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Invertase inhibition based electrochemical sensor for the detection of heavy metal ions in aqueous system: Application of ultra-microelectrode to enhance sucrose biosensor's sensitivity

Dipali Bagal-Kestwal[a,1], Meena S. Karve[a,*], Bhalchandra Kakade[b,2], Vijayamohanan K. Pillai[b,2]

[a] Department of Chemistry, University of Pune, Pune, Maharashtra, India
[b] Physical and Materials Chemistry Division, National Chemical Laboratory, Pune 411008, India

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ABSTRACT

We are reporting fabrication and characterization of electrochemical sucrose biosensor using ultra-microelectrode (UME) for the detection of heavy metal ions (Hg(II), Ag(I), Pb(II) and Cd(II)). The working UME, with 25 μm diameter, was modified with invertase (INV, EC: 3.2.1.26) and glucose oxidase (GOD, EC: 1.1.3.4) entrapped in agarose–guar gum. The hydrophilic character of the agarose–guar gum composite matrix was checked by water contact angle measurement. The atomic force microscopy (AFM) images of the membranes showed proper confinement of both the enzymes during co-immobilization. The dynamic range for sucrose biosensor was achieved in the range of 1×10^{-10} to 1×10^{-7} M with lower detection limit 1×10^{-10} M at pH 5.5 with 9 cycles of reuse. The spectrophotometric and electrochemical studies showed linear relationship between concentration of heavy metal ions and degree of inhibition of invertase. The toxicity sequence for invertase using both methods was observed as $Hg^{2+} > Pb^{2+} > Ag^{+} > Cd^{2+}$. The dynamic linear range for mercury using electrochemical biosensor was observed in the range of 5×10^{-10} to 12.5×10^{-10} M for sucrose. The lower detection limit for the fabricated biosensor was found to be 5×10^{-10} M. The reliability of the electrochemical biosensor was conformed by testing the spike samples and the results were comparable with the conventional photometric DNSA method.

1. Introduction

Enzymes have attracted the attention of analytical chemists as highly efficient protein catalysts by virtue of their unique properties, such as selectivity and sensitivity towards specific substrates and functioning in aqueous media at ambient temperatures (Evtugyn et al., 1998). Enzyme inhibition is an extremely important area of research in medical as well as analytical field. Many methods used in environmental monitoring are based on enzyme inhibition, which are mainly focused on the detection of pollutants, such as pesticides, heavy metal ions and other toxic compounds. For example, lead, mercury, other heavy metal ions are extremely poisonous not only to human beings but also for all living organisms due to their inhibitory action on enzymes. Example: Pb[II] can easily react with the sulfhydral (-SH) groups in a protein.

$$\text{Protein-SH} + Pb^{2+} + \text{HS-Protien} \rightarrow \text{Protein-S-Pb-S-Protein} + 2H^{+}$$

It has been reported that the toxic effect of metal ions on enzymes is usually due to their binding to -thiol groups present in or near the active site/center producing an irreversible inhibition (Zhylyak et al., 1995). Numerous enzymes showing significant inhibition by different metal ions have already been targeted for the development of different enzyme biosensors. Verma and Singh had presented the well account of different types of sensors for the analysis of heavy metal ions including enzyme-based sensors (Verma and Singh, 2006). Biosensor based on invertase (INV) (Mohammadi et al., 2004, 2005; Bertocchi et al., 1999), urease (Ilangovan et al., 2006), acetylcholine esterase (AChE) (Ghosh et al., 2006) enzymes have been used for the detection of heavy metal ions due to their high specificity and sensitivity.

Heavy metals such as lead, cadmium and mercury, are hazardous to ecosystems and can cause serious danger to human population because of their ability to accumulate in complex organs like liver, heart, brain, etc. There are several methods for heavy metal detection including spectroscopic (AAS, AES, ICP–MS, etc.) (Wang et al., 2007; Lee and Lee, 2002) or other methods described by Anderson et al. (1996), which are based on ion selective electrode (ISE),

* Corresponding author at: Biochemistry Division, Department of Chemistry, University of Pune, Pune, Maharashtra, India. Tel.: +91 20 25601225x521; fax: +91 20 25601728.
E-mail addresses: dipalibagal@yahoo.com (D. Bagal-Kestwal), mskarve@chem.unipune.ernet.in (M.S. Karve).
[1] Tel.: +91 20 25601225x521; fax: +91 20 25601728.
[2] Fax: +91 20 25902636.

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polarography, etc. These methods are either expensive or not useful when there is a need to detect metal ions with low concentrations. Biosensors can be the ideal sensors for toxicity measurement of heavy metals, due to their inherent biological specificity.

Recently, enzyme sensors have been the target of intense research and development and have proven to be both rapid and highly selective (Karube and Nomura, 2000). The limited use of enzymatic procedure involving native enzyme, is often due to the limited availability, high price for purified enzyme preparations, and low stability of pure enzymes solutions during storage due to cumulative effect of various factors. Also these soluble biocatalysts are difficult to separate from reagents and reaction products; therefore, it is impossible to reuse them. These disadvantages can be overcome with the use of immobilized enzymes; where they are artificially bound to a water-insoluble support or with water-soluble polymers, which retain their catalytic activity (fully or partially).

