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RESEARCH PAPER

Effects of Succinic Anhydride Modification on Laccase Stability and Phenolics Removal Efficiency

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Abstract: Chemical modification is a useful method to change the properties of enzymes. Laccase is a phenol oxidase belonging to a multicopper protein, which catalyzes the oxidation of many phenolics. DeniLite IIS, a commercial laccase preparation from the Novozymes China Company, was purified by ammonium sulfate fractional precipitation. Succinic anhydride (SA) was used as a modifier for the chemical modification of the purified laccase. The effects of modification were characterized using the 6-trinitrobenzene sulfonic acid method, ultraviolet spectroscopy, and fluorescence spectroscopy. The pH stability, thermal stability, and the phenolics removal efficiency for the native and modified laccases were compared. The results showed that the average amino modification yield of the modified laccase was 85% and the modified laccase had a blue shifted ultraviolet peak and fluorescence emission peak as well as a decrease in the ultraviolet absorbance and an increase in the fluorescence intensity. Although chemical modification with SA did not change the optimum temperature for the catalysis of the laccase, it caused the optimum pH of the catalysts to shift from 4.5 to 5.5 and the enzymatic activity increased by 60%. Compared with the native laccase, the modified laccase exhibited remarkably higher pH stability and thermal stability and its catalysis efficiency (k_{cat}) and substrate affinity (k_{cat}/K_m) increased by 53% and 122%, respectively. The phenolics removal efficiency (*o*-, *m*-, *p*-dihydroxybenzene) of the modified laccase increased by 48%, 57%, and 18%, respectively. These results indicate that the modified laccase with higher stability and higher efficiency is suitable for application in industrial production and for the treatment of phenolics-polluted water.

Key words: succinic anhydride; laccase; chemical modification; stability; kinetics; phenolics removal efficiency

Laccases (Lac, EC 1.10.3.2) belong to a group of polyphenol oxidases containing copper atoms in the catalytic centre and they are usually referred to as multicopper oxidases [1]. It was first found in the exudates of the Japanese lacquer tree and a few years in fungi and bacteria. Laccases from different sources have different molecular weights and oxidation capacities [2]. There are four copper atoms in the active centre of laccase. According to their spectroscopic properties these four copper atoms are divided into three types, types I, II, and III. Type I copper plays an important role in the oxidation capacity of laccase [3]. Laccase has a wide range of substrate specificity and it can use oxygen directly as an oxidant to catalyze substrate oxidation. The four copper atoms in laccase transport electrons synergistically during catalysis [4].

Many reports have shown that laccase is an interesting prospect for application in detoxification, decolorization, and biological examinations [2]. It is destined to become an industrially-relevant enzyme. Phenolics are major organic pollutants in water environments and most phenolics are toxic to living organisms. Since laccase can use various types of aromatics such as phenolics as substrates, many studies have been carried out on phenolic wastewater treatment by laccase [5].

In general, low stability and the potential for a drastic reduction in enzymatic activity has always been considered a hindrance to the practical application of enzymatic systems. Laccase as a biocatalyst also has these shortcomings [6]. Therefore, the enhancement of laccase stability is important and urgent. Many methods have been used to modify the properties of enzymes such as genetic engineering including site-directed mutagenesis and molecular evolution, chemical modification including monofunctional polymer substitution and small molecule modification, and cofactor introduction

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[7–9]. Chemical modification leads to the introduction of functional groups and specificity-determining groups that are inaccessible by conventional mutagenesis techniques and improvements in enzyme activity and/or stability are achieved at low cost using relatively straightforward methods [8,10]. Therefore, chemical modification is an alternative (and complementary) approach to genetic modification for modifying their activity-stability properties. Recently, some attempts have been made to enhance laccase stability and efficacy by chemical modification. The chemical reagents used include polyethylene glycol (PEG) [11,12], dextran [13], citraconic anhydride (CA) [14], ethylene-glycol-N-hydroxy succinimide (EGNHS) [15], glutaraldehyde [14], and polyalkyleneoxide-*co*-maleic anhydride [6], and they were used as modifiers of fungal laccases. As the structure and function of laccase is now understood more clearly, it is known that lysine residues are not a part of the active site of laccase [2,4]. Because of its strong electrophilicity, the ε-NH_2 of lysine can react with many electrophiles. Therefore, laccase can be chemically modified by dicarboxylic anhydrides. Our earlier studies on horseradish peroxidase [15], porcine pancreas lipase (PPL) [16], and papain [17] modified with PA have shown that the stability and the efficacy of the modified enzyme increased.

