Published in final edited form as:

Electroanalysis. 2008 August 5; 20(18): 2040. doi:10.1002/elan.200804287.

Sensitive electrochemical detection of horseradish peroxidase at disposable screen-printed carbon electrode

Ai-Cheng Lee^{a,b}, Guodong Liu^b, Chew-Kiat Heng^c, Swee-Ngin Tan^d, Tit-Meng Lim^{a,*}, and Yuehe Lin^{b,*}

^aDepartment of Biological Sciences, National University of Singapore, 14 Science Drive 4, Singapore 117543

^bPacific Northwest National Laboratory, Richland, Washington 99352

^cDepartment of Paediatrics, National University of Singapore, 5 Lower Kent Ridge Road, Singapore 119074, Singapore

^dAcademic Group of Natural Sciences and Science Education, Nanyang Technological University, 1 Nanyang Walk, Singapore 637616, Singapore

Abstract

A rapid, simple and sensitive electrochemical assay of horseradish peroxidase (HRP) performed on disposable screen-printed carbon electrode was developed. HRP activities were monitored by square-wave voltammetric (SWV) measuring the electroactive enzymatic product in the presence of *o*-aminophenol and hydrogen peroxide substrate solution. SWV analysis demonstrated a greater sensitivity and shorter analysis time than the widely used amperometric and differential-pulsed voltammetric methods. The voltammetric characteristics of substrate and enzymatic product as well as the parameters of SWV analysis were optimized. Under optimized conditions, a linear response for HRP from 0.003 - 0.1 U/mL and a detection limit of 0.002 U/mL (1.25×10^{-15} mol in 25 μ L) were obtained with a good precision (RSD = 8%; $n = 6$). This rapid and sensitive HRP assay with microliters-assay volume could be readily integrated to portable devices and point-of-care (POC) diagnosis applications.

Keywords

horseradish peroxidase; *o*-aminophenol; screen-printed carbon electrode; electrochemical; point-of-care

1. Introduction

Peroxidase enzymes have been studied for decades, and are of central importance to many applications in the bioscience and biotechnology. Among the members of the large class of peroxidase, horseradish peroxidase (HRP) is undoubtedly one of the most useful and important enzymes used in a wide variety of bioassays. It is frequently used as secondary detection reagents (*i.e.* label) in numerous biotechnological applications, including immunohistochemical analysis [1] and enzyme-linked immunosorbent assays (ELISAs) [2], *in-situ* hybridization [3], blot analysis [4], immuno [5-7] and DNA biosensors [8].

*Corresponding author: Email: yuehe.lin@pnl.gov. Tel: 01-509-371-6241 (Y. Lin); and Email: dbsltm@nus.edu.sg. Tel: 65-65162913 (T.M. Lim).

The HRP labels have been assayed conventionally by optical method based on measurement of the characteristic optical properties, e.g. luminescent or fluorescent light and absorbance of its enzymatic product. Chromogenic substrates such as 3,3',5,5'-tetramethylbenzidine (TMB) [9], 2'-azino-*bis*-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) [10], *o*-phenylenediamine dihydrochloride (OPD) [4] and diaminobenzidine (DAB) [3] generate a colored reaction product while the use of chemiluminescence detection reagents including luminol [2] and lucigenin [11] produce light. The fluorogenic substrates include *p*-hydroxyphenylacetic acid [12], 3-(*p*-hydroxyphenyl)-propionic acid [13], and homovanillic acid [14]. These substrates generally achieve the detection limit ranging from 10^{-19} to 10^{-13} mol of HRP [15].

