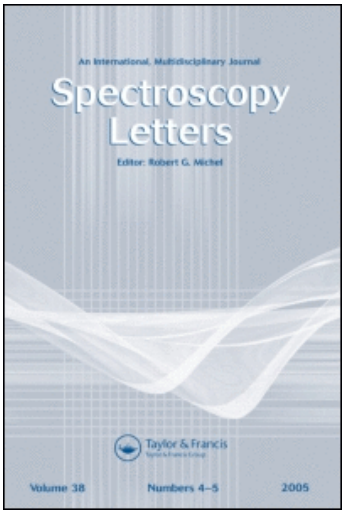

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pH-CONTROLLED CHANGE OF TOBACCO PPO II ACTIVE SITE

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ABSTRACT

PPO II is a novel enzyme that plays a key role in the plant defense system. PPO II has the active site of a pair of antiferromagnetic coppers with phenolate oxygen bridged at neutral pH medium. At low pH, the coordination ability of phenolate oxygen becomes weak and two H_2O molecular replaces the phenolate oxygen bridge in the active center and the absorption of PPO II is changed to a novel state as that of mushroom PPO. A new PPO II active site is formed between pH 8.5 and 9.0 suddenly. The phenolate oxygen bridge has been broken, hydroxide coordination with one of coppers is formed. The other copper is still coordinated with the

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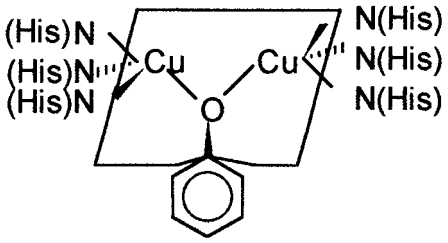
phenolate oxygen. The CD data shows at the pH 3.0 and pH 9.0 the second structure keeps stabile as the resting PPO II and is broken at high pH 10.0.

Key Words: Polyphenol oxidase; Tobacco; Absorption spectrum; pH; Active center

INTRODUCTION

The polyphenol oxidases (PPO) are a group of copper-proteins distributed through all the phylogenetic scale widely from bacteria to mammals[1]. The common feature of this group is their capacity to catalyze the oxidation of polyphenols through the molecular oxygen[2]. PPO II (EC 1. 10. 3. 1) is a novel copper enzyme purified from tobacco (*Nicotiana tobacum*), which inhibits the culture of *E. Coli*. and acts as a key role in plant defense system[3]. PPO II has a molecular weight of 35,600 Da and contains two antiferro-magentically coupled copper ions. The structure of PPO II active center is as the scheme 1[4]. The optimum pH and optimum temperature of PPO II are about pH = 6.5 and 40°C respectively. PPO II does not show the activity of the catalytic oxidation to p-diphenol or m-diphenol, and only possesses lower activity to chlorgenic acid[3]. The oxidation mechanism is proposed rather superoxide oxidation process (unpublished experiments) than peroxide oxidation process as other report[4,5]. PPO II is activated after one azide molecule binds in terminal mode with PPO II[6] and azide induces the catalytic mechanism from superoxide to peroxide process (unpublished data) for azide anion, an electron-repulsive group, induces the change from superoxide to peroxide. The electronic absorption spectrum shows the MLCT from the pair of coppers to the phenolate oxygen of residue of Tyr and PPO II belongs to type-3 copper protein[7].



Scheme 1. Active site of PPO II.

The pH-linked conformational transition of enzyme's model complex has been widely researched for owing to the problem encountered in biomimetic inorganic chemistry. Torelli S. reported the polyphenol oxidase model complex of $[Cu_2(BPMP)(OH)][ClO_4]_20.5C_4H_8O$ and $[Cu_2(BPMP)(H_2O)_2][ClO_4]_20.5C_4H_8O$ are reversibly interconverted upon acid/base titration (pK 4.95) and $[Cu_2(H_2BPMP)(OH)_2][ClO_4]_20.5C_4H_8O$ is reversibly formed, and the bridge of hydroxide is broken in the acid and base condition[8]. PPO II has the active center that phenolate oxygen is bridged with the pair of coppers. Here we report the pH-controlled change of tobacco PPO II active center.

Materials and Experiment

Materials

The fresh tobacco leaves (*Nicotiana tobacum*) were harvested directly from the field, washed and then kept in refrigerator below 4°C for about 24 hours. DEAE-Sephadex A-50, CM-Sephadex C-50 and Sephadex G-75 were purchased from Pharmacia Corporation Sweden. Other chemicals were analytical reagents.

Protein Preparation

Polyphenol oxidase II sample was prepared using the efficient method of acetone powder, purified by 30% and 80% ammonium sulfate precipitation and then the column chromatography of DEAE-Sephadex A-50, CM-Sephadex C-50 and Sephadex G-75 respectively[4]. PPO II obtained in above process has been detected by PAGE, SDS-PAGE and MALDI-TOF-MS spectra (LDI 1700 Linear Scientific Inc) as a single enzyme and the molecular weight 35,600 Da. PPO activity was determined by UV/VIS spectroscopy method in 50 mM catechol, 50 mM H_3PO_4-NaOH buffer pH = 6.5 at room temperature. The unit of enzyme activity was defined as the amount of enzyme that caused an absorbance increase of 0.01 unit per minute at 420 nm. The protein concentration was determined using the method mentioned by Bradford[9].

