Journal of Industrial Technology, Kangwon Natl. Univ., Korea, No. 37:1 (2017) 16~20 ISSN 1229-9588

Bacillus sonorensis KCTC13918로부터 새로운 laccase유전자 (*soncotA*)의 클로닝과 대장균에서의 발현

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Cloning and expression of new laccase gene (*soncotA*) from *Bacillus sonorensis* KCTC13918 in *E. coli*

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Received 13 April 2017; Revised 2 August 2017; Accepted 21 August 2017

Abstract

A new putative laccase gene (*soncotA*) which show 78% homology with that from *Bacillus licheniformis* (*liccotA*) was isolated from draft genome sequence of *Bacillus sonorensis* KCTC 13918. A 1,545 bp of PCR product corresponding 514 amino acids was cloned into *Nde*I-*Not*I site of pET21c and expressed as soluble form in *E. coli*. About 59 kDa size of recombinant laccase was purified into homogenity by Ni-NTA column and laccase activity was confirmed by zymography. The enzymatic properties of recombinant laccase were characterized. The specific activity of *B. sonorensis* laccase was 0.033 fold lower than that of *Bacillus licheniformis* laccase. The finding of new laccase gene broadened the enzymatic diversity of *Bacillus* species laccases.

Keywords: Genome mining, *Bacillus sonorensis,* Laccase, Recombinant expression

1. Introduction

Laccases (benzenediol: oxygen oxidoreductase, EC1.10.3.2) are multi-copper-containing enzymes which reduce molecular oxygen to water and simultaneously perform one-electron oxidation of various substrates such as diphenols, methoxy-substituted monophenols and aromatic and aliphatic amines[11, 15].

Laccase has applications in many fields, like medical diagnosis, pharmaceutical industry. Laccase has also applications in the agriculture area by clearing herbicides, pesticides and some explosives in soil. It is also used in the preparation of some important drugs, like anticancer drugs, and added in some cosmetics to reduce their toxicity. Laccase also has the ability to form polymers of valuable importance[13, 16]. Laccases have been isolated in higher plants, prokaryotes, insects, and are widespread in fungi[11]. In contrast to fungal laccases, bacterial laccases are highly active and much more stable at high temperatures, at high pH as well as high concentrations of chloride and copper ions and the immobilized spore laccases are more compatible with almost all industrial processes[6].

Several new laccase genes from *Bacillus* species were isolated and its enzymatic properties were determined[3, 7, 10, 14, 17, 18]. Recently, bioinformatic analysis of published genome data revealed high diversity of bacterial genes for laccase-like enzymes. More than 1,200 putative genes were retrived from chromosomes and plasmids of diverse bacteria [2]. *Bacillus sonorensis* was isolated from Sonoran desert soil. It most closely related to *Bacillus licheniformis*. *B. sonorensis* can be distinguished from *B. licheniformis* by salt tolerance, pigmentation, multilocus enzyme electrophoresis, reassociation of genomic DNA and

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sequence differences in protein-coding genes and 16S rRNA[12]. We were interested in discovering novel laccases from this *B. sonorensis*. Draft genome sequencing of *Bacillus sonorensis* KCTC13918 (NBRC101234) revealed putative laccase gene[1, 9]. In this report, putative laccase gene (soncotA) was cloned, and functionally expressed in *E. coli*. Its enzymatic characteristics were kinetically determined and compared with previously known *B. licheniformis* laccase (liccotA).

2. Results

2.1 Gene cloning of laccase gene

About 1.5 kb of PCR product was obtained from PCR amplification of putative laccase gene using *B. sonorensis* genomic DNA as template [Fig. 1]. The putative laccase gene was designated as *soncotA* and subsequently cloned into *Nde*I-*Not*I site of pET21c. For comparison of other *bacillus* laccase, synthetic laccase gene encoding previously known *Bacillus licheniformis* (GenBank Acc No. ADZ57281) was synthesized and cloned into *Nde*I-*Xho*I site of pET21c. The resulting recombinant pET21c plasmids were confirmed by DNA sequencing and transformed into *E. coli* BL21(DE3)plysS strain.

