R8K	This study
pJAM831	Km^r; pET24b with *lccA* with R6K R7K R8K for expression in *E. coli*	This study
pJAM835	Km^r; pET24b with *lccA* with *Δpro* for expression in *E. coli*	This study
Primers		
HvoB0205 *lccA* up	5′-TGGGCGTCATATGacagactggtctaggcggcgg-3′	[33]
HvoB0205 *lccA* up Δpro	5′ -GCGGCTCATATGgcgtcaccgacgttg-3′	This study
HvoB0205 *lccA* down	5′-AAAAGCTTtcaggccacttcgtcgccgcttc-3′	[33]
LccA R6K R7K R8K FW	5′-cagactggtctaAgAAgAAgttcttacagacaggc-3′	This study
LccA R6K R7K R8K Rev	5′-gcctgtctgtaagaacTTcTTcTtagaccagtctg-3′	This study

Ap^r ampicillin resistance, *Nv^r* novobiocin resistance, *Km^r* kanamycin resistance; Δpro, propeptide deleted from LccA. *Uppercase letters* in primer sequences represent nucleotides that were altered from genome sequence to introduce site-directed mutation(s) or restriction enzyme site

primer pairs designed to change the arginine (R) residues at positions 6, 7, and 8 in the deduced polypeptide to lysine (K). Quickchange polymerase was used for PCR with buffer and nucleotide concentrations according to Stratagene. PCR was performed with an iCycler (Bio-Rad Laboratories). PCR products were treated with DpnI and transformed into *E. coli* XL-1 Blue to generate plasmid pJAM830 (*lccA* R6K R7K R8K or *Δtat*). The *lccAΔtat* gene from pJAM830 was further modified by ligation into NdeI and BlpI sites of pET24b to generate pJAM831 for expression in *E. coli*. PCR was also performed using primer pairs designed to amplify *lccA* without its propeptide sequence (*Δpro*) using *H. volcanii* DS70 genomic DNA as the template. PCR generated-DNA fragments of appropriate size (1.6 kb) for *lccAΔpro* were isolated from 0.8 % (w/v) SeaKem GTG agarose (FMC Bioproducts, Rockland, ME) gels in TAE buffer using the QIAquick gel extraction kit (Qiagen) and ligated into the NdeI to HindIII sites of pET24b to generate plasmid pJAM835 for expression in *E. coli*. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA), and the fidelity of cloned DNA sequences were confirmed by Sanger DNA Sequencing (ICBR Genomics Facility, University of Florida).

Microbial growth and enzyme purification

Escherichia coli Rosetta (DE3) was transformed with plasmids pJAM831 (*lccAΔtat*) and pJAM835 (*lccAΔpro*) to generate US04 and US05, respectively (Table 1). Strains were grown in 2.8-l Fernbach flasks at room temperature (200 rpm) in 500 ml of LB medium supplemented with 250 μM $CuSO_4$, 50 $\mu g{\cdot}ml^{-1}$ km and 30 $\mu g{\cdot}ml^{-1}$ Cm. Isopropyl *β*-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM to log-phase cells (OD_{600} of 0.5). Cultures were further incubated at room temperature (150 rpm, 4 h). Cells (80 ml culture) were harvested by centrifugation (10 min at 9,200 × *g* and 4 °C), resuspended in 20 mM Tris–HCl buffer at pH 7.5, and lysed by French press (2,300 psi). Cell lysate was clarified by centrifugation (10 min at 9,200 × *g* and 4 °C), filtered (0.45-μm filter), and dialyzed (2×, 2.5 h at 4 °C)

against 20 mM Tris–HCl buffer at pH 7.5 supplemented with 2 M NaCl. Samples were equilibrated to low salt by dialysis (2×, 2.5 h at 4 °C) against T buffer (25 mM Tris–HCl, pH 8.4) supplemented with 0.2 M NaCl. Proteins were filtered (0.2 μm) and applied to a MonoQ 5/5 column (Pharmacia) equilibrated with T buffer. Active fractions were eluted at 625–675 mM NaCl by a linear gradient of NaCl (0–1 M) in T buffer at 0.5 ml min^{-1}. LccA of US04 was further purified by Superdex 200 HR 10/30 chromatography (Pharmacia) in T buffer supplemented with 0.15 M NaCl (at 0.3 ml min^{-1}). Fractions with laccase activity were pooled, separated by reducing 12 % SDS-PAGE, and stained in gel with Coomassie Blue R-250 to monitor purity. Samples were equilibrated by dialysis against T buffer supplemented with 0.2 M NaCl prior to storage at 4 °C.