Electrochemical bioassays have gained considerable attention and evolved dramatically over the past two decades as they could offer an attractive alternative to spectrophotometry and fluorescence detection [16-17]. It has the inherent advantages, which can be used in color or turbid media without interference from the many absorbing and fluorescing compounds in biological samples. The simple and low cost detection system with adequate sensitivity is ideally suited for the portability requirement of decentralized point-of-care (POC) testing or field detection of chemicals and bioagents. Common HRP substrates developed for electrochemical HRP-labeled bioassays include TMB [18-20] OPD [8,21], hydroquinone [6, 22], hydroxymethyl ferrocene [23], osmium complex [5]. The use of phenol-based substrates such as *p*-aminophenol [24], *m*-aminophenol [25] and *o*-aminophenol (*o*-AP) [26] have also been reported. In acidic solutions the favored path after electro-oxidation of *o*-AP monomers to *o*-AP^{•+} radical cations was shown to be the rapid dimerization of *o*-AP^{•+} with C-N coupling, resulting in cyclic dimer of 3-aminophenoxazone (3-APZ) as the major product (Scheme 1) [27]. The 3-APZ could undergo a reversible redox reaction (Scheme 2) [28]. Among various techniques used in electrochemical analysis, amperometric method was studied extensively to measure the electrocatalytic current generated by the HRP enzymatic reaction [5-7,20,22,23, **Error! Reference source not found.**29-31]. Amperometric detections of HRP-labeled immunoreagents using *o*-AP substrate were demonstrated for the determination of transferrin [7], *Schistosoma japonicum* antigen [30] and Complement 3 in human serum [31]. The SWV analysis has higher sensitivity than other electrochemical techniques, such as cyclic voltammetry (CV) and differential-pulsed voltammetry (DPV) [32]. To date, SWV characteristics of *o*-AP-HRP system and its performance on HRP assay have not been systematically explored.

o-AP was used as electrochemical substrate for the first time [26] as mediator for the electron transfer between HRP and H₂O₂. The resulting electroactive enzyme product, 3-APZ was measured by linear-sweep voltammetry (LSV) using a dropping mercury electrode (DME). The assay has very good sensitivity. However, the DME is not suitable for POC application. Disposable screen-printed electrodes [8,23,34] have gained attention in recent years due to its simplicity, low cost and suitability for POC and portable sensing devices. A disposable sensor array has been designed and developed for simultaneous and independent measurements of nucleic acids, incorporating HRP enzyme as a marker [34]. The array consists of a 96- well plate whose bottom has been modified with an array of 96 screen-printed sensors, each of which constitutes a carbon working electrode and an Ag/AgCl pseudoreference electrode. The combination of screen-printed electrode array and SWV analysis enables multiplex and high throughput of samples analysis.

In this paper, we present a SWV analysis performed on screen-printed electrode to determine HRP tracers' activity by using *o*-AP-H₂O₂ substrate system. We compared amperometric, DPV and SWV techniques; SWV was found to be the most appropriate technique in terms of sensitivity and analysis time of HRP assay. This simple, rapid and sensitive HRP assay with microliters-assay volume could be readily integrated to portable devices and POC diagnosis applications.

2. Experimental

2.1. Materials

HRP (1080 U/mg, $R_z = 3.0$) and *o*-AP were purchased from Sigma. Stock HRP solution was prepared in 0.01 M PBS buffer (pH 7.4) and stored at 4 °C. A pH 5.7 Britton-Robinson (BR) buffer was prepared from an acidic solution containing 0.04 M each of H_3PO_4 , HOAc and H_3BO_3 . The stock substrate solution of *o*-AP was prepared daily or prior to use in the BR buffer at a final concentration of 0.01 M. The *o*-AP working substrate solution containing 2×10^{-4} M of H_2O_2 (*o*-AP- H_2O_2) was prepared from the stock solution prior to use. The enzymatic product solution was prepared by allowing the reaction of 10 μ L of HRP and 100 μ L of *o*-AP- H_2O_2 to proceed for 5 minutes unless otherwise stated. The control solution was prepared identically as of product solution by replacing HRP with PBS buffer. All other chemicals and reagents are of analytical grade and were prepared with ultrapure water (18 Ω .cm).

2.2. Instrumentation

CV, SWV and DPV were performed using an electrochemical analyzer CHI 620C while CHI 660A (CH Instruments, Austin, TX) was used for amperometric measurements. All analyzers were under computer control. A three-electrode configuration was employed for all electrochemical measurements. The screen-printed carbon electrode (SPCE) (AndCare, Durham, NC) consisted of three built-in electrodes on a 1.3 cm $\times$ 3.0 cm $\times$ 0.05 cm plastic substrate with screen-printed carbon serving as a working electrode (0.4 cm diameter) and counter electrodes, and silver as the reference electrode. The SPCE was electronically connected to the analyzer by a sensor connector SC-01P (AndCare, Durham, NC).

