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Expression, refolding, and characterization of a small laccase from *Thermus thermophilus* HJ6

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ABSTRACT

An open reading frame of the *Thermus thermophilus* HJ6 hypothetical laccase, which composed of 729 bases, was cloned and expressed as a fusion protein with six histidine residues in *Escherichia coli* SoluBL21™ cells. The resulting insoluble bodies were separated from cellular debris by centrifugation and solubilized with 6 M guanidine HCl. The solubilized protein was refolded by a simple on-column refolding procedure using Ni-chelation affinity chromatography and then the refolded protein was purified by gel filtration chromatography. It showed a single band with a molecular mass of 27 kDa in SDS–PAGE. The results from UV–visible absorption and electron paramagnetic resonance (EPR) analysis suggested that the enzyme had the typical copper sites, type-1, 2, and 3 Cu(II) of laccase. The purified enzyme exhibited the laccase activity with the optimal catalytic temperature at 75 °C. The optimum pH for the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and syringaldazine was 4.5 and 6.0, respectively. The recombinant protein showed high thermostability, and the half-life of heat inactivation was about 50 min at 85 °C. The enzyme oxidized various known laccase substrates, its lowest K_m value being for syringaldazine, highest k_{cat} value for guaiacol, and highest k_{cat}/K_m for 2,6-dimethoxy-phenol. The enzyme reaction was strongly inhibited by the metal chelators and the thiol compounds.

Introduction

Laccases (benzenediol:oxygen oxidoreductase; EC 1.10.3.2) belong to the family of the blue multicopper oxidases, a large group of enzymes characterized by the presence of at least four copper ions distributed in cupredoxins domains. The type-1 copper (T1 copper) is responsible for pulling electrons from a substrate, which are subsequently delivered to a metal cluster formed by the three other copper ions (one T2 copper and two T3 coppers). This trinuclear cluster is capable of binding oxygen, which, in this redox system, is the final electron acceptor [1,2]. These enzymes catalyze the oxidation of a wide range of substrates including phenolics, polyphenolics, aromatic amines, lignin, and other compounds with the concomitant reduction of dioxygen to water [3–5]. They are sometimes also referred to as polyphenol oxidases (PPOs[1]) and are a part of the multicopper oxidase family [6].

Laccases are widely distributed in nature and have been described in fungi [7,8], plants [9], insects [10], and more recently, in bacteria [11–14] and archaea [15]. The physiological function of plant and fungal laccases has been exhaustively studied and shown to be related to the synthesis and degradation of lignin, respectively [16]. Among the increasing number of bacterial laccases reported, several with distinctive functions have been described, including roles in morphogenesis and sporulation processes, pigment production, and resistance to copper and phenolic compounds [12,17–19]. In addition to physiological functions, due to their broad substrate specificity, laccases have attracted considerable interest for application in many fields of industrial and

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[1] *Abbreviations used:* PPOs, polyphenol oxidases; CV, column volume; EPR, electron paramagnetic resonance; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; ORF, open reading frame; MCO, multi-cooper oxidase.

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environmental processes, including pulp bleaching, textile dye bleaching, effluent detoxification, washing-powder components, wine clarification, transformation of antibiotics and steroids, biosensing and biofuel cells [20–22].

One of the general prerequisites for an enzyme to be applicable in industrial processes is thermostability or thermotolerance [23]. In general, thermostable enzymes usually have higher resistance to chemical denaturants, high alkalinity or extreme acidity, increased substrate solubility and higher reaction rates at elevated temperatures, and less susceptible to microbial contaminations [23,24]. Enzymes from thermophiles, in particular, are promising for industrial applications, as they have high intrinsic thermal and chemical stability. So far, the most thermostable laccase, with a half-life of thermal inactivation at 80 °C of over 14 h, has been found in the extremely thermophilic bacterium *Thermus thermophilus* HB27 [25]. Recently, a cooper-activated metallo-oxidase, McoA, from the thermotolerant bacterium *Aquifex aeolicus* was reported to demonstrate heat stability at 80 and 90 °C, with activity lasting for up to 9 and 5 h, respectively [26]. In order to obtain a new thermostable laccase, we found a putative laccase gene, which encodes a new member of the multi-copper oxidase family from the genome databases of the thermophilic bacterium *T. thermophilus* HB8 (GenBank No. AP008226), HB27 (AE017221), and JL-18 (CP003252). In this study, we cloned, sequenced, and expressed the gene encoding a new small laccase (*Tt*SLAC) from *T. thermophilus* HJ6 in *Escherichia coli*. *T. thermophilus* HJ6 is an aerobic chemoorganotroph, gram negative rod, extremely thermophilic bacterium that grows between 80 and 95 °C, and optimally at 80 °C [27]. We also report the refolding and biochemical characterizations of the recombinant *Tt*SLAC protein.

