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Review Article

Ethanol Determination using Immobilized Enzymes

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Abstract

Several analytical methods have been developed during the years for use in food, forensic, fuels, clinical, and pharmaceutical industries. These include the use of chemical, spectroscopic and chromatographic methods. Though these methods are precise and reliable, they are complex, expensive and time consuming. Such drawbacks could be overcome by the use of enzymatic methods. Enzymes are highly specific biological catalysts and find a widespread application in biosensors. Their nature, specificity and catalytic properties make them excellent tools for chemical analysis. Biosensors based on the enzymes such as alcohol oxidase and horseradish peroxidase are intended to be discussed in this review, concerning their applications and detection mechanism in determination of compounds..

Keywords: Enzymes; Biosensors; Alcohol oxidase; Horseradish peroxidase.

Introduction

In recent years, biosensing has become a pioneering technique in many fields, from environmental to biomedical applications, to detect various chemical and biological components [8]. These reagentless analytical devices know as biosensor differs entirely from other chemical sensors in terms of no requirement of sample processing, before or after analysis. Conventional analytical approaches such as chromatography and several other techniques may fulfill these requirements, but requires expensive instruments and are tedious and time- consuming. Hence biosensors can be a suitable option for an economic, quick and accurate analyses of different parameters [14].

According to a proposed IUPAC definition, a biosensor is an analytical device capable of providing quantitative analytical information using a biological recognition element in direct spatial contact with a transducer element. The biological recognition element interacts with the target analyte and generates a signal that could be detected by the transducer. The transducer or the detector element then converts the signal into a measurable electrical signal. Electrochemical, piezoelecric, mass selective, optical, thermometric and gravimetric transducers are normally used. Based on the biological component, biosensor is of two types- catalytic and affinity biosensors. The catalytic biosensors uses enzymes as the biological recognition element and the affinity biosensors uses specific binding proteins, receptors, whole cells, nucleic acids, lectins, antibodies or antibody- related substances [3,7,22,8,14]. Of all these biological components, enzymes find immense application in biosensor construction. This review focuses on the two enzymes extensively used in the detection of ethanol namely, alcohol oxidase and horseradish peroxidase.

Alcohol Oxidase

Alcohol oxidase (AOX), also known as ethanol oxidase [20] is an oligomeric enzyme and homo- octameric flavoprotein produced by methylotrophic yeasts during growth on methanol. It belongs to the group of glucose-methanol- choline (GMC) family and consists of eight identical sub- units arranged in a quasi-cubic arrangement, each containing a strongly bound cofactor, flavin adenine dinucleotide (FAD) molecule [2,25,23]. Though its physiological role is the oxidation of methanol, it is also able to oxidise other short chain alcohols such as propanol, butanol and ethanol to their corresponding aldehydes with a cocomitant

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release of hydrogen peroxide. Oxidation of alcohols by alcohol oxidase is catalysed by using molecular oxygen as the electron acceptor according to the following equation 1 [3, 2, 23, 25, 12].

$RCH_2OH + O_2 + AOX \rightarrow RCHO + H_2O_2$ ....(1)

Based on substrate specificity, alcohol oxidase is of four types: Short chain alcohol oxidase (SCAO), Long chain alcohol oxidase (LCAO), Aromatic alcohol oxidase (AAO) and Secondary alcohol oxidase (SAO). AOX that catalyzes the oxidation of lower chain length alcohol substrates in the range of C_1-C_8 belongs to short chain alcohol oxidase. Those that catalyze alcohol substrates with carbon chain of length above C_6 comes under long chain alcohol oxidase also known as fatty alcohol oxidase or long chain fatty alcohol oxidase. Secondary alcohol oxidase catalyses the oxidation of secondary alcohols to ketones. Cholesterol oxidase (ChOx) and polyvinyl alcohol oxidase (PAO) comes under this category. AOX such as vanillyl alcohol oxidase, veratryl alcohol oxidase falls under aryl alcohol oxidase or aromatic alcohol oxidase. These enzymes catalyse the oxidation of aromatic primary alcohol to aromatic aldehydes [20].

AOX biosensors monitoring the consumption of oxygen or hydrogen peroxide are commonly based on the electrochemical principles of detection. Clark type O_2 electrodes are widely used for this purpose. The Clark electrode consists of a platinum cathode and a silver/ silver chloride anode in contact with an electrolyte solution of potassium chloride. It is covered at the tip by a semi- permeable membrane, usually polypropylene membrane. Oxygen diffuses through the membrane to the cathode producing a measurable current proportional to the oxygen tension in solution [2]. Wen et. al., 2007 developed a biosensor containing eggshell membrane as the immobilization platform. Alcohol oxidase was immobilized on the eggshell membrane using chitosan and clark- type oxygen electrode was used as the transducer. Ethanol concentration in beverages can be determined using this biosensor. The decrease in oxygen level upon exposure to ethanol is related to the ethanol concentration [9].