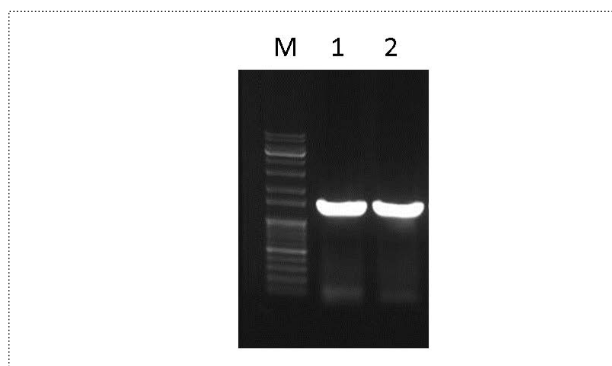

Fig. 1 PCR products of *Bacillus* laccases gene on agarose gel. Lane M: 1kb plus size marker, lane 1: *B. licheniformis licotA*, lane 2: *B. sonorensis soncotA*

2.2 Expression and purification of recombinant laccase

Small scale expression of recombinant laccase was initially tested by 4 ml growth and confirmed by SDS-PAGE and ABTS active staining. The recombinant laccases were expressed as soluble form. Judging from 514 deduced amino acid sequences, an estimated 59 kDa size of corresponding laccase bands were shown from both LiccotA (lane 1) and SoncotA (lane 2) in SDS-PAGE gel analysis [Fig. 2]. The expression level of recombinant laccases in SDS-PAGE gel were almost identical, but *B. sonorensis* soncotA produced only detectable level of laccase activity which showed about 0.1 fold lowered laccase activity than liccotA of *B. licheniformis*. At this time, we don't know the plausible reason why *B. sonorensis* laccase showed relatively low laccase activity compared to *B. licheniformis* laccase. For further analysis of these two laccases, large scale expression employing 200 ml culture were performed and purified into homogeneity by his-tag affinity Ni-NTA column method (data not shown). The result of microplate method using purified recombinant laccase are also coincidence analysis as shown in Fig. 3.

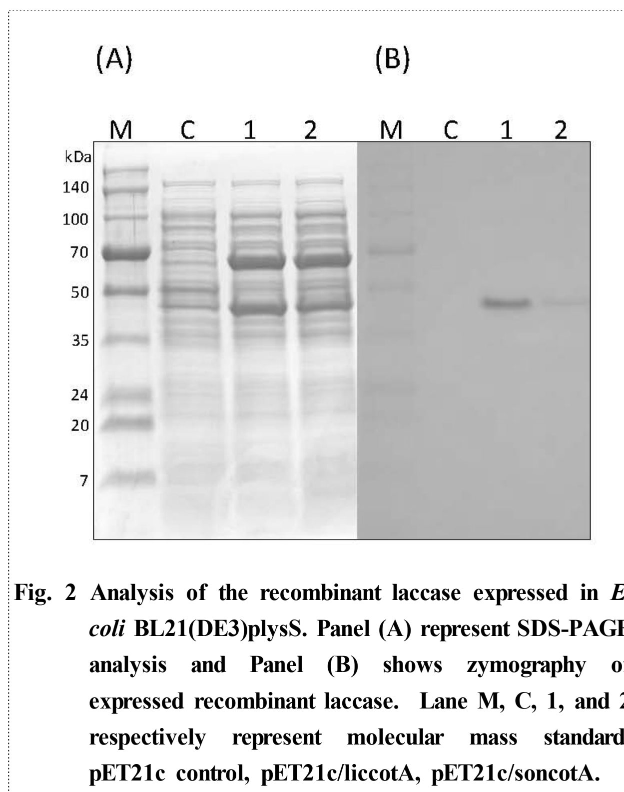

Fig. 2 Analysis of the recombinant laccase expressed in *E. coli* BL21(DE3)plysS. Panel (A) represent SDS-PAGE analysis and Panel (B) shows zymography of expressed recombinant laccase. Lane M, C, 1, and 2 respectively represent molecular mass standard, pET21c control, pET21c/liccotA, pET21c/soncotA.

