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A nylon membrane based amperometric biosensor for polyphenol determination

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ABSTRACT

A nylon membrane based amperometric biosensor employing banana fruit polyphenol oxidase (PPO) is presented for polyphenol detection. Nylon membrane was first activated and then coupled with chitosan. PPO was covalently attached to this membrane through glutaraldehyde coupling. The membrane bioconjugate was characterized by scanning electron microscopy (SEM) and Fourier Transform Infrared (FTIR) study and then mounted onto Au electrode using parafilm to construct a working electrode. Once assembled along with Ag/AgCl as reference and Pt as auxiliary electrode, the biosensor gave optimum response within 15 s at pH 7.5 and 30 °C, when polarized at +0.4 V. The response (in mA) was directly proportional to polyphenol concentration in the range 0.2–400 μM. The lower detection limit of the biosensor was 0.2 μM. The biosensor was employed for determination of polyphenols in tea, beverages and water samples. The enzyme electrode showed 25% decrease in initial activity after 150 reuses over 6 months, when stored at 4 °C.

1. Introduction

Polyphenolic compounds are natural constituents of fruits and vegetables [1] and thus greatly influence food quality and storage as well as human health [2]. However, the effect of polyphenols on human health is contradictory. Some polyphenols play role in prevention of cardiovascular diseases, cancer and diabetes [3–6], while certain members of polyphenol family are considered as endocrine disrupting compounds [7–9]. Consequently, it is of great significance to determine polyphenolic compounds to study food quality, healthcare and pollution monitoring [10,11]. The available detection methods for polyphenols include mainly chromatographic techniques such as high-performance liquid chromatography (HPLC) and spectrometry [1]. However, these methods are cumbersome and expensive as they require several operations including pretreatment of the sample and costly equipments, which make them time-consuming as well [9,12].

Immobilized enzyme based biosensors present a fast and reliable alternative owing to their simplicity, specificity, fast response and reusability, which make them cost effective [13]. Numerous biosensors have been proposed for detection of polyphenolic compounds employing polyphenol oxidase (PPO). PPO, also known as catecholoxidase or tyrosinase, is widely distributed in plant kingdom [14]. It is a copper containing metalloprotein, which is known to catalyse two types of reactions using oxygen: (i) the *o*-hydroxylation of monophenols such as tyrosine and *o*-cresol to *o*-diphenols and (ii) the dehydrogenation of *o*-dihydroxyphenols such as catechol and L-3,4-dihydroxy phenylalanine (L-DOPA) to *o*-diquinones [15–17]. Various matrices have been used to immobilize PPO such as polyethersulphone membrane [10], laponite clay coating [18], gelatin [19], carbon paste modified electrodes [20], cobalt (II) phthalocyanine (CoPc) modified cellulose-graphite composite on a polycarbonate support [21], eggshell membrane [22], ZnO nanorod clusters/nanocrystalline diamond electrode [23], 2,2′-bipyridine chloro (p-cymene) ruthenium (II)]chloride mediator complex and 1,2-diamino benzene (DAB) [24], Fe_3O_4 nanoparticle-chitosan bionanocomposite film [25], polyvinyl chloride membrane [26], menthyl monomer (MM) with pyrrole [27], polyaniline [28], 1-(4-nitrophenyl)-2,5-di(2-thienyl)-1*H* pyrrole [29], graphite screen printed electrodes modified with ferrocene [30] and thionine modified carbon paste electrode [9]. Compared to commercial enzyme, PPO purified from a plant source is economical and more stable and thus more suitable for construction of polyphenol biosensors. PPO has been purified and characterized from banana fruit as early as 1981 [31]. It has also been extracted from the leaves and stems of the banana for the production of L-DOPA [32].

Nylon is an inherently hydrophilic membrane, which is compatible with aqueous and alcoholic solutions and solvents. It is made of repeating units linked by peptide bonds (or amide bonds) and frequently referred to as polyamide. It is cationic and maintains its positive charge over a wide pH range. Nylon membrane also offers narrow pore size distribution and good mechanical rigidity. To improve the hydrophilicity and to increase the number of reac-

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tive sites (i.e. primary amino groups), the nylon membrane was activated by epichlorohydrin and then coupled with chitosan [33]. The present study, describes for the first time, the covalent immobilization of banana fruit PPO on chitosan coated nylon membrane through glutaraldehyde coupling and its application in construction of a polyphenol biosensor.

2. Materials and methods

2.1. Materials

L-DOPA from HiMedia (Mumbai, India), glutaraldehyde, polyvinyl pyrrolidone (PVP), epichlorohydrin and chitosan from SRL (Mumbai, India) and nylon membrane from Amresco, Ohio, US were used. All other chemicals were of analytical grade. Tea leaves (Brand: "Tata tea" manufactured by Tata global Beverages Limited, Kirloskar Business park, Block-C, Hebbal, Bengaluru, India. "Darjeeling" manufactured by Twining Private Limited, Kolkata, West Bengal, India. "Red label" and "Taj Mahal" manufactured by Brooke Bond, Hindustan Unilever Limited, (HUL), Backbay Reclamation, Mumbai, India) and alcoholic beverages of various commercial brands ("Vodka" manufactured by Radika Khaitan Limited, Badhali, Ambala, India. "Royal challenge" manufactured by United Spirits Limited Unit Merrut, Cant U.P, India. "Kingfisher Beer" manufactured by Millennium Beer, Industries Limited, Dharuhera, Rewari, Haryana, India. "Whisky" manufactured by Bagpiper, Mathura Road, Palwal, Haryana, India.) (All 2010 products) were purchased from local market. Water samples were collected from different sources like tap water, well and canal samples from a nearby rural area of Rohtak. L-DOPA was added into freshly prepared 0.1 M sodium phosphate buffer pH 7.0 to give a final concentration of 10 mM, for assay of PPO.

