丁二酸酐修饰对漆酶稳定性和除酚效率的影响

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摘要: 采用 $(\text{NH}_4)_2\text{SO}_4$ 分步沉淀法对诺维信中国公司生产的漆酶制剂 DeniLite IIS 进行了纯化, 并用丁二酸酐 (SA) 对纯化酶进行了化学修饰, 运用三硝基苯磺酸法、紫外光谱法及荧光光谱法对修饰效果进行了初步表征, 比较了天然酶和修饰酶的 pH 稳定性、热稳定性及除酚效率。结果表明, 修饰酶的平均氨基修饰度为 85%, 其紫外吸收峰和荧光发射峰均出现蓝移, 而且紫外吸收减小、荧光强度增加。尽管采用 SA 化学修饰未能改变漆酶的最适反应温度, 但使其最适反应 pH 值由 4.5 提高到 5.5, 并且使酶活提高 60%。与天然酶相比, 修饰酶的 pH 稳定性和热稳定性更高, 催化效率 (k_{cat}) 和酶与底物的亲和力 ($k_{\text{cat}}/K_{\text{m}}$) 分别提高了 53% 和 122%, 对邻、间和对苯二酚的除酚效率分别提高了 48%, 57% 和 18%。这预示着这些修饰漆酶可望应用于工业生产和酚类污染废水的治理。

关键词: 丁二酸酐; 漆酶; 化学修饰; 稳定性; 动力学; 除酚效率

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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 multi-copper 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 ($k_{\text{cat}}/K_{\text{m}}$) 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

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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 have 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 [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], ethyl-

ene-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 -NH₂ 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 spectrophotomet-

rically 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 °C 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 μ mol of ABTS oxidized per minute at 25 °C. Analysis was 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 °C. The 45%–80% ammonium sulfate cut-off fraction was dissolved in 0.010 mol/L acetate buffer at pH = 4.5 and it was then dialyzed in the same acetate buffer overnight and lyophilized at –50 °C.

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 °C and the solution was dialyzed against 0.010 mol/L acetate buffer at pH = 4.5 overnight at 4 °C 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 out using a UV-4000 spectrophotometer (HITACHI, Japan). The DM was calculated by the following equation:

$$DM = (\Delta A_{\text{Lac}} - \Delta A_{\text{SA-Lac}}) \times 100\% \quad (1)$$

where ΔA_{Lac} and $\Delta A_{\text{SA-Lac}}$ represent the absorbance at 420 nm for the native and the SA-modified laccases, respectively, at the same enzyme molar concentration.

1.6 Spectral analysis

UV-Vis spectra of the native and modified laccase at the same molar concentration were recorded at 25 °C with a UV-4000 spectrophotometer. Fluorescence spectra using 272 nm as the excitation wavelength were measured at 25 °C with a F-4500 spectrofluorometer (HITACHI, Japan).

1.7 Determination of optimum pH, temperature, and kinetic parameters

The optimum pH, temperature, Michaelis-Menten constant (K_m), and the maximum initial velocity (V_{max}) were determined by changing each of the parameters and keeping all the other conditions constant. The activities of the native and modified enzymes were studied at 25 °C at pH 3.0–5.5 using 0.1 mol/L acetate buffer and 6.0–8.0 using 0.10 mol/L phosphate buffer. The activities of the native and modified enzyme were studied at 40–80 °C and their optimum pHs, respectively. Kinetic studies were carried out by changing the substrate concentration from 0.10 to 1.00 mmol/L and the initial velocity of catalysis (v_i) was measured by recording the absorbance increase (ΔA) at 10 s intervals over 1 min and plotting a straight line of ΔA versus t . The K_m and V_{max} values were obtained by a Hanes-Woolf (Eq. 2) plot and the turnover number (k_{cat}) was calculated according to Eq. 3.

$$[S]/v = 1/V_m \times [S] + K_m/V_m \quad (2)$$

$$k_{\text{cat}} = V_m/[E_0] \quad (3)$$

The pH stability profile at 25 °C was studied by incubating the enzyme in a pH range of 3.0–5.5 using 0.10 mol/L acetate buffer and 6.0–8.0 using 0.10 mol/L phosphate buffer over 1 h and then assaying the residual enzyme activity at the optimum pH. The activity of the native laccase at pH 4.5 was taken as 100%.

The thermal stability profile at the optimum pH was studied by incubating the enzyme at 20–60 °C over 1 h and then assaying the residual enzyme activity. The activity of the native laccase without thermal incubation was taken as 100%.

