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Purification, crystallization and preliminary X-ray structure analysis of the laccase from *Ganoderma lucidum*

Andrey V. Lyashenko,[a] Oksana Belova,[b] Azat G. Gabdulkhakov,[a] Alexander A. Lashkov,[a] Alexandr V. Lisov,[b] Alexey A. Leontievsky[b] and Al'bert M. Mikhailov[a]*

[a]A. V. Shubnikov Institute of Crystallography, Russian Academy of Sciences, Leninskiy Prospect 59, Moscow 119333, Russia, and [b]G. K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino 142290, Russia

Correspondence e-mail: amm@ns.crys.ras.ru

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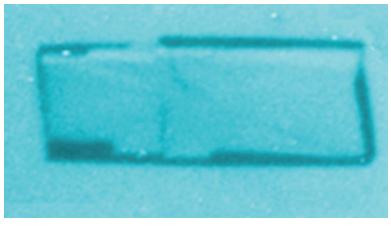

The ligninolytic enzymes of the basidiomycetes play a key role in the global carbon cycle. A characteristic property of these enzymes is their broad substrate specificity, which has led to their use in various biotechnologies, thus stimulating research into the three-dimensional structures of ligninolytic enzymes. This paper presents the purification, crystallization and preliminary X-ray analysis of the laccase from the ligninolytic basidiomycete *Ganoderma lucidum*.

1. Introduction

Copper-containing proteins are widespread in living organisms. They contain one or more Cu atoms per protein molecule and in most cases are involved in redox processes. An important group of Cu-containing proteins are the so-called blue copper oxidases: multi-copper oxidoreductases which combine various oxidation reactions of reduced substrates with the reduction of oxygen (Farver & Pecht, 2007). For example, CotA oxidase is involved in the formation of melanin in *Bacillus subtilis* spore coats, higher plant ascorbate oxidase participates in ascorbic acid metabolism and bilirubin oxidase supports bilirubin homeostasis (Sakurai & Kataoka, 2007). Laccase is the most well known enzyme of the blue copper oxidase group. It is known to occur in bacteria, imperfect fungi, ascomycetes, basidiomycetes, lichens, plants and insects (Claus, 2003; Asada *et al.*, 2004; Baldrian, 2006; Lisov *et al.*, 2007). Fungal laccases have been relatively more studied. A typical fungal laccase is a monomeric glycoprotein with a molecular mass of 50–70 kDa and a carbohydrate content of 15–30%. Homodimeric laccases have also been reported (Ng & Wang, 2004; Yaver *et al.*, 1996). Fungal laccases are acidic proteins with an isoelectric point of lower than 5.0 and contain four Cu atoms arranged into three metal centres. The type 1 metal centre contains one Cu atom and is assigned to the absorption band in the region of 600 nm in the enzyme absorption spectrum. The type 2 metal centre also contains one Cu atom. The type 1 and 2 metal centres are responsible for the characteristic EPR spectrum of the enzyme. The type 3 metal centre contains two Cu atoms which are EPR-invisible owing to strong antiferromagnetic coupling. However, the type 3 copper centre shows a shoulder at 330–345 nm in the absorption spectrum (Solomon *et al.*, 1996). The type 1 copper site shuttles electrons from the reducing substrate to the other copper sites. The type 1 Cu atom is typically coordinated by two histidines and one cysteine residue. The type 2 and 3 metal centres make up a united cluster responsible for oxygen reduction. The type 2 copper is three-coordinated by two histidine ligands and one water molecule. The type 3 coppers are each four-coordinated by three histidine ligands and a bridging hydroxide. The role of the type 2 and 3 copper centres is to reduce oxygen to water (Morozova *et al.*, 2007).

