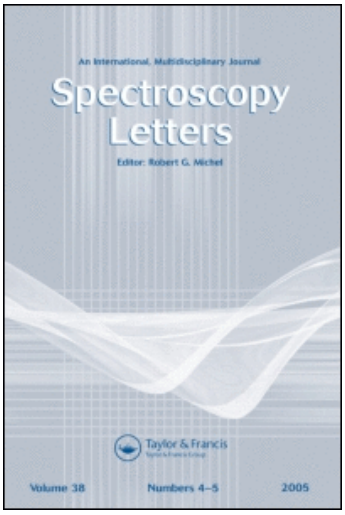


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# UV/VIS SPECTRA STUDIES ON THE POLYPHENOL OXIDASE II (PPO II) FROM TOBACCO

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## ABSTRACT

From the UV/VIS spectra, the type of the copper involved in the polyphenol oxidase II (PPO II) has been investigated and also the basic coordination environment has been discussed. The UV/VIS spectra characters of native enzyme, reduced, oxidized state and adding fluoride anion demonstrate the absence of type 1 copper and the existence of type 3 copper. The type 3 coppers are coordinated with the oxygen of phenolate. There is a clear absorption of 580 nm which assigned as $O_2(2-)$ $(\pi^*)\rightarrow Cu(II)$ $(dx^2-y^2)$CT transition in PPO II after adding $H_2O_2$. There is a clear $F-Cu^{++}$ CT transition absorption about 380 nm after adding fluoride anion.

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*Key Words*: UV/VIS spectra; Polyphenol oxidase; Tobacco; Copper blue protein



## INTRODUCTION

Polyphenol oxidase (PPO) is one kind of multicopper protein which is widely distributed throughout the bacteria to mammals. This protein is responsible for enzymatic browning by oxidation of the polyphenol to its quinone derivatives[1]. The copper plays an essential role as a cofactor in a number of enzyme systems including cytochrome oxidase, superoxidase dismutase and polyphenol oxidase, and others because of the multiple valences of $Cu^{+}$ and $Cu^{++}$ in the system. The multicopper oxidases such as polyphenol oxidase, ascrobate oxidase, and laccase catalyze the reduction of dioxygen to water. There are three kinds of Cu atoms to present in those enzymes, i.e., type 1 or blue ($A_{//} \leqslant 95 \times 10^{-4}\,cm^{-1}$), type 2 or normal ($A_{//} > 140 \times 10^{-4}\,cm^{-1}$), and type 3 or coupled binuclear through a ligand of bridging oxygen (EPR undetectable)[3], and copper presented in hemocyanin[10]. The various copper sites can also give rise to characteristic electronic absorption features, for example, type 1 site exhibits an intense $S \rightarrow Cu^{2}+$ charge transfer band near 600 nm ($\varepsilon = 5000\,m^{-1}.cm^{-1}$) which was attributed to $\sigma$(S)—Cu(II) LMCT and several weaker features from the ultraviolet to near-IR region[4] and type 3 site oxidized displays abroad near UV absorption band maximizing at 330 nm and a d→d band at 740–745 nm[5,6]. No absorption features have been assigned to the type 2 site except for the normal absorption of copper ion. In the monomeric $Cu^{2+}$ system with N, O ligation, ligand→metal charge transfer (CT) features generally occur at $> 20\,000\,cm^{-1}$($< 500$ nm) and the d→d transition occur at $< 20\,000\,cm^{-17}$. The d-d features provide in sight into ligand stereochemistry and electronic structure. Copper(II) can adopt square-planar, square-pyramidal, trigonal-bipyramidal, octahedral, and tetrahedral geometry and the d-d spectra shown by these coordination geometry are clearly distinctive only in the case of the tetrahedral environment.[20,21] In the tetragonal five- and six-coordinate $Cu^{++}$ complexes with N ligands, the d-d absorption features occur at energies between 13400 and $17500\,cm^{-1}$ [16] and in square-planar $Cu^{++}$ amino acid complexes, the transitions occur in the range from 15400 to 17700 and 17500 to $20600\,cm^{-1}$ [2] and the d-d band in the flattened tetrahedral type 1 $Cu^{++}$ site in plastocyanin are at much low energy, ranging from 13900 to $5000\,cm^{-1}$.[4]

Among the copper proteins, laccase (LC) and ascorbate oxidase, (AO) were widely studied because they include the three kinds of copper. Uma M. Sundaran et al.[8] studied the TlHg LC (laccase with the type 1 copper replaced by non-redox active metal Hg) and T2D (laccase with the type 2

copper removed), the blue center at 600 nm disappeared with the absent of type 1 copper. This paper describes an attempt at determining the structure and ligand environments of the copper sites in PPOII on the basis of their electronic spectra. Although this method alone is somewhat limited, we are optimistic because these copper-containing enzymes show such distinct and unusual spectra and the potential donor atoms are limited.