Biosensors based on the principle of enzyme inhibition have been applied for a wide range of significant analytes such as organo-phosphorous pesticides (OP), organo-chlorine pesticides, derivatives of insecticides, heavy metal ions and glycoalkaloids (Hosseinpour et al., 2003). Enzymes such as alkaline phosphatase (Koncki et al., 2006; Satoh et al., 1993; Satoh and Iijima, 1995), glucose oxidase (GOD) (Kukla et al., 1999), as well as peroxidase (Veselova and Shekhovtsova, 1999, 2000), acetyl choline esterase (Jeanty et al., 2001) and invertase have been incorporated with electrochemical and optical transducer to detect the range of metal ions in the nanomolar range including arsenic, bismuth, beryllium, zinc, mercury, cadmium, lead and copper.

Invertase (glycoprotein) is an ideal enzyme for studies of inhibition owing to its low cost, good stability and high specific activity (Mohammadi et al., 2004; Trojanowicz et al., 2004). Glucose produced through catalysis of sucrose by the soluble/immobilized invertase reacts with glucose oxidase and produces H_2O_2. The activity of invertase decreases in presence of metal ions and causes lowering of product formation. The extent of inhibition is directly proportional to the concentration of metal ions in solution.

In the present studies, attempts have been made towards the development of amperometric sucrose biosensor in conjunction with co-immobilized invertase–glucose oxidase (EC: 3.1.1.26 and EC: 1.1.3.4) in a composite matrix. The use of new biopolymer composite (agarose–guar gum) as an immobilization matrix allowed the development of novel electrochemical sensor for sucrose. Agarose and guar gum were selected as the matrix for immobilization of the enzymes because of an unusual combination of its properties, which includes an excellent membrane-forming ability, high permeability toward water, good adhesion, biocompatibility, non-toxicity and high mechanical strength. Co-immobilization of INV–GOD by physical entrapment procedure without any chemical modifications was achieved in the novel composite of agarose–guar gum polysaccharide (Bagal and her co-workers (2006, 2007)). The principle of sucrose estimation by enzymatic method involves hydrolysis of sucrose by invertase enzyme releasing glucose. The glucose thus produced is oxidized by glucose oxidase releasing H_2O_2 which is amperometrically estimated by electrochemical reduction at +0.35 V using Ag/AgCl reference electrode. Thus the amount of sucrose present in the solution is determined by the rate at which hydrogen peroxide is produced.

2. Experimental details

2.1. Reagents and materials

The acid invertase was derived from watermelon fruit (*Citrullus vulgaris*) and partial purification was carried out in our laboratory (Bagal, 2007). Glucose oxidase (EC: 1.1.3.4, 100–200 Units mg^{-1}, from *Aspergillus niger*) and agarose were purchased from Sigma Chemicals (USA). Guar gum, glucose, sucrose, 4-aminoantipyrine, 3,5-dinitrosalicycilc acid (DNSA) and other chemicals were purchased form Sisco Research Laboratory (SRL), Pvt. Ltd., India and Qualigens Fine Chemicals Ltd., Mumbai, India.

Different buffers and solutions were prepared in the double glass distilled water or deionized water obtained using Milli-Q system (Millipore, Bedford, MA). All other chemicals were of analytical grade and used without further purification. The solutions were degassed with N_2 (20 min prior to start the experiment) to create oxygen free environment.

2.2. Instruments

Shimadzu UV–visible 1601 Spectrophotometer was used for the DNSA method. Electrochemical analyzer (CH Instrument 400) was used for analysis including a cyclic voltammetry in combination with potentiostat PGSTAT30 analyzer form Autolab with data acquisition system. Nanosurf® Mobile-S atomic force microscopy (AFM) instrument provided by Sinsil International, Baroda, India, was used for elucidating surface morphology of the membranes and water contact angle measurements were taken on Telescopic Rame Hart Goniometer (Model 100-00-230) instrument, USA.

2.3. Optimization of sucrose biosensor using ultra-microelectrode

In present studies, sucrose biosensor was fabricated first and the analytical performance of the sensor was checked for the novel biosensor. Under optimum conditions the applicability of the fabricated biosensor was checked for the estimation of heavy metal ions in aqueous system.

2.4. Biosensor preparation

The platinum ultra-microelectrode (25 μm) was polished with 0.05 mm alumina slurry on a piece of polishing cloth holding in the 90 °C to the polishing pad using figure of eight motion for 2–3 min. Further it was cleaned ultrasonically in distilled water and dried at room temperature. The sol–gel of agarose–guar gum was prepared from individual gel solutions of 3% agarose and 1% guar gum in 3:1 proportion at 50 °C. The ultra-microelectrode was directly dipped into the agarose–guar gum sol–gel mixture containing 10 Units of acid invertase and 15 Units of GOD. A thin membrane was formed on the electrode surface within 2 h. The leaching test for both enzymes entrapped in polymer film was carried out spectrophotometrically, as well as electrochemically. The AG sol–gel forms thin, transparent, self-adhesive membrane at the surface of ultra-microelectrode (UME). The enzyme-modified ultra-micro electrodes prepared by this procedure were stored at 4 °C in airtight container to prevent microbial attack.