2.3. Voltammetric and Amperometric Measurements

The voltammetric (CV, SWV and DPV) measurements were carried out by loading a sample droplet of 25 μ L covering the entire working area of SPCE. All measurements were performed at room temperature (25 °C). CV scanning was performed from +0.50 V to -0.60 V at a scanning rate of 0.10 V/s. SWV and DPV scanning were performed from 0.00 to -0.60 V with a step potential of 0.004 V, an amplitude of 0.025 V, and a frequency of 15 Hz for SWV and a pulse width of 0.05 seconds for DPV. Amperometric measurements were performed in a 2-mL electrochemical cell with the SPCE immersed in BR buffer under continuous magnetic stirring rate of 100 rpm. The desired working potential (-0.40 V) was applied, and the transient currents were allowed to decay to a steady-state value followed by the injection of 100 μ L enzymatic product solution.

3. Results and Discussion

3.1. Cyclic Voltammetric Characteristics of HRP-*o*AP- H_2O_2 at the SPCE

HRP is known to catalyze the redox reactions of the following type:-

Here, $Sub_{(red)}$ refers to the reduced state of the substrate which is oxidized to $Sub_{(ox)}$ as the enzymatic product. In the current study, HRP is expected to catalyze the oxidation of *o*-AP in acidic medium (BR buffer at pH 5.7) by H_2O_2 (Scheme 1) and produce the electroactive enzymatic product, 3-APZ which could be reduced at the SPCE (Scheme 2) during the SWV scanning. The resulting reduction currents output is related to the concentration of HRP.

Figure 1 showed the cyclic voltammograms of *o*-AP- H_2O_2 substrate solution (curve a) and the substrate solution after 20 minutes of enzyme catalytic reaction (curve b) at the SPCE in the

potential range of +0.50 V to -0.60 V at a sweep rate of 0.10 V/s in BR buffer. A well-defined anodic oxidation peak at the potential of +0.12 V was observed in *o*-AP-H₂O₂ substrate solution in the absence of HRP (curve a). This peak had a broad peak observed at +0.10 V on its negative sweep with asymmetry peak current and ΔE_p of ca. 0.02 V. This redox reaction could be assigned to the electro-oxidation of *o*-AP to its cation radicals as the *o*-AP undergoes the electrochemical oxidation. The addition of HRP (0.25 U/mL) to the substrate solution resulted in the disappearance of the well-defined peak (curve b) which could be due to the decrease in the concentration of *o*-AP.

Another pair of broad redox peak at lower potential (E_{pa} : -0.36 V, E_{pc} : -0.38 V) with relatively small peak currents was observed for the substrate solution (curve a). However, the redox peaks developed were well-defined peaks (E_{pa} : -0.34 V, E_{pc} : -0.36 V) after the HRP catalytic reaction preceded 20 minutes (curve b). The peak potentials shifted 0.02 V towards the positive direction while the ΔE_p of 0.02 V was maintained. This pair of redox peaks which is the characteristic of the reversible redox reaction of 3-APZ (Scheme 2), could be used to determine the activity of HRP enzyme tracer. The well-separated characteristic peaks of 3-APZ product (E_{pc} : -0.36 V) and *o*-AP substrates (E_{pa} : +0.12 V) offers a better specificity for the HRP assay compared to two of the most commonly used substrates such as TMB [37] and hydroquinone [6,37]. The characteristic peaks of substrate and product for TMB and hydroquinone have been observed at similar potential. In these cases, the presence of HRP was monitored by a decrease in the oxidation (consumption of substrate) and an increase in the reduction current (formation of product). Residues of such anodic and cathodic peaks were observed in the blank substrate solution which could be due to the auto-oxidation and electro-oxidation of *o*-AP, respectively.

3-APZ could also polymerize to form an electroactive polymer on the electrode [28]. A control test was conducted in which the enzymatic product solution was discarded and replaced by BR buffer without electrode cleaning. Significant decrease of product peak current was observed indicating that 3-APZ remained predominant in the enzymatic reaction. The resulting residue response, which could be attributed to the deposition of reduction product of 3-APZ was removed from the electrode surface simply by applying a constant potential to the electrode at +1.00 V for 2 minutes in BR buffer. This regeneration step effectively cleaned the electrode surface as negligible carryover of peak current was observed in the following measurement. Single-sweep scanning was therefore performed throughout this work and the electrode was cleaned electrochemically after each measurement.