Enzyme activity

Oxidation of syringaldazine (SGZ) (at 1 mM) was monitored in triplicate by absorbance increase at 526 nm by UV/Visible-spectroscopy using a BioTek Synergy HT multi-detection microplate reader with a 96-well plate format. Standard assay was at 45 °C in T buffer supplemented with 185 mM NaCl. Copper sulfate (1 mM) was included for assay of the *E. coli* produced LccA. One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of substrate per min ($\varepsilon = 65{,}000\ M^{-1}\ cm^{-1}$ at 526 nm). Laccase activity was also detected in-gel. Proteins were separated by native electrophoresis (using 10 % Tris–glycine gels) followed by immersion of gels in T buffer supplemented with 1 mM SGZ. Laccase activity was rapidly visualized in-gel as a pink color. For detection of total protein, gels were counterstained in Coomassie Blue R-250 and destained according to supplier (Bio-Rad). Images of activity and protein stain were acquired using a VersaDoc 1000 imaging system (Bio-Rad). Protein concentration was determined based on the method of Bradford using Bio-Rad Protein Assay with bovine serum albumin as a standard. All assays and protein purifications were performed at least in triplicate.

N-terminal sequencing

LccA proteins, purified from US04 and US05, were separated by reducing 12 % SDS-PAGE and transferred by electroblotting to Immobilon-P PVDF membranes (Amersham Biosciences) at 100 V for 100 min at 4 °C. Proteins were stained in membrane with 0.2 % (w/v) Coomassie Blue R-250 in 40 % acetic acid for 30 s and rinsed with deionized water. LccA protein bands were excised and subjected to automated Edman degradation for N-terminal sequencing as previously described [33].

UV–Vis, EPR, CD, and DSC

UV–Visible spectra were acquired using a Beckman Coulter DU spectrophotometer with LccA protein at 50 μM in T buffer containing 0.2 M NaCl. X-band EPR spectra were measured with a Bruker Elexsys E580 EPR spectrometer equipped with a continuous helium flow cryostat (Oxford ESR900) using the standard rectangular TE_{102} cavity. In the EPR experiments, purified protein was at 100 μM and was supplemented to 20 % (vol/vol) glycerol in T buffer with 0.2 M NaCl to prevent protein aggregation upon freezing. Sample (100 μl) was inserted into 3 × 4 mm (ID × OD) quartz tubes, flash-frozen in pre-cooled isopentane (near its melting point of 113 K), and inserted into a cryostat pre-cooled to 20 K. Instrumental parameters were as follows: microwave power (P_{mw}), 0.63mW; modulation amplitude, 20 G (for US02) and 10 G (for US05); modulation frequency, 100 kHz; receiver gain, 60 dB; receiver time constant, 41 ms; conversion sampling time, 82 ms; temperature, 20 K; points per spectrum, 1024; averages per spectrum, 16 (for US02) and four (for US05); microwave frequencies (ν), 9.441771 GHz (for US02) and 9.443192 GHz (for US05); G/point, 1.953 (for US02) and 1.563 (for US05). All spectra were normalized and baseline corrected against a background of buffer solution under non-saturating conditions. Spectra were simulated using the EasySpin toolbox in Matlab™ [31].

Circular dichroism (CD) data were acquired on an Aviv 202 spectrometer from Aviv Biomedical (Lakewood, NJ) similar to previously described [13]. LccA protein was at 0.1 mg per ml in T buffer supplemented with 0.2 M NaCl. The parameters used for CD experiments are summarized in Suppl. Table S1. The CD signal from the buffer was subtracted from the protein signal and converted to mean residue ellipticity (deg cm^2 $dmol^{-1}$) using Eq. (1).

$$[\theta] = \frac{\theta}{10.N.C.l} \quad (1)$$

In Eq. (1), θ is the ellipticity (deg), defined as $\theta = \tan^{-1}$ (b/a), where a and b are the major and minor axes of the resulting elliptically polarized light; N is the number of amino acid residues in the protein (548 based on the mature LccA protein sequence); C is the protein molar concentration, and l is the path length in cm with $l = 0.1$ cm, based on the path length of Hellma CD cuvettes (Müllheim, Germany) used in this experiment.