2.5. Atomic force microscopy studies for membranes

Atomic force microscopy was used to measure surface height profile as a function of distance, where a cantilever tip attached spring is dragged across the sample. The AFM imaging of the enzyme containing self-assembled AG membranes was performed at different magnifications. Finally the AFM images were taken by thermostable microscope AFM instrument at 10,000 Å magnifications. The vibrating mode was selected for scanning during the investigations. Blank and enzyme entrapped membranes were prepared with same composition of the biopolymers, under identical conditions as discussed by Bagal et al. (2007).

2.6. Contact angle measurements for hydrophilicity

The hydrophilicity studies of the INV–GOD immobilized membrane were carried out by surface contact angle measurements (Wamser and Gilbert, 1992; Hosseinpour et al., 2003). Contact angle formed by a drop of distilled water on the invertase–glucose oxidase co-immobilized membrane surface was measured by Rame Hart Goniometer (Model 100-00-230). The studies were performed by sessile water drop (1.0 μL) on the AG composite membranes before and after enzyme immobilization.

2.7. Electrochemical characterization

The voltammetry studies were carried out in specially designed glass cell with volume capacity of 5.0 mL. The platinum (Pt) wire of 0.5 mm diameter served as a counter electrode (CE) and a saturated silver chloride (Ag/AgCl [3 M KCl]) act as a reference electrode (RE). Invertase–glucose oxidase modified platinum ultra-microelectrode (UME, diameter = 25 μm and total area 2.35 mm^2) was used as a working electrode (WE) for sucrose biosensor during inhibition-based studies. The scan rate from 50 to 500 mV s^{-1} (vs. Ag/AgCl) was used to measure the substrate-dependent current intensity produced during enzymatic reaction. All three electrodes were placed in tri-arm cell containing 0.2 M phosphate buffer, pH 5.5, degassed with N_2 and amperometric measurements were recorded using potentiostat.

There was no peak observed for enzymes along with matrix upon the cyclic sweep in different potential range. The AG-modified platinum UME was optimized for various parameters like enzyme concentration, influence of pH, effect of applied potential, operational as well as storage stability, etc. The pH sensitivity of the matrix-coated ultra-microelectrode was investigated in the pH range 3.5–7.5 using 0.2 M acetate and phosphate buffer solutions. The successive measurements were carried out for enzyme-modified UME to optimize number of operational cycles of the sensor. Further, it was employed for the estimation of sucrose in the solution. The modified UME was inserted in the buffered sucrose and the current intensity was measured. The calibration graph was plotted using substrate concentration [S] vs. current (μA). The linear range and lower detection limit were also calculated using calibration graph. The electrochemical probe was preserved at 4 °C and its sensitivity towards sucrose was checked after regular intervals of time.

2.8. Invertase inhibition based amperometric biosensor

2.8.1. Estimation of heavy metal ions

In order to determine heavy metal ion concentration, the fabricated electrochemical biosensor was optimized in terms of substrate concentration and enzyme activities. The degree of inhibition was measured by cyclic voltammetry. The cyclic voltammograms for enzyme-modified microelectrode were studied after incubation with each metal ion. The UME was then gently washed with assay buffer to remove traces of metal ions. UME was placed in the measuring cell containing buffered substrate to measure the output (current density) for sucrose hydrolysis. All inhibition measurements were performed with a high concentration of sucrose (1×10^{-7} M) with 10 min contact time and reaction time of 10 min. These conditions were chosen so as to obtain steady-state out put (current). The oxidation current corresponding to product formation decreased after the addition of metal ion inhibitors. The extent of inhibition was correlated with the concentration of inhibitor using following reaction sequence:

$$\text{Sucrose} + H_2O \xrightarrow[\text{Inhibitor}]{\text{Invertase}} \text{D-Glucose} + \text{D-Fructose} \quad \text{(I)}$$

$$\text{D-Glucose} + O_2 \xrightarrow{\text{GOD}} \text{D-Gluconic acid} + H_2O_2 \quad \text{(II)}$$

The experiments were conducted in two steps. In first, a known concentration of sucrose was added to electrochemical cell containing tri-electrodes and steady-state current corresponding to H_2O_2 oxidation was measured as (I_1). In second step, the sucrose biosensor (working platinum UME) was directly incubated into cell containing known concentration of inhibitors (Hg(II)/Ag(I)/Pb(II)/Cd(II)) with 10 min contact time and then washed with 0.2 M phosphate buffer pH 5.5. After this, the working electrode was placed in electrochemical cell containing substrate and assay buffer. The steady-state current was recorded for enzyme–substrate catalyzed reaction (I_2). The steady-state current (I_2) was expected to be smaller than (I_1), as heavy metal ions inhibited the enzyme action. The degree of inhibition for each metal ion was calculated by performing similar experiments. Percent inhibition was determined as follows:

$$I\% = \frac{I_1 - I_2}{I_1} \times 100\%$$

where I_1 is the resultant current due to spontaneous hydrolysis of sucrose into glucose and fructose in presence of invertase and I_2 is the reduced current recorded after 10 min contact with respective heavy metal ions (inhibitor). The oxidation current is corresponding to the decrease in product formation after the addition of metal ion inhibitors. The inhibitors used for this study include Hg(II), Ag(I), Pb(II) and Cd(II) from their respective chloride salts.

