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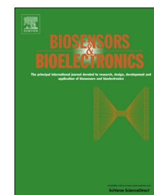

Detection of methyl salicylate using bi-enzyme electrochemical sensor consisting salicylate hydroxylase and tyrosinase

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ABSTRACT

Volatile organic compounds have been recognized as important marker chemicals to detect plant diseases caused by pathogens. Methyl salicylate has been identified as one of the most important volatile organic compounds released by plants during a biotic stress event such as fungal pathogen infection. Advanced detection of these marker chemicals could help in early identification of plant diseases and has huge significance for agricultural industry. This work describes the development of a novel bi-enzyme based electrochemical biosensor consisting of salicylate hydroxylase and tyrosinase enzymes immobilized on carbon nanotube modified electrodes. The amperometric detection using the bi-enzyme platform was realized through a series of cascade reactions that terminate in an electrochemical reduction reaction. Electrochemical measurements revealed that the sensitivity of the bi-enzyme sensor was $30.6 \pm 2.7\ \mu A\ cm^{-2}\ \mu M^{-1}$ and the limit of detection and limit of quantification were 13 nM (1.80 ppb) and 39 nM (5.39 ppb) respectively. Interference studies showed no significant interference from the other common plant volatile compounds. Synthetic analyte studies revealed that the bi-enzyme based biosensor can be used to reliably detect methyl salicylate released by unhealthy plants.

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1. Introduction

The rapid increase in world population has put enormous pressure on food and agriculture industry (Ingram, 2011; Keinan and Clark, 2012; Smil, 2001). Demand for food is projected to double by 2050, which requires that agricultural and food production must be increased to meet that demand (Godfray et al., 2010). Agricultural productivity is directly dependent on minimizing the crop losses and improving overall quality. The economic losses to agriculture due to pathogenic diseases of crops could be attributed to excessive and unnecessary spraying of chemicals (which increases cost), untimely application of fungicides (leads to crop damage) and lack of suitable technology to warn about the onset of pathogenic diseases. All of these can be addressed by a rapid detection technology that is capable of detecting diseases in early stages. Precision agriculture with detailed information about severity and specific location of a pest or pathogen infection can be realized through early detection systems. The commonly available methods for identifying infections in crops are the molecular biology based direct methods (i.e. phytopathogens detection) such as polymerase chain reaction (PCR), immunofluorescence (IF), fluorescence *in-situ* hybridization (FISH), enzyme-linked immunosorbent assay (ELISA) and flow cytometry (FCM) (Fang and Ramasamy, 2015; Sankaran et al., 2010), and indirect methods (i.e. phytodisease symptom detection) such as thermography, fluorescence and hyperspectral imaging (Mahlein et al., 2012). Among the other less explored indirect methods of plant disease detection, volatile profiling of the infected plants have been suggested as a useful strategy to selectively identify the type and severity of crop infections as plants release specific volatile compounds during a biotic stress event such as fungal infections (Fang et al., 2014; Laothawornkitkul et al., 2008). Commonly used techniques for volatile organic compound (VOC) analysis such as gas-chromatography mass-spectrometry (GC–MS) and head-space solid phase micro-extraction (HS-SPME) confine the usage mostly to the lab and are prohibitively expensive for on-field use (Kessler and Baldwin, 2001; Pawliszyn, 1999; Schmelz et al., 2003; Zhang and Pawliszyn, 1993). On the other hand, electrochemical biosensors have been largely overlooked for this application. Electrochemical biosensors are portable, can provide reliable real-time measurement and possess other attributes such as high accuracy, short

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response time, high sensitivity and specificity and low limit of detection (LOD) (Bakker, 2004).

Methyl salicylate (MeSA) was discovered as one of the most distinct VOCs released by stressed plants through Shikimate biosynthesis pathway (Kessler and Baldwin, 2001; Loake and Grant, 2007). For example, MeSA was observed to be released by pepper plants upon infection by a fungal pathogen *Phytophthora capsici* (Buttery et al., 1969), by tobacco plants during the infection of tobacco mosaic virus (TMV) (Seskar et al., 1998), and by soybean plants during the infection of soybean aphid (Zhu and Park, 2005). Unlike other green leaf volatiles (GLVs), MeSA is an allelochemical that can be produced not only at infection site but throughout the plant (stem, root, leaf etc.), which makes it easier to detect (Dudareva et al., 2006; Pichersky et al., 2006). Previous studies indicate that detection of methyl salicylate can be achieved either by direct electrochemical reaction of salicylate (the hydrolyzed form of methyl salicylate) (Cascalheira et al., 2004). With the presence of salicylate hydroxylase, consumption of NADH, O_2 and generation of CO_2 can also be used for salicylate detection (Neto et al., 1999). In addition, in our previous work, we reported the application of a bi-enzyme biosensing platform based on alcohol oxidase and horseradish peroxidase enzymes as recognition molecules for MeSA detection through the detection of methanol, a hydrolysis product of MeSA (Fang et al., 2016). In this work, a more selective enzyme combination for bi-enzyme biosensor based on salicylate hydroxylase and tyrosinase was used to improve the sensitivity and prevent unwanted cross-reactions that may result in false positive signals in our previous bi-enzyme system.