The ability of laccases to oxidize reducing substrates depends on the redox-potential ratio of the type 1 Cu atom and the substrate: the efficiency of oxidation increases with decreasing redox potential of the substrate (Xu, 1996). There are two types of laccase substrates: (i) electron donors and (ii) electron and proton donors. Typical representatives of the first group are 2,2′-azino-bis-(3-ethylthiazoline-

6-sulfonate) (ABTS) and Fe^{II}. The pH optimum for the oxidation of such substrates is lower than 2.5. The other group of substrates are substituted phenols and aromatic amines. In a typical laccase reaction, a phenolic substrate undergoes one-electron oxidation to form aryloxy radicals. These reactive species can convert to quinones, which are coupled in a non-enzymatic reaction to form polymers (Thurston, 1994). The optimum pH for oxidation of such substrates is around 5.0. Laccases can catalyze reactions with polymeric matter (lignin, proteins and humic acids) in which polymers undergo various transformations: oxidation of side substituents, cleavage of ether bonds of aromatic subunits in lignin and polymerization/depolymerization reactions (Zavarzina *et al.*, 2004). Laccases can extend their substrate range in the presence of synthetic or natural molecules, which are called mediators (Bourbonnais & Paice, 1990; Li *et al.*, 1999). The most efficient mediators include synthetic compounds such as ABTS, TEMPO and various aryl-NO structures, *e.g.* 1-hydroxybenzotriazole. Phenolic mediators of natural origin have also been reported to exist (Camarero *et al.*, 2008; González *et al.*, 2009). Owing to their broad substrate specificity and the advantages of using mediators, laccases have become a useful tool in biotechnology. Thus, laccases and laccase–mediator systems are used in enzymatic dye bleaching, detoxification of pollutants and bioremediation, delignification and biobleaching of pulp and in enzymatic synthesis (Abadulla *et al.*, 2000; Camarero *et al.*, 2004; Gianfreda & Rao, 2004).

Recent studies have reported the crystal structures of several laccases from various organisms and the determination of their three-dimensional structures by X-ray diffraction analysis. Data have been obtained for laccases from basidiomycetes such as *Coprinus cinereus* (Ducros *et al.*, 2001), *Trametes versicolor* (Piontek *et al.*, 2002), *Coriolus zonatus* (Lyashenko, Bento *et al.*, 2006), *Cerrena maxima* (Lyashenko, Zhukhlistova *et al.*, 2006; Lyashenko, Zhukova *et al.*, 2006), *Panus tigrinus* (Ferraroni *et al.*, 2005), *Rigidoporus lignosus* (Garavaglia *et al.*, 2004) and *Trametes hirsuta* (Polyakov *et al.*, 2009), and the ascomycetes *Melanocarpus albomyces* and *Thielavia arenaria*, the CotA bacterial laccase from the endospore coat of *Bacillus subtilis* (Enguita *et al.*, 2003) and the small laccase from *Streptomyces coelicolor* (Skálová *et al.*, 2007). The crystal structure of a laccase of unknown organism from a metagenome has also been determined (Komori *et al.*, 2009).

The white-rot basidiomycete *Ganoderma lucidum* is a popular remedy in Oriental medicine and is known to be a producer of antitumour and immunomodulatory agents (Lin & Zhang, 2004). To date, three isozymes of *G. lucidum* laccase have been purified and characterized (Ko *et al.*, 2001), the gene for *G. lucidum* laccase has been cloned and expressed, and the antioxidative properties of the enzyme have been studied (Joo *et al.*, 2008).

This paper presents the crystallization and preliminary structural analysis of laccase from the white-rot fungus *G. lucidum*.

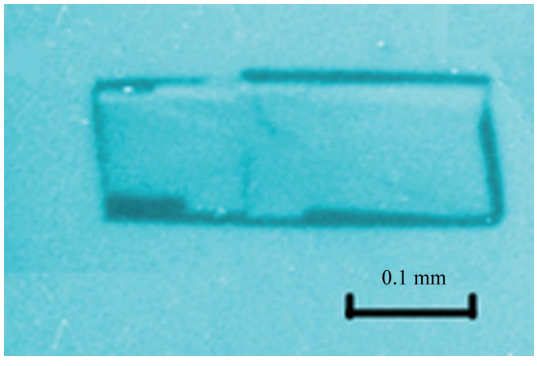

Figure 1
Crystal of *G. lucidum* laccase.

