Carbon Paste Biosensor Based on Crude Soybean Seed Hull Extracts for Phenol Detection

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In this study, a novel biosensor based on enzyme extracts from soybean seed hulls has been prepared, which demonstrated promising results in the detection of hydrogen peroxide and phenol. The biosensor preparation is straightforward and inexpensive, and the response time is 50 s. The optimum conditions of pH and temperature are a pH of 7.4 and a temperature of 20 °C. Contrary to expectations, the biosensor showed narrow pH and temperature optimums. The effects of enzyme loading and type of mediator were also investigated. The biosensor showed a linear response up to 500 μM phenol.

Keywords: *Soybean seed coat peroxidase; amperometric biosensor; phenol detection*

INTRODUCTION

Phenols are common environmental contaminants originating mainly from their use as widespread spectrum biocides in agriculture, in wastes from coal or petroleum refining, and in plastics and resin industries (EPA, 1980). Due to their suspected carcinogenity and the increasingly stringent environmental legislation for waste disposal**,** several efforts are being devoted to their monitoring and removal from wastewaters (Matatovmeytal and Sheintuch, 1998). Laboratory assays such as high-performance liquid chromatography (HPLC) or gas chromatography (GC) or colorimetric (Buchwald, 1996) and fluorometric methods (Tsuruta et al., 1996) can be used for phenol monitoring but may be unsuitable outside the laboratory environment. Biosensors have found a niche in the portable sensors and quality control markets (Deshpande and Rocco, 1994). Featuring specific selectivity, fast response, portable size, lower cost, and accurate measurements, biosensors as novel analytical devices have demonstrated potential in various fields such as medicine, pharmaceuticals, environmental control, and bioproduction for analytical purposes (Schimd and Scheller, 1989).

Previously, several amperometric biosensors for phenolics have been reported, which were based on horseradish peroxidase (HRP) (Lindgren et al., 1997), tyrosinase from mushroom, banana, and potato (Wang et al. 1992) polyphenol oxidases from egg plant (Navaratne and Rechnitz, 1990), lignin peroxidase, flavin monoxygenase, or microbial cells (Wollenberger et al., 1994). Vieira and Fatibello (1997) have recently reported a phenol biosensor based on crude sweet potato extracts containing tyrosinase. The biosensor was constructed by the immobilization of sweet potato crude extract with glutaraldehyde and bovine serum albumin onto an oxygen membrane.

Schmitz et al. (1997) and Vierling and Wilcox (1996) reported that soybean (*Glycine max*) seed coats contain large amounts of peroxidase enzyme. Comparisons between high and low peroxidase activity seeds demonstrated a lengthy (336 h) release of anionic peroxidase by Ep (Harovinton) cultivars following incubation in an aqueous environment. In its purified state, soybean seed coat peroxidase exhibited good catalytic activity toward phenolic substrates including eugenol, caffeic acid, and ferulic acid. These authors also showed that, using guaiacol as a substrate at various pH levels and temperatures, soybean seed coat peroxidase had a greater enzyme stability and a wider range of action than other peroxidase enzymes. Kenausis et al. (1997) have demonstrated the construction of a soybean peroxidase (SBP) biosensor for hydrogen peroxide, glucose, and lactate. Purified SBP was cross-linked with poly(vinylpyridine) and [Os(bpy)(2)Cl](+/2+) (bpy = bipyridine) and covered by layers of immobilized glucose oxidase or a lactate oxidase layer for glucose or lactate. McEldoon and Dordick (1996) reported on the high thermal stability of SBP [melting temperature of 90.5 °C at pH 8.0 in the presence of 1 mM $CaCl_2$ compared to horseradish peroxidase (HRP)]. The SBP enzyme is also stable in a broad pH range extending into both the acidic and the basic regimes, which is also atypical with other peroxidases in general.

