Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Purification, crystallization and preliminary crystallographic analysis of recombinant Lac15 from a marine microbial metagenome

Honghua Ge,[a,b] Peisong Xu,[a,b]‡ Ying Xu,[a,b]‡ Zemin Fang[a,b] and Yazhong Xiao[a,b]*

[a]Modern Experiment Technology Center and School of Life Sciences, Anhui University, Hefei 230039, People's Republic of China, and [b]Anhui Provincial Engineering Technology Research Center of Microorganisms and Biocatalysis, Hefei 230039, People's Republic of China

‡ These authors contributed equally to this work.

Correspondence e-mail: xiaoyz@yahoo.cn

Received 9 May 2011
Accepted 24 June 2011

Laccases are members of the blue multi-copper oxidase family that can oxidize a wide range of aromatic compounds. A new bacterial laccase (Lac15) has recently been obtained from a marine microbial metagenome from the South China Sea and characterized. In this work, recombinant Lac15 was overexpressed in *Escherichia coli*, purified and crystallized using the hanging-drop vapour-diffusion method. An X-ray diffraction data set was collected to 2.2 Å resolution. The crystal belonged to space group *C*121, with unit-cell parameters a = 123.41, b = 91.36, c = 86.157 Å, β = 112.10°.

1. Introduction

Laccases (benzenediol:oxygen oxidoreductases; EC 1.10.3.2) are copper-containing oxidases that can oxidize a wide range of substrates, preferably phenolic compounds, with concomitant reduction of molecular oxygen to water, bypassing the stage of hydrogen peroxide production (Solomon *et al.*, 1996; Hoegger *et al.*, 2006). Laccases have been the subject of increasing attention owing to their established or potential applications in biotechnology, and fundamental studies have been performed on their structure and catalytic mechanism (Giardina *et al.*, 2010).

Laccases have been found in plants, insects, bacteria and nearly all wood-rotting fungi. In plants, laccases participate in lignin biosynthesis (Bao *et al.*, 1993), whereas in fungi they are involved in the degradation of lignin, detoxification and pathogenesis and also in fungal development and morphogenesis (Eggert *et al.*, 1997; Leonowicz *et al.*, 2001; Baldrian, 2006; Dittmer *et al.*, 2004). In insects, laccases appear to play a role in sclerotization of the cuticle in the epidermis (Dittmer *et al.*, 2004). Bacterial laccases are probably involved in morphogenesis, pigment biosynthesis and copper homeostasis (Hullo *et al.*, 2001; Suzuki *et al.*, 2003).

Although whole-genome analyses suggest that laccases are widespread in bacteria (Alexandre & Zhulin, 2000; Sharma *et al.*, 2007), few of them have been biochemically characterized and little is known about their applications (Arias *et al.*, 2003; Koschorreck *et al.*, 2009). To date, only fungal laccases have been used in biotechnological applications. However, the development of bacterial laccases would be of significant importance because bacterial genetic tools and biotechnological processes are well established.

A new bacterial laccase, Lac15, with alkalescence-dependent activity and excellent chloride tolerance has recently been obtained from a marine microbial metagenome from the South China Sea and characterized (Fang *et al.*, 2011). Lac15 shares less than 40% sequence identity with all characterized bacterial multicopper oxidases. It also has low sequence similarity to other laccases of known structure, with an identity of 22% to the typical bacterial laccase CotA from *Bacillus subtilis* (PDB entry 1gsk; Enguita *et al.*, 2003), 26% to LacB from *Trametes* sp. AH28-2 (PDB entry 3kw7; Ge *et al.*, 2010) and 27% to an ascomycete fungal laccase from *Thielavia arenaria* (PDB entry 3pps; Kallio *et al.*, 2011).

