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ORIGINAL PAPER

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A hyperthermophilic laccase from *Thermus thermophilus* HB27

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Abstract A copper-inducible laccase activity was detected in *Thermus thermophilus* HB27. The enzyme was partially purified and separated by SDS-PAGE. After staining, a gel slice containing a ~53-kDa protein was excised and treated with trypsin, and the in-gel digests were analyzed by mass spectrometry. By mass fingerprinting, the peptides were found to share identity with the TTC1370 protein of the thermophile, which was tentatively annotated as a laccase in the whole genome analysis, albeit experimental evidence was lacking. The assigned mass nearest to the N-terminal sequence was that from Gln^{23} to Lys^{31}. By signal peptide prediction, TTC1370 protein was assumed to be a secretory protein starting from Gln^{23}. The DNA encoding the mature protein was then cloned and expressed in *Escherichia coli*. The recombinant enzyme, expressed as an apoprotein, was dialyzed against copper-containing buffer to yield a holoprotein. The holoprotein was purified to homogeneity, which displayed a blue color typical of laccases and oxidized canonical laccase substrates such as guaiacol and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate). The enzyme was most notable for its striking thermophilicity; the optimal reaction temperature was ~92°C and the half-life of thermal inactivation at 80°C was > 14 h, ranking it as the most thermophilic laccase reported thus far.

Keywords CueO · Peptide mass fingerprinting · Thermostability · Tat system

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Abbreviations ABTS: 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonate) · MALDI-TOF MS: Matrix-assisted laser desorption ionization time-of-flight mass spectrometry · SGZ: Syringaldazine · *Tth*-laccase: Laccase from *Thermus thermophilus* HB27

Introduction

Laccase (EC 1.10.3.2) is a multicopper blue oxidase that couples the four-electron reduction of oxygen with the oxidation of a broad range of organic substrates, including phenols, arylamines, and even certain inorganic compounds by a one-electron transfer mechanism (Mayer and Staples 2002; Claus 2004). The reaction is accomplished by four monoelectronic oxidations of the substrate, catalyzed by the type-1 copper. The electrons are further transferred to the trinuclear cluster, where reduction of molecular oxygen and release of water take place. Laccases participate in (i) cross-linking of monomers, (ii) degradation of polymers, and (iii) ring cleavage of aromatic compounds. Because of their wide reaction capabilities as well as the broad substrate specificity, the enzymes possess great biotechnological potential. Promising applications include textile-dye bleaching (Claus et al. 2002), pulp bleaching (Palonen and Viikari 2004), bioremediation (Murugesan 2003; Wesenberg et al. 2003), polymer synthesis (Huttermann et al. 2001), and biosensors (Peter and Wollenberger 1997). To date, laccases have mostly been isolated and characterized from plants and fungi (Mayer and Staples 2002), and only fungal laccases are used currently in biotechnological applications (Alexandre and Zhulin 2000). In contrast, little is known about bacterial laccases although recent rapid progress in the whole genome analysis suggests that the enzymes are widespread in bacteria (Alexandre and Zhulin 2000; Martins et al. 2002; Claus 2003). Because genetic tools and biotechnological processes are well established, developing bacterial laccases would be significantly important. In addition to the above points of view, it is also

important to consider the stability of the enzymes in order to use them under harsh industrial conditions and to prevent protein degradation during storage (Lasa and Berenguer 1993). Since laccases use molecular oxygen for the substrate oxidation, the genome databases of aerobic thermophilic bacteria were searched for laccases and an open reading frame designated TTC1370 in the extremely thermophilic bacterium *Thermus thermophilus* HB27 (Henne et al. 2004) was identified. This organism grows optimally at 68°C and at temperatures up to 85°C (Oshima and Imahori 1974). The gene was tentatively annotated as "laccase" by the genome project (Henne et al. 2004), but experimental evidence of its catalytic activity was lacking, hence detection of laccase activity in the thermophile was attempted. After such activity was detected, the enzyme (*Tth*-laccase) was partially purified. Peptide mass fingerprinting of tryptic digests of the enzyme was carried out, revealing identity with TTC1370. The gene was then cloned, sequenced, and expressed in *Escherichia coli*. Recombinant enzyme was purified to homogeneity and characterized for its physical and catalytic properties.

Materials and methods

Chemicals

2,6-Dimethoxyphenol and 2,2′-azino bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) were purchased from Wako (Osaka, Japan); guaiacol and SGZ (syringaldazine) were purchased from Sigma (St. Louis, MO, USA); restriction enzymes and DNA ligase were purchased from Takara Bio (Shiga, Japan); KOD-Plus DNA polymerase was purchased from Toyobo (Osaka, Japan); oligonucleotides were purchased from Hokkaido System Science (Hokkaido, Japan); pCR-Blunt II-TOPO vector and competent *E. coli* BL21 (DE3) cells were purchased from Invitrogen (Carlsbad, CA, USA); Overnight Express autoinduction system 1, BugBuster, Benzonase nuclease, and pET-32a(+) were purchased from Merck (Rahway, NJ, USA).

Expression and partial purification of native *Tth*-laccase

T. thermophilus HB27 was grown in 250 ml of TYM medium [0.8% (w/v) tryptone, 0.4% (w/v) yeast extract, 0.2% (w/v) NaCl, pH adjusted to 7.0 with NaOH] in the presence of $CuSO_4$ (0, 0.1, 0.5, or 1 mM final concentration) at 72°C for 20 h. Cells were pelleted by centrifugation (5,000 *g*; 10 min; 4°C) and resuspended in 10 ml of BugBuster protein extraction reagent and 1 μl of Benzonase nuclease. The solution was incubated at room temperature for 20 min with mild shaking, and cell debris was removed by centrifugation (20,000 *g*; 20 min; 4°C). The supernatant was loaded onto a cation-exchange HiTrap SP column (1 ml, Amersham Biosciences, Chalfont, UK) pre-equilibrated with 20 mM Tris–HCl (pH 7.0). The column was washed with the buffer, and bound proteins were eluted with a linear gradient of NaCl (0–0.3 M in 20 ml) in 20 mM Tris–HCl (pH 7.0). Active fractions were pooled and ammonium sulfate was added to give a final concentration of 1 M. The proteins were then loaded onto a hydrophobic-interaction Butyl-Toyopearl 650S column (4 ml, Tosoh, Tokyo, Japan) pre-equilibrated with 20 mM Tris–HCl (pH 7.0), with 1 M ammonium sulfate. The column was washed with 20 mM Tris–HCl (pH 7.0), 1 M ammonium sulfate, and bound proteins were eluted with a linear gradient of ammonium sulfate (1–0 M in 20 ml) in 20 mM Tris–HCl (pH 7.0).

