INVESTIGATIONS ON THE REDUCTION OF MOLECULAR OXYGEN ON POLYPHENOL OXIDASE-MODIFIED ELECTRODE

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Polyphenol oxidase (PPO, E.C.1.10.3.1) from *Nicotiana tobacum*, PPO II, shows a different behavior from that of PPOs from other sources. A one-electron oxygen reduction on PPO II – Nafion modified glassy carbon electrode surface has been observed by square-wave voltammetry. The cathodic peak has been found to be proportional to the concentration of dissolved oxygen. The optimum salt concentration was 0.2 mol/l. The study of its dependence on pH (4–9) indicates that the reduction of oxygen is accompanied by a one-proton-transfer process. Temperature shows little influence on the cathodic peak. The electrode storage has also been studied to evaluate its stability.

Keywords: Square-wave voltammetry; Polyphenol oxidase; Oxygen detection; Nafion modified electrode; Enzymes; Modified electrodes; Electrochemistry.

Polyphenol oxidases (PPO) are a group of copper-proteins widely distributed through the whole phylogenetic scale from bacteria to mammals[1,2]. The common feature of the enzymes is their capability to catalyze the oxidation of polyphenols through molecular oxygen to corresponding quinones. Due to their specific substrates, the PPO family can also be divided into tyrosinase, catechol oxidase and laccase[3]. The immobilization of PPO on different matrices has been intensively studied, many of which can afford excellent amperometric biosensors which may be widely used in the determination of phenol derivatives[4–11] or enzyme inhibitors, such as cyanide[12,13], L-cysteine[14], hydrazine[15] and some pesticides[16,17]. On the other hand, some metalloproteins, such as myoglobin[18], glucose oxidase[19,20] and blue copper oxidases[21] have been encapsulated to determine the dissolved or gaseous oxygen. However, little has been reported for PPO in the direct detection of molecular oxygen, the co-substrate in PPO catalytic processes. The difficulty may be the following: the electrochemical response to molecular oxygen mainly comes from two origins, the reduction of oxygen to water (four-electron process) or to hydrogen peroxide (two-electron pro-

cess). The former process gives a relatively interference-free signal but has a drawback of low sensitivity due to the large background current[22]. As to the latter, the sensitivity improved, while it is more prone to be interfered with a highly positive overpotential[23]. The oxidation catalyzed by PPO usually adopts the above two routines, which leads to a disadvantage in the detection of oxygen.

PPO II (molecular weight 35.6 kDa) has been isolated from tobacco; it belongs to catechol oxidase with unusual spectroscopic properties[24,25]. The electrochemical experiments have shown the process of its combination with oxygen; it can be calculated to be a one-electron-transfer process. In the present communication, the relationships between the current of the cathodic peak and the oxygen concentration are investigated and its stable conditions are also evaluated.

EXPERIMENTAL

Instruments and Reagents

The electrochemical measurements were performed on an LK 98 microcomputer-based electrochemical system. A three-electrode compartment cell was used for square-wave voltammetry. A glassy carbon (GC) electrode (4 mm in diameter) was used as a working electrode, a platinum plate (Pt) as counter-electrode and an Ag/AgCl electrode as reference electrode. The unsaturated oxygen concentration was determined by a JPB-607 portable dissolved oxygen analyzer (Shanghai REX Instrument Factory, P. R. China).

Nafion solution (5% solution in ethanol) and mushroom tyrosinase were purchased from Sigma Corporation, U.S.A. The chromatographic materials, DEAE-Sephadex A-50 and Sephadex G-75, were purchased from Pharmacia Corporation, Sweden. All other chemicals were of analytical grades. A protein sample from the fresh tobacco leaves (*Nicotiana tobacum*) was prepared by the methods described earlier[24], with some modification of the order in the column chromatography: DEAE-Sephadex A-50, followed by Sephadex G-75 and a second DEAE-Sephadex A-50. Protein concentration was determined using the method of Bradford[26].