UV/VIS Spectra

UV/VIS spectra of PPO II were recorded on an UV-2100 spectrophotometer in 10 mm quartz cuvettes (SHIMADZU corp.). The sample of

PPO II was prepared at the concentration of 0.3 mg/mL respectively at the different pH and the buffer concentration is 0.05 M.

CD Spectrum

Performed with a JASCO J-720 spectropolarimeter from 190 nm to 250 nm, CD spectra were experimented by using 1 mm quartz cuvettes. The sample was prepared at the concentration of 0.1 mg/mL at different pH and the buffer concentration is 0.01 M.

RESULTS AND DISCUSSION

As is shown in Fig. 1, electronic spectrums of PPO II at pH 6.5, 7.5 and 8.5 are almost same with each other, while the spectrums at pH 9.0, 9.5, 10.5 and 11.5 have been changed suddenly. The absorption between 300 nm and 400 nm and above 410 nm has decreased between pH 8.5 and pH 9.0 and the absorption of 415 nm increases simultaneously. All the lines intersect at 410 nm and 435 nm respectively, which means a new structure of active site is formed. Fig. 2 shows the absorption changes at low pH of 0.5,

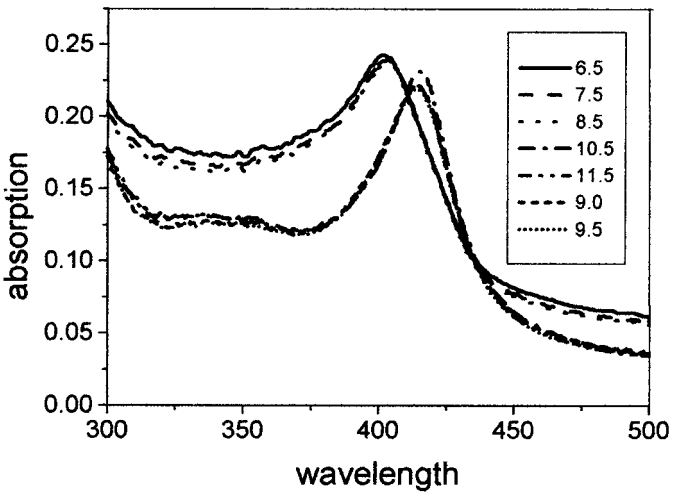


Figure 1. The absorption spectrum of PPO II at high pH. The spectra were recorded on a UV-2100 spectrophotometer in 10 mm quartz cuvettes. The sample of PPO II was prepared at the concentration of 0.30 mg/mL at pH 6.5, 7.5, 8.5, 9.0, 9.5, 10.5, and 11.5 at room temperature.

1.5, 2.5 and 3.5. A new absorption appears at 445 nm and the absorption at 406 nm decreased rapidly with the decrease of pH value. The electronic absorption spectrums at low pH have a point of intersection at about 427 nm. The absorption spectrums at pH 4.5 and pH 5.5 are similar as that at pH 6.5. The pH value is important role in sustain the second structure of protein. As shown in Fig. 4, at high pH 10.0, the second structure of PPO II has been changed from the CD spectrums between 190 nm and 250 nm (dash dot line), while the CD spectrum at pH 9.0 is similar as that at pH 6.0. In low pH 3.0, the second structure of PPO II keeps as same as that at pH 6.0[10]. The hydrogen bonds have been broken at high pH 10.0, which induces the conformation change of PPO II at high pH.

The UV/VIS absorption spectrum of PPO II from 300 nm to 800 nm is different to the PPO from other source. There were some clear absorption peaks or shoulders at about 420 nm, 560 nm, 660 nm and other absorption 330 nm and 380 nm also could be observed based on the Gaussian analysis and the CD spectra between 300 nm and 500 nm at pH 6.5[7] (unpublished data). At low pH (pH = 0.5), There exists a wide absorption at about 380 nm (Fig. 2), which is similar as that of mushroom PPO [11,12]. This means that the absorption of PPO II changes from MLCT (from copper d orbital to

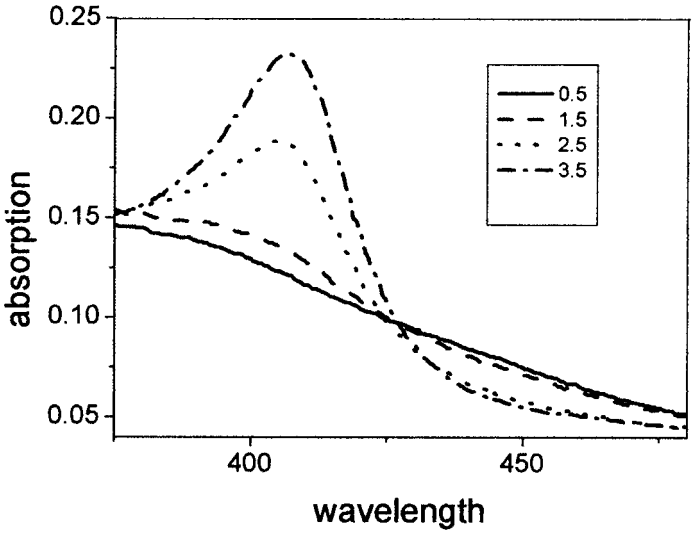


Figure 2. The absorption spectrum of PPO II at low pH. The spectra were recorded on an UV-2100 spectrophotometer in 10 mm quartz cuvettes. The sample of PPO II was prepared at the concentration of 0.30 mg/mL at pH 3.5, 2.5, 1.5, and 0.5 at room temperature.

