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Crystallization and preliminary X-ray diffraction analysis of a putative two-domain-type laccase from a metagenome

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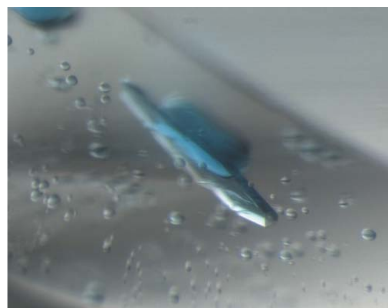

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A putative two-domain-type laccase retrieved from a metagenome was successfully crystallized using the sitting-drop vapour-diffusion method. Data were collected to a resolution of 1.7 Å at 100 K using synchrotron radiation. The crystal belonged to space group $P2_12_12_1$, with unit-cell parameters a = 74.67, b = 100.95, c = 124.11 Å. The self-rotation function showed the presence of a noncrystallographic threefold axis in the structure. The presence of one trimer in the asymmetric unit yielded a Matthews coefficient ($V_{\rm M}$) of 2.05 $Å^3$ Da^{-1} and a solvent content of 40%.

1. Introduction

Laccases (benzenediol:oxygen oxidoreductases; EC 1.10.3.2) are multicopper proteins that oxidize a range of phenolic and non-phenolic compounds using molecular oxygen as an electron acceptor to produce water. Known laccases have broad substrate specificity and thus may be used in a variety of biotechnological applications, including textile dye bleaching (Claus *et al.*, 2002), pulp bleaching (Palonen & Viikari, 2004), bioremediation (Murugesan, 2003; Wesenberg *et al.*, 2003), polymer synthesis (Huttermann *et al.*, 2001) and biosensors (Peter & Wollenberger, 1997). Laccases of fungal origin are the most well known, but recent studies have revealed the widespread presence of these enzymes in bacteria [*e.g.* CotA from *Bacillus subtilis* (Hullo *et al.*, 2001; Martins *et al.*, 2002), CueO from *Escherichia coli* (Grass & Rensing, 2001) and a laccase from *Thermus thermophilus* HB27 (Miyazaki, 2005)]. However, all of these enzymes are three-domain-type laccases. In addition to these bacterial laccases, other laccases that possess different structures have been identified. SLAC was identified in the genomic sequence of *Streptomyces coelicolor* (Machczynski *et al.*, 2004) and lacks the second domain of typical three-domain-type laccases. EpoA, an extra-cytoplasmic phenol oxidase, from *S. griseus* is also a two-domain laccase and is produced upon induction with copper in the medium (Endo *et al.*, 2002, 2003).

In silico screening of a metagenomic sequence database created in our laboratory identified a partial gene fragment with a copper-binding motif. Since the sequence did not have an open reading frame, we performed DNA sequencing to determine the entire sequence. We then cloned the full-length gene to reveal the coding of a protein of 359 amino-acid residues with a calculated molecular mass of 40 402 Da, which is small compared with most laccases. We consider this protein to be a novel putative two-domain-type laccase. In this paper, we report the crystallization and preliminary X-ray analysis of this two-domain-type laccase from metagenomic DNA.

2. Methods and results

2.1. Gene cloning, overexpression and protein purification

During the course of metagenomic library screening for various genes (Suenaga *et al.*, 2007; Mori, Mizuta *et al.*, 2008; Mori, Suenaga *et al.*, 2008), we constructed a metagenomic sequence database. In the search for various potentially useful enzymes, we identified a gene containing a copper-binding motif. We determined the full length of the gene to find that the deduced amino-acid sequence comprised

Table 1
Summary of crystallographic data.

Values in parentheses are for the highest resolution shell.

Source	SPring-8 BL38B1	SPring-8 BL38B1
Wavelength (Å)	1.0000	1.3500
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)	a = 74.67, b = 100.95, c = 124.11	a = 74.61, b = 100.92, c = 124.05
Resolution (Å)	50.00–1.70 (1.76–1.70)	50.00–2.00 (2.07–2.00)
No. of observed reflections	755765	410066
No. of unique reflections	103567 (10202)	62317 (5088)
Completeness (%)	100.0 (99.9)	97.1 (80.4)
$I/\sigma(I)$	25.6 (2.9)	22.0 (4.0)
R_{merge} (%)	8.0 (42.6)	8.7 (24.0)
Resolution for phasing (Å)		50.00–2.50
No. of Cu sites		12
Phasing power		1.189
Figure of merit (centric/acentric)		0.364/0.081