Materials and methods

Chemicals, strains, and plasmids

DNA primers and substrates were prepared by Bioneer (Daejeon, Korea). Taq DNA polymerase was purchased from Takara (Tokyo). Chemicals used for the determination of laccase activity were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of reagent grade. *E. coli* DH5α and BL21 (DE3) codon plus (Novagen, Inc., San Diego, CA, USA) were used as the cloning and expression host cells, respectively. The plasmid pET-21a was purchased from Novagen as the expression vector for the laccase.

Cloning and DNA sequencing

Chromosomal DNA of *T. thermophilus* HJ6 was prepared using the GeneAll® GENEx Genomic kit (GeneAll Biotechnology, Seoul, Korea). To obtain the *Tt*SLAC gene from HJ6, PCR was performed using two primers (Primer-1, 5′-CAGGCCTTCTGGAGGGGGTGGCCCC-3′ and Primer-2, 5′-GTGCCACGGGCCCCTGTACGCTGCC-3′) which were designed based on the upstream sequence of the initiation codon (ATG) and the downstream sequence of the termination codon (TAA), for a multicopper oxidase from the genome databases of the thermophilic bacterium *T. thermophilus* HB8 (GenBank No. AP008226), HB27 (AE017221), and JL-18 (CP003252).

DNA amplification was carried out using Taq DNA polymerase for 30 cycles at 95 °C for 30 s, 55 °C for 60 s, and 72 °C for 60 s. To prepare templates for sequencing, the amplified 0.73-kbp fragment was cloned into pGEM®-T as a *Tt*SLAC candidate and the construct was designated as pGEM-TtSLAC. DNA sequencing analysis was performed by an ABI Prism 3700 genetic analyzer (Perkin–Elmer Applied Biosystems, Foster City, CA, USA). The nucleotide sequence of the *Tt*SLAC gene from *T. thermophilus* HJ6 was submitted to the GenBank nucleotide sequence database under the accession number GU233492.

Construction of the enzyme

To construct the expression vector using an *Xho* I recognition site of pET-21a plasmid DNA, *Xho* I recognition site within a *Tt*SLAC in pGEM-TtSLAC was silent mutated using overlap extension PCR. PCR techniques were applied using two complementary mutagenic primers, S1 (5′-GACCGCCTCAAGGCGCTCCAG-3′) and S2 (5′-CTGGAGCGCCTTGAGGCGGTC-3′), and two outer primers, S3 (5′-GGTGGCCATATGACCCTCCTGCGCACCCC-3′, the *Nde* I site is underlined) and S4 (5′-CCGCTCGAGACGGGGGGGAAGCATGACG-3′, the *Xho* I site is underlined). As for C398T mutant construction, S3 primer and mutagenic primer S1 were annealed with the pGEM-TtSLAC and the intermediate DNA was produced by PCR. S4 primer and mutagenic primer S2 were then annealed with the pGEM-TtSLAC and the second intermediate was produced by PCR. PCR amplification was conducted for 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 74 °C for 1 min using Pfu DNA polymerase (INTRON Biotech., Korea). The intermediate products were joined by PCR with two outer primers S3 and S4, and the amplified mutagenic fragment contained C398T mutation was digested with restriction enzymes *Nde* I and *Xho* I. The resulting fragment was inserted into pET-21a that contains a region coding for His-tag sequence (Novagen). The resulting plasmid was designated as pET-*Tt*SLAC. The C398T mutation was verified by nucleotide sequencing.