3.2. Comparison of Voltammetric and Amperometric Detection of the Enzymatic Product

The favorable redox characteristics of the enzymatic product on the SPCE provide a facile electrochemical quantification method to assay the activity of HRP. A comparative study of the SWV, DPV and amperometric techniques was carried out for the measurement of enzymatic product at SPCE by SWV, DPV and amperometric techniques (Fig. 2). Final product solution with the same concentration was used in each measurement. The stock product solution was produced from 5 minutes enzymatic reaction of 0.45 U/mL HRP in the substrate solution containing 3×10^{-4} M *o*-AP and 2×10^{-4} M H₂O₂. During the amperometric measurements, 100 μ L of the stock product solution was injected into a 2 mL-BR buffers with slow mixing (Fig. 2c). The stock product solution was diluted accordingly in BR buffer before loaded to the SPCE for SWV (Fig. 2a) and DPV (Fig. 2b) analysis. A well-defined reduction peak of the enzymatic product was observed in both SWV and DPV responses after a negative potential scanning from 0.00 to -0.60 V. The SWV response was typically 2-fold higher than that of DPV under the identical product concentrations. The amperometric analysis showed a relatively small response of ca. 10% of DPV response. SWV analysis significantly enhanced the detection of enzymatic product by ca. 17-fold compared to the commonly used amperometric method. Also, the amperometric detection of *o*-AP enzymatic product required substantially longer analysis

time, typically 6 to 8 minutes compared to those of SWV and DPV. Prolong duration was required to obtain the steady-state base line current before and after sample injection due to diffusion of product species in stirring solution while SWV and DPV scanning was completed within seconds. The SWV analysis offered the most favorable analytical characteristics and was subsequently used to determine the amount of enzymatic product, which is related to the amount of HRP enzyme.

3.3. Factors affecting SWV analysis of HRP-*o*-AP-H₂O₂ enzyme-substrate system

The working potential range, accumulation effect, *o*-AP substrate concentration and the level of background current were investigated to evaluate the characteristic performance of the SWV analysis for *o*-AP substrate and HRP enzymatic product.

3.3.1. Initial Scanning Potential—In the CV study (Fig. 1, curve a), we observed residual characteristic redox peaks of the enzymatic product even in the blank substrate solution, which could be attributed to the auto-oxidation and electro-oxidation [27-28] of *o*-AP forming the same oxidation product, 3-APZ as of the enzyme-catalyzed oxidation reaction. These residue peaks observed in the blank substrate would contribute as background current in the SWV analysis of HRP activity. Electro-oxidation of *o*-AP to its cation radicals occurred at the potential of ca. +0.12 V (Fig. 1, curve a) compared to the reversible redox reaction of its oxidation product at ca. -0.36 V and -0.38 V. These results suggested that the working potential range of SWV scans, especially the initial scanning potential would significantly affect the level of background current and ultimately the sensitivity of HRP assay.

Figure 3 shows the effect of the initial scanning potential on the (i) SWV response (shown as the bar chart) of the substrate and enzymatic product, and (ii) signal-to-noise ratio (SNR), depicted by the line graph. The response of substrate solution decreased slowly upon decreasing the initial scanning potential from +0.30 to +0.10 V and decreased more steeply from +0.10 to 0.00 V; then it remained at almost similar response level at lower initial scanning potential. This trend observed is consistent with the one obtained by CV analysis (Fig. 1, curve a) in which the oxidation of *o*-AP started at 0.00 V. The non-enzymatic electro-oxidation of *o*-AP yielded more oxidized product and resulted in higher response of substrate solution at potential above 0.00 V. SWV detection of enzymatic product with initial scanning potential higher than 0.00 V showed a poor SNR of below 2 compared to those of 0.00 V and -0.10 V with SNR of 30 and 40 respectively. Further experiments thus employed an initial scanning potential of -0.10 V. It is noted that the responses of product after subtracting the current response of blank substrate remained similar for all initial scanning potentials as the concentration of product solution used in the test was kept constant.

