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Glycosylated yellow laccases of the basidiomycete *Stropharia aeruginosa*

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ABSTRACT

Here we describe the identification, purification and characterisation of glycosylated yellow laccase proteins from the basidiomycete fungus *Stropharia aeruginosa.* Biochemical characterisation of two yellow laccases, Yel1p and Yel3p, show that they are both secreted, monomeric, N-glycosylated proteins of molecular weight around 55 kDa with substrate specificities typical of laccases, but lacking the absorption band at 612 nm typical of the blue laccase proteins. Low coverage, high throughput 454 transcriptome sequencing in combination with inverse-PCR was used to identify cDNA sequences. One of the cDNA sequences has been assigned to the Yel1p protein on the basis of identity between the translated protein sequence and the peptide data from the purified protein, and the full length gene sequence has been obtained. Biochemical properties, substrate specificities and protein sequence data have been used to discuss the unusual spectroscopic properties of *S. aeruginosa* proteins in the context of recent theories about the differences between yellow and blue laccases.

1. Introduction

Oxidoreductases (E.C.1) comprise a superfamily of enzymes including fungal lignin degrading enzymes. Oxidoreductases are frequently used in industry to catalyse a wide array of reactions such as asymmetric oxyfunctionalisations, polymer synthesis and modification, oxidative degradation of pollutants and many others [1]. However, most of these reactions require expensive co-factors such as NAD, NADP and FAD, which must be either supplied or regenerated, adding additional steps and further increasing costs [2,3]. Laccases (E.C. 1.10.3.2) are oxidative enzymes belonging to the family of multicopper oxidases that do not require expensive co-factors; instead they catalyse a one electron oxidation of a substrate coupled with a four electron reduction of molecular oxygen to water. The enzymes use molecular oxygen, rather than peroxide, as an oxidant, and they thus have the capacity to be more environmentally-friendly. This potential for reduced costs along with the green credentials of laccases and their ability to utilise a range of substrates, including phenolics, lignins and alkenes, make them attractive alternatives to other oxidoreductases in a range of processes such as bioremediation and synthetic chemistry.

Laccases are one of the oldest-known group of enzymes and were discovered as a component of the resin ducts of the lacquer tree *Rhus venicifera* by Yoshida at the end of 19th century [4]. It is now known that laccases are a diverse group of proteins that can be found in fungi, plants, bacteria and insects [5,6]. Most laccases are blue, resulting from a charge transition from the sulphur atom of a cysteine residue to a Cu^{2+} ion at the catalytic T1 site giving rise to an absorption band at around 600 nm [7]. However, it is now known that not all laccases are blue and both yellow and white laccases have also been identified, along with other unusual laccases such as heterodimeric enzymes [8]. Unlike the four copper atoms found in blue and yellow laccases, white laccases contain one copper, two zinc ions and one iron ion per protein molecule [9]. Yellow laccases are believed to have greater industrial potential than blue laccases because of expanded substrate specificity, often giving them the capacity to oxidise non-phenolic lignins [10]. There are several

Abbreviations: ABTS 2,2′, azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); L-DOPA, L-3,4-dihydroxyphenylalanine; ConA, Concanavalin A; CBS, Centraalbureau voor.

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Table 1
Protein purification steps used to isolate the Yel1p and Yel3p *S.aeruginosa* yellow laccases.

No.	Purification step	Laccase annotation	Laccase specific activity (U mg^{-1})	Enrichment
1.	Extract		0.60	–
2.	Salting out		9.50	16
3.	Q Sepharose		16.6	28
4.	Concanavalin A		156	260
5.	Phenyl Toyopearl flow-through 0.8 M $(NH_4)_2SO_4$	Yel1p	158	263
6.	Phenyl Toyopearl elution 0.28 M $(NH_4)_2SO_4$		198	330
7.	Phenyl Toyopearl elution 0.064 M $(NH_4)_2SO_4$	Yel3p	120	200
8.	Mono Q 0.4 M NaCl	Yel1p	323	539
9.	Superdex 75	Yel1p	394	657
10.	Superdex 75	Yel3p	191	318

theories attempting to explain the yellow colour of certain laccases. Early reports proposed that yellow laccase proteins are essentially identical to blue laccases, but with a lignin-derived mediator in the catalytic site that causes the disappearance of the blue colour and is responsible for the expansion of substrate specificity [10,11]. However, most recent reports suggest that alterations to the catalytic copper coordinating sphere are the likely explanation for the yellow colour [12]. In addition to laccases, yellow variants of other copper proteins, namely multicopper oxidases have also been described [13]. These results show that yellow forms of copper proteins are more common than first thought [13].

We had previously identified a potentially interesting laccase-like activity in the basidiomycete fungus *Stropharia aeruginosa*. Thus our first objective was to purify and characterise the enzyme(s) responsible for this activity [14]. The second objective was to clone and characterise the gene coding sequence(s). As in a previous study [15], we employed 454 transcriptome pyrosequencing to identify the cDNA sequences when more usual PCR-based cloning methods proved unsuccessful, probably as a consequence of gene duplications, the multiplicity of small exons and frequency of alternative transcripts.

2. Materials and methods

2.1. Organism

S. aeruginosa CBS 839.87 purchased from Centraalbureau voor Schimmelcultures (Netherlands) was cultured in high nitrogen content medium (HNC) containing: 2% (w/v) glucose, 0.5% (w/v) mycological peptone (Oxoid), 0.2% (w/v) yeast extract (Sigma), 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4$, 6 ppm (w/v) NaCl, stationary, at 25 °C, for four weeks.

