Biosensors and Bioelectronics 24 (2009) 1159–1163

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Hydrogen peroxide biosensor based on direct electrochemistry of soybean peroxidase immobilized on single-walled carbon nanohorn modified electrode

Lihong Shi[a,b], Xiaoqing Liu[a,b], Wenxin Niu[a,b], Haijuan Li[a,b], Shuang Han[a,b], Jiuan Chen[a,b], Guobao Xu[a,*]

[a] State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, 5625 Renmin Street, Changchun, Jilin 130022, China
[b] Graduate University of the Chinese Academy of Sciences, Chinese Academy of Sciences, Beijing 100864, China

ARTICLE INFO

Article history:
Received 7 April 2008
Received in revised form 17 June 2008
Accepted 1 July 2008
Available online 12 July 2008

Keywords:
Single-walled carbon nanohorns
Soybean peroxidase
Direct electrochemistry
Hydrogen peroxide
Carbon nanotubes

ABSTRACT

Single-walled carbon nanohorns (SWCNHs) were used as a novel and biocompatible matrix for fabricating biosensing devices. The direct immobilization of acid-stable and thermostable soybean peroxidase (SBP) on SWCNH modified electrode surface can realize the direct electrochemistry of enzyme. Cyclic voltammogram of the adsorbed SBP displays a pair of redox peaks with a formal potential of −0.24 V in pH 5 phosphate buffer solution. The formal potential has a linear relationship with pH from 3 to 9 with a slope of −48.7 mV/pH, close to the value of −55.7 mV/pH expected at 18 °C for the reversible transfer of one proton and one electron. Bioactivity of SBP remains good in SWCNH microenvironment, along with effective catalysis of the reduction of H_2O_2. In the absence of a mediator, this H_2O_2 biosensor exhibited a high sensitivity (16.625 μA L/mmol), a linear range from 0.02 to 1.2 mmol L^{-1}, and a detection limit of 5.0×10^{-7} mmol L^{-1}, as well as acceptable preparation reproducibility and excellent stability.

1. Introduction

The determination of hydrogen peroxide is of great importance in many different fields, such as food, clinical, pharmaceutical, industrial and environmental analyses. Among many techniques developed for this purpose, a third-generation amperometric biosensor, based on the direct electron transfer between an electrode and immobilized peroxidase, is especially promising because of its practical advantages including operation simplicity, low expense, and suitability for real-time detection (Ferapontova, 2004). Until now, horseradish peroxidase (HRP), belonging to class III of the plant peroxidase superfamily, has been the commonly used enzyme for the construction of a mediator-free biosensor (Ferapontova et al., 2001; Jia et al., 2002; Kong et al., 2003; Tang et al., 2003; Lu et al., 2006; Wang et al., 2006). However, the life of HRP-based biosensors is often limited by the inherent instability of HRP (Schubert et al., 1991). Soybean peroxidase (SBP) is another member of class III plant peroxidase superfamily. It is a 326-amino acid containing glycoprotein with a molecular mass of ~37 kDa. It has similar overall structure to HRP with a degree of sequence homology of 57%. The common features between SBP and HRP include Fe(III) protoporphyrin IX (heme) as the prosthetic group, catalytic mechanism, conserved catalytic residues, four disulfide bonds, two Ca^{2+} binding sites located distal and proximal to heme, eight glycans, and a single tryptophan. Noteworthily, SBP is less susceptible to heme loss and permanent inactivation by hydrogen peroxide than HRP (McEldoon and Dordick, 1996; Henriksen et al., 2001; Kamal and Behere, 2002; Ryan et al., 2006). It can maintain its bioactivity over a wide pH range (3–10), at high temperature (<70 °C), and in a variety of organic solvents (McEldoon et al., 1995; Kamal and Behere, 2002; Guto et al., 2007). Furthermore, SBP is more economical for practical applications because it can be obtained readily from cheap soybean seed coats. Hence, SBP particularly shows great promise for biocatalytical and biosensing application. Under the existence of various mediators, some stable biosensors based on this enzyme have been fabricated by using redox-conducting hydrogel (Vreeke et al., 1995), sol–gel thin film (Wang et al., 1999), grafting copolymer (Wang et al., 2001), and so on. Only Zhang et al. (2002) reported the direct electrochemistry and electrocatalysis of SBP by immobilizing the enzyme in lipid films.