2.8.2. Response time for modified electrode

The modified platinum ultra-microelectrode was inserted in the electrochemical cell filled with sucrose (1×10^{-7} M) and assay buffer having a final volume of 5.0 mL. The electrochemical behaviour of the bare and modified electrode was observed in presence of 0.2 M phosphate buffer pH 5.5. The signal was measure at fixed applied potential +0.35 V, obtained after interaction of INV–GOD immobilized in AG membrane with sucrose in presence of assay buffer, where it plays role of carrier medium for electrons. The change in peak intensity (*i*p) was recorded till it become stable (approximately after 2 min).

2.8.3. Mercury inhibition and estimation

To evaluate the applicability of the fabricated enzyme ultra-microelectrode for sucrose, initially, the experimental conditions were standardized with mercury metal ions. For this purpose, different concentrations of mercury were checked. The sensor response was studied for the inhibitory effect of Hg^{2+} on invertase entrapped in novel composite hydrogel with respect to contact time. The mercury sample was added in the test cuvette, into which the enzyme-modified ultra-microelectrode was incubated for 10 min at room temperature. Then the electrode was washed carefully with assay buffer without disturbing the thin semi-permeable membrane present on the surface of electrode. The electrode was placed in electrochemical cell containing 0.2 M phosphate buffer pH 5.5. All inhibition measurements were performed with sucrose, with 10 min incubation time and sensor response was observed. The progress curve for the sucrose hydrolysis was recorded. A standard curve of invertase–GOD modified ultra-microelectrode for mercury was plotted by incubating electrode with different concentrations of mercury ion. Further estimation of mercury ion was done to find out the lower detection limit for mercury ion.

2.8.4. Sensitivity towards other inhibitors

Similar studies were carried out using other heavy metal ions including Ag(I), Pb(II) and Cd(II). The fabricated ultra-

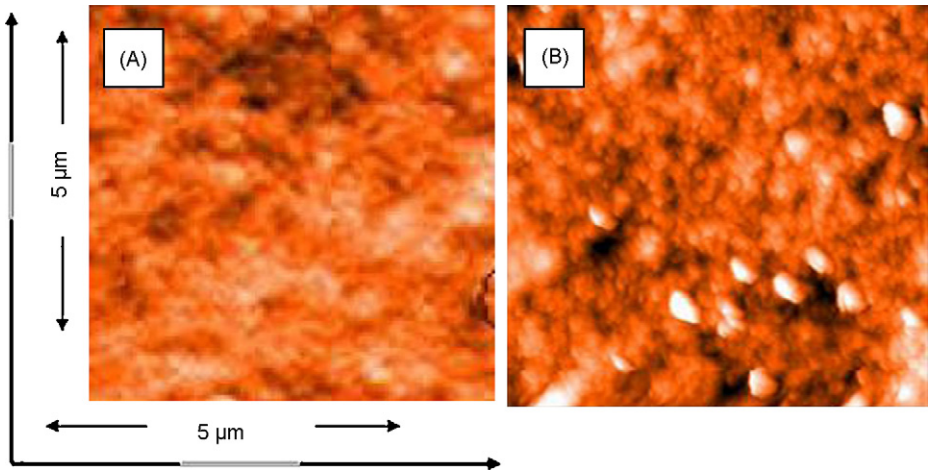

Fig. 1. AFM images of (A) blank A–G membrane and (B) A–G membrane with GOD and acid invertase.

microelectrode was used to estimate the ionic concentration of these metal ions. Same methodology was applied during studies. The values obtained for limits of detection, linear range and I_{50} were also calculated. The toxicity sequence for the invertase immobilized in the agarose guar gum membrane was calculated considering I_{10} values for each heavy metal ion.

3. Results and discussion

In present context, the novel strategy for modification and use of UME allows the detection of sucrose hydrolysis at a lower potential and achieve a current intensity response. Further depending on the analytical performance, the fabricated sucrose biosensor was used for detection of heavy metal ions such as cadmium, silver, lead and mercury. The reduction in invertase activity was monitored by UME probe to detect the traces of heavy metal ions in aqueous medium.

3.1. Preparation of AG-modified enzyme ultra-microelectrode

A film of a novel agarose–guar gum coating material was prepared on the surface of ultra-microelectrode. 3% (w/w) agarose and 1% (w/w) guar gum sol–gel was prepared individually (Bagal and Karve, 2006). The sol–gel of A and G was mixed in 3:1 proportion along with partially purified watermelon acid invertase (10 Units) and GOD (15 Units). The mixture was ultra sonicated and tip of ultra-microelectrode was directly inserted in the sol–gel of the matrix containing both enzymes. Total area of the UME coated was calculated by considering the thickness of the membrane (5 μm) and was found to be 1.429 mm^2. The UME along with stand was subjected for drying in clean room for 2 h. The surface-modified UME was used for sucrose estimation and inhibition studies.

