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Research Article

Effect of pH on the conformational structure of cytochrome c and subsequent enzymatic cross-linking catalyzed by laccase

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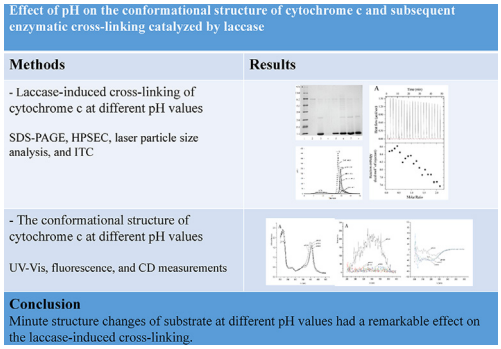
GRAPHICAL ABSTRACT



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ABSTRACT

Background: The aim of the present study was to investigate the effect of substrate conformational structure changes on the laccase-induced protein cross-linking. The effects of laccase amount, pH, and ferulic acid (FA) on the enzymatic cross-linking of substrate, Cyt C, were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. High-performance size exclusion chromatography, laser particle size analysis and isothermal titration calorimetry (ITC) were also applied to investigate the cross-linking product and enthalpy changes. Structural changes of Cyt C at different pH values were analyzed by ultraviolet–visible (UV–vis), fluorescence, and circular dichroism (CD) measurements.

Results: Complete cross-linking, partial cross-linking, minute cross-linking, and no cross-linking occurred at pH 2.0, 4.0, 6.0, and 8.0, respectively. ITC analysis demonstrated that the enzymatic cross-linking of Cyt C was an endothermic process. The UV–vis, fluorescence, and CD measurements exhibited that the tertiary structure of Cyt C was disrupted, and part of the α-helical polypeptide region unfolded at pH 2.0. The structural flexibilities decreased, and the tertiary structure of Cyt C became increasingly compact with the increase in pH values from 4.0 to 8.0. The gradual changes in the structure of Cyt C at different pH values were in accordance with the cross-linking results of Cyt C catalyzed by laccase.

Conclusions: The results demonstrated that minute structure changes of substrate had a remarkable effect on the laccase-induced cross-linking. The findings promote the understanding of the substrate

Abbreviations: Cyt C, cytochrome *c*; FA, ferulic acid; ABTS, 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; CD, circular dichroism; HPSEC, high-performance size exclusion chromatography; ITC, isothermal titration calorimetry; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Trp, tryptophan residue; Tyr, tyrosine residue; UV–vis, ultraviolet-visible.

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requirement of laccase in protein cross-linking and are instructive for the modulation of laccase-induced protein cross-linking.

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1. Introduction

The covalent cross-linking of protein molecules is widely used in many fields. In food technology, food matrix proteins are cross-linked to develop structures with desirable properties or improve functionality [1,2]. In tissue engineering, proteins are cross-linked to generate artificial scaffold as structural support or encapsulate cells in a 3D microenvironment [3]. In enzyme engineering, enzyme proteins are cross-linked for recycling and stability improvement [4]. Additionally, the cross-linked proteins have been proven to be efficient for the delivery of pharmaceuticals or biologically active molecules [3,5,6]. Protein cross-linking can be prepared by radical polymerization, chemical method, or enzymatic method. In particular, the enzymatic method has a strong appeal for protein cross-linking due to its substrate specificity, mildness, non-toxicity, and excellent biocompatibility.

Enzymatic cross-linking of proteins can be achieved by either transferases (e.g., transglutaminases) or the oxidoreductase family (e.g., tyrosinases or laccases) [7,8,9,10,11]. Laccase (E.C. 1. 10. 3. 2) is commonly present in higher plants and fungi [12,13]. It can catalytically oxidize various aromatic substrates, such as polyphenols, different substituted phenols, diamines, and aromatic amines [14,15,16,17]. On the basis of its wide range of substrates, laccase is employed as a valuable biotechnology tool in many fields, such as degrading phenolic and aromatic pollutants, designing biosensors for drug and pesticide analysis, bleaching pulp, and decolorizing dyes. Some researchers have focused their attention on the exploitation of laccase-mediated protein cross-linking [18,19,20]. Laccase oxidizes tyrosine residues in proteins via a one-electron hydrogen abstraction mechanism, thereby generating free radicals that spontaneously condense with one another, leading to cross-linking, as presented in Fig. 1.

In previous studies, laccase was employed to catalyze the cross-linking of different proteins with varying conformational structures, such as casein, bovine serum albumin, β-lactoglobulin, and wheat proteins [18]. These studies demonstrated that the 3D structure of protein substrate plays a key role in enzymatic cross-linking. However, detailed information on the effect of substrate conformational structure changes on the laccase-induced cross-linking has not been investigated.

In this study, cytochrome c (Cyt C) was chosen as the substrate model. Cyt C is a convenient substrate model for enzymatic cross-linking, because its amino acid sequence and native conformation are well known. Cyt C is a small protein and only contains four potentially reactive tyrosine residues, which makes it easy to clarify the results. In addition, its conformational structure changes can be readily followed by various spectroscopic techniques.

In this paper, the effects of laccase amount, pH, and ferulic acid (FA) on the cross-linking of Cyt C catalyzed by laccase were investigated. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), high-performance size exclusion chromatography (HPSEC), laser particle size analysis and isothermal titration calorimetry (ITC) were applied to investigate the cross-linking results. In addition, pH-induced structural changes of Cyt C were analyzed by ultraviolet–visible (UV–vis), fluorescence, and circular dichroism (CD) measurements. This study is the first to explore the effect of substrate structure changes on the laccase-induced cross-linking and makes a contribution to understanding the molecular details of enzymatic cross-linking.

2. Materials and methods

2.1. Enzyme and reagents

Laccase (protein content 2.5% in enzyme reagent, from *Trametes versicolor*) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich (Shanghai, China). Cyt C (from pig heart) and FA were purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). Unstained Protein Molecular Weight Marker was purchased from Thermo Fisher Scientific (Shanghai, China). Other chemicals were purchased from China National Pharmaceutical Group Co., Ltd. (Shanghai, China).