It is proposed that SBP catalyzes the polymerization of phenols in the presence of hydrogen peroxide in a manner similar to other peroxidases such as HRP. A possible sequence of reactions catalyzed by SBP is shown in eqs 1−7 below. These equations are based on a mechanism originally reported for HRP (Buchanan et al., 1998) assuming that SBP would follow a similar scheme. The mechanism is as follows:

$$\mathrm{SBP} + \mathrm{H_2O_2} \rightarrow \mathrm{SBPI} + \mathrm{H_2O} \quad (1)$$

$$\mathrm{SBPI} + \mathrm{PhOH} \rightarrow \mathrm{SBPII} + \mathrm{PhO^*} \quad (2)$$

$$\mathrm{PhO^*} + \mathrm{PhO^*} \rightarrow \text{Ph-polymers} \quad (3)$$

$$\mathrm{SBPIII} \rightarrow \mathrm{SBP} + \mathrm{O_2^-} \quad (4)$$

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$$SBPII + H_2O_2 \rightarrow SBPIII + H_2O \quad (5)$$

$$SBPII + PhOH \rightarrow SBP + PhO^* + H_2O \quad (6)$$

$$SBPII + H_2O_2 \rightarrow SBPIII + H_2O \quad (7)$$

Peroxidases carry out the polymerization of phenols through the production of free radicals, PhO*. As shown in eqs 1−7, SBP is oxidized by hydrogen peroxide, creating another catalytically active intermediate SBPI. This form of the enzyme then oxidizes the substrate (phenol) to create a phenoxy radical and another intermediate, SBPII. This second compound is capable of oxidizing another phenolic substrate, producing another free radical, and returning to its native form. It has been shown that the second compound can also be oxidized by hydrogen peroxide, creating a third compound that is inactive (Buchanan and Nicell, 1997). This third compound can spontaneously return to the native form of the enzyme as shown in eq 4. The free radicals produced by the reaction are then free to couple and form polymers. These phenolic polymers are much less soluble than the monomer form and therefore could be removed by conventional filtration or centrifugation (Alberti and Klibanov, 1981; Nicell et al., 1992).

The objectives of this study were to investigate the application of soybean seed hull extracts that contain SBP (EC 1.11.1.7) for the development of an amperometric biosensor for phenol. The hulls are currently sold for animal feed and are therefore of low commercial value.

Studies with HRP revealed that intermediate enzyme forms in eqs 1−7 can be reduced back to native state by electroactive mediators such as ferrocene or TCNQ (Ruzgas et al., 1995). Thus, this reduction can be considered in a catalytic cycle for amperometric biosensor detection as shown below:

$$H_2O_2 + PhOH \xrightarrow{SBP} H_2O + PhO^* \quad (8)$$

$$PhO^* + M_{red} \rightarrow PhOH + M_{ox} \quad (9)$$

$$M_{ox} + ne \xrightarrow{Appl.Pot.} M_{red} \quad (10)$$

Here, M_{ox} is the oxidized form of the electroactive mediator, M_{red} is the reduced form of the electroactive mediator, PhO* is the phenoxy radical, ne is the number of electrons transferred (gained), and Appl. Pot. is the applied operating potential in the electrochemical cell.

Thus, the phenolic radicals produced are capable of being reduced electrochemically. The reduction current should be proportional to the concentration of the phenolic radicals.

EXPERIMENTAL PROCEDURES

Chemicals. Dimethyl ferrocene (DMF), tetrathiafulvalene (TTF), 7,7,8,8,-tetracyanoquinodimethane (TCNQ), phenol, graphite powder, and mineral oil were purchased from Sigma-Aldrich Chemical Co. Hydrogen peroxide solutions were prepared by appropriately diluting 30% H_2O_2 in 0.1 M phosphate buffer (pH 7.0). All other chemicals used were of analytical grade.