3.2. Physical characterization

3.2.1. AFM studies of agarose–guar gum membrane

The major advantage of AFM is to carry out measurements on non-conducting substrates, and to determine particle height as well, with atomic level precision and flexibility (Wang et al., 2007). In present studies the atomic force microscopic images were obtained by using non-contact vibrating mode. The confinement of co-immobilized enzymes (acid invertase and glucose oxidase) in agarose–guar gum (AG) composite matrix was confirmed by studying the surface topography of the membranes with AFM images at 10,000× magnifications. Fig. 1A showed the AFM image of agarose–guar gum membrane without any enzyme (blank membrane) while Fig. 1B showed the A–G membrane containing invertase and glucose oxidase enzyme. The white domains of the both enzymes are visible in the image. The images indicates that both enzyme molecules are not only properly entrapped in the composite membrane but also well exposed at the surface, which can be clearly seen in Fig. 1B.

3.2.2. Contact angle measurements for hydrophilicity

Contact angle measurements are sensitive to surface modifications and by this technique one can probe any changes in surface

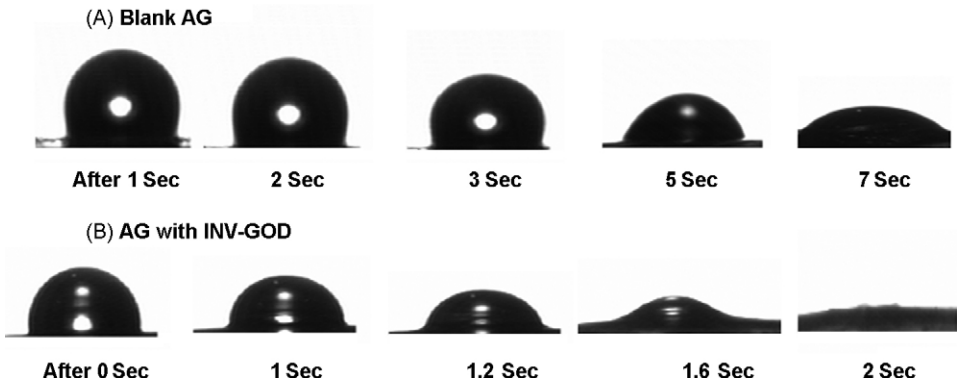

Fig. 2. Contact angle measurement for (A) blank A–G membrane and (B) A–G membrane with GOD and invertase.

hydrophobicity due to incorporation of the enzyme molecules in the composite matrix. The water contact angle measurements were done on thin membranes on a Si(III) substrate before and after immobilization. It was observed from the AFM images (Fig. 1) that the protein/enzymes was not only adsorbed but also well embedded, during physical entrapment in the agarose–guar gum composite membrane. In case if the enzymes would have decreased the hydrophilicity of the membrane then the contact angle would have increased. But it was found that the contact angle of the enzyme entrapped membrane had decreased (less than 90°) as compared to the blank agarose–guar gum membrane (Fig. 2). During the measurement the enzyme entrapped membrane showed swelling within few seconds (see Fig. 2B). Hydrophilic property of the matrix is useful in case of enzyme stability where enzymes and matrix are directly interacting with the non-functional probe (UME). The hydrogen bonding plays important role in the stabilization of enzyme on the probe during multiple use and longer storage.

3.3. Spectrophotometric studies of heavy metal ions

During our spectrophotometric investigations, it was observed that the mercury inhibits invertase irreversibly, similar to the mode of inhibition of yeast acid invertase by mercury as reported by Amine et al. (2006). Kinetic studies were carried out for Hg(II), Ag(I), Pb(II) and Cd(II). The sensitivity of invertase for each heavy metal ion was obtained by spectrophotometerically (Somogyi, 1952). The inhibition could be successfully correlated with the concentration of inhibitor. The I_{50} and I_{10} concentration defines the effective analyte concentration producing 50% and 10% enzyme inhibition respectively which was calculated from the decrease in the output intensity when the enzymes based biosensor was treated with analyte. The toxicity sequence for watermelon invertase was found to be $Hg^{2+} > Pb^{2+} > Ag^{+} > Cd^{2+}$.

Addition of metal chelating ligand, EDTA, does not restore the activity of invertase. This kind of inhibition is known to be irreversible and same was observed in case of mercury ion (Bertocchi et al., 1999; Amine et al., 2006; Mohammadi et al., 2005).

3.4. Inhibition based electrochemical sucrose sensor

The composite membrane of hydrogel without enzyme was coated on the surface of the ultra-microelectrode and after drying, the cyclic voltammogram was recorded in presence of assay buffer using scan rate 100 mV s^{-1}. The CV profiles for (A–G)–INV–GOD modified electrode was compared with bare and blank agarose–guar gum composite film coated Pt ultra-microelectrode (Fig. 3). The changes in the voltammetric profile of working UME in presence and absence of substrate are illustrated in Fig. 3. No electrochemical interference was observed due to matrix coating on ultra-microelectrode. This confirms that both agarose and guar gum were electrochemically inactive and chemically inert. For the immobilized enzymes, leaching was checked in residual solution of electrode washing, in presence of assay buffer and substrate by using DNSA photometric method. During study, no traces of INV and GOD were found in washing, ensuring proper entrapment of both glycol-proteins in polysaccharide matrix of agarose and guar gum.