The effect of accumulation of enzymatic product at the SPCE under constant potential condition in the range of 0.00 to +0.45 V for a duration of 1 minute prior to SWV scan was investigated (data not shown). No significant signal amplification of the product response was observed for the potential range investigated. Hence, no accumulation step was employed in the subsequent experiments.

3.3.2. Working concentration of *o*-AP—It is well known that the rate of enzymatic reaction increases with the substrate concentration at fixed concentration of enzyme. However, higher substrate concentration could result in an increase of background current caused by the non-enzymatic oxidation of *o*-AP as discussed earlier. Figure 4 shows the SNR of SWV detection of enzymatic product using *o*-AP concentration varying from 0.03 – 5×10⁻³ M. The product response was measured by end-point assay with 5 minutes of enzymatic reaction. Each product response was compared to the corresponding blank substrate response to get SNR of detection at each *o*-AP concentration. Optimum SNR was achieved at 0.06×10⁻³ M *o*-AP

concentration. The SNR decreased from 35 to 6 upon increasing the *o*-AP concentration from $0.06 - 5 \times 10^{-3}$ M which could be attributed to the increase of non-enzymatic substrate response, especially at the concentrations above 0.5×10^{-3} M. Further lowering of *o*-AP concentration resulted in decreasing SNR to 23 due to the smaller response of product. The appropriate working range appears to be in the range of $0.06 - 0.15 \times 10^{-3}$ M for low-range HRP detection; the substrate solution containing 0.06×10^{-3} M *o*-AP and 2×10^{-4} M H_2O_2 was used subsequently.

Besides electro-oxidation and enzymatic-catalyzed oxidation, substrates such as TMB, OPD, *o*-AP could undergo auto-oxidation when exposed to air and light. The stability of *o*-AP solution was investigated. The *o*-AP stock solution when stored at 4 °C and protected from light could be used within 5 hours after preparation. After 5 hours, it was not used for the detection of low HRP activity as the blank was too high and thus resulted in high SNR for the assay. Hence, *o*-AP and *o*-AP- H_2O_2 solutions were prepared just before use and carefully stored to reduce auto-oxidation of *o*-AP by oxygen and light. In the current study, measurement of samples was always accompanied with blank test for the confirmation of positive values.

3.4. Analytical Characteristics of SWV Detection of HRP

The SWV detection of HRP was performed under the optimized conditions containing 0.06×10^{-3} M *o*-AP and 2×10^{-4} M H_2O_2 . Figure 5 shows the typical SWV responses of increasing HRP concentrations (from bottom to top, the concentration of HRP in PBS buffer is 0.1, 0.05, 0.025, 0.012, 0.006, 0.003 and 0 U/mL, respectively). Well-defined peaks proportional to the concentration of HRP were observed. The corresponding linear-plot of calibration curve is shown as an insert in Figure 5. The linear calibration range was 0.003 – 0.1 U/mL, and the relative standard deviation (RSD) was 8% ($n=6$; at 0.025 U/mL). A detection limit of 0.002 U/mL (2 ng/mL; 1080 U/mg) was obtained on the basis of $3 \times \text{SD}$ of determination of the zero standard. This detection limit is corresponding to 1.25×10^{-15} mol of HRP molecules in 25 μL . The detection limit obtained in this electrochemical assay is better than those reported with fluorescence detection [14,35-36]. The proposed HRP assay was performed at room temperature with 5 minutes of enzymatic reaction and 10 seconds of SWV analysis time using the SPCE. This condition is relatively more practical than that reported earlier with 30 minutes of enzymatic reaction at 37 °C using a mercury dropping electrode [26]. The present detection at the SPCE has potential to be integrated with lab-on-chip application.

4. Conclusions

We have demonstrated a rapid, simple and sensitive SWV analysis of HRP performed at the SPCE. The SWV analysis was superior to those widely used amperometric and DPV techniques in term of its sensitivity and response time. The formation of 3-APZ product dominated by HRP-catalyzed oxidation of *o*-AP could be achieved by controlling the initial scan potential to avoid those formed by electro-oxidation. Appropriate working potential of SWV and *o*-AP working concentration minimized the background response which in turn significantly improved the HRP assay sensitivity. Enzymatic product of *o*-AP exhibited a well-separated peak compared to the blank substrate. This characteristic offers a better specificity for the HRP assay compared to other electrochemical substrates. The analytical approach described herein could be used for any bioassay with HRP tracers and is readily to be integrated to portable devices and POC diagnosis applications.

