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Development of an amperometric sulfite biosensor based on SO_x/PBNPs/PPY modified ITO electrode

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ABSTRACT

A sulfite oxidase (SO_x) (EC 1.8.3.1) purified from *Syzygium cumini* leaves was immobilized onto prussian blue nanoparticles/polypyrrole composite (PBNPs/PPY) electrodeposited onto the surface of indium tin oxide (ITO) electrode. An amperometric sulfite biosensor was fabricated using SO_x/PBNPs/PPY/ITO electrode as working electrode, Ag/AgCl as standard and Pt wire as auxiliary electrode connected through a potentiostat. The working electrode was characterized by Fourier transform infrared (FTIR) spectroscopy, cyclic voltammetry (CV), scanning electron microscopy (SEM) and electrochemical impedance spectroscopy (EIS) before and after immobilization of SO_x. The biosensor showed optimum response within 2 s, when operated at 20 mV s^{-1} in 0.1 M Tris–HCl buffer, pH 8.5 and at 35 °C. Linear range and minimum detection limit were 0.5–1000 μM and 0.12 μM (S/N = 3) respectively. There was good correlation (r = 0.99) between red wine samples sulfite value by standard DTNB method and the present method. The sensor was evaluated with 97% recovery of added sulfite in red wine samples and 2.2% and 4.3% within and between batch coefficients of variation respectively. The sensor was employed for determination of sulfite level in red and white wine samples. The enzyme electrode was used 200 times over a period of 3 months when stored at 4 °C.

1. Introduction

Determination of sulfite is important; particularly from biological and industrial point of view, as it is widely used as a preservative and antioxidant in food, beverages and pharmaceuticals. Sulfite oxidase (SO_x) (EC 1.8.3.1) is a metallo-enzyme, which utilizes a molybdopterin as a cofactor and belongs to a superfamily of enzymes including dimethylsulphoxide (DMSO) reductase, xanthine oxidase and nitrite reductase. The human SO_x is located in mitochondrial intermembranous space and involved in the transfer of electrons from sulfites into the electron transfer chain (ETC) via cytochrome c. Studies have shown that human serum contains sulfite concentration in range of 0–10 μM [1,2]. Food and Drug Administration (FDA), USA, has instrumented warning labels on any food containing more than 10 mg/kg or beverages containing more than 10 mg/L of sulfite [3,4]. The catabolism of sulfur containing amino acids, methionine and cysteine contributes to the bulk of the sulfite load in the body. The quantitative determination of sulfite in different types of samples has been reported employing a range of analytical techniques such as iodimetry [5], colorimetric/DTNB method [6], spectrophotometry [7–10], anodic stripping voltammetry [11], reciprocal oscillographic chronopotentiometry [12], ion chromatography [13], enthalpimetry [14], chemiluminescence [15,16], gravimetry [17], ion selective electrode [18,19], gas chromatography [20] and HPLC [21]. However, these analytical techniques are labor-intensive, time consuming sample and reagent preparations and lack precision/sensitivity. Hence development of an enzyme biosensor for detection of sulfite is of considerable interest, since it would allow fast, selective and accurate determination of sulfite, without the need for significant sample preparation. A number of sulfite biosensors have been reported viz. glassy carbon electrode coated with thin mercury film [22], polytyramine-platinized glassy carbon electrode [12], polyaniline-sulfonic acid [23], polypyrrole films [24], on conducting polyaniline film [25], self-assembled monolayer of 11-mercaptoundecanol (MU) cast on a gold electrode [26], on gold nanoparticles/chitosan/multiwalled carbon nanotube/polyaniline modified gold electrode [27]. The disadvantages of these reported biosensors include limited detection limit due to poor electron flow and low storage stability of the working electrode. Polypyrrole (PPY) is useful in construction of biosensor, because of its high conductivity, good environmental stability and large variety of applications. Composites containing PPY and any inorganic nanoparticles such as AgNPs, AuNPs, CuSNPs and prussian blue nanoparticles (PBNPs) have been synthesized. Among those inorganic materials, PBNPs have received much attention and play many important roles in the fields of magnetic molecules,

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electrochromic devices and rechargeable batteries owing to their interesting electrochemical, photophysical, and magnetic properties [28,29]. PBNPs–PPY composite film is expected to exhibit many advantages like high activity, excellent electrochemical activity and selectivity in detection of H_2O_2 [30–32]. The present work describes the construction of sulfite biosensor based on *Syzygium cumini* leaf SO_x immobilized on PBNPs–PPY electrodeposited onto ITO electrode and its application for measurement of sulfite in red and white wine samples

2. Experimental

2.1. Chemicals

Sephadex G-100 and DEAE-Sephacel from Sigma–Aldrich, St. Louis, USA, sodium sulfite, potassium ferricyanide, ferric chloride and pyrrole from Sisco Research Laboratory (SRL), Mumbai, India were used. All other chemicals used were of Analytical Reagent (AR) grade. The mature leaves of *S. cumini* (Jamun) plants growing on road side of new campus of M.D. University, Rohtak were collected, brought to the laboratory immediately in a ice bath, washed thoroughly with Double distilled water (DW), dried between folds of filter paper and stored at −20 °C until use. Indium tin oxide coated glass plate (ITO) (2 cm × 1 cm) was a gift from Metrohm India Limited. Different brands of red and white wines were purchased from market of different states of India. DW was used throughout the experiments.