N-terminal amino acid sequencing of native *Tth*-laccase

Partially purified proteins were separated in an SDS-PAGE gel (12.5%), blotted onto Immobilon transfer membrane (Millipore, Bedford, MA, USA), and stained with Coomassie Blue R-250. A piece of the membrane containing the major ~53-kDa protein was used for N-terminal sequencing on a Procise 494 HT protein sequencing system (Applied Biosystems, Foster City, CA, USA).

Peptide-mass fingerprinting of native *Tth*-laccase

The ~53-kDa protein isolated by SDS-PAGE (12.5%) was digested with trypsin. Digests were desalted with a μZipTipC18 tip (Millipore) and subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis on a Voyager-DE STR (Applied Biosystems). Peptide-mass fingerprinting was carried out by using MS-Fit 3.1.1, Protein Prospector software Version 3.2.1 (Clauser et al. 1999). The following amino acid modifications were considered: N-terminal glutamine to pyroglutamate, oxidation of methionine, N-terminal acetylation, and acrylamide-modified cysteine.

N-terminal sequencing and MALDI-TOF MS analysis of recombinant *Tth*-laccase

N-terminal sequence of the purified recombinant laccase was determined on Procise 494 HT protein sequencing system and the mass was analyzed on a Voyager-DE STR.

Gene cloning

PCR cloning was carried out using a set of oligonucleotide primers: 5′-CATATGCAAGGCCCTTCCTTCCCC-3′

and 5′- AAGCTTAACCCACCTCGAGGACTCC-3′. The product (~1.5 kbp) was cloned into pCR-Blunt II-TOPO, the resultant plasmid digested with *Nde*I and *Eco*RI, and the insert subcloned into the corresponding sites of pET-32a(+) to yield the expression plasmid pET32TL1. The nucleotide sequence of the insert was verified by using a BigDye Terminator Version 3.1 cycle sequencing kit (Applied Biosystems) and a PRISM 310 genetic analyzer (Applied Biosystems).

Expression and purification of recombinant *Tth*-laccase

Competent *E. coli* BL21 (DE3) cells were transformed with pET32TL and grown on an LB [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl] agar plate containing 100 μg/ml ampicillin at 37°C. A single colony was picked and grown with shaking in 1 l of LB medium containing Overnight Express autoinduction system 1 and 100 μg/ml ampicillin, at 30°C for 24 h. Cells (wet weight, 11 g) were pelleted by centrifugation (5,000 *g*; 10 min; 4°C) and resuspended in 50 ml of 20 mM sodium acetate (pH 6.0). To the solution, 5 ml of 10 BugBuster and 5 μl benzonase nuclease were added and incubated with mild shaking at room temperature for 20 min. Cell debris was removed by centrifugation (20,000 *g*; 20 min; 4°C) and the supernatant (crude proteins) heated at 65°C for 20 min to precipitate thermolabile proteins derived from *E. coli*. After centrifugation (20,000 *g*; 20 min; 4°C), the supernatant was taken and dialyzed against 20 mM sodium acetate (pH 6.0), 0.1 mM $CuSO_4$. Precipitates were removed by centrifugation (20,000 *g*; 20 min; 4°C). Subsequent protein purification was performed at room temperature unless stated otherwise. The supernatant was loaded onto a HiTrap SP column (5 ml, Amersham Biosciences) pre-equilibrated with 20 mM sodium acetate (pH 6.0). The column was washed with the equilibration buffer and bound proteins were eluted with a linear gradient of NaCl (0–0.3 M in 100 ml) in the equilibration buffer. Active fractions were pooled and solid ammonium sulfate was added to give a final concentration of 1 M. The proteins were then loaded onto a Butyl-Toyopearl column (4 ml, Tosoh) pre-equilibrated with 20 mM sodium acetate (pH 6.0), 1 M ammonium sulfate. The column was washed with the equilibration buffer, and bound proteins were eluted with a linear gradient of ammonium sulfate (1–0 M in 80 ml) in 20 mM sodium acetate (pH 6.0). Active fractions were pooled and dialyzed against 20 mM sodium phosphate (pH 7.0). The proteins were then loaded onto a hydroxyapatite Toyopearl-HA column (4 ml, Tosoh) pre-equilibrated with 20 mM sodium phosphate (pH 7.0). The column was washed with the equilibration buffer, and bound proteins were eluted with a linear gradient of sodium phosphate (20–0.5 M in 20 ml). Active fractions were pooled and dialyzed against 20 mM sodium acetate (pH 6.0).

Protein determination

Concentration of proteins was determined using the molar absorption coefficient, ϵ_{279} = 46,065 M^{-1} cm^{-1}, which was calculated from the amino acid sequence (Gill and von Hippel 1989).

Enzyme activity and stability

All measurements were carried out in triplicate. To assure accurate temperature control, experiments were performed using a thermal cycler. Kinetic constants were determined at 90°C for the ABTS or SGZ substrates. The pH dependence for the activity was determined at 70°C in Britton and Robinson buffer (50 mM borate, 50 mM acetate, 50 mM phosphate; Britton 1952) using ABTS and SGZ. The temperature dependence of the activity was determined in 20 mM sodium acetate (pH 5.0) at temperatures from 65°C to 99°C using ABTS as a substrate. Thermostability of the enzyme was measured in 20 mM sodium acetate (pH 6.0) at 60, 70, 75, 80, 85, 90, 95, and 100°C by incubating the enzyme in thin-wall microtubes for 10 min, followed by chilling the tubes in an ice-water bath. A portion of the sample was used to determine the activity using ABTS as a substrate. The half-life of thermal inactivation of the enzyme was determined at 80°C in 20 mM sodium acetate (pH 6.0). At various time intervals, aliquots were removed and used for the measurement of residual activity at 70°C.

