Bull. Chem. Soc. Ethiop. **2007**, 21(2), 179-188. ISSN 1011-3924
Printed in Ethiopia

APPLICATION OF *o*-TOLIDINE AS SUBSTRATE FOR THE ELECTROCHEMICAL DETERMINATION OF HEMOGLOBIN OR HYDROGEN PEROXIDE

Wei Sun*, Hong Jiang, Jianghua Zhong and Kui Jiao

College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao 266042, P.R. China

(Received May 22, 2006; revised July 22, 2006)

ABSTRACT. In this paper hemoglobin (Hb) was used to catalyze the oxidative reaction of *o*-tolidine (OT) with H_2O_2. The oxidative product of OT with H_2O_2 was an azo substrate, which was electroactive and had a sensitive linear sweep voltammetric reductive peak at -0.52 V (*vs.* SCE) on hanging mercury drop working electrode (HMDE) in pH 5.0 Britton-Robinson (B-R) solution. The conditions of Hb catalytic reaction and voltammetric detection were optimized. Under the optimal conditions, the electrochemical behaviour of the oxidative product was carefully investigated and the electrode process of the product on mercury electrode was proposed. Based on the increase of the reductive peak current of the oxidative product with the concentration of the H_2O_2 or Hb, a new electrochemical method for the determination of trace amount of H_2O_2 or Hb was proposed. The calibration graph had a linear range of 6.0×10^{-8} to 4.0×10^{-5} M for H_2O_2 and 1.0×10^{-9} to 7.0×10^{-7} M for Hb with the detection limit of 1.0×10^{-8} M H_2O_2 and 5.0×10^{-10} M Hb (3σ), respectively. This new proposed method was further attempted to determine the content of H_2O_2 in fresh rainwater with satisfactory results.

KEY WORDS: Hemoglobin, Hydrogen peroxide, *o*-Tolidine, Linear sweep voltammetry

INTRODUCTION

The determination of hydrogen peroxide is of great importance for its relationship with clinical assay, environmental protection and industrial application [1]. Many oxidases can oxidize the corresponding substrates in the presence of dissolved oxygen and produce a certain amount of hydrogen peroxide. Different kinds of methods have been proposed for H_2O_2 determination [2-5] such as titrimetry, spectrophotometry, fluorometry and electrochemistry. Hemoglobin (Hb) is an important respiratory protein in the red cells, which consists of four polypeptide subunits with an iron-porphyrin heme as the active center. It is the carrier of oxygen transfer in human blood. Hb has the same active group as peroxidase and it can be used as mimetic peroxidase to catalyze the oxidation reaction with the reduction of hydrogen peroxide. Zhang *et al.* [6] had reported a new method for the determination of Hb based on its catalytical oxidation of *o*-phenylenediamine and the product was detected by a stopped flow spectrophotometric method. Huang *et al.* [7] had used Hb as mimetic enzyme of peroxidase with H_2O_2 and 4-aminoantipyrine/different kinds of chlorophenolic derivatives for the spectrophotometric determination of H_2O_2 and glucose in human serum. Electrochemical methods had been widely used for the investigation of Hb and H_2O_2, respectively. Different kinds of biosensor had been constructed for H_2O_2 by the catalysis of immobilized horseradish peroxidase (HRP) to construct mediated or mediated-free HRP-based sensor [8-12]. The electrochemistry of Hb was also investigated by different kinds of solid electrode to know the direct electron transfer process between the redox center of Hb and the surface of electrode [13-15].

In this paper, electrochemical method was used to investigate the Hb catalyzed reaction of H_2O_2 with *o*-tolidine (OT, 3,3'-dimethylbenzine) for the first time. OT has been used as the spectrophotometric and electrochemical substrate for HRP-based enzyme immunoassay [16-17].

*Corresponding author. E-mail: sunwei_1975@public.qd.sd.cn

Jiao *et al.* had carefully studied the OT-H_2O_2-HRP catalytial system and applied to the electrochemical enzyme immunoassay for the detection of different kind of antigen such as cucumber mosaic virus and tobacco mosaic virus [18]. Hb has the similar active center of iron porphyrin heme as HRP and has been used as mimetic enzyme as HRP. In these experiments, it was found that Hb could also catalyze the oxidation of OT with H_2O_2. The oxidative product was electroactive and had well-defined electrochemical response on the mercury electrode, which can be detected by linear sweep voltammetry. The reductive peak current increased with the increase of the concentration of Hb or H_2O_2. Based on this principle, a new electrochemical method for the determination of Hb or H_2O_2 was proposed. The optimal conditions of Hb-H_2O_2-OT catalytical reaction were selected and the electrochemical behavior of the oxidative product was carefully investigated. The proposed method was further used for the determination of H_2O_2 content in fresh rainwater with satisfactory results.

