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Crystallization and preliminary X-ray diffraction analysis of the small laccase from *Streptomyces coelicolor*

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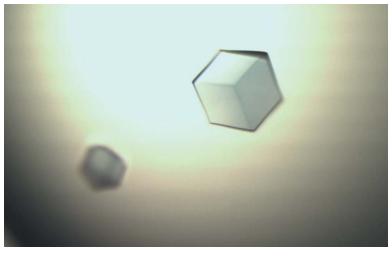


The small bacterial laccase from the actinobacterium *Streptomyces coelicolor* which lacks the second of the three domains of the laccases structurally characterized to date was crystallized. This multi-copper phenol oxidase crystallizes in a primitive tetragonal lattice, with unit-cell parameters $a = b = 179.8$, $c = 175.3$ Å. The crystals belong to either space group $P4_12_12$ or $P4_32_12$. The self-rotation function shows the presence of a noncrystallographic threefold axis in the structure. Phases will be determined from the anomalous signal of the natively present copper ions.

1. Introduction

Laccases (EC 1.10.3.2) are multicopper oxidases that catalyze the reduction of molecular oxygen to water accompanied by the oxidation of a substrate (Sakurai & Kataoka, 2007). The laccases play many different biological roles and exhibit rather broad substrate specificity. It is therefore difficult to define them by their reducing substrates, which include polyphenols, methoxy-substituted phenols, aromatic diamines and a range of other compounds. They contain four copper ions localized as one sole ion (type 1 copper), which gives them their characteristic blue colour, and a separated trinuclear cluster (types 2 and 3 copper ions; Malmström, 1982; Machczynski *et al.*, 2004). Laccases of known three-dimensional structure consist of three domains and the copper ions are located in and between the C- and N-terminal domains. The laccase from the actinobacterium *Streptomyces coelicolor* was named a 'small' laccase as it consists of only two domains, which are similar to domains 1 and 3 of the classical laccases. Expression of the small *S. coelicolor* laccase in *Escherichia coli* and its characterization have been described previously (Machczynski *et al.*, 2004).

Intensive research efforts into the exploitation and development of laccases as industrial catalysts have been driven by their ability to catalyze the oxidation (by O_2) of industrial effluents and other substances of industrial interest. The studied applications can be divided into the following classes depending on the final objective (Couto & Herrera, 2006; Wells *et al.*, 2006; Mayer & Staples, 2002; Riva, 2006): (i) bioremediation agents for the detoxification of industrial effluents and soil pollutants, (ii) the transformation of raw materials into functional materials, (iii) biosensors for medical diagnostics to detect of a wide range of reducing substances, and (iv) catalysts in organic speciality chemical synthesis. Like other industrial enzymes, laccases may replace hazardous chemicals, reduce energy consumption and create novel chemistry. Ideally, their successful application will thus contribute to reductions in the economical and environmental costs of the processes.