UV-visible absorption spectrum

The UV-visible absorption spectrum of the recombinant *Tth*-laccase (10.2 μM) was recorded at room temperature in 20 mM sodium acetate (pH 6.0) using a V-550 UV/VIS spectrophotometer (Jasco, Tokyo, Japan).

Results and discussion

Identification of a laccase activity in *T. thermophilus* HB27

Laccases use molecular oxygen as a substrate, hence only aerobic organisms should have the enzyme. Because most hyperthermophiles grow anaerobically, there is little chance of identifying laccases from hyperthermophiles. Laccase sequences in genomes of aerobic thermophiles were sought. A BLAST search (Altschul et al. 1990) was carried out using a thermostable laccase-like spore coat protein (CotA) sequence from *Bacillus subtilis* (NCB Accession No. P07788) as a query. An open reading frame, TTC1370 (NCB Accession No. AAS81712), was identified in *T. thermophilus* HB27. It was tentatively annotated as "laccase." Just downstream of the TTC1370, an open reading frame TTC1371 (NCB

Accession No. AAS81713) was identified, which was tentatively annotated as "copper-exporting ATPase." This suggested that TTC1370 might also encode a copper-related protein, though there was no experimental support for such annotation of TTC1371. Therefore, the thermophile was grown to search for laccase activity. In ordinary TYM medium, the activity was not detected in the cell extract or culture supernatant. The medium was then supplemented with $CuSO_4$, since various laccases are induced by copper ion (Galhaup and Haltrich 2001; Hullo et al. 2001; Outten et al. 2001). A dose-dependent laccase activity was detected in the cell extract in the range 0.1–1 mM $CuSO_4$. Relative activities were 0, 0.014, 0.039, and 0.078 for 0, 0.1, 0.5, and 1 mM $CuSO_4$, respectively. No cell growth was observed in the presence of 10 mM $CuSO_4$.

Amino acid sequence analysis of native *Tth*-laccase

The laccase expressed in the thermophile in the presence of 1 mM $CuSO_4$ was partially purified by column chromatography. Proteins were separated by SDS-PAGE (Fig. 1), blotted onto a polyvinylidene fluoride (PVDF) membrane, and stained with Coomassie Blue G-250. Subsequently, a piece of membrane containing a protein band at ~53 kDa was excised and subjected to N-terminal sequencing. However, no specific sequence was determined, suggesting the existence of post-translational modification at the N-terminus. The partially purified sample was then separated again in an SDS-PAGE, gel stained with Coomassie Blue G-250, and a gel slice containing the ~53-kDa protein was excised. The gel was then treated with trypsin (in-gel digestion) and resultant fragments were analyzed on a MALDI-TOF MS. Peptide-mass fingerprinting was carried out and a total of 121 mass data matched to the theoretical peptides resulting from in silico digestion of TTC1370, which covered 73% of the entire sequence (340 of 462 total amino acids) as shown in Fig. 2. Therefore, the band at ~53 kDa was attributed to the TTC1370 protein. However, no mass corresponding to the sequence from Met^1 to Ala^{22} was identified. Taking amino acid modifications (i.e., N-terminus acetylated, N-terminal pyroglutamate, oxidation of methionine, and acrylamide-modified cysteine) into account, a mass corresponding to the peptide from Gln^{23} to Lys^{31} was identified, with the N-terminal Gln^{23} modified to pyroglutamate. In accordance with the prediction of the presence of a signal peptide by the method of Bendtsen et al. (2004) (available via Internet at http://www.cbs.dtu.dk/services/SignalP/), the TTC1370 protein appeared to be cleaved at the peptide bond between Ala^{22} and Gln^{23}. Since the bond is not the target site of trypsin, it is likely that, in the thermophile, the precursor protein was digested with a signal peptidase to expose Gln^{23} as a new N-terminus, which was then modified to pyroglutamate to yield the mature protein. Failure to determine the N-terminal sequence also supported the modification.

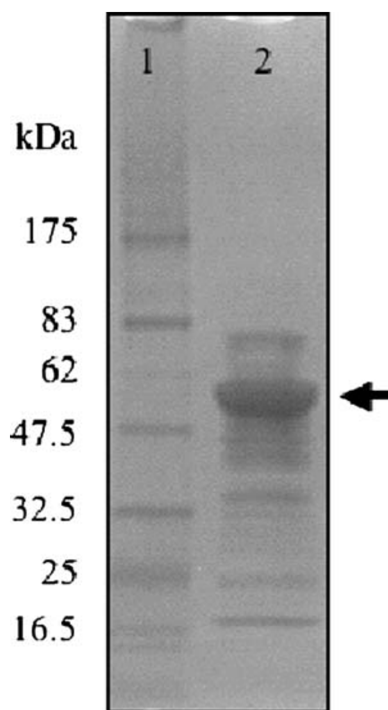

Fig. 1 SDS-PAGE analysis of the partially purified native *Tth*-laccase. *Lane 1* is molecular standard (prestained protein marker, broad range, New England Biolabs, Beverly, MA, USA); *Lane 2* is partially purified *Tth*-laccase. *Arrow* indicates the position of the ~53 kDa *Tth*-laccase

Gene cloning and sequence analysis

The DNA encoding the mature region of *Tth*-laccase was amplified by PCR and the gene fragment inserted into a vector. The nucleotide and the deduced amino acid sequences were identical to those reported by the genome project (Henne et al. 2004).

The amino acid sequence of *Tth*-laccase was next compared with related proteins. Multiple alignment was performed using CLUSTAL W (Thompson et al. 1994). *Tth*-laccase showed rather low but irrefragable partial identities to laccase-related proteins; 30.8% with blue copper protein CueO (NCB Accession No. P36649) from *E. coli* and 25.2% with CotA from *B. subtilis* (NCB Accession No. P07788). Although the overall similarities were rather low, residues involved in the copper-binding motifs were highly conserved (Fig. 3, the motifs indicated by thick bars). The *Tth*-laccase is also similar to laccase from *Streptomyces lavendulae* REN-7 (Suzuki et al. 2003) (NCB Accession No. BAC16804). Interestingly enough, a homologous sequence was not identified in the most closely related organism, *T. thermophilus* HB8, whose complete genome sequence has recently been reported (NCB Accession No. NC_006461). By Southern hybridization, the lack of a homologue in *T. thermophilus* HB8 and also in *T. aquaticus* YT1 (data