EXPERIMENTAL

Reagents

Hemoglobin (bovine Hb, Biochemical grade, Tianjin Chuanbei Bioproduct Company) was used as received without further purification and its molecular weigh was 64500. A 1.0 x 10^{-4} M Hb stock solution was prepared by directly dissolving it in doubly distilled water and stored at 4 ^{o}C. The working solution was obtained by diluting the stock solution to the suitable concentration daily. A 1.0 x 10^{-2} M *o*-tolidine (OT, Shanghai Chemical Reagent Company) solution was prepared by dissolving 0.2125 g OT in 100 mL anhydrous ethanol just before use. H_2O_2 solution was prepared by appropriate dilution of commercial 30 % H_2O_2 with doubly distilled water and standardized by titration with $KMnO_4$. 0.2 M Britton-Robinson (B-R) buffer solution was used to control the acidity of solution, which was prepared by mixing 12.35 g of boracic acid, 13.55 mL of 99 % phosphoric acid and 11.8 mL of 99 % acetic acid in 1000 mL doubly distilled water. All the other reagents were of analytical reagents grade and doubly distilled water was used for preparing the solutions.

The fresh rainwater was collected, filtered and directly applied to the general procedure just after the collection.

Apparatus

A Varian model Cary 50 probe UV-Vis spectrophotometry (Varian, Austria) was used for recording the absorption spectrum. All the cyclic voltammetric experiments were carried out using a DS-2004 electrochemical analyzer (Shandong Dongsheng Electronic Instrument, China) with a DS-991 hanging mercury drop electrode (HMDE, Shandong Dongsheng Electronic Instrument, China) as working electrode, a saturated calomel reference electrode (SCE) and a platinum wire auxiliary electrode. Linear sweep voltammetric measurements were performed on a JP-303 polarographic analyzer (Chengdu Instrument Factory, China) with a dropping mercury working electrode (DME), a platinum wire auxiliary electrode and an saturated calomel reference electrode (SCE). All the pH values were measured with a pHS-25 acidimeter (Shanghai Leici Factory, China). A model GSY-II thermostat bath (Beijing Medical Instrument Plant, China) was used to control the reaction temperature.

Procedure

Quantitation of Hb with electrochemical detection. Into a 10 mL colorimetric tube was placed 2.0 mL of 1.0 x 10^{-2} M OT solution, 2.0 mL of 4.0 x 10^{-3} M H_2O_2 solution, 0.5 mL of 0.2 M pH 4.4 B-R buffer solution, different amount of Hb solution, 4.0 mL of anhydrous ethanol in

sequence and diluted to 10 mL with doubly distilled water. The solution was mixed homogenously after reacted for 30 min in a 37 ^oC water bath, the reaction solution was cooled with ice-water to stop the catalytic reaction. Then 5.0 mL of the reaction solution was transferred to another 10 mL colorimetric tube and mixed with 1.0 mL 0.2 M pH 5.0 B-R buffer solution, then diluted to scale. The linear-sweep voltammograms of above solutions were recorded by a JP-303 polarographic analyzer, and the reductive peak current of the oxidative reaction product at the potential of -0.52 V (*vs.* SCE) was measured.

Quantitation of standard H_2O_2 or H_2O_2 in fresh rainwater. Into a 10 mL colorimetric tube was placed 2.0 mL of 1.0 x 10^{-2} M OT solution, 1.0 mL 1.0 x 10^{-6} M Hb, 0.5 mL of 0.2 M pH 4.4 B-R buffer solution, different amount of H_2O_2 standard solution or fresh rain water samples and 4.0 mL of anhydrous ethanol in sequence, then diluted to 10 mL with doubly distilled water and mixed homogenously. After reaction for 30 min in a 37 ^oC water bath, the reaction solution was cooled with ice-water to stop the catalytic reaction. Then 5.0 mL reaction solution was mixed with 1.0 mL of 0.2 M pH 5.0 B-R buffer solution and diluted to scale. The linear-sweep voltammograms of above solutions were recorded by a JP-303 polarographic analyzer and the reductive peak current at -0.52 V (*vs.* SCE) was measured.