A MicroCal VP-DSC microcalorimeter (Northampton, MA) was employed in differential scanning calorimetry (DSC) experiments. Sample cells were injected with 20 μM LccA protein in T buffer with 0.2 M NaCl. Reference cells were injected with T buffer with 0.2 M NaCl (no protein). Temperature was increased from 30 to 100 °C at a

scan rate of 60 °C h^{-1}. Results were plotted as differential power or heat flow in mW as a function of temperature in °C. Heat flow was subsequently converted to heat capacity (C_p, kcal mol^{-1} deg^{-1}). The peak maximum for each plot was estimated to be the melting temperature (T_m) of the protein, the temperature when half of the protein is in the denatured state.

Copper content

The copper content of purified protein samples was measured according to Method 200.7 [21]. The samples were diluted and analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) according to service protocol (Garratt-Callahan Company, CA).

Results and discussion

Expression of an archaeal laccase in *E. coli*

Biotechnological applications often require the development of methods for large-scale purification of enzymes, either directly from the native organism or from expression of cloned genes in a recombinant host. Our previous work [33] demonstrated that LccA is readily purified from the extracellular fraction of *H. volcanii* as an active laccase (with the encoded polypeptide cleaved to generate an enzyme with an N-terminal residue corresponding to Ala32 of Hvo_B0205). Conversely, when *lccA* was expressed with its wild-type coding sequence in *E. coli* using plasmid pET24b under the control of the T7 promoter, the levels of LccA protein (denatured-SDS PAGE and native state) and laccase activity (oxidation of syringaldazine or SGZ) were undetectable. The mechanism of how LccA is secreted and processed to remove an N-terminal propeptide in native host *H. volcanii* remains to be determined. However, analysis of the Hvo_B0205 sequence revealed conserved motifs suggesting that LccA is secreted by the TAT system and cleaved by a leader peptidase in *H. volcanii*. Most of the proteins predicted to be secreted in haloarchaea have conserved TAT signal sequences [6]. Thus, the TAT system is thought to be the major mechanism of protein translocation in this group of archaea [6]. While the TAT pathway is known to transport fully folded proteins with their cofactor bound before translocation in *E. coli*, the Sec system is the predominant system for protein translocation in this as well as most other bacteria [3]. The preference for Sec substrates may explain the undetectable levels of LccA produced in *E. coli*.

To overcome the limited synthesis of LccA in recombinant *E. coli*, the *lccA* gene sequence was mutated to encode LccA with either a modified twin-arginine translocation (TAT) motif (R6K R7K R8K or *Δtat*) or deletion of its N-terminal propeptide (ΔMet1 to Ala31 of the deduced polypeptide or *Δpro*). With this approach, the *H. volcanii* laccase was produced at high levels in recombinant *E. coli* grown in medium supplemented with 250 μM $CuSO_4$ (Fig. 1a). The reason for the limited production of LccA with its wild-type coding sequence in *E. coli* is unknown, since this bacterium does use TAT targeting and translocation for protein export. However, either deletion of the N-terminal propeptide or modification of the TAT motif overcame this limitation and resulted in high-level synthesis of LccA in the soluble cellular fraction. Kiiskinen and Saloheimo [17] have demonstrated a similar strategy based on the expression of an ascomycete laccase without its N-terminal propeptide in a heterologous host (*Saccharomyces cerevisiae*) and obtained higher protein yield than when the original gene sequence was expressed. Likewise, the *silA*-encoded laccase from the bacterium *Streptomyces ipomoea* could be synthesized at detectable levels in the cytosolic fraction of *E. coli* using an N-terminal His-tag fused to its TAT signal sequence after purification by Ni^{2+}-affinity chromatography [25].

LccA purified from recombinant *E. coli*

Unlike most organisms, both the cytosol and extracellular milieu of haloarchaea are of high ionic strength. Thus, haloarchaeal enzymes often require the presence of high concentrations of salt for stability and activity. Halophilic proteins usually feature an excess of acidic amino acids (e.g., p*I* of 4.31 for LccA) with the negative surface charge important in maintaining enzyme solubility in high salt [24]. The unusual properties of halophilic proteins can pose problems for expression in heterologous hosts such as *E. coli*, since halophilic proteins typically misfold and aggregate in conditions of low ionic strength. In this study, activation of LccA required the cell lysate, of recombinant *E. coli* expressing LccA, to be dialyzed against buffer supplemented with high salt (2 M NaCl). *E. coli*-expressed LccA was not active prior to dialysis in high-salt buffer. However, after high-salt activation, LccA was dialyzed and stored in low-salt (200 μM) buffer to maintain optimal laccase activity for months at 4 °C. Like LccA, the citrate synthase of *H. volcanii* is also reactivated after synthesis in *E. coli* by overnight incubation in high-salt (2 M NaCl) buffer [5].