1.8 Removal efficiency of phenolics

The phenolics (*o*-, *m*-, *p*-DHB) were dissolved in 0.10 mol/L acetate buffer at pH 4.5 and 5.5 to prepare the 4.0 mmol/L phenolics solutions. The 5.0 ml of 0.10 μ mol/L native or modified laccase was added to 25 ml of the pheno-

lics solutions to react in the dark. Aliquots were withdrawn at 10 min intervals and the absorbance was determined at the maximum absorption wavelength of the corresponding phenolics. Acetate buffer was used as a reference. The phenolics removal efficiency (RE) was calculated using the following equation:

$$RE = (A_0 - A) / A_0 \times 100\% \quad (4)$$

where A_0 is the initial absorbance of the phenolics and A is the absorbance of the phenolics after treatment by the native or modified laccase.

2 Results and discussion

2.1 Purification, chemical modification, and characterization

The crude laccase was fractionated at 45%–80% saturated ammonium sulfate precipitation. The recovery yield of the laccase activity was 87% and the specific activity of the purified laccase was 33 U/mg. SA was used for the chemical modification of the purified laccase, which specifically reacted with the ε -amino groups of the laccase protein. The average amino modification yield of the SA-modified laccase was 85%. *Myceliophthora thermophila* laccase has eight lysine residues [14] and approximately seven lysine residues were modified in the SA-modified laccase. SDS-PAGE electropherograms of the protein samples are shown in Fig. 1(a). Both the native laccase and the modified laccase were found to be homogeneous. Because the modifier SA is a small molecular substance, the difference between the native laccase and the modified laccase is not significant. Figure 1(b) shows that the molecular weight of the native laccase is 85.0 kD. The molecular weight of the native laccase was found to be identical to the reported value [23].

Proteins have ultraviolet absorption because they contain an aromatic and a heterocyclic conjugated system. The microenvironments of these conjugated rings (chromophores) determine the conformation of the protein. With regard to the position, strength, and shape of the absorption peaks the ultraviolet absorption spectra of these chromophores change with the conformation of the protein [24]. Differences in the UV-Vis spectra of the native laccase and the modified laccase are shown in Fig. 2(a). Compared with the native laccase the modified laccase shows a slight blue shift in its ultraviolet peak from 266–269 nm and its absorbance is 26% less. Nearly no absorbance difference or wavelength shift is observed for the 238 nm peak. It is known that changes in the ultraviolet spectrum of proteins in the 230–240 nm region are related to the transformation of the α -helical while the absorbance decrease and the red shift of the peak in that region indicates a decrease in the α helix [25,26]. These results mean that the SA modification causes microenvironmental changes in the amino residues with aromatic and heterocyclic groups but few alterations in the α -helical conformation of the protein.

Tryptophan and tyrosine residues are the major fluorescence groups and the microenvironments of these groups can affect the fluorescence intensity and peak position obviously [27]. From Fig. 2(b), a pronounced difference in the fluorescence spectra between the native and the modified laccases is evident. The modified laccase shows a shift in the maximum fluorescence emission peak from 369 to 382 nm and it shows a 10% increase in fluorescence intensity.

Similar changes in the enzyme's ultraviolet and fluorescence spectra are caused by chemical modification with acid anhydride, and this has also been observed in our previous work [16,17]. These changes further verify that laccase is modified with SA. After the chemical modification, the conformation of the enzyme was altered to a certain degree,

Fig. 1. SDS-PAGE electrophoresis (a) and estimation of the molecular weight (b) of the native and the modified laccases. Lane 1, protein molecular weight standards; lane 2, the crude laccase; lane 3, the native laccase; lane 4, the modified laccase.

Fig. 2. UV-Vis (a) and fluorescence (b) spectra of the native (1) and the modified (2) laccases.

which caused the tryptophan and tyrosine residues to become more tightly enclosed and consequently the ultraviolet absorption decreased and the fluorescence intensity increased but the α -helical conformation was not influenced.

2.2 Effect of pH

The activities of the native and the modified laccases at different reaction pHs are shown in Fig. 3(a). The optimum pH of the native laccase was at 4.5 but for the modified laccase the optimum pH was 5.5. This is possible because of the reaction between the acid anhydride and the lysine residues of the laccase, which introduces carboxyl groups with an anionic charge into the enzyme molecule. This shifts the optimum pH to less acidic values [28]. The chemical modification of papain with anhydrides gave similar results [17,28]. An increase in the optimum pH of the laccase will be beneficial for its application. Compared with the native laccase in terms of its enzymatic activities in Fig. 3(a), the modified laccase has higher activity and its maximum activity is 60% higher. It has been reported that the modification of *Myceliophthora thermophila* laccase with CA and the modification of *Trametes hirsuta* laccase with glutaraldehyde leads to a three-fold increase and a 1.2-fold increase in enzymatic activity, respectively [14].