Preparation of PPO II–Nafion-Modified Electrode

Prior to the preparation of the modified electrode, GC electrode was polished successively with 1, 0.3 and 0.05 μm α-alumina slurry, rinsed thoroughly with doubly distilled water between polishing steps, sonicated successively in 1:1 nitric acid, acetone and doubly distilled water and then allowed to dry at room temperature. The PPO II (or tyrosinase) film electrodes were prepared as follows: 3 μl of 20 mg/ml PPO II (or tyrosinase) sample and 3 μl of a Nafion stock solution (5%) were mixed, poured on the surface of the GC electrode, and dried in air at 4 °C for 6 h. The electrode was then used without any electrochemical activation and no pretreatment on the electrode was needed before each measurement.

RESULTS AND DISCUSSION

Electrochemical Behavior of PPO II on the Nafion-Modified Electrode

Square-wave voltammetry of PPO II has been performed in the phosphate buffer solution (PBS, pH 6.0) in the presence and absence of oxygen, as a simplified plot shows in Fig. 1. A cathodic peak appears at –0.23 V in the presence of oxygen (curve *1*). When passing nitrogen into the solution, the peak disappears (curve *2*). The peak emerges again (curve *3*) after blowing air into the solution. This property is absolutely reversible after the elimination and addition of molecular oxygen and shows that the observed peak involves reduction of molecular oxygen. The special phenomenon is not found for either tyrosinase–Nafion- or Nafion-modified electrode (figures not shown). Further, the number of the transferred electrons (n) for the reduction peak can be calculated according to the following equation[27,28]:

$$W_{1/2} = (RT/nF)\{3.53 + 3.46\xi_{sw}^2/(\xi_{sw} + 8.1)\} , \qquad (1)$$

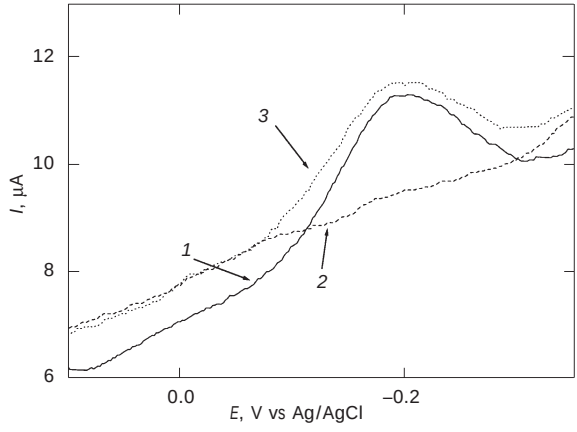

FIG. 1

Square-wave voltammograms of PPO II in the presence and absence of oxygen. Square-wave parameters: modulation amplitude, 25 mV; modulation period, 60 ms; modulation steps, 2 mV. The oxidation process has been omitted for clarity. The solid line was scanned with native solution (*1*), the dash line was scanned after bubbling nitrogen through the solution for 30 min (*2*) and the dot line was scanned after bubbling air through the solution for another 30 min (*3*). The following parameters were constant during the experiments unless special conditions were indicated: the cell solution was 0.05 M phosphate buffer (pH 6.0) containing 0.2 M NaCl. The temperature was 18 °C and the air pressure was ca. 1.0×10^5 Pa. The scan rate was 10 mV/s

where $W_{1/2}$ is the half-band width of the peak; $\xi_{sw} = nFE_{sw}/RT$, E_{sw} is the amplitude of the square wave (25 mV here) and F is the Faraday constant. $W_{1/2}$ has been measured to be 112 mV, thus n is ca. 0.85, approximate by 1. Therefore, the peak can be attributed to the reduction of oxygen to superoxide with one-electron transfer. The current of reduction peak is large enough for the analysis of oxygen in aqueous solution and makes its use for the detection of oxygen possible.

Cyclic voltammetries (CV) with the electrode show, when the scan rate (v) changes between 10 and 100 mV/s, the peak current proportional to $v^{1/2}$ (R = 0.99; figure not shown). The peak potential and the peak separation are both independent of the scan rate, indicating that the process is a diffusion-determined process.