Table 1
X-ray data statistics.

Values in parentheses are for the last resolution shell.

Wavelength (Å)	1.05
Temperature (K)	100
Oscillation (°)	1
Total rotation range (°)	140
Crystal-to-detector distance (mm)	220
No. of crystals	1
Exposure time per image (s)	10
Space group	$P2_1$ (No. 4)
Unit-cell parameters (Å, °)	a = 54.388, b = 94.630, c = 104.133, $\alpha = \gamma$ = 90, β = 102.933
Asymmetric unit content	2 molecules
V_M (Å^3 Da^{-1})	2.25
Solvent content (%)	45.4
Resolution limits (Å)	27.52–3.1 (3.2–3.1)
Completeness (%)	91.2 (79.1)
No. of reflections	42126 (2123)
No. of unique reflections	17187 (1389)
$R_{observed}(I)$† (%)	34.5 (42.2)
Mosaicity (°)	0.6
Wilson B factor (Å^2)	35.1
Mean $I/\sigma(I)$	5.12 (1.91)
Multiplicity	2.45 (1.52)

† $R_{observed}(I) = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

2. Purification of the enzyme

G. lucidum OE99 from the collection of IBPM RAS was grown in 750 ml flasks in 'high-nitrogen' liquid mineral medium (Kirk *et al.*, 1978) with 1 m*M* $CuSO_4{\cdot}5H_2O$ and 1 m*M* $MnSO_4{\cdot}H_2O$ as laccase inducers. Upon the achievement of maximum laccase activity (8 d of cultivation), the culture broth was harvested. The laccase was purified by anion-exchange chromatography of the culture liquid on DEAE Sevacell 52 equilibrated with 20 m*M* sodium acetate buffer pH 5.0 (buffer *A*). The enzyme was eluted with 0.5 *M* NaCl in buffer *A* at a flow rate of 1 ml min^{-1}. The active fractions were combined, dialyzed against buffer *A* and loaded onto a DEAE Sepharose CL column. Elution was performed with a linear gradient of 0–0.5 *M* NaCl in buffer *A*. The active fractions were combined, dialyzed against buffer *A*, loaded onto a Mono Q column and eluted under the same conditions using an ÄKTA FPLC system (General Electric, USA). As a final stage, gel filtration on a HiLoad 26/60 Superdex 200 column was used. As a result, an electrophoretically homogeneous laccase preparation of molecular mass 64 kDa was obtained and was used for subsequent crystallization experiments.

3. Crystallization

Initial screening was performed at 298 K employing Crystal Screen, Crystal Screen 2, Index, Crystal Screen Cryo and Crystal Screen 2 Cryo crystallization solutions (Hampton Research). Crystals of *G. lucidum* laccase were obtained by mixing 2 µl protein solution (28 mg ml^{-1} in 20 m*M* sodium acetate buffer pH 5.0) with 2 µl reservoir solution. Crystallization was performed on siliconized glass slides (Hampton Research, USA) in Linbro plates at 277 K using the hanging-drop vapour-diffusion method. The reservoir solution consisted of 0.49 *M* sodium phosphate, 0.91 *M* potassium phosphate pH 6.9 (condition No. 18 from Index) and 5%(*w*/*v*) polyethylene glycol 3350. Crystals (Fig. 1) grew in two weeks.