Since the discovery of carbon nanotubes (CNTs) by Iijima (1991), they have been the targets of numerous investigations. The excellent electrical conductivity, high surface area, significant mechanical strength, and good chemical and thermal stability make CNTs attractive materials for electroanalysis (Wang and Musameh, 2003; Valcarcel et al., 2007). Especially, they have been successfully used as "molecular wires" to realize direct electron transfer between redox enzymes and electrode surfaces, which can establish a foundation for fabricating new kinds of mediator-free

* Corresponding author. Tel.: +86 431 85262747; fax: +86 431 85682747.
E-mail address: guobaoxu@ciac.jl.cn (G. Xu).

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doi:10.1016/j.bios.2008.07.001

biosensors (Wang et al., 2002; Gooding et al., 2003; Lin et al., 2004; Patolsky et al., 2004; Wang, 2005). However, the production of CNTs with conventional methods will be inevitably accompanied by the formation of large amounts of by-products including metal catalyst particles, carbonaceous, and other amorphous material impurities (Thess et al., 1996; Journet et al., 1997; Flahaut et al., 2005). Even by using many purification techniques, metal particles residues still remain in CNTs, which may influence the analytical results in electrochemical applications (Moore et al., 2004; Banks et al., 2006; Sljukic et al., 2006; Jones et al., 2007).

For biosensing utility, the recently discovered single-walled carbon nanohorns (SWCNHs) may be explored as a complement and/or advantageous replacement of nanotubes (Iijima et al., 1999). Because SWCNHs are produced with high yield by vaporizing pure graphite rods via CO_2 laser ablation without using any catalyst, an advantage of SWCNHs over carbon nanotubes is that SWCNHs are essentially metal-free (Iijima et al., 1999; Isobe et al., 2006). SWCNHs are round-shaped aggregates of graphitic tubes, and these aggregates have a homogeneous diameter of 80–100 nm. Each individual tube features closed end with corn-shaped cap, 2–4 nm in diameter and 30–50 nm in length, which permits assembly into "dahlia-like" spherical nanostructures. Therefore, SWCNHs can provide various pore structures including microporosity, mesoporosity, and macroporosity (Yang et al., 2005). The unique internal and interstitial nanopore structures of SWCNHs, coupled with their high thermal stability and conductive graphitic structures, make them promising candidates in gas adsorption and storage (Murata et al., 2002; Bekyarova et al., 2003; Tanaka et al., 2004), catalyst support (Bekyarova et al., 2005), and drug delivery (Murakami et al., 2004; Ajima et al., 2005).

In present work, SWCNHs were used to realize the direct electrochemistry of SBP. Based on the direct electrochemistry of SBP, a stable and sensitive reagentless biosensor for hydrogen peroxide was fabricated.

2. Experimental

2.1. Reagents

Professor S. Iijima generously offered SWCNHs (>95%). SBP (50–150 purpurogallin (20 s) units mg^{-1}) was purchased from Sigma. A 30% hydrogen peroxide solution was purchased from Beijing Chemical Reagent (Beijing, China), and a fresh solution of H_2O_2 was prepared daily. Phosphate buffer solution (50 mmol L^{-1}) was made up from KH_2PO_4 and adjusted to desired pH by adding 1.0 mol L^{-1} KOH or HCl solution. All other chemicals were of analytical grade and used as received without further purification. All aqueous solutions were prepared in doubly distilled water.

2.2. Apparatus

Electrochemical measurements were preformed with a three-electrode system comprising a glassy carbon (GC) working electrode (3.0 mm in diameter), a platinum wire auxiliary electrode (99%, Aldrich) and an Ag/AgCl (saturated KCl) reference electrode. The electrodes were connected to a CHI832 electrochemical workstation (CHI Inc., USA). Prior to the experiment, the GC electrode was carefully polished with 0.05 μm alumina slurry, sonicated in distilled water, and dried with high-purity nitrogen. All solutions were bubbled with high-purity nitrogen for about 20 min before each electrochemical experiment, and kept under a nitrogen atmosphere during the experiments. The morphologies of electrode modifiers were determined with a JEOL 2010 transmission electron microscope (TEM) operated at an accelerating voltage of 200 kV.

2.3. Preparation of SWCNH modified GC electrode and SBP/SWCNH modified GC electrode

2.0 mg of the SWCNHs were dispersed in dimethylformamide (DMF) (with the aid of ultrasonic agitation) to give a black suspension of 2.0 mg mL^{-1}. The SWCNH modified electrode was prepared by dropping 3 μL of SWCNHs solution on the polished GC electrode and then allowed to dry for over 12 h at room temperature. In order to get more uniform films, the modified electrode was covered with a small bottle during the evaporating process of DMF. After the SWCNH modified electrode was washed with water, it was immersed in a 2.0 mg mL^{-1} SBP solution (pH 5) at 4 °C for over 6 h to obtain the SBP/SWCNH modified GC electrode. The SBP/SWCNH modified GC electrode was stored under dry condition at 4 °C when not used.