Apparatus. The voltammograph system from Bioanalytical Systems, Inc. consisted of a cell stand (MF-9064), a cyclic voltammograph (CV-27), a low-current module (PA-1), an RXY recorder (MF-9044), and a three-electrode cell. In the cell, a platinum wire (0.05 × 4.5 cm) auxiliary electrode, an Ag/AgCl reference electrode, and a carbon paste Teflon electrode with a cavity (3 mm diameter, 5 mm depth) were used to perform cyclic voltammetry or constant potential amperometric measurements at −100 mV versus Ag/AgCl. A jacketed cell of the same volume as the regular cell but connected to a temperature-controlled water bath was used to perform tests at different temperatures.

Preparation of Chemically Modified Carbon Paste Electrodes. The technique used for construction of carbon paste electrodes was based on that reported by Almeida and Mulchandani (1993) and was as follows: Plain carbon paste (CP) was made by thoroughly mixing 40 mg of carbon graphite with 10 μL of mineral oil until a uniform paste was formed in a glass plate. Chemically modified carbon paste (CMCP) was made by combining DMF, graphite powder, and mineral oil. Ten milligrams of DMF was previously dissolved in 100 μL of toluene and toluene allowed to totally vaporize. To this dried DMF were added 50 mg of graphite powder and 14.5 μL of mineral oil to create a paste. The CP was first pressed into the cavity of the Teflon electrode (BAS) followed by CMCP. The electrode surface was then polished on a wax paper to produce a flat, shiny surface with an area of $\sim 7 \times 10^{-6}$ m^2, and a 0.5 mm dent was created on the surface using a specially machined plexiglass tool. Soybean seed hulls (ADM Agri-Industries, Windsor, ON) were mixed together in a large batch and stored dry. A measured amount of the hulls were immersed in phosphate buffer (10 mM, pH 6.0) overnight on ice. The samples were centrifuged at 3000 rpm for 15 min, and the supernatant was divided into 1 mL aliquots and frozen at −20 °C. This supernatant was used as the crude enzyme extract. Five microliters crude extracts were mixed with 1.5 μL (1% w/v) gluteraldehyde and 1.5 μL of bovine serum albumin, introduced into the electrode cavity, and left to dry for 60 min. The enzyme activity of the crude extracts was obtained from a related study in the author's laboratory and reported as units of guaiacol oxidation to tetraguaiacol, where 1 U is the rate of change of absorbance (at 490 nm) per minute in an assay mixture containing 50 μL of 0.3% (v/v) H_2O_2, 50 μL of 1% guaiacol, 200 μL of H_2O, and 10 μL of test solution. On the basis of this assay, the crude extracts had a measured activity of 0.24 U/μL. Some biosensors were made with TTF or TCNQ as the electroactive mediator instead of DMF by following a similar protocol as described above.

RESULTS AND DISCUSSION

Optimization of Conditions. Because hydrogen peroxide is a cosubstrate in the reaction, the biosensor response was optimized with respect to operating conditions. The response of the mediated enzyme extract electrode is affected by several parameters. These include composition of the carbon paste matrix, operating potential, enzyme loading of the matrix, pH, and temperature. The composition of the carbon paste, which contained graphite, mineral oil, and DMF, was an optimum composition based on previous studies (Almeida and Mulchandani, 1993). Enzymes have previously been incorporated into the paste by either mixing them into the paste or cross-linking on top of the paste using glutaraldehyde and then retaining behind a dialysis membrane or electropolymer film. Mixing the enzyme into the paste is simple. However, this can require large quantities of enzyme. Mixing of extracts into the paste did not result in adequate sensor response, possibly due to the lower activities in the crude extracts. The deposition of the enzyme by cross-linking with glutaraldehyde was found to be effective. We found no additional membrane or electrochemical film was required, and the biosensor was stable for up to 3 weeks as shown later.