RESULTS AND DISCUSSION

UV-Vis absorption spectra

Figure 1 showed the UV-Vis absorption spectra of the solution of OT and H_2O_2 in the absence and presence of different amount of Hb. Curve 1 was the mixture of OT with H_2O_2 and it showed no absorbance in the scanned wavenumber range. After the addition of different amount of Hb solution and reacted at 37 ^oC for 30 min, a new absorption peak appeared at 414 nm and the absorbance value increased with the increase of the concentration of Hb (curve 2-5), which indicated that the concentration of the Hb catalytic product increased step by step. Since the oxidative reaction produced an azo product and the azo group of –N=N– had the maximum absorbance band at 414 nm. So the change of absorbance in the spectra indicated a new compound was formed in the selected reaction conditions.

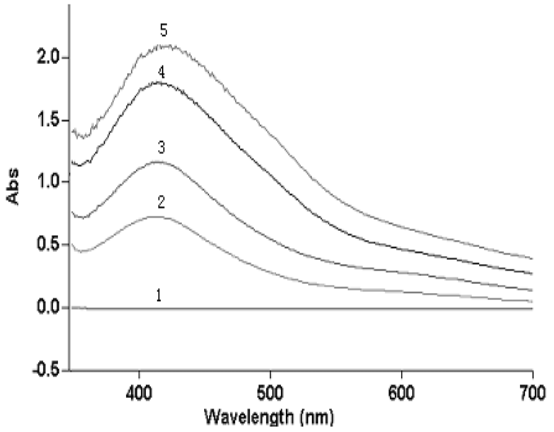

Figure 1. UV-Vis absorption spectra of Hb-H_2O_2-OT reaction solution in the absence and presence of different amounts of Hb. 1.0 x 10^{-3} M OT + 4.0 x 10^{-4} M H_2O_2; 2-5.1 + 0.5, 1.0, 5.0, 10.0 x 10^{-7} M Hb, respectively.

Electrochemical behaviors of the Hb catalytical reaction product

According to our previous report [17, 18], the oxidation of OT by H_2O_2 in the presence of horseradish peroxidase (HRP) got a stable azo product with HRP as the catalyst. Since Hb has a similar active center as HRP, so the reaction of OT and H_2O_2 with Hb can also produce the same azo product, which was considered to produce from the condensation of two molecules of the *o*-toluidine quinonediimine. The Hb catalytical reaction equation was deduced in Figure 2.

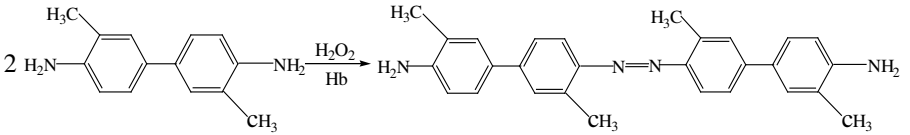

Figure 2. The catalytic oxidation reaction scheme of Hb-OT- H_2O_2.

Ni *et al.* had reported the electrochemical behaviour of some azo compounds on the mercury electrode, which can easily take place reductive reaction with two electrons in the acidic buffer solution [19]. The electrochemical behaviour of the formed azo product was also carefully studied. Figure 3 showed the cyclic voltammgrams of the Hb-H_2O_2-OT reaction system in the potential range of -0.25 V to -0.75 V. Curve 1, 2, 3 and 4 was cyclic voltammograms of B-R buffer, B-R with Hb, B-R with H_2O_2, and B-R with OT, respectively. It can be seen that no electrochemical response was appeared. Curve 5 showed the cyclic voltammograms of the reaction solution under the selected conditions. A pair of redox peaks appeared in the selected potential range, which was attributed to the electrochemical response of the Hb catalytic reaction product. The electrochemical behaviours of the product on the mercury electrode were carefully studied. From the cyclic voltammogram it could be seen that the anodic peak potential (Epa) as -0.374 V and the cathodic peak potential (Epc) as -0.512 V. So the apparent formal potential, E^0 = (Epa+Epc)/2, was calculated as -0.443 V. The anodic peak current (Ipa) was 0.263 μA and the cathodic peak current (Ipc) was 0.450 μA. The peak potential separation (ΔEp = Epa-Epc) was calculated as 0.138 V with the peak current of Ip,a/Ip,c ≈ 0.6. The result indicated that the electrode process of the oxidative product on the mercury electrode was a quasi-reversible process.

