Journal of Metals, Materials and Minerals, Vol. 25 No.1 pp.7-16, 2015

Peroxidase Inhibition Assay for the Detection of Some Thiols by Carbon Screen Printed Electrodes Based on Square Wave Voltammetry

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Abstract

Cassava peroxidase (CSP) from leaves of cultivar KU50 was used as an alternative peroxidase for the electrochemical detection of thiram and thiourea. Square-wave voltammetry (SWV) incorporated with screen-printed carbon electrode was applied for the investigation of peroxidase activity. It was based on the measurement of 3, 3′-diaminobenzidine (DAB) electro active product catalyzed by CSP and horseradish peroxidase (HRP) in 0.04 M Britton-Robinson buffer, pH 6, containing urea hydrogen peroxide in 96-well microplate. The reaction mixture in the presence of either thiourea or thiram was performed in order to study their effects on DAB oxidation catalyzed by both peroxidases. From the result of SWV signals obtained from PalmSens potentiometric instrument, it was found that the reduction peak of DAB-product catalyzed by peroxidase occurred at -0.24±0.02 V versus Ag/AgCl electrode. In the presence of thiols, decrements of SWV current peak heights were clearly observed as inhibitory effects by both peroxidases. Thiram was a stronger inhibitor than thiourea. CSP inhibition assays elucidated well-correlated relationship between thiram log concentration and its relative activity as a linear function which could potentially be applied for thiram detection in the range of 10-100 μM under the SWV optimal condition.

Keywords: Cassava, Peroxidase; Voltammetry; Square wave voltammetry; Screen printed electrode

DOI: 10.14456/jmmm.2015.2

Introduction

Peroxidases, a heme-containing oxidoeductase, have been utilized in many applications ranging from bioremediation and biocatalysis through to diagnostics and biosensors. Horseradish peroxidase (HRP) is a commonly available enzyme, which is used as a tracer in ELISA (enzyme-linked immunosorbent assay) [1, 2] and a biomolecular recognition of biosensors for the determination of hydrogen peroxide [3] and the detection of some peroxidase inhibitors [4]. Moreover, various techniques for electrochemical measurement have been reported for the determination of enzyme activities and enzyme catalyzed product based on mediator reactions. Mediators are low molecular weight molecules that can transfer electrons between redox center of the enzymes and working electrodes, thus facilitate electrical communication between them. The use of mediators made it possible to decrease the applied potential and greatly reduce the influent signals caused by easily oxidizable interfering compounds present in real samples. Some mediators such as ferrocence [5], ferrocence carboxylic acid [6] and 3, 3′, 5, 5′-tetramethyl benzidine [7] act as effective mediators for monitoring enzyme activity by voltammetric measurements. The analytical advantages of voltammetric techniques include excellent sensitivity with linear concentration range for both inorganic and organic species (10^{-12} to 10^{-1} M). A large number of useful solvents and electrolytes and a wide range of temperatures can be employed with rapid analysis time in seconds and this can be used to determine several analytes, simultaneously. In addition, the ability to determine kinetic parameters, a well-developed theory

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and thus the ability to reasonably estimate the values of unknown parameters can be achieved [8]. Furthermore, there are different potential waveforms that can be generated for measuring small currents such as normal pulse voltammetry (NPV), differential pulse voltammetry (DPV) and square-wave voltammetry (SWV). Among these techniques, SWV shows excellent sensitivity with lowest background currents and the speed of analysis is very fast. The applicable uses of electrochemical measurement for enzyme inhibition assays have been reported in many literatures. They focused on the development of inhibitor-based sensors as a tool for the determination of contaminants and pollutants in food and environmental samples. For examples, the detection of malathion and 2, 4-dichloro phenoxyacetic acid based on alkaline phosphatase inhibition with the amperometric measurement and the limit of detection (LOD) of 0.5-6 μg/L was defined [9]. Also the biomonitoring of methomyl pesticide by laccase inhibition and square-wave voltammetry with the LOD of 0.235 μM was reported [10]. In addition, the screen printed electrode immobilized with peroxidase was developed for amperometric sulfide detection by using inhibition based enzyme detector [11].