A relationship between the cathodic peak of PPO II and the oxygen concentration has been investigated at 18 °C. Different oxygen concentrations are obtained by bubbling pure N_2 through the cell to remove a part of the dissolved oxygen and monitored by the oxygen analyzer. Figure 2 shows CV changes of various oxygen concentrations. The current of the cathodic peak increases with increasing oxygen concentration. A linear relationship between the current and the oxygen concentration can be observed from 1.7 to 7.1 mg/l (ca. 84% of the saturation concentration) with a linear correlation coefficient R = 0.992. The current tends to be saturated when the oxygen concentration was above 7.1 mg/l, which limits its detectable scope. The difficulty can be overcome by increasing the working area of the

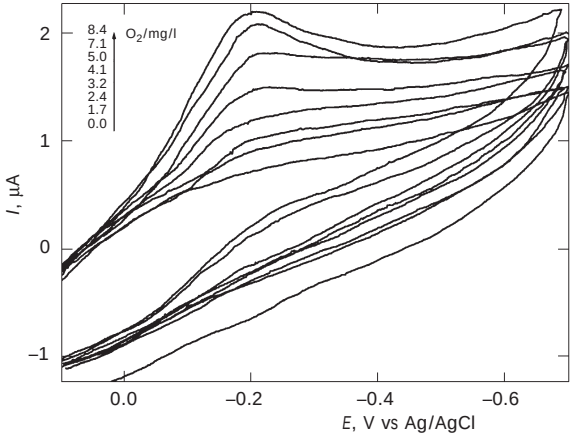

FIG. 2
The relationship between CVs and oxygen concentration in the electrolyte

GC electrode (in the present work the area is 0.1256 cm^2) and thus the current can be increased.

Characteristics of the Electrode

The dependence of the current of the cathodic peak on the salt content has been investigated. The salt concentration corresponding to the highest current of the cathodic peak is ca. 0.2 mol/l. The current goes down to ca. 92% of the highest value when the solution is free of NaCl. At higher salt concentrations, it decreases rapidly to 88% at 0.4 mol/l, 59% at 0.6 mol/l, 35% at 0.8 mol/l and 27% at 1.0 mol/l. As it is known, the saturation concentration of dissolved oxygen decreases with the increasing amount of dissolved salt. However, the decrease in the current is much larger than that in the oxygen concentration. Thus, the electrode is dramatically sensitive to the salt content, probably due to the inhibition of chloride ion on PPO [29].

The potentials of the irreversible cathodic peaks have been found to shift with pH changes. The slope of the potential vs pH dependence is –66 mV/pH in the range of pH 4.0–9.0 (Fig. 3 inset, R = 0.997), close to the standard slope for the H^+-determined process, indicating that protons take part in the reduction process.

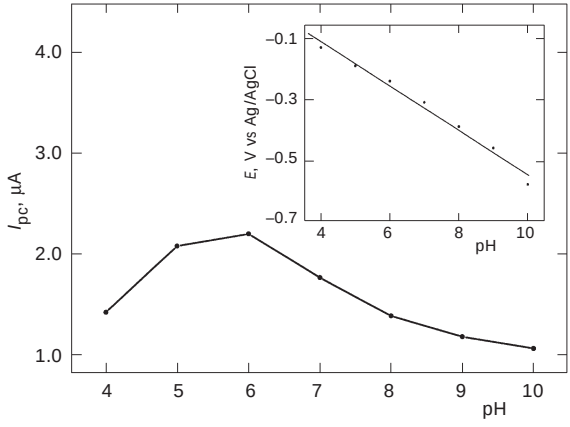

FIG. 3
The plot of the current of the cathodic peaks vs pH. Inset: The relationship between the potential of the peaks and pH. The electrolyte buffers were 0.1 M Tris-HCl (pH 8.0–10.0) or 0.1 M phosphate buffer (pH 4.0–7.0), both containing 0.2 M NaCl

The linear-fit equation for the potentials on the electrode is

$$E \text{ [V vs SHE]} = 0.366 - 0.066 \text{ pH} . \qquad (2)$$

There is a precipitation in the rate of the potential between pH 9.0–10.0 (ca. 120 mV), which may be due to conformational changes in the catalytic center[30], accompanied by a change in its catalytic mechanism. The optimum pH for the cathodic current is 6.0 (see Fig. 3), coinciding with that for PPO II with catechol as the substrate[24].