2.2. Determination of laccase activity

The activity of laccase was determined following the method described by Mollania et al. [12] with a few modifications, using 1 mM ABTS as substrate in 100 mM citrate buffer (pH 4.0). In brief, the reaction was started by adding 0.1 mL of laccase solution

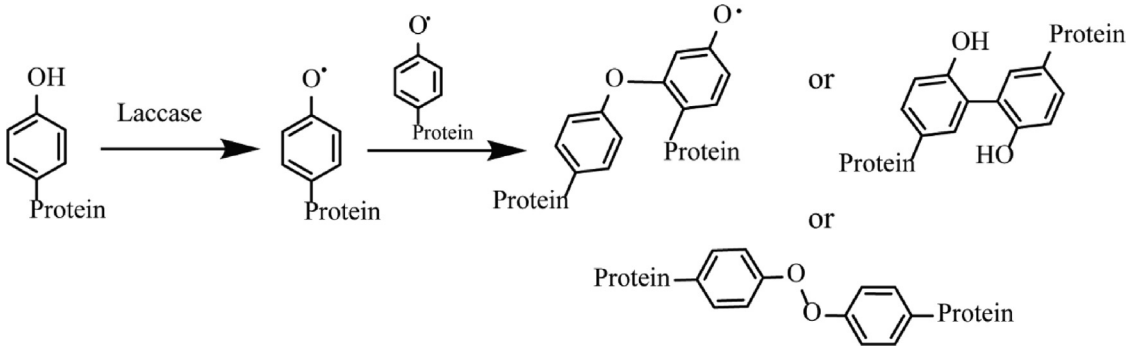

Fig. 1. Reaction scheme of laccase-generated cross-linking of protein.

(0.5 mg/mL) to the 2 mL of substrate solution. After incubation at 20°C for 3 min, 2 mL of 100 mM HCl was added to terminate the reaction. The oxidation of ABTS was monitored at 420 nm (the molar extinction coefficient ε420 = 36,000 $M^{-1}cm^{-1}$). A unit (U) of activity was defined as the amount of enzyme that oxidized 1 μmol ABTS per min.

The effect of pH on the activity of laccase was investigated at pH 2.0–8.0. The results of relative activity about pH are presented in a normalized form, with the highest value of each group being assigned 100% activity.

The pH stability of laccase was determined by measuring the residual activity of the enzyme exposed to pH 2.0–8.0 buffer. Residual activity was calculated by using the initial activity as 100%. The activity of laccase at 20°C and respective pH value without any incubation was defined as the initial activity.

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was performed on a mini-protein tetra system electrophoresis apparatus (PowerPac Basic, Bio-Rad, USA) to investigate the cross-linking levels of the protein substrates. The gels consisted of 4% stacking gel and 12% resolving gel. In general, 10 μL of the respective samples and molecular weight marker were added to the SDS-PAGE gel. Gels were stained with Coomassie Brilliant Blue. After destaining, photos of the gels were obtained by using a gel imager (Gel Doc™ XR + Imaging System, Bio-Rad, USA). The relative band intensities of Cyt C were determined by using the Image Lab™ software to quantitatively analyze band changes and are presented in a normalized form, with the Cyt C band intensity without laccase being assigned 100% intensity.

2.4. Cyt C cross-linking catalyzed by laccase

Cross-linking experiments were performed by incubating 200 μL of 2 mg/mL Cyt C solution with 200 μL of 5 mg/mL laccase (734 U) in 200 mM phosphate-citrate buffer at 25°C. After incubation for 24 h, 50 μL of the solution was removed, boiled with 2 × SDS loading buffer, and finally analyzed by SDS-PAGE.

The cross-linking studies were carried out under different conditions: different laccase amounts (2.5, 5, 10, and 15 mg/mL), different pH values (pH 2, 4, 6, and 8), and in the presence of mediator (2 mM FA). A control experiment was simultaneously carried out to investigate the possibility of the cross-linking of Cyt C at pH 2.0 in the absence of laccase.

2.5. Laser particle size analysis

About 200 μL of 2 mg/mL Cyt C solution was incubated with 200 μL of 5 mg/mL laccase (734 U) in 200 mM phosphate-citrate buffer at pH 2. After incubation at 25°C for 24 h, 200 μL of solution was removed and diluted sixty times by phosphate-citrate buffer for further determination. Particle size was determined with a laser diffraction particle size analyzer (Better size 3000 plus, China) by using dH_2O as calibration background.

2.6. High-performance size exclusion chromatography (HPSEC) analysis

Cross-linking experiment was performed by incubating 2 mL of 2 mg/mL Cyt C solution with 2 mL of 5 mg/mL laccase (734 U) in 200 mM phosphate-citrate buffer (pH 2.0 and 4.0, respectively). After incubation at 25°C for 24 h, samples were filtered through a 0.22 μm membrane and analyzed by HPSEC using a high-performance size exclusion chromatography system (Agilent Technologies Inc.) coupled with a UV/Vis detector operating at 220 nm. Samples were injected onto a TSKgel G4000PWxl column (TOSOH Bioscience, Japan). Analysis was performed at 20°C by injecting 10 μL and using 0.05 M potassium phosphate buffer (pH 6.0) as the eluent at a flow rate of 0.5 mL/min.

2.7. Isothermal titration calorimetry (ITC) analysis

For ITC measurements, the higher concentration laccase and Cyt C were diluted in buffer solution to make a final concentration with 75 μM and 770 μM, respectively. All ITC experiments were performed at 25°C using an iTC-200 microcalorimeter (Malvern Panalytical) with a reaction cell and a syringe injector of 300 μL and 60 μL, respectively. The Cyt C solution was loaded into the syringe and injected into the reaction cell which initially contained 300 μL of laccase solution in 20 steps of 2 μL aliquots each (0.4 μL for the first injection only). The titrant was injected at 150 s intervals to ensure that the titration peak returned to the baseline before the next injection. Each injection lasted 4 s. For sake of homogeneous mixing in the cell, the stirrer speed was kept constant at 700 rpm. Data were fitted using the single-site binding model within the Origin software package (MicroCal, Inc.).

2.8. UV–vis analysis

The solution of Cyt C (1 mg/mL) in phosphate-citrate buffer (0.2 M) of different pH values (pH 2, 4, 6, and 8) was incubated at 25°C for 4 and 24 h. After incubation, the solution was diluted five times by phosphate-citrate buffer for further determination. The UV–vis spectra of the solution of Cyt C at pH 2, 4, 6, and 8 were scanned at the range of 200–500 nm by using a UV–vis spectrophotometer (UV-2600, Shimadzu, Japan).

2.9. Fluorescence emission analysis

The solution of Cyt C (1 mg/mL) in phosphate-citrate buffer (0.2 M) of different pH values (pH 2, 4, 6, and 8) was incubated at 25°C for 4 and 24 h. The fluorescence emission spectra of the solution of Cyt C at pH 2, 4, 6, and 8 were measured by using a luminescence spectrometer (LS55, Perkin Elmer, USA) at 280–400 nm with the excitation wavelength at 280 nm (2.5–5.0 nm slits), excitation and emission slit width of 8 nm each, and scan speed of 200 nm/min.

2.10. CD analysis

The solution of Cyt C (1 mg/mL) in phosphate-citrate buffer (0.2 M) of different pH values (pH 2, 4, 6, and 8) was incubated at 25°C for 4 and 24 h. After incubation, the solution was diluted four times by phosphate-citrate buffer for further determination. CD measurements were performed at 200–280 nm with a spectropolarimeter (Model410, AVIV, USA).

