Biosensors and Bioelectronics 81 (2016) 39–45

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A novel bi-enzyme electrochemical biosensor for selective and sensitive determination of methyl salicylate

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ARTICLE INFO

Article history:
Received 5 November 2015
Received in revised form
29 January 2016
Accepted 30 January 2016
Available online 17 February 2016

Keywords:
Agricultural biosensor
Bi-enzyme electrode
Methyl salicylate
Alcohol oxidase
Horseradish peroxidase

ABSTRACT

An amperometric sensor based on a bi-enzyme modified electrode was fabricated to detect methyl salicylate, a volatile organic compound released by pathogen-infected plants via systemic response. The detection is based on cascadic conversion reactions that result in an amperometric electrochemical signal. The bi-enzyme electrode is made of alcohol oxidase and horseradish peroxidase enzymes immobilized on to a carbon nanotube matrix through a molecular tethering method. Methyl salicylate undergoes hydrolysis to form methanol, which is consumed by alcohol oxidase to form formaldehyde while simultaneously reducing oxygen to hydrogen peroxide. The hydrogen peroxide will be further reduced to water by horseradish peroxidase, which results in an amperometric signal via direct electron transfer. The bi-enzyme biosensor was evaluated by cyclic voltammetry and constant potential amperometry using hydrolyzed methyl salicylate as the analyte. The sensitivity of the bi-enzyme biosensor as determined by cyclic voltammetry and constant potential amperometry were 112.37 and 282.82 μA cm^{-2} mM^{-1} respectively, and the corresponding limits of detection were 22.95 and 0.98 μM respectively. Constant potential amperometry was also used to evaluate durability, repeatability and interference from other compounds. Wintergreen oil was used for real sample study to establish the application of the bi-enzyme sensor for selective determination of plant pathogen infections.

1. Introduction

Agricultural economic losses due to pest and pathogen infections amount to $40 billion annually in the U.S. alone (Roberts et al., 2006). Minimizing crop damages through proper disease management practices demand new technologies for detecting infections at very early stages. Currently there is no rapid detection method available to detect the existence of plant diseases on the field. Among the various off-field methods explored for the detection of pathogenic diseases in crops, profiling of voltaic organic compounds (VOCs) released by plants has been identified as an useful route to non-destructively analyze the sample. The chemical signature of infected plants contains unique information related to the nature of the infection and hence can be used as a reliable marker to identify pathogenic infections in crops. An infected plant would produce different amount of VOCs as opposed to a healthy plant (Fang et al., 2014). The VOCs in infected plants are produced through various biosynthetic pathways, including the octadecanoid pathway leading to fatty-acid derived green leaf volatiles (GLVs), monoterpenes, diterpenes, sesquiterpenes, isothiocyanates and a large diversity of aromatic metabolites (Schoonhoven et al., 2005). Among the various compounds in the volatile signature of plants, methyl salicylate (MeSA) is released in large quantities during pathogenic infections and infestation and therefore is a suitable target compound (marker) for detecting biotic stresses of plants. Plants produce MeSA through Shikimate biosynthesis pathway, during a biotic stress event such as the pathogenic infection and herbivorous infestation. For instance, the production of MeSA was observed from *Tetranychus urticae* infested lima beans (De Boer and Dicke, 2004; De Boer et al., 2004; James and Price, 2004; Pickett et al., 2006). MeSA production was also reported from maize and pepper infected by *Fusarium* and *Phytophthora capsici* respectively (Buttery et al., 1969; Piesik et al., 2011). MeSA is an allelochemical that is released not just at the site of pathogen infection but throughout the plant through a systematic response (Dudareva et al., 2006; Pichersky et al., 2006). It is one of the key markers for volatile based detection of fungal diseases such as fruit blight, leaf blight, leather rot etc., which