According to Thai national policy, it has been established to encourage the research trend for the utilization of all parts of many crops to increase the economic returns to the farmers. Research on cassava leaf production and potential uses of cassava leaves has been initiated and investigated [12, 13]. Kasetsart 50 (KU50) is the most important Thai cassava cultivar with high starch content and also extensively grown in every part of Thailand. In this study, cassava peroxidase (CSP) isolated from cassava leaves with specific characteristics was attempted to be used as an alternative peroxidase. The sensitive and rapid electrochemical technique such as square wave voltammetry was applied for the detection of peroxidase activities and inhibitory effects of thiourea and thiram on both CSP and HRP. In addition, the capability for the detection of thiourea and thiram based on peroxidase inhibition assay under specific condition was also carried out.

Materials and Experimantal Procedures

Materials

Enzymes: Cassava peroxidase (CSP) was isolated from cassava leaves (*Manihot esculenta* Crantz of cv. KU50) and horseradish peroxidase Type II (E.C.1.11.1.7) was purchased from Sigma (U.S.A.).

Material for electrode deposition: polyvinylchloride (PVC) flexible sheets (0.3 mm)

Chemicals: Acetonitrile and methanol were HPLC solvent from J. T. Baker (U.S.A). Ammonium sulfate was from Fluka (Switzerland), Concanavalin-A, DEAE-cellulose, dimethyl sulfoxide (DMSO), 3,3', 5,5'-tetramethylbenzidine (TMB),3,3'-diaminenzidine (DAB), urea hydrogen peroxide (UHP) and thiourea was from Sigma-Aldrich (U.S.A.). Acetic acid, boric acid, phosphoric acid, sulfuric acid and sodium hydroxide were from Sigma (U.S.A.). Thiram was from Dr. Ehrenstofer GmbH (Germany). Most of chemicals used for preparing buffers and reagents were of analytical grade. Ultra-pure water obtained from reverse osmosis Milli-Q system (R≈18 MΩ) was used for buffers and reagents preparation.

Method

Peroxidase extraction

Five hundred grams of fresh leaves of cassava were extracted and partially purified as previously reported [4]. They were cut into small pieces and blended in cold 50 mM sodium phosphate buffer, pH 6.5 containing 0.1 M phenyl-ethylsulfonyl fluoride and 2% (w/v) of polyvinyl polypyrrolidone with the ratio of 1:1 (w/v). After filtration and centrifugation, the supernatant was collected and purified by ammonium sulfate precipitation, DEAE cellulose and Concanavalin -A column chromatography.

Spectrophotometric method for peroxidase activity assay

The activity of peroxidase was determined using spectrophotometric method modified from Bos et al. [4]. Both peroxidases were stored in 50 mM sodium phosphate buffer, pH 6.0 containing 0.2 M NaCl and were diluted with 0.1 M phosphate buffer, pH 6.0 at 25°C to prepare 1.5 units/ml working solution prior to the use in all experiments. Substrate solution was prepared by dissolving 1 mg of TMB in 200 μL of DMSO, then it was gently diluted in 0.1 M acetate-citric acid, pH 6.0. After 10 minutes, 1 mg of UHP in 500 μl of 0.1 M acetate-citric acid, pH 6.0 was added and the final volume was adjusted to 10 mL. The reaction solution consisted of 10 μl of enzyme solution was pre-incubated with 100 μl of 0.1 M acetate-citric acid buffer, pH 6.0 in a microtiter well at 25°C for 3 min. The reaction was then initiated by the addition of 200 μl of substrate solution and incubated for 3 min.

The enzymatic reaction was stopped by the addition of 40 µl of 2 M sulfuric acid. The absorbance at 450 nm was then measured by Titertek Multiscan plus plate reader (Labsystems Ins., Finland). One unit of enzyme is the amount of enzyme, which oxidizes 1 µmole of TMB per minute. The molar extinction coefficient was 67,300 M^{-1} cm^{-1} [14]. The optimal pH for the catalysis of substrates containing TMB and UHP by both peroxidases in the pH range of 3-9 was investigated. The assay was carried out at 25°C and buffers used were 0.1 M Britton-Robinson buffer (3.0-7.0) : 0.1 M boric acid, 0.1 M phosphoric acid and 0.1 M acetic acid (1:1:1); 0.2 M of sodium hydroxide was added for pH adjustment, 0.1 M acetate-citric acid (pH 4.0-7.0), 0.1 M phosphate (7.0-8.0), and Tris-glycine (7.0-9.0). Moreover, the optimal temperature of both peroxidases in the range of 5-70°C in 0.1 M Britton-Robinson buffer, pH 6 was also carried out.