Protein expression and purification from inclusion bodies

The solubilization of inclusion bodies was carried out based on the procedure of Glykys et al. [28]. *E. coli* SoluBL21™ cells (Genlantis, Inc., San Diego, CA, USA) harboring constructed pET-TtSLAC were used to inoculate 10 ml LB cultures supplemented with 100 μg/ml ampicillin and were grown overnight at 37 °C to saturation. One liter LB cultures with 100 μg/ml ampicillin were inoculated with 10 ml from the overnight cultures and were grown at 37 °C in the presence of 1 mM $CuCl_2$ to an OD_{600} of approximately 0.5. The temperature was lowered to 25 °C and IPTG was added to a final concentration of 0.3 mM. After 16 h of growth, the cells were pelleted by centrifugation and the cell pellets were frozen at −20 °C. The thawed pellets were sonicated on ice and the lysate was separated by centrifugation at 27,000×*g* for 10 min. The supernatant was discarded and the insoluble fractions containing the laccases were resuspended in 20 ml of ice-cold buffer containing 20 mM Tris–HCl pH 8.0, 500 mM NaCl, 2 M urea and 2% Triton X-100. The insoluble material was twice separated by centrifugation at 27,000×*g* for 10 min. The resulting washed pellets were resuspended in 50 ml Binding buffer (20 mM Tris–HCl pH 8.0, 500 mM NaCl, 6 M guanidine HCl, 5 mM imidazole and 1 mM 2-mercaptoethanol) and stirred for 30–60 min at room temperature. The solution was centrifuged at 27,000×*g* for 10 min to remove unsolubilized material.

The solubilized laccases were loaded onto HisTrap HP column (GE Healthcare, bed volume, 5 ml) for simultaneous purification and refolding of the protein. In a typical run, 50 ml of inclusion bodies solution was injected to the column and washed with 10 column volume (CV) of binding buffer followed by 10 CV of Washing buffer (20 mM Tris–HCl pH 8.0, 500 mM NaCl, 6 M urea, 20 mM imidazole and 1 mM 2-mercaptoethanol). Refolding was completed by changing the washing buffer to a Refolding buffer (20 mM Tris–HCl pH 8.0, 500 mM NaCl and 20 mM imidazole) using a linear gradient over 30 CV. Experiments were performed with and without the addition of 1 mM $CuCl_2$ to the refolding

buffer. After washing with 5 CV of refolding buffer, the protein was eluted with 10 CV of Elution buffer (20 mM Tris–HCl pH 8.0, 500 mM NaCl and 500 mM imidazole). The fractions with refolded laccase were pooled and concentrated to 5 ml using an Amicon Ultra-15 Centrifugal Filter Unit (10 kDa MWCO). The concentrated samples were loaded on a HiPrep Sephacryl S-100 HR 26/60 column (GE Healthcare) and eluted with buffer (20 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM $CuCl_2$). The purity of the recombinant protein was confirmed by SDS–PAGE. Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard.

UV/visible and EPR spectra

The purified protein (1.47 mg/ml) was dialyzed against copper absence buffer (50 mM sodium acetate buffer, pH 6.0). The UV–visible absorption spectra of the dialyzed protein were obtained at room temperature using a UV–Vis spectrophotometer (Optizen 2120UV, Mecasys, Korea). The electron paramagnetic resonance (EPR) spectrum was recorded on a Bruker EMX spectrometer (X-band) (Bruker BioSpin, Karlsruhe, Germany) at 77 K with a modulation amplitude of 2 G, modulation frequency 100 kHz, microwave power 19.5 mW, and microwave frequency 9.64 GHz. The protein sample contained 0.27 mM *Tt*SLAC in 10% glycerol, 90% 50 mM sodium phosphate buffer (pH 6.0).

Enzyme assay

Laccase activity was assayed by measuring the oxidation of 2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Formation of the cation radical was monitored at 420 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$). An aliquot of enzyme solution was incubated in 1 ml of 50 mM sodium acetate buffer (pH 4.5) containing 1 mM ABTS at 75 °C. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μM of ABTS per min. All assays were performed in triplicate.

Temperature and pH optimum

The temperature dependence of the activity was determined in 50 mM sodium acetate (pH 4.5) at temperatures ranging from 30 to 90 °C using ABTS as a substrate. To determine the optimum temperature for laccase reaction, its activity was measured at temperatures ranging from 30 to 90 °C. The optimum pH for its reaction was estimated by monitoring activity at pHs of 3.0–9.0 using the following buffers: 50 mM sodium acetate buffer for pH 3.0–6.0; 50 mM sodium phosphate buffer for pH 6.0–7.5; 50 mM Tris–HCl buffer for pH 7.5–9.0. The thermostability of laccase was investigated by pre-incubation of the enzyme solution for various times in the absence of substrate in 50 mM sodium acetate buffer (pH 4.5), at temperatures 75 °C, 80 °C, and 85 °C, respectively. Residual activities were determined under laccase activity assay with ABTS as substrate.