Abbreviations: AOD, alcohol oxidase; CNT, carbon nanotube; CV, cyclic voltammetry; DMF, dimethylformamide; GC–MS, gas chromatography-mass spectrometry; GC, glassy-carbon; GLV, green leaf volatile; HRP, horseradish peroxidase; LOD, limit of detection; LOQ, limit of quantification; MeOH, methanol; MeSA, methyl salicylate; PB, phosphate buffer; PBSE, pyrenebutanoic acid succinimidyl ester; RDE, rotating disc electrode; RSD, relative standard deviation; SA, salicylate; VOC, volatile organic compound

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http://dx.doi.org/10.1016/j.bios.2016.01.095
0956-5663/

primarily affect the cucurbit crops. Therefore it is a suitable target analyte (marker) for development of biosensors to detect pathogenic diseases in crops. Moreover, the hydrolyzed form of MeSA (consisting methanol) can be amperometrically detected on an enzymatic bioelectrode, providing the possibility of highly selective quantitative detection based on bio-electrochemical redox reactions. Although gas chromatography-mass spectrometry (GC–MS) is the most commonly used technique to study the volatile signature of infected plants, this technique requires the sample to be collected in the field and analyzed elsewhere in a laboratory (Ewen et al., 2004; Lui et al., 2005; Prithiviraj et al., 2004; Vikram et al., 2006). Moreover the high cost of the instrumentation and complexity of the analysis prohibit it from being used in the field for real time monitoring of plant diseases (Fang and Ramasamy, 2015). On the other hand amperometric electrochemical sensors such as the one described in this work, offer many unique advantages for this application due to the following characteristics: accuracy, rapid detection, robustness, non-invasive detection, selective and ultra-low detection limits (Bakker, 2004). They cost less and require no skilled analysts to infer the results.

In this article, we report the successful development of a bi-enzyme based amperometic biosensor for selective determination of methyl salicylate. The biosensor platform is a glassy-carbon electrode, the surface of which is modified with multiwalled carbon nanotubes (CNTs). The CNT acts as both immobilization support for the enzymes and as amperometric transducer. The bio-recognition element consists of two enzymes namely alcohol oxidase (AOD) and horseradish peroxidase (HRP) immobilized on a matrix of multiwalled CNTs. The immobilization was achieved through an established molecular tethering approach developed and extensively demonstrated by us for other bio-electrochemical systems (Calkins et al., 2013; Lau et al., 2012; Ramasamy et al., 2010; Umasankar et al., 2014; Umasankar and Ramasamy, 2013). As illustrated in Fig. 1, the detection is carried out in three steps. First, MeSA is hydrolyzed in potassium hydroxide (KOH) to form potassium salicylate (SA) and methanol (MeOH). The pH was adjusted to 7.6 by adding phosphoric acid. Next the enzyme alcohol oxidase (AOD) converts methanol into formaldehyde via its native biochemical reaction, during which a simultaneous reduction of O_2 to H_2O_2 takes place (Ozimek et al., 2005; Patel et al., 1981). The third step involves the bio-electrochemical reduction of H_2O_2 to water using the second enzyme horseradish peroxidase (HRP), which results in an amperometric signal for detection on the electrode (Akkara et al., 1991; Ghindilis et al., 1997; Veitch, 2004). The amperometric signal is proportional to the concentration of the hydrolyzed MeSA in the electrolyte. The setup offers a simple bi-enzyme biosensor for the detection of an important plant allelochemical namely methyl salicylate.