Fabrication of screen-printed electrodes (SPEs)

Electrodes were screen-printed onto a PVC substrate, 15x20 cm^2. SPEs were printed in 3 arrays of 8 couples (working and reference electrodes). Screen-printing was performed semi automatically by the use of DEK 248 CERD and polyester screen mesh screens. Initially, a carbon conductive layer (screen mesh size 90T and MCK carbon ink) was applied with curing temperature of 55°C for 2 h, followed by a silver/silver chloride ink (screen mesh size 90T and silver/silver chloride ink) layer with curing at temperature of 55°C for 2 hours. Finally, a non-conducting insulation material was covered over non-working surface. The working and reference area of each electrode was defined as 1.5 × 3.0 mm^2 and 2.0 × 3.0 mm^2, respectively. The electrode preparation was performed at sensor laboratory, pilot plant development and training institute, King Mongkut's University Technology, Thonburi.

Portable set of electrochemical devices

Reaction solutions containing peroxidase and 3, 3'-diaminobenzidine (DAB) as a mediator in the presence and absence of thiourea or thiram in 0.04 M Britton-Robinson buffer, pH 6 were performed in a well of 96-well microtiter plate. Electrochemical measurements in both cyclic voltammetry and square wave voltammetry were carried out using SPEs cooperated with PalmSens potentiometric instrument and PSTrace software as shown in Figure 1.

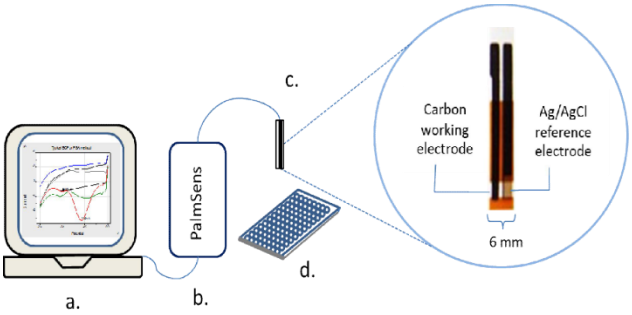


Figure 1. The portable set of electrochemical devices consisted of a. notebook computer, b. PalmSens potentiometric instrument, c. screen printed electrode and 96-well microtiter plate

Cyclic voltammetry (CV)

CV was used for the investigation of the electrochemical characteristic of DAB at SPE surface. It was performed by starting an initial potential at 1.1 V and a potential was switched in the range of -0.6 to +0.8 V with a scan rate of 20 m Vs^{-1} using a PalmSens potentiostat with PSTrace software operating system.

Square-wave voltammetry (SWV) for peroxidase inhibition study

Voltammetric measurement based on the redox reaction of DAB was modified from previous report [15]. SWV was used for the determination of oxidized DAB concentration corresponding to peroxidase activity. The stock solution of 5,000 µM DAB was prepared by dissolving 1 mg of DAB in 920 µL of RO water and two-fold serial dilutions were performed to get the concentration of 2,500, 1250, 625 and 312 µM. UHP stock solution was prepared by dissolving 4 mg of UHP in 4.2 mL of RO water to get a concentration of 10,000 µM and it was diluted to 500 µM as a concentration of working solution. Peroxidase was kept in a storage buffer (50 mM sodium phosphate buffer, pH 6.0 containing 0.2 M NaCl) and it was diluted to0.3 units/mL in 0.1 M phosphate buffer, pH 6.0 as working solution for peroxidase assay. Ten microliters of peroxidase working solution was added into a microtiter well and followed by the addition of 100 µL of 0.04 M Britton-Robinson (BR) buffer, pH 6.0 containing 5% methanol and 50 µL of DAB solution. It was thoroughly shaken and incubated for 3 minutes, and then the reaction was initiated by the addition of

50 μL UHP solution. After incubation at 25°C for 3 min, the enzyme reaction was stopped by the addition of 40μLof 0.2 M sulfuric acid. Before the electrochemical measurement was performed, the SPE was rinsed with RO water and allowed to dry at room temperature. The working and reference area of electrodes were immersed in the reaction solution and SWV current response of DAB product was measured. For the preliminary study of this work, HRP was used to find optimal conditions for SWV measurement using a portable set of electrochemical devices. The SWV reductive current peak with a good peak shape and high response was found. For current measurement, the optimal condition was set at the potential range of 1.0 to -0.8 V with a step potential of 10 mV, pulse at 75 mV and frequency of 50 Hz at the scan rate of 50 mVs^{-1}. In addition, the detection range of the SWV measurement was investigated by the graphical relationship between DAB concentration and the current response of its product catalyzed by peroxidase.

