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Optical method for detecting oxygen via the chromogenic reaction catalyzed by polyphenol oxidase

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ABSTRACT

The present work describes a method for detecting the ingress of gas phase oxygen into packed food. It uses the enzyme polyphenol oxidase (PPO)from Mushroom and Mediterranean dwarf palm. The PPO is incorporated into an indubiose film along with a non-toxic polyphenol such as gallic acid or chlorogenic acid. If exposed to oxygen, the test spot undergoes an irreversible and visible color change from pale to deep brown due to the PPO catalyzed oxidation of the respective polyphenol by oxygen. The color change can be detected visually or by spectrophotometry at 470 nm. The effect of the amount of oxygen or substrate, type of enzyme substrate, enzyme source, temperature and duration of storage on the response were studied. Air oxygen can be detected within 30 min under optimized condition. The smallest amount of oxygen that can be detected with acceptable response time (120 min) is 5%. The test is highly selective for oxygen and the detector is stable over time. The detector may be used in any application as long as the presence or absence of oxygen in a sealed space is determined prior to the application using the detector.

1. Introduction

Oxygen infiltration concern for food industry packaging's based on results of studies that show vacuum packed food product can be seriously degraded due to accidental or intentional infiltration of oxygen [1]. This may cause serious and unnoticeable deterioration that can be a serious hazard for the consumer. Several methods for oxygen detection exist [2–4]. The optical chemical sensors are based on the luminescence of excited chemical dyes which are sensitively quenched by molecular oxygen [5–8]. Other methods are based on chemicals dyes, as leuco indigo and leuco thio-indigo [9] or methylene blue [10,11] involving the oxidation process by molecular oxygen. However, there is currently a general tendency to avoid the use of the chemicals in food packaging because they have a potential risk to human health and may have a toxic effect [12,13].

Previous studies have described biological oxygen detectors [14]. Detector based on blue copper containing oxidases described by Gardiol and al [15], consists of *Rhus vernicifera* laccase enclosed with ascorbate as substrate under nitrogen gas in low-density polyethylene bag. This detector has the ability to detect oxygen in the confined space by means of a blue color resulting from oxidation by the oxygen of the reduced oxidase. The drawback of this detector is that it is liquid encapsulated in a plastic bag permeable to oxygen which increases considerably the response time to oxygen to 24 h at atmospheric air (20.9% oxygen).

On the other hand, it is known that polyphenol oxidase (PPO) enzyme catalyze the oxidation of the *ortho*-diphenols to *ortho*-quinones by oxygen molecules [16–18]. The resulting quinines products are highly reactive and polymerize to give colored substances while oxygen is reduced to water molecule (Fig. 1).

The PPO enzyme are reported for several applications; e.g., Agrifood, waste treatment, biotechnology and detectors manufacturing for phenolic substances [19,20]. In order to find an alternative to chemical oxygen detector with a low response time, herein we present a new oxygen detector using an optical absorption spectral change reaction caused by oxidation of nontoxic substrate as chlorogenic acid or gallic acid via an enzymatic catalysis based on polyphenol oxidase. The PPO was extracted and partially purified from Mushrooms (*Agaricus bisporus*) and Mediterranean dwarf palm (*Chamaehrops humilis)*. The

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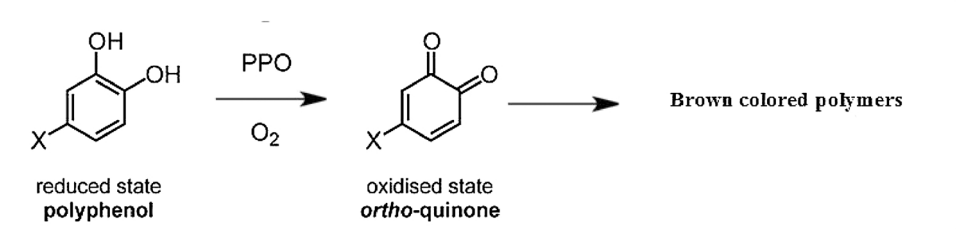

Fig. 1. Schematic representation of the PPO catalyzed oxidation of polyphenol by oxygen molecule.

preparation and the characterization of the obtained detector are described. Therefore, various tests were carried out in order to investigate the effect of oxygen concentration, the type of the enzyme substrate, the source of the enzyme, temperature and storage time on detector's response. The performances of the investigated detector are discussed considering the visual and spectrophotometric monitoring of their responses with respect to oxygen infiltration.