3. Results and discussion

3.1. Cross-linking of Cyt C catalyzed by laccase at different conditions

3.1.1. Cross-linking of Cyt C catalyzed by different amounts of laccase

Reactions of the cross-linking of Cyt C (2.0 mg/mL) catalyzed by different amounts of laccase (2.5, 5, 10, and 15 mg/mL) were carried out in phosphate-citrate buffer (0.2 M) at pH 6.0 and 25°C for 24 h. The resulting samples were analyzed by SDS-PAGE (Fig. 2).

As shown in Fig. 2, the laccase band and the Cyt C band were observed at approximately 66.2 (Lane 2) and 14.0 kDa (Lane 3), respectively. With the increase in the laccase amount from 2.5 mg/mL to 15 mg/mL (367 U to 2202 U), no obvious decrease

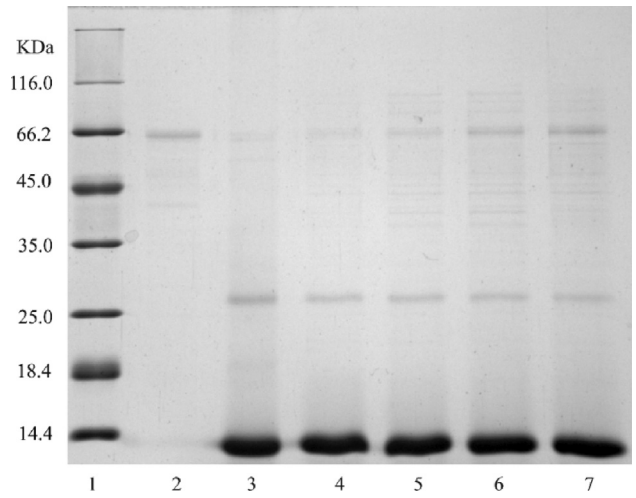

Fig. 2. Images of SDS-PAGE for the cross-linking of Cyt C catalyzed by different amounts of laccase. Lane1, molecular weight marker; 2, laccase (5 mg/mL); 3, Cyt C (2 mg/mL); 4–7, laccase ± Cyt C, laccase concentration 2.5, 5, 10, and 15 mg/mL, respectively, pH 6.0, 24 h.

occurred for the relative band intensities of Cyt C (Fig. 2, Lanes 4–7). The results demonstrated that the amount of laccase was not the critical factor of enzymatic cross-linking of Cyt C.

The efficiency of laccase to cross-link proteins depends on the presence of tyrosine residues in the primary sequence and the accessibility of tyrosine residues that is dictated by the conformational structure of substrate protein, namely, the degree of folding of the substrate protein [21,22,23]. Cyt C from pig heart contains 104 amino acid residues, including four tyrosine residues. No cross-linking occurred indicating that none of the four tyrosine residues in Cyt C were accessible for the active site of laccase at pH 6.0. Tyr_{67} and Tyr_{97} were located within the helix region 61–69 and 87–100, respectively. After folding of the polypeptide chain, the Tyr_{67}-monophenol group was located in the distal portion of the hydrophobic heme pocket of Cyt C and involved in the hydrophobic interaction with porphyrin ring and important hydrogen bonding interactions within the heme pocket [24], leading to the inaccessibility of the Tyr_{67}-monophenol group to the active site of laccase. The accessibility of the active site of laccase to the Tyr_{97}-monophenol group might be limited due to the hindrance of the rigid helix polypeptide chain. Tyr_{48} and Tyr_{74} were located within two Ω loops (i.e., 40–57 and 71–85) that participate in the formation of a hydrogen bond network in the distal side of the heme. The limited flexibility of peptide chain might lead to the inaccessibility of Tyr48 and Tyr74 to the active site of laccase [24,25].

3.1.2. Cross-linking of Cyt C catalyzed by laccase at different pH values

Reactions of the cross-linking of Cyt C (2.0 mg/mL) catalyzed by laccase (5 mg/mL) were carried out in different pH values of phosphate-citrate buffer (pH 2.0, 4.0, 6.0, and 8.0; 0.2 M) at 25°C for 24 and 48 h. The resulting samples were analyzed by SDS-PAGE (Fig. 3).

As presented in Fig. 3, the bands that represented Cyt C completely disappeared and no new bands appeared over 24 and 48 h of incubation with laccase at pH 2.0 (Lane 4), indicating complete cross-linking of Cyt C catalyzed by laccase at pH 2.0. The resulting aggregates were too large to penetrate into the gels during SDS-PAGE analysis. No change occurred to the Cyt C band after Cyt C was incubated without laccase at pH 2, 25°C, for 24 h (Fig. 3A Lane 8), suggesting that Cyt C could not spontaneously cross-link without laccase. At 4.0, the relative band intensities of Cyt C significantly decreased to 63% and 38% for 24 h and 48 h, respectively (Lane 5), indicating partial cross-linking of Cyt C catalyzed by laccase at pH 4.0. At pH 6.0, the relative band intensities of Cyt C slightly decreased to 93% over the 48 h of incubation, indicating minute cross-linking of Cyt C at pH 6.0. At pH 8.0, no decrease was observed for the relative band intensities of Cyt C band and no new bands appeared over the 48 h of incubation period with laccase (Lane 7), which represented that no cross-linking occurred. In conclusion, with the shift of pH from 2.0 to 8.0, the cross-linking of Cyt C gradually declined. Laccase could catalyze the complete cross-linking of Cyt C at pH 2.0 but could not catalyze the cross-linking of Cyt C at pH 8.0. These results exhibited that pH had a significant effect on the cross-linking of Cyt C.

pH affected both the activity of laccase and structure of Cyt C. On the basis of our determination of the activity of laccase, laccase exhibited the maximum activity and the best pH stability at pH 4.0, the minimum activity at pH 8.0, and the worst pH stability at pH 2.0. Laccase retained about 39%, 84%, 77%, and 48% of its initial activity after 3 h of incubation at pH 2.0, 4.0, 6.0, and 8.0, respectively. The relative activity of laccase was 30%, 100%, 32%, and 26% at pH 2.0, 4.0, 6.0, and 8.0, respectively. Laccase exhibited higher activity and better stability at pH 4.0 and 6.0 than at pH 2.0. However, complete cross-linking occurred at pH 2.0, partial cross-linking occurred at pH 4.0, minute cross-linking occurred at