## EXPERIMENTAL SECTION

### Enzyme Extraction

The fresh tobacco leaves were harvested directly from the field, washed and then kept below 4℃ in for about 24 hours. DEAB-Sephadex A-50, Sephadex G-75 and CM-Sephadex C-50 were purchased from Pharmacia Corporation Sweden. Other chemicals were analytical reagent grade.

A 300 g sample of fresh tobacco leaves was homogenized with 150 ml of ice-cold acetone/water/triton X-100 (80:19:1, V/V) for about 24 hours (−18℃), then filtered through a porous glass filter. The residue was washed three times with 150 ml acetone (−18℃). The resulting acetone powder was dried about one-half hour at room temperature.

The sample of the acetone powder was suspended in 200 ml of 0.05 phosphate buffer (pH = 6.5), containing 0.01 M PVP, treated in ultrasonic instrument for about one hour and then the suspension was centrifuged (5600 × g, 15 min). The ammonium sulfate was added into the supernatant and up to 30% (m/m), stirred for about four hours in 4℃ and then centrifuged (5600 × g, 20 min) again. The pellet was discarded and ammonium sulfate was added to the supernatant and up to 80% (m/m). The solution was stirred for about 12 hours and centrifuged (5600 × g, 30 min). The resulting pellet was homogenized in 50 mM Tris-HCl buffer pH = 7.5 and dialyzed for about 20 hours against the same buffer.

The final solution obtained in the above step was loaded onto a DEAE-Sephadex A-50 column (3.6 × 70 cm) and conducted the chromatographic separation, then the activity band was collected and concentrated to about l0 ml. Aller that it was dialyzed in 50 mM $H_3PO_4$-NaOH buffer pH = 6.5 about 20 hours, and the solution was fed the top of Sephadex G-75 column (l.6 × 100 cm) again. In the same way, the activity portion was concentrated to about l0 ml, then dialyzed in 50 mM $H_3PO_4$-NaOH buffer pH = 6.5 about 20 hours. The sample was loaded to the CM-Sephadex C-50 (2.6 × 60 cm). The part with activity was concentrated to about 10 ml and dialyzed in water, and then kept in fridge at −18℃. At the end PPOII was determined by SDS-PAGE and PAGE to be single band.

The PPO activity was determined by UV spectroscopy in 50 mM catechol and 50 mM $H_3PO_4$-NaOH buffer pH = 6.5. The unit of enzyme activity can be defined as the amount of enzyme which causes an absorption increase of 0.0l unit per minute at 420 nm. The protein concentration can be determined using Bradford method.[9]

UV/VIS Spectra: UV/VIS absorption spectra were recorded on a UV2100 spectrophotometer(SHIMADAZU Corp.) in 10 mm quartz curettes at room temperature of 298 K. The concentration of the sample recorded from 190 nm to 800 nm was 1.05 mg/ml (29.5 uM). The resting protein was prepared at 4℃ and the concentration of 0.64 mg/ml (18.0 uM), and other spectra were recorded in the same concentration with the existence of 10 mM $F^-$, 50 mM $H_2O_2$, and 10 mM ascorbic acid respectively.

## RESULTS AND DISCUSSION

Copper (II) can adopt square-planar, square-pyramidal, triangle-bipyramidal, octahedral, and tetrahedral geometry structure in copper protein.[10] Three kinds of coordination atoms are likely involved in these