m/z submitted	MH+ matched	Δ ppm	Start	End	Peptide Sequence	Modifications
686.4005	686.4201	-28.6073	247	252	(R)AEVLVR(L)	
710.4660	710.4929	-37.7677	409	414	(R)LLVPLR(E)	
745.3925	745.4208	-38.0134	60	65	(R)LTLENR(L)	
858.4725	858.4797	-8.3867	202	209	(R)LLNASNAR(Y)	
902.5234	902.5311	-8.5634	13	21	(R)SQGGLLSLK(L)	
969.4557	969.4682	-12.8144	1	9	(-)QGPSFPEPK(V)	pyroGlu
1003.6217	1003.6304	-8.7003	301	309	(K)NPKPLPLPK(A)	
1180.6580	1180.6578	0.1890	146	155	(R)EAEEHLLVLK(D)	
1235.6786	1235.6788	-0.1940	259	268	(R)FLLQALPYDR(G)	
1249.6281	1249.6285	-0.3004	326	336	(R)LVLTEDMMAAR(F)	
1265.6156	1265.6234	-6.1456	326	336	(R)LVLTEDMMAAR(F)	1Met-ox
1379.6864	1379.6860	0.2759	337	347	(R) FFINGQVFDHR(R)	
1395.7961	1395.7960	0.1119	22	35	(K)LSATPTPLALAGQR(A)	
1535.7973	1535.7872	6.5795	337	348	(R)FFINGQVFDHRR(V)	
1565.9078	1565.9055	1.4463	310	324	(K)ALSPFPTLPAPVVTR(R)	
1621.7741	1621.7545	12.0822	419	431	(R)TVFHCHIVEHEDR(G)	
1650.8853	1650.8855	-0.1527	36	51	(R)ATLLTYGGSFPGPTLR(V)	
1722.0113	1722.0067	2.6856	310	325	(K)ALSPFPTLPAPVVTRR(L)	
1820.8969	1820.8985	-0.8934	105	119	(K)ELAGTFWYHPHLHGR(V)	
1936.0336	1936.0445	-5.6359	66	82	(R)LPEPTNLHWHGLPISPK(V)	
1993.1446	1993.1446	-0.0198	177	195	(K)EGDLVLVNGALRPTLVAQK(A)	
2292.0792	2292.1018	-9.8513	156	176	(K)DLALQGGRPAPHTPMDWMNGK(E)	
2308.0752	2308.0967	-9.2924	156	176	(K)DLALQGGRPAPHTPMDWMNGK(E)	1Met-ox
2324.0680	2324.0916	-10.1444	156	176	(K)DLALQGGRPAPHTPMDWMNGK(E)	2Met-ox
2566.1890	2566.2393	-19.6339	83	104	(K)VDDPFLEIPPGESWTYEFTVPK(E)	
2566.1890	2566.2005	-4.4934	419	440	(R)TVFHCHIVEHEDRGMMGVLEVG(-)	1Cys-am
2582.2048	2582.1954	3.6521	419	440	(R)TVFHCHIVEHEDRGMMGVLEVG(-)	1Met-ox, 1Cys-am
2610.2406	2610.2961	-21.2593	326	347	(R)LVLTEDMMAARFFINGQVFDHR(R)	
2678.4649	2678.5133	-18.0611	120	145	(R)VAPQLFAGLLGALVVESSLDAIPELR(E)	
3359.4906	3359.5905	-29.7505	269	300	(R)GAMGMMDMGGMAHAMPQGPSRPETLLYLIAPK(N)	
3375.4985	3375.5854	-25.7398	269	300	(R)GAMGMMDMGGMAHAMPQGPSRPETLLYLIAPK(N)	1Met-ox
3391.4902	3391.5803	-26.5869	269	300	(R)GAMGMMDMGGMAHAMPQGPSRPETLLYLIAPK(N)	2Met-ox
3407.4577	3407.5753	-34.4941	269	300	(R)GAMGMMDMGGMAHAMPQGPSRPETLLYLIAPK(N)	3Met-ox
3423.4586	3423.5702	-32.5890	269	300	(R)GAMGMMDMGGMAHAMPQGPSRPETLLYLIAPK(N)	4Met-ox
3439.4396	3439.5651	-36.4757	269	300	(R)GAMGMMDMGGMAHAMPQGPSRPETLLYLIAPK(N)	5Met-ox
3455.4788	3455.5600	-23.5001	269	300	(R)GAMGMMDMGGMAHAMPQGPSRPETLLYLIAPK(N)	6Met-ox
3688.7946	3688.9478	-41.5225	213	246	(R)LALQDHPLYLIAADGGFLEEPLEVSELLLAPGER(A)	

Fig. 2 Summary of the peptide-mass fingerprinting of the tryptic fragments of *Tth*-laccase. Modifications taken into consideration were pyroglutamate (*pyroGlu*), oxidized methionine (*Met-ox*), and acrylamide-modified cysteine (*Cys-am*)