SBP in soluble form has a broad pH optimum between 6.0 and 9.0. To determine the optimum pH for the use of the SBP biosensor, tests were carried out in 0.1 M

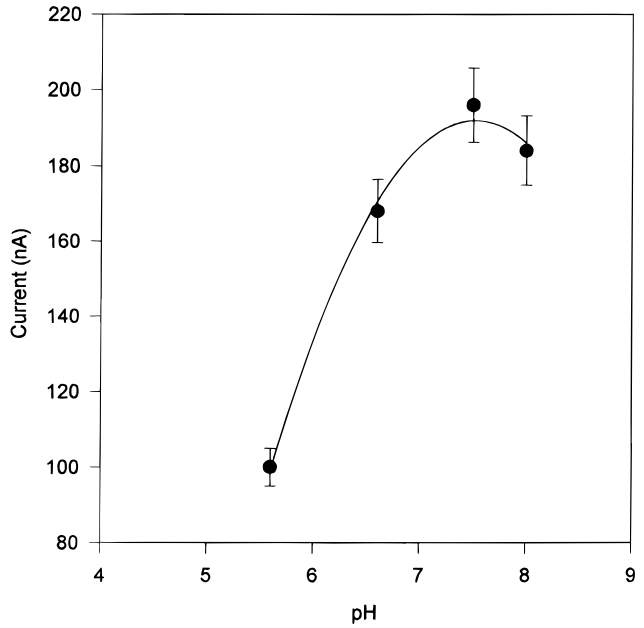

Figure 1. Effect of pH on response of biosensor to 0.05 μM H_2O_2 injections. Conditions: temperature, 22 °C; activity of crude extract loaded on biosensor, 2.4 U (as defined in the text); operating potential, −100 mV versus Ag/AgCl reference. The error bars indicate the standard error for triplicate measurements with the same electrode.

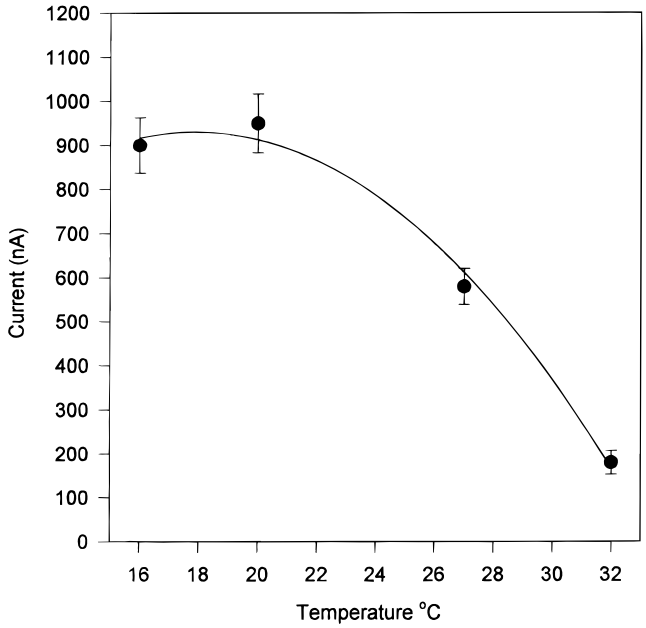

Figure 2. Effect of temperature on response of biosensor to 0.05 μM H_2O_2 injections. Conditions: pH 7.4; activity of crude extract loaded on biosensor, 2.4 U (as defined in the text); operating potential, −100 mV versus Ag/AgCl reference. The error bars indicate the standard error for triplicate measurements with the same electrode.

phosphate buffer solutions of different pH values. The response to an input of 0.05 μM H_2O_2 is shown in Figure 1. On immobilization of the crude enzyme extracts on the biosensor surface, an optimum pH of 7.45 was noted. The error bars indicate standard error based on three consecutive tests with the same biosensor. The biosensor also did not display a broad pH optimum. The differences in pH optimum between the soluble form of the enzyme and the immobilized soybean hull extracts may be due to differences in physicochemical properties of the enzyme in the bound state.

The effect of temperature was also contrary to the expected broad thermal stability of the soybean peroxidase enzyme. The response to injections of 0.05 μM H_2O_2 is shown in Figure 2. The response was optimum at 18−20 °C and decreased at higher temperatures. This could be due to possible deactivation of the enzyme with temperature.