Detection of some thiol compounds based on the inhibition assay using electrochemical measurement

The effect of thiourea and thiram on both peroxidases activity was tested by peroxidase inhibition assay. The stock solution of thiourea and thiram was prepared in methanol at a concentration of 10 mM and diluted to 1-100 μM in 0.04 M BR buffer, pH 6 containing 5% methanol as working solutions. The peroxidase inhibition assay was performed as described previously except that 100 μL of thiourea or thiram working solution was used instead of 100 μL of 0.04 M BR buffer. The change of current responses in the presence and absence of target compounds were measured and calculated for the percentage (%) of relative activity. They were graphically plotted as a function of thiourea and thiram concentrations. The detection range of thiourea or thiram could be observed from the inhibition curve.

Results and Discussion

Characteristics of alternative plant peroxidase

In this study, we investigated peroxidase from leaves of cassava which is an economic plant in Thailand. Its leaves are considered as non-valuable parts of cassava. CSP obtained from cassava leaves was a heme-containing cationic glycoprotein with the Soret maximum at 400 nm [4]. It was successfully extracted from fresh cassava leaves as crude with approximately 200 units/g of leaf. After purification by precipitation and column chromatography, the cationic glycoprotein CSP was prepared with 15 folds of purification and the specific activity was found to be approximately 8,000 units/mg of protein. For spectrophotometric measurement, CSP and HRP showed their optimal pH in 0.1 M BR buffer at pH 6 but their pH optimum was found to be at pH 7 when the reaction was performed in 1.2 M acetate-citric acid. CSP showed its pH working range of 5-7 whereas that of HRP was in a wider pH range of 4-8. As a result of temperature effect, it was found that both peroxidases showed the same optimal temperature at 55°C in 0.1 M BR buffer, pH 6 but the temperature working range of CSP with its activity higher than 78% was remarkably observed from 5 to 70°C. It was a broader range than that of HRP which was in between 25-55°C as shown in Table 1.

Table 1. Some biochemical characteristics of cassava and horseradish peroxidases.

Peroxidases	Characteristics				
	Source	Molecular weight[1]	Isoforms[1]	pH working range[2]	Temperature working range[3]
Cassava peroxidase (CPS)	Leaf of Manihot esculenta Crantz cv. KU50	38, 44 kDa	>5	5-7	5-70°C
Horscradish peroxidase (HRP)	Root of Armoracia rusticana	44kDa	4	4-8	25-55°C

[1] The biochemical characteristics reported by Jongmevasna et al., 2013
[2] The activity was performed in at 25°C using 3, 3′, 5, 5′-tetramethylbenzidine and urea hydrogen peroxide as peroxidase substrates
[3] The activity was performed in 0.1 M Britton-Robinson buffer, pH 6 using 3, 3′, 5, 5′-tetramethylbenzidine and urea hydrogen peroxide as peroxidase substrates

Screen-printed electrodes (SPEs) used for electrochemical measurement

PVC is an inexpensive material which has beneficial properties to be used as a support for disposable SPE. It is resistant to chemical and moisture and thus, not affected by the curing temperature and ink solvent during the preparation step. Moreover, the printed electrode sheet could be easily cut into individual electrodesusing scissors. In order to ensure the accuracy of SWV measurement by the use of the prepared SPEs, the repeatability of SPEs fabricated by semi-automatic screen printer was examined before use. Five electrodes were selected by random sampling and they were used for the determination of oxidized DAB concentration corresponding to peroxidase activity. Five reactions and SWV measurements with duplicate readings were performed using fixed concentrations of DAB (125 µM) and UHP (100 µM) in the reaction as previously described. The repeatability of electrochemical measurements obtained from prepared SPEs was found to be in an acceptable range. The relative standard deviation (RSD) of peak position (V) and height (nA) wereless than 8% and 12%, respectively.