Kinetic calculations

Rates of substrate oxidation were examined by spectrophotometry using the molar extinction coefficients of various substrates, which were determined in 50 mM sodium acetate buffer (pH 4.5) at 75 °C. The K_m value was determined measuring the initial velocity, and the apparent k_{cat} was determined from $k_{cat}t = V_{max}/[\text{enzyme}]$. All kinetic studies were performed at least twice and the kinetic data was calculated according to the procedure of Michaelis–Menten equation by EZ-Fit program (Perrella, 1988).

Effect of inhibitors and metal ions on enzyme activity

Effects of potential inhibitors on laccase activity were determined with 1 mM ABTS as the substrate in 50 mM sodium acetate buffer (pH 4.5) and the presence of an inhibitor. The effects of L-cysteine, NaN_3, ethylenediaminetetraacetic acid (EDTA), tropolone, dithiothreitol (DTT), 2-mercaptoethanol, *p*-coumaric acid, and acetyl acetone on its activity were determined after 5 min of incubation of the enzyme with the various inhibitors at 75 °C. After pre-incubating the enzyme solutions containing each metal ions in 50 mM sodium acetate buffer (pH 4.5) at 25 °C for 15 min, substrate ABTS (1 mM) was then added, and the enzyme activity was measured as described above under standard conditions. The ions tested were 1 and 100 mM of $CuSO_4$, $MnCl_2$, $MgCl_2$, $CaCl_2$, $FeSO_4$, $ZnSO_4$, $CoCl_2$, Ni_2SO_4, NaCl, KCl, and $AlCl_3$.

Results and discussion

Identification of TtSLAC gene

The PCR product for the entire open reading frame (ORF) of a putative laccase gene was amplified from the chromosomal DNA of *T. thermophilus* HJ6, cloned into the pGEM-T vector, and sequenced (for detail see "Materials and methods"). The ORF encoded a protein comprising 242 amino acids with a predicted molecular weight of 26.2 kDa and an estimated isoelectric point of 8.92.

Fig. 1 shows schematic drawings of the alignment of a putative laccase from *T. thermophilus* HJ6 with its homolog. The deduced amino acid sequence of a putative laccase from *T. thermophilus* HJ6 showed some similarities with those of hypothetical protein yifH from *E. coli* (identity 33.1%), hypothetical laccase from *Geobacillus stearothermophilus* (identity 37.6%), and polyphenol oxidase (RL5) from bovine rumen metagenome (identity 26.5%) (Fig. 1). Although the overall similarities were rather low, amino acid residues belonging to the three copper sites which have been identified in the RL5 enzyme [29] were almost conserved in this protein (Fig. 1). Thus, the ORF was designated as the small laccase (*Tt*SLAC) gene and classified as a member of the multi-cooper oxidase (MCO) family. The nucleotide sequence of the *Tt*SLAC gene from *T. thermophilus* HJ6 was submitted to the DDBJ/EMBL/GenBank nucleotide sequence database under the accession number GU233492. We suspected that ORF encoded the laccase, and to prove this supposition, we produced it as a fusion with a hexa-histidine (His_6) tag at the C terminus and investigated its biochemical characteristics.

Protein expression and refolding

The *Tt*SLAC was overexpressed in *E. coli* SoluBL21™ cells, but the majority of the protein was found in inclusion bodies. Several refolding protocols were used to attempt to refold the insoluble protein. The *Tt*SLAC protein was readily soluble in either 6 M guanidine HCl or 8 M urea, and DTT was added to ensure any disulfides were reduced. The largest amount of protein was obtained when the *Tt*SLAC was refolded on a metal-chelating column with $CuCl_2$ (1 mM) (for detail see "Materials and methods"). Fig. 2 shows the ratio of protein in the soluble and insoluble fractions, and the refolded and purified *Tt*SLAC protein. The purified *Tt*SLAC gave a single band of ~27 kDa in SDS/PAGE (Fig. 2). The molecular mass (about 26.3 kDa) calculated from the amino acid sequence

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HJ6       ---------MTLLRTPLPVPHG----FTTREGGVS-EGPFRSLNLSAATGDDPERVAENQRRVLAAFGHPP--VAGLRQVHGTEVHPVEGP--------  75
E.coli    --------MSKLIVPQWPQPKGVAACSSTRIGGVS-LPPYDSLNLGAHCGDNPDHVEENRKRLFAAGNLPSK-PVWLEQVHGKDVLKLTGEPY-------  83
G.stea    MPDIFQQEARGWLRCGAPPFAGAVAGLTTKHGGES-KGPFASLNMGLHVGDDRTDVVNNRRRLAEWLAFPLERWVCCEQVHGADIQKVTKSDRGNGAQDF  99
Rumen     --------MIELEKLDFAKSVEGVEAFSTTRGQVDGRNAYSGVNLCDYVGDDALRVLDARLTLAMQLGVDLDDLVMPRQTHSCRVAVIDERFRALDIDEQ  92
                                  *  *          *     **    *                    * *