Laccase activity was monitored in recombinant *E. coli* strains expressing full-length and site-directed variants of *lccA* using syringaldazine (SGZ) as a substrate. SGZ is an effective reagent for detecting laccase activity [14]. As previously reported [33], *E. coli* strains carrying pET24b or pET24b with the full-length *lccA* coding sequence did not produce detectable levels of laccase activity. In contrast,

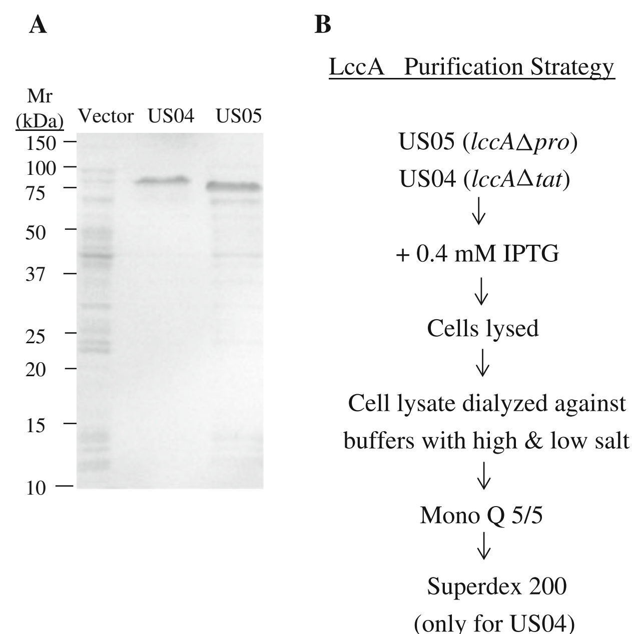

Fig. 1 Synthesis of *H. volcanii* LccA in recombinant *E. coli.* **a** Reducing SDS-PAGE analysis of cell lysate derived from *E. coli* Rosetta(DE3) with plasmid pET24b (vector), pJAM831 (US04 *lccAΔtat*) or pJAM835 (US05 *lccAΔpro*). Cells were grown for 4 h in the presence of 0.4 mM IPTG prior to harvesting. Proteins (1 µg per lane) were separated by reducing SDS-PAGE and stained with Coomassie Brilliant Blue R-250. **b** Strategy for purification of LccA from the cell lysate of recombinant *E. coli.* US05 (*lccAΔpro*) expresses LccA with the propeptide deleted. US04 (*lccAΔtat*) expresses LccA as an R6K R7K R8K variant

laccase was readily detected at specific activities of ~0.6 U mg^{-1} of protein in the soluble fraction of lysate prepared from *E. coli* strains carrying pET24b with either *lccAΔtat* (US04) or *lccAΔpro* (US05) (Table 2). Thus, modification of the *lccA* coding sequence (particularly the N-terminal propeptide and TAT signal sequence motif) resulted in enhanced levels of laccase activity in recombinant *E. coli*.

LccA was readily purified as an active enzyme from the soluble fraction of recombinant *E. coli* strains US04 (*lccAΔtat*) and US05 (*lccAΔpro*) (Fig. 1b). LccA was purified 41-fold from US04 (*lccAΔtat*) with a specific activity of 25 U·mg^{-1} protein and final yield of 13 % (Table 2). Similarly, LccA from US05 (*lccAΔpro*) was purified 56-fold with a specific activity of 33 U mg^{-1} protein and final yield of 54 % (Table 2). These specific activity values for laccase are comparable to LccA purified by single-step chromatography from the extracellular matrix of *H. volcanii* US02 (52 U mg^{-1} protein with a 22-fold purification and final yield of 28 %) [23]. Of the two *E. coli* strains, the LccA from US05 (*lccAΔpro*) required only single-step chromatography for purification from cell lysate while US04 (*lccAΔtat*) required two column steps. In comparison, when the *B. subtilis* CotA laccase is purified from recombinant *E. coli*, it is produced inside the cell but requires refolding from inclusion bodies for its activity [7].