Kuswandi et. al., 2014 constructed a biosensor in the form of a dip stick containing alcohol oxidase immobilized onto polyaniline film. The presence of alcohol could be determined using this biosensor via color change from green to blue when exposed to alcoholic samples. For quantitative results, the color change of the films can be scanned and analysed using image analysis software [3]. Xiao et. al., 2004 was the first to report an organic phase optical biosensor that could work in a solvent mixture of acetonitrile and phosphate aqueous buffer as well as n- hexane. It consists of an optical oxygen transducer with the spongiform of immobilized alcohol oxidase and horseradish peroxidase in silica gel/ Hydroxyethyl Carboxymethyl Cellulose- 4- tert-butylpyridinium (HECMC- PAB)/ Octadecylsilica (ODS) [27]. Alcohol oxidase biosensors is also used in the determination of aspartame in soft drinks and commercial sweetner tablets. Dilek et. al., 2004 developed a bienzyme system consisting of carboxyl esterase and alcohol oxidase. Aspartame in the soft drinks will be first cleaved by esterase to methanol, which will be then oxidised by alcohol oxidase. The oxygen consumption during this enzymatic reaction will be measured by an oxygenmeter and it corresponds to the aspartame concentration in the sample. [5]

Horseradish peroxidase

Horseradish peroxidase (HRP), a heme containing enzyme, [1, 18] exists in the roots of the horseradish plant, a hardy perennial herb cultivated in the temperate regions of the world. Owing to the commercial use of the enzyme, production of HRP from horseradish roots occurs on a relatively large scale [18]. Due to its specificity, flexibility, sensitivity in range of analyte detection and availability in pure form, it finds wide application in analytical technologies [1]. Since HRP has the ability to reduce hydrogen peroxide (H_2O_2) and some other peroxidases by an electron donor, HRP- based biosensors can be used to control and monitor these peroxidases in food products, air and water ozonisation processes, dairy, environmental, pharmaceutical industries and bleaching operations in textile and paper industries [1,24]. Donor molecules such as phenols, ferrocene, hydroquinone, Prussian blue, methylene blue, catechol, methylene green, potassium

hexacyanoferrate, nile blue, thionine, toluidine blue, thioaminosoles, aromatic amines and iodide can be used as mediators for reaction with horseradish peroxidase [17,26]. When mediators and redox enzyme couples are used together, the type of mediator should be carefully chosen, for some mediators participate in both coupled redox enzyme reactions and may generate false signals. The distracted electron transfer in turn reduces the signal accuracy and sensitivity of the biosensor. For example, the most commonly used mediator ferrocenemethanol, can react with HRP and flavin adenine dinucleotide- dependent oxidase simultaneously [15].

The principle of detection in these biosensors is quite simple. The detection mechanism depends on how the electrode is modified and whether the mediator is used or not. In case where enzyme or protein, in some modified electrodes shows direct electrochemistry, mediator is not required. Here direct electrochemistry of HRP enzyme and hemoglobin (Hb) protein plays a vital role [26]. If an HRP- modified electrode is placed in a solution containing peroxide and set at a negative potential, the peroxide oxidises the enzyme and the electrode reduces back to its native form. As a result of this, proportionality between the reduction current and the peroxide concentration will be observed. . The mechanism of reduction of a peroxide molecule at HRP-modified electrode is shown in figure 1 [1].