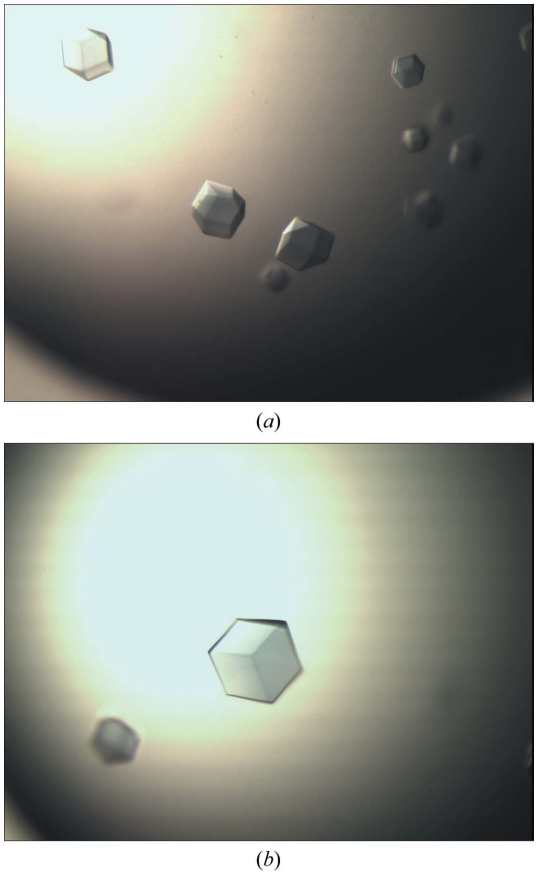
2. Materials and methods

2.1. Expression and purification

The M145 derivative of *S. coelicolor* A3(2) was obtained from the National Collection of Type Cultures (NCTC) in London. Genomic DNA was prepared from *S. coelicolor* grown on a plate with GYM

Streptomyces medium using a FastDNA SPIN Kit for Soil from Qbiogene Inc. The gDNA was used as template in a polymerase chain reaction (PCR) with the primers 5′-primer (AGG ATT CAC CAT GGA CAG GCG AGG CTT TAA C) and 3′-primer (ACT CGA GTC AGT GCT CGT GTT CGT GTG CGG C). The Phusion DNA polymerase from Finnzymes was used for the PCR in combination with GC buffer owing to the GC-rich nature of the template DNA. As given by the primers, the amplified DNA sequence preserved the native secretion signal, TAT, found in *S. coelicolor*. The PCR product was cloned into an expression vector essentially as described by Christensen *et al.* (1988), using standard molecular-biology techniques. After verification by DNA sequencing, the construct was transformed into protoplasts of *Aspergillus oryzae* for expression driven by the TAKA amylase promoter. The transformed strain of *A. oryzae* was typically grown for 4 d at 310 K in MY51 medium (30 g maltodextrin, 2 g $MgSO_4$, 10 g KH_2PO_4, 2 g K_2SO_4, 2 g citric acid, 10 g yeast extract, 0.5 ml trace metals, 1 g urea and 2 g ammonium sulfate pH 6.0 per litre) with 2% maltose and 500 µ*M* copper(II) sulfate for production of the laccase. The fermentation broth was then sterile filtered to remove fungal hyphae.

The resulting filtrate was used as the starting material in purification of the laccase. Briefly, the protein was bound to a pre-equilibrated column of Source 15Phe in the presence of 1.2 *M* ammonium sulfate. After a thorough wash, the bound protein was eluted from the column using MilliQ water. Fractions with a clear blue colour indicative of the laccase enzyme were pooled. The pH of the pooled fractions was adjusted to 9.0 and the pool was diluted with deionized water to reduce the conductivity to 2.5 mS cm^{-1}. The laccase-containing pool was then applied onto a Q-Sepharose FF column pre-equilibrated with 50 m*M* H_3BO_3–NaOH pH 9.0. After washing the column thoroughly with the equilibration buffer, the column was eluted with a linear NaCl gradient (0–0.5 *M*) in the equilibration buffer over five column volumes. Collected fractions containing pure laccase, as estimated by SDS–PAGE and spectroscopy, were pooled. The pooled enzyme solution was stored at 253 K until use. All purification steps were carried out at room temperature.



(*a*)

(*b*)

Figure 1
Crystals of laccase from *S. coelicolor* grown using (*a*) Jeffamine ED-2001 as precipitant (crystal size ∼100 µm), (*b*) PEG 550 monomethyl ether as precipitant (crystal size ∼200 µm).

2.2. Crystallization

The Hampton Research Index Screen was used for initial screening for crystallization conditions of the laccase, which was concentrated to 7 mg ml^{-1} in 0.05 *M* H_3BO_3–NaOH pH 9.0 at 298 K. Clusters of needles grew in several conditions. After optimization, lentil-shaped crystals with a pentagonal or hexagonal appearance and a diameter of ∼130 µm were obtained with 0.2 *M* ammonium sulfate, 8%(*w*/*v*) polyethylene glycol (PEG) 3350, sodium acetate trihydrate pH 4.5 using 1 + 1 µl drops and 1 ml reservoir using the hanging-drop vapour-diffusion technique with a protein concentration of 14.8 mg ml^{-1} and a temperature of 291 K. These crystals diffracted poorly at an in-house X-ray source and some of them disintegrated within one month.

