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Construction of D-amino acid biosensor based on D-amino acid oxidase immobilized onto poly (indole-5-carboxylic acid)/zinc sulfide nanoparticles hybrid film

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ABSTRACT

D-Amino acid oxidase (DAAO) purified from goat kidney was immobilized covalently via N-ethyl-N-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxy succinimide (NHS) chemistry onto poly indole 5-carboxylic acid (Pin5-COOH)/zinc sulfide nanoparticles (ZnSNPs) hybrid film electrodeposited on surface of an Au electrode. A highly sensitive D-amino acid biosensor was constructed using this enzyme electrode as working electrode, Ag/AgCl as reference electrode, and Pt wire as auxiliary electrode connected through potentiostat. The biosensor showed optimum response within 3 s at pH 7.5 and 35 °C, when polarized at 0.15 V vs. Ag/AgCl. There was a linear relationship between biosensor response (mA) and D-alanine concentration in the range 0.001–2.0 mM. The sensitivity of the biosensor was 58.85 $\mu A\,cm^{-2}\,mM^{-1}$ with a detection limit of 0.001 mM (S/N = 3). The enzyme electrode was used 120 times over a period of 2 months when stored at 4 °C. The biosensor has an advantage over earlier enzyme sensors that it has no leakage of enzyme during reuse and is unaffected by the external environment due to the protective layer of poly indole-5-carboxylic acid film. The biosensor was evaluated and employed for measurement of D-amino acid level in fruits and vegetables.

1. Introduction

D-Amino acids level is increased in foods under high temperature or extreme pH or due to adulteration or microbial contamination as D-amino acids are major components of the bacterial cell wall [1–3]. Changes in D-amino acids level in brain are associated with several neurological and psychiatric diseases and thus have a major impact on the organism as a whole. Therefore, the determination of D-amino acid in biological materials is important [4–7]. A number of methods are available for determination of D-amino acids such as high performance liquid chromatography (HPLC), gas chromatography (GC), or capillary electrophoresis [8–10]. The analysis of D-amino acids by GC/HPLC is cumbersome for samples in small quantity, due to the multiple steps necessary for sample cleanup and derivatization. Thus, for routine and quality control measurements, a simple and easily applicable analytical method is required. Biosensing methods meet these requirements. A number of D-amino acid biosensors have been reported based on Prussian blue (PB) film electrodeposited onto gold electrode [11], gel matrix of hydroxyethyl cellulose [12], L- and/or D-amino acid oxidase immobilized onto rhodonised carbon electrode [13], amine-modified silica gel [14], activated controlled pore glass [15], eggshell membrane [16], Prussian blue and SWCNT [17], graphite working electrode [18], purified *Rodotorrula gracillis* DAAO mutants were covalently immobilized on an Amberzyme Oxirane support by a coupling procedure involving protein-free amino groups [19] and D-amino acid oxidase on a poly (*o*-phenylenediamine) and Nafion-modified platinum–iridium disk electrode [20]. In these biosensors, the enzyme electrode brings about the stereospecific oxidative deamination of D-amino acids catalyzed by immobilized DAAO as follows:

$$\text{D-Amino acid} + O_2 + H_2O \xrightarrow{\text{DAAO}} \alpha\text{-keto acid} + NH_3 + H_2O_2 \qquad (1)$$

The H_2O_2 generated is measured electrochemically, which is directly proportional to the concentration of D-amino acid. However, these biosensors suffer from low stability, reusability and sensitivity and thus require to be improved. The use of nanomaterials in construction of enzyme electrodes has led to the improvement in their stability, reusability, sensitivity and anti-interference ability. Among the various metal oxide nanoparticles available, zinc sulphide nanoparticles (ZnSNPs) are chemically more stable and technically better than other chalcogenides (such as ZnSe) [21,22]. These nanomaterials are considered as a promising host material due to their excellent role in catalysis owing to their

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quantum size and magnetic functionality [23–25]. Further these nanoparticles provide a favorable microenvironment for enzymes to exchange electrons directly with an electrode, thus improving the sensitivity of amperometric biosensor.

Among the various conducting polymers employed in construction of enzyme electrodes, such as polyacetylene, polythiophene and polypyrrole, polyaniline, polyindole (Pin) is a better polymer, as it can be easily polymerized into a film which possesses high conductivity and chemical stability. Further, the polyindole film has fairly better thermal stability, higher redox activity and lower degradation rate compared to polypyrrole and polyaniline film [26,27].

