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Short communication

Biosensing approach for alcohol determination using immobilized alcohol oxidase

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Abstract

Alcohol oxidase (AOD) was immobilized in polypyrrole (PPy) and a random copolymer containing 3-methylthienyl methacrylate and *p*-vinylbenzyloxy poly(ethyleneoxide) matrices. Immobilization of enzyme was performed via entrapment in conducting polymers during electrochemical polymerization of pyrrole through the thiophene moiety of the copolymer. Three different alcohols, namely methanol, ethanol and *n*-propanol, were used as substrates. Maximum reaction rates, Michaelis–Menten constants, optimum temperature and pH values, operational stabilities and shelf life of the enzyme electrodes were investigated.

Keywords: Alcohol oxidase; Immobilization; Electrochemistry; Primary alcohols; Aldehydes

1. Introduction

The quantitative measurement of alcohol is very important for clinical and forensic purposes in the analyses of human breath and blood. Moreover, the pulp, the food and beverages industries are very much interested in fast analytical methods to control fermentation process and product quality. The determination of alcohol, especially ethanol, is important also in agricultural and environmental analysis, e.g. for assessment of ethanol at a spill site or in ground waters (Patel et al., 2001).

Many analytical methods have been developed during the years for the determination of ethanol and other aliphatic alcohols. These include the use of chemical methods such as redox titrations, colorimetric methods, specific gravity and refractive index measurements, chromatographic and spectroscopic methods. Although, some of these methods are precise and reliable, they are complex, time consuming and require previous separation processes, expensive instrumentation and trained operators. Such disadvantages can be overcome by the use of enzymatic methods. The ability of a single enzyme molecule to catalyze reaction of numerous substrate molecules also provides an amplification effect, which enhances the sensitivity of analysis. A further advantage is that most enzyme catalyzed reactions can be followed by simple widely available spectroscopic or electrochemical methods (Azevedo et al., 2005; Curulli et al., 2004).

One of the most used enzymes is alcohol oxidase (AOD) (EC 1.1.3.13). AOD is an oligomeric flavoprotein with eight identical sub-units arranged in a quasi-cubic orientation, each containing a non-covalently bound flavin adenine dinucleotide molecule (FAD) as a cofactor. AOD catalyses the oxidation of low molecular weight alcohols by molecular oxygen (O_2) into the corresponding aldehydes with the concomitant production of hydrogen peroxide (H_2O_2). During this reaction, the AOD cofactor (FAD) is first reduced to its hydrogenated form ($FADH_2$) and re-oxidized to its native form by molecular oxygen resulting in the formation of hydrogen peroxide (Azevedo et al., 2004a,b).

$$RCH_2OH + O_2 \xrightarrow{AOD} RCHO + H_2O_2$$

When coupled to the oxidation or reduction or dyes, enzymatic methods may be used in colorimetric determinations. A well-known example is the estimation of ethanol with a combination of AOD, peroxidase (POD) and dyes such as *o*-dianisidine or ABTS trade name for 2,2′-azino-di[3-ethylbenzthiozoline-6-

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sulfonic acid]. The method involves two enzymatic reactions (Verduyn et al., 1984; Majkich-Singh and Berkes, 1980).

$$\text{Substrate} + O_2 \xrightarrow{\text{AOD}} \text{RCHO} + H_2O_2$$

and,

$$H_2O_2 + o\text{-dianisidine (reduced)} \xrightarrow{\text{Peroxidase}} H_2O + o\text{-dianisidine (oxidized)}$$

Biosensors are devices capable of recovering analytical information by utilizing biological component as a part of the sensor (Besombes et al., 1997). A key factor in the construction of a biosensor is the enzyme immobilization (Zhang et al., 2003). Immobilization of enzymes makes heterogeneous catalysis possible which has great advantages: it is possible to use a single batch of enzymes repetitively and stop the reaction by the removal of immobilized enzyme from the solution. Also in many cases, the enzyme is stabilized by bonding (Cirpan et al., 2003a). Additional advantages include easy analyte determination in complex mixtures and use of small volumes. The enzyme will still be active and largely uncontaminated, thus can be used again. Also due to longer life, predictable decay rate and elimination of reagent preparation are further advantages of immobilization (Yildiz et al., 2005a).

