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Analytical Methods

Measurement of polyphenol oxidase activity using optical waveguide lightmode spectroscopy-based immunosensor

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ABSTRACT

Polyphenol oxidase (PPO) is an important quality index during food processing involving heat-treatment and sensitive determination of PPO activity has been a critical concern in the food industry. In this study, a new measurement of PPO activity exploiting an optical waveguide lightmode spectroscopy-based immunosensor is presented using a polyclonal anti-PPO antibody that was immobilized *in situ* to the surface of a 3-aminopropyltriethoxysilane-treated optical grating coupler activated with glutaraldehyde. When analysed with a purified PPO fraction from potato tubers, a linear relationship was found between PPO activities of 0.0005607–560.7 U/mL and the sensor responses obtained. The sensor was applicable to measurement of PPO activity in real samples that were prepared from potato tubers, grapes and *Kimchi* cabbage, and the analytical results were compared with those obtained by a conventional colorimetric assay measuring PPO activity. When tested for long-term stability, the sensor was reusable up to 10th day after preparation.

1. Introduction

Polyphenol oxidase (PPO, EC 1.14.18.1) is a tetramer that contains four atoms of copper per molecule, and binding sites for two aromatic compounds and oxygen. The enzyme catalyses the *o*-hydroxylation of monophenols to *o*-diphenols. It can also further catalyse the oxidation of *o*-diphenols to produce *o*-quinones (Mayer, 2006; Worthington Biochemical Corporation, 2013). Browning of fruits and vegetables, and processed products including juices is an important indicator for quality deterioration and is caused by rapid polymerisation of *o*-quinones into black, brown or red pigments called polyphenols. However, PPO-induced browning is not always an undesirable reaction; the familiar brown colour of tea, coffee and cocoa is developed by enzymatic browning by PPO. Conventionally, PPOs are classified into monophenol oxidases (tyrosinases) and *o*-diphenol:oxygen oxidoreductases (catechol oxidases). A mixture of these enzymes is present in nearly all plant tissues, and can also be found in bacteria, fungi, insects and animals (Mayer, 2006; Sugumaran & Lipke, 1983). Due to importance of PPOs in the food industry, many studies dealing with PPO measurement have been reported. Most of them exploited colorimetric methods to determine PPO activity, and used natural and synthetic substrates such as L-3,4-dihydroxyphenylalanine (L-DOPA) and diazo derivatives of phenol (Fan et al., 2011; Gauillard, Richard-Forget, & Nicolas, 1993; Haghbeen & Wue, 2003). In addition, activity measurements using optical and amperometric biosensors (Akyilmaz, Yorganci, & Asav, 2010; Li, 2010), and nondestructive prediction of PPO activity by visible near-infrared hyperspectral imaging (Gaston, Frias, Cullen, O'Donnell, & Gowen, 2010) have been reported. Nonetheless, development of more sensitive analytical measures for PPO activity is still required to conduct quality control for enzymatic browning reactions more efficiently, hence aiding produce management decision makers in the food industry (Gaston et al., 2010).

Main advantages of labelled immunosensors that exploit the complex formation between antigen and antibody, and use enzyme or fluorescent labels for signal generation in noncompetitive or competitive format have reportedly been known as intrinsic high-sensitivity and versatility in system setup (Lin & Ju, 2005; Osipov, Zaitseva, & Egorov, 1996). On the other hand, they are labile to interference caused by colouring substances in samples and are, in most cases, complex in measurement procedure (Kim, 2002). In contrast, label-free immunosensors that do not require probe molecules for signal generation measure changes in physical parameters like refractive index as results of immune response (Kim, 2002; Oh, Kim, Park, Lee, & Choi, 2004). They are normally simple in measurement procedure and inert to interference found

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in the case of the labelled counterparts (Kim, Park, & Kim, 2004). Optical waveguide lightmode spectroscopy (OWLS)-based immunosensor that exploits science of light guided in structures smaller than wavelength of the light has recently been developed as a device of integrated optics (Vörös et al., 2002). As a label-free technique for studying processes at the solid/liquid interface, it has been reported to have sensitivity even higher than those for already reported label-free techniques of ellipsometry (Benesch, Askendl, & Tengvall, 2000; Elwing, 1998), scanning angle reflectometry (Huetz et al., 1995) and SPR (Homola, Yee, & Gauglitz, 1999). Therefore, it is strongly urged that OWLS-based techniques be applied to biological processes that require use of immunoanalytical devices.

The aim of this study is to develop a highly sensitive OWLS-based immunosensor that is able to determine PPO activity through measurement of the complex formation between an immobilized anti-PPO antibody and PPO in a sample. For this purpose, a flow-type immunosensor system that had a He–Ne laser source emitting one monochrome (λ = 632.8 nm) was constructed. Then, main characteristics of the immunosensor were first described in details.

2. Experimental

2.1. Transducer and reagents

An optical grating coupler (OGC) sensor chip (OW 2400) for use as transducer was acquired from MicroVacuum (Budapest, Hungary). The diffraction grating of the sensor chip had a surface relief depth of ~20 nm, grating periodicity of 2400 lines/mm, and grating area dimensions of ~2 mm (length) and 16 mm (width). Refractive index (n_F) and thickness (d_F) of the waveguide layer under the diffraction grating were 1.77 and 170–220 nm, respectively. Dimensions of the substrate glass slide under the waveguide layer that had a refractive index (n_S) of 1.53 were 48 mm (length), 16 mm (width) and 0.55 mm (thickness). An amino-terminal silane compound, 3-aminopropyltriethoxysilane (APTES), and glutaraldehyde that were used for silanization and the following activation of the sensor chip, respectively, were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were the products of Sigma–Aldrich or guaranteed reagents from various suppliers and double distilled water was used throughout the study.