PPO from Mushrooms has been extensively studied and used in various applications [21,22].It should be pointed out that to the best of our knowledge the enzyme extracted from palm was studied in one variety *(Acanthophoenix rubra)*[23] while PPO from Mediterranean dwarf palm (*Chamaehrops humilis)* has never be studied. This innovative aspect has been comprehensively investigated in our laboratory, particularly the aspects concerning the kinetics and molecular characteristic of this new extracted PPO.

2. Experimental

2.1. Reagents

Chlorogenic acid, gallic acid, sodium dihydrogen phosphate, sodium hydrogen phosphate, poly (vinyl-pyrrolidone), ascorbic acid, triton X100, indubiose (3,6-Anhydro-a-L-galacto-b-D-galactan), were procured from Sigma-Aldrich, inc [www.sigmaaldrich.com]. Enzyme polyphenol oxidase (PPO) was extracted and partially purified from Mushrooms and from Mediterranean dwarf palm.

2.2. Instruments

A domestic blender and a centrifuge (Sigma 3–18 K) were used for the enzyme extraction and purification. The evolution of the absorbance as a function of time was monitored using a UV-MINI 1240 Shimadzu spectrophotometer. A16-megapixel CCD digital camera (Canon S 500) was used for the photographic documentation.

2.3. Enzyme extraction and purification

Enzymes polyphenol oxidase PPO was extracted from Mushrooms and Mediterranean dwarf palm, and then partially purified. All the steps for the enzyme extraction were performed at 4 °C, unless otherwise stated. To prepare the partially purified enzyme, 20 g of finely chopped Mushroom or Mediterranean dwarf palm were homogenized for 3 min in a blender with 100 mL of chilled phosphate buffer (0.1 M, pH 6.8) containing ascorbic acid (100 mM) and 0.5%(w/v) of poly (vinyl-pyrrolidone). The homogenate was collected through cheese cloth and the filtrate centrifuged at 16,000*g* for 30 min. The proteins in the supernatant were collected than fractionated with solid ammonium sulfate and the precipitate of 30–80% saturation was collected by centrifugation at 15,000*g* for 30 min. The pellet was re-dissolved in buffer solution (100 mM phosphate buffer, pH 6.8) and dialyzed at 4 °C against phosphate buffer for 24 h with 4 changes of the water during dialysis. Protein in dialysis was estimated by employing the method described by Bradford [24].

2.4. Measurement of the PPO activity

The activity of the enzyme was tested by UV–vis spectrometry at 470 nm. The activity was assayed in 3 mL of reaction mixture, 1 mL of enzyme extract, 1 mL of substrate and 2 mL of phosphate buffer solution (100 mM). A unit of enzyme activity was defined as the change of 0.001 in the absorbance value per min under the conditions of the assay [25–27].

2.5. Determination PPO specificity

The PPO substrate specificity was performed using catechol, chlorogenic acid and gallic acid at varying concentrations for determining the best activity of the enzyme. Michaelis constant (K_M) and maximum velocity (V_{max}) values of each enzyme for each substrate were calculated from a plot of 1/V against 1/[S] by the method of Lineweaver and Burk [28]. All measurements were taking at room temperature (nearly 25 °C) unless otherwise stated.

2.6. Preparation of detectors

A 2.5 w/v% solution of the hydrogel in phosphate buffer solution (100 mM) was prepared. Then, the solution was heated at 50 °C for 3 min. Under nitrogen atmosphere, 1 mL of the enzyme solution (0.45 mg mL^{-1}) and 1mLof the substrate (30 mM) are mixed with 2 mL of the liquid indubiose hydrogel. The mixture can be cast on the PET support by dip coating (1 mm of thickness) or placed in a vacuum within a container impervious to oxygen and allowed to solidify at ambient temperature. Four detectors were prepared;B1 and B2 based on Mushroom PPO, gallic acid and chlorogenic acid incorporated into the indubiose hydrogel respectively. And two others, B3 and B4 detectors, based on Mediterranean dwarf palm PPO, gallic acid and chlorogenic acid incorporated into the indubiose hydrogel respectively.

2.7. Enzyme detectors activity

The activity of the enzymes used in detectors was tested by UV–vis spectrometry. The measurement was taking using a spectrophotometer vacuum. The activity was assayed in 3 mL of reaction mixture; 1 mL enzyme, 1 mL substrate and 2 mL of hydrogel. A unit of enzyme activity was defined as the change of 0.001 in the absorbance value per min under the conditions of the assay [25–27]. The type and concentration of enzyme substrate, enzyme source, the temperature and duration of storage on the enzyme detectors activity were then studied.

2.8. Statistical analyses

Statistical analyses of all experimental data on PPO activity for different parameters were done with Microsoft Office Excel 2010. All assays were performed in three replicates and values were expressed as mean ± SD.