In this study, the immobilization of alcohol oxidase was performed via entrapment within polypyrrole (PPy) and CP-co-polypyrrole matrices. CP (3-methylthienyl methacrylate and *p*-vinylbenzyloxy poly(ethyleneoxide)) was synthesized and characterized in a previous study (Yildiz et al., 2005c). The conducting polymers, PPy and CP-co-PPy were synthesized using sodium dodecyl sulfate (SDS) as the supporting electrolyte. Using three different substrates, namely methanol, ethanol and *n*-propanol, optimum conditions such as pH, temperature, kinetic parameters (K_m and V_{max}), operational stabilities and shelf life were investigated.

2. Experimental

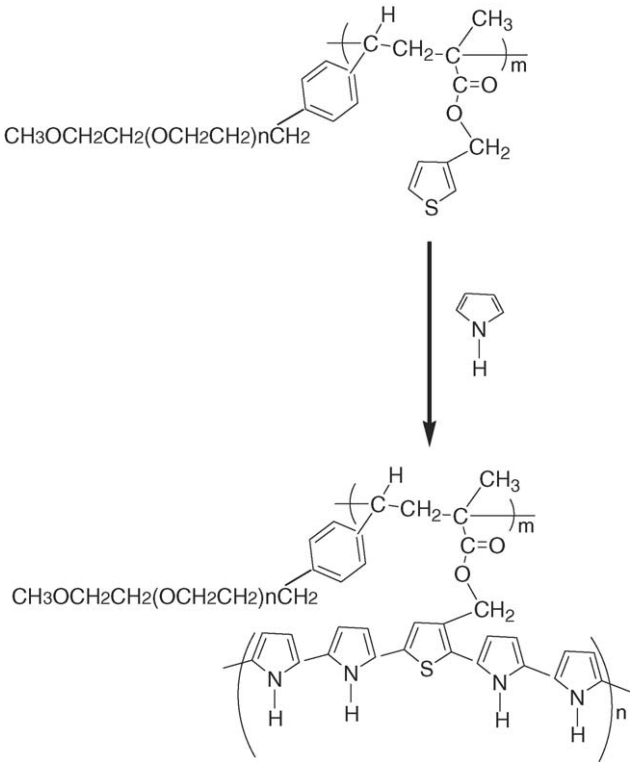
2.1. Materials

Alcohol oxidase (EC: 1.1.3.13), peroxidase, type II (EC: 1.11.1.7), *o*-dianisidine and sodium dodecyl sulfate were purchased from Sigma. Pyrrole (Merck) was distilled before use and stored at 4 °C. Sulfuric acid and hydrogen peroxide were supplied by Merck.

Potentioscan Wenking POS-73 potentiostat and Shimadzu UV-Model spectrophotometer were used.

2.2. Immobilization of alcohol oxidase in CP-co-PPy and PPy matrices

Immobilization process was achieved by the electropolymerization of pyrrole on bare and CP coated platinum electrodes. The electrolysis solution contains 5 mL AOD (100 units), 0.6 mg/mL SDS as the supporting electrolyte and 5 mL citrate buffer (pH 7.5). Polymerization reactions were carried out by applying 1.0 V. After polymerization was completed, electrodes were washed with distilled water and kept in pH 7.5 buffer at 4 °C (Scheme 1).



Scheme 1. Structure of CP-co-PPy conducting copolymer.

2.3. Determination of alcohol oxidase activity

The activity determination was performed by using a modified version of Janssen's study (Janssen et al., 1965). For free alcohol oxidase activity, alcohol oxidase activity, alcohol solutions, were placed in test tubes and incubated at 25 °C. After addition of 0.1 mL enzyme solution to 0.5 mL alcohol, enzyme and substrate were allowed to react for specific times. Then, 0.1 mL POD (U/mL) (for catalyzing the reaction between hydrogen peroxide and *o*-dianisidine (2.4 mL, 0.21 mM)) were also added. The reaction was stopped with the addition of 0.5 mL, 2.5 M sulfuric acid. Spectrophotometric measurements were performed at 530 nm and the hydrogen peroxide standard calibration curve was used in order to define enzyme activity as the oxidation of 1 μmol of alcohol to corresponding aldehyde and hydrogen peroxide per minute at pH 7.5 at 25 °C. For enzyme electrodes, the same procedure was applied.

2.4. Determination of kinetic parameters

In order to determine maximum velocity of the reaction (V_{max}) and the Michaelis–Menten constant (K_m) for each electrode, activity assay was applied for different concentrations of methanol, ethanol and *n*-propanol solutions.