Investigation of catalytic activation of a *Bacillus* laccase after temperature treatment

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Abstract

Laccases belong to a family of multicopper oxidases that have strong oxidation abilities towards phenolic compounds. Interestingly, new isolated laccase showed the remarkable behavior of activation after thermal treatment. The activity increased at least 300% also the kcat was successfully enhanced (2.5 fold) after 50 min incubation at 70 °C. Due to the limited knowledge presented on this interesting phenomenon, the present paper concerns the differences which are induced in enzyme structure after thermal treatment. The local changes in secondary structures were also obseved. Oligomerization studies of the enzyme using gas-phase electrophoretic mobility macromolecule analysis (GEMMA) did not prove any oligomerization.

Keywords: Laccase; Intrinsic fluorescence; gas-phase electrophoretic mobility macromolecule analysis, Thermoactivation

Introduction

Laccases(monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1) are a member of an important group, the multicupper oxidases, that catalyze the oxidation of a variety of aromatic substances to less hazardous compounds [1-3]. Due to harsh industrial process conditions, improved biochemical properties such as thermostability and

thermotolerance will facilitate novel and efficient applications [4, 5]. Stimulation of activity by incubation of the enzyme at higher temperatures has been demonstrated for some laccases from fungal origins. The e behavior of the laccase after incubation at high temperatures showed remarkable differences in comparison to CotA from *Bacillus subtilis.*

Methods

The recombinant plasmid pET21a (+) containing laccase gene was transformed into E. coli BL21 (DE3). Laccase activity was determined spectrophotometrically at 525 nm by following the oxidation of 0.05 mM syringaldazine in 100 mM potassium phosphate buffer pH 7.0 (ε= 65,000 M-1 cm-1)[6]. Thermotolerance of the isolated laccase enzyme was determined in two temperatures (70 °C and 80 °C) by incubating the enzymes in 60 µl aliquots for 1 to 240 minutes. After the incubation, the tubes were chilled on ice and the residual laccase activity was measured with SGZ. Laccase activity without thermal treatment was marked as 100%. Thermal activation was also determined in the presence of CaCl2, CaSO4, CuCl2, CuSO4 (2 mM) and CH3COONa (4 mM) as divalent and monovalent ions. Some biochemical experiments such as gas-phase electrophoretic mobility macromolecule analysis (GEMMA), size-exclusion chromatography, intrinsic fluorescence measurement and circular dichroism studies were performed to investigate possible oligomerization or conformational differences between non-incubated and incubated laccase enzyme at higher temperatures.

Results and discussion

The enzyme without any thermal incubation showed the kcat and KM values of 20 s-1 and 5 µM, respectively. These parameters were changed to 50 s^{-1} and 5 µM after incubation of the laccase (Fig. 1).

Thermal activation of laccase was also investigated in the presence of different ions: $CaCl_2$, $CaSO_4$, $CuCl_2$, $CuSO_4$ (2 mM) and CH_3COONa (4 mM). Laccase solution without any extra ions (just in the presence of phosohate buffer) served as the control. In the presence of divalent ions, no activation was observed in comparison to control after incubation at 70 °C; but in the presence of CH_3COONa, thermal activation was obvious. GEMMA analysis proved no oligomerization occurs in Bacillus laccase. The result showed that the laccase keeps its monomeric form both before and after heating (Fig. 2).

High temperature induces minor changes in laccase conformation.

The increase in fluorescence intensity indicates a more compact conformation of enzyme after incubation at 70 °C up to 50 min. It can be mentioned that increasing the time of incubation upto 240 minutes, led to reduction in fluorescence intensity. The inset presents variation in fluorescence intensity versus time.

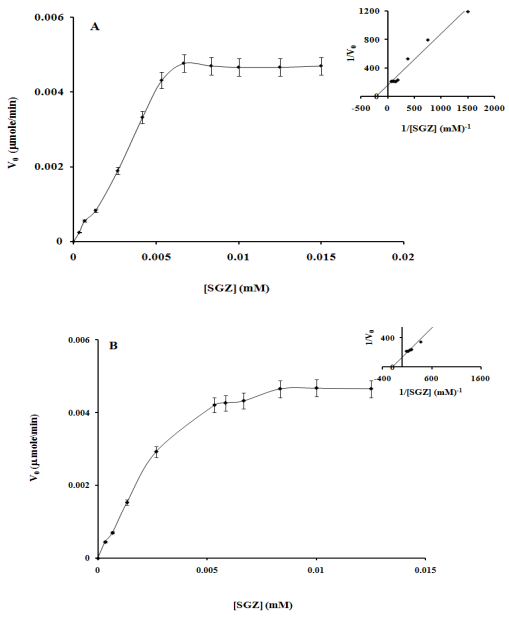

Fig. 1. The kinetic properties were determined spectrophotometrically by measuring the oxidation of SGZ (A) before (B) after preincubation of the enzyme at 70 °C for 50 min.