The reductive peak current increased with the increase of the scan rate and the plot of the peak current against the scan rate in the range of 100 to 500 mV/s was a straight line with the linear regression equation as Ipc(nA) = 0.27 ν (mV/s) + 383.43 (n = 6, γ = 0.994). And the relationship of peak current with the square root of the scan rate ($\nu^{1/2}$) was not linear but an upward curve. In multiple sweep cyclic voltammetric experiments the peak current decreased gradually with the increase of the number of scan cycle. The results indicated that the reaction product could be strongly absorbed on to the mercury electrode. The plot of the reductive peak potential against the pH of buffer was a straight line with a slope of -0.061 in the pH range of 3.0-7.0. According to the equation: -0.059 x/n = -0.061, where n is the electron transfer number and x is the hydrogen ion number participating in the reaction, so x = 2 and n = 2.

From the above results it can be deduced that under the optimal conditions, the azo product appeared to undergo two-electron adsorptive quasi-reversible reduction on the Hg electrode, so the electrochemical reduction procedure of the product was proposed in Figure 4.

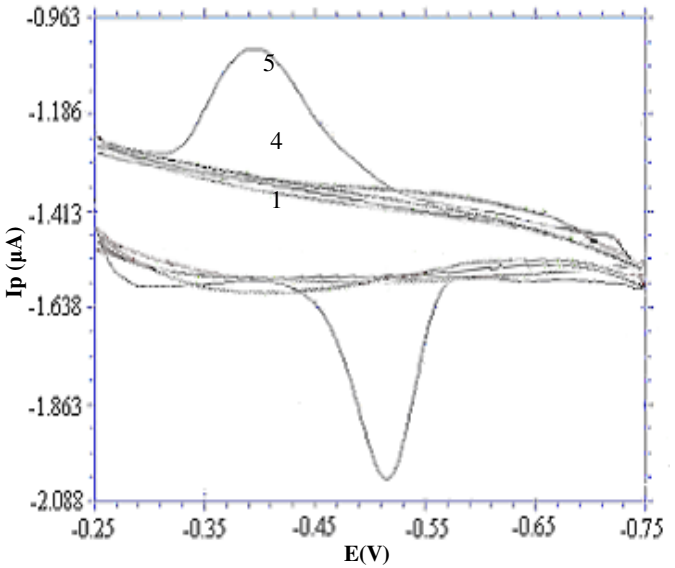

Figure 3. Cyclic voltammograms of the oxidation product under the selected condition with scan rate as 300 mV/s. 1. B-R buffer, 2. B-R with Hb, 3. B-R with H_2O_2, 4. B-R with OT, 5 B-R + OT + H_2O_2 + Hb.

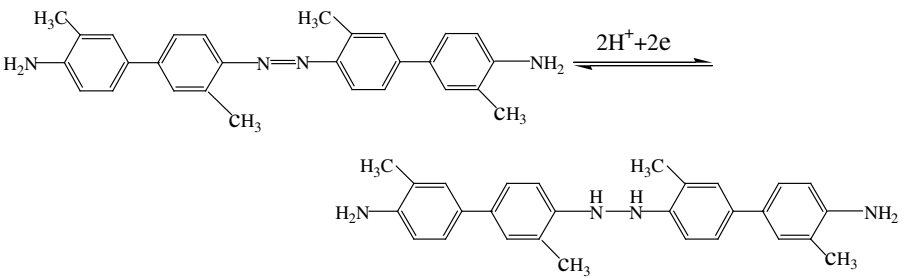

Figure 4. The electro-reduction scheme of the oxidative product.

linear-sweep voltammogram

In this paper, linear sweep voltammetry with second order derivation treatment, which was more sensitive than the normal linear sweep voltammetry, was used throughout in the analytical procedure. Figure 5 showed the typical voltammograms of Hb-OT-H_2O_2 catalytic system. Curve 1 was the voltammogram of the B-R buffer solution, which had no voltammetric peak in the potential range. Curve 2 was that of OT-H_2O_2 solution and had a blank voltammetric peak at -0.52 V (*vs.* SCE), which was due to the slow oxidation of OT by H_2O_2 and the high sensitivity of this electrochemical method. Curve 3 was the voltammogram of OT-Hb solution. It was similar as curve 2, which was due to the slowly oxidation of OT by oxygen in the air. Curve 4, 5 and 6 were the mixture of OT-H_2O_2 solution with different amount of Hb. After reacted at 37 ^oC for 30 min, a well-defined voltammetric reductive wave was appeared at -0.52 V, which was owing to electrode reduction of the catalytical reaction product. The more the Hb added, the

higher the peak current got. The peak current was in proportion to the concentration of Hb or H_2O_2, so this method can be applied to the determination of H_2O_2 or Hb content in real samples.