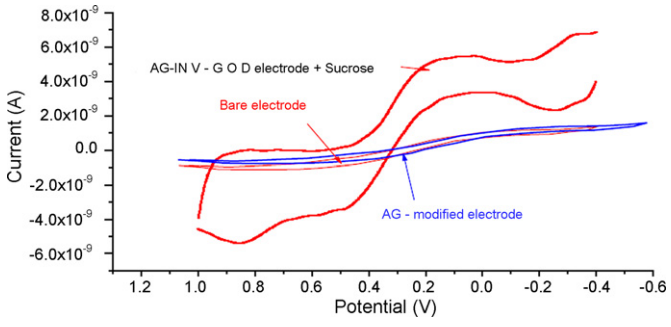

Fig. 3. Electrochemical profile for modified and unmodified platinum ultra-micro electrode.

Table 1
Type of inhibition and I_{10} values for heavy metal ion on invertase using spectrophotometric method

	Metal ions	Mode of inhibition	Dynamic concentration range (M)	I_{10} values (M)
1.	Hg(II)	Irreversible competitive	1×10^{-8} to 1×10^{-6}	1.0×10^{-8}
2.	Pb(II)	Irreversible competitive	5×10^{-8} to 3×10^{-6}	11.2×10^{-8}
3.	Ag(I)	Reversible competitive	5×10^{-7} to 5×10^{-6}	1×10^{-7}
4.	Cd(II)	Reversible competitive	5×10^{-7} to 5×10^{-6}	1×10^{-7}

3.5. Sucrose sensor based on Pt-UME

All amperometric measurements were performed at a constant applied potential of +0.35 V in order to detect the hydrogen peroxide produced by the enzyme reaction. The scan rate dependent cycles were studied in the range of 50–500 mV s^{-1}. Maximum current intensity was observed at scan rate 100 mV s^{-1}, with low background noise disturbance. The redox peak currents were proportional to the scan rate in the above range indicating the typical surface controlled quasi reversible process. The fabricated sensor was employed for sucrose estimation and its performance was checked under optimum experimental conditions as mentioned in Table 1. The cyclic voltammograms with increase in sucrose concentration have been shown in Fig. 4. The Pt ultra-microelectrode based sensor showed high sensitivity for sucrose as compared to previously fabricated sensor using disc Pt electrode (Mohammadi et al., 2002). The enzyme-modified platinum electrode can be reused for 9 cycles with 85% retention of its initial response.

3.6. Analytical performance of a sensor

The fabricated electrochemical UME biosensor was optimized for various parameters, including enzyme concentration, response time, influence of pH, effect of applied potential, operational as well as storage stability, etc. The fabricated sucrose electrode showed short response time, i.e. 15 s. This fast response may be due to the thin membrane (thickness around 5.0 μm) formed on the surface of ultra-microelectrode. Another reason for the fast response can be due to the tunable porosity of the agarose–guar gum hydrogel, which provides good semi-permeability to the self-adhesive membranes. The 1×10^{-7} M substrate concentration was used throughout the experiments. An increase in enzyme concentration can enhance the electrode response but a relatively thick enzyme layer can also acts as a diffusional barrier (Scheller et al., 1995). In order to optimize the concentration of both enzymes for better film stability and response, each enzyme (INV and GOD) concentration in the A–G composite was varied and optimized spectrophotometrically as well. The optimum pH for sucrose estimation was observed to be 5.5, where invertase enzyme as well as glucose oxidase were showing the maximum catalytic activity. The results also indicate that the co-immobilization procedure does not alter the inherent properties of both entrapped biomolecules. These observations are in good agreement with pre-