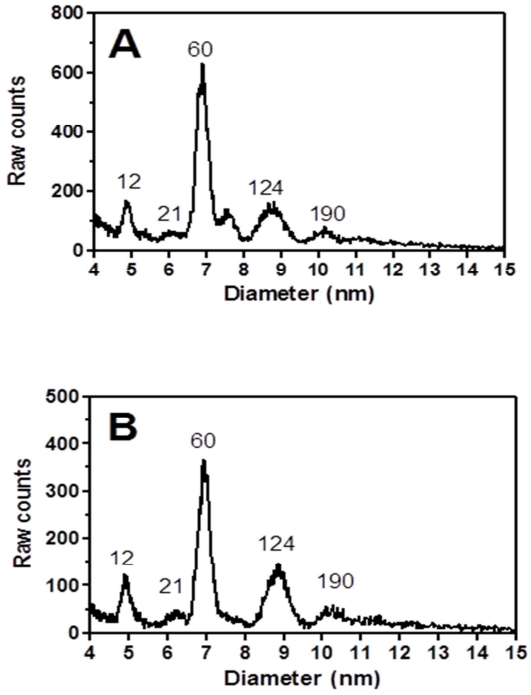

Fig. 2. Gas-phase electrophoretic mobility macromolecule analysis (GEMMA) analysis of 0.02 mg/ml Laccase protein incubated at room temperature (A) and 70 ºC for 50 minutes (B).

The results represent obvious local changes in the amount of secondary structures when non-incubated and incubated laccase were compared. The helix amount of native enzyme (44.5%) increased to 48% after incubation at 70 ºC. Higher percentage of α-helix is usually suspected for more structural rigidity which could be seen here after thermal treatment (Fig.3).

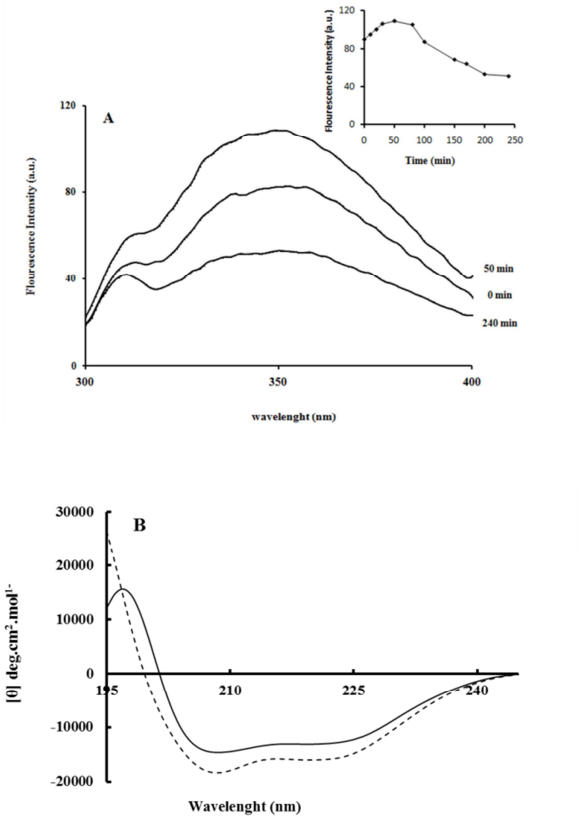

Fig. 3. Analyzing structural modifications of laccase from Bacillus sp. HR03 after incubation at 70 ºC. A) Fluorescence intensities of laccase at 340 nm upon incubation at 70 ºC for 10 to 240 minutes. The excitation wavelength was 280 nm. Compactness was increased upto 50 minutes of incubation. Elongating the time reduced fluorescence intensity. The inset shows variation in fluorescence intensity versus time. B) Circular dichroism spectra of laccase after 50 minutes incubation at 70 ºC (dashed line) compared with native form (solid line). Comparison of incubated and non-incubated enzymes shows increased secondary structures after incubation.

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