2.3. Enzymatic properties and kinetic parameters

The overall enzymatic characterization of two laccases were shown in Table 1. In general, the catalytic efficiencies (K_{cat}/K_m) are considered as a measurement of the enzymatic specificity. To investigate the kinetic parameters for the enzymatic activity of SoncotA laccase, the initial reaction rates at various substrate concentrations were determined, and kinetic parameters of ABTS substrate determined for *Bacillus* laccases were summarized in Table 1. The kinetic parameters of SoncotA fall below the ranges reported for other bacterial laccases [11]. K_m value of SoncotA was 9.7 M compared with 33.9 M of

Fig. 3 Comparison of recombinant *B. sonorensis* laccase (SonCotA) with *B. licheniformis* laccase (LicCotA) in microplate assay. Lane C: pET21c control, lane 1: pET21c/LicCotA, lane 2: pET21c/SonCotA

LiccotA, while K_{cat}/K_m value (0.0016) of SoncotA was about 0.16 fold lower than that (0.0099) of LiccotA. To determine the optimal pH for recombinant laccases, we measured the enzyme activity at various pH values (pH3.0~9.0), using ABTS, SGZ, 2,6-DMP as substrate. The pH value for optimal activity of SoncotA was determined to be around pH 3.0, slightly acidic than LiccotA (pH 4.0). The optimal temperature for the enzyme was 70℃, slightly lower than that of LiccotA (80℃).

Table 1 Comparison of kinetic constants of *Bacillus* laccases with ABTS as substrate

	Specific Activity (U/L/ug)	V_{max}(uM/ug/s)	K_m (uM)	$K_{cat}(s^{-1})$	K_{cat}/K_m
LicCotA	17.325	0.10404	33.87055	0.33504	0.00989
SonCotA	0.524	0.00450	9.675272	0.01575	0.00163

3. Materials and Methods

3.1 Bacterial strains, plasmids, and growth conditions

Bacillus sonorensis KCTC13918 was obtained from Korea Collections for Type Culture (KCTC, Jeongeup, Korea). The expression vector pET21c was manipulated in *E. coli* strain XL10-gold, which was used as a host for cloning. *E. coli* BL21(DE3)plysS which harboring the recombinant plasmids were used for expressing recombinant laccases. All *E. coli* transformants were cultured in LB broth containing 100 μg/ml ampicillin at 37℃.

3.2 Gene cloning and PCR

Specific forward and reverse primer set which covers upstream and downstream part of *soncotA* were synthesized from draft genome

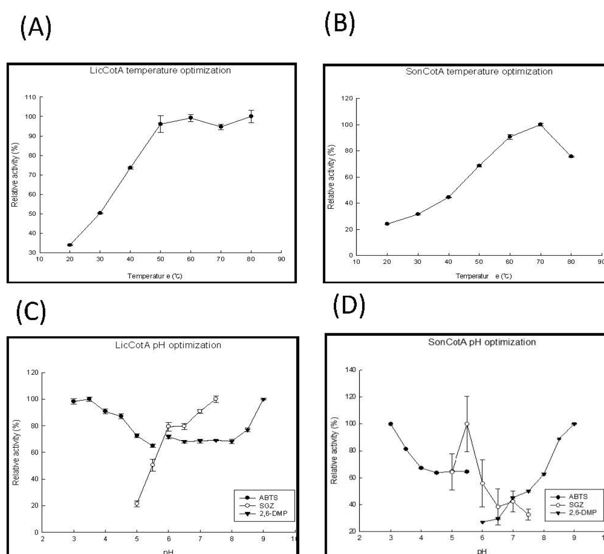

Fig. 4 Effect of temperature and pH on the activity of the purified recombinant laccase at 37℃. ABTS, pH 3.0-6.5; SGZ, pH 6.0-8.5; 2,6-DMP, pH 6.5-9.0. Panel (A) and (B) show the effects of temperature on LiccotA and SoncotA, while Panel (C) and (D) represent optimum temperatures of LiccotA and SoncotA