Electrochemical characteristic of 3, 3'-diaminobenzidine (DAB)

Cyclic voltammetry (CV) of DAB

It was reported that the oxidation of DAB catalyzed by HRP and hydrogen peroxide yielded a stable product, 4, 4'-diimino-bicyclohexylidene-2, 5, 2', 5'-tetraene-3, 3'-diamine providing electroactive property in Britton-Robinson (BR) buffer. It could be measured using voltammetric techniques and its cyclic voltammogram was found to be irreversible with a maximum response of voltammetric peak at a potential of -0.62 V (vs. SCE) [15]. They suggested that the enzyme-oxidized product could be produced in BR buffer pH 3.5 and it could be reduced through a two-electron transfer process in pH 5. This finding agreed with our work. There was only one oxidation peak of DAB catalyzed by peroxidase at a potential of +0.36 V (vs. Ag/AgCl) in 0.04 M BR buffer, pH 6.0 containing 5% methanol and urea hydrogen peroxide (without the addition of sulfuric acid) as shown in Figure 2a. Moreover, it was reported that the oxidation potential of DAB monomer was found at the potential of +0.454 V (vs. Ag/AgCl) in ethanol solution [16]. They suggested that the charge transfer process of DAB monomer oxidation was a single electron process and gave a DAB monocation, while the oxidation process of benzidine was a two successive charge transfer to give a benzidine dication as presented in Scheme 1. In the case of sulfuric acid addition, our result showed that there were two oxidation peaks at the potentials of -0.19 V and 0.45 V (vs. Ag/AgCl) in the reaction containing 5% methanol and their reduction peaks were also observed when the CV scan was reversed as shown in Figure 2b. This indicated that the solution containing sulfuric acid might interrupt the electron process of DAB oxidation with its CV that that was observed in both one and two electrons transfer processes.

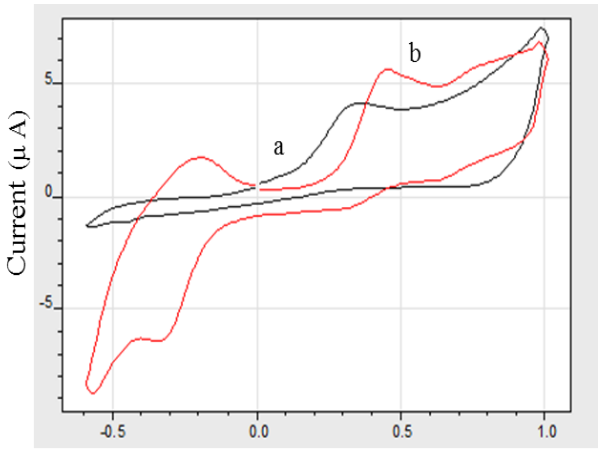


Figure 2. Cyclic voltammograms (CV) of DAB in 0.04 M Britton-Robinson buffer pH 6 containing HRP and UHP. The oxidative peak was at a potential of 0.36 V in the solution without the addition of sulfuric acid (a) and two oxidative peaks were at potentials of -0.19 V and 0.45 V in the solution with the addition of sulfuric acid (b). (CV measurements were obtained from screen-printed electrode cooperated with PalmSens at a scan rate of 20mVs^{-1} in the potential range of -0.6 to 0.8 V).

Square-wave voltammetry (SWV) of DAB

After the enzymatic reaction was incubated in BR buffer pH 6.0 for 3 minutes and the reaction was stopped by the addition of sulfuric acid, the oxidized DAB concentration corresponding to peroxidase activity was determined by SWV. The voltammetric measurement at the potentials ranging from 0.0 to -0.6 V with a step potential of 10 mV, pulse at 75 mV and frequency of 50 Hz at a scan rate of 50 mVs^{-1} was performed for the determination of reductive current response of DAB product at carbon screen printed electrode (SPE). From the results shown in Figure 3, a votammetric peak with the peak height of 2.2 µA was obtained from the solution (e) containing HRP, DAB, UHP and H_2SO_4. It was the highest peak of current response compared to the control reactions such as