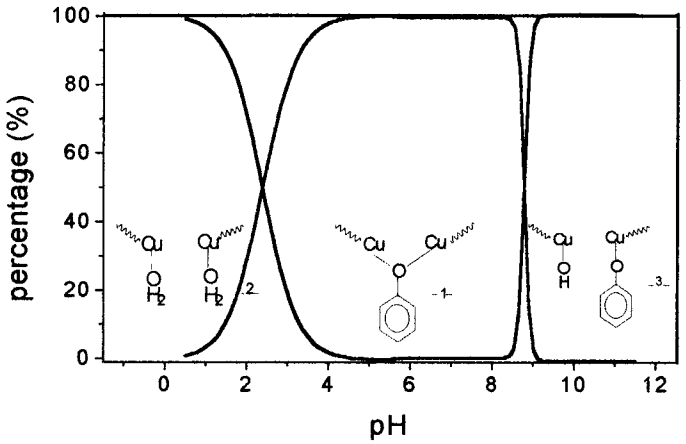


Figure 3. The species distribution curves as a function of pH. (1) is the active site structure at neutral solution, (2) is that at low pH solution, and (3) is that at high pH solution.

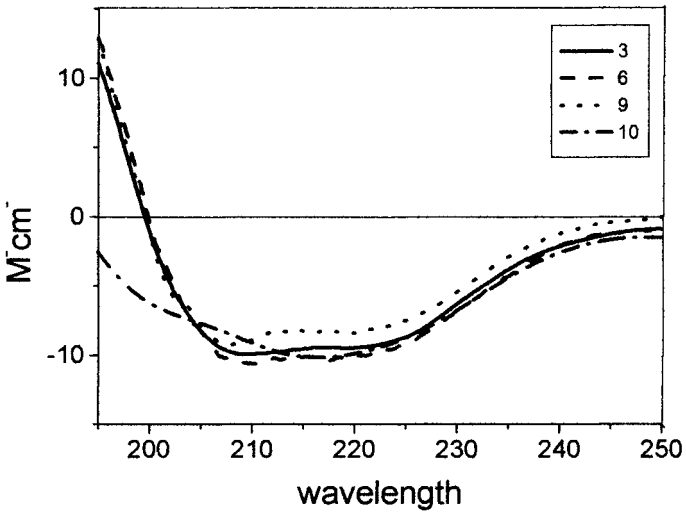


Figure 4. The far-ultraviolet CD spectrum of PPO II at different pH. The CD spectra (195 nm–250 nm) were experimented using 1 mm quartz cuvettes performed with a JASCO J-720 spectropolarimeter for the 195 nm–250 nm region, the sample was prepared at the concentration of 0.1 mg/mL at different pH of 3.0, 6.0, 9.0, and 10.0.

phenolate oxygen) at pH 6.5[7] to LMCT (from H_2O oxygen to copper) at low pH condition. The bridge of the phenolate oxygen at the active site is replaced by two H_2O molecules[4,7,8]. A new active site (see Fig. 3, 2) that have the active center of two H_2O molecules oxygen binding each coppers respectively is formed when the pH is lower than 3.5. Fig. 3, 1–3 shows the species distribution curves as a function of pH, which is estimated by the absorption change of PPO II. The active site changes at the high pH condition and a new active site structure is formed. At pH 9.0, 9.5, 10.5 and 11.5, the absorption decreases about half between 300–400 nm, which shows that the phenolate oxygen bridge may be broken and the phenolate oxygen coordinates with only one of the coppers. In the same hydroxide coordination with the other copper is formed (see Fig. 3, 3). The far-ultraviolet CD spectrums at different pH show that the high pH (pH = 10.0) breaks the second structure of PPO II, while the low pH (pH = 3.0) don't change the second structure (Fig. 4). The CD data demonstrates that the change of the second structure is not the reason that absorption is changed that large.

CONCLUSION

This work first reports the pH-controlled change of polyphenol oxidase active site. In low pH, a new active site of each H_2O molecular oxygen binds of one the pair of coppers is formed, which is different from the native PPO II active center of the pair of coppers with phenolate oxygen bridged. A new active site is formed suddenly between pH 8.5 and 9.0, which is the hydroxide anion binds one of the pair of coppers and phenolate oxygen binds with the other copper. CD data shows that the change of the second structure is not the reason that affects the absorption spectrum.

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