HJ6       --GLWEGDGLLTRTPGLLLRVGVADCYPLLLYHPKG-AVGALHAGWRGVVGGILPKALERLEAVYRLDPTEVHLAIGPGIGGACYQVGEEVVARFAEAGL 172
E.coli    --ASKRADASYSNTPGTVCAVMTADCLPVLFCNRAGTEVAAAHAGWRGLCAGVLEETVS----CFADNPENILAWLGPAIGPRAFEVGGEVREAFMAVDA 177
G.stea    ATAVLGVDGLYTDEAGVLLALCFADCVPIYFVAPSAGLVGLAHAGWRGTAGGIAGHMVRLWQTREHIAPSDIYVAIGPAIGPCCYTVDDRVVDSLRPTLP 199
Rumen     EAALEGVDALVTRLQGIVIGVNTADCVPIVLVDSQAGIVAVSHAGWRGTVG-RIAKAVVEEMCRQGATVDRIQAAMGPSICQDCFEVGDEVVEAFKKAHF 191
                 *       *       *** *          *   ******                                ** *      *   *


HJ6       FTFRE--DPAAPGKYLLDLEKALLLQARRAGLREERIYRVGLCTHCAPNLFSHRRDR-GRTGRMWGLVMLPPR-- 242
E.coli    KASAA--FIQHGDKYLADIYQ--LARQRLANVGVEQIFGGDRCTYTENETFFSYRRD-KTTGRMASFIWLI---- 243
G.stea    PESPLPWRETSPGQYALDLKEANRLQLLAAGVPNSHIYVSERCTSCEEALFFSHRRDRGTTGRMLAFIGRREEWT 274
Rumen     NLNDIVVRNPATGKAHIDLRAANRAVLVAAGVPAANIVESQHCSRCEHTSFFSARRLGINSGRTFTGIYRK---- 262
                          *          *     *     *     *   *      **
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Fig. 1. Alignment of the deduced amino acid sequence of *Tt*SLAC with its homologs. Multiple sequence alignments were made using a Clustal W online tool for the protein sequences. HJ6, *Tt*SLAC from *T. thermophilus* HJ6 (this work); *E. coli*, hypothetical protein YifH from *E. coli* (GenBank™ accession number AAG57706); *G. stea*, hypothetical laccase from *Geobacillus stearothermophilus* (KFX35128); Rumen, polyphenol oxidase (RL5) from rumen metagenome (AM269758); An asterisk (*) denotes that residues at that position are exactly the same.

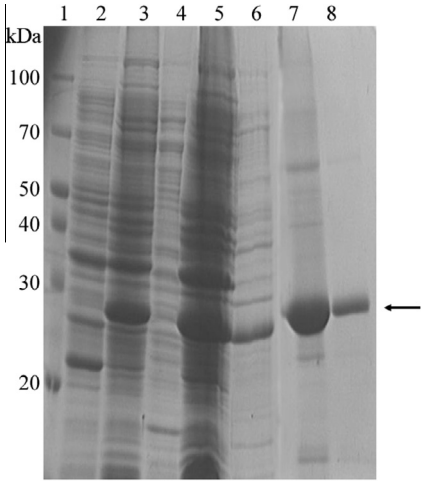

Fig. 2. SDS–PAGE analysis of *Tt*SLAC expression and purification. *Tt*SLAC was subjected to SDS–PAGE on 0.1% SDS–12% PAGE and stained with Coomassie Brilliant Blue R-250. Lane 1, molecular mass marker; Lane 2, whole cell extract of the uninduced cell culture; Lane 3, whole cell extract of the induced cell culture; Lane 4, soluble protein fraction from induced cells; Lane 5, insoluble protein fraction from induced cells; Lane 6, insoluble protein fraction from induced cells solubilized in 6 M guanidine HCl; Lane 7, refolded *Tt*SLAC protein from a metal-chelating column; Lane 8, purified *Tt*SLAC protein after gel filtration chromatography.

was in agreement with the value obtained by SDS–PAGE (Fig. 2). The molecular mass of purified protein was determined to be about 25 kDa by gel filtration chromatography with a HiPrep Sephacryl S-100 column, indicating that the *Tt*SLAC was a monomeric enzyme (data not shown). The monomer molecular mass of *Tt*SLAC (~27 kDa) is the lowest one among the bacterial, archaeal, and eukaryotic laccases described thus far (36–110 kDa) [13,30], with the RL5 laccase, from Rumen metagenome, having a monomeric molecular mass of 28,282 Da [29].