The effect of enzyme loaded on the biosensor surface on the response of the biosensor to hydrogen peroxide injections is shown in Figure 3. The figure shows that the biosensor response increased with increasing quantities of enzyme activity. However, the amount of extract that could be loaded on the surface was limited by practical considerations, as higher amounts of extract resulted in poorly constructed electrodes. Thus, an enzyme activity of 2.4 U was used in further biosensor experiments.

Two other electroactive mediators, TTF and TCNQ, were also utilized in the construction of the biosensor. The effect of mediator on biosensor response is shown in Figure 4. Clearly, the biosensor constructed with TCNQ did not respond as well as the DMF- and the TTF-based biosensors. However, both TTF and DMF provide an adequate response at the operating potential of −100 mV versus Ag/AgCl reference.

Analytical Characteristics of the Biosensor. Calibration of the biosensor was carried out in the same electrochemical cell in 10 mL of phosphate buffer (0.1 M, pH 7.4). Steady-state current was measured by sequential injections of hydrogen peroxide, and a response time of 50 s was observed. Figure 5 shows the experimental results. Each point in the standard calibration curve was based on the average of three measurements. Linear concentration range was given from 0 to 0.06 μM. The limit of detection of the biosensor to H_2O_2 was found to be 0.005 μM.

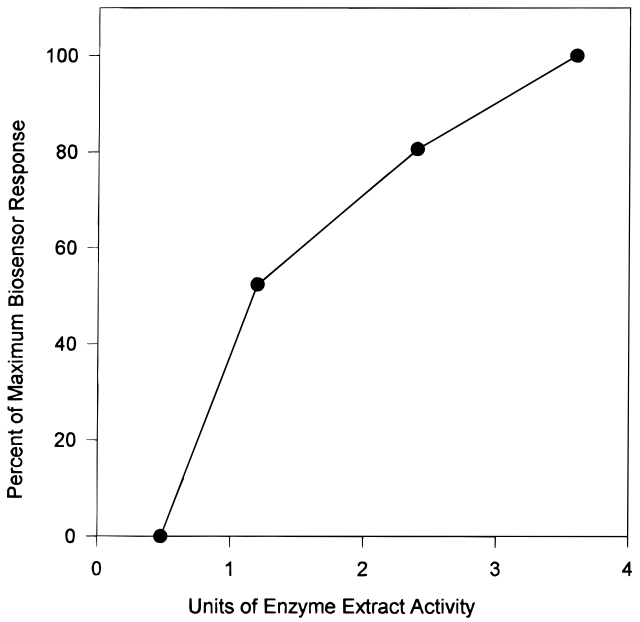

Figure 3. Effect of enzyme activity on response of biosensor to 0.05 μM H_2O_2 injections. Conditions: temperature, 22 °C; pH 7.4; operating potential, −100 mV versus Ag/AgCl reference. The results are the average of duplicate measurements with four biosensors prepared identically but with different enzyme extract loadings. One unit (U) of activity is defined as the rate of change of absorbance (at 490 nm) per minute in an assay mixture containing 50 μL of 0.3% (v/v) H_2O_2, 50% of 1% guaiacol, 200 μL of H_2O, and 10 μL of test solution.