3. Results and discussion

3.1. Characterization by TEM

The morphologies of bare SWCNHs and SBP/SWCNH composite were characterized by TEM. As shown in Fig. 1A, the bare SWCNHs formed dahlia-like assemblies with a diameter of about 100 nm. The individual SWCNH structural unit is shown clearly. After the SWCNHs were immersed in SBP solution for several hours, TEM image of the resulting SWCNHs is obscure and it is difficult to observe the clear structure of single SWCNH (Fig. 1B), indicating that SBP has adsorbed onto SWCNH (Guan et al., 2005). The immobilization of SBP in SWCNHs matrix might be mainly based on physisorption and π–π stacking interactions (Pagona et al., 2006, 2007; Cioffi et al., 2007).

3.2. Electrochemistry of the SBP/SWCNH modified electrode

Cyclic voltammetry was used to characterize the modification of electrode surface. Fig. 2 shows cyclic voltammograms of different modified electrodes in 50 mmol L^{-1} phosphate buffer solution (pH 5) at a scan rate of 50 mV s^{-1}. A pair of well-defined redox peaks are observed at the SBP/SWCNH modified GC electrode, and no redox peak is observed at the SWCNH modified GC electrode. Therefore, the redox peaks in Fig. 2a are attributed to the reduction and oxidation of the immobilized SBP. The formal potential (estimated as the average of the anodic and cathodic peak potentials) of SBP was −0.24 V, which was consistent with that of SBP entrapped in lipid films by Zhang et al. (2002). This formal potential value was also similar to those of other heme-containing proteins including HRP, myoglobin, and hemoglobin at the same pH value of buffer solution (Nassar et al., 1996; Hu and Rusling, 1997; Han et al., 2002; Liu et al., 2005; Lu et al., 2006).

The cyclic voltammetric curves of the SBP/SWCNH modified GC electrode at various scan rates were shown in Fig. 3. The ratio of cathodic to anodic peak currents is nearly unity. According to the equation $\Gamma = Q/nFA$ (where Γ is the surface concentration of electroactive SBP and Q is the charge consumed in reaction, obtained from integrating the redox peak area in the cyclic voltammograms under background correction), the surface concentration of the electroactive SBP on modified electrode was estimated to be $\sim 5.22 \times 10^{-11}$ mol cm^{-2} (assuming an one electron transfer reaction). It was found that the peak currents increased along with increasing scan rate, while the peak-to-peak separation and the formal potential were almost constant. The cathodic peak current for SBP was linearly proportional to the scan rate ranging from 20 to 300 mV s^{-1}, indicating that the redox reaction is a surface controlled process. In addition, cyclic voltammograms of the

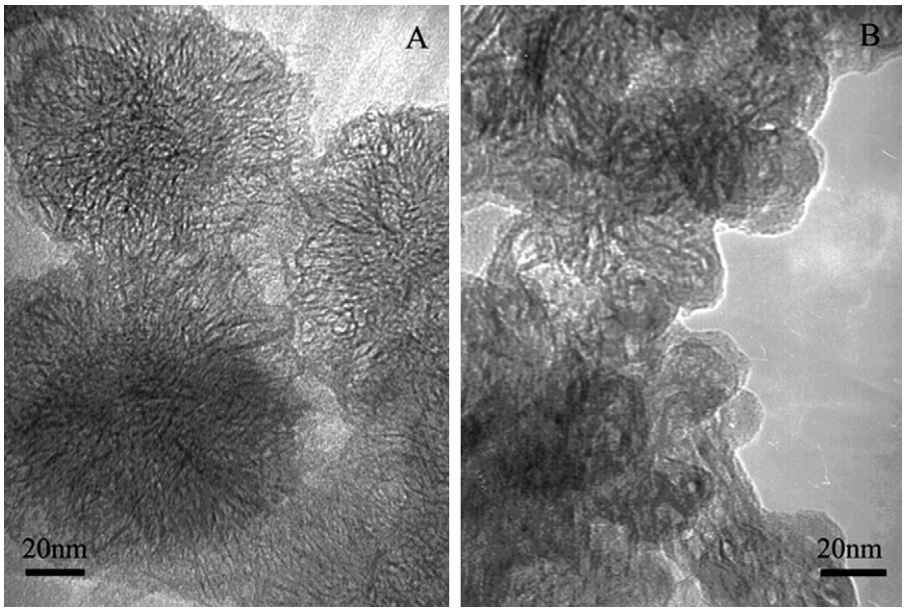

Fig. 1. TEM images of (A) bare SWCNHs and (B) SBP/SWCNH composite.