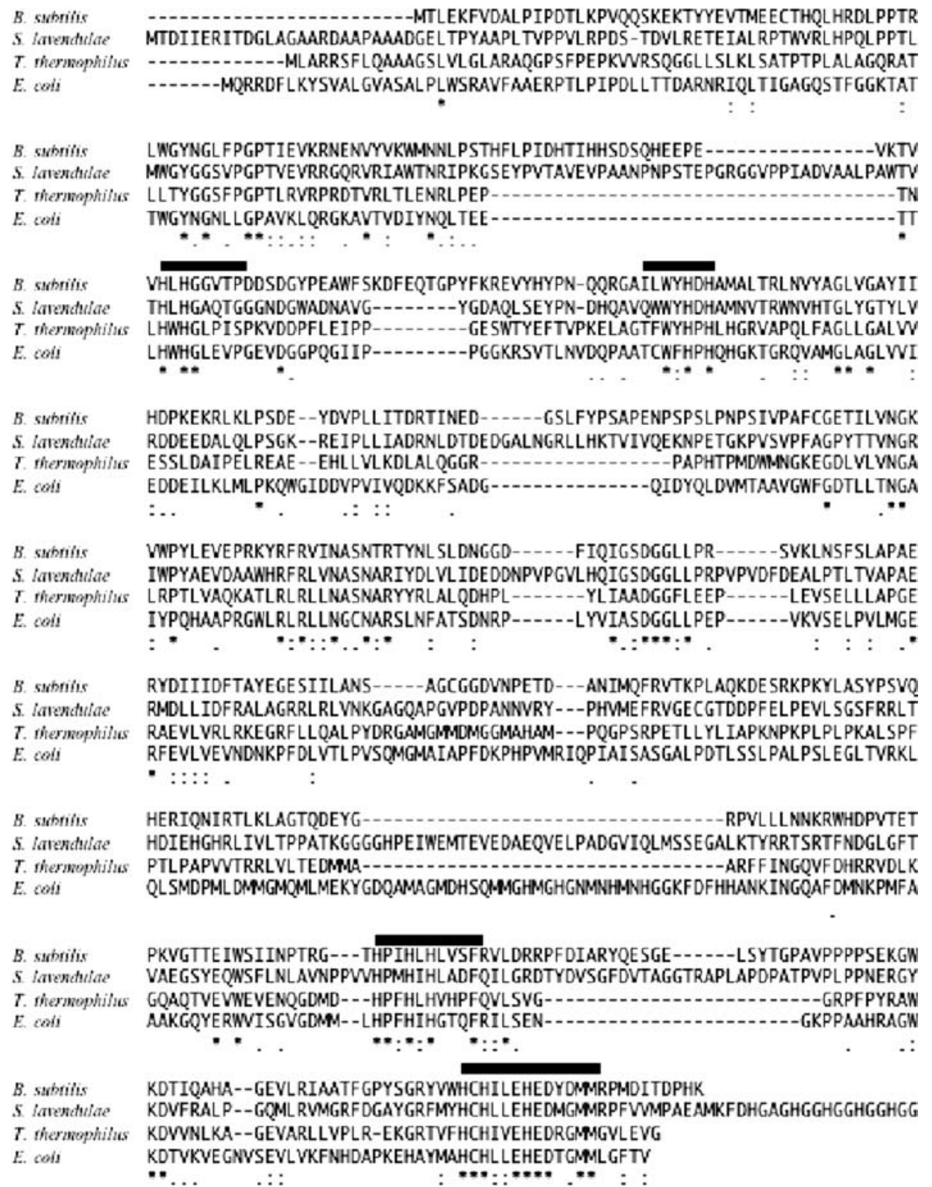

Fig. 3 Multiple amino acid sequence alignment of laccase-related proteins. Amino acid sequences used for the alignment were *B. subtilis* CotA (NCB Accession No. P07788), *S. lavendulae* REN-7 laccase (NCB Accession No. BAC16804), *T. thermophilus* laccase (GenBank Accession No. AB200322), and *E. coli* CueO (NCB Accession No. P36649). Homologous residues are shown in *dots* and identical ones are indicated by *asterisks* below the residues. Conserved copper-binding motifs are indicated by the *thick bar* above the residues

not shown) was confirmed. Therefore, laccase does not distribute universally over the *Thermus* genus. It is not clear whether the laccase gene jumped into the *T. thermophilus* HB27 genome from other organisms or if a pre-existing laccase gene was lost from *T. thermophilus* HB8 and *T. aquaticus* YT1.

Apart from the mature sequence, a Tat (twin-arginine protein transport) sequence motif (Sargent et al. 1998) was identified in the signal sequence (MLARRSFLQAAAGSLVLGLARA): Ala[3] to Gln[9], A *RR*S *FL*Q (*italicized* residues are highly conserved in the motif). A hydrophobic region (Ala[10] to Ala[20]) and basic amino acid (Arg[21]), both of which are well conserved in the Tat system, were also identified. The Tat system is a molecular machine dedicated to the translocation of fully folded proteins across energy-transducing membranes (Sargent et al. 1998). It has been reported that complex, cofactor-containing Tat substrates acquire their cofactors prior to export, and that substrate proteins must be folded before transport can proceed. Therefore, it is not surprising that metal-containing *Tth*-laccase folds into an active form in the cytoplasm and is translocated to the periplasm via the Tat system, just as the homologous CueO is secreted to the periplasm via the Tat system in *E. coli* (DeLisa et al. 2004). Although there is no experimental evidence to support the existence of this translocation system in *T. thermophilus* HB27, it appears very likely from the sequence data that this thermophile possesses a Tat system and that laccase is a substrate.

Expression and purification of recombinant *Tth*-laccase

The mature-protein-encoding region of the *Tth*-laccase gene was subcloned into a conventional expression