Recombinant Lac15 expressed in *Escherichia coli* was stable at pH values in the range 5.5–9.0 and at temperatures from 288 to 328 K; it showed high activity at pH 6.5–9.0 with an optimum pH of 7.5 and with highest activity occurring at 318 K. Interestingly, Lac15 showed excellent chloride tolerance, with its activity retaining its original level even at 1000 m*M* chloride. Although the native function and *in vivo* substrate of Lac15 remain unclear, it is able to oxidize a range of substrates *in vitro*, including several industrial dyes of the reactive azo class under alkalescent conditions. Because of its alkalescence-dependent activity, high chloride tolerance and dye-decolorization ability, the new laccase Lac15 could be an alternative for specific industrial applications.

In order to better understand the details of the Lac15 oxidation mechanism, we purified and crystallized Lac15 expressed in *E. coli* and performed preliminary X-ray analysis of the crystal.

2. Materials and methods

2.1. Cloning, expression and purification

Lac15 was cloned and expressed as described previously (Fang *et al.*, 2011). Briefly, cDNA corresponding to Lac15 (GenBank accession No. HM623889) without the 22-amino-acid signal peptide was cloned into expression vector pET22b (incorporating a C-terminal hexahistidine tag). The pET22b-Lac15-encoding plasmid was transformed into *E. coli* BL21 (DE3) and the cells were grown at 310 K in Luria–Bertani medium containing 100 µg ml^{-1} ampicillin. Expression of Lac15 was induced at an OD_{600} of 0.4–0.6 by adding 0.5 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by incubation at 289 K for 16 h.

The cultured cells were harvested by centrifugation and lysed by sonication in buffer *A* (20 m*M* Tris–HCl pH 7.6, 200 m*M* NaCl). The lysate was clarified by centrifugation at 15 000*g* for 30 min. The soluble fraction was applied onto nickel-chelating resin (Amersham Biosciences) pre-equilibrated with buffer *A*. The resin was washed with buffer *B* (buffer *A* plus 40 m*M* imidazole) and the target protein was eluted using buffer *C* (buffer *A* plus 200 m*M* imidazole).

The eluate was exchanged into buffer *D* (20 m*M* Tris–HCl pH 7.6, 50 m*M* NaCl) and further purified by chromatography on a MonoQ anion ion-exchange column (GE Healthcare) in buffer *D*. The target protein was eluted using a linear gradient of 50–500 m*M* NaCl in 20 m*M* Tris pH 7.6 and was then applied onto a gel-filtration column (16/60 Superdex 200, GE Healthcare) in buffer *A*. The purified protein was concentrated to 25 mg ml^{-1} in buffer *E* (20 m*M* Tris pH 7.6, 20 m*M* NaCl) by centrifugal ultrafiltration (Millipore). The protein concentration was determined by the Bradford method (Bio-Rad protein assay) using bovine serum albumin as a standard. The presence and purity of the recombinant Lac15 was then analyzed on SDS–PAGE (better than 95% purity) and it was judged to be suitable for crystallization.

Figure 1
Lac15 crystals of approximately 0.15 × 0.06 × 0.05 mm in size.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.97916
Space group	*C*121
Unit-cell parameters (Å, °)	a = 123.41, b = 91.36, c = 86.15, $\alpha = \gamma = 90$, $\beta = 112.10$
Resolution range (Å)	40–2.2 (2.32–2.20)
No. of unique reflections	44251
Molecules in asymmetric unit	2
Solvent content (%)	49.74
$R_{\rm merge}$† (%)	8.3 (47.2)
Average $I/\sigma(I)$	10.4 (3.1)
Multiplicity	4.1
Completeness (%)	98.4 (97.9)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean intensity of the i observations of reflection hkl.

2.2. Crystallization and data collection

The protein was diluted to different concentrations with buffer *E* for initial screening and optimization. A Mosquito Crystallization Robot (TTP LabTech) was used to set up initial crystallization trials with Crystal Screen, Crystal Screen 2 and PEG/Ion Screen reagent kits (Hampton Research) using the sitting-drop vapour-diffusion method at 277 K. Each sitting drop, consisting of 0.15 µl protein solution (5–15 mg ml^{-1}) and an equal volume of reservoir solution, was equilibrated against 150 µl reservoir solution. Further crystal-optimization experiments were performed using the hanging-drop vapour-diffusion method. The crystals grew to their full dimensions in the final conditions after one month. The crystals were harvested using cryoloops and briefly immersed in a cryoprotectant solution consisting of reservoir solution with 20% glycerol. The crystals were subsequently flash-cooled, stored in liquid nitrogen and transferred to the SSRF (Shanghai Synchrotron Radiation Facility) for X-ray diffraction analysis and data collection.