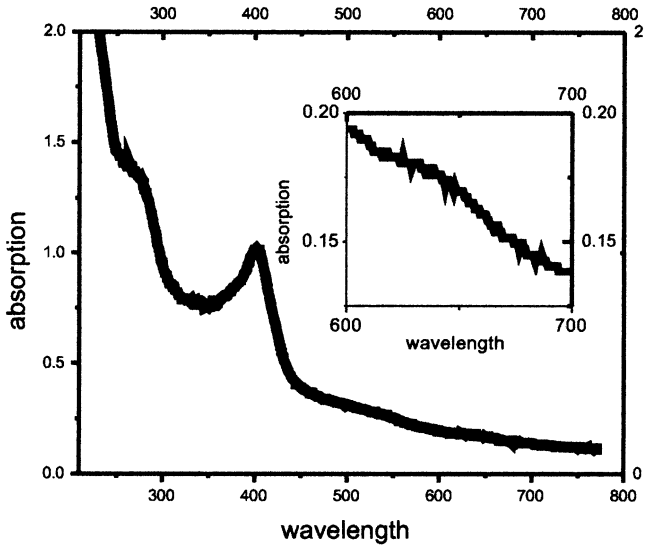


*Figure 1.* Absorption spectra of PPOII at room temperature. The concentration of the sample was 1.05 mg/ml, The experiment was processed in room temperature and at 0.05 M $H_3PO_4$-NaOH buffer at pH = 6.5. Inset: the absorption of d-d transition at 640 nm.

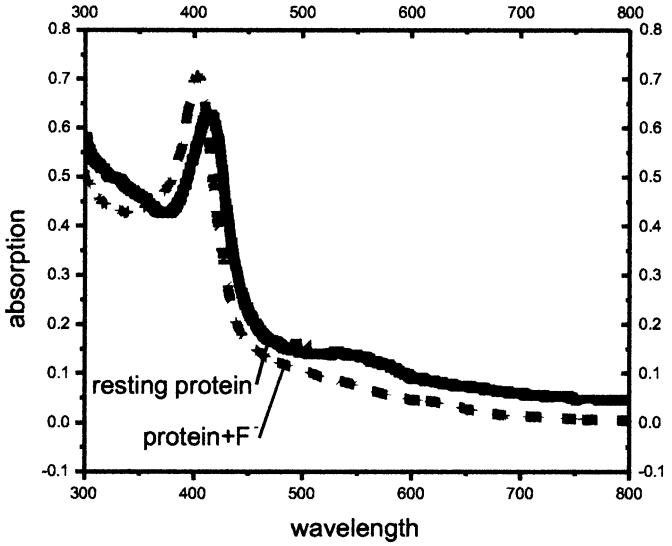


*Figure 2.* Absorption spectra of PPOII and PPOII +10 mM $V_C$ at room temperature. The concentration of the protein was 0.64 mg/ml.

proteins, namely oxygen (carboxylate, phenolate, and water), nitrogen (amine, amide anion, and imidazole), and sulfur (thioether and thiolate). We imagine that the bonding of copper to the phenolate anion, thioether, or the thiolate anion, with particular coordination geometry can give some unusual features of UV/VIS spectra. Figure 1 is the UV/VIS spectra of PPOII purified from tobacco. There are some clear absorption peaks at about 640 nm, 540 nm, 420 nm and 330 nm. Also this curve shows the normal protein absorption of 280 nm, 220 nm and about 250 nm. The absorption peak of 420 nm is very strong and the absorption peak of 640 nm is very weak which prove that the former should be the contribution of charge transfer transition and the later should be the forbidden d-d transition.

According to the former study of copper protein. The absorption of the type 1 copper was intensity around 600 nm. The most likely absorption of type 1 in PPOII is that of 420 nm, but the energy was much higher than the copper blue center absorption of about 600 nm (The energy of the former is higher 7740 $cm^{-1}$ than that of the later). No reports found were given the absorption of type 1 Copper at about 400 nm now. The absorption of type 1 copper disappeared after the copper protein was fully reduced by ascorbic acid[2] in respect of the reduction of $Cu^{++}$ to $Cu^{+}$ and the disappear of LMCT. Figure 2 shows the result of PPO II reduced by ascorbic acid and which doesn't show reduction of around 420 nm which prove the absence of

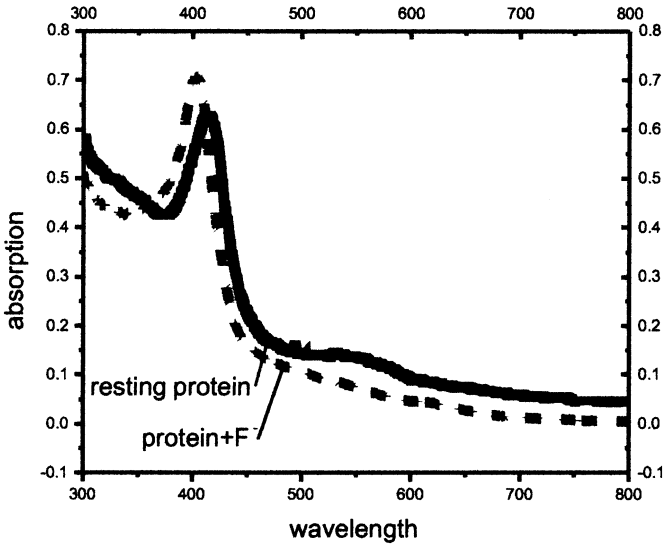


*Figure 3.* Absorption spectra of PPOII and PPOII +10 mM $F^-$ at room temperature. The concentration of both sample contained 0.64 mg/ml PPO II.