4. Data collection

An X-ray data set was collected to 3.1 Å resolution on beamline X13 of the Deutsches Elektronen-Synchrotron (EMBL/DESY, Hamburg, Germany) using a MAR CCD 165 detector (MAR Research, Germany), an oscillation range $\Delta\varphi$ of 1° and a crystal-to-detector distance of 220 mm. The crystals were directly flash-cooled in a stream of cold nitrogen gas at 100 K using an Oxford Cryosystems cooling device (Oxford Cryosystems Ltd, England). The wavelength was 1.05 Å. Prior to freezing in liquid nitrogen, the crystals were transferred into a cryoprotectant solution consisting of 1 *M* sodium/potassium phosphate pH 6.9 and 20%(*v*/*v*) anhydrous glycerol. All data were processed and scaled using *XDS* (Kabsch, 2010). 500 images were obtained during the collection of X-ray diffraction data. All data were indexed, merged and processed using the *XDS* program with XYCORR, INIT, COLSPOT, IDXREF, DEFPIX, XPLAN, INTEGRATE and CORRECT options. For a semiautomatic determination of the space group, the minimal value of the *XDS* 'quality-of-fit' function was used. The crystals of the laccase from *G. lucidum* belonged to space group $P2_1$. Detailed data statistics are presented in Table 1.

5. X-ray structure analysis

The structure was resolved by the molecular-replacement technique using the *Phaser* program with rigid-body refinement option (McCoy, 2007). X-ray diffraction data from 10 to 3.1 Å resolution were used in this step. The X-ray structure of one monomer only of ligand-free laccase from *T. versicolor* at 2.4 Å resolution (PDB entry 1kya; Bertrand *et al.*, 2002) was utilized as a search model. Metal ions, water molecules and carbohydrates were excluded from the starting model. Two molecules were found in the asymmetric unit. The Matthews coefficient (Matthews, 1968) was 2.25 $Å^3\,Da^{-1}$ and the solvent content was 45.4% (Table 1).

The top molecular-replacement models were assessed using *Z*-score and log-likelihood gain (LLG) statistics. Before refinement, 5% of the observations were chosen at random and were set aside for cross-validation analysis and to monitor the various refinement strategies. After ten cycles of restrained refinement in *REFMAC* (Murshudov *et al.*, 2011), the *R* factor was 40.49% and R_{free} was 47.53%.

After manual correction of the model using *Coot* (Emsley & Cowtan, 2004), we performed three further cycles of restrained refinement in *REFMAC*. The *R* factor was 37.18% and R_{free} was 43.26%. We identified four copper ions in the $(|F_o| - |F_c|)$ electron-density map from *Coot* (Fig. 2). The contour level of the copper ions was 4.5σ.

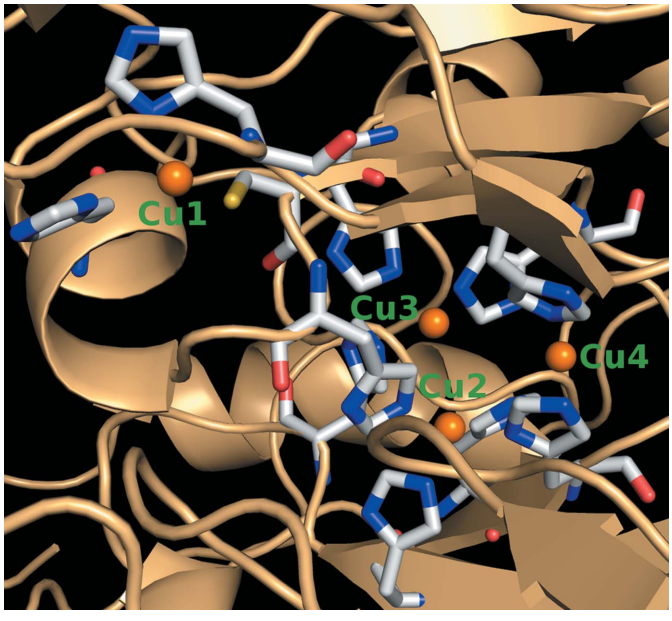

Figure 2
Structure of the laccase molecule from *G. lucidum*. The cartoon representation was generated with *PyMOL* (DeLano, 2002).

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