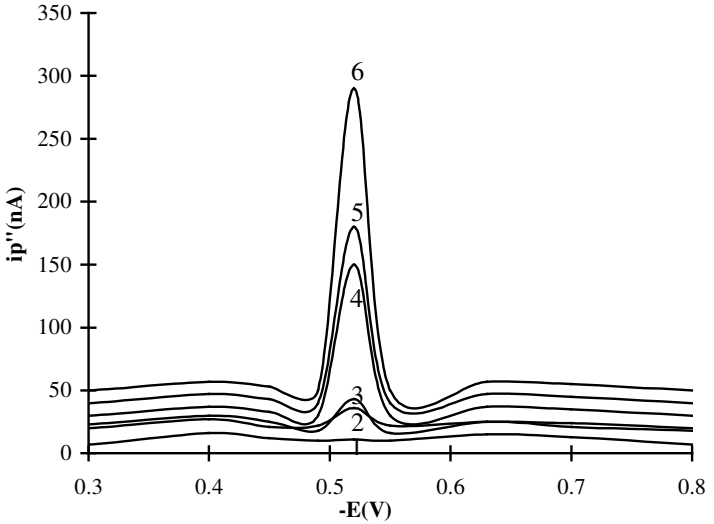

Figure 5. Linear sweep voltammogram of Hb-H_2O_2-OT catalytic solution: 1. 0.2 M pH 4.4 B-R buffer; 2. 1+1.0 x 10^{-3} M OT + 4.0 x 10^{-4} M H_2O_2; 3. 1 +1.0 x 10^{-3} M OT + 1.0 x 10^{-7} M Hb; 4-6. 2 + 0.6, 1.0, 2.0 x 10^{-7} M Hb, respectively.

Optimization of reaction and detection conditions

The activity of Hb was greatly affected by the kind of the buffer and the pH value of the buffer solution. By keeping other conditions constant, the effect of the pH of B-R buffer solution on the Hb catalytic reaction was tested in the pH range from 2.0 to 7.0 and the results are shown in Figure 6, which indicated that the maximum peak current was got at pH 4.4. When the acidity or alkalinity of the buffer solution is too high, Hb will be denatured and lost its activity. Therefore, pH 4.4 was chosen as optimal reaction pH.

The impact of various buffer on the catalytic reaction was also examined and the results showed that the activity of Hb in B-R buffer was much higher than that of HOAc-NaOAc, NH_3-NH_4Cl, KH_2PO_4-Na_2HPO_4, etc. The amount of B-R buffer solution added was investigated and 0.5 mL of 0.2 M B-R buffer solution in 10 mL final reaction solution was chosen. The voltammetric behavior of the Hb-catalytic reaction product was also greatly influenced by the acidity of the supporting electrolyte. So the effect of the pH of the supporting electrolyte on the electrochemical response of the azo product was also checked and the results showed that in pH 5.0 B-R buffer solution the peak current reached the maximum, so pH 5.0 B-R buffer was chosen to adjust the acidity of supporting electrolyte for voltammetric detection.

The influence of reaction temperature on the Hb catalytic reaction was examined in the range from 20 ^{o}C to 70 ^{o}C and the results are shown in Figure 7. When the temperature was lower than 50 ^{o}C, the peak current of the catalytic product was increased with the increase of temperature and it reached maximum at 50 ^{o}C, when the temperature was exceeded 50 ^{o}C, the peak current decreased correspondingly, which was due to the denaturing of protein at high temperature. The result was identical to the references' results [20]. However, in this paper the

reaction temperature was selected at 37 °C. Since Hb was extracted from bovine serum and 37 °C was the body temperature of biology, which can keep the longest time of bioactivity of Hb. At the same time, it is possible to decrease the self-decomposition of H_2O_2 at high temperature. The peak current reached maximum for 30 min at 37 °C, and remained constant for at least 2 hours, so 30 min was selected as the reaction time for Hb-catalytic reaction.

