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Fabrication of an amperometric ascorbate biosensor using egg shell membrane bound *Lagenaria siceraria* fruit ascorbate oxidase

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ABSTRACT

An ascorbate oxidase (EC 1.10.3.3) purified from *Lagenaria siceraria* fruit was immobilized onto egg shell membrane through glutaraldehyde coupling with 73.3% retention of its initial activity and a conjugation yield of 0.097 mg/cm^2. The membrane consisting of ascorbate oxidase was mounted over an Au electrode to construct a working electrode for ascorbate biosensor. The biosensor showed optimum response i.e. current in mA within 10 s at pH 6.0, 40 °C and 0.6 V using Ag/AgCl reference and Cu wire as auxiliary electrode. There was a linear relationship between L-ascorbic acid concentration in the range 1×10^{-5} M and 4×10^{-4} M and current. The biosensor was employed for determination of L- ascorbic acid in serum, fruit juices and vitamin C tablets. The analytical recovery of added ascorbate in sera was 98.2% and 96.7%. Within batch and between batch coefficients of variations (CV) in ascorbate of sera were <3.6% and <4.49% respectively. L-Ascorbate values obtained for fruit juices and vitamin C tablets by present method and by DCPIP (2,6-dichlorophenolindophenol) method, showed a good correlation (r = 0.993). The biosensor has advantages such as fast response time (10 s), good repeatability (200 assays) and long-term stability (50% of its initial sensitivity after 4 months of storage).

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1. Introduction

Ascorbic acid or vitamin C being reductive in nature has widespread use as an antioxidant agent in foodstuffs and soft drinks. As an antioxidant, vitamin C's primary role is to neutralize free radicals. Since ascorbic acid is water soluble, it can work both inside and outside the cells to combat free radical damages. Vitamin C is an excellent source of electrons therefore it can donate electrons to free radicals such as hydroxyl and superoxide radicals and quench their reactivity" [1]. Vitamin C plays an important role in a number of metabolic functions including the activation of vitamin B, folic acid, the conversion of cholesterol to bile acids and the conversion of the amino acid, tryptophan to the neurotransmitter, serotonin. It is used as therapeutic agent in many diseases and disorders. Vitamin C protects the immune system by reducing the severity of allergic reactions and helps fight off infections [2]. Deficiency of vitamin C is known to cause anemia, scurvy, infections, bleeding gums, muscle degeneration, poor wound healing, atherosclerotic plaques and capillary hemorrhaging, neurotic disturbances consisting of hypochondriasis, hysteria and depression followed by decreased psychomotor performances [3,4]. Among the various methods available for determination of ascorbic acid such as colorimetric [5], spectrophotometric methods [6], high performance liquid chromatography [7] and sequential injection spectrophotometry [8], electrochemical methods are considered as one of the most potential approach, because of its high sensitivity, simplicity, rapidity, sufficiently short response time and durability. However, direct oxidation of ascorbic acid at bare electrodes is irreversible and requires a high overpotential. This high overpotential results in electrode fouling, poor reproducibility, low selectivity and low sensitivity. Thus, various chemically modified electrodes have been proposed for determination of ascorbic acid such as β-cyclodextrin–ferrocene inclusion complex modified carbon paste electrode [9], glassy carbon modified with nickel(II) macro cycle containing dianionic tetraazaannulene ligand [10], nylon net membrane [11], electrochemically etched platinum microelectrode [12], multilayer films of carbon nanotubes and redox polymer on screen-printed carbon electrodes [13], polypyrrole nanowire modified electrode [14], carbon nanotube-modified carbon fiber microelectrodes [15], micelle membrane coated on both aminated glassy carbon electrode and gold electrode [16], Cu(II) zeolite-modified electrode [17] and dopamine using a poly(acriflavine)-modified electrode [18]. In all these electrodes, ascorbate oxidase has been immobilized onto various supports either through absorption or entrapment or encapsulation, which allows leakage of enzyme resulting into low stability of electrode. Covalent immobilization of enzyme not only overcomes this problem but also leads to better bimolecular activity and greater stability. We have reported a method for covalent immobilization of enzymes onto egg shell membrane, which is expected to overcome this problem. Further

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an egg shell membrane has excellent gas and water permeability, biocompatibility, low cost and stability [19]. Being an inert support, it has been used as a good support for immobilization of enzyme in biosensor construction [20].

The present report describes the construction and application of an ascorbate biosensor by immobilizing an ascorbate oxidase purified from bottle gourd fruit onto egg shell membrane and then mounting this membrane onto Au electrode.

2. Materials and methods

2.1. Reagents

Sephadex G-100, DEAE-Sephacel and glutaraldehyde (grade 1, 25%) from Sigma–Aldrich, ammonia and nickel chloride from Sisco Research Laboratory (India) were used. Leghorn white eggs were purchased from local market. All other chemicals were of analytical reagent (AR) grade. The commercial vitamin C tablets (marketed under the brand name "Lamcea and Becozyme c forte" manufactured by Bayer, EU, Turkey) and fruits (lemon, grape, orange and apple) were purchased from local market. Fresh serum samples of healthy individuals were collected from hospital of Pt. BDS University of Health & Medical Science, Rohtak and stored at −20 °C until use. Fresh green fruits of bottle gourd (*Lagenaria siceraria*) of 10–15 cm diameter were collected from nearby village during the month of June–July (30 ± 5 °C) in ice bath, washed in distilled water and stored at 4 °C until use.