Spectroscopic characterization

To determine the state of its catalytic center, the *Tt*SLAC was characterized spectroscopically. The purified *Tt*SLAC showed a typical UV–visible spectrum for multi-copper oxidase with a peak absorption at about 610 nm, originating from the T1 Cu–S (Cys) bond, and a shoulder at 330 nm, indicative of the presence of a hydroxyl group bridging the T3 copper ions [30] (Fig. 3A). The ratio of A_{280} to A_{610} is 22.5, which is similar to those of the laccases of *Ceriporiopsis subvermispora* and *Coriolus versicolor* [31,32]. When the T1 copper content was estimated by A_{610} ($\varepsilon = 4600\ M^{-1}\ cm^{-1}$ [33]), a value of 0.24 mol of T1 Cu per mol of protein was indicated. The EPR spectrum of the enzyme (Fig. 3B) showed the existence of two copper atoms of T1 and T2 Cu(II), each in a different coordination environment. The parameters of the T2 Cu(II) signal were $g_{II} = 2.23$, $A_{II} = 0.0194\ cm^{-1}$, and $g_{\perp} = 2.05$, and were nearly similar to those of Rumen polyphenol oxidase (RL5) with 2.23, $0.0195\ cm^{-1}$, and 2.05, respectively [29]. The parameters the T1

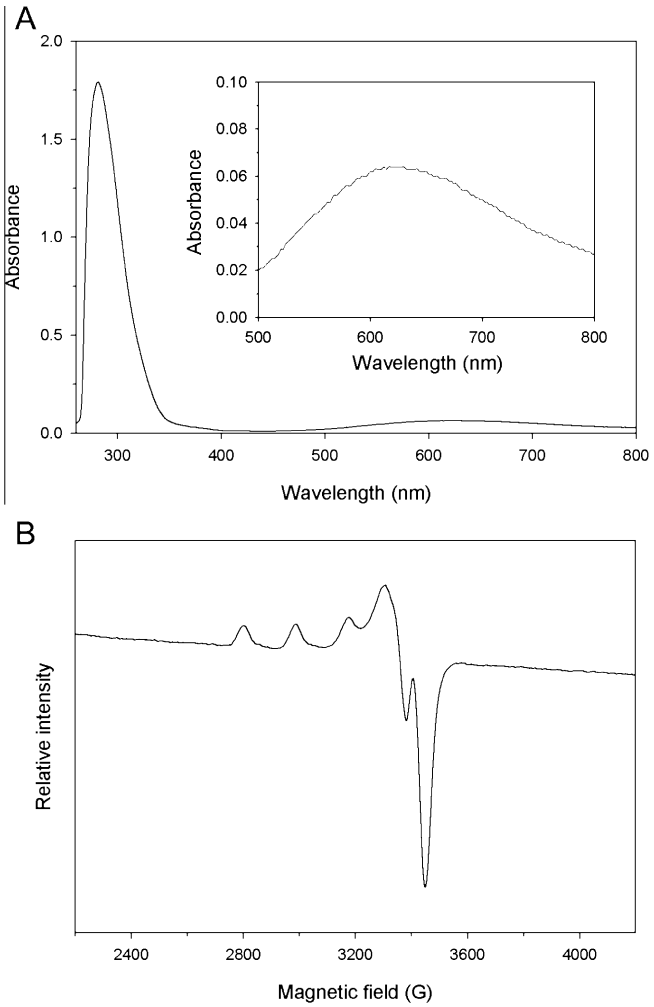

Fig. 3. Spectroscopic properties of *Tt*SLAC. (A) UV–visible absorption spectrum of *Tt*SLAC. (B) EPR spectrum of *Tt*SLAC. The enzyme was prepared as described in "Materials and methods" section.

Cu(II) signal were not accurately determined because of an overlap with the T2 Cu(II) signal. These spectral characteristics revealed that the *Tt*SLAC is a protein containing T1, T2, and T3 coppers, as are other blue copper enzymes.