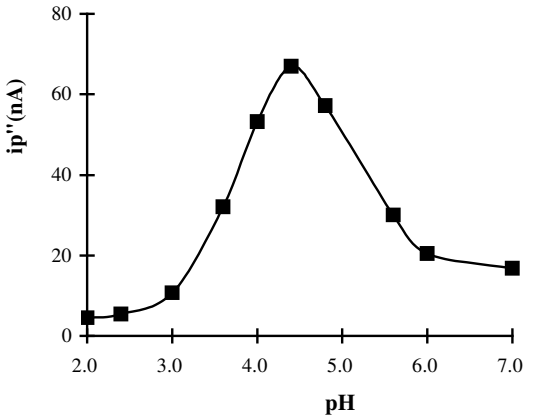

Figure 6. The influence of buffer pH on the catalytic reaction. The concentrations of OT, H_2O_2 and Hb were 1.0 x 10^{-3} M, 6.0 x 10^{-5} M, and 1.0 x 10^{-7} M, respectively.

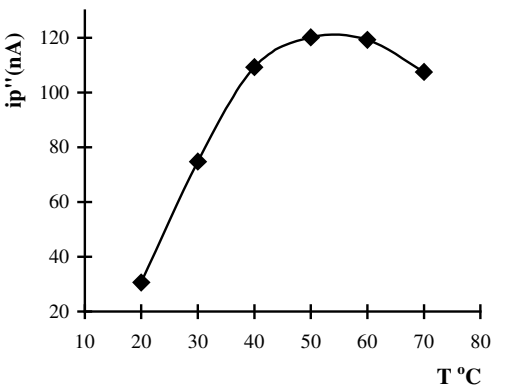

Figure 7. The influence of temperature on the catalytic reaction. The concentrations of OT, H_2O_2 and Hb were 1.0 x 10^{-3} M, 1.0 x 10^{-4} M, and 1.0 x 10^{-7} M, respectively.

The optimal concentration of OT was investigated when the concentrations of H_2O_2 and Hb were fixed at 4.0 x 10^{-4} M and 1.0 x 10^{-7} M, respectively. When the concentration of OT were at 1.0 x 10^{-3} M the peak current reached maximum and then decreased with the further increase of the OT concentration, which was due to the poor solubility of the reaction product. So 1.0 x 10^{-3} M OT was used in the following procedure.

The optimal instrumental conditions for the detection were also selected as follows: the initial potential: -0.30 V; the mercury drop standing time: 13 s and the potential scanning rate: 1000 mV/s.

Effect of coexisting substances

Since the proposed method can be directly used for the H_2O_2 or Hb determination. So the influences of some coexisting substances on the determination of 4.0 x 10^{-6} M H_2O_2 were studied. These substances such as metal ions, amino acids, glucose, etc, may exist in the water sample or body fluid samples. The results are listed in Table 1. It can be seen that few substances interfere with this assay and good selectivity can be obtained with this method.

Table 1. Influences of coexisting substances on the determination of 4.0 x 10^{-6} M H_2O_2.

Coexisting substances	Concentration (mol/L)	Relative error Δip" (%)	Coexisting substances	Concentration (mg/L)	Relative error Δip" (%)
Co^{2+}	1.0 x 10^{-5}	3.17	Citric acid	10.0	3.57
NH_4^+	1.0 x 10^{-5}	4.11	L-Valine	10.0	5.07
Zn^{2+}	1.0 x 10^{-5}	-3.95	Urea	10.0	-2.17
Na^+	1.0 x 10^{-5}	0.39	L-Arginine	10.0	3.25
K^+	1.0 x 10^{-5}	2.38	L-Glutamine	10.0	0.19
Cu^{2+}	1.0 x 10^{-5}	2.84	L-Lysine	10.0	-3.26
Mn^{2+}	1.0 x 10^{-6}	-0.90	L-Leucine	10.0	2.78
Fe^{2+}	1.0 x 10^{-6}	1.24	Glucose	10.0	-0.37

Analytical performance

Calibration curve for Hb assay. Hb was used as the biocatalyst and the existence of Hb greatly accelerated the catalytic reaction. The more the Hb added, the higher the peak current got. So this new electrochemical method could be used for Hb detection. Under the selected conditions with 1.0 x 10^{-3} M OT and 4.0 x 10^{-4} M H_2O_2, the peak current increased with the increase of Hb concentration, the results are shown in Figure 5. The linear sweep voltammetric peak current was in linear with the Hb concentration in the range of 1.0 x 10^{-9} - 7.0 x 10^{-7} M with the linear regression equation as ip"(nA) = 25.47 + 1.54 C (nM) (n = 16, γ = 0.995), where ip" was the second order derivation of the linear sweep voltammetric reductive peak current and C the concentration of Hb. The relation standard deviation for 11 parallel determination of 1.0 x 10^{-9} M Hb was 3.05 % and the detection limit was 5.0 x 10^{-10} M (3σ). So the proposed method established a potential electrochemical method for the determination of Hb in biological samples.