The native and modified laccases were incubated for 1 h

Fig. 3. Effects of reaction pH (a) and incubation pH (b) on the enzymatic activities of the native (1) and modified (2) laccases.

at 25 °C at different pHs and their activities were measured and are shown in Fig. 3(b). The native laccase was the most stable at pH = 4.5 and it lost a significant amount of activity when the pH deviated from 4.5, and it lost almost half its activity at pH = 5.5. However, the modified laccase exhibited much higher activity within a pH range of 2.5–7.0 and almost lost half its activity at pH = 8.0. These results indicate that the modified laccase has a higher pH stability than the native laccase.

An increase in the catalytic activity and pH stability of the enzyme is related to a change in its conformation. To determine the relationship between the enzymatic property and the conformation of laccase the ultraviolet (Fig. 4) and fluorescence (Fig. 5) spectra of the native and modified laccases at different pHs were determined. At about 270 nm, as shown in Fig. 4, the conformations of the native and modified laccases change with a change in the environmental pH from their optimum pH but the absorption peak does not shift. When viewed in terms of the peak related to the α helix in Fig. 4, the native laccase shifts from 238 to 243 nm and the absorbance decreases from 0.987 to 0.520 with a change in pH from 4.5 to 1.0. However, the peak of the modified laccase shifts from 238 to 240 nm and the absorbance decreases from 0.981 to 0.625 with a change in pH from 5.5 to 1.0. Obviously, the amplitude of variation in the ultraviolet absorption and the red shift of the peak related to

Fig. 4. UV-Vis spectra of the native (a) and modified (b) laccases at different pHs.

the α helix of the modified laccase is smaller than that of the native laccase. This indicates that the conformation of the modified laccase under acidic conditions is more stable than that of the native laccase. Figure 5 also shows a similar feature but the fluorescence intensity increases and the maximum fluorescence emission peak shifts slightly to a longer wavelength. These spectra reveal that chemical modification with SA increases the pH stability of the laccase.

2.3 Effect of temperature

The activities of the native and modified laccases were examined at different temperatures for catalysis. Figure 6(a) shows that the optimum temperature of the modified laccase was 65 °C, which is the same as that of the native laccase and the activity of the modified laccase was always higher than that of the native laccase at the same temperature. When the temperature was above 65 °C the modified laccase lost its activity more slowly compared with the native laccase.

The native and modified laccases were incubated for 1 h at different temperatures and then their activities were measured (Fig. 6(b)). Both the laccases lost their activities with an increase in incubation temperature but the degree of decrease for the modified laccase was lower than that of the

Fig. 5. Fluorescence spectra of the native (a) and modified (b) laccases at different pHs.

native laccase. After treatment for 1 h at 65 °C the activities of the native and modified laccases were reduced by 80% and 41%, respectively. Therefore, the modified laccase had a stronger thermal stability than the native laccase. Laccases from different origins were modified with different chemical modifiers and the difference in their thermal stabilities was large. *Trametes hirsuta* laccase showed a 7% and a 50% increase in thermal stability at 50 °C after modification with 2 kDa and 5 kDa PEG, respectively, and it gave a 50% decrease after modification with 1.1 kDa [12]. *Trametes sp.* laccase that was modified with polyalkyleneoxide-co-maleic anhydride gave a 20-fold and a 10-fold increase in the thermal stability at 25 °C and 50 °C, respectively [6]. *Myceliophthora thermophila* laccase modified with CA gave a 10-fold increase in the thermal stability at 75 °C [14].

2.4 Kinetic studies

Enzymatic activity is closely related to catalysis efficiency and the substrate affinity of enzymes. The kinetic parameters for the catalysis of the native and modified laccases were determined at their optimum pH, respectively. A Hanes-Woolf plot (Fig. 7) showed a good linear relationship between $[S]/v$ and $[S]$. According to the slopes and intercepts of the native and modified laccases the corresponding kinetic parameters K_m , V_m , k_{cat} , and k_{cat}/K_m were 0.0954 mmol/L, 5.34 $\mu\text{mol}/(\text{L}\cdot\text{min})$, 50.9 s^{-1} , and 534 $\text{L}/(\text{mmol}\cdot\text{s})$

Fig. 6. Effects of reaction temperature (a) and incubation temperature (b) on the enzymatic activities of the native (1) and modified (2) laccases.