The final fractions of LccA purified from *E. coli* US04 (*lccAΔtat*) and US05 (*lccAΔpro*) were estimated to be 74 kDa based on SDS-PAGE (Fig. 2a) and gel filtration (data not shown). The 74-kDa molecular mass is similar to that previously described for LccA purified from the extracellular medium of *H. volcanii* and reveals a monomeric configuration when the molecular mass estimated by gel filtration is compared to the deduced polypeptide sequence. Interestingly, the LccA protein in the cell lysate and early MonoQ chromatography fractions of US04 (*lccA*Δtat) was estimated at 85 kDa. A reduction of ~10 kDa was observed from the MonoQ anion-exchange chromatography step to the Superdex 200 gel filtration step suggesting that the N terminus of LccA is processed during purification from US04 (*lccAΔtat*) (Fig. 2a). Indeed, N-terminal sequencing of LccA from US04 (*lccAΔtat*) after gel filtration revealed an N-terminal sequence comparable to LccA from *H. volcanii* US02 and corresponding to Ala32 of the deduced polypeptide sequence. Long-term storage of the MonoQ fractions of the 85-kDa LccA in 0.6 M NaCl (at 4 °C) did not result in cleavage to 74 kDa, suggesting the N-terminal propeptide is optimally removed from the *E. coli* produced-LccA when exposed to the low salt (0.15 M) buffer used for gel filtration. In contrast, LccA was expressed in US05 (*lccAΔpro*) without a propeptide by fusing an ATG start codon to the Ala32 codon

Table 2 Purification of LccA from recombinant *E. coli* strains US05 and US04

Fraction	Total activity[a] (U)	Protein (mg)	Yield (%)	Sp Act (U mg^{-1})	Purification (fold enrichment)
US05 (*lccAΔpro*)					
Cell lysate	9.2	16	100	0.59	1
MonoQ 5/5	5.0	0.15	54	33	56
US04 (*lccAΔtat*)					
Cell lysate	22	36	100	0.60	1
MonoQ 5/5	9.7	0.87	45	11	18
Superdex 200	2.7	0.11	13	25	41

[a] Per 80-ml culture broth

of Hvo_B0205. Similarly to the other LccA proteins, N-terminal sequencing of the LccA from *E. coli* US05 (*lccAΔpro*) revealed an N-terminal residue corresponding to Ala32 of Hvo_B0205. However, the LccA proteins from *H. volcanii* US02 and *E. coli* US04 (*lccAΔtat*) strains are thought to be processed by the host leader peptidase to remove the 31-residue N-terminal propeptide. In contrast, the LccA from US05 (*lccAΔpro*) is likely cleaved by a methionine aminopeptidase between Met1 and Ala2 (where Ala2 corresponds to Ala32 of LccA open reading frame, HVO_B0205). Methionine aminopeptidases remove N-terminal methionine residues from proteins that have penultimate residues with small side chains (e.g., Ala) [19].

In-gel activity stain (after native electrophoresis) revealed the LccA proteins purified from *E. coli* US04 (*lccAΔtat*) and US05 (*lccAΔpro*) were active against SGZ similar to LccA purified from *H. volcanii* US02 (Fig. 2b). However, the number of LccA isoforms detected by this method varied among the strains. In particular, there was a significant reduction in the banding pattern of LccA purified from US04 (*lccAΔtat*) compared to LccA purified from *H. volcanii* US02 or *E. coli* US05 (*lccAΔpro*). Only two active bands were detected, for the US04-purified MonoQ fractions of LccA, with both of these bands migrating slower than the three LccA isoforms detected for the other two strains (US02 and US05) (Fig. 2c). In parallel with the 10-kDa reduction in the molecular mass of LccA during purification from US04 (as determined by SDS-PAGE, described above), the migration pattern of LccA from US04 differed between the MonoQ and Superdex 200 chromatography fractions based on in-gel activity stain of proteins separated by native PAGE, with the final fraction migrating as a single isoform (Fig. 2c). The observed reduction in specific activity of LccA when purified from US04 might be correlated with these differences in isoform patterns compared to US05 and US02. Since LccA from US05 purifies similar to its native counterpart (LccA from *H. volcanii* US02), with comparable yield and specific activity (Table 2), further experiments on the biochemical properties of LccA from recombinant *E. coli* were carried out using protein purified from US05 (*lccAΔpro*).

Copper content and spectroscopic analyses

Typical metal content of laccases includes a type-1 Cu site (T1), a type-2 Cu site (T2), and a dinuclear type-3 Cu site (T3), with T2 and T3 arranged in a trinuclear cluster (TNC). The T1 Cu site contains the blue copper, whose tight coordination to a cysteine is responsible for an intense SCys → Cu(II) charge transfer transition at around 600 nm, giving the typical blue color to the enzyme. T2 shows a characteristic electron paramagnetic resonance (EPR) spectrum, clearly distinct from that of T1, whereas the T3 copper dimer is anti-ferromagnetically coupled and EPR-silent [27]. In this study, a variety of spectroscopic methods were used to analyze copper coordination in the T1, T2, and T3 sites.

