Amperometric Detection of Polyphenols Using Peroxidase-Immobilized Gold Electrodes

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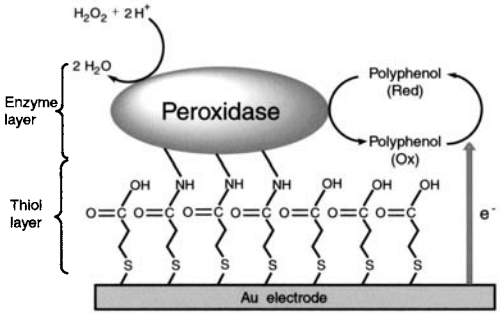
Horseradish peroxidase (HRP) is covalently immobilized on a self-assembled monolayer of mercaptopropionic acid on vapor-deposited gold electrode. The electrode allows the polyphenol detection down to 2 μmol dm^{-3} with a linear relationship up to 25 μmol dm^{-3}. The reduction current of oxidized polyphenol intermediate formed during the enzymatic reaction in the presence of H_2O_2 is proportional to the concentration of polyphenol. The present method is applicable to the determination of the total polyphenol content in wine and tea.

Polyphenols are widely distributed in plant-derived foods including wine, tea, cacao and fruits, and recently attract much attention because of their possible health benefits arising from their antioxidant activity, such as free radical scavengers and inhibition of lipoprotein oxidation.[1–5] While the spectroscopic method, the Folin–Ciocalteu method,[6] has been used for determining the total polyphenol content in foods, simpler and time-saving methods are required for certain purposes, e.g., real-time monitoring of a polyphenol level in food processing.[6] Biochemical sensors utilizing the redox cycling of enzyme have been proposed as sensitive and selective devices for monitoring many kinds of substances.[7,8] The rapid detection of the total amount of phenolic components was achieved by biosensors based on polyphenol oxidase and peroxidase without the sample pretreatment.[9–12] We here propose a useful amperometric method using horseradish peroxidase (HRP)-immobilized gold electrodes for the determination of total polyphenol content, which has several advantages over the Folin–Ciocalteu method, ex. shorter detection time and smaller sample volume.

HRP-immobilized gold electrodes (HRP | MPA | Au)| were prepared according to the following procedure. A self-assembled monolayer (SAM) of mercaptopropionic acid (MPA: Dojin Laboratory Co.) was formed by immersing a vapor-deposited gold/mica substrate into 1 mM (M = mol dm^{-3}) ethanolic solution of the thiol for 20 h. HRP (EC 1.11.1.7. 830 U mg^{-1}: Sigma) was covalently immobilized on the MPA-adsorbed Au substrates by immersing the substrate into 10 mM phosphate buffer (pH 6.9) containing 50 μM HRP and 1 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC: Sigma) for 48 h at 4 °C. Reproducible electrode responses were not obtained without using EDC, confirming the formation of binding between amino groups on HRP surface and carboxyl groups on the SAM. Amino groups on HRP surface responsible for binding cannot be specified now. The amount of the immobilized HRP was estimated to be 2.4×10^{-12} mol cm^{-2} from spectrophotometric measurements[13] and did not significantly increase when the reaction time for the HRP immobilization was prolonged more than 48 h. Although the immobilization of enzymes as monolayers on SAMs is not suitable for the increase in sensitivity, this method can be applied to fabricate biosensor devices composed of well-organized array of enzyme and mediator or multiple enzymes using mixed thiol SAMs. The HRP | MPA | Au substrate was mounted on a circular window on the side of a home-made cylindrical glass cell by using an elastic o-ring whose diameter is 10 mm (the geometrical electrode area: 0.8 cm^2). A freshly prepared 10 mM sodium phosphate buffer (pH 6.9) containing 0.2 mM H_2O_2 was placed into the cell and a steady-state current was detected at +0.0 V vs Ag | AgCl (saturated KCl) at room temperature under stirring at 580 rpm.



Scheme 1. Reaction pathway for the detection of polyphenol at a HRP|MPA|Au electrode.