2.2. Preparation of anti-PPO antibody

The preparation of whole PPO from potato tubers was conducted in *Escherichia coli* BL21(DE3) as an insoluble inclusion body as follows. That is, PPO cDNA was amplified by PCR that was carried out with oligonucleotide primers designed against the known sequence in the NCBI database. To express the PPO gene, recombinant plasmid pET-PPO was transformed into BL21(DE3). The transformant was grown in LB medium that contained 50 μg/mL of kanamycin until the optical density at 600 nm reached 0.8. Isopropyl thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM thereafter. BL21(DE3) cells that harboured pET-PPO showed an extra protein band after induction with IPTG. The PPO expressed in *E. coli* was resuspended in lysis buffer that comprised urea to solubilise the insoluble inclusion body. The soluble supernatant obtained was purified by His-affinity column chromatography packed with Ni–NTA resins and the eluted major peak fractions in which PPO protein was present in several milligrams as weight basis were used for production of the antibody.

The anti-PPO antibody was prepared, making use of the PPO purified, as described previously (Moon, Kim, & Kim, 2006) and reactivity of the prepared antibody was confirmed as follow by an ELISA in which a yellow–orange product that was produced by reacting *o*-phenylenediamine (OPD) substrate with HRP was measured at 492 nm (Bovaird, Ngo, & Lenhoff, 1982; Ryu, Jang, & Kim, 2009). Fifty microlitres of a purified PPO solution that was diluted to the concentration of 100 ng/well with a coating buffer (0.1 M carbonate buffer, pH 9.6) were added to a 96-well plate. The plate was then incubated at 37 °C for 2 h. After discarding the coating solution, 200 μL of 3% non-fat skim milk were dispensed to the plate to block the unoccupied well surface. After incubating at 37 °C for 1 h, the blocking solution was discarded. The plate was washed once with 0.1 M Tris-buffered saline (pH 7.4, containing 0.1% Tween 20, TBS-T) and blotted on Kimwipes to remove the remaining solution. Fifty microlitres of the antibody solution or control serum at each dilution were added to the plate and then it was incubated at 37 °C for 1 h. After washing the plate three times with 300 μL of TBS-T, 50 μL of a diluted HRP-conjugated secondary antibody solution were added to the plate and it was incubated at 37 °C for 1 h. The plate was washed five times with TBS-T and blotted as described above thereafter. Fifty microlitres of a substrate solution that contained OPD and H_2O_2 in 100 mM citrate–phosphate buffer were added to the plate. After incubating the plate for 10 min, 100 μL of a stop solution (0.05 M H_2SO_4) were added to the plate and the absorbance at 492 nm was measured with a microplate reader.

2.3. Preparation of analyte

PPO for use as analyte was isolated from potato as follows. After washing with tap water, periderm portion of potato tubers was cut off and 100 g of the corresponding portion were minced finely and frozen quickly under liquid nitrogen. The frozen sample was crushed using a Waring blender and then was added with 400 mL of 25 mM Tris–HCl (pH 7.0, comprising 20 mM L-ascorbic acid). The resultant suspension was treated with a Polytron homogenizer (PT 300, Kinematica AG, Lucerne, Switzerland) for 1 min and then was filtered through Miracloth. The filtrate was centrifuged at 12,000 rpm at 4 °C for 30 min. The resultant supernatant was treated with 22.6% $(NH_4)_2SO_4$, adjusted to pH 7.0 with 5 M NaOH, stirred slowly for 30 min and centrifuged at 12,000 rpm at 4 °C for 30 min. The precipitate was suspended with 5 mL of 25 mM Tris–HCl (pH 7.0), treated with a Dounce homogenizer and centrifuged at 12,000 rpm at 4 °C for 5 min. The supernatant was loaded on a column that was packed with Sephadex G-50 resins and was equilibrated by passing 30 mL of 25 mM Tris–HCl (pH 7.0). Elution was conducted with the same buffer solution at 1.5 mL/min and 30 fractions that had 1.5 mL of eluate were collected.

PPO activity in the collected fractions was measured colorimetrically as follows exploiting L-DOPA as substrate. A mixture that included 1.4 mL of 10 mM L-DOPA and 100 μL of an enzyme solution was reacted at 20 °C for 3 min. Absorbance at time zero and after 3 min were measured at 475 nm in triplicate. One unit of enzyme was defined as the amount of enzyme that increased 0.001 of absorbance reading per minute. The enzyme activity of PPO5 fraction from the above elution was 5607 U/mL.

Protein contents in the collected fractions were determined by microprotein assay of Bradford (1976) as follows. Fifty milligrams of Coomassie brilliant blue G250 were dissolved in 25 mL of ethanol. To the dye solution, 50 mL of 85% H_3PO_4 were added, and the resultant mixture was diluted to 500 mL using distilled water and was filtered to prepare Bradford working buffer. To 5 mL of the buffer solution, aliquots of 100 μL of standard BSA solutions (0–100 μg/100 μL) and appropriately diluted PPO solutions were added individually. After swirling gently, the resultant mixtures were incubated at 20 °C for 5 min and absorbance at 595 nm were