Inductively coupled argon plasma optical emission spectroscopy was used to measure the total copper content of the purified LccA proteins. *E. coli* US05-derived LccA had a lower copper content (1.12 mol per mole of protein) than its counterpart produced from *H. volcanii* US02

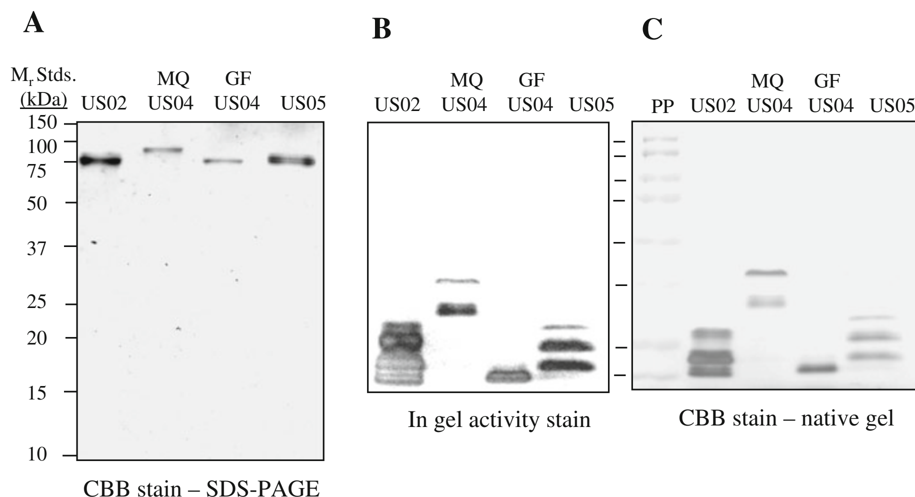

Fig. 2 Electrophoretic comparison of LccA purified from *H. volcanii* and recombinant *E. coli* strains. LccA protein from *H. volcanii* US02, *E. coli* US04 (*lccAΔtat*) MonoQ (MQ) and gel filtration (GF) fractions, and *E. coli* US05 (*lccAΔpro*) are indicated. Proteins (1 µg per lane) were separated by reducing SDS-PAGE (**a**) and native gel electrophoresis (**b** and **c**). LccA protein was detected by staining for total protein by Coomassie Brilliant Blue R-250 (CBB) and in gel activity using SGZ as the substrate as indicated. M_r stds., indicate Prestained Kaleidoscope Standards (used for estimation of molecular mass of LccA polypeptide). *PP* Precision Plus unstained proteins (included in native gel for comparison of protein migration, not estimation of molecular mass)

(3.8 mol per mole of protein), suggesting improper copper loading in the *E. coli*-produced protein.

UV–visible spectroscopy was used to further analyze the Cu centers of the LccA proteins. Laccase enzymes typically have a UV–visible spectrum that includes a band at ~600 nm that corresponds to the Cu-Cys interaction at the T1 Cu center and a shoulder at 330 nm, indicative of the presence of a T3 Cu center [30]. While comparing UV–visible spectra of LccA from US02 with US05, both proteins had a similar shoulder at ~330 nm, which corresponds to the T3 Cu center. However, the peak at 600 nm, corresponding to a T1 Cu center, was weak for *E. coli*-derived LccA (US05) compared to LccA from *H. volcanii* US02, suggesting that copper is not fully incorporated in the former protein (Fig. 3). Consistent with the UV–visible spectra, the LccA fractions from *H. volcanii* strain US02 are visibly blue, whereas the *E. coli*-derived LccA lacks this distinct color even at comparable concentrations of protein, indicating poor copper incorporation into the enzyme.

The EPR spectra were also determined for LccA proteins purified from *H. volcanii* US02 and *E. coli* US05 strains (Fig. 4) (see also supplemental information for simulations, Fig. S1 to S5). The *H. volcanii* US02-purified LccA spectrum (solid line, Fig. 4) exhibited a predominant type I (T1) copper signal with spectral parameters of $g_\perp = 2.046$, $g_{II} = 2.226$, and $A_\perp = 233$ MHz (see the four hyperfine-split g_{II}-signals at 292, 300, 307, and 315 mT together with the main g_{II}-line at 330 mT). The low-field region of the spectrum also included a residual type II (T2) Cu(II) signal (see insert with partial spectrum of the US02 sample below 290 mT multiplied by a factor of 10 and showing a peak at 273 mT) (Fig. 4). Conversely, the *E. coli* US05-derived LccA (broken line, Fig. 4) predominantly showed a T2 Cu(II) signal (see three visible g_{II}-signals at 273, 292, and 311 mT together with the $g_\perp$-line at 329 mT and an off-principal axis or overshoot line at 336 mT) with no sign of T1 Cu(II). The signal could be simulated with $g_\perp = 2.052$, $g_{II} = 2.238$, and $A_\perp = 576$ MHz. Furthermore, this signal was distinct from that of free aqueous Cu(II) in the buffer solution, which showed parameters of $g_\perp = 2.048$, $g_{II} = 2.237$, and $A_\perp = 571$ MHz, although it cannot be excluded that the preparation contained some small background of free Cu(II). If we assume that the low-field peak at 272 mT in the spectrum of LccA purified from *H. volcanii* US02 corresponds to copper bound in the T2 site we can estimate a 1:2.2 molar ratio of copper for T2: T1 sites in this preparation based on the double integration of the spectral simulations (see supplemental information, Fig. S5).