SBP/SWCNH modified GC electrode showed a strong dependence on solution pH. As shown in Fig. 4, an increase of pH in solution resulted in a negative shift in potential for both reduction and oxidation peaks of SBP. The formal potential has a linear relationship with pH from 3 to 9 with a slope of −48.7 mV/pH. This value is close to the value of −55.7 mV/pH expected at 18 °C for the reversible transfer of one proton and one electron (Bond, 1980). The pH dependence of formal potential for heme proteins may come from the influence of the protonization of trans and residue ligands around the heme and the protonization of the water molecules coordinated with the central iron ion (Nassar et al., 1997).

3.3. Electrocatalysis and detection of H_2O_2 at SBP/SWCNH modified GC electrode

As mentioned above, a pair of well-defined redox peaks are observed at the SBP/SWCNH modified GC electrode in 50 mmol L^{-1} pH 5.0 phosphate buffer solution (Fig. 2a). Upon addition of 0.2 mmol L^{-1} H_2O_2 to electrolyte, an obvious electrocatalytic response was observed (Fig. 2c). The reduction peak current of the SBP/SWCNH modified GC electrode dramatically increased and the oxidation peak current decreased. Since the SWCNH modified GC electrode showed negligible catalytic activity toward the reaction of H_2O_2 (Fig. 2d), the obvious catalytic current shown in Fig. 2c is mainly due to the presence of SBP. It indicates that SWCNHs provide a favorable microenvironment for proteins and play important role in facilitating the electron exchange and preserving activity of proteins.

Before using as biosensor for H_2O_2 detection, the SBP/SWCNH modified GC electrode was investigated at different applied potentials and pH values to obtain optimum detection conditions. From the dependence of steady-state amperometric responses on the applied potentials, an applied potential of −0.25 V was chosen to obtain good sensitivity and minimize interference. The influence of pH on steady-state amperometric responses was studied in the

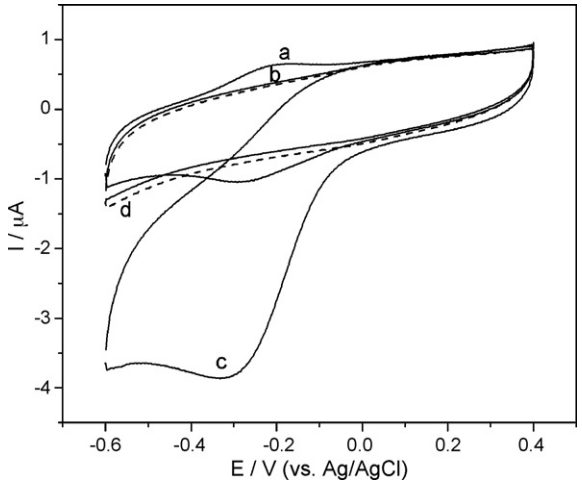

Fig. 2. Cyclic voltammograms of SBP/SWCNH modified GC electrode (a and c) and SWCNH modified GC electrode (b and d) in the absence (a and b) and presence (c and d) of 0.2 mmol L^{-1} H_2O_2: supporting electrolyte, 50 mmol L^{-1} pH 5.0 phosphate buffer solution; scan rate, 50 mV s^{-1}.

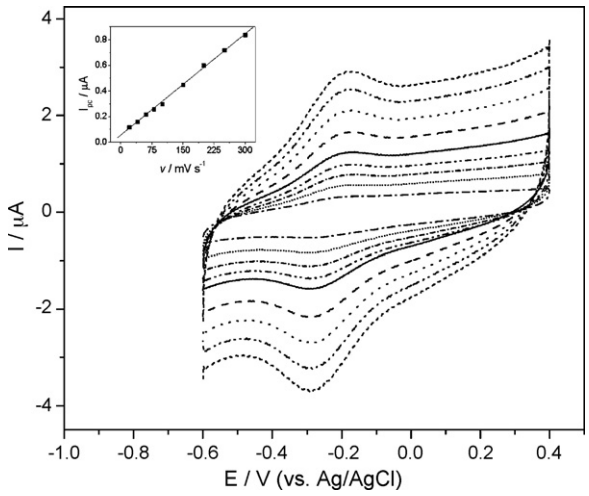

Fig. 3. Cyclic voltammograms of the SBP/SWCNH modified GC electrode in 50 mmol L^{-1} pH 5.0 phosphate buffer solution at various scan rates from 20 to 300 mV s^{-1}. Inset is the plot of cathodic peak current versus scan rate.