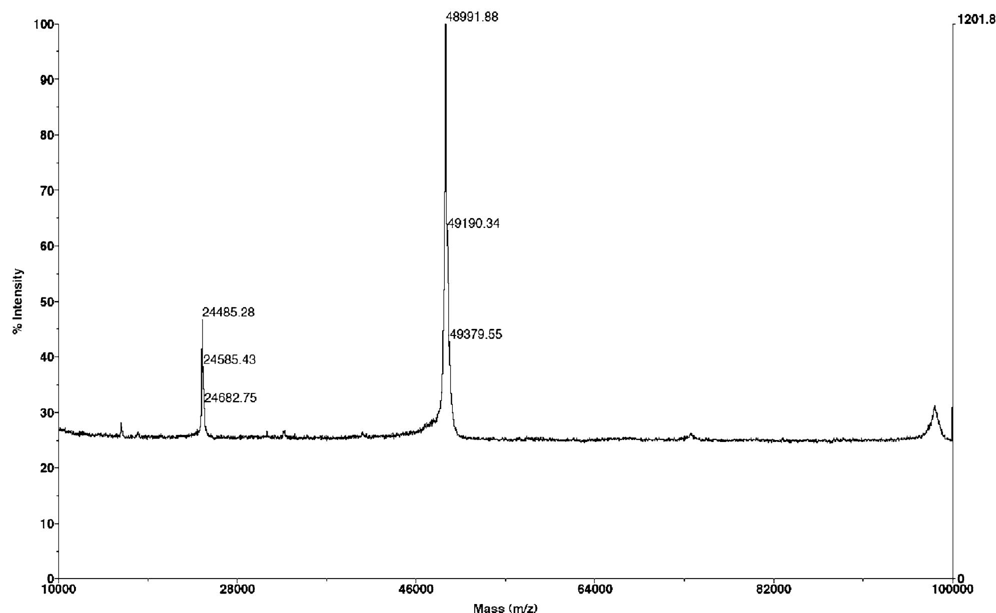

Fig. 4 MALDI-TOF MS analysis of recombinant *Tth*-laccase

plasmid pET-32a(+) under control of the strong T7 *lac* promoter (Studier and Moffatt 1986). Cells were grown in LB medium containing the Novagen Overnight Express autoinduction system. (Though copper ion could have been added to the medium to assist proper folding of laccase, it was not, in order to avoid expression of the host *E. coli*'s copper-inducible CueO protein, which is functionally homologous to laccase). Collected cells were lysed in BugBuster reagent. The reagent contains non-ionic detergents that are capable of cell wall perforation without denaturing soluble protein. Debris was then removed by centrifugation and the supernatant heated at high temperature (65°C for 20 min), which enormously simplified protein purification. After the heat treatment, the protein was dialyzed against copper-containing buffer to incorporate the ion into the protein. During dialysis, the protein acquired a blue color. After dialysis, three-step column chromatography was applied to yield a purified protein. Approximately 10 mg of purified protein was routinely obtained from 1 l of culture.

Physical properties of recombinant *Tth*-laccase

The purified recombinant enzyme showed a molecular mass of ~53 kDa by SDS-PAGE. This is similar to that of the native enzyme (data not shown). However, the value was slightly higher than that calculated from the deduced amino acid sequence (48,986.16). The mass was therefore analyzed by MALDI-TOF MS. As shown in Fig. 4, a sharp peak corresponding to the m/z 48,991.86 (average molecular mass of 48,990.87) was identified as a singly charged ion. Peaks at m/z 49,190.34 and 49,379.55 corresponded to the masses of protein and mono-sinapinic and di-sinapinic acid adducts. A peak at m/z 24,485.28 was for the doubly charged ion and peaks at m/z 24,585343 and 24,682.75 were those bound to mono-sinapinic and di-sinapinic acid. These experimental values were consistent with the theoretical value, indicating that the recombinant *Tth*-laccase comprised the amino acid sequence from Met^{1} to Gly^{441}. This was also supported by N-terminal sequencing of first five residues: MQGPS. The apparent high mass on the SDS-PAGE therefore did not reflect the true mass but was an artifact. This is probably due to the abnormally high pI of the enzyme, as suggested by other electrophoresis experiments. The enzyme did not migrate into a commercial (Invitrogen) isoelectric focusing (IEF) gel (for pH 3–10). Native PAGE was also unsuccessful. From

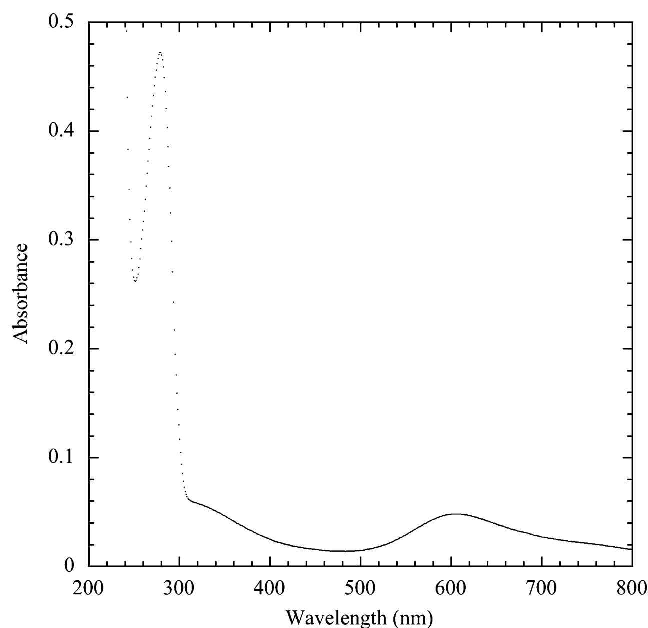

Fig. 5 UV-visible absorption spectrum of recombinant *Tth*-laccase

the amino acid sequence, the pI of the *Tth*-laccase was estimated to be 7.18 (Skoog and Wichman 1986). However, these experiments clearly showed that the actual pI is much higher than 7.18. Underestimation of pI was also reported for CotA from *B. subtilis*; the predicted value was 5.9 from the sequence but was determined to be 7.7 by experiment (Martins et al. 2002).

UV-visible absorption spectrum of recombinant *Tth*-laccase

The purified protein exhibited a blue color common to other laccases. As shown in Fig. 5, the UV-visible absorption spectrum showed peaks around 278 and 605 nm, corresponding to the aromatic side chains and the T1 or blue copper center, respectively, and a shoulder at ~330 nm, corresponding to the T3 binuclear copper center. These spectroscopic properties are common to other laccases. The ratio of A_{278}/A_{607} was 9.9, which is comparable to other laccases.

Catalytic properties of recombinant *Tth*-laccase

As shown in Fig. 6a, the enzyme requires copper ion for activity. The dependence of the activity on Cu^{2+} concentration was sigmoidal with the midpoint (i.e.,

