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Short communication

Development of cholesterol biosensor based on immobilized cholesterol esterase and cholesterol oxidase on oxygen electrode for the determination of total cholesterol in food samples

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Abstract

The development of a cholesterol biosensor by co-immobilization of cholesterol esterase (ChEt) and cholesterol oxidase (ChOX) on oxygen electrode is described. The electrode consists of gold cathode and Ag/AgCl anode. The enzymes were immobilized by cross-linking with glutaraldehyde and Bovine Serum Albumin (BSA). The immobilized enzymatic membrane was attached to the tip of the electrode by a push cap system. The optimum pH and temperature of the sensor was determined, these are 6 and 25 °C respectively. The developed sensor was calibrated from 1–75 mg/dl of cholesterol palmiate and found linear in the range of 2–50 mg/dL. The calibration curve was drawn with V_i (ppm/min)(initial velocity) vs different concentrations of cholesterol palmiate (mg/dL). The application of the sensor to determine the total cholesterol in different real food samples such as egg, meat was investigated. The immobilized enzymatic layer can be reused over 30 times and the stability of the enzymatic layer was studied up to 9 weeks.

Keywords: ChEt; Cholesterol biosensor; ChOX; Food sample analysis; Stability

1. Introduction

Cardiovascular diseases in people are increasing day by day and cardiac arrest is a major cause of death world over. There are several causes for this but one of the most important reasons is hypercholesterolemia i.e. the increased concentration of cholesterol in the blood [1,2]. Cholesterol belongs to the sterol group of fats. It is present in egg yolk, dairy products, goat meat etc. Therefore, cholesterol is one of the most frequently determined analytes in clinical as well as in analysis of food samples. The development of efficient rapid analytical methods for their estimation in food and clinical samples is important. HPLC [3], gas–liquid chromatography [4,5] methods used for the determination of total cholesterol offer sensitivity and selectivity but are neither suitable for rapid nor cost effective detection. Enzymatic procedures have practically replaced the chemical methods based on the classical Libermann–Burchard reaction, used traditionally for free and total cholesterol determination [6]. Owing to the advantages of simplicity, rapidness and cost effectivity, a few cholesterol biosensors have been developed which are based on cholesterol oxidase (ChOX) and cholesterol esterase (ChEt) [7,8], cholesterol oxidase [9–14], Cytochrome P450scc [15], fiber-optic biosensor [16,6], acoustic wave [17].

Most of the above reported cholesterol biosensors were applied for clinical analysis. Application of this sensor in the field of food sample analysis is very limited. In this paper our aim is to develop a fast, economic, simple cholesterol biosensor based on immobilized ChEt and ChOX on a polycarbonate membrane attached to the tip of the oxygen electrode and use it for determination of cholesterol in food. For quality control, determination of cholesterol in food is important as food high in cholesterol may increase its level in the blood [18].

The current enzymatic methods are based on ChEt and ChOX, which catalyze the following reactions [19,20].

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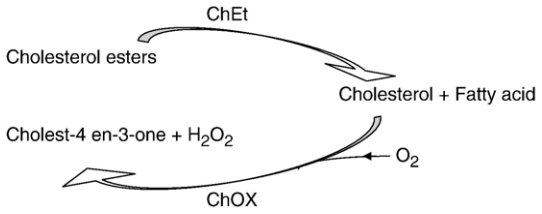

2. Materials and methods

2.1. Materials

Cholesterol esterase (EC 3.1.1.1.3,3.6 U mg^{-1}), cholesterol palmiate, nonaethylene glycol monododecyl ether used as a surfactant were purchased from Sigma, USA. Cholesterol oxidase (EC 1.1.3.6, 15 Umg^{-1}) was procured from SRL, India. Mono and di-sodium phosphate, glutaraldehyde, Bovine Serum Albumin (BSA), sodium chloride were obtained from Merck, Germany. Polycarbonate membrane (pore size 0.4 μm) was purchased from Sartorius, Germany. All the food and meat samples were purchased from local market of Jadavpur, Kolkata, India.