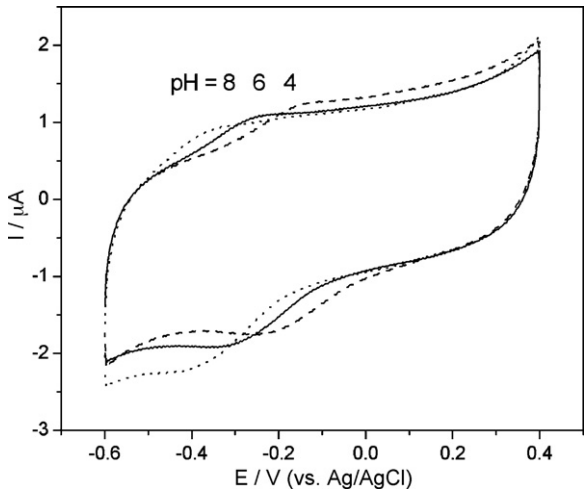

Fig. 4. Cyclic voltammograms of the SBP/SWCNH modified GC electrode in 50 mmol L^{-1} phosphate buffer solution at different pH values. Scan rate, 100 mV s^{-1}.

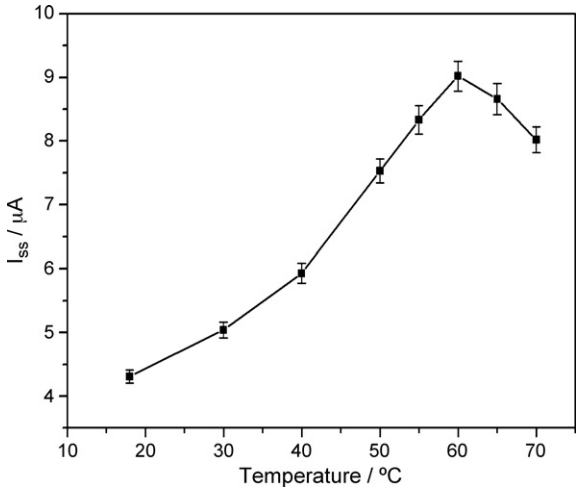

Fig. 6. Effect of temperature on the amperometric responses for 0.2 mmol L^{-1} H_2O_2 at the SBP/SWCNH modified GC electrode: applied potential, −0.25 V; supporting electrolyte, 50 mmol L^{-1} pH 5.0 phosphate buffer solution.

pH range from 3 to 8. The maximum steady-state amperometric response appeared at pH 5.0. So a pH of 5.0 was selected for further experiments in order to obtain maximum sensitivity.

Under optimum conditions, the characteristics of the biosensor were investigated by amperometric measurement. As shown in Fig. 5, the current responses increase linearly with increasing the concentrations of H_2O_2 from 0.02 to 1.2 mmol L^{-1}. The linear regression equation was I (μA) = 0.834 + 16.625c (mmol L^{-1}) with a correlation coefficient of 0.999. The sensitivity (16.625 μA L/mmol) of the SBP-based biosensor is much higher than those of various HRP-based biosensors (Ferapontova et al., 2001; Jia et al., 2002; Kong et al., 2003; Tang et al., 2003; Lu et al., 2006). It indicates that the entrapped SBP in SWCNHs matrix exhibits a higher catalytic activity. The detection limit was 5.0×10^{-7} mol L^{-1} at a signal-to-noise ratio of 3. The apparent Michaelis–Menten constant (K_m^{app}), which provides an indication of the enzyme–substrate kinetics and a way to compare one biosensor with others, could be obtained from the electrochemical version of the Lineweaver–Burk equation (Kamin and Wilson, 1980): $1/I_{SS} = 1/I_{max} + K_m^{app}/I_{max}c$, where I_{ss} is the steady-state current after the addition of substrate, c is the bulk concentration of the substrate, and I_{max} is the maximum current