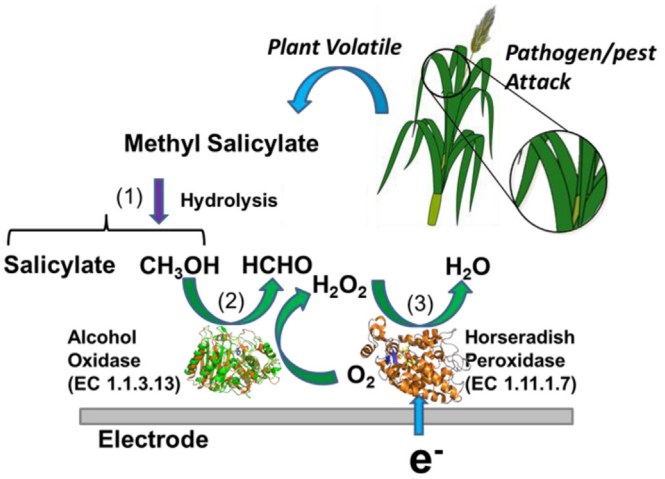

Fig. 1. Schematic illustration of methyl salicylate detection by bi-enzyme modified electrode. The process begins with the hydrolysis of methyl salicylate to form salicylate and methanol (1), oxidation of methanol and production of hydrogen peroxide (2) and direct electron transfer from electrode to hydrogen peroxide by horseradish peroxidase (3). Not drawn to scale.

2. Materials and methods

2.1. Materials

Alcohol oxidase (EC 1.1.3.13) from *Pichia pastoris* was purchased from Sigma-Aldrich and used as received. Horseradish peroxidase (specific activity 281 U/mg) was purchased from Calbiochem Inc. Multiwalled carbon nanotube (CNT) was obtained from DropSens Inc. Pyrenebutanoic acid succinimidyl ester (PBSE) was purchased from AnaSpec Inc. (Fremont CA). Dimethylformamide (DMF) was purchased from Acros Organics Inc. Chemicals for the interference study such as *cis*-3-hexenol, hexyl acetate and *cis*-hexenyl acetate were obtained from TCI America (Portland, OR) and used as received. Wintergreen oil purchased from Piping Rock Health Products was used as obtained for the real sample study. Methyl salicylate (MeSA) was used as received from Sigma-Aldrich Inc. All other chemicals used in the work were of the analytical grade. 100 mM phosphate buffer (PB) (pH 7.6) was used as the electrolyte for all experiments. All the aqueous solutions were prepared using 18.2 MΩ nano pure de-ionized (DI) water. Electrolyte (buffer) solutions were oxygenated by purging with pure oxygen for 15 min before each experiment.

2.2. Apparatus

Cyclic voltammetry (CV) and constant potential amperometry were performed using CHI 920 c potentiostat. A conventional three-electrode system consisting a Pt wire as the counter electrode and 3 M Ag/AgCl as the reference electrode was used for electrochemical measurements. The working electrodes were glassy carbon (GC) electrode, modified multiwalled CNT or a glassy carbon rotating disk electrode (RDE) from Pine Instrument Inc. All experiments were carried out at 25 ± 2 °C.

2.3. Electrode preparation and electrochemical measurement

GC and RDE electrodes were surface cleaned by polishing on a polishing pad with 0.05 μm alumina polishing powder before each experiment. The electrodes were then cleaned using ultrasonic cleaner and rinsed by DI water to remove the fine alumina powder adhered to the electrode surface. The CNT suspension was prepared by ultrasonication of 1 mg of multiwalled CNTs in 1 mL DMF for 1 h. The CNT modified electrodes were prepared by drop casting 8 μL (in 8 steps of 1 μL) for GC electrode and 12 μL (in 3 steps of 4 μL) for RDE followed by drying at 70 °C. CNT modified electrodes were placed on the ice and allowed to cool down before 2 and 4 μL of 10 mM PBSE in DMF were added on GC and RDE respectively. The electrodes were incubated for 15 min to allow the formation of non-covalent linkage between CNT and PBSE. Then the electrodes were rinsed by DMF and 100 mM PB (pH 7.6) sequentially to remove excessive PBSE (Ramasamy et al., 2010). HRP solution was prepared by dissolving 5 mg HRP in 1 mL 20 mM PB (pH 7.6). The bi-enzyme solution was simply prepared by mixing 5 μL of alcohol oxidase solution and 5 μL HRP solution. 10 μL of bi-enzyme solution was drop casted on the electrodes and incubated on ice for 30 min for enzyme immobilization. The electrodes were rinsed with 100 mM PB (pH 7.6) to remove any unimmobilized enzyme. For CV measurements, the potential range used was from 0.2 V to 0.7 V at a scan rate of 20 mV s^{-1} with a