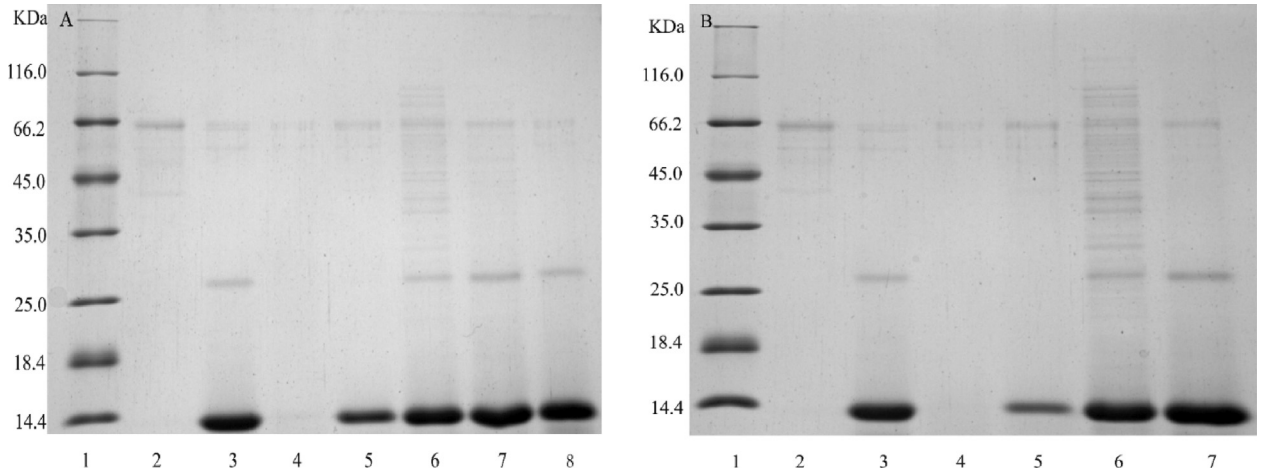

Fig. 3. Images of SDS-PAGE for the cross-linking of Cyt C catalyzed by laccase at different pH for 24 (A) and 48 h (B). Lane1, molecular weight marker; 2, laccase (5 mg/mL); 3, Cyt C (2 mg/mL); 4–7, laccase (5 mg/mL) ± Cyt C (2 mg/mL), pH 2, 4, 6, and 8, respectively, 24 (A) and 48 h (B); 8, Cyt C (2 mg/mL) without laccase, pH 2, 25°C, 24 h (A).

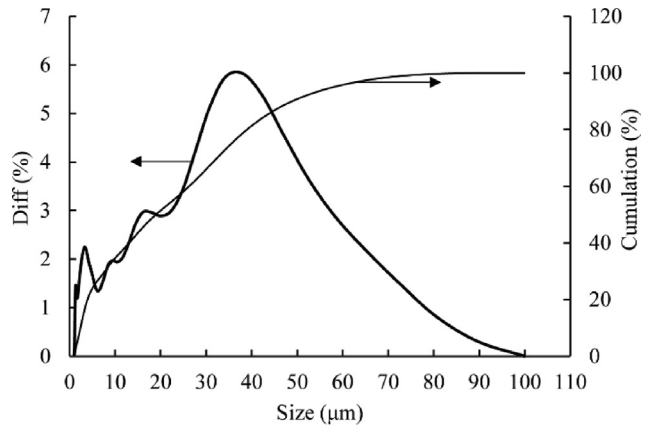

Fig. 4. Laser particle size analysis for the cross-linking of Cyt C catalyzed by laccase at pH 2.0 for 24 h.

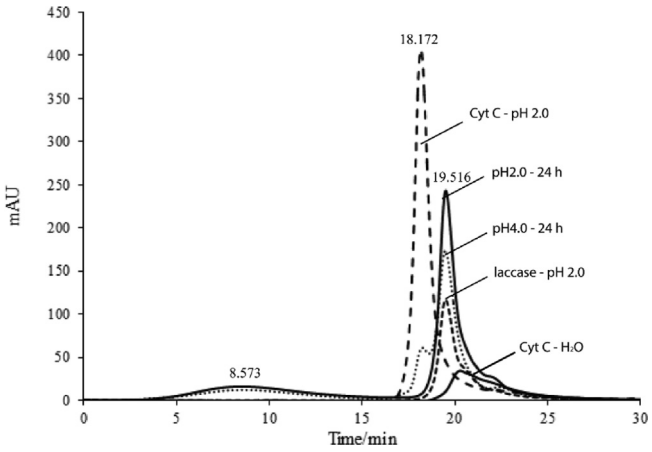

Fig. 5. HPSEC analysis for the cross-linking of Cyt C catalyzed by laccase at pH 2.0 and 4.0 for 24 h.

pH 6.0, and no cross-linking occurred at pH 8.0. Moreover, the increase in laccase amount did not improve cross-linking at pH 6.0 (as presented in Fig. 2). From the laccase activity and cross-linking results, we could deduce that the different cross-linking results at different pH values may be attributed to the change in structure of Cyt C rather than the difference in laccase activity.

Cross-linking products of Cyt C catalyzed by laccase at pH 2.0 and 4.0 were too large to analyze by SDS-PAGE. Insoluble particles were observed with the cross-linking reaction at pH 2.0. A laser diffraction particle size analyzer was used to measure the particle size of cross-linking solution at pH 2.0 (Fig. 4).

As shown in Fig. 4, the cross-linking particles at pH 2.0 were distributed in the range of 0–100 μm. The particles were too large to penetrate into the gels during SDS-PAGE analysis. Meanwhile, the particles were formed by covalent cross-linking, which could not be broken by β-mercaptoethanol of the loading buffer, so no new bands appeared with the decrease in Cyt C.

HPSEC was used to investigate the cross-linking reactions catalyzed by laccase at pH 2.0 and 4.0. The elution profile of cross-linking samples at pH 2.0 and 4.0 is shown in Fig. 5.

As presented in Fig. 5, the peaks of retention times of 18.172 and 19.516 min corresponded to Cyt C and laccase, respectively. Upon cross-linking of Cyt C with laccase at pH 4.0, the peak at 18.252 min corresponding to Cyt C strongly decreased while a novel peak appeared at a shorter retention time of 8.573 min. The peak corresponding to Cyt C completely disappeared for the reaction at pH 2.0. The novel peak eluting at a retention time of 8.573 min was part of the cross-linking product. Most cross-linking particles were removed by microfiltration before measurement. The retention time of Cyt C (in dH_2O) was at about 20.290 min. After Cyt was incubated in pH 2.0 buffer for 24 h, a shorter retention time, 18.172 min, was observed. The incubation at pH 2.0 might induce structural changes in Cyt C, which resulted in a larger hydrodynamic radius and, consequently, led to a shorter elution time [26].

3.1.3. Cross-linking of Cyt C catalyzed by laccase in the presence of FA at different pH

Reactions of the cross-linking of Cyt C (2.0 mg/mL) catalyzed by laccase (5 mg/mL) with and without FA were carried out in phosphate-citrate buffer (pH 2.0, 4.0, 6.0, and 8.0, 0.2 M) at 25°C. The resulting samples were analyzed by SDS-PAGE (Fig. 6).