2.2. Protein purification and biochemical characterisation

Protein was isolated directly from the culture medium, protein purification steps are summarised in Table 1. The medium was filtered through Miracloth (Calbiochem) and protein was precipitated overnight with 35% (w/v) $(NH_4)_2SO_4$ at 4 °C from 5 L of HNC medium. Precipitated protein was pelleted by centrifugation, dissolved in 100 mM potassium phosphate, pH 6.5 (buffer A) and dialysed overnight against buffer A. The solution was then mixed on a roller mixer as a 1/5 (v/v) mixture with pre-equilibrated Q Sepharose (Sigma) for 30 min to uniformly bind pigment to the anion exchanger. Unbound protein was recovered by centrifugation and stored. After washing, bound protein was eluted from the Q Sepharose using 0.5 M NaCl in Buffer A, recovered by centrifugation from the pigmented chromatographic medium and combined with the unbound fraction. The resultant protein was loaded onto ConA Sepharose (Sigma) equilibrated with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ in buffer A. Bound proteins were eluted with 100 mM α-D-methylmannopyranoside (Sigma) in buffer A. The ConA eluate was saturated with $(NH_4)_2SO_4$ to a final concentration of 0.8 M. Precipitated proteins were pelleted at 20,000 × *g* and discarded. The supernatant was loaded onto Phenyl Toyopearl (Tosoh), equilibrated with 0.8 M $(NH_4)_2SO_4$ in buffer A. Bound proteins were eluted with a decreasing gradient 0.8 M–0 M $(NH_4)_2SO_4$. Active fractions were pooled, concentrated with spin concentrators (Millipore) and denoted as Yel3p. The non-bound fractions (Yel1p) were collected, pooled, concentrated with spin concentrators and the buffer exchanged to 100 mM Tris–HCl pH 9.0 (Buffer B). Yel1p was loaded onto a pre-packed MonoQ (GE Healthcare) chromatographic column equilibrated with buffer B. The protein was eluted with a gradient of 0–2 M NaCl in buffer B. Protein polishing was performed with Superdex 75 (GE Healthcare) equilibrated with buffer A containing 0.15 M NaCl. Molecular weights were determined with the same column calibrated with gel filtration molecular weight markers 12,000–200,000 (Sigma). All purification steps from the ConA step onwards were performed using a BioLogic DuoFlow system (Bio-Rad). Protein assays were performed according to the Bradford method [16] using a calibration curve prepared for bovine serum albumin (BSA). SDS-PAGE was performed according to the method of Laemmli [17].

Purified protein samples (5 μg) were digested with 200 ng trypsin (Sigma) in 50 mM ammonium bicarbonate containing 2 M urea overnight at 30 °C and stopped with 1% (w/v) formic acid. The samples were run on a tandem mass spectrometer at the University of Liverpool proteomics facility; the data were sequenced manually using the PepSeq module of the MassLynx package (Waters).

2.3. Biochemical analysis

2.3.1. Laccase activity, kinetic analysis, absorbance spectrum

One unit of laccase activity was defined as the amount of enzyme required to oxidise 1 μmol of substrate per minute. Enzyme activity was determined in triplicates *via* the monitoring of the oxidation of a variety of substrates at 42 °C for 15 min. The assay mixture (1 mL) under standard conditions contained: 890 μL of 0.1 M McIlvaine buffer pH 3.0; 100 μL of substrate at 1 mM final concentration and 10 μL of appropriately diluted enzyme. The following molar extinction coefficients were used to determine activity with the various substrates: ABTS 36,000 M^{-1} cm^{-1}; *o*-dianisidine 11,300 M^{-1} cm^{-1}; and syringaldazine 65,000 M^{-1} cm^{-1}. All measurements were made using a Unicam UV2 UV/vis Spectrometer. The kinetic measurements included variable concentrations of the substrates. Thermostability tests included a pre-incubation step of 1 h at temperatures in the range 4–90 °C, whereas pH stability included pre-incubation step of 24 h in 0.1 M Britton Robinson buffer in the pH range 2–12 at 4 °C before the activity measurements were performed under standard conditions. The assays used to determine the temperature activity profiles were performed over the temperature range 4–80 °C. pH activity profiles were performed in 0.1 M Britton–Robinson buffer in the pH range 2–12. Other parameters of the activity assay were kept to standard conditions. Kinetic data were calculated on the basis of non-linear fitting with Graphpad Prism 5 (Graphpad software). The absorbance spectra of purified proteins were measured with a Nanodrop 1000 (Thermo Scientific) using 1 μL of purified protein sample.

2.3.2. Dye decolourisation

Decolourisation was determined in triplicate by spectrophotometric monitoring of the oxidation of different dyes (Sigma). Dyes and the wavelengths corresponding to their maximal absorbance are summarised in Table 2. Each decolourisation reaction contained 1 mL of 50 μM dye in 0.1 M Britton–Robinson buffer pH 3.0 and 0.1 U_{ABTS} of purified protein. Decolourisation reactions were performed at 42 °C and decrease in absorbance was monitored at hourly intervals over a 6 h period.

2.4. Molecular biology methods

Partial gene sequences of yellow laccases were identified using transcriptome sequencing. Inverse-PCR was then used to obtain the full length cDNA sequences using primers based on the partial DNA sequence. The full length cDNA was then assembled, amplified and cloned into pJET 1.2 vector and sequenced. Unless stated

Table 2
Decolourisation of dyes by purified *S. aeruginosa* yellow laccases Yel1p and Yel3p.

Dye	λ (nm)	Decolourisation by Yel1p after 6 h (%)	Decolourisation by Yel3p after 6 h (%)
Amaranth	522	5	8
New coccine	506	7	13
Orange G	475	8	31
Tartrazine	428	8	11
Remazol Brilliant Blue R	592	19	59
Reactive Black 5	590	12	19
Reactive Orange 16	494	7	13