2.2. Extraction and purification of SO_x from leaves of S. cumini

SO_x from frozen jamun leaves was extracted as described [33], with modification by homogenizing leaves with cold 0.1 M Tris–HCl (pH 8.0) in 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 supernatant was collected and treated as crude enzyme. It was tested for sulfite oxidase activity and protein concentration by Lowry method. Crude enzyme was purified as described using a combination of 0–80% ammonium sulfate precipitation, gel filtration on Sephadex G-100 and ion-exchange chromatography on DEAE Sephacel using linear gradient of KCl (0.1–0.6 M) [33]. Finally, the enzyme was purified by 57-fold with 19.5% yield.

2.3. Assay of SO_x

The assay of SO_x was carried out as described with modifications and based on quantification of H_2O_2 generated from sulfite, using a color reaction consisting of 4-aminophenazone, phenol and peroxidase as chromogenic system [34]. The reaction mixture containing 1.8 mL of 0.1 M Tris–HCl buffer pH 8.5, and 0.1 mL enzyme preparation was preincubated at 35 °C for 5 min. The reaction was started by adding 0.1 mL of 0.4 mM sodium sulfite. After incubation at 35 °C for 10 min in dark, 1.0 mL color reagent was added and kept at room temperature for 15 min. to develop the color. A_{520} was read and H_2O_2 content generated during the reaction was interpolated from a standard curve between H_2O_2 conc. and A_{520} prepared in 0.1 M Tris–HCl buffer, pH 8.5.

One unit of enzyme is defined as amount of enzyme required to catalyze the formation of 1.0 nmol of H_2O_2 from oxidation of sulfite per min under standard assay conditions.

Preparation of color reagent: The color reagent was prepared according to the method of Bais et al. and consisted of 50 mg 4-aminophenazone, 100 mg solid phenol and 1 mg horseradish peroxidase per 100 mL of 0.4 M sodium phosphate buffer, pH 7.0 [35]. It was stored in amber colored bottle at 4 °C and discarded after one week.

2.4. Preparation of prussian blue nanoparticles (PBNPs)

PBNPs were prepared by mixing equimolar amount of $FeCl_3$ and $K_4[Fe(CN)_6]$ aqueous solutions. 2 mL of $FeCl_3$ (2 mM) solution was poured slowly drop wise into 2 mL of $K_4[Fe(CN_6]$ (2 mM) solution under vigorous stirring. A blue coloured solution was gradually formed indicating the formation of PBNPs. Then PBNPs were subjected to sonication for about 30 min [36].

2.5. Preparation of enzyme electrode (SO_x/PBNPs/PPY/ITO electrode)

Firstly, the ITO coated glass plate was washed with DW and dried at room temperature. Then, ITO coated glass electrode was immersed into 0.1 M phosphate buffer (PB) solution (pH 7.0) containing 0.2 M pyrrole and 0.1 M KCl and subjected to cyclic voltammetry (CV) in a potential range, −0.2 to (+0.6 V) (vs Ag/AgCl) for electro-polymerization and deposition of polypyrrole on the surface of electrode (PPY/ITO). The thickness of the PPY film was controlled by the scanning times (12–15 times) in electrodeposition. Then, PBNPs were electrodeposited onto PPY/ITO electrode by immersing it into a mixture of 20 mL of 0.1 M KCl and 5 mL of PBNPs colloidal solution and applying same potential as above. After rinsing with DW, PBNPs/PPY/ITO electrode was dried in air.

The purified SO_x was immobilized onto the surface of PBNPs/PPY/ITO electrode through adsorption by layering its 100 μL solution in PB pH 7.0 containing 3.0 μg protein and keeping it undisturbed for about 12 h at 4 °C. ITO coated glass plate was finally washed with 0.1 M Tris–HCl buffer (pH 8.5) to remove unbound enzyme. The protein concentration in remaining solution and wash out solution were determined.

2.6. Characterization of enzyme electrode

The modified ITO electrode, at different stages of its construction was characterized by scanning electron microscopic (SEM) images in Scanning Electron Microscope (Make: Joel Japan, Model: JSM-6510, at Advanced Instrumentation Research Facility, JNU, New Delhi). Fourier transform infrared (FTIR) spectra in FTIR spectometer (Make: Thermoelectron, USA, Model: iS10) and cyclic voltammetric and electrochemical impedance spectroscopic (EIS) studies on potenitostat/galvanostat (Make: Autolab, Eco Chemie BV, Netherlands, Model: AUT83785 with the GPES 4.9 software) with three electrode system, SO_x/PBNPs/PPY/ITO electrode as the working electrode, a Pt wire as the auxiliary electrode and an Ag/AgCl (saturated 3 M KCl) electrode as the reference electrode. The EIS measurements were carried out in 1 mM $K_3[Fe(CN)_6]$ in the 0.1 M PB pH 7.0 at ambient temperature, at the polarization potential of 0.2 V (vs Ag/AgCl) in the frequency range 0.1–10^4 Hz and with the amplitude of 10 mV. The resulting enzyme electrode (SO_x/PBNPs/PPY/ITO electrode) was stored in refrigerator at 4 °C, when not in use. To record FTIR spectra of hybrid material deposited onto ITO electrode, it was scrapped off the ITO electrode, mixed with dried KBr and its pellet was formed by hydraulic pellet press. Then this pellet was kept into the socket of the FTIR spectrometer and its spectrum was recorded.

2.7. Response measurement of SO_x/PBNPs/PPY/ITO electrode

Cyclic voltammetric measurements were carried out in a three electrode cell containing reaction mixture 20 mL KCl as electrolyte (0.1 M), 5 mL Tris–HCl buffer (0.1 M, pH 8.5) and 0.1 mL of sulfite