359 residues, which is small compared with most three-domain-type laccases commonly found in fungi and bacteria. Since the protein appeared to contain a signal peptide, the mature region of the protein was amplified by PCR using a set of oligonucleotide primers, 5′-TTTTCATATGGCAGAGAGGGAATTTGATATGACTATTGAG-3′ and 5′-TTTTTAAGCTTCCTCCCTGGACGACGTTGACGTT-GTTC-3′. The fragment was digested with *Nde*I and *Hin*dIII and subcloned into the corresponding sites of the pET-29(a)+ vector. The expression plasmid, designated pET29mgLAC1, was introduced into *E. coli* BL21(DE3) cells and transformants were selected on an LB agar plate containing 50 µg ml^{-1} kanamycin. A single colony was selected with a sterile toothpick and seeded into 35 ml LB medium supplemented with Overnight Expression autoinduction reagent, 50 µg ml^{-1} kanamycin and 2 m*M* $CuSO_4$. Cells were cultivated at 310 K for 17 h, collected by centrifugation (5000*g*, 20 min, 277 K) and resuspended in 4 ml water. After complete mixing of the cells, 0.1 ml 10× BugBuster was added and mixed by agitation at room temperature for 30 min. The cell lysate was collected by centrifugation (20 000*g*, 20 min, 277 K), heated at 343 K for 20 min and chilled in an ice–water bath. Precipitated materials were removed by centrifugation (20 000*g*, 20 min, 277 K) and the heat-treated supernatant was loaded onto a Qiagen (Hilden, Germany) Ni–NTA column (1 ml) pre-equilibrated with 20 m*M* sodium phosphate pH 7.4, 0.5 *M* NaCl and 25 m*M* imidazole. The column was washed with 10 ml equilibration buffer and bound proteins were eluted by increasing the concentration of imidazole from 25 m*M* to 0.5 *M* in the equilibration buffer over 20 ml. The buffer was changed to 20 m*M* Tris–HCl pH 8.0 by repeated ultrafiltration using a Millipore (Billerica, Massachusetts, USA) Amicon Ultra-15 device. We determined the protein concentration using a molar absorption coefficient at 280 nm of 59 360 M^{-1} cm^{-1}, which was calculated based on the amino-acid sequence using the method of Gill & von Hippel (1989). The purified protein solution was concentrated to 8.5 mg ml^{-1}.

2.2. Protein crystallization

Crystallization screening was carried out with Crystal Screens I and II and PEG/Ion Screen (Hampton Research) and the Cryo I and II sparse-matrix crystallization screens (Emerald Biosystems) using the sitting-drop vapour-diffusion method at 293 K. Protein droplets prepared by mixing 1 µl protein solution and 1 µl reservoir solution were equilibrated against 100 µl reservoir solution at 293 K. The crystals were obtained using a reservoir solution consisting of 0.2 *M* potassium chloride, 0.05 *M* HEPES pH 7.5 and 35%(*v*/*v*) pentaerythritol propoxylate (5/4 PO/OH). Plate-like crystals appeared and grew to approximate dimensions of around 0.05 × 0.1 × 0.4 mm after two weeks (Fig. 1).

2.3. Data collection and crystallographic analysis

For data collection, crystals were transferred directly from the mother liquor to the nitrogen cold stream at 100 K. X-ray diffraction data were collected from a single crystal on beamline BL38B1 (λ = 1.0000 Å) at SPring-8 using a Rigaku Jupiter210 CCD detector. The crystal-to-detector distance was maintained at 160 mm with an oscillation range per image of 0.5°, covering a total oscillation range of 180°. Diffraction from the crystal extended to a resolution of 1.7 Å. Determination of the unit-cell parameters and integration of reflections were performed using the *HKL*-2000 program package (Otwinowski & Minor, 1997). The crystal is orthorhombic and belongs to space group $P2_12_12_1$, with unit-cell parameters a = 74.67, b = 100.95, c = 124.11 Å. A total of 755 765 reflections were integrated to a resolution of 1.7 Å and were then merged to obtain 103 567 unique

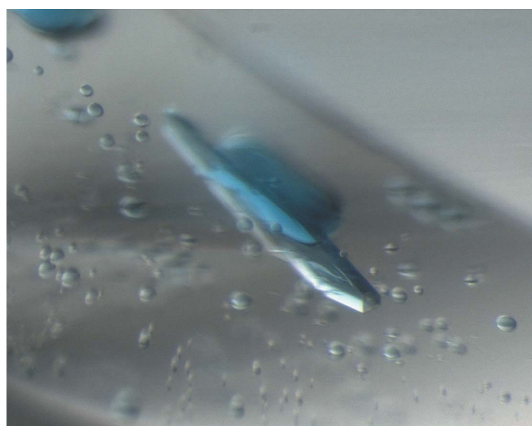

Figure 1
A crystal of the two-domain-type laccase from a metagenome. The crystal dimensions are 0.05 × 0.1 × 0.4 mm.