We describe herein the construction of an improved amperometric biosensor for determination of D-amino acid in fruits and vegetables by immobilizing covalently a DAAO (purified from goat kidney) onto polyindole-5-carboxylic acid/zinc sulphide nanoparticles (Pin5-COOH/ZnSNPs) hybrid film electrodeposited on the surface of Au electrode.

2. Experimental design

2.1. Analytical methods

Cyclic voltammetry was performed using modular electrochemical system (Autolab, model: AUT83785, manufactured by Eco Chemie, The Netherlands) equipped with PSTAT10 module and driven by a GPES software. A three electrode configuration was used with DAAO/Pin5-COOH/ZnSNPs modified Au electrode as a working electrode, Ag/AgCl as reference electrode and a Pt wire as a counter electrode. Scanning electron microscope (SEM) (Zeiss EV040) for morphological studies, UV–visible spectrophotometer (Shimadzu, Model 1700) for UV–visible spectra, transmission electron microscope (TEM) (JEOL 2100 F) for TEM study, X-ray diffractometer (XRD), (122 Rigaku, D/Max2550, Tokyo, Japan) for X-ray diffraction studies and Fourier transform infra-red spectrometer (FTIR) (Thermo Scientific, USA) for FTIR spectra were used.

2.2. Reagents

Sephadex G-100 and DEAE–Sephacel from Sigma–Aldrich, USA, D-alanine, ferrous chloride and ferric chloride from SRL, Mumbai, India were used. All other chemicals used were of analytical reagent grade. Gold electrode, fruit juices and goat kidney were purchased from local market. Double distilled water (DW) was used in all experimental studies.

2.3. Extraction and purification of D-amino acid oxidase

DAAO was purified from the goat kidney cortex region as previously described [28]. The cortex tissue was homogenized with cold 0.01 M Tris HCl (pH 8.0) in a 3:1 ratio (w/v) in a chilled pestle and mortar. The extract was filtered through a muslin cloth and the filtrate was centrifuged at 10,000 × *g* for 30 min at 4 °C. The pellet was discarded and the supernatant was collected and treated as crude enzyme. It was tested for DAAO activity as described [28] with modification and protein content by Lowry method. The enzyme was purified as previously described [28] using ammonium sulfate precipitation (0–80%), gel filtration on Sephadex G-100 and ion-exchange chromatography on DEAE Sephacel using a linear gradient of KCl (0.1–0.6 M). This resulted into 57-fold purification of enzyme with 19.5% yield. The purified enzyme had a specific activity of 184.6 units/mg.

2.4. Assay of D-amino acid oxidase

The assay of DAAO was carried out in dark as described [28] with modification. The reaction mixture containing 1.7 ml of 0.01 M Tris HCl buffer (pH 8.0), 0.1 ml of FAD (10^{-3} M), 0.1 ml of $CuSO_4$ (10^{-2} M) and 0.1 ml enzyme was pre-incubated at 37 °C for 2 min. The reaction was started by adding 0.1 ml D-alanine solution (10^{-4} M). After incubating it at 37 °C for 5 min, 1.0 ml color reagent was added and kept at room temperature for 15 min to develop the color. Absorbance was read at 520 nm against control and the amount of H_2O_2 generated during the reaction was determined from a standard curve of H_2O_2.

One unit of enzyme is defined as amount of enzyme required to catalyze the formation of 1.0 micromole of H_2O_2 from oxidation of D-alanine per min/ml under standard assay conditions.

The color reagent consisted of 50 mg 4-aminophenazone, 100 mg solid phenol and 1.0 mg horseradish peroxidase (RZ = 1.0) per 100 ml of 0.4 M sodium phosphate buffer pH 7.0. It was stored in amber colored bottle at 4 °C and prepared fresh after every week.

2.5. Construction of DAAO/Pin5-COOH/ZnSNPs modified Au electrode

2.5.1. Preparation of ZnSNPs

ZnSNPs were synthesized by chemical precipitation method [24]. Aqueous solutions of 0.5 M zinc acetate ($Zn(CH_3COO)_2{\cdot}H_2O$) and 0.5 M sodium sulphide (Na_2S) were used for synthesis of ZnSNPs. To 100 ml zinc acetate solution, sodium sulphide solution was added drop-wise under constant stirring until white colored precipitates appeared. The stirring was allowed further for 15 min at room temperature. ZnSNPs were washed several times with DW. The prepared nanoparticles were kept at 60 °C for drying.