After screening for diffraction quality, a complete data set to 2.2 Å resolution was collected using a single crystal maintained at 100 K on beamline BL17U of the SSRF at a wavelength of 0.97916 Å using 1° oscillations with a crystal-to-detector distance of 220 mm and an exposure time of 0.8 s per image. The diffraction data were processed with *iMOSFLM* (Battye *et al.*, 2011) and scaled with *SCALA* from the *CCP*4 program suite (Winn *et al.*, 2011). Table 1 gives a summary of the data-collection statistics.

3. Results and discussion

Recombinant Lac15 from a marine microbial metagenome was expressed in *E. coli* and purified to homogeneity. The enzyme was crystallized in the substrate-free form.

Within three weeks, small crystals appeared in several drops containing PEG 3350 or PEG 4000 at pH 6.5 (0.1 *M* MES or 0.1 *M* Bis-Tris). We optimized the conditions by varying the precipitant concentration and protein concentration using the hanging-drop method. Finally, the best crystals (approximately 0.15 × 0.06 × 0.05 mm in size; Fig. 1) were produced by mixing 2.0 µl protein solution (10 mg ml^{-1})

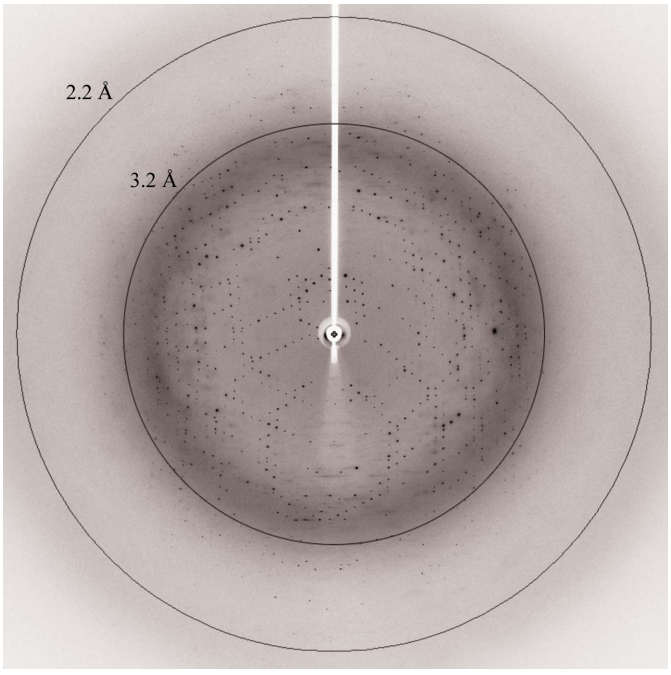

Figure 2
X-ray diffraction image of the Lac15 crystal. The resolution circles are at approximately 3.2 and 2.2 Å resolution.

and an equal volume of reservoir solution consisting of 0.1 *M* MES pH 6.5, 18% PEG 3350 and incubating at 277 K.

The crystal was flash-cooled to 100 K and diffraction data were collected to 2.2 Å resolution (Fig. 2). Crystal parameters and data-collection statistics are summarized in Table 1. The Lac15 crystal belonged to space group *C*121, with unit-cell parameters $a = 123.41$, $b = 91.36$, $c = 86.15$ Å, $\beta = 112.10^{\circ}$. Assuming the presence of two 46 kDa molecules in the asymmetric unit, a Matthews coefficient of 2.45 $Å^3\ Da^{-1}$ and a solvent content of 49.74% were calculated (Matthews, 1968).