type 1 copper in PPO II. $F^-$ binds to the type 2 copper with a high binding constant and then the type 1 copper site.[13] After the binding of F- to type 1 copper, the absorption of type 1 would decrease,[15] while in PPOII the absorption of 420 nm didn't decrease after adding $F^-$ to the protein. This is other proof that PPO II doesn't contain type 1 copper. Figure 3 shows the figure after $F^-$ was added to the system, and the absorption of 420 nm was blue-shifted about 10 nm, the peak about 370 nm was the CT transition of fluoride to the metal[14] and this means the fluoride had been coordinated with the copper.

Figure 4 shows $H_2O_2$ was added in the system, PPOII shows a clear weak intensity absorption at about 580 nm contributed by $O_2$(2-) ($\pi^*$)→Cu(II) ($dx^2-y^2$) to copper charge transfer transition which was stabile and didn't disappear after 48 hours (the figure was not given), which prove that the peroxide binding to the copper site and the protein contains type 3 copper.[19] The peroxide was binding with type 3 copper of PPOII from the clear absorption 580 nm and complex absorption change around high-energy absorption about 330 nm.

From Figure 1, the bands at about 540 nm, 420 nm, and 330 nm are not readily associated either with d-d band because of the intensity

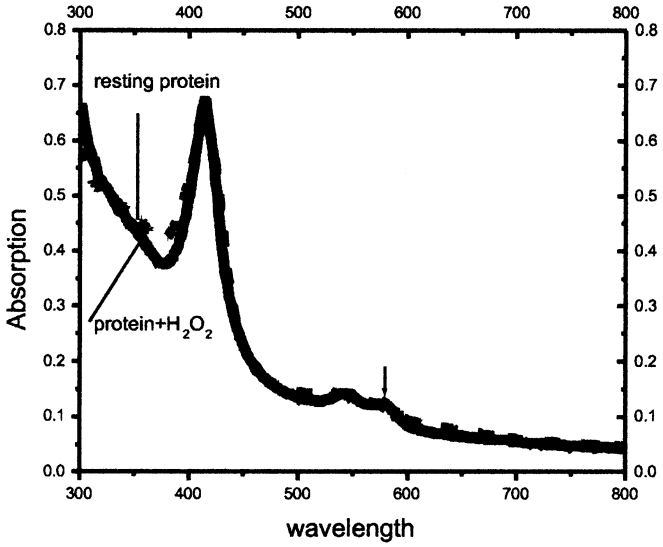


*Figure 4.* Absorption spectra of PPOII and PPOII $+H_2O_2$ room temperature. The concentration of the protein sample was 0.64 mg/ml and the sample of protein $+H_2O_2$ contained 1.05 mg/ml PPO II and 50 mM $H_2O_2$.



absorption and the natural amino acid contained in protein. The model complex of copper glycinate shows no CT transition absorption below 40000 $cm^{-1}$, and these bands can't be O($\sigma$)−Cu(II) LMCT.[11] The 8-hydroxyquinoline complex of Cu(II) also shows these two bands of about 420 nm and 330 nm but displaced by about 2000 $cm^{-1}$ to high energy, since the quinoline ring allows more delocalization than the benzene ring, these transitions are likely to be MLCT not LMCT.[11] The LMCT transition should disappear after the reduction from $Cu^{++}$ to $Cu^{+}$ ion, while the absorption of 420 nm didn't disappear in Figure 4. Here we assigned the absorption of 540 nm, 420 nm, and 330 nm were Cu(II) (d)-phenolate($\Pi^*$) transition energies ($cm^{-1}$) in PPOII[12] which was just as the coordination environment of copper in the protein of hemocyanin.[10]

## CONCLUSION

This work is about the relationship of UV/VIS spectra, the type of copper protein and some coordination formation of the copper in PPO II.

The UV/VIS spectra study of PPO II shows the result of the existence of type 3 and absence of type 1 copper, the type 3 copper was bridged by phenolate oxygen, and there exists copper d orbital to phenolate oxygen MLCT. After adding hydrogen peroxide to PPO II, peroxide was coordinated with the pair of copper ions in the active center.

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