2.2. Extraction of PPO from banana fruit

Pulp of fresh ripened banana fruit (25 g) was homogenized with 100 mL 0.1 M sodium phosphate buffer (pH 7.0) containing 2.5 g of PVP-360 as stabilizer, in a liquefier for 2 min at 4 °C. The suspension was filtered through a Whatman filter paper no. 1 on ice and centrifuged at 25,000 × *g* for 30 min at 4 °C. The supernatant (crude enzyme) was collected and stored at 4 °C until used [33].

2.3. Assay of PPO

The assay of PPO in crude extract was carried out by measuring the change in absorbance (ΔA) at 475 nm in a UV visible spectrophotometer (Shimadzu, Model 1700) due to conversion of L-DOPA into dopachrome. The reaction mixture containing 100 μL of crude enzyme and 3.0 mL of 10 mM L-DOPA solution in 0.1 M sodium phosphate buffer (pH 7.0) was incubated at 25 °C [34]. Under the specified assay conditions, one enzyme unit is defined as:

$$\text{Unit activity} = \frac{\Delta A_{475}/\text{min} \times \text{total vol. of reation mixture}}{E \times \text{vol. of enzyme}}$$

[E = 3.6 (extinction coefficient of L-DOPA); total volume = 3.1; enzyme volume = 0.1 mL]

The protein concentration in PPO preparation was determined by Lowry method [35], using bovine serum albumin as standard.

2.4. Activation/pretreatment of nylon membrane

Chitosan-coated nylon membrane, which possesses a large number of reactive groups $-CH_2OH$ and $-NH_2$, was prepared by coupling chitosan onto the nylon membrane. A rectangular piece of nylon membrane (4 cm × 4 cm) was soaked into 1 M HCl (10 mL) and stirred for 24 h at room temperature. After partial hydrolysis of amide bonds, the membrane was put into a 20% epichlorohydrin solution, adding NaOH to adjust the pH of the solution to pH 11, and then, the resulting mixture was stirred for 10 h at 60 °C. The activated membrane was shaken in 10 mL chitosan solution (prepared by dissolving 0.15 g chitosan in 10 mL 1% acetic acid solution) for 1 h at room temperature. The chitosan solution was sucked and then membrane was incubated in an oven at 80 °C for 7 h. Non-covalently bound chitosan was removed by washing the membrane with 1% acetic acid solution and deionized water [33].

2.5. Immobilization of PPO onto the chitosan coated nylon membrane

The chitosan coupled membrane was activated by dipping it into a 2.5% glutaraldehyde in 0.02 M sodium phosphate buffer, pH 7.0 and kept it for 2 h at room temperature. The membrane was removed and washed thoroughly in 0.02 M sodium phosphate buffer, pH 7.0. The activated membrane was dipped into 1 mL of enzyme solution containing 13 enzymatic U and kept overnight at 4 °C for covalent immobilization of enzyme. The nylon membrane containing immobilized enzyme was washed in the reaction buffer and tested for enzyme activity.

2.6. Scanning electron microscopy (SEM) of nylon membrane

The nylon membrane (1 cm × 1 cm) with and without enzyme was subjected to scanning electron microscopy (SEM) at Chemistry Dept. of MDU, Rohtak, to confirm the immobilization.

2.7. Fourier Transform Infrared (FT-IR) study of nylon membrane

The nylon membrane (1 cm × 1 cm) before and after immobilization of enzyme was placed between two KBr disks for the mid-IR characterization, using a FTIR spectrometer (mode iS10, Thermoelectron, USA) instrument.

2.8. Construction and response measurement of amperometric polyphenol biosensor

An amperometric polyphenol biosensor was constructed by mounting PPO-nylon membrane biocomposite onto Au wire electrode (1.5 cm × 0.05 cm) with a parafilm and connecting this working electrode along with a silver/silver chloride (Ag/AgCl) reference electrode and Pt as auxiliary electrode to a three-electrode electrochemical cell system employing potentiostat/galvanostat (Autolab, Eco Chemie, The Netherland. Model: AUT83785). To measure the response of the three electrode system, it was immersed into a beaker containing mixture of 2.9 mL of 0.1 M phosphate-EDTA buffer, pH 7.0 and 0.1 mL L-DOPA (10 mM). The electrode was polarized by applying different potential in the range +0.1 to +0.6 V against Ag/AgCl. The potential, at which the maximum current generated, was noted using the linear sweep method. The following electrochemical reactions occurred during measurement:

$$\text{L-DOPA} + 1/2O_2 \xrightarrow{\text{PPO}} \text{Benzoquinone derivative} + H_2O$$

In terms of electron transfer, the reaction can be written as:

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$$

The depletion of oxygen at the electrode caused by electrochemical reaction also involves consumption of electrons, resulting in an electrochemical signal, which was directly proportional to the concentration of polyphenols in the sample.