Considering the participation of protons and electrons, the process of the reduction of molecular oxygen can be described as

$$O_2 + H^+ + e \rightarrow HO_2^{\bullet} . \qquad (3)$$

O_2 binds to PPO II before it is catalytically reduced since a corresponding peak has not been observed for the naked-Nafion-modified electrode. The standard midpoint potential for dissolved oxygen at 1 mmol/l is –0.32 V (vs SHE) and ca. –0.35 V under the experimental conditions (ca. 0.26 mmol/l of dissolved oxygen). Compared with Eq. (*2*), the potential on PPO II-modified electrode is ca. 0.72 V higher than the standard state and thus the energetic disadvantage may be overcome.

The temperature dependence of the current was also studied. All experiments were carried out in the saturation concentration of dissolved oxygen at different temperatures for the sake of comparison. The plot of the cathodic peak I_{pc} against temperature is shown in Fig. 4, along with the rela-

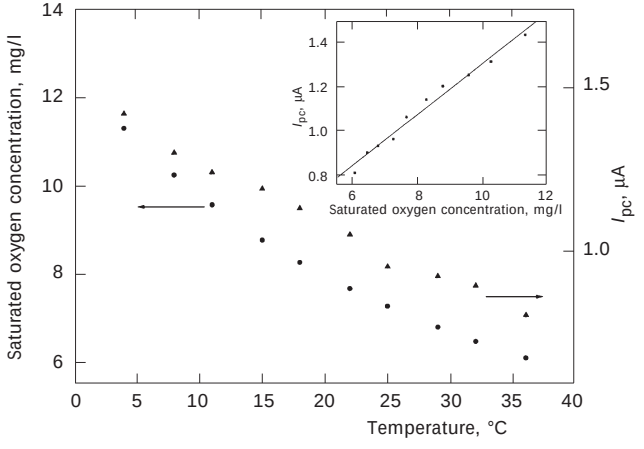

FIG. 4
The effect of temperature on the current of the cathodic peaks. Inset: The plot of cathodic peaks vs saturated oxygen concentration at different temperatures

tionship between the saturation concentration of dissolved oxygen and temperature. The two curves have the same trends. Further, when I_{pc} is plotted against the saturation concentration of dissolved oxygen, a rather fine linear curve is obtained (Fig. 4 inset, R = 0.990). Hence temperature hardly affects the reduction of oxygen and the decrease in I_{pc} mainly corresponds to the decrease in the amount of dissolved oxygen.

The cathodic peak is stable for 4 weeks. The current of the cathodic peak was recorded at 18 °C in a solution saturated with oxygen, after 1, 3, 5, 7, 10, 14, 21, 28 days. The electrode was stored at 4 °C during the 4 weeks. The current is very stable for the first 10 days or so, and then it decreases slowly in the following 18 days. The final peak current can still reach 73% of the initial value. Inhibition of the culture of some bacteria by PPO II [24] may keep the current steady.

In conclusion, PPO II deposited with Nafion on GC electrode can catalyze electrochemical reduction of molecular oxygen. The calculation and pH experiments show that the process is diffusion-controlled and involves a transferred electron and a proton. The process is considered to be a reductive step to protonated superoxide. The electrode is able to detect the dissolved oxygen concentration in the common range. The reduction potential of molecular oxygen on the PPO electrode is significantly raised compared with the normal electrode, which makes the one-electron reduction possible. The electrode shows high stability in the temperature range from 4 to 36 °C. The stability study shows that the signal is stable for a rather long time, which may be due to the inhibition of a bacterial culture.

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