2.5.2. Preparation of Pin5-COOH/ZnS nanocomposites

It was prepared as described [24] with modification. Dispersion was prepared by mixing of 10.0 g ZnSNPs and 1.0 ml indole monomers in 50.0 ml distilled water. Then 4.5 g $FeCl_3$ (as oxidizing agent for polyindole synthesis) was added to the ZnSNPs dispersion under continuous stirring. After 2 h of stirring, the particles were cleaned by DW, filtered, extracted for 10 h and dried at 50 °C.

2.5.3. Electrodeposition of Pin5-COOH/ZnSNPs onto Au electrode

Prior to the surface modification, the Au electrode (0.2 cm^2) was cleaned with piranha solution [H_2SO_4:H_2O_2 in 3:1 ratio (v/v)] for 20 min and then rinsed thoroughly with DW. Then the electrode was polished with alumina slurry. To electrodeposited nanocomposite film of Pin5-COOH/ZnSNPs on surface of Au electrode, the polished Au electrode was immersed into 25 ml, 0.01 M Tris HCl buffer pH 7.5 containing 0.1 g Pin5-COOH/ZnSNPs nanocomposite and then applied 10 polymerization cycles in the potential range, −0.1 V to +0.6 V with the applied scan rate of 50 mV s^{-1}. The resulting Pin5-COOH/ZnSNPs/Au modified electrode was washed thoroughly with DW to remove unbound matter and stored in dry petri plate at 4 °C until use.

2.5.4. Preparation of enzyme electrode (DAAO/Pin5-COOH/ZnSNPs/Au)

The purified DAAO (25 IU/ml) was immobilized onto the Pin5-COOH/ZnSNPs modified Au electrode surface through EDC/NHS activation chemistry by dipping modified electrode in a mixture of 0.5 ml of 0.2 M EDC and 0.5 ml 0.2 M NHS, by adjusting pH 6.0 and keeping at room temperature for 1 h. The EDC/NHS treated electrode was dipped into 1.5 ml of purified enzyme and kept at 4 °C overnight. The resulting enzyme electrode was washed with 0.01 M Tris HCl buffer, pH 7.5, 4 times to remove residual/unbound enzyme. The resulting DAAO/Pin5-COOH/ZnSNPs/Au electrode was used as working electrode. This working electrode was characterized by SEM, FTIR and EIS at different stages of its construction.

2.5.5. Electrochemical measurement

Electrochemical measurements were carried out in a potentiostat/galvanostat with a three electrode system. Prior to response measurements, the steady state current was achieved by polarizing the working electrode at 0.15 V in the reaction buffer. On the addition of 100 μl (10 mM D-alanine), it was oxidized to α-keto acid, producing an electroactive H_2O_2, which was split into $2H^+ + O_2 + 2e^-$ under a potential of 0.15 V. The flow of e^-, i.e. current, was measured in mA.

2.6. Response measurement of DAAO/Pin5-COOH/ZnSNPs/Au electrode and its optimization

The measurements of D-amino acid were performed by employing cyclic voltammetry. The biosensor was optimized by studying its optimum pH, temperature and response time. To determine optimum pH, the reaction buffers of different pH in the range, pH 5.0–10.0 at an interval of 0.5 were used (0.01 M sodium succinate for pH 5.0 and 5.5, 0.01 M sodium phosphate for pH 6.0 and 6.5 and 0.01 M Tris HCl for pH 7.0–10.0). Similarly, the optimum temperature was studied by incubating the reaction mixture at 20–50 °C, with an interval of 5 °C. Optimum response time was determined by measuring the biosensor response (mA) at different time from 2 s to 12 s with an interval of 2 s.

2.7. Amperometric measurement of D-amino acid in fruit juices

D-Amino acid (DAA) level in different fruit juices was measured by the present biosensor in the same manner as described above for response measurement under optimal working conditions, except that D-alanine was replaced with the fruit juice. The current was recorded and the amount of DAA was interpolated from the standard curve between D-alanine concentration and current (mA) prepared under the optimal working conditions (Fig. 1a inset and b).

2.8. Storage stability of enzyme electrode

The long-term storage and stability of the biosensor was investigated over 2 months, when enzyme electrode was stored dry in a refrigerator at 4 °C. The activity of enzyme electrode was measured after every one week.