As shown in Fig. 6A, the Cyt C band completely disappeared over 4 h of incubation with laccase in the absence of FA (Fig. 6A, Lanes 4–8), which represented the efficient cross-linking of Cyt C catalyzed by laccase at pH 2.0. When we compared Fig. 6B with Fig. 6A, we found that there was no significant difference in the

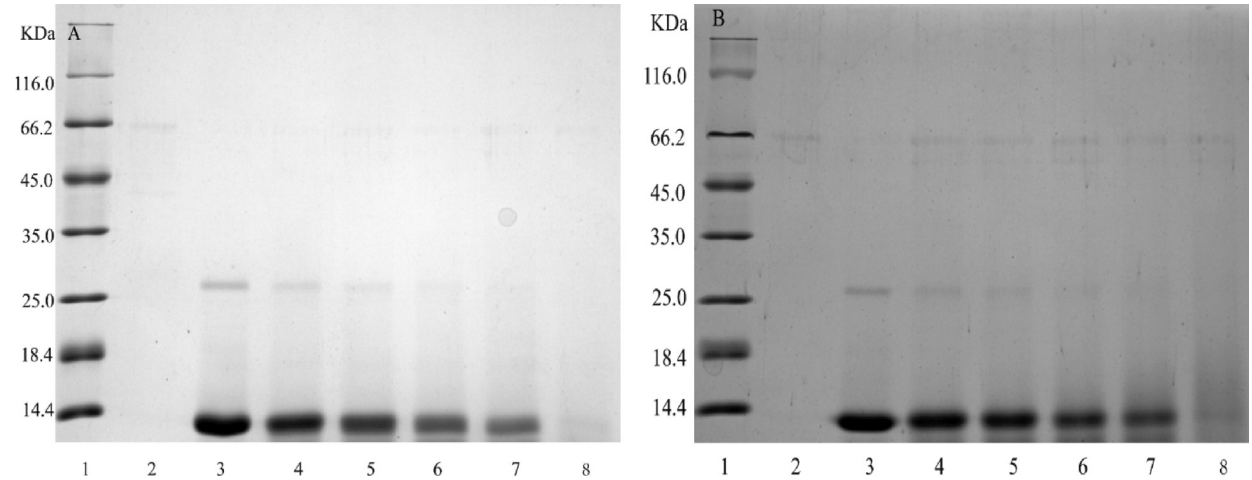

Fig. 6. Images of SDS-PAGE for the cross-linking of Cyt C catalyzed by laccase in the absence (A) and presence (B) of FA at pH 2.0. Lane1, molecular weight marker; 2, laccase (5 mg/mL); 3, Cyt C (2 mg/mL); 4–8, laccase (5 mg/mL) ± Cyt C (2 mg/mL), reaction times of 10 min, 20 min, 40 min, 1 h, and 4 h, respectively, in the absence (A) and presence (B) of FA (2 mM).

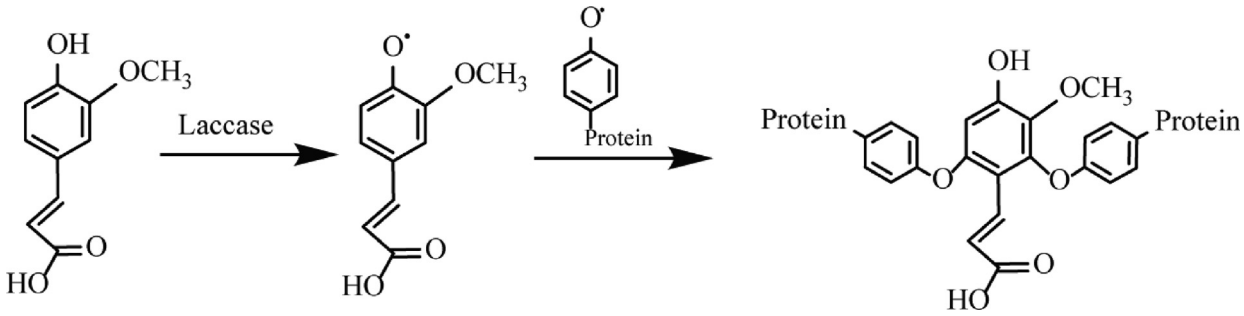

Fig. 7. Reaction scheme of laccase-generated cross-linking of protein in the presence of FA.

relative band intensities with and without FA, indicating that the inclusion of mediator did not improve the cross-linking of Cyt C at pH 2.0. FA, the low-molecular-weight phenolic compound, is usually used as a mediator to promote the cross-linking of proteins. It was easily catalyzed by laccase and formed free radicals, which cross-reacted with the free radicals of two proteins generated by laccase [18,27]. The reaction scheme of the laccase-generated cross-linking of protein in the presence of FA is presented in Fig. 7. The lack of improvement may be due to the changes in structure of Cyt C at pH 2.0 led to the easier accessibility of Tyr residues to the active site of laccase, thereby generating a large quantity of free radicals, which could easily polymerize without the need for a mediator.

Fig. 8 presents the SDS-PAGE analysis results for the enzymatic cross-linking of Cyt C at pH 4.0, 6.0, and 8.0. Upon comparing Lanes 4–6 with Lanes 7–9 of Fig. 8A, the relative band intensities of Cyt C with FA were slightly lower than that of Cyt C without FA, indicating that the mediator slightly improved the cross-linking of Cyt C at pH 4.0. As shown in Fig. 8B, a dramatic decrease in the relative band intensities of Cyt C occurred over 4, 24, and 48 h of incubation in the presence of FA (Lanes 4–6), indicating that FA greatly improved the cross-linking of Cyt C at pH 6.0. Thus, FA, as the mediator, could act as a bridging agent and facilitate the cross-linking of free radicals from Cyt C. As shown in Fig. 8C, no change was observed for the relative band intensities of Cyt C over 48 h of incubation with laccase in the absence and presence of FA at pH 8.0