Acknowledgments

The work was done at Pacific Northwest National Laboratory (PNNL) supported partially by grant number NS058161-01 from the National Institutes of Health CounterACT Program through the National Institute of Neurological Disorders and Stroke and partially by grant number U54 ES16015 from the National Institute of

Environmental Health Sciences (NIEHS), NIH. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the Federal Government. The research described in this paper was partly performed at the Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the DOE's Office of Biological and Environmental Research and located at PNNL. PNNL is operated by Battelle for DOE under Contract DE-AC05-76RL01830. One of the authors, A. C. Lee gratefully acknowledges the award of National University of Singapore Postgraduate Research Scholarship and a PNNL fellowship to perform this work at PNNL.

6. References

- Seeger TF, Bartlett B, Coskran TM, Culp JS, James LC, Krull DL, Lanfear J, Ryan AM, Schmidt CJ, Strick CA. *Brain Res* 2003;985:113. [PubMed: 12967715]
- Lin J, Yan F, Hu X, Ju H. *J. Immunol. Meth* 2004;291:165.
- Barroso-Chinea P, Aymerich MS, Castle MM, Perez-Manso M, Tunon T, Erro E, Lanciego JL. *J. Neurosci. Meth* 2007;162:119.
- Uhl J, Newton RC. *J. Immunol. Meth* 1988;110:79.
- Salinas E, Torriero AAJ, Battaglini F, Sanz MI, Olsina R, Raba J. *Biosens. Bioelectron* 2005;21:313. [PubMed: 16023958]
- Lei CX, Gong FC, Shen GL, Yu RQ. *Sens. Actuators, B* 2003;96:582.
- Liu GD, Hu KS, Li W, Shen G, Yu RQ. *Analyst* 2000;125:1595. [PubMed: 11064939]
- Azek F, Grossiord C, Joannes M, Limoges B, Brossier P. *Anal. Biochem* 2000;284:107. [PubMed: 10933863]
- Dotsikas Y, Loukas YL, Siafaka I. *Anal. Chim. Acta* 2002;459:177.
- Gill P, Forouzandeh M, Rahbarizadeh F, Ramezani R, Rasaei MJ. *J. Immuno. Immunochem* 2006;27:303.
- Myhre O, Andersen JM, Aarnes H, Fonnum F. *Biochem. Pharmacol* 2003;65:1575. [PubMed: 12754093]
- Zhu C, Li D, Zheng H, Zhu Q, Chen Q, Xu J. *Anal. Sci* 2000;16:253.
- Li QG, Xu JG, Huang XZ, Chen GZ. *Talanta* 1994;41:2049. [PubMed: 18966169]
- Zaitis K, Ohkura Y. *Anal. Biochem* 1980;109:109. [PubMed: 7469007]
- Akhavan-Tafti H, deSilva R, Eickholt R, Handley R, Mazelis M, Sandison M. *Talanta* 2003;60:345. [PubMed: 18969057]
- (a) Yantasee W, Lin Y, Wang J. *Frontier in Bioscience* 2005;10:492. (b) Yantasee W, Lin Y, Zemanian TS, Fryxell GE. *Analyst* 2003;128:467. [PubMed: 12790199]
- (a) Liu G, Lin Y. *Electrochem. Commun* 2006;8:251. (b) Wang J, Lin Y. *Anal. Chim. Acta* 1993;271:53. (c) Liu G, Lin Y. *Anal. Chem* 2006;78:835. [PubMed: 16448058]
- He YN, Chen HY, Zheng JJ, Zhang GY, Chen ZL. *Talanta* 1997;44:823. [PubMed: 18966807]
- Liu G, Timchalk C, Lin Y. *Electroanalysis* 2006;18:1605.
- Fanjul-Bolado P, González-García MB, Costa-García A. *Anal. Bioanal. Chem* 2005;382:297. [PubMed: 15891870]
- Wu L, Chen J, Du D, Ju H. *Electrochim. Acta* 2006;51:1208.
- Ionescu RE, Gondran C, Gheber LA, Cosnier S, Marks RS. *Anal. Chem* 2004;76:6808. [PubMed: 15538808]
- Dai Z, Serban S, Ju H, El Murr N. *Biosens. Bioelectron* 2007;22:1700. [PubMed: 16950612]
- Sun W, Jiao K, Zhang S, Zhang C, Zhang Z. *Anal. Chim. Acta* 2001;434:43.
- Zhang S, Jiao K, Chen H, Wang M. *Talanta* 1999;50:95. [PubMed: 18967699]
- Zhang S, Jiao K, Chen H. *Electroanalysis* 1999;11:511.
- Jackowska K, Bukowska J, Kudelski A. *J. Electroanal. Chem* 1993;350:177.
- Barbero C, Silber JJ, Sereno L. *J. Electroanal. Chem* 1989;263:333.
- Messina GA, Torriero AAJ, De Vito IE, Olsina RA, Raba J. *Anal. Biochem* 2005;337:195. [PubMed: 15691499]
- Liu GD, Wu ZY, Wang SP, Shen GL, Yu RQ. *Anal. Chem* 2001;73:3219. [PubMed: 11476218]
- Liu GD, Yan JT, Shen GL, Yu RQ. *Sens. Actuators, B* 2001;80:95.