sequence of *Bacillus sonorensis* KCTC13918 *Bacillus sonorensis* laccase (GenBank Acc No. NZ_AYTN01000063). The PCR primers were as follows. SoncotA-F(*Nde*I): 5'-GCGGAATTCCATATGAAACTTGAAAAATTTGTCGAC-3', soncotA-R(*Not*I): 5'-GTAGGCGGCGGCCGCTGATTGGATGCGAAAATCTGTGAC-3'. For comparison of laccase activity in *Bacillus* species, another primer set for cloning of synthetic *Bacillus licherniformis cotA* (designated as *liccotA*) gene was synthesized by Bioneer Co. (Taejeon, Korea) and used as a template for cloning. PCR primers for *liccotA* are as follows: liccotA-F(NdeI):5'-GCGGAATTCCATATGAAACTGGAAAAATTCGTTGAC, liccotA-R(XhoI): 5'-GCGCTCGAGTTGATGACGAACATCTGTCAC-3'. The PCR was performed using ExTaq polymerase (Takara, Japan) as follows: 2 min at 94℃ (1 cycle), 15 sec at 94℃, 30 sec at 55℃, 2 min at 72℃ (25 cycles).

3.3. Transformation and expression

After PCR amplification, the laccase genes were digested with *Nde*I/*Not*I or *Nde*I/*Not*I and ligated into pET21c expression vector

between the *Nde*I and *Not*I or *Xho*I sites. Ligation mixtures were used to transform competent *E. coli* XL10-gold cells. Recombinant plasmid DNAs were isolated and confirmed by DNA sequencings,and then transformed into competent BL21(DE3)pLysS cells for the expression of recombinant laccase.

3.4. Purification of recombinant laccases

The cell lysate was obtained from 200 ml culture cell pellet by sonication in Ni-NTA column binding buffer containing 20 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, pH 7.4. The Ni-NTA column was washed with 40 mM imidazole containing same buffer, and eluted in 250 mM imidazole buffer. The eluted sample was extensively dialyzed against 0.1M citrate-phostaphate buffer (pH4.0) and used for enzyme characterization.

3.5 Enzyme assay and characterization

The standard reaction mixture (1 ml) contained 100 mM citrate-phosphate buffer (pH4.0), 0.5 mM ABTS and 93 ug purified recombinant laccase. The reaction mixtures were incubated at 37℃ and 180 rpm for 5 min in a shaking incubator. Thereafter, the reaction mixture was measured at 420 nm. Enzyme activity measurements were performed on a Multiskan spectrum microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a 96-well plate. All assays were performed in triplicate. One unit of laccase activity was defined as the amount of enzyme that produced 1 mmol of product per minute under the standard assay conditions[5]. Laccase activity in SDS-PAGE gel could be determined by incubation of recombinant laccase in 100mM citrate-phosphate buffer (pH4.0) containing 2 mM ABTS after extensive washing with 100 mM citrate-phosphate buffer.

4. Conclusion

In this report, we identified a new putative laccase gene (*soncotA*) which show 78% homology with *Bacillus lichenifomris* (*liccotA*) from draft genome sequence of *Bacillus sonorensis* KCTC 13918. Recombinant expression of a 1,545 bp of PCR product corresponding 514 amino acids resulted in a soluble form of about 59 kDa active laccase enzyme in *E. coli*. The characterization of enzymatic properties of recombinant laccase was described. At this time, we could not directly compare the wild type of laccases from *B. sonorensis* strain with recombinant laccase. Further study about remarkable low laccase activity of SoncotA should be followed. In conclusion, the finding of new laccase SoncotA broadened the enzymatic diversity of *Bacillus* species laccases and could be used for evolutionary relationship.

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