In addition to EPR, the LccA proteins from *H. volcanii* US02 and *E. coli* US05 were analyzed by CD spectroscopy (Fig. 5) and differential scanning calorimetry (DSC) (Fig. 6). Common CD spectral features were detected for the LccA proteins including a minimum mean residual ellipticity at around 211–214 nm, which is indicative of β-sheet structures [16] and comparable to other published multicopper oxidases [9, 11]. In addition, the melting temperatures (T_m) determined by DSC for LccA from US02 and US05 (≈70 °C) were not significantly different, since minimum error attributed to data resolution is ±0.3 °C. These results suggest that production of LccA in *E. coli* US05 does not affect the stability or secondary

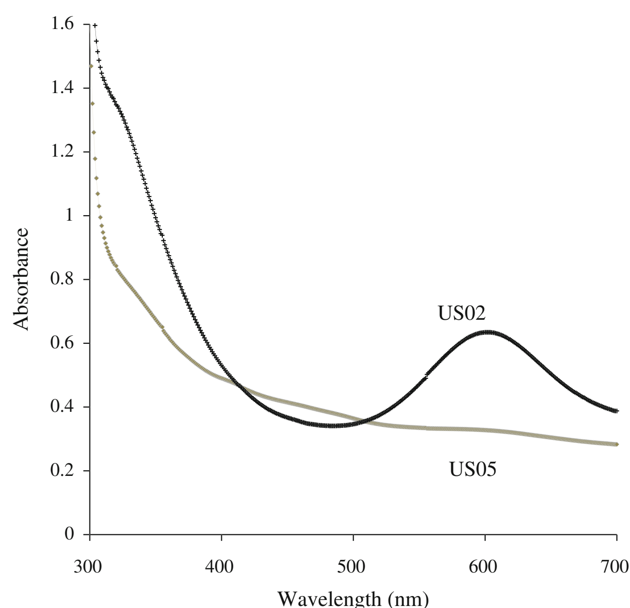

Fig. 3 UV-visible absorbance spectra of LccA purified from recombinant *H. volcanii* (US02, *grey line*) and *E. coli* (US05, *black line*)

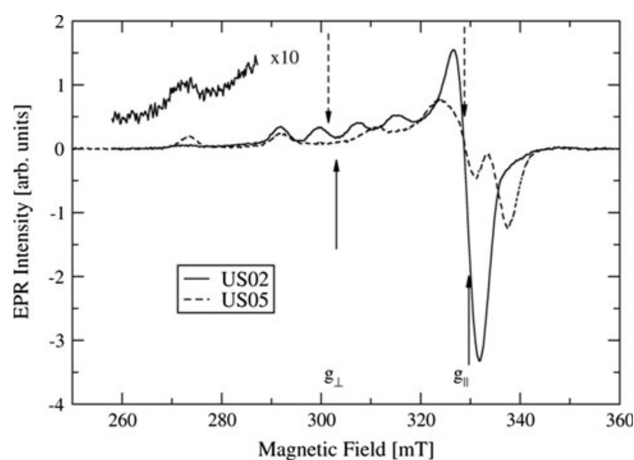

Fig. 4 EPR spectra of *H. volcanii* (US02, *solid line*) and *E. coli* (US05, *broken line*)-derived LccA. The insert displays the low-field portion of the *H. volcanii* US02-derived LccA spectrum (below 290 mT) multiplied by a factor of 10. See "Materials and methods" for details on spectra acquisition