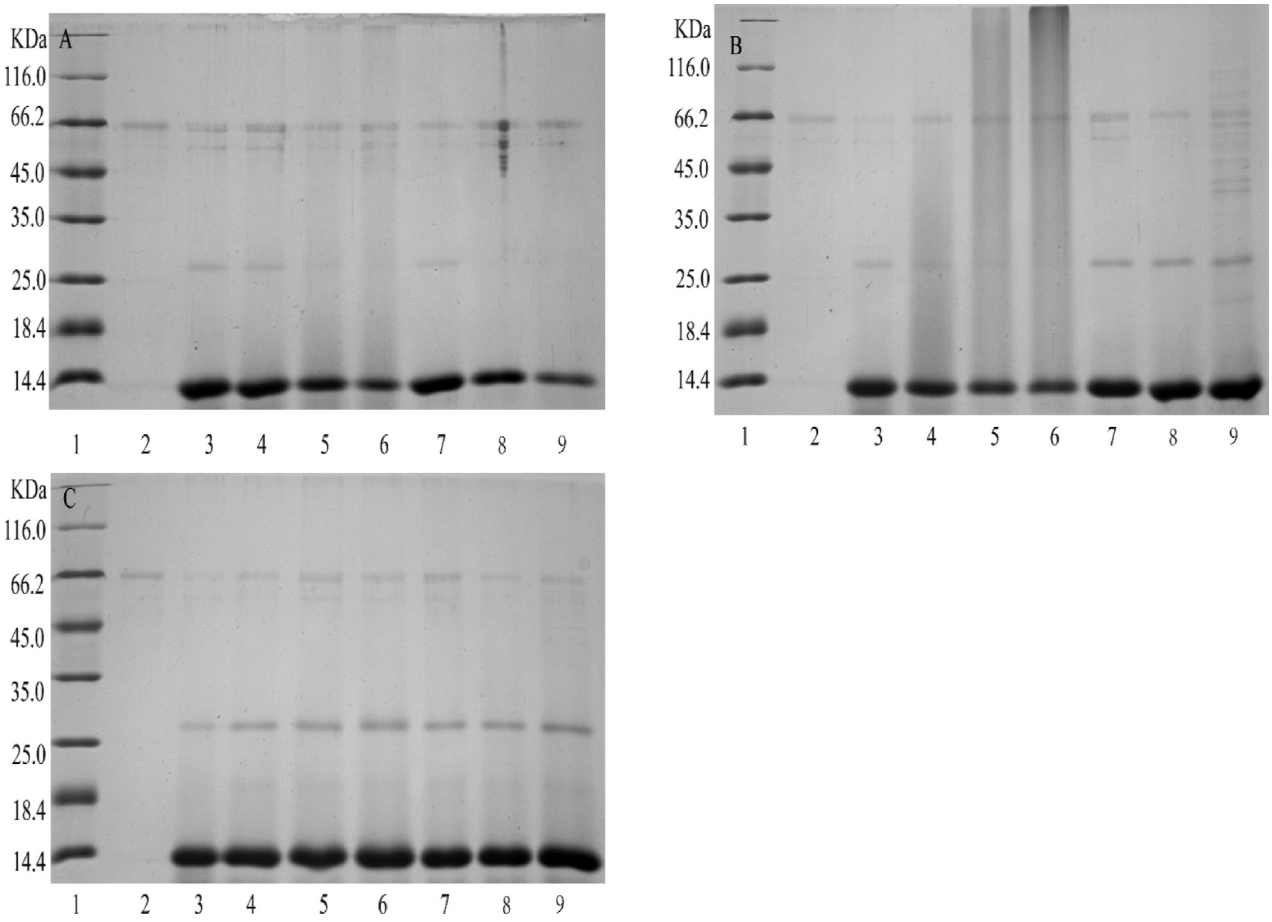

Fig. 8. Images of SDS-PAGE for the cross-linking of Cyt C catalyzed by laccase in the presence and absence of FA at pH 4.0 (A), 6.0 (B), and 8.0 (C). Lane 1, molecular weight marker; 2, laccase (5 mg/mL); 3, Cyt C (2 mg/mL); 4–6, laccase (5 mg/mL) + Cyt C (2 mg/mL) + FA (2 mM), at pH 4.0 (A), 6.0 (B), and 8.0 (C), for the reaction times of 4, 24, and 48 h, respectively; 7–9, laccase (5 mg/mL) + Cyt C (2 mg/mL), at pH 4.0 (A), 6.0 (B), and 8.0 (C), for the reaction times of 4, 24, and 48 h, respectively.

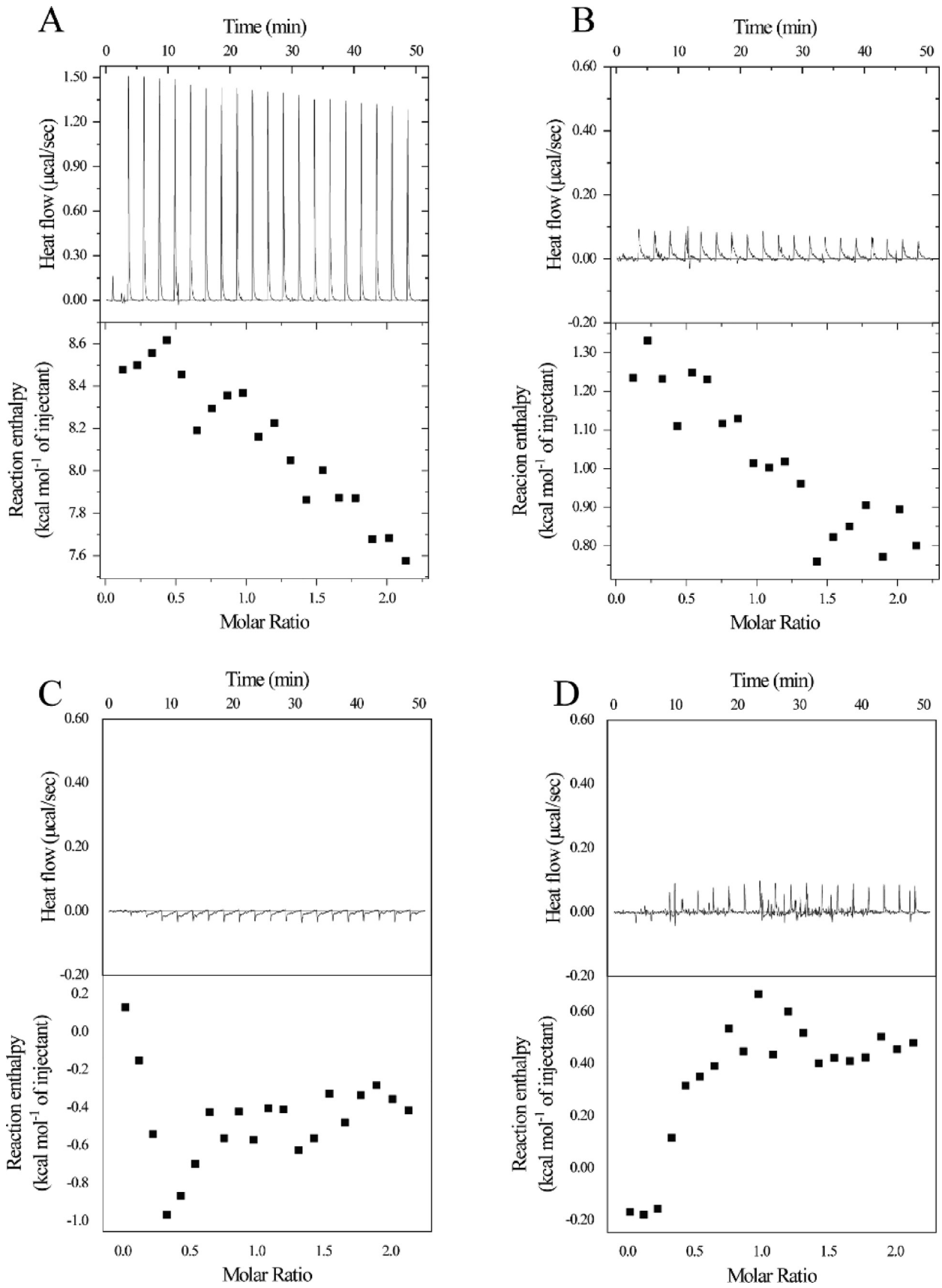

Fig. 9. ITC plot for titration of Cyt C into laccase in pH 2.0 (A), pH 4.0 (B), pH 6.0 (C), and pH 8.0 (D) buffer.