Salicylate, a main compound formed after hydrolysis of MeSA, can be electrochemically detected using salicylate hydroxylase (SH) as the recognition element with high selectivity. The enzyme is immobilized through an in-house developed tethering chemistry that binds the enzyme to the multi-walled carbon nanotubes on the surface of glassy-carbon electrodes. The CNT provides high surface area for enhanced sensitivity while at the same time, provides electronic conductivity inside the biosensor platform. SH is an FAD-dependent monooxygenase that converts salicylate to catechol in the presence of NADH and oxygen (Katagiri et al., 1965; Yamamoto et al., 1965). Although salicylate acts as the natural substrate for SH, other pseudo-substrates such as benzoate derivatives can also be catalyzed by SH. This issue is addressed by employing a second enzyme – tyrosinase (TYR) as a part of the recognition element, in order to build an enzyme cascade that provides highly selective MeSA detection on the electrode. The reaction scheme of the enzyme cascade and the mechanism behind electrochemical detection are illustrated as steps 1–4 in Fig. 1. Salicylate produced from the hydrolysis of methyl salicylate (step 1 in Fig. 1) reacts with SH and generate catechol as the intermediate (step 2) (Yamamoto et al., 1965). Catechol can be further oxidized enzymatically by TYR to produce 1,2-benzoquinone (step 3) (Rassaei et al., 2012). The biochemically generated 1,2-benzoquinone can then be electrochemically reduced to catechol by the electrode thereby regenerating catechol (step 4) (Enache and Oliveira-Brett, 2011). Therefore, the amperometric detection of salicylate can be realized through measuring the reduction current of 1,2-benzoquinone. Since the MeSA detection is realized through salicylate (see Fig. 1) and that the methanol formed during hydrolysis of MeSA does not impact the MeSA detection (as found in our previous work) (Fang et al., 2016), the hydrolyzed form of MeSA, i.e. salicylate was used in this work as the analyte for fundamental studies.

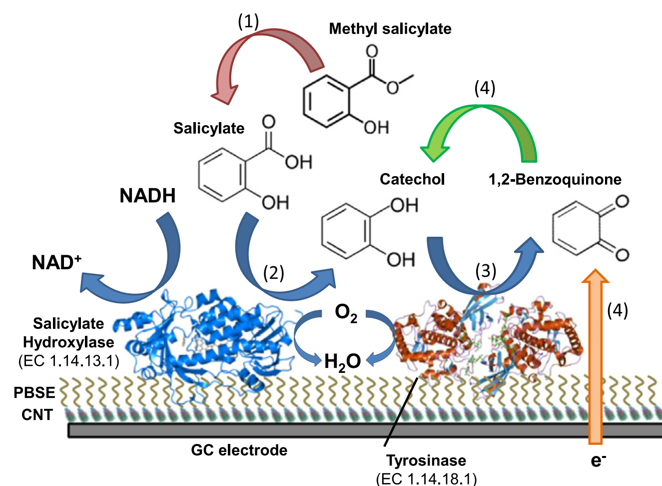

Fig. 1. Schematic illustration of methyl salicylate detection on bi-enzyme (salicylate hydroxylase and tyrosinase) based carbon nanotube and PBSE modified biosensor. Methyl salicylate was hydrolyzed manually to generate salicylate and methanol (1). Salicylate, the main analyte was catalyzed by salicylate hydroxylase to generate catechol in presence of NADH and oxygen (2). Catechol is oxidized by tyrosinase to form 1,2-benzoquinone (3). The detection of methyl salicylate is finally realized by measuring the reduction of 1,2-benzoquinone to catechol on the electrode surface (4).

2. Materials and methods

2.1. Materials

Tyrosinase (E.C. 1.14.18.1) derived from mushroom (lyophilized powder, $\geq$ 1000 unit/mg solid), methyl salicylate and farnescene were purchased from Sigma-Aldrich and used as received. Humulene and trimethylbenzene were obtained from Aldrich for the experiments. Multiwalled carbon nanotubes (MWCNTs) were obtained from DropSens Inc. 1-pyrenebutanoic acid succinimidyl ester (PBSE) was purchased from AnaSpec Inc. (Fremont CA). Dimethylformamide (DMF), salicylate and NADH were purchased from Acros Organics. FAD and dichlorobenzene were purchased from Alfa Aesar and Eastman respectively. Methanol and phosphoric acid were obtained from Fisher Scientific. All reagents used in this project were analytical grade. 0.1 M 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 deionized (DI) water. Solutions were oxygenated by purging with purified oxygen for 15 min before each experiment.

2.2. Recombinant synthesis of salicylate hydroxylase

Salicylate hydroxylase enzyme is not commercially available and therefore was synthesized recombinantly in this work. Gene *nahG* that codes salicylate hydroxylase in *Pseudomonas putida* can be found from previous publications (You et al., 1991). The *nahG* gene was codon optimized for expression in *Escherichia coli* and synthesized by GenScript with histidine tag (6X) at N- terminal of the sequence. The recombinant plasmid pTrc99A-*nahG* was constructed by cloning the *nahG* gene into pTrc99A that harbors ampicillin resistance gene (amp^R) as an antibiotic selection marker (Fig. S1). The expression of *nahG* gene was under the control of P_{lac} and was inducible by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). Recombinant plasmid was transformed to *E. coli* XL1-blue through electroporation for the purpose of enzyme expression. The resultant transformants of *E. coli* XL1-blue was cultured in test tubes, where each contains 3 mL of LB media (with 100 μg/mL of ampicillin). The strain was cultured overnight aerobically at 37 °C. Each of the overnight culture was further

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