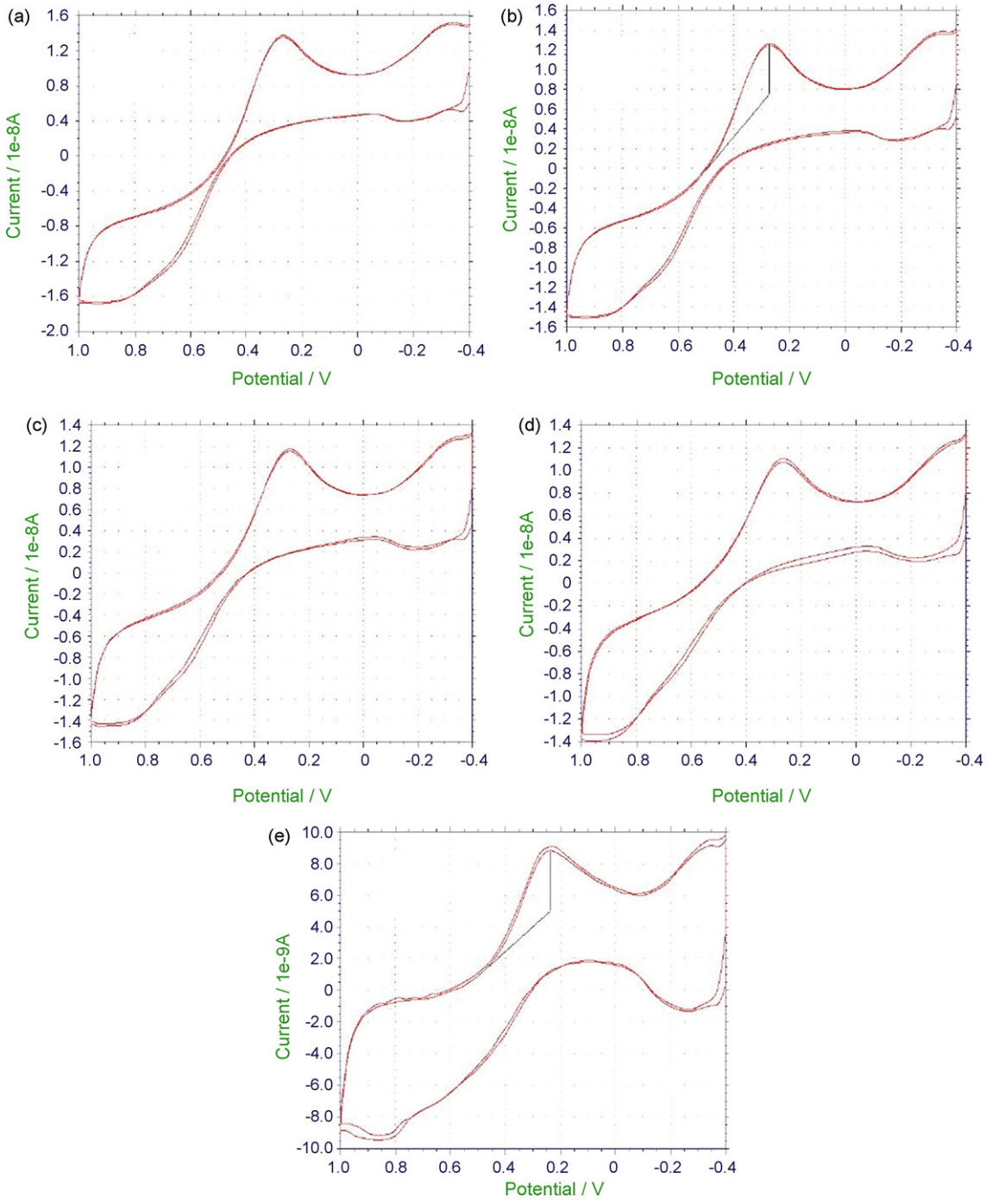

Fig. 4. Cyclic voltammogram profiles for sucrose concentration (a) 1×10^{-10} M, (b) 1×10^{-9} M, (c) 1×10^{-8} M, (d) 1×10^{-7}, and (e) 1×10^{-6} using scan rate 100 mV s^{-1}.

vious reports (Hamid et al., 1988; Bertrand et al., 1981). A pH of 5.5 was therefore selected as pH optima for the phosphate buffer (0.2 M) and used for both enzymes for further studies (Table 2.).

Table 2
Performance of sucrose sensor using platinum ultra-microelectrode

Parameter studies	Values
Bio-molecules (invertase: GOD)	10 Units:15 Units
Response time	15 s
Optimum pH	Acidic (pH 5.5, 0.2 M)
Optimum stability	9 Cycles
Sensor lifetime	57 days

The sucrose sensing ultra-microelectrode showed lifetime of 57 days with retention of 68% initial response and repeat use of 9 cycles with 85% response. The higher lifetime of the micro sensor can be attributed to the microenvironment provided by A–G hydrogel which contains approximately 90% water. The linear current response was found in the concentration range of 1×10^{-10} to 1×10^{-7} M as shown in Fig. 4. The results were comparable with earlier reports, where lipid coated enzymes were employed for the sensor (Mori et al., 1999). The fabricated sensor showed higher sensitivity as compared to PVA-SbQ modified electrode reported by Sohn et al. (1997). The lower detection in case of sucrose was experimentally possible up to 1×10^{-10} M using fabricated enzyme-modified UME.

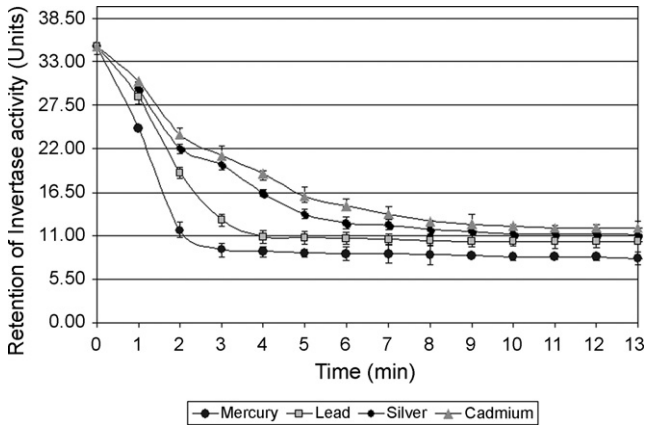

Fig. 5. Profile for contact time for invertase-modified probe during inhibition process. The pre-incubation of UME was carried out with 25×10^{-9} M mercury, 10×10^{-8} M lead, 15×10^{-8} M silver and 10×10^{-8} M cadmium respectively during study. The enzyme–substrate reaction was carried out using 50×10^{-8} M sucrose.