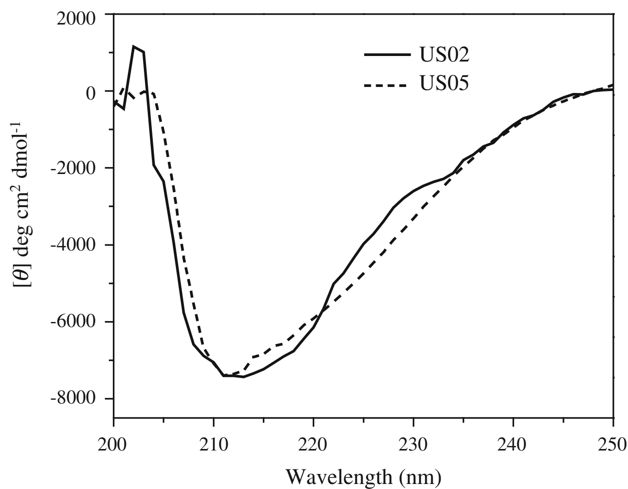

Fig. 5 CD spectra in the far-UV region of LccA purified from recombinant *H. volcanii* (US02, *solid line*) and *E. coli* (US05, *broken line*), reflecting rich β sheet content and comparable secondary structures

structure of this protein when compared to the production of LccA in *H. volcanii* US02.

Based on the spectral analyses, we suggest that one factor limiting high-level synthesis of LccA activity in recombinant *E. coli* is the lack of full content of copper needed for optimal enzyme activity. Poor copper coordination is a common problem with metallooxidases produced in recombinant *E. coli* as demonstrated not only in this but other studies including synthesis of the *B. subtilis* CotA [22], *E. coli* CueO [12], and *P. aerophilum* McoP [11]. One possibility for the poor copper incorporation into LccA is that copper was lost when LccA was purified from the lysate of *E. coli* (compared to the culture broth of

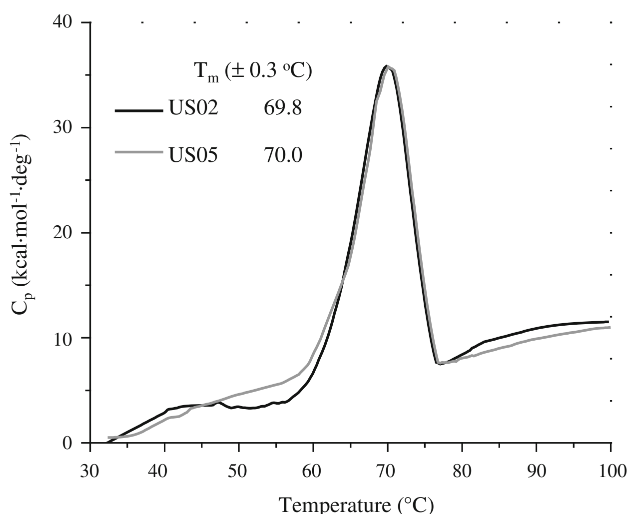

Fig. 6 DSC spectra of LccA purified from recombinant *H. volcanii* (US02, *black line*) and *E. coli* (US05, *grey line*), reveal similar melting temperatures

H. volcanii). Alternatively, LccA (although not in aggregates) was not properly folded when produced in *E. coli*. Unlike LccA purified from the culture broth of *H. volcanii*, salt was required for the activation of the 'salt-loving' LccA when it was synthesized in the low ionic strength cytosol of *E. coli*. It is also possible that a molecular chaperone is used to load copper into the T1, T2, and T3 sites of MCOs, such as LccA, which is not conserved between bacteria and archaea. Further understanding the molecular mechanism of copper incorporation into multicopper oxidases is likely to facilitate high level synthesis of these types of metalloenzymes in recombinant systems.

Conclusions

Here we report construction of a recombinant host (*E. coli*) for synthesis of an archaeal laccase (LccA). Modification of the N-terminal propeptide of LccA resulted in high-level production of the LccA protein in *E. coli* (as visualized by SDS-PAGE). Brief exposure of the cell lysate of these *E. coli* strains to high-salt buffer activated LccA without the need for extensive refolding of the protein. The yield of LccA in an active form was somewhat low when purified from the cell lysate of *E. coli* (1.9 mg l^{-1} culture) compared to the culture broth of *H. volcanii* (8 mg l^{-1} culture). However, the laccase activity of the cell lysate of these recombinant *E. coli* strains could be useful for some applications that do not require highly pure enzyme (can generate laccase activity at nearly 300 U l^{-1} culture within 1–2 days from inoculum to cell lysate). Another advantage of developing the recombinant *E. coli* system is that it now adds the flexibility of producing LccA in cell lysate (*E. coli* US05) as well as in culture broth (*H. volcanii* US02).

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