Quantitative determination of H_2O_2. Under the optimal conditions, the calibration graph for the determination of H_2O_2 was constructed with 1.0 x 10^{-3} M OT and 1.0 x 10^{-7} M Hb. The increase in the concentration of H_2O_2 resulted in the increase of the reductive peak current, and good linearity can be found as follow: (1) in the range of 6.0 x 10^{-8} to 6.0 x 10^{-7} M with the linear regression equation as ip"(nA) = 13.17 + 0.82 C (μM) (n = 9, γ = 0.995); (2) in the range of 8.0 x 10^{-7} to 4.0 x 10^{-5} M with the linear regression equation is ip"(nA) = 19.93 + 0.11 C (μM) (n = 10, γ = 0.994), where ip" was the second order derivation of reductive peak current and C was the concentration of H_2O_2. The relative standard deviation for 11 parallel determinations of 6.0 x 10^{-8} M H_2O_2 was 3.86 % and the detection limit was 1.0 x 10^{-8} M (3σ).

The proposed electrochemical method for the determination of H_2O_2 with OT-Hb was compared with some reported method based on some heme proteins as catalyst and summarized in Table 2. It can be seen that this method had the comparable wider linear range and lower detection limit. Since the Hb is more cheaper and stable than the enzyme of HRP, so it can serve as the potential mimetic enzyme in the catalytical reaction.

Table 2. Comparison of the analytical characteristics for the determination of H_2O_2.

Catalyst	Method	Linear range (10^{-8} M)	LOD (10^{-8} M)	References
HRP	Fluorometry	0.4 - 8.0	0.21	[21]
HRP	Fluorometry	17 - 1700	1.7	[22]
HRP	Spectrophotometry	21 - 1500	-	[23]
Hemin	Spectrophotometry	32 - 3200	-	[24]
Hb	Fluorometry	10 - 8000	2.6	[25]
Hb	Voltammetry	20 - 1000	5.0	[26]
Hb	Voltammetry	6.0 - 4000	1.0	This method

Sample determination of H_2O_2 in fresh rain water. The proposed method was further applied to the determination of the content of H_2O_2 in fresh rainwater and the analytical results were listed in Table 3. The rainwater was collected and immediately analyzed according to the general procedure. The recovery was in the range of 98.02 - 105.07 % and the result showed that this method was practicable and reliable.

Table 3. Analytical results of the content of H_2O_2 in fresh rainwater.

Sample	Found ($\times 10^{-5}$ M)			Average ($\times 10^{-5}$ M)	RSD (%)	Added ($\times 10^{-5}$ M)	Detected ($\times 10^{-5}$ M)	Recovery (%)
1	2.15	2.27	2.17	2.20	3.71	10.00	12.71	105.07
	2.31	2.09	2.23					
2	4.21	4.18	4.29	4.18	2.58	10.00	13.98	98.02
	4.16	4.25	3.98					
3	3.44	3.36	3.49	3.51	3.04	10.00	13.45	99.43
	3.57	3.67	3.51					

CONCLUSIONS

In this paper Hb was used as the biocatalyst to catalyze the reduction of hydrogen peroxide with the subsequent oxidation of *o*-tolidine (OT). The azo product can be reduced on the mercury electrode and further detected by the linear sweep voltammetry. Based on the voltammetric reductive peak current of the reaction product, a new electrochemical method was proposed for the determination of microamount of H_2O_2 or Hb. Although the mercury is toxic, the mercury electrode has the instinctic advantages than the solid electrode. Dropping mercury electrode (DME) has a high hydrogen over-voltage and possesses a highly reproducible, readily renewable and smooth surface, which can provide highest sensitivity to establish a sensitive analytical method. So DME was used in this paper. The method was sensitive, reliable and practicable. It was successfully applied to the H_2O_2 concentration determination in fresh rainwater with satisfactory results.

AKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (20405008) and the Doctoral Foundation of QUST (0022125).

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