3.7. Invertase biosensor for mercury estimation

3.7.1. Mercury inhibition

The inhibition effect of mercury on invertase was checked amperometrically. The experimental setup was similar to that used in case of sucrose sensor as discussed earlier. The platinum ultra-microelectrode with (A–G)–invertase–GOD was used. The self-adhering A–G novel composite matrix with co-immobilized enzymes provides protection to the electrode from poisoning. The sensor requires relatively low concentration of invertase (10 Units) to produce higher sensitivity. Similarly, other three metal ions Ag(I), Pb(II) and Cd(II) were studied (Fig. 5). The linear dynamic range and lower detection limits were calculated for each heavy metal ion. During these investigations 10 Units of invertase was immobilized, to achieve maximum sensitivity with fast response of the sensor. This is in accordance with the observations reported by Myrback (1959) where low concentration of enzyme helps to improve the sensitivity towards the inhibitor.

3.7.2. Contact time for inhibition based sensor

INV–GOD based UME was incubated with metal ions at room temperature. The contact time was varied from 1 to 15 min to understand the mode and influence of metal of enzyme activity. The percentage inhibition of the invertase was calculated by considering the initial response as 100%. The contact of UME was carried out with 25×10^{-9} M mercury, 10×10^{-8} M lead, 15×10^{-8} M silver and 10×10^{-8} M cadmium respectively during studies. The invertase activity was determined in presence of 50×10^{-8} M sucrose. Our observations showed that 10 min contact time was the duration sufficient enough to inactivate the invertase to 55.5%, while further incubation seems to have no adverse effect on enzyme (as shown in Fig. 5). The gradual decrease in output (within initial 3 min) indicates that mercury and lead ions may have induced changes in the catalytically active conformation of invertase enzyme. While incase of silver and cadmium, the enzyme–inhibitor association was time dependent. Hence 10 min contact time was regulated for each metal ion during further studies.

3.7.3. Linear range and limit of detection

The linear concentration dependent response for mercury was observed in the range of 5×10^{-10} to 1.25×10^{-9} M, while for other metal ions it was as listed in Table 3. Experimentally it was possible to detect the mercury ion up to 5×10^{-10} M. The limit of detection was observed to be comparable to those reported by Bertocchi et al. (1999). While Maslowska and Leszczynska (1985), have reported the LOD for mercury 1×10^{-11} M using soluble or free invertase enzyme by amperometry. Recently Mohammadi et al. (2002), also reported mercury determination in the range of 2.5–12 ng mL^{-1} with lower detection limit 1 ng mL^{-1} using amperometric sucrose biosensor. Similar studies were carried out for lead, silver and cadmium ions. Effect of varying concentration of each inhibitor was studied to determine limit of detection, by employing the same modified ultra micro-electrode. The mode of inhibition was checked spectrophotometrically. The dynamic range, I_{10} values and lower detection limit for each heavy metal ions was also calculated. Under the experimental conditions described above, mercury could be determined with highest sensitivity as compared to other metal ions, i.e. 0.5 ng mL^{-1}, which leads to 27.5% inhibition of invertase.

Table 3
Electrochemical determination of inhibitory effect of heavy metal ions on invertase sensor response

	Metal ions	Dynamic concentration range (M)	Lower detection limit (M)
1.	Hg^{2+}	5×10^{-10} to 12.5×10^{-10}	5×10^{-10}
2.	Pb^{2+}	5×10^{-8} to 2.5×10^{-7}	3×10^{-8}
3.	Ag^{+}	5×10^{-8} to 5×10^{-7}	5×10^{-8}
4.	Cd^{2+}	2.5×10^{-8} to 12.5×10^{-8}	2.5×10^{-8}

The sequence of inhibitory effect of metal ions for invertase enzyme using the fabricated sucrose sensor was studied and compared with the photometric studies. The relative toxicity sequence of metal ions for invertase enzyme from sensor was found to be same as found in case of spectrophotometric study. The samples with known concentration (spike) of heavy metal ions were used to check the reliability of the sensor. The values obtained from the UME based sensor were found to be in good agreement with values obtained from spectrophotometric studies (DNSA method).

4. Conclusion

The constructed electrochemical sucrose biosensor exhibited good sensitivity and reproducibility for heavy metal ion detection. At fixed potential, a good linear relationship between the current intensity and sucrose concentration was achieved. As agarose–guar gum membrane provides protection to electrode, no fouling of electrode was observed with increase in concentration of analyte detection. Fast diffusion of substrate and inhibitor across the membrane was possible due to high hydrophilic property which might have contributed to the high sensitivity of the UME biosensor. The kinetic parameters of inhibition by metal ions were studied by DNSA method. A higher sensitivity was achieved amperometrically by employing platinum ultra-microelectrode instead of disc Pt electrode. The experimental results proved the enhanced analytical performance of the biosensing system in order to estimate sucrose and metal ions. The results suggest that the (A–G)-enzyme-modified UME based sucrose sensor is a promising tool, for the estimation of Hg[II] and other heavy metal ions. The results indicate that a portable, digital sensor can be fabricated especially for the determination of Hg^{2+}. Future developments will include adopting suitable methods to achieve selective detection of heavy metal ions and real onsite sample analysis.

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