32. Wang, J. Stripping Analysis. Deerfield Beach; VCH: 1985.
33. Nassef HM, Radi A-E, O'Sullivan CK. J. Electroanal. Chem 2006;592:139.
34. Wojciechowski M, Sundseth R, Moreno M, Henkens R. Clin. Chem 1999;45:1690. [PubMed: 10471692]
35. Li Y, Liu H, Dong Z, Chang W, Ci Y. Microchem. J 1996;53:428.
36. Chen X, Li D, Yang H, Zhu Q, Zheng H, Xu J. Anal. Chim. Acta 2001;434:51.
37. Volpe G, Compagnone D, Draisci R, Palleschi G. Analyst 1998;123:1303.

Scheme 1.

Scheme 2.

Fig. 1. Cyclic voltammograms of *o*-AP in the absence and presence of HRP using the SPCE in a pH5.7 BR buffer solution containing 0.04 M each of H_3PO_4 , HOAc and H_3BO_3 : (a). 1.2×10^{-5} M *o*-AP + 2×10^{-4} M H_2O_2 ; (b). as in (a) + 0.25 U/mL HRP; Potential scanning rate: 0.1 V/s; scanning potential range: +0.50 V to -0.60 V. Curve b was recorded after the enzyme catalytic reaction preceded for 20 minutes.

Fig. 2.

Responses at the SPCE to *o*-AP enzymatic product catalyzed by HRP in BR buffer recorded using (a) SWV, (b) DPV and (c) amperometric techniques. Equal concentration of product solution was used. SWV and DPV scanning were performed from 0.00 to -0.60 V with a step potential of 0.004 V, an amplitude of 0.025 V, and a frequency of 15 Hz for SWV and a pulse width of 0.05 seconds for DPV. Potential was applied at -0.4 V for amperometric analysis. All curves were shown with *o*-AP background current subtraction.

Fig. 3.

Effect of initial scanning potential on SWV responses of enzymatic product (grey bars) and blank substrate (white bars) solution. Signal-to-noise ratio (line graph) represents the ratio of response of product to that of blank substrate. Conditions of SWV measurement were the same as in Figure 2.

Fig. 4. Optimization of substrate concentration. Signal-to-noise ratio represents the ratio of response of product to that of blank substrate. Product solution was produced by 5 minutes of 0.1 U/mL HRP enzymatic reaction in the *o*-AP substrate solution containing 2×10^{-4} M H_2O_2 . Conditions of SWV measurement were the same as in Figure 2.

Fig. 5.

Typical SWV responses of increasing HRP concentration in PBS buffer. From bottom to top, the concentration of HRP in PBS buffer is 0.1, 0.05, 0.025, 0.012, 0.006, 0.003 and 0 U/mL, respectively. Insert shows the calibration curve of HRP. Enzymatic reaction preceded 5 minutes at room temperature in the substrate solution containing 0.06×10^{-3} M *o*-AP and 2×10^{-4} M H_2O_2 in BR buffer (pH 5.7).