(Lanes 4–9). The results indicated that laccase could not catalyze the cross-linking of Cyt C, and the inclusion of the mediator was unable to improve cross-linking at pH 8.0. None of the tyrosine residues in Cyt C was accessible to the active site of laccase at pH 8.0.

3.2. ITC analysis

ITC was a straightforward and powerful tool for studying virtually any enzyme reaction [28,29]. In this study, we applied ITC to measure enthalpy changes of the enzymatic cross-linking at different pH values. Heat flow and reaction enthalpy diagrams were presented in Fig. 9 for the titration of Cyt C solution into laccase solution.

It has been found that there were high and narrow endothermic heat flow peaks at pH 2.0 (Fig. 9A), indicating that the interaction between Cyt C and laccase, including the binding and enzymatic cross-linking, accompanied the rapid endothermic change. Low and wide heat flow peaks were observed at pH 4.0 (Fig. 9B). The heat flow clearly decreased at pH 4.0 with the decline of cross-linking efficiency, which suggested the decrease in the interaction between Cyt C and laccase. There were no distinct heat flow peaks with changes in the composition during the titration at pH 6.0 and 8.0 (Fig. 9C-D). These heat flow changes corresponded to the cross-linking results at different pH values exhibited by SDS-PAGE and could demonstrate the different interactions between laccase and Cyt C at different pH values.

3.3. Conformational structure changes of Cyt C at different pH

Cyt C after incubation at different pH values was measured by using a UV–vis spectrophotometer, luminescence spectrometer, and CD spectropolarimeter to investigate the effect of pH on the conformational changes of Cyt C and further elucidate the influence of substrate conformational structure on the laccase-induced cross-linking.

3.3.1. UV–vis analysis

The solution of Cyt C in phosphate-citrate buffer (0.2 M) of different pH values (pH 2, 4, 6, and 8) was incubated at 25°C for 4 and 24 h. The solution of Cyt C was scanned by UV–vis spectrophotometry. The UV–vis spectra are shown in Fig. 10.

Cyt C is a small globular protein with a covalently linked heme group [30]. The heme is located in a hydrophobic pocket and interacts with amino acid residues by hydrophobic interaction and salt forces. The Fe^{3+} of the heme forms coordination bonds with His18 and Met80. The characteristic absorption peak of heme occurs at about 409 nm, called the Soret peak [31,32], which can reflect the change in the heme pocket.

As shown in Fig. 10, the Soret peaks were observed at about 409 nm for Cyt C, and the absorption intensity increased with increasing pH values from 4.0 to 8.0 over 4 and 24 h of incubation. At pH 2.0, the maximum Soret peak blue shifted to 395 nm. The blue shift could be attributed to the significant conformational change in the heme pocket [24], namely, the loosening of its tertiary structure and disruption of the Met_{80}-Fe and His_{18}-Fe ligation after the incubation of Cyt C at pH 2.0. The increase in the Soret peak with the increase in pH values from 4.0 to 8.0 suggested that protein flexibilities decreased and the structure of Cyt C became increasingly compact, giving rise to the enhancement of the hydrophobicity of heme.

3.3.2. Fluorescence emission analysis

The fluorescence emission spectra of Cyt C after incubation at pH 2.0, 4.0, 6.0, and 8.0 are presented in Fig. 11.

Fluorescence properties of proteins mainly result from aromatic amino acid residues present in their structures, especially tryptophan (Trp) and Tyr residues [33]. Cyt C has one Trp (Trp_{59}) and four Tyr residues for intrinsic fluorescence. The fluorescence spectral characteristics of side chains of Trp_{59} are sensitive to the microenvironment in Cyt C [34]. Thus, information regarding the microenvironment of Trp and the conformational changes of Cyt C can be obtained by comparing the fluorescence spectra of Cyt C after incubation at different pH values.

As presented in Fig. 11, excited by irradiation at 280 nm, Cyt C after incubation at pH 4.0, 6.0, and 8.0 did not emit fluorescence. Cyt C contains one tryptophan residue, namely, Trp_{59}. After the folding of the polypeptide chain of Cyt C, Trp_{59} did not emit fluorescence excited by irradiation at 280 nm, because it could transfer the emitting fluorescence to the adjacent heme via energetic resonance [34], leading to fluorescence quenching. However, after 4 and 24 h of incubation at pH 2.0, Cyt C emitted fluorescence at about λ^{max} 350 nm. This phenomenon indicated that exposure of Cyt C to pH 2 induced acid denaturation, which led to the disruption of the tertiary structure into unfolded polypeptide. The unfolding of the polypeptide chain caused the increase in distance between the heme and Trp_{59}, leading to the exposure of Trp_{59} to

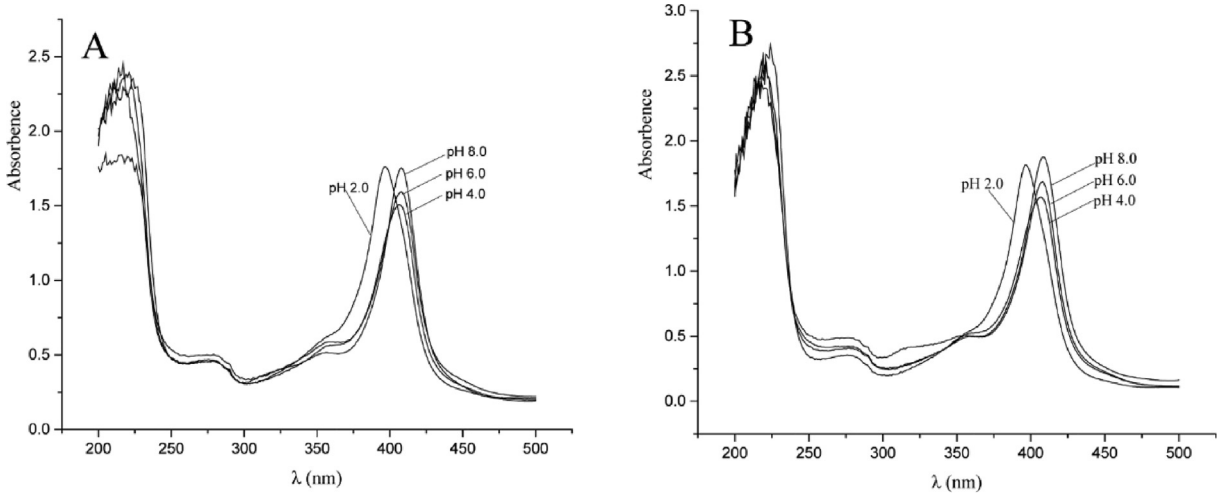

Fig. 10. UV–vis spectra of Cyt C incubated at pH 2.0, 4.0, 6.0, and 8.0 for 4 (A) and 24 h (B).