solution (a) BR buffer containing only H_2SO_4, solution (b) BR buffer containing DAB and H_2SO_4 and solution (c) BR buffer containing HRP, DAB and H2SO4 where current response could not be detected. For solution (b) and (c), the results indicated that DAB could not be directly reduced by itself or catalyzed by HRP in the test solution. However, a low signal of current peak height could be observed from the solution (d) containing BR buffer, DAB, UHP and H2SO4 without peroxidase. From these results, it can be elucidated that DAB could be slightly reduced by hydrogen peroxide generated from UHP in the solution. Therefore, the oxidized form of DAB in test solution (e) and its concentration was corresponded to peroxidase activity. According to our procedure for SWV experiment, DAB was successfully used as a mediator for monitoring peroxidase activity by electrochemical measurement performed at SPE. The linear relationship between DAB concentration and the ratio of DAB concentration to current density was found. The linearity was in the range of 31-1,000 μM of DAB concentration with R^2 of 0.992 as shown in Figure 4. Under the specific condition at the fixed concentration of peroxidase substrates (500 μM of DAB and 100 μM of UHP) the reductive current at 0.045 cm working electrode of the oxidized substrate catalyzed by CSP after 3 minutes could generate the current density of 25.6 μA/cm^2. In comparison with other mediators, the current density of DAB under our condition was lower than that of ophenylenediamine (59.5 μA/cm^2), but it was higher than that of phenol (7.14 μA/cm^2) catalyzed by HRP-H_2O_2 reaction detected with 0.21 cm^2 graphite-epoxy composite (GCE) reported by Kergaravat et al [17]. Nevertheless, the current density of our system would be improved to reach the maximum density at the optimal ratio of DAB and H_2O_2 concentrations for peroxidase catalysis

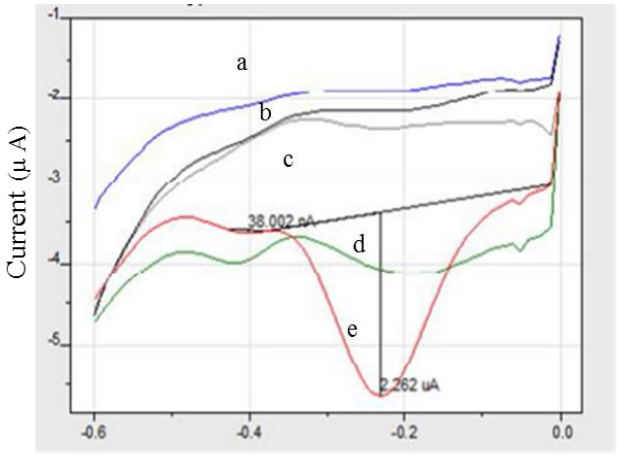
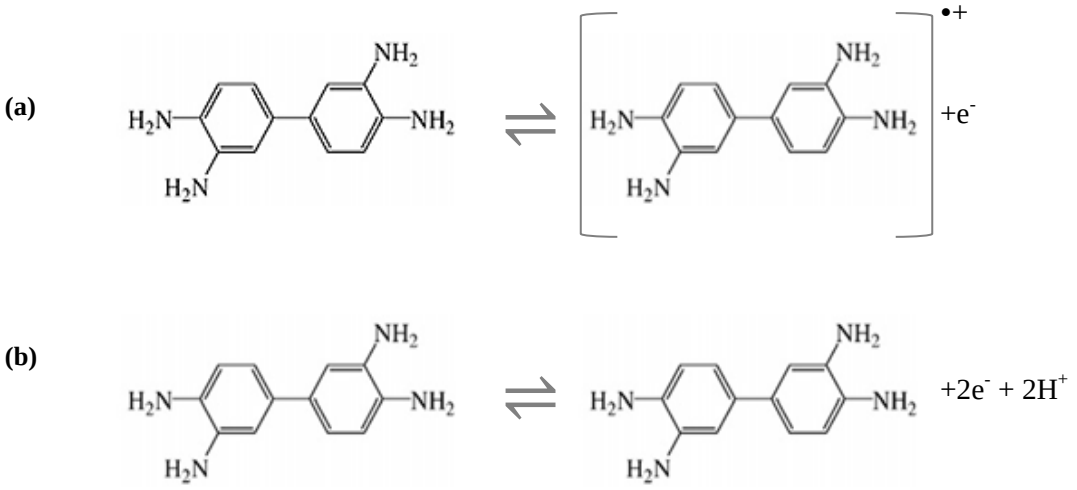


Figure 3. Square-wave voltammograms (SWV) of DAB in 0.04 M Britton-Robinson buffer pH 6, containing BR + H_2SO_4 (a), BR + DAB + H_2SO_4 (b), BR + HRP+ DAB + H_2SO_4 (c), BR + DAB + UHP + H_2SO_4 (d) and HRP + DAB + UHP + H_2SO_4 (e). (The results were obtained from screen printed electrode cooperated with PalmSens. SWV measurement was performed in the potential range of 1.0 to -0.8 V with a step potential of 10 mV, pulse at 75 mV and frequency of 50 Hz at the scan rate of 50 mVs^{-1}.).