Catalytic properties

As shown in Fig. 4A, the enzyme requires copper ion for activity. The dependence of activity on Cu^{2+} concentration was sigmoidal with the midpoint (i.e., apparent binding constant) at 0.154 mM. Other metal ions (Mg^{2+}, Mn^{2+}, Ca^{2+}, Ni^{2+}, or Zn^{2+} each at 1 mM) failed to support the activity (data not shown). The effect of pH on enzyme activity was examined at pH values ranging from 3.0 to 9.0 with ABTS and syringaldazine as substrate. The *Tt*SLAC has different pH optima, which are substrate dependent. The optimum pH for the oxidation of ABTS was 4.5 and that for syringaldazine oxidation was 6.0 (Fig. 4B). These results were similar to the findings for the laccase of *T. thermophilus* HB27 [25]. The optimum temperature for ABTS oxidation was 75 °C, which is close to the optimal growth temperature of *T. thermophilus* HJ6 (Fig. 4C). The heat inactivation of the enzyme was estimated by measuring the residual laccase activity after heat treatment at three different temperatures. The *Tt*SLAC showed high thermostability, and the half-life of heat inactivation was about 50 min at 85 °C (Fig. 4D). The heat stability of *Tt*SLAC was higher than those previously reported for other laccases from *Cryptococcus albidus* (21 min at 65 °C) [34], *Cerrena unicolor* (90 min at 70 °C) [35], and *Trametes hirsuta* (70 min at 75 °C) [36].

Substrate specificity

To investigate the kinetic parameters for the enzymatic activity of *Tt*SLAC, the initial reaction rates at various substrate concentrations were determined, and kinetic parameters of different substrates determined for *Tt*SLAC were summarized in Table 1. The *Tt*SLAC was able to oxidize ABTS and various phenolic compounds. The substrate preference of *Tt*SLAC was as follows: 2,6-dimethoxy-phenol > syringaldazine > guaiacol > catechol > ABTS > 4-methylcatechol > pyrogallol. Among the substrates tested in this study, the *Tt*SLAC showed the highest affinity (the lowest K_m value) toward syringaldazine and the highest molecular catalytic constant (k_{cat}) toward catechol. 2,6-DMP was the best substrate considering its highest k_{cat}/K_m value (26.63 $mM^{-1}\ s^{-1}$). The catalytic efficiency (k_{cat}/K_m) for 2,6-DMP was much lower than that of the thermostable fungal laccase from *Trametes hirsute* (250 $mM^{-1}\ s^{-1}$) [36] and was higher than that of the thermostable bacterial laccase from *Bacillus pumilus* (16.17 $mM^{-1}\ s^{-1}$) [37]. As in the case of typical laccases, the *Tt*SLAC did not oxidize L-tyrosine and veratryl alcohol, which are standard substrates for tyrosinase and arylalcohol oxidase, respectively.

Effects of inhibitors

The sensitivity of the enzyme toward several putative laccase inhibitors is shown in Table 2. The metal chelators tested, NaN_3, EDTA, tropolone, and *p*-coumaric acid had strong inhibitory effects, whereas acetyl acetone produced little inhibition on the enzyme