Scheme 1 represents the detection mechanism of polyphenol at the HRP | MPA | Au electrode. The enzymatic cycle reaction between HRP immobilized on the electrode surface and H_2O_2 in solution results in the formation of oxidized HRP, which is reduced back to the reduced form by taking electrons from polyphenols in solution. The formed oxidized polyphenol intermediate can be electrochemically reduced back to its initial form at the gold electrode. The reduction current should be proportional to the concentration of polyphenols in solution.

Figure 1 shows an increase in the steady-state reduction current, I_{lim}, at 0 V on the stepwise addition of aliquots of (+)-catechin solution into 10 mM phosphate buffer containing 0.2

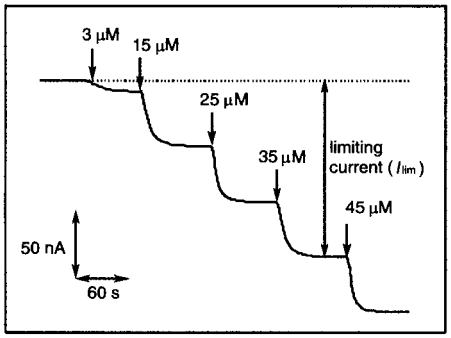


Figure 1. Steady-state current response detected at 0 V with a HRP|MPA|Au electrode on the stepwise addition of (+)-catechin solution into 10 mM phosphate buffer (pH 6.9) containing 0.2 mM H_2O_2 under stirring. At the points indicated by arrows, (+)-catechin solution was injected to reach the concentration to the value written at the foot of the arrow.

mM H_2O_2. The time required for I_{lim} to reach a 90% level of the steady-state value ranged from 45 to 90 s and was independent on the polyphenol concentration. No reduction current was observed in the absence of H_2O_2 in the buffer or the immobilized HRP, indicating that the I_{lim} originates from the oxidized polyphenol produced through the mediated reduction of H_2O_2 by HRP. The I_{lim} increased with the concentration of H_2O_2 and leveled off over 0.1 mM, indicating that the present measurements were carried out under the substrate-saturated condition.

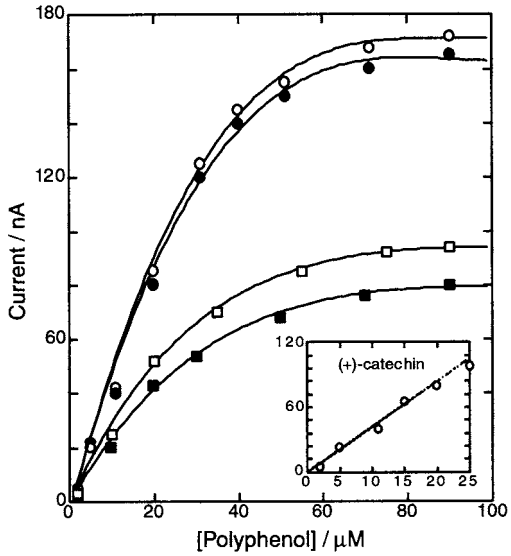


Figure 2. I_{lim} as a function of the concentration of (+)-catechin (○), (-)-epicatechin (●), caffeic acid (□), and 3,4-dihydroxybenzoic acid (■). The current was measured with a HRP|MPA|Au electrode at 0 V in 10 mM phosphate buffer (pH 6.9) under stirring.

Figure 2 shows the change of I_{lim} as a function of the concentration of several polyphenols. The I_{lim} value increased linearly with the concentration of (+)-catechin up to 25 μM with a correlation coefficient of 0.995 and deviated downward from the linear relationship at the higher concentration region due to the HRP reaction kinetics. The lowest detection limit was 2.0 μM at a signal-to-noise ratio of three. The I_{lim} vs polyphenol concentration plots for (–)-epicatechin, caffeic acid, and 3,4-dihydroxybenzoic acid are also shown in Figure 2. For these polyphenols, the linear relationship was obtained in a similar concentration range to that for (+)-catechin. However, the sensitivity of the polyphenol detection, which corresponds to the slope of the I_{lim} vs polyphenol concentration plot, was different and the higher sensitivity was found for (+)-catechin and (–)-epicatechin than for caffeic acid and 3,4-dihydroxybenzoic acid. Under the substrate-saturated condition, the electrode responses seem to be rate-limited by the formation and the electrochemical reduction of the oxidized polyphenol. Because the former process is correlated with the oxidation potential of polyphenols,[12] most of monophenols, which have more positive oxidation potentials than polyphenols, were not detected. The importance of the latter process is supported by the fact that the larger I_{lim} was detected at the more negative potential for all polyphenols. CV measurements revealed that the oxidized intermediates of (+)-catechin and (–)-epicatechin are more easily reduced than that of caffeic acid, and hence the higher sensitivity was obtained at 0 V for the former polyphenols. The