The commercial laccase preparation, DeniLite IIS, which is produced by Novozymes is the laccase from *Myceliophthora thermophila* expressed genetically in *Aspergillus oryzae*. Although DeniLite IIS is in great demand, it is necessary to enhance the stability and efficacy of laccase as the core composition of this preparation. In this paper, the laccase in DeniLite IIS was purified and the purified laccase was chemically modified by succinic anhydride (SA). The modified laccase was characterized by average amino modification yield detection, ultraviolet spectroscopy, fluorescence spectroscopy, pH stability, thermal stability, and the removal efficiency of phenolics. This work will be beneficial to promote the practical application of laccase.

1 Experimental

1.1 Chemicals

Crude laccase (120 LAMU/g) designated DeniLite IIS was kindly provided by the Novozymes China company (Tianjin). Bovine serum albumin (BSA, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), tris(hydroxymethyl) aminomethane (Tris), acrylamide, *N*,*N*'-methylenebis(acrylamide), sodium dodecylsulfate (SDS), *N*,*N*,*N*',*N*'-tetramethylethylenediamine (TEMED), and ammonium persulfate were obtained from Sigma (St. Louis, Missouri, USA). All other chemicals such as SA, *o*-, *m*-, *p*-dihydroxybenzene (*o*-, *m*-, *p*-DHB) et al., were analytical reagents from China. The water used was double-distilled.

1.2 Laccase activity assay

The activity of laccase was determined spectrophotometrically using ABTS as a substrate. The detailed method was improved according to Bourbonnais and Paice [18]. The reaction mixture contained 1.0 ml 0.50 mmol/L ABTS, 1.5 ml 0.10 mol/L acetate buffer (pH = 4.5), and 0.5 ml the appropriately diluted enzyme solution. After incubation at 25 ºC for 3 min, the increase in absorbance was monitored at 420 nm. The molar extinction coefficient ε was 36 000 L/(mol·cm). One unit of enzyme activity was defined as 1 μmol of ABTS oxidized per minute at 25 ºC. Analysis were carried out in duplicate. The data given here are average values.

1.3 Purification of crude laccase and electrophoresis

Crude laccase was dissolved in 0.10 mol/L acetate buffer at pH = 4.5 and precipitated fractionally with ammonium sulfate at 4 ºC. The 45%–80% ammonium sulfate cut-off fraction was dissolved in 0.01 mol/L acetate buffer at pH = 4.5 and it was then dialyzed in the same acetate buffer overnight and lyophilized at –50 ºC.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Bio-RAD MINI-PROTEAN II, as described by Laemmli [19] in a 12% (*m*/*v*) polyacrylamide slab gel. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

The protein concentration was estimated, as described by Lowry et al. [20] using BSA as a standard.

1.4 Chemical modification of the crude laccase

The chemical modification of laccase with SA was performed according to our previously described method [15–17,21]. The native laccase lyophilized powder was dissolved in 0.025 mol/L phosphate buffer at pH = 7.5 to yield a 1 mg/ml enzyme solution. SA was dissolved in 0.025 mol/L phosphate buffer at pH = 7.5 with 4% (*v*/*v*) dimethylsulfoxide to prepare a 2 mmol/L SA solution. The SA solution (10.0 ml) was added gradually with constant stirring to 10.0 ml of the laccase solution. The reaction was proceeded for 1 h at 4 ºC and the solution was dialyzed against 0.010 mol/L acetate buffer at pH = 4.5 overnight at 4 ºC to remove excess reagents and then lyophilized.

1.5 Degree of modification

The degree of modification (DM) is expressed by the average amino modification yield, which was determined by the 6-trinitrobenzenesulfonate (TNBS) method [22]. A solution without enzyme protein was used as a reference. The measurement of the TNBS reaction mixture absorbance was carried

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