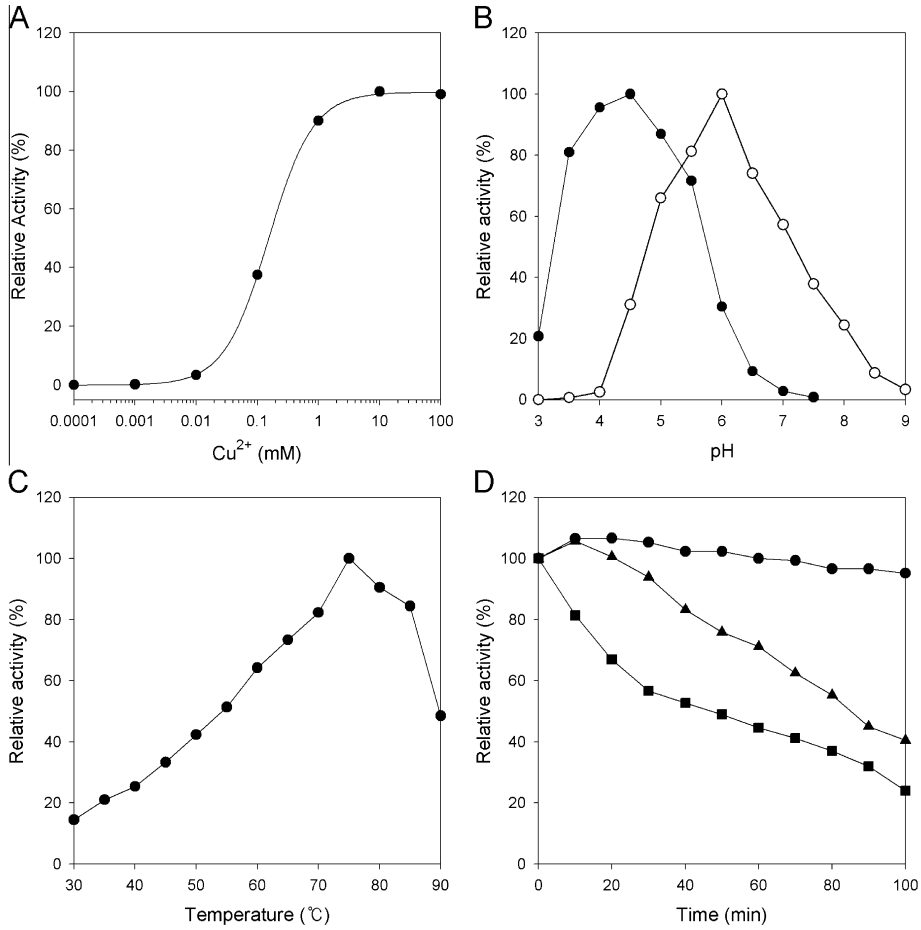

Fig. 4. Catalytic properties of *Tt*SLAC. (A) Copper dependence of activity. (B) pH dependence of activity. Closed circles indicate activity to ABTS; open circles indicate activity to syringaldazine. (C) Temperature dependence of activity. (D) Thermostability of *Tt*SLAC. Enzyme (1.2 μM *Tt*SLAC in 50 mM sodium acetate, pH 4.5) was incubated for various lengths of time at 75 °C (●), 80 °C (▲) and 85 °C (■), and the residual activity of samples were measured at 75 °C and pH 4.5.

Table 1
Kinetic properties of recombinant *Tt*SLAC.

Substrate	ε_{max} (M^{-1} cm^{-1})	Wavelength (nm)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
2,6-Dimethoxy-phenol	35,645	470	0.11	2.93	26.63
Syringaldazine	65,000	530	0.04	0.35	8.75
Guaiacol	12,100	465	0.73	3.75	5.14
Catechol	2211	450	0.83	3.06	3.68
ABTS	36,000	420	0.49	1.48	3.02
4-Methylcatechol	2091	420	0.96	1.48	1.54
Pyrogallol	4400	450	1.83	1.09	0.59

Table 2
Effects of inhibitors on the activity of recombinant *Tt*SLAC in presence of 1 mM ABTS as substrate carried out in triplicates.

Compound	Concentration (mM)	Inhibition (%)
L-Cysteine	1	55.3
	10	100.0
Sodium azide	1	67.3
	10	100.0
EDTA	1	72.9
	10	100.0
Tropolone	1	50.5
	10	100.0
Dithiothreitol	1	94.4
	10	100.0
2-Mercaptoethanol	1	100.0
	10	100.0
p-Coumaric acid	1	58.0
	10	75.8
Acetyl acetone	1	17.1
	10	27.0

reaction. In the presence of thiol compounds, L-cysteine, DTT, and 2-mercaptoethanol, the activity of *Tt*SLAC was also strongly inhibited. The maximum inhibitory effect was observed for 2-mercaptoethanol which completely inhibited enzyme activity at a low concentration (1 mM). Inhibition of laccase activity by thiol compounds has been reported for several laccases [34] and was presumed to be the result of coordination of the thiol to the copper atoms in the enzyme active site [38].

Conclusions

We have identified a gene that codes for small laccase from *T. thermophiles* HJ6 and also described the refolding and biochemical characterizations of the recombinant *Tt*SLAC. Despite the fact that *Tt*SLAC was the smallest one among the laccases reported so far, it had fully the three copper sites that are detectable in the classical laccases and exhibited the high thermostability. Recently, several studies on applications of thermostable laccases have been reported in the bleaching of wheat straw pulp [39] and the decolorization of the dye [35,40]. The results from this study suggested that recombinant *Tt*SLAC could be used as an effective tool for industrial application at high temperature.

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