Scheme 1. The electron redox process of DAB oxidation catalyzed by peroxidase and H2O2 in BR buffer. One electron transfer of DAB (a) and two electronstransfer of DAB (b)

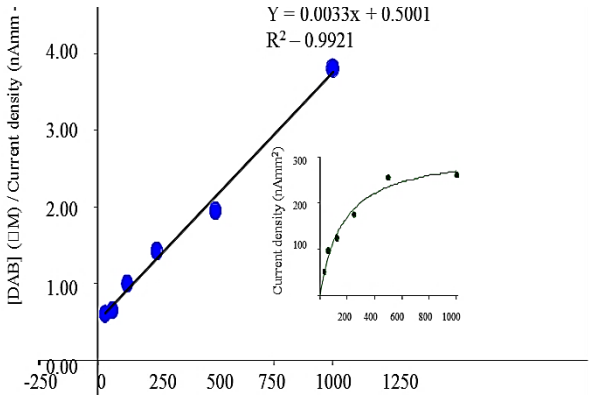


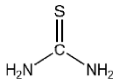
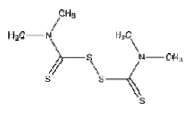
Figure 4. The linear relationship between the ratio of DAB concentration and current density and DAB concentration. (The reaction solution was 0.04 M Britton-Robinson buffer, pH 5, containing 10 µL HRP with a constant concentration of UHP at 100 µM and varied concentrations of DAB in the range of 31.2-1,000 µM. It was incubated for 3 minutes and stopped by the addition of 2 M sulfuric acid and the current peak height at E -0.24 volts was determined by SWV measurement obtained from screen printed electrode cooperated with PalmSens.)

Detection of thiourea by SWV measurement and peroxidase inhibition

As a result of inhibition assay, the SWV current response decreased when thiourea concentration was increased in the reaction. It indicated that thiourea showed its ability to be an inhibitor of both peroxidases for catalyzing the oxidation of DAB in the presence of UHP and the inhibitory effect could be detected by electrochemical measurement under the designed condition. Unfortunately, we did not design the electrochemical measurement to monitor the initial velocity of peroxidase-catalyzed reaction. Basically, the SWV could be used for the determination of kinetic parameters as previously described by Kergaravat et al. [1]. The evaluation of electrochemical characteristics of seven cosubstrates for HRP catalysis was performed using SWV technique. If the SWV was performed as an interval of 10 s to monitor the inhibitory effect of thiourea on the initial velocity of the DAB oxidation, then the type of inhibition could be classified and compared to the results from spectrophotometric measurement [4].

For dose response curves of thiourea inhibition toward CSP and HRP activity for the catalysis of DAB oxidation, SWV measurement could detect the inhibitory effect when the concentration of thiourea was higher than 10 µM. From the semi-log plot, thiourea showed its ability to be a strong inhibitor for HRP than CSP. HRP activity was dramatically decreased in the presence of thiourea with the IC50 of 40±8 µM whereas IC50 of CSP was found to be 100±8 µM. The inhibition curve could be used for the detection of thiourea in the range of 10-100 µM as shown in Table 2. However, there was another report suggested that the amperometric measurement of HRP electrode using 1, 1-dimethylferrocence (DMFc) as electron transfer mediator could detect thiourea in 1.5 % (v/v) methanol solution in the range of 0.05-1.0 mM [18]. In principle, the degree of inhibition of peroxidase activity depends on types and chemical structure of cosubstrate and inhibitors [19].

Table 2. Summary of electrochemical measurement for the detection of some thiols based on peroxidase inhibition assay

Peroxidase inhibition assay	Cassava peroxidase (CSP)		Horseradish peroxidase (HRP)	
	Detectable range (µM)	IC50 (µM)	Detectable range (µM)	IC50 (µM)
Thiourea (THU) 	10-10	100 ±16	5-100	50 ±8
Thiram (TIR) 	10-100	40 ±8	5-100	16 ±4

IC50: Inhibition concentration at 50% of thiol was estimated from the semi-log inhibition curve at 100 µM UHP and 250 µM DAB