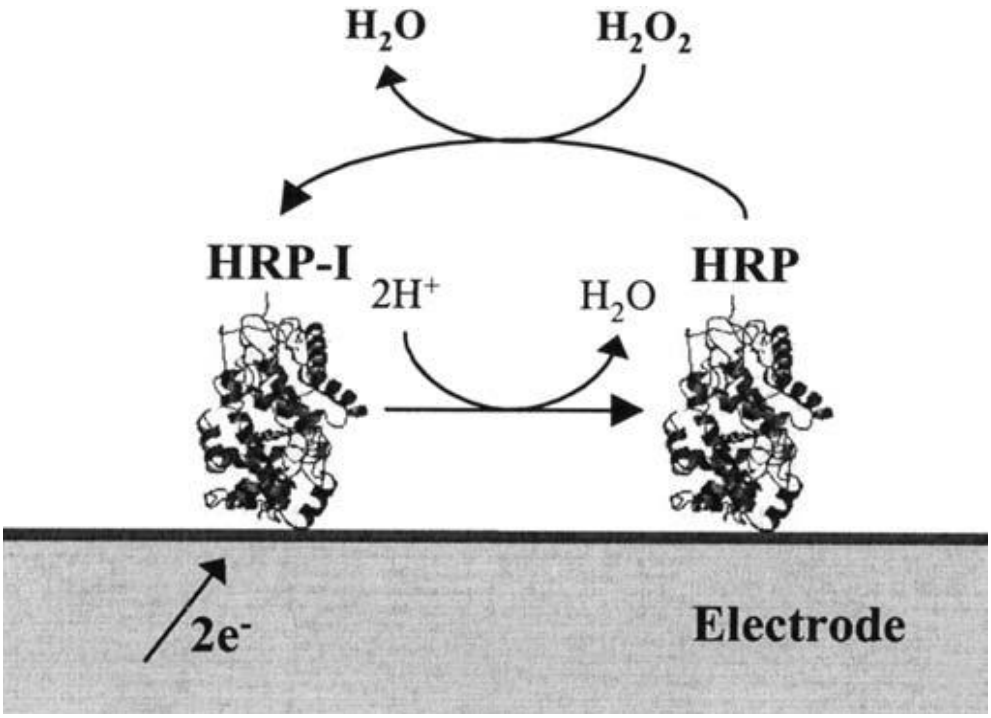

Figure 1. Direct electroenzymatic reduction of a peroxide molecule at HRP- modified electrode

Besides peroxidases, HRP biosensors can also be used for the detection of metal ions. Mambo et. al., 2014 reported a HRP/ MT-MWCNT (Horseradish peroxidase- Maize tassel-Multiwalled Carbon Nanotube) biosensor for the detection of zinc ions in aqueous solution. A decrease in enzyme activity below normal standards indicates the possibility of heavy metal poisoning [16]. Furthermore, biosensors with HRP immobilized on graphite electrodes is also been reported for the detection of phenol and its derivatives. The detection in this case depends on the electrochemical reduction of phenoxy radicals with the reducing current proportionate to their concentration in solution [21].

Apart from this, another simple technique designed for the determination of ethanol is the sequential injection analysis (SIA) system. It is a novel automated analytical technique reported to be of good performance. Alhadeff et. al., 2007 designed a SIA system with two enzymatic microreactors packed with AOX and HRP, immobilized on aminopropyl glass beads separately. When samples of alcoholic beverages are injected, the two enzymatic reactors works in line with the SIA system followed by colorimetric detection [6].

HRP in combination with other enzymes finds application in the determination of sugars

such as glucose. Nuno et. al., 2011 developed a glucose paper test strip based on the enzymes HRP and glucose oxidase (GOD). The enzymes in combination with the color generating precursors will be mixed in a solution of gelatin and then will be deposited onto a cellulose paper by drop casting method. These paper strips were tested in solutions containing different concentrations of glucose and was reported to be cheap and simple showing quick response in less than one minute [19].

Haiying et. al., 1997 fabricated a bienzymatic sensor based on the immobilization of HRP and lactate oxidase in a novel composite membrane of polyvinyl alcohol and regenerated silk fibroin. Phenazine methosulphate was used as an electron transfer mediator in this bienzymatic configuration that is sensitive to lactate. In addition to this, biosensors based on the immobilization of only HRP onto a composite membrane of polyvinyl alcohol and regenerated silk fibroin have also been developed. Methylene blue and meldola blue were reported to be efficient electron transfer mediators in this type of biosensors sensitive to hydrogen peroxide [10,11,28].

Biosensors based on the immobilization of HRP onto polyaniline film (PANI) finds to be accurate in detecting hydrogen peroxide even at lower concentrations of 0.7 nm. The detection mechanism in this type of biosensor is based on the change of conductance of PANI via enzymatic reaction of HRP. Hydrogen peroxide in the test sample first oxidises HRP, which then oxidises PANI thus resulting in the change of conductivity proportional to the target concentrations of hydrogen peroxide. The detection limit of this biosensor is one of the lowest concentrations of hydrogen peroxide that has ever been reported [13]. Dan et. al., 2010 demonstrated the feasibility of detecting sulphides via inhibition effect on an enzyme electrode based on HRP. Herein, the HRP is incorporated into the laponite/ chitosan (chit) - modified glassy carbon electrode. When this electrode is tested in phosphate buffer solution (PBS) containing hydrogen peroxide, the cathodic current is found to be increasing with increasing hydrogen peroxide concentration. This shows that it has good bioelectrocatalytic activity towards hydrogen peroxide. Upon addition of sulphide to the test solution containing hydrogen peroxide, the cathodic current decreased dramatically. As suggested by Zhao et. al., 1996, the mechanism underlying this inhibition is that sulphides were able to directly attack the heme group present in HRP, causing severe inactivation by blocking the active site of HRP and therefore inhibiting the reduced current of hydrogen peroxide [4,29].

Conclusions

Hence the studies carried on determination of compounds using AOX and HRP biosensors, along with their detection mechanism is summarized. At present, bi- enzymatic biosensors based on the immobilization of AOX coupled with HRP is proposed to be the most promising one for detection of ethanol. Commercialization of these bi- enzymatic biosensors will certainly benefit the requirements of rapid, economic and reliable methods for ethanol determination in various fields.

Conflict of interest

Authors declare there are no conflicts of interest.

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