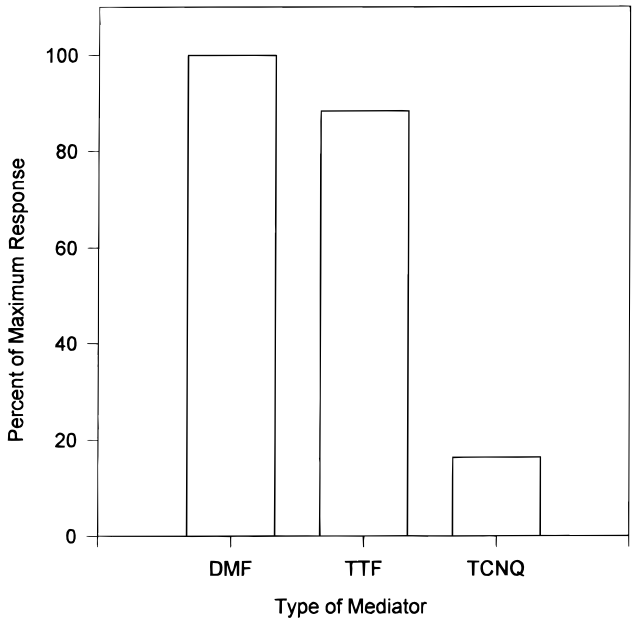

Figure 4. Effect of electroactive mediator on response of biosensor to 0.05 μM H_2O_2 injections normalized with respect to response with DMF biosensor. Conditions: temperature, 22 °C; activity of crude extract loaded on biosensor, 2.4 U (as defined in the text); pH 7.4; operating potential, −100 mV versus Ag/AgCl reference.

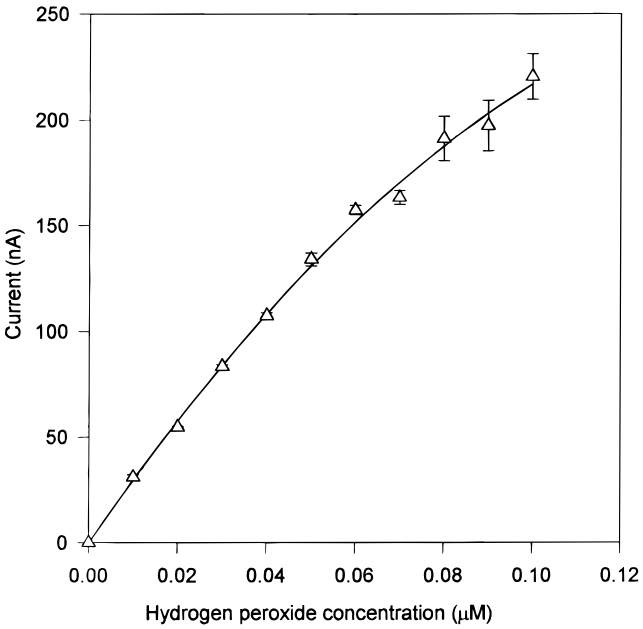

Figure 5. Calibration of biosensor response with respect to H_2O_2 additions. Conditions: temperature, 22 °C; activity of crude extract loaded on biosensor, 2.4 U (as defined in the text); operating potential, −100 mV versus Ag/AgCl reference; pH 7.4. Two biosensors were prepared, and each was tested three times immediately after preparation. The error bars indicate the standard error for triplicate measurements with the same electrode.

Measurement of Phenol Using SBP Biosensor. The prepared biosensors responded to phenol concentrations in the range of 0−0.7 mM. These results are shown in Figure 6. The biosensor response was linear up to 0.5 mM phenol, and the minimum detection limit was found to be 50 μM. Thus, the biosensor responds effectively to injected phenol. However, it was found that the biosensor was not sensitive to low concentrations of phenol. This could be due to the low concentrations