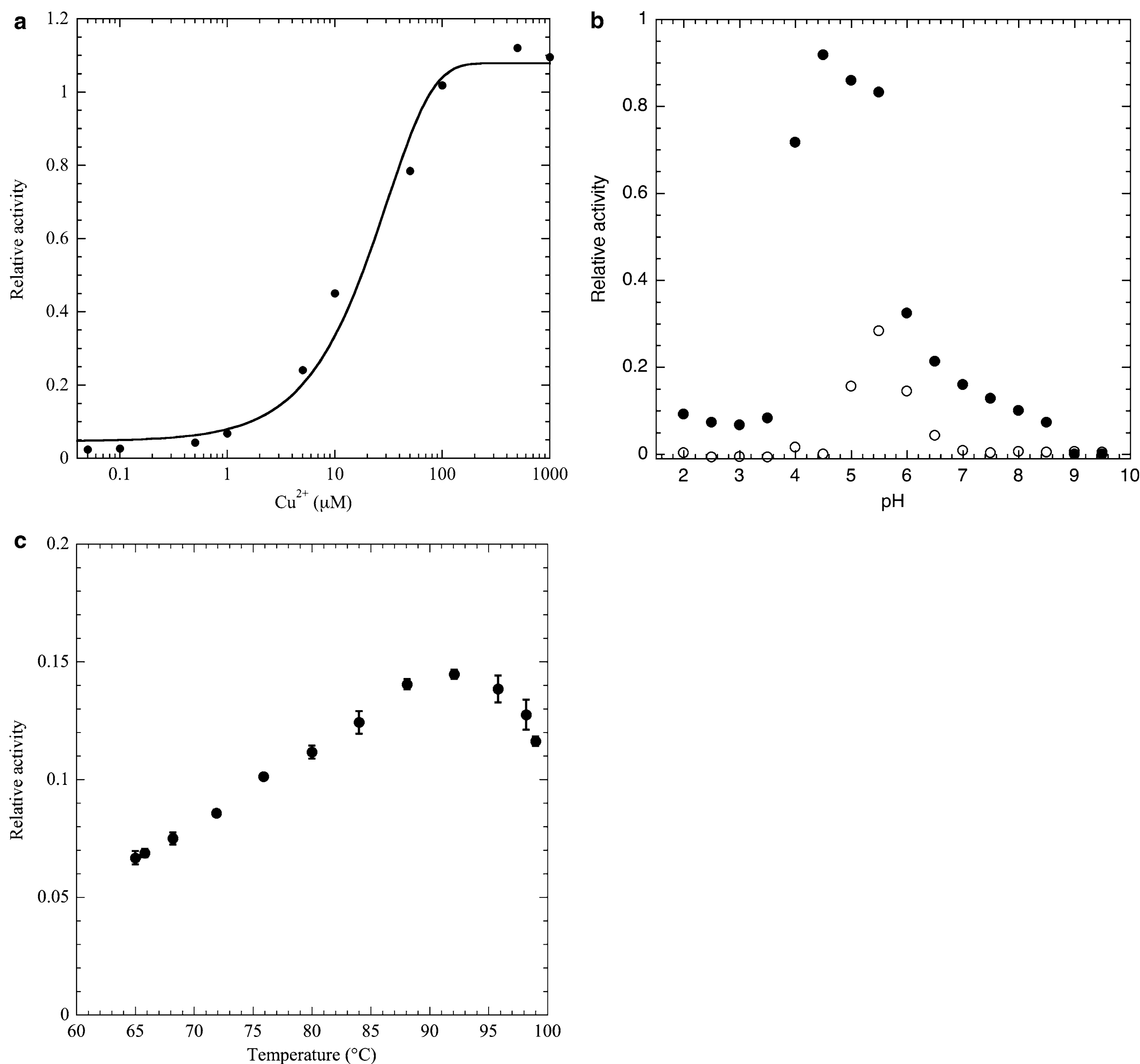

Fig. 6 Catalytic properties of recombinant Tth-laccase. **a** Copper dependence of activity. **b** pH dependence of activity. *Closed circles* indicate activity to ABTS; *open circles* indicate activity to SGZ. **c** Temperature dependence of activity

apparent binding constant) at 30.4 μM. Other metal ions (Mg^{2+}, Mn^{2+}, Ca^{2+}, Ni^{2+}, or Zn^{2+} each at 1 mM) failed to support the activity. In addition, the reaction in the presence of 0.1 mM $CuSO_4$ was completely inhibited by the addition of 1 mM EDTA. These results clearly indicate the existence of an extra copper-binding site, which was essential for expressing the activity. Solano et al. (2001) classified laccases into three groups depending on the activity to 2,6-dimethoxyphenol. The first group contains laccases that oxidize substrates without copper supplementation. The second group of proteins requires copper for activity, with an initial burst of activity followed by rapid inactivation. The third group contains laccases that show a very slow oxidation rate. From the time course of the reaction using the 2,6-dimethoxyphenol, *Tth*-laccase appeared to belong to the second group (data not shown). In CueO from *E. coli*, which also belongs to the second group, structural analysis revealed the presence of a labile copper ion in addition to the canonical T1-3 copper sites (Roberts et al. 2003). The residues surrounding the labile copper are Met^{355}, Asp^{360}, Asp^{439}, and Met^{441}. A mutational study revealed that M355L and D360A variants were the most impaired while D439A and M441L retained considerably more activity. In *Tth*-laccase, corresponding residues were not identified by simple sequence alignment because of the lack of sequence similarity (Fig. 3). However, there is a methionine-rich region from Met^{271} to Met^{283}, which may be involved in copper binding.

In the presence of copper ion, *Tth*-laccase oxidized canonical laccase substrates such as guaiacol, 2,6-dimethoxyphenol, ABTS, and SGZ. Kinetic parameters (i.e., Michaelis constant, K_m, and turnover number, k_{cat}) were determined for ABTS and SGZ. The dependence of the rate on the substrate concentration followed Michaelis–Menten kinetics. From Lineweaver–Burk plots, apparent K_m and k_{cat} were determined to be 0.90 mM and 24.6 s^{-1} for ABTS and 1.88 mM and 6.47 s^{-1} for SGZ, respectively. As for pH dependence, the enzyme displayed maximal activity for ABTS and for SGZ oxidation at pH 4.5 and 5.5, respectively (Fig. 6b). The temperature dependence of laccase activity was measured between 65°C and 99°C using ABTS as substrate at pH 5.0, and the optimal temperature was determined to be 92°C (Fig. 6c). This is much higher than the optimal temperature of 75°C for CotA from *B. subtilis*, which had been the most thermophilic laccase-like protein so far reported (Martins et al. 2002).

Thermostability of recombinant *Tth*-laccase

As shown in Fig. 7a, the enzyme was resistant to incubation at 85°C for 10 min. Even at 100°C for 10 min, the enzyme retained two-thirds of its initial activity, indicating the extreme robustness of the enzyme. The half-life of the enzyme activity was next estimated at 80°C. A plot of the log of residual activity versus time was linear, indicating a first-order decay process under the condition (Fig. 7b). From the plot, the rate constant was calculated (k = 0.0007982 min^{-1}), and a half-life of inactivation of 868 min was determined. The half-life was seven-fold that of CotA from *B. subtilis* (k = 0.0056 min^{-1} and half-life 112 min); the *Tth*-laccase is the most thermostable laccase reported thus far.

