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Evidence for a halotolerant-alkaline laccase in *Streptomyces psammoticus*: Purification and characterization

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Abstract

An unusual halotolerant-alkaline laccase from *Streptomyces psammoticus* has been purified to homogeneity through anion exchange and gel filtration chromatography steps with an overall purification fold of 12.1. The final recovery of the enzyme was 22.1%. The molecular mass of the purified laccase was about 43 kDa. The enzyme was active in the alkaline pH range with pH optima at 8.5 and 97% activity retention at pH 9.0. The optimum temperature was 45 °C. The enzyme was stable in the pH range 6.5–9.5 and up to 50 °C for 90 min. The enzyme was tolerant to NaCl concentrations up to 1.2 M. It was inhibited by all the putative laccase inhibitors while the enzyme was activated by metal ions like Fe, Zn, Cu, Na and Mg. Fe enhanced the enzyme activity by twofold (204%). The enzyme showed lowest K_m value with pyrogallol (0.25 mM) followed by ABTS (0.39 mM). The purified enzyme was a typical blue laccase with an absorption peak at 600 nm.

Keywords: Laccase; Halotolerance; Alkaline enzyme; Purification; Characterization; *Streptomyces psammoticus*

1. Introduction

The research on laccases has been intensified in the recent years due to its applications in diverse biotechnological sectors. This versatile enzyme plays significant role in pulp biobleaching [1], dye decolorization [2], phenol and aromatic compounds degradation [3,4], wine and fruit juice stabilization [5], etc. It is the low substrate specificity of laccases that makes them a best choice for industrial applications. Laccases act on phenols, substituted phenols, aromatic amines, etc. The catalytic cycle of laccases involves one-electron oxidation of four reducing substrate molecules concomitant with the four-electron reduction of molecular oxygen to water [6]. They belong to the family of blue copper oxidases, which also includes other proteins such as ascorbate oxidase, ceruloplasmin, etc. [7].

Laccases have been found commonly in fungi and some higher plants. White rot fungi are the leading laccase producers and the purification of enzyme from this group has been largely reported [8–10]. Laccases have also been purified and characterized from certain non-filamentous bacteria like *Azospirillum lipoferum*, *Marinomonas mediterranea* and *Bacillus subtilis* [7,11,12].

Actinomycetes are believed to be the potent producer of laccases, next to fungi. Purification and characterization of laccases from actinomycetes, especially, different *Streptomyces* sp. have been accounted. The laccase-like phenol oxidase from *Streptomyces griseus* has been reported to have a highly unique homotrimer structure [13] while the small laccase (SLAC) from *Streptomyces coelicolor* has been described as a dimer, lacking the second domain [14]. Laccase from *Streptomyces lavendulae* has been reported as thermostable, being stable at 70 °C [15]. Arias et al. [1] have described a laccase from *Streptomyces cyaneus* that was capable of oxidizing non-phenolic compounds in the presence of mediators. The enzymes with remarkable properties are of great interest owing to their significance in various applications. In this paper we report for the first time the purification and characterization of a halotolerant-alkaline laccase from a novel mangrove isolate *Streptomyces psammoticus*, with wide substrate range and having potential for vivid biotechnological applications.

2. Materials and methods

2.1. Microorganism and culture conditions

S. psammoticus MTCC 7334 was isolated from a mangrove swamp [16]. The culture was maintained on starch–casein agar slants and 1-week-old fully grown slants were used for inoculum preparation. The culture was aseptically

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transferred to the inoculum medium with the following composition (g L^{-1}): glucose, 5.0; yeast extract, 3.0; $(NH_4)_2SO_4$, 0.1; $MgSO_4$, 0.1, $CaCO_3$, 0.02; 10 mL of trace elements solution that contained (g L^{-1}): $FeSO_4$, 1.0; $ZnSO_4$, 0.9; $MnSO_4$, 0.2. The culture was allowed to grow in the above medium for 48 h and used as the inoculum. Laccase production was carried out by the solid-state cultivation, using rice straw [17]. The substrate was moistened with a salt solution containing (g L^{-1}): yeast extract, 3.0; $(NH_4)_2SO_4$, 0.2; $MgSO_4$, 0.2; $CaCO_3$, 0.04; CuSO4, 0.5. The initial moisture content and incubation temperature were maintained at 65% and 32 °C, respectively. After 48 h of fermentation, the crude enzyme was extracted from the fermented solid matter using 50 mM sodium phosphate buffer, pH 7.0 (buffer A), centrifuged at 10,000 rpm for 20 min at 4 °C and the supernatant was taken for purification steps.

2.2. Laccase assay

Laccase activity was measured by monitoring the oxidation of 500 μM 2,2′-azino-bis-[3-ethyl benzothiazoline-6-sulfonic acid] (ABTS) (SIGMA) buffered with 0.2 M sodium phosphate buffer (pH 7.5) at 420 nm for 1 min [18]. One unit of enzyme activity was defined as 1 μM of ABTS oxidized per minute. To calculate enzyme activity an absorption coefficient of 3.6×10^4 M^{-1} cm^{-1} was used.

2.3. Protein estimation

Total soluble protein was determined by Lowry's method [19] using bovine serum albumin as the standard.