2.2. Extraction and purification of ascorbate oxidase

Ascorbate oxidase was extracted and purified from fresh green fruits of bottle gourd (*L. siceraria*) using a combination of 65% ammonium sulphate precipitation, gel filtration on Sephadex G-100 and ion-exchange chromatography on DEAE-Sephacel as described in [21] with slight modification. The purified enzyme exhibited a single band in simple polyacrylamide gel electrophoresis (PAGE) using coomassie blue as protein stain, indicating its apparent homogeneity (results not given). The purified enzyme had an activity 9.6 unit/ml.

2.3. Assay of ascorbate oxidase

The assay of ascorbate oxidase was carried out as described by Oberbacher and Vines [22] with slight modification. The reaction mixture contained 290 μmol phosphate/EDTA buffer (pH 5.6), 0.5 μmol L-ascorbic acid and 100 μg of enzyme in a total volume of 3.1 ml. The blank contained 3.00 μmol of phosphate/EDTA buffer pH 5.6 and 0.5 μmol ascorbic acid in a total volume of 3.1 ml. A_{265} was read in a UV and visible spectrophotometer (Make: Shimadzu 1700, Japan). The activity of enzyme was calculated as follow:

$$\text{Activity (U/ml)} = \frac{(\Delta A_{265}/\text{min}) \times 3.1 \times \text{dilution factor}}{e \times 0.1}$$

where e = 13.386 (extinction coefficient of dehydroascorbate); total volume = 3.1; enzyme volume = 0.1 ml.

2.4. Pretreatment and activation of egg shell membrane

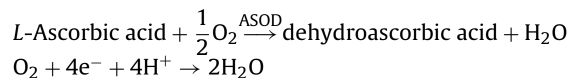
It was carried out as described in [23]. An egg was covered with 150 ml of 3 M HCl in a 1 l flask. As the acid dissolves the egg, carbon dioxide gas is liberated and white foam of calcium chloride rises slowly in the flask. When the reaction was completed egg shell membrane was carefully separated from egg after removing egg yolk. Egg shell membrane was washed with distilled water to remove all residual particles and then cut into rectangular pieces (dimension 4 cm × 2 cm). A piece of membrane was washed again with distilled water and transferred to cleaned test tube containing reagent A (10 ml of liquid ammonia and 50 mg of nickel chloride). After incubation in reagent A for 5 h, membrane was washed with distilled water to remove excess of reagent A. This pretreated membrane was mounted onto one end of Au electrode (1.5 cm × 0.05 cm) with a parafilm. Affixed egg shell membrane was treated with reagent B (2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.0) for 2 h at ambient temperature (RT). The electrode with affixed membrane was taken off from reagent B and washed many times with distilled water.

2.5. Immobilization of enzyme/preparation of working electrode

Ascorbate oxidase was immobilized on pretreated egg shell membrane through glutaraldehyde coupling as described in [23]. The enzyme (25 μl) was placed onto activated egg shell membrane and kept at 4 °C in dark for 10 h to allow covalent coupling between enzyme and membrane. The membrane was washed 4–5 times with buffer (0.1 M phosphate EDTA buffer, pH 6.0) to remove unbound enzyme. The immobilization occurred by coupling of $-NH_2$ groups on surface of enzyme with -CHO groups introduced on egg membrane through glutaraldehyde. Fig. 1 depicts the chemical reaction involved in immobilization of enzyme(s) on egg shell membrane.

2.6. Construction of amperometric ascorbate biosensor and response measurement

An amperometric ascorbate biosensor was constructed by connecting the working electrode with a silver/silver chloride (Ag/AgCl) reference electrode and Cu as auxiliary electrode through a three terminal electrometer (Keithley, 6215A/E Japan). To test the activity of the three-electrode system, it was immersed into a mixture of 2.9 ml 0.1 M phosphate–EDTA buffer, pH 5.6 and 0.1 ml ascorbic acid (0.005 M). The electrode was polarized applying different potential in the range 0.1–0.8 V and the current (mA) generated, was measured. The following electrochemical reactions occur during measurement:



$$L\text{-Ascorbic acid} + \frac{1}{2}O_2 \xrightarrow{\text{ASOD}} \text{dehydroascorbic acid} + H_2O$$
$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$$

where ASOD = ascorbate oxidase.

2.7. Optimization of ascorbate biosensor

The optimum working conditions of biosensor/kinetic properties of immobilized ascorbate oxidase were studied at 0.6 V (voltage at which maximum current was generated) and compared with those of free enzyme. To determine the optimum pH, the pH of reaction buffer was varied from pH 3.0 to 6.5 using the following buffer; each at a final concentration of 0.1 M: pH 3.0–5.0 sodium citrate and pH 5.5–6.5 phosphate–EDTA buffers. Similarly, the optimum temperature was studied by incubating reaction mixture at different temperatures ranging from 15 °C to 50 °C at an interval of 5 °C in a controlled temperature water bath. To study response time, the current was measured at 2 s, 4 s, 6 s, 8 s, 10 s and 12 s. To study the effect of substrate concentration, the ascorbic acid concentration was varied from 1 μM to 500 μM. K_m and I_{max} were calculated from Lineweaver–Burk (LB) plot.

2.8. Amperometric determination of ascorbic acid in serum and fruit

Fresh serum samples (0.5 ml) from apparently healthy persons were collected at local Pt BDS Postgraduate Institute of Medical Science hospital, Rohtak. To 0.2 ml of serum sample, 0.07 mg $NaNO_2$ and then 1 ml phosphate–EDTA buffer were added. To prepare

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