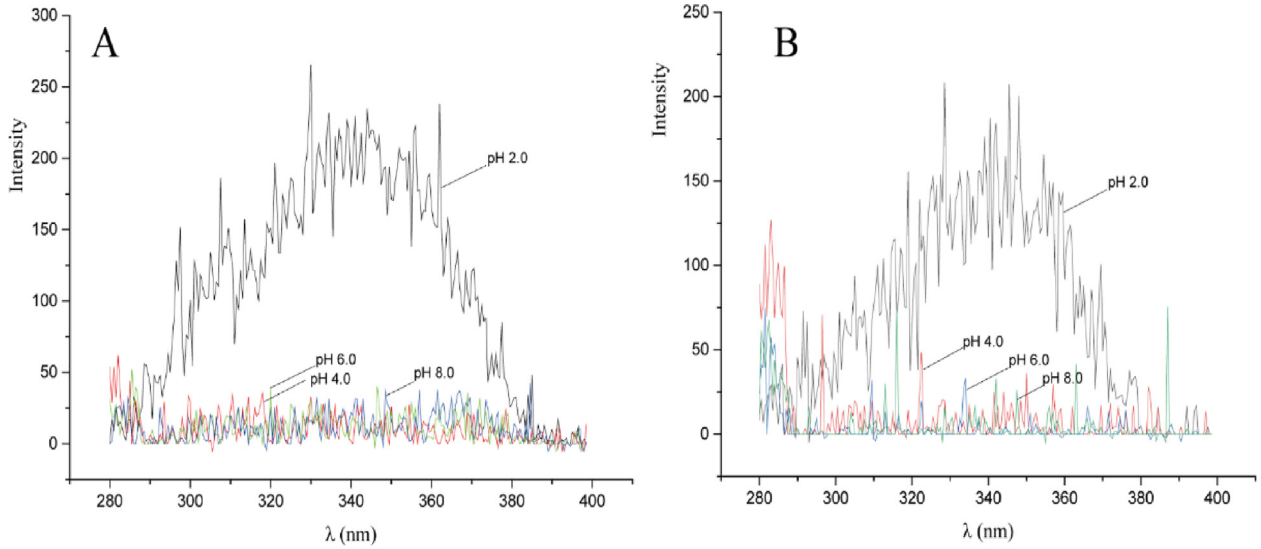

Fig. 11. Fluorescence emission spectra of Cyt C incubated at pH 2.0, 4.0, 6.0, and 8.0 for 4 (A) and 24 h (B).

the polar environment and the disruption of fluorescence quenching.

3.3.3. CD analysis

CD spectra were used to monitor the conformational change of the secondary structure in Cyt C [35,36,37,38,39]. The CD spectra of Cyt C after incubation at pH 2.0, 4.0, 6.0, and 8.0 are presented in Fig. 12.

As shown in Fig. 12, the characteristic and negative peak of α-helix in Cyt C was located at around 220 nm [40]. The spectra of Cyt C over 24 h of incubation at pH 4.0, 6.0, and 8.0 were typical of proteins with α-helix structures. However, Cyt C over 24 h of incubation at pH 2.0 showed a significant reduction of the CD signal at 220 nm, suggesting a remarkable decrease in the α-helical content. These results suggested that exposure of Cyt C to pH 2.0 led to acid-induced denaturation and loosening of part of the α-helical polypeptide region.

The results of UV, fluorescence, and CD measurements revealed differences in the conformational structure of Cyt C at pH 2.0, 4.0, 6.0, and 8.0. The changes in the conformational structure of Cyt C were in accordance with the cross-linking results of Cyt C catalyzed by laccase. At pH 2.0, the tertiary structure of Cyt C was destroyed, and part of the α-helical polypeptide region unfolded. The disruption of the Cyt C structure at pH 2.0 led to the increased exposure of Tyr residues to the reactive site of laccase. Efficient complete cross-linking occurred at pH 2.0. With the increase in pH from 4.0 to 8.0, no alterations of the overall molecular conformation occurred, but the structural flexibility decreased. Therefore, partial cross-linking, minute cross-linking, and no cross-linking were observed at pH 4.0, 6.0, and 8.0, respectively.

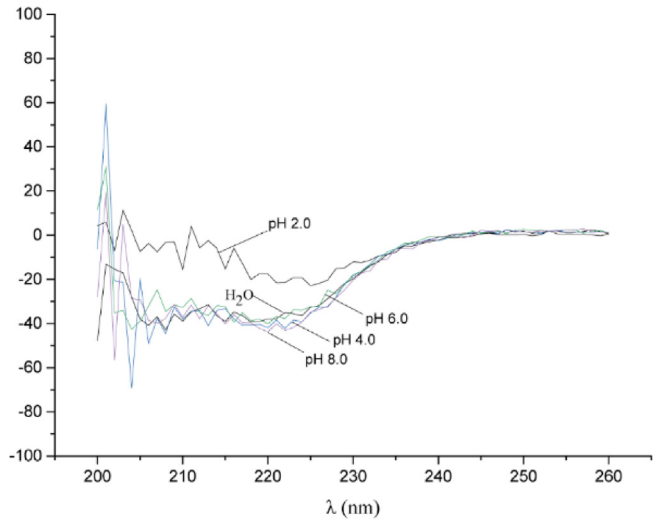

Fig. 12. CD spectra of Cyt C incubated at pH 2.0, 4.0, 6.0, and 8.0 for 24 h.

4. Conclusions

In this study, the effect of substrate conformational structure on protein cross-linking catalyzed by laccase was demonstrated. Cyt C as a model substrate was cross-linked at different conditions. Results revealed that pH played an important role in the cross-linking of Cyt C catalyzed by laccase. Additionally, the effect of FA on cross-linking was dependent on pH. UV, fluorescence, and CD measurements demonstrated the conformational structure changes of Cyt C at different pH values. Our findings show that laccase favors proteins with a more flexible structure, and the reactivity of laccase could be essentially modulated by structure changes in substrate. The potential of laccase in practical applications could be facilitated by some measures, such as heating and acid or base addition, which can change the conformational structure of the substrate. In the future studies, the Tyr sites of Cyt C catalyzed by laccase could be exploited to further reveal the catalysis mechanism of laccase.

Author contributions

- Study conception and design: D-X Li, J-Q Zhou
- Data collection: Z-Y Qi
- Analysis and interpretation of results: Z-Y Qi, J-Y Liu
- Draft manuscript preparation: Z-Y Qi, J-Q Zhou
- Revision of the results and approved the final version of the manuscript: D-X Li, Z-Y Qi, J-Y Liu, J-Q Zhou

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Conflict of Interest

None of the authors of this study has any financial interest or conflict with industries or parties.

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