Since Lac15 shares less than 27% sequence identity with related structures in the PDB, a heavy-atom-derivative search and molecular-replacement trials are both currently under way.

The authors would like to thank Professor Maikun Teng and Liwen Niu at USTC for generous assistance. We also thank Dr Ye Yuan and Minhao Wu for assistance during data collection. This work was supported by the Natural Science Foundation of the Department of Education of Anhui Province (Grant No. KJ2011A015), Anhui Provincial Natural Science Foundation (Grant No. 11040606M66), the National High Technology Research and Development Program of China (2011AA09070305), the Scientific Research Foundation for Returned Scholars, Ministry of Education of China, the Innovative Research Team Program of 211 Project in Anhui University.

References

Alexandre, G. & Zhulin, I. B. (2000). *Trends Biotechnol.* **18**, 41–42.
Arias, M. E., Arenas, M., Rodríguez, J., Soliveri, J., Ball, A. S. & Hernández, M. (2003). *Appl. Environ. Microbiol.* **69**, 1953–1958.
Baldrian, P. (2006). *FEMS Microbiol. Rev.* **30**, 215–242.
Bao, W., O'Malley, D. M., Whetten, R. & Sederoff, R. R. (1993). *Science*, **260**, 672–674.
Battye, T. G. G., Kontogiannis, L., Johnson, O., Powell, H. R. & Leslie, A. G. W. (2011). *Acta Cryst.* D**67**, 271–281.
Dittmer, N. T., Suderman, R. J., Jiang, H., Zhu, Y.-C., Gorman, M. J., Kramer, K. J. & Kanost, M. R. (2004). *Insect Biochem. Mol. Biol.* **34**, 29–41.
Eggert, C., Temp, U. & Eriksson, K. E. (1997). *FEBS Lett.* **407**, 89–92.
Enguita, F. J., Martins, L. O., Henriques, A. O. & Carrondo, M. A. (2003). *J. Biol. Chem.* **278**, 19416–19425.
Fang, Z., Li, T., Wang, Q., Zhang, X., Peng, H., Fang, W., Hong, Y., Ge, H. & Xiao, Y. (2011). *Appl. Microbiol. Biotechnol.* **89**, 1103–1110.
Ge, H., Gao, Y., Hong, Y., Zhang, M., Xiao, Y., Teng, M. & Niu, L. (2010). *Acta Cryst.* F**66**, 254–258.
Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S. & Sannia, G. (2010). *Cell. Mol. Life Sci.* **67**, 369–385.
Hoegger, P. J., Kilaru, S., James, T. Y., Thacker, J. R. & Kües, U. (2006). *FEBS J.* **273**, 2308–2326.
Hullo, M. F., Moszer, I., Danchin, A. & Martin-Verstraete, I. (2001). *J. Bacteriol.* **183**, 5426–5430.
Kallio, J. P., Gasparetti, C., Andberg, M., Boer, H., Koivula, A., Kruus, K., Rouvinen, J. & Hakulinen, N. (2011). *FEBS J.* **278**, 2283–2295.
Koschorreck, K., Schmid, R. D. & Urlacher, V. B. (2009). *BMC Biotechnol.* **9**, 12.
Leonowicz, A., Cho, N. S., Luterek, J., Wilkolazka, A., Wojtas-Wasilewska, M., Matuszewska, A., Hofrichter, M., Wesenberg, D. & Rogalski, J. (2001). *J. Basic Microbiol.* **41**, 185–227.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Sharma, P., Goel, R. & Capalash, N. (2007). *World J. Microbiol. Biotechnol.* **23**, 823–832.
Solomon, E. I., Sundaram, U. M. & Machonkin, T. E. (1996). *Chem. Rev.* **96**, 2563–2606.
Suzuki, T., Endo, K., Ito, M., Tsujibo, H., Miyamoto, K. & Inamori, Y. (2003). *Biosci. Biotechnol. Biochem.* **67**, 2167–2175.
Winn, M. D. *et al.* (2011). *Acta Cryst.* D**67**, 235–242.