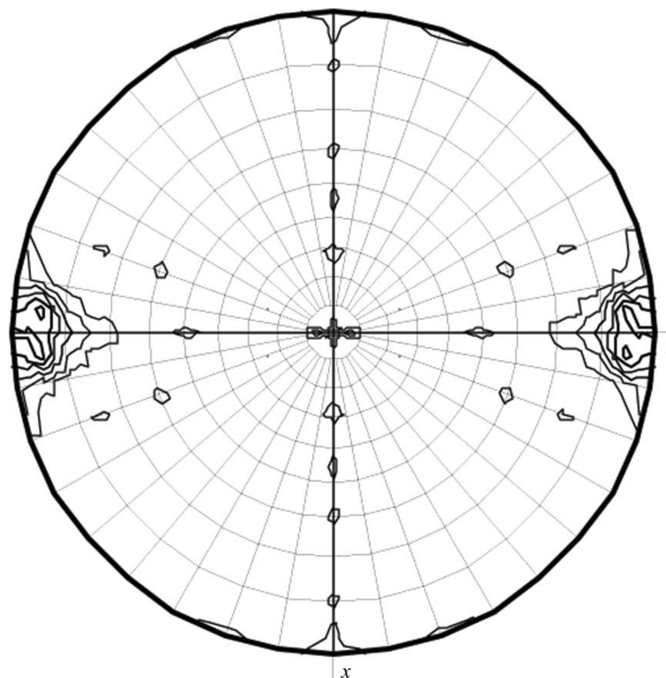

Figure 2
χ = 120° section of the self-rotation function calculated using *MOLREP* in the resolution range 15–3.5 Å with an integration radius of 30 Å.

reflections with an overall R_{merge} of 0.080 and a completeness of 100.0%. The data-collection statistics are summarized in Table 1.

The self-rotation function calculated using the *MOLREP* program (Vagin & Teplyakov, 1997) shows peaks corresponding to a non-crystallographic threefold axis (Fig. 2). According to the Matthews coefficient (V_M) calculation one trimer is present in the asymmetric unit, with an estimated solvent content of 40% (Matthews, 1968).

The structure was solved by the single-wavelength anomalous diffraction (SAD) technique using Cu atoms. A SAD data set (λ = 1.3500 Å) was collected to a resolution of 2.0 Å (Table 1). Heavy-atom refinement, density modification and initial structure modelling were performed using the *autoSHARP* program (Vonrhein *et al.*, 2007). Refinement and model building are currently ongoing using the programs *REFMAC* (Murshudov *et al.*, 1997) and *Coot* (Emsley & Cowtan, 2004).

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References

Claus, H., Faber, G. & König, H. (2002). *Appl. Microbiol. Biotechnol.* **59**, 672–678.

Emsley, P. & Cowtan, K. (2004). *Acta Cryst.* D**60**, 2126–2132.

Endo, K., Hayashi, Y., Hibi, T., Hosono, K., Beppu, T. & Ueda, K. (2003). *J. Biochem.* **133**, 671–677.

Endo, K., Hosono, K., Beppu, T. & Ueda, K. (2002). *Microbiology*, **148**, 1767–1776.

Gill, S. C. & von Hippel, P. H. (1989). *Anal. Biochem.* **182**, 319–326.

Grass, G. & Rensing, C. (2001). *Biochem. Biophys. Res. Commun.* **286**, 902–908.

Hullo, M.-F., Moszer, I., Danchin, A. & Martin-Verstraete, I. (2001). *J. Bacteriol.* **183**, 5426–5430.

Huttermann, A., Mai, C. & Kharazipour, A. (2001). *Appl. Microbiol. Biotechnol.* **55**, 387–394.

Machczynski, M. C., Vijgenboom, E., Samyn, B. & Canters, G. W. (2004). *Protein Sci.* **13**, 2388–2397.

Martins, L. O., Soares, C. M., Pereira, M. M., Teixeira, M., Costa, T., Jones, G. H. & Henriques, A. O. (2002). *J. Biol. Chem.* **277**, 18849–18859.

Matthews, B. W. (1968). *J. Biol. Chem.* **33**, 491–497.

Miyazaki, K. (2005). *Extremophiles*, **9**, 415–425.

Mori, T., Mizuta, S., Suenaga, H. & Miyazaki, K. (2008). *Appl. Environ. Microbiol.* **7**, 6803–6805.

Mori, T., Suenaga, H. & Miyazaki, K. (2008). *Biosci. Biotechnol. Biochem.* **72**, 1611–1614.

Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997). *Acta Cryst.* D**53**, 240–255.

Murugesan, K. (2003). *Indian J. Exp. Biol.* **41**, 1239–1248.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Palonen, H. & Viikari, L. (2004). *Biotechnol. Bioeng.* **86**, 550–557.

Peter, M. G. & Wollenberger, U. (1997). *EXS*, **80**, 63–82.

Suenaga, H., Ohnuki, T. & Miyazaki, K. (2007). *Environ. Microbiol.* **9**, 2289–2297.

Vagin, A. & Teplyakov, A. (1997). *J. Appl. Cryst.* **30**, 1022–1025.

Vonrhein, C., Blanc, E., Roversi, P. & Bricogne, G. (2007). *Methods Mol. Biol.* **364**, 215–230.

Wesenberg, D., Kyriakides, I. & Agathos, S. N. (2003). *Biotechnol. Adv.* **22**, 161–187.