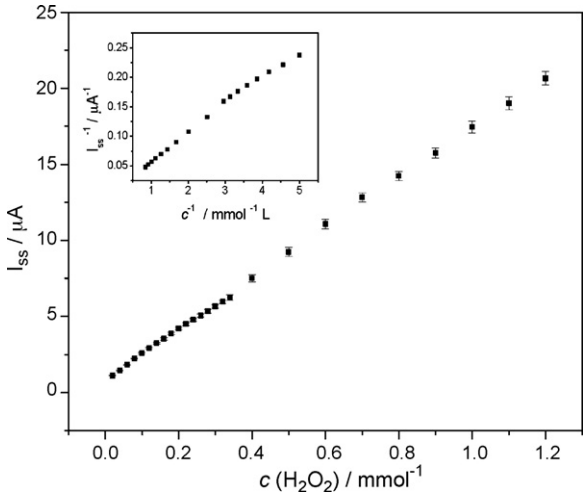

Fig. 5. Calibration curve for detection of H_2O_2 at the SBP/SWCNH modified GC electrode: applied potential, −0.25 V; supporting electrolyte, 50 mmol L^{-1} pH 5.0 phosphate buffer solution. Inset is the corresponding Lineweaver–Burk plot.

measured under saturated substrate conditions. According to the analysis the slope and intercept for the plot of the reciprocal of the steady-state current versus reciprocal of H_2O_2 concentration, K_m^{app} of the present biosensor was calculated to be 4.88 mmol L^{-1}. This value is lower than 5.12 mmol L^{-1} for SBP–sol–gel thin film modified electrode (Wang et al., 1999), suggesting that SBP molecules entrapped in SWCNHs are of high affinity.

3.4. Selectivity, stability and reproducibility of the SBP/SWCNH modified electrode

In order to test the applicability of this H_2O_2 sensor with real samples, interferences from some potential substances were examined under the optimum conditions. The interference degree can be evaluated by comparing the current for a mixture of 0.4 mmol L^{-1} interfering substance and 0.2 mmol L^{-1} H_2O_2 with that for 0.2 mmol L^{-1} H_2O_2 alone. Results showed the values for glucose, L-cystine, L-tyrosine, ascorbic acid and nitrite all lay in the range of 0.99–1.05. The good selectivity of this biosensor is largely attributed to the low working potential of −0.25 V used for determining H_2O_2.

The SBP/SWCNH modified GC electrode could retain constant current values upon the continuous cyclic voltammetric sweep at 50 mV s^{-1} in 50 mmol L^{-1} phosphate buffer solutions. After the sensor was stored under dry condition in a refrigerator at 4 °C for a week, there is no obvious decrease in the currents for the direct electron transfer and the response to H_2O_2. After 2 months, the sensor retained 96% of its initial current response for the determination of 0.2 mmol L^{-1} H_2O_2. Especially, the SBP-based biosensor possessed good thermal stability. For the determination of 0.2 mmol L^{-1} H_2O_2 at different temperatures, the current response firstly increased with increasing temperature, and then decreased gradually at temperature higher than 60 °C (Fig. 6). This result is very similar to that of previous reports (Vreeke et al., 1995; Wang et al., 1999). This biosensor lost only 2.1% of its activity per hour while operating at 60 °C. The better thermostability of SBP-based biosensor than HRP-based biosensor can be ascribed to the tightly bound heme of SBP that is beneficial for maintaining a catalytically active conformation of the protein (Kamal and Behere, 2002).

The relative standard deviation is 2.7% for nine successive measurements of 0.2 mmol L^{-1} H_2O_2. For the fabrication reproducibility of five electrodes from the same batch, the relative standard devi-

ation is 6.3% for the response to 0.2 mmol L^{-1} H_2O_2, showing the proposed biosensors possess good reproducibility.

4. Conclusions

SWCNHs provided a favorable microenvironment for direct electrochemistry of SBP. Based on direct electrochemistry of SBP, a mediator-free H_2O_2 biosensor was constructed. The as-prepared mediator-free biosensor exhibited high sensitivity, excellent thermostability, and good long-term stability. SWCNH is a potential matrix to immobilization of biomolecules and fabrication of electrochemical biosensors.

Acknowledgements

We are very grateful to Professor S. Iijima (Solution Oriented Research for Science and Technology in Japan Science and Technology Agency) for generous offer of SWCNHs. This project was supported by the National Natural Science Foundation of China (No. 20505016), the Hundred Talents Program of Chinese Academy of Sciences, and the Department of Sciences & Technology of Jilin Province (20070108).

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