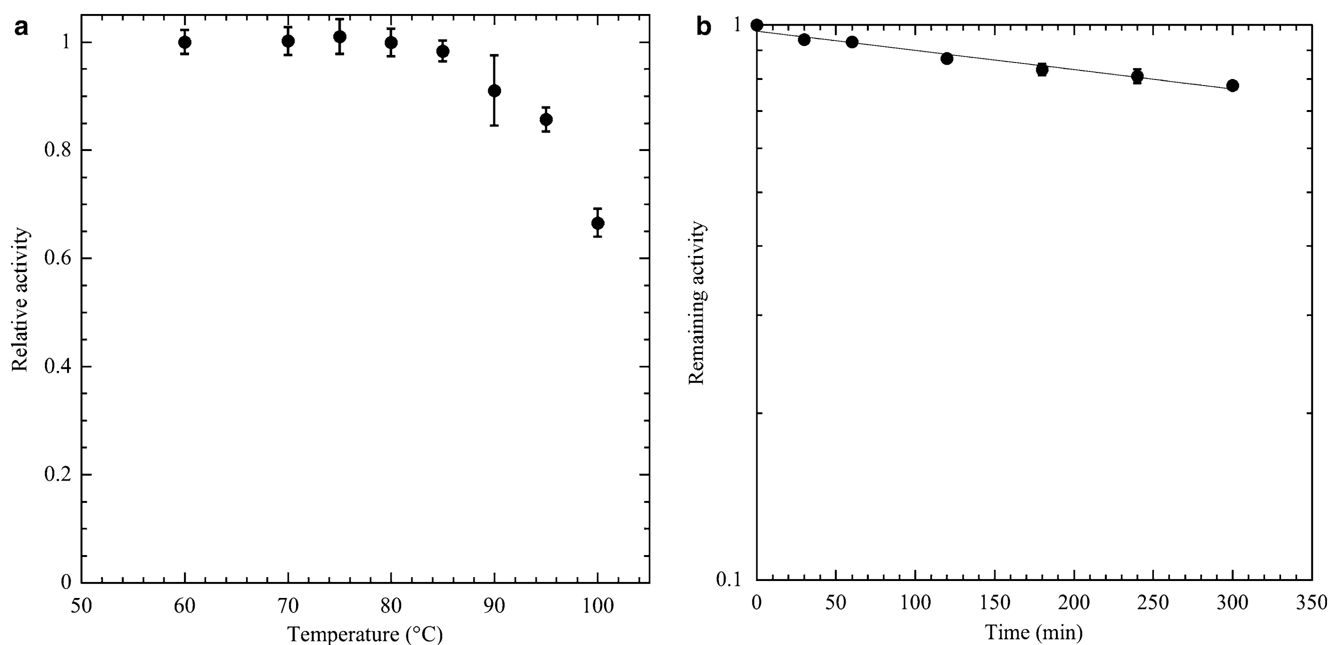

Fig. 7 Thermostability assay for recombinant *Tth*-laccase. **a** Thermostability of recombinant *Tth*-laccase. **b** Loss of activity upon incubation at 80°C

Sequence analysis with regard to extreme thermostability

In order to unveil the mechanism of the striking thermostability, a simple sequence analysis of some thermostable laccase homologues was done; *Tth*-laccase; CotA from *B. subtilis* (Martins et al. 2002); laccase from *S. lavendulae* REN-7 (Suzuki et al. 2003); a laccase-like protein (NCB Accession No. NC_003364) from the hyperthermophilic archaeon, *Pyrobaculum aerophilum* IM2 (Volkl et al. 1993; Fitz-Gibbon et al. 2002); and a laccase-like protein (NCB Accession No. NC_000918) from the hyperthermophilic bacterium *Aquifex aeolicus* VF5 (Deckert et al. 1998). Note that the last two sequences were identified as homologues in a BLAST search and that there is no experimental evidence to support their hypothesized laccase activity or thermostability. These organisms grow optimally at 100°C and at up to ~95°C, respectively, temperatures much higher than that optimal for *T. thermophilus*, which grows at temperatures up to 85°C. Therefore, these proteins might be more thermostable than *Tth*-laccase. Some parameters that might affect protein thermostability are considered. Laccase from *S. lavendulae* REN-7 is thermostable and retains its original activity after 20 min of incubation at 70°C (Suzuki et al. 2003). As for proline content (Suzuki et al. 1987), *Tth*-laccase has the highest at 10% and the others contain less: 8.97% for CotA, 9.35% for *S. lavendulae* laccase, 7.60% for the *P. aerophilum* protein, and 4.99% for the *A. aeolicus* protein. Since the proteins from hyperthermophiles have the smallest values, it is uncertain if proline content is actually related to thermostability. The ratios of arginine to lysine are high for *Tth*-laccase and *S. lavendulae* laccase, but low for CotA and PAE1888. As for cysteine, CotA contains four residues and it is known that Cys^{229} and Cys^{322} form a disulfide bridge, which may account for CotA's thermostability. The aliphatic index (defined as the relative volume of a protein occupied by aliphatic side chains of alanine, valine, isoleucine, and leucine) of proteins of thermophilic bacteria is known to be significantly higher than that of ordinary proteins (Ikai 1980). Aliphatic index values were calculated using the ProtParam tool maintained by the Swiss Institute of Bioinformatics (available via Internet at http://www.expasy.org/tools/protparam.html) (Gasteiger et al. 2003). The index for the recombinant *Tth*-laccase was calculated to be 96.39, that for CotA to be 77.89, that for laccase from *S. lavendulae* REN-7 to be 79.18, that for *P. aerophilum* to be 89.80, and that for *A. aeolicus* to be 79.32. Again, proteins from the hyperthermophiles have lower values than that of the *Tth*-laccase.

Concluding remarks

Although laccases are widespread in many organisms, including bacteria (Alexandre and Zhulin 2000; Martins et al. 2002; Claus 2003), laccase-homologous sequences identified in thermophiles are very rare. Two hyperthermophiles that seem to contain homologues were found: *P. aerophilum* IM2 and *A. aeolicus* VF5. However, values of none of the parameters examined that can affect thermostability support the hypothesized high stability of these homologues. It is of interest to confirm the stabilities of these proteins by experiment. In any case, *Tth*-laccase is the most thermophilic laccase thus far reported and may have great potential in industrial applications.

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