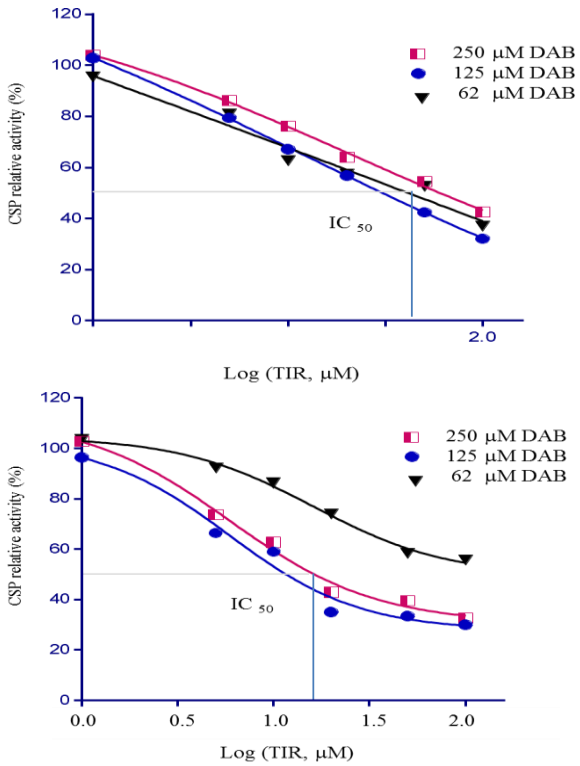


Figure 5. Dose response curve of thiram on CSP (a) and HRP activity inhibition (b). (The reaction solution was 0.04 M Britton-Robinson buffer, pH 6, containing 10 µL of CSP at fixed concentration of DAB (62, 125 and 250 µM) and 100 µM of UHP in the presence of 1-100 µM of thiram

Detection of thiram by SWV measurement and peroxidase inhibition

For electrochemical measurement based on peroxidase inhibition assay, it indicated that the proposed method had a potency for the detection of thiol compounds using SWV measurement incorporated with SPE and PalmSens. According to the linear inverse relationship between the percentage of relative CSP activity and log concentration of thiram in the reaction, thiram could be detected in the concentration range of 5-100 µM as shown in Table 2. The linearity of inhibition lines obtained from the reaction containing various concentrations of DAB were not significantly different among them. This might indicate that the inhibitory effect of thiram towards CSP did not depend on concentration of DAB in that range (Figure 5a). On the contrary, the inhibition of thiram on HRP activity was observed as a higher degree than its effect on CSP activity, the thiram IC50 for HRP (16±4µM) was less than that of CSP (50±8 µM). Moreover, the inhibitory effect of thiram on HRP activity seemed to be dependent on DAB concentration and it demonstrated the relationship as a non-linear curve which could be used for the detection of thiram in the concentration range of 5-100 µM (Figure 5b).

Thiram is a dithiocarbamate fungicide that has been widely used for the protection of fruits, vegetables and crops from fungal diseases and also used as accelerator for rubber vulcanization. The Integrated Risk Information System has set up the toxicological constant of thiram at 0.005 mg/kg/day. For routine analysis, HPLC has been used for the determination of thiram in crop matrices as well as in soil and water in environmental compartments [20]. Thiram was determined based on its maximum absorption and retention time of HPLC system which required large volume of mobile phase and time-consuming. Although its chemical structure consists of thiol groups like thiourea, there have been no reports on the interaction between thiram and peroxidases. This work is intended to investigate peroxidase reaction in the presence of thiram and propose a rapid voltammetric measurement with an adequate sensitivity based on enzyme inhibition.

Conclusions

In order to increase alternative peroxidase utilization in chemical analysis, the ability of CSP isolated from cassava leaves for the detection of some thiols based on peroxidase inhibition assay was investigated and the electrochemical measurement by using carbon screen printed electrode was developed. It was found that the portable set of electrochemical device and SWV technique used in this study showed the applicability of the method for the detection of some thiols such as thiourea and thiramin the level of µM. These findings could be an illustration of applicable uses of CSP as a valuable tool in various chemical and biotechnological applications.

Acknowledgments

The authors would like to gratefully acknowledge the laboratory facilities and financial support provided by Biosensor and Chemical Sensor Laboratory, King Mongkut's University of Technology Thonburi, Electrochemical and optical Spectroscopy Research unit, Department of Chemistry, Department of Biochemistry, and Program in Biotechnology, Faculty of Science and Graduate School of Chulalongkorn University. The acknowledgement is also expressed to Miss Krissanaporn Churangsikul for screen printed electrode preparation.

Contributions

WJ performed the research and drafted the manuscript; OC contributed to the chemical analysis section. MHP coordinated the experiments, drafted and revised the manuscript. All authors read and approved the final manuscript.

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