The screening for crystallization conditions was further extended to Hampton Research Crystal Screen (Jancarik & Kim, 1991) and Crystal Screen 2 (Cudney *et al.*, 1994), but this did not lead to crystals of better quality. The effect of additives on crystal formation was tested by use of the Additive Screen (Hampton Research), additives 1–48, to attempt to improve the crystallization conditions of the lentil-shaped crystals. The additives had only a small effect, producing crystals of similar morphology and some clusters of needles.

Four months after the initial setup of the Hampton Research Index Screen, light blue protein crystals of distinct morphology were formed in condition No. 39 [30%(*w*/*v*) Jeffamine ED-2001 pH 7.0, 0.1 *M* HEPES pH 7.0]. After optimization, reproducible crystals with a diameter of up to 140 µm (Fig. 1*a*) were obtained with 45%(*w*/*v*) Jeffamine ED-2001 titrated to pH 7.0 and 0.05 *M* HEPES pH 7.0 using 1 + 1 µl drops and 1 ml reservoir with the hanging-drop vapour-diffusion technique and a protein concentration of 14.8 mg ml^{-1} at a temperature of 298 K, with a growth time of one to two weeks. Some of these crystals also disintegrated within one month, but intact crystals diffracted well at an in-house X-ray source.

Six months after set-up of the experiment, a crystal was found in Crystal Screen 2 condition No. 46 [0.1 *M* NaCl, 0.1 *M* bicine pH 9.0, 20%(*v*/*v*) PEG monomethyl ether 550]. Modified conditions with increased precipitant concentration led to crystals with dimensions of up to 200 µm and a growth time of one week (Fig. 1*b*) using 1 + 1 µl drops and 1 ml reservoir with the hanging-drop vapour-diffusion technique, a protein concentration of 15.0 mg ml^{-1} and a temperature of 298 K; the reservoir solution contained 0.1 *M* NaCl, 0.1 *M* glycine pH 9.0 and 39%(*v*/*v*) PEG monomethyl ether 550. Drops with a 2:1 protein:reservoir ratio and the same conditions led to larger crystals with dimensions of up to 400 µm with a two-week growth time.

Crystals grown in both the PEG monomethyl ether 550 and the Jeffamine conditions occur with the same morphology (probably a rhombic dodecahedron; Fig. 1), the same crystal lattice and comparable diffraction quality. The crystals from the PEG monomethyl ether 550 conditions seem to be more stable over time.

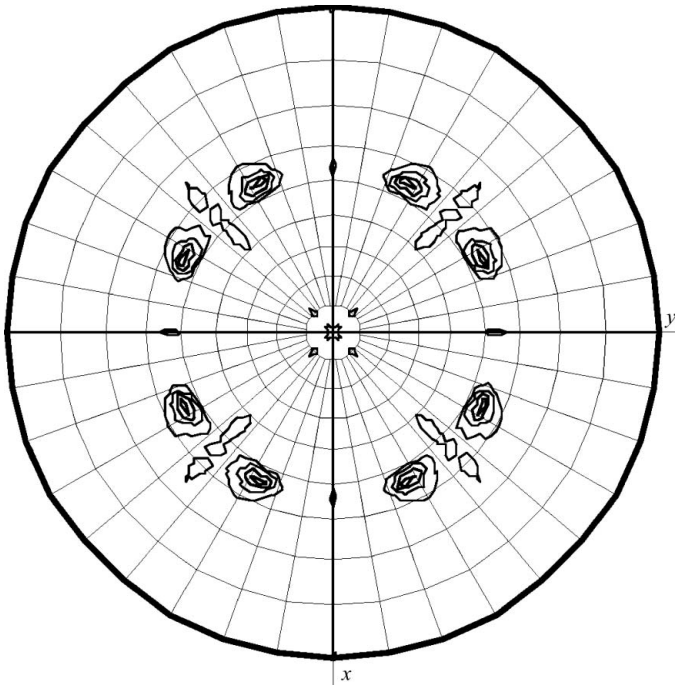


Figure 2
Stereographic projection of the $\chi = 120^\circ$ section of the self-rotation function calculated using data in the resolution range 32.9–3.7 Å and an integration radius of 66.5 Å (*MOLREP*; Vagin & Teplyakov, 1997).