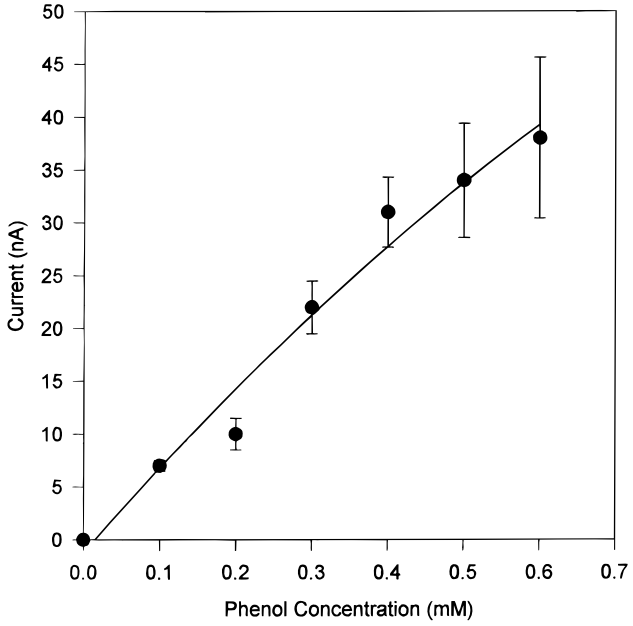

Figure 6. Observed response of biosensor to phenol additions. Conditions: temperature, 22 °C; activity of crude extract loaded on biosensor, 2.4 U (as defined in the text); operating potential, −100 mV versus Ag/AgCl reference; concentration of H_2O_2, 10 μM. The results are the average of duplicate determinations with the same biosensor.

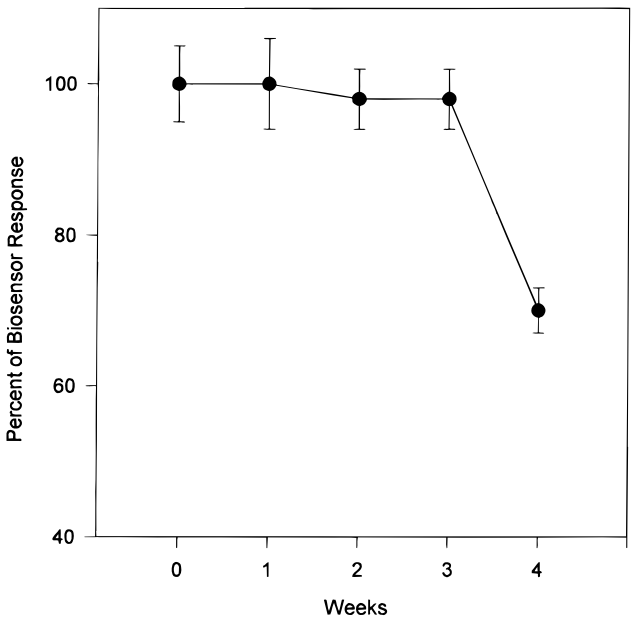

Figure 7. Long-term stability of biosensor response. Conditions: temperature, 22 °C; activity of crude extract loaded on biosensor, 2.4 U; operating potential, −100 mV versus Ag/AgCl reference. The error bars indicate the standard error for four measurements with the same electrode.

of enzyme in the biosensor. Increasing the enzyme activity or using purified enzymes may lead to increased sensitivity. However, this was not attempted in this study as the intent was to investigate if functional phenol biosensors based on crude enzyme extracts can be prepared. The resulting biosensor holds potential for more studies in this direction.

Long-Term Stability of the Biosensor. Figure 7 shows the effects of long-term operation on stability of the biosensor response. One biosensor constructed with DMF was tested four times each week to determine how

well it performed after extended periods of time. The useful lifetime of the biosensor was found to be 3 weeks with a 10% decrease in response. These results demonstrate that in comparison to other enzyme-based biosensors, the SBP biosensor is stable over 20 days of operation.

SUMMARY

A novel biosensor based on crude seed hull enzyme extracts has been prepared for monitoring phenol and hydrogen peroxide. The biosensor has demonstrated very promising results as an effective instrument to monitor both hydrogen peroxide and phenol. The advantages of this technique are very rapid response and a stable and inexpensive biosensor that can be operated for up to 3 weeks. In terms of response to phenol detection, the developed SBP biosensor is less sensitive than other previously reported biosensors based on purified SBP or HRP or on crude extracts of sweet potato (Lindgren et al., 1997; Ruzgas et al., 1995; Vieira and Faitbello, 1997), which have detection limits in the micromolar range for phenols. The main reason for this is the low activity of the enzyme extracts in our study. Further work on the improvement of biosensor sensitivity and applications for the detection of chlorophenols and other substituted phenols is in progress.

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