2.2. Apparatus

Oxygen consumption during the reactions was measured polarographically at −0.7 V using an oxygen electrode. The probe consists of a silver anode and a gold cathode (with a sensitive end of 0.5 mm diameter). This probe is connected to the DO meter (Orion, 850 plus). When a polarizing voltage (−0.7 V) is imposed across the cathode/anode, by the principles of electrode chemistry, it reduces oxygen at the cathode, causing a measurable current to flow. This current is in proportional to the difference in partial pressure of oxygen (pO_2) across the membrane, which is in turn proportional to the absolute pressure out side the membrane, assuming pO_2 inside the membrane is zero. The higher the oxygen concentration in the reaction medium, and the higher the external pO_2 the more is the current flow. The DO meter used this signal to calculate the dissolved oxygen concentration in the reaction medium in parts per million (ppm). For constant stirring of the sample a magnetic stirrer (REMI India, model 1 MLH) was used.

2.3. Preparation of cholesterol palmiate solution

Cholesterol palmiate is insoluble in water. So, it is important to make the homogeneous solution using either propanol/triton-X or nonaethylene glycol monododecyl ether. Standard solution of cholesterol palmiate was prepared by dissolving 400 mg of cholesterol palmiate followed by addition of nonaethylene glycol monododecyl ether by stirring till the solution was clear and colorless at 65 °C. Then hot phosphate buffer saline (PBS) was added and volume made up to 100 ml. The solution was allowed to reach its normal temperature before being used and was stored at 4 °C.

2.4. Immobilization of enzymes

A 10 μl (10×4) aliquot from a 40 μl mixture prepared by mixing 25 μl of BSA (10%), 10 μl of a bifunctional cross-linker, glurataldehyde (2.5%) and 5 μl of PBS containing different amount of cholesterol oxidase (2.5 μl) and 150 U ml^{-1} of cholesterol esterase (2.5 μl) was spread over the polycarbonate membrane and dried at room temperature (25 °C) for 1 h [21]. The membrane was washed thoroughly with PBS (pH 7) to remove any excess glutaraldehyde and stored in PBS at 4 °C when not used.

2.5. Development of the sensor

The immobilized enzyme membrane was mounted on the tip of the oxygen electrode with a push cap system. A rubber washer was used between the membrane layer and push cap to make it leak proof. The enzyme electrode was connected to the DO meter with a socket. The buffer saturated with air had an oxygen concentration 7–8 ppm at 25 °C. Zero oxygen concentration was obtained by an oxygen scavenging solution of sodium sulfite.

2.6. Assay procedure

The enzyme electrode was polarized for about 30–40 min every time before use. The DO meter was calibrated with the help of the manufacturer's instruction manual. Assay was started by adding different concentrations of cholesterol plamiate. In order to restore 100% oxygen saturation, the enzymatic membrane was washed several times with PBS before the following assay. Oxygen diffusion from air decreases the efficiency of the assay when carried out with enzyme in the solution [22,23]. To overcome this problem, an air sealed reaction chamber of glass (capacity 12 ml) was used. But with enzyme immobilized on an electrode, environmental oxygen does not interfere as the sensor measures oxygen at the level of the film [23]. For sample measurement, the background O_2 concentration was subtracted every time. All the experimental data given are average of 5 times repeats.

Buffer and all other solutions were prepared with double distilled water, immediately before use.

2.7. Sample preparation

Sample extraction was performed by using a modification of the method of Folch et al. [24] and Ishikawa et al. [25]. Fresh goat meat and chicken meat were gutted and the muscle tissue was cut into small pieces. About 5 g of each sample was homogenized in a mixer–grinder (REMI, India) with 50 ml of chloroform–methanol mixture. The homogenized tissue was then filtered. For sensor measurement the mixture solution was evaporated at 80 °C in water bath. After that the sample residue was dissolved in nonaethylene glycol monododecyl ether following the same procedure as the standard cholesterol palmiate preparation.

2.8. Cholesterol estimation by spectrophotometric method

By the colorimetric enzymatic kit (CHOD-PAP) method (Human ®, Germany) the desired colour of cholesterol in the