Fig. 7. Hanes-Woolf plots of the native and modified laccases at 25 °C. (1) Native laccase at pH = 4.5; (2) Modified laccase at pH = 5.5. $[E_0] = 1.75 \times 10^{-9}$ mol/L.

for the native laccase and 0.0655 mmol/L, 8.17 $\mu\text{mol}/(\text{L}\cdot\text{min})$, 77.8 s^{-1} , and 1188 $\text{L}/(\text{mmol}\cdot\text{s})$ for the modified laccase, respectively. k_{cat} represents the catalytic efficiency and a larger k_{cat} indicates a higher catalytic efficiency. Therefore, the catalysis efficiency of the modified laccase increased by 53% compared with the native laccase. $k_{\text{cat}}/K_{\text{m}}$ is normally used to express the substrate affinity of the enzyme and a larger $k_{\text{cat}}/K_{\text{m}}$ value indicates a higher substrate affinity. The substrate affinity of the modified laccase increased by 122% compared with that of the native laccase. These kinetic parameters indicate that the improvement in the modified laccase was due to the increase in catalytic

efficiency and substrate affinity. The effect of chemical modification on laccases has been reported previously. The k_{cat} values of *Trametes versicolor* modified with dextran was found to decrease by 1.4% and its $k_{\text{cat}}/K_{\text{m}}$ value was found to decrease by 5.5% [13]. The k_{cat} values of *Trametes versicolor* modified with PEGs of different molecular mass were found to increase but its $k_{\text{cat}}/K_{\text{m}}$ values were found to decrease obviously [12].

2.5 Phenolics removal efficiency

Phenolics are major hazardous pollutants in water environments and they are harmful to human health. For example, *o*-DHB has a remarkable inhibitory effect on the central nervous system and it affects the DNA synthesis of leukocytes, which then leads to a chromosomal aberration. The treatment of phenolic wastewater with laccases is promising. In our experiments, the characteristic peaks of *o*-, *m*-, *p*-DHB, and their oxidation products that were catalyzed by laccase were determined by UV-Vis spectroscopy. They were at 279, 275, and 288 nm respectively for *o*-, *m*-, *p*-DHB and at 468, 477, and 508 nm for the corresponding oxidation products. Because of the difference in the ultraviolet peaks of the phenolics their corresponding oxidation products had large differences as well. The absorbances at the corresponding phenolic ultraviolet peaks were used to represent the phenolics concentration in the catalytic system. The *o*-, *m*-, *p*-DHB solutions were treated with native and modified laccases and the phenolics removal efficiencies were determined at 10 min intervals (Fig. 8). Both the native and the modified laccase gave the highest removal efficiency for *p*-DHB and the lowest removal efficiency for

Fig. 8. Effect of reaction time on the removal efficiency of phenolics from aqueous solutions by the native laccase for *o*-DHB (1), *m*-DHB (2), and *p*-DHB (3) and the modified laccases for *o*-DHB (4), *m*-DHB (5), and *p*-DHB (6).

m-DHB. The modified laccase exhibited a remarkably improved efficiency for all the phenolics compared with the native laccase. For example, the removal efficiencies for *o*-, *m*-, *p*-DHB at 1 h were 72.5%, 40.2%, and 93.2%, respectively, and these values are 48%, 57%, and 18% higher compared with the native laccase, respectively. Shin-ya et al. [6] reported that the chemical modification of *Trametes* *sp* laccase with polyalkyleneoxide-*co*-maleic anhydride improved the removal efficiencies of phenolics including mono-, di-, and tri-chlorophenols, cresol, and xylene by 14%–231%.

3 Conclusions

The chemical modification of amino groups in an enzyme with acid anhydrides is a simple and effective method and may provide a very valuable strategy to modify enzymes with new and useful characteristics related to their stability and catalytic effect. In this paper, a modified laccase with useful properties was obtained by the modification of laccase with SA. These properties include better combination with the substrate, higher activity, stronger pH, thermal stability and a higher phenolics removal efficiency. These good effects are beneficial for the application of laccase in industries such as papermaking, printing and dyeing, and phenolic wastewater treatment etc. We speculate that seven lysine residues in the protein molecule are modified by SA. However, it is not known which lysine residues react with SA. Another point of interest is how the higher conformation of the protein changed with the degree of modification. This work will be carried out in the future because it will help us further understand what the effect of SA modification is on protein conformation and enzymatic properties.

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