2.4. Purification of laccase

The proteins were precipitated from the supernatant with ammonium sulfate (0–80% saturation). Laccase activity was detected in 30–60% saturated fractions. Laccase active fractions were pooled, centrifuged (10,000 rpm, 20 min) and the precipitate was dissolved in minimal amount of buffer A and dialyzed against the same buffer overnight at 4 °C. The dialyzate was loaded on to a DEAE cellulose anion exchange column (10 cm × 2.8 cm), which was equilibrated and washed with 50 mM sodium phosphate buffer, pH 6.0 (buffer B). The enzyme was eluted with a linear gradient of NaCl (0–1.0 M) at the flow rate of 0.5 mL/min and the eluate was monitored for absorbance at 280 nm, conductivity and laccase activity. Fractions containing laccase activity were then pooled and concentrated with an Amicon ultrafiltration stirred cell containing a 10 kDa molecular mass cutoff membrane (MWCO), and applied to a Biogel P 100 (Bio-Rad) column (30 cm × 3 cm) that was pre-equilibrated with buffer A. The column was eluted with the same buffer at a flow rate of 0.5 mL/min. The fractions containing laccase activity were pooled and stored at 4 °C until further use.

2.5. Enzyme characterization

2.5.1. Analytical electrophoresis sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and activity staining

Molecular weight of the purified laccase was determined by SDS-PAGE as per the method of Laemmli [20]. The strength of the gel was 12% (w/v) and the protein bands were stained with Coomassie brilliant blue R 250. Medium range (14.3–97.4 kDa) molecular weight markers (Genei) were used to determine the molecular mass of purified laccase. The markers were: phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soyabean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa).

Activity staining of purified laccase was performed using 1,8 diaminonaphthalene (DAN) as per the method of Hoopes and Dean [21]. After staining with DAN, the gel was counterstained with Coomassie brilliant blue R.

2.5.2. Isoelectric focusing

Isoelectric pH of the purified laccase was determined using rotofor (Biorad). Focusing was carried out at a constant power of 12 W for 2 h. Twenty samples were collected and analyzed for enzyme activity and pH. The ampholyte used was in the pH range of 3.0–10.0.

2.5.3. Effects of pH and temperature on laccase activity and stability

The optimum pH of the enzyme was determined within a pH range of 4–10 using ABTS as substrate in the following buffers: acetate (pH 4–5.5); phosphate (pH 6–8); Tris–HCl (pH 8.5); glycine–NaOH (pH 9–10) at 30 °C. The effect of temperature on enzyme activity was measured in the range of 25–60 °C at pH 8.5. The effect of pH on enzyme stability was determined by incubating the enzyme in different buffers in the pH range of 6.0–10, at optimum temperature. Thermal stability was determined by incubating the enzyme in Tris–HCl buffer, pH 8.5 at different temperatures. For experiments on pH and thermal stability, the residual activities were measured under the standard assay conditions at frequent time intervals for 90 min.

2.5.4. Effect of salt on enzyme activity

Effect of NaCl on purified laccase was determined at different concentrations of NaCl ranging from 0.2 to 2.0 M concentration. Salt tolerance of the purified laccase was determined by incubating the enzyme at different concentrations of NaCl, up to 2 M for 24 h at 45 °C. The residual activity was calculated under the standard assay conditions at frequent time intervals. Laccase activity in the absence of NaCl was also determined, for comparison.

2.5.5. Effects of inhibitors and metal ions

Eight potential laccase inhibitors were selected to evaluate their effect on the purified laccase from *S. psammoticus*. The enzyme was incubated with various inhibitors for 10 min at optimum temperature and the laccase activity was measured with 0.5 mM ABTS. The effect of various metal ions on laccase activity was also evaluated. The final concentration of the inhibitors and metal ions were decided specifically for each of the compound.

2.5.6. Total carbohydrate content

The total carbohydrate content of the enzyme was estimated by Dubois method [22] using glucose as the standard.

2.5.7. Spectral studies

The spectral studies of the purified laccase were carried out using a UV–vis spectrophotometer (Shimadzu UVPC-2401).

2.5.8. Substrate specificity and kinetic studies

The substrate specificity of purified laccase was tested with a range of substrates that included non-phenolic compounds, substituted phenols and aromatic amines. All the substrates were studied at a final concentration of 1 mM and the oxidation of each of the substrate was measured at its maximum absorbance wavelength and the enzyme activity was calculated using the respective extinction coefficient values. The kinetic constant K_m was determined for the enzyme with substrates such as ABTS, pyrogallol and syringaldazine.

3. Results and discussion

3.1. Purification of laccase

Ammonium sulfate precipitation (30–60% saturation) increased the specific activity from 1.78 to 7.6 U/mg. A purification fold of 4.3 was achieved by this step (Table 1). The main increase in specific activity was obtained after the chromatographic step using DEAE cellulose, in which the laccase fractions were eluted with 0.11–0.54 M NaCl gradient. The specific activity has increased to 20.3 U/mg with a fold purification of 11.4. The anion exchange chromatography also helped to remove the dark color from the protein solution. Subsequent purification by gel filtration using Biogel P 100 served to further increase the fold purification to 12.1, although