2.3. Data collection and X-ray analysis

Preliminary diffraction data from a laccase crystal grown using the Jeffamine ED-2001 condition were collected on beamline ID 23-1 at the ESRF in Grenoble using an ADSC Quantum Q315r detector. Cryoprotection was not necessary owing to the high polymer concentration in the crystallization condition. The data were processed to 3.0 Å resolution with *MOSFLM* (Leslie, 1992) and merged with *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). The data-collection and processing statistics are listed in Table 1.

The crystal has a primitive tetragonal lattice, with unit-cell parameters $a = b = 179.8$, $c = 175.3$ Å, $\alpha = \beta = \gamma = 90^\circ$, and belongs to space group $P4_12_12$ or $P4_32_12$.

The self-rotation function was calculated in *MOLREP* (Vagin & Teplyakov, 1997) to 3.7 Å resolution and with a radius of integration of 66.5 Å. A peak of the function above 2.6σ for $\chi = 120^\circ$ suggests the presence of a noncrystallographic threefold axis in the structure at $\theta = 55^\circ$ and $\varphi = 27^\circ$ (Fig. 2).

Molecular-replacement trials were performed in *MOLREP* using the structure of laccase from *Coprinus cinereus* (Ducros *et al.*, 2001; PDB code 1hfu) and also using its individual domains 1 and 3. A computational model based on sequence analysis and modelling and its individual domains were also tested as models for molecular replacement, in all cases without any positive results.

An attempt to solve the phase problem using single-wavelength anomalous dispersion (SAD) of natively present copper ions was performed with the radiation wavelength not optimized for the copper anomalous signal. Several copper-ion positions were found using *SHELXC* (Sheldrick, 2004), *SHELXD* (Schneider & Sheldrick, 2002) and *MLPHARE* (Collaborative Computational Project, Number 4, 1994). However, the experimental phases obtained using *SHELXE* (Sheldrick, 2002) and other phase-modification algorithms were not sufficient for structure solution.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P4_12_12$ or $P4_32_12$
Unit-cell parameters (Å, °)	$a = 179.8$, $b = 179.8$, $c = 175.3$, $\alpha = \beta = \gamma = 90$
Resolution range (Å)	33.13–3.00 (3.16–3.00)
No. of observations	3849668
No. of unique reflections	57991 (8334)
Data completeness (%)	99.9 (100.0)
Redundancy	15.6 (16.1)
Wavelength (Å)	1.072
Average $I/\sigma(I)$	20.6 (6.4)
$R_{\rm merge}$†	0.147 (0.447)
$R_{\rm r.i.m.}$‡	0.152 (0.461)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \overline{I(hkl)}|/\sum_{hkl}\sum_i I_i(hkl)$ (Weiss, 2001), where $I_i(hkl)$ and $\overline{I(hkl)}$ are the observed individual and mean intensities of a reflection with indices *hkl*, respectively, $\sum_i$ is the sum over the individual measurements of a reflection with indices *hkl* and $\sum_{hkl}$ is the sum over all reflections. ‡ $R_{\rm r.i.m.} = \sum_{hkl}[N/(N-1)]^{1/2} \times \sum_i |I_i(hkl) - \overline{I(hkl)}|/\sum_{hkl}\sum_i I_i(hkl)$ (Weiss, 2001), where N is the redundancy of a reflection with indices *hkl*.

The results from the self-rotation function and heavy-atom site analyses suggest the presence of trimers in the crystal structure rather than the expected dimers found by other authors. There are examples of laccases that are functional in a trimeric form, but this behaviour was not predicted in the case of our *S. coelicolor* enzyme. A multiple-wavelength anomalous dispersion experiment using a tunable source of X-ray radiation is planned.

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