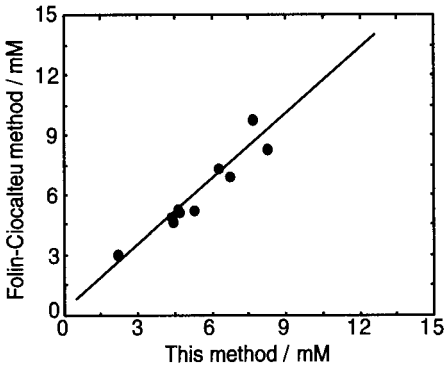


Figure 3. Correlation between the present method and the Folin-Ciocalteu method for the determination of the total polyphenol content in wine and tea. Caffeic acid was used as the standard.

response of the HRP | MPA | Au electrode was dependent on pH and a maximal response appeared at about pH 6.5.

We used HRP | MPA | Au electrodes to measure the total polyphenol content of four wine and six tea samples. The obtained content values were compared with those determined by the Folin–Ciocalteu method as shown in Figure 3. In both methods, the sensitivity of the detection is actually not equal for all polyphenols contained in wine and tea and thus the content values depend on the standard polyphenolic compound used. For the caffeic acid standards, a linear relationship with a slope of 1.00 and a correlation coefficient of 0.900 was obtained between the values estimated by the two methods, indicating the applicability of the present method to monitor a change in the polyphenol content and to compare the polyphenol content between samples.

References and Notes

1 C. Sanchez-Moreno, J. A. Larruri, and F. Saura-Calixto, *Food Rev. Int.*, **32**, 407 (1999).
2 L. Fremont, L. Belguendouz, and S. Delpal, *Life Sci.*, **64**, 2511 (1999).
3 Y. Kono, K. Kobayashi, S. Tagawa, K. Adachi, A. Ueda, Y. Sawa, and H. Shibata, *Biochim. Biophys. Acta*, **1335**, 335 (1997).
4 Z. Anqi, T. C, Ping, S. L. Yan, K. K. H. Walter, and Z.-Y. Chen, *J. Nutr. Biochem.*, **8**, 334 (1997).
5 C. A. Rice-Evans, N. J. Miller, and G. Paganga, *Free Radical Biol. Med.*, **20**, 933 (1996).
6 V. L. Singleton, R. Orthofer, and R. M. Lamuela-Raventos, *Methods Enzymol.*, **299**, 152 (1999).
7 D. L. Wise, "Bioinstrumentation," Butterworth, Boston (1990).
8 F. Scheller and F. Schubert, "Biosensors," Elsevier, Amsterdam (1992).
9 A. Lindgren, J. Emneus, T. Ruzgas, L. Gordon, and G. Marko-Varga, *Anal. Chim. Acta*, **347**, 51 (1997).
10 T. Ruzgas, J. Emneus, L. Gorton and G. Marko-Varga, *Anal. Chim. Acta*, **311**, 245 (1995).
11 S. Cosnier, J.-J. Fombon, P. Labbe, and D. Limosin, *Sens. Actuators, B*, **59**, 134 (1999).
12 Y. -T. Kong, S. Imabayashi, K. Kano, T. Ikeda, and T. Kakiuchi, submitted to *Am. J. Enol. Vitic.*
13 S. Imabayashi, Y. -T. Kong, and M. Watanabe, submitted to *Electroanalysis*.