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A novel and simple cell-based electrochemical biosensor for evaluating the antioxidant capacity of *Lactobacillus plantarum* strains isolated from Chinese dry-cured ham

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ABSTRACT

The analysis of antioxidants in foodstuffs has become an active area of research, leading to the recent development of numerous methods for assessing antioxidant capacity. Here we described the fabrication and validation of a novel and simple cell-based electrochemical biosensor for this purpose. The biosensor is used to assess the antioxidant capacity of cell-free extracts from *Lactobacillus plantarum* strains isolated from Chinese dry-cured ham. The biosensor relies on the determination of cellular reactive oxygen species (ROS) (the flux of H_2O_2 released from RAW 264.7 macrophage cells) to indirectly assess changes in intracellular oxidative stress level as influenced by *L. plantarum* strains. A one-step acidified manganese dioxide (a-MnO_2) modified gold electrode (GE) was used to immobilize RAW 264.7 macrophage cells, which were then encapsulated in a 3D cell culture system consisting of alginate/ graphene oxide (NaAlg/GO). The biosensor exhibited a rapid and sensitive response for the detection of H_2O_2 released from RAW264.7 cells. The detection limit was 0.02 μM with a linear response from 0.05 μM to 0.85 μM and the biosensor was shown to have good stability and outstanding repeatability. This technique was then used for evaluating the antioxidant ability of extracts from *L. plantarum* NJAU-01. According to the electrochemical investigations and assays of SEM, TEM, and ROS, these cell-free extracts effectively reduced the oxidative stress levels in RAW264.7 cells under external stimulation. Extracts from *L. plantarum* strains at a dose of 10^{10} CFU/mL showed the highest antioxidant activities with a relative antioxidant capacity (RAC) rate of 88.94%. Hence, this work provides a simple and efficient electrochemical biosensing platform based on RAW264.7 cells for fast, sensitive and quantitative assessment of antioxidant capacity of *L. plantarum* strains. The method demonstrates its potential for rapid screening for evaluating antioxidant properties of samples.

1. Introduction

Reactive oxygen species (ROS) are generated naturally *in vivo* during metabolic processes. In normal living organisms ROS is kept in balance as it has important physiological effects on transcription factors, cell proliferation and differentiation (Wang et al., 2014). However, the accumulation of excess ROS in organisms, as a result of a harmful environmental stress, could cause damage to nucleic acids, proteins and lipids, impede normal cellular metabolism, and induce various diseases such as aging, initiate cancer, heart disease and arteriosclerosis (Dickinson and Chang, 2011).

Therefore, overproduction of ROS can be extremely harmful to body health (Pi et al., 2017). Thus, most living organisms have developed enzymatic and non-enzymatic antioxidant defense systems to counteract the deleterious effects of ROS (Ahmad et al., 2017). However, these cellular antioxidative systems are not always able to prevent living organisms from oxidative damage. Hence, exogenous synthetic and natural antioxidants capable of removing free radicals and other pro-oxidants have been largely studied (Blois, 1958). The safety of synthetic antioxidants has recently been questioned due to their potential hepatotoxicity and carcinogenicity (Luo and Fang,

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2008). Therefore, as a safer alternative, extracts from mixed *Lactobacillus plantarum* strains isolated from traditional Chinese dry-cured ham, have recently received much attention for their *in vitro* scavenging activity against hydroxyl and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radicals, and for their resistance to hydrogen peroxide (Li et al., 2012).

Many methods have been devoted to evaluating antioxidant activity in the area of food technology, including photometric (Pazourek et al., 2005; Prenesti et al., 2007), fluorimetric (Ou et al., 2001; Qiao et al., 2015), chromatography (Gimeno et al., 2015) and electrochemical methods (Gao et al., 2015; Ignatov et al., 2002; Kamel et al., 2008; Reybier et al., 2010; Wang et al., 2015). Among these methods, the electrochemical strategy has been used for assessing antioxidant capacities because of its high sensitivity, fast response time, simple operation, low cost and accuracy (Andlauer and Heritier, 2011; Bordonaba and Terry, 2012; Cata et al., 2016; Chakraborty and Raj, 2009; Romero et al., 2017). In particular, the differential pulse voltammetry (DPV) technique is suitable for rapid real-time monitoring as it has high temporal resolution properties as well as good dynamic kinetic characteristics for electrochemical redox process (Ervin and Kariuki, 2014; Tufan et al., 2014; Zhang et al., 2015; Zhao et al., 2017). However, most of these electrochemical studies have focused on the modification of electrodes for detecting radical scavengers or for DNA damage, while methods based on living cells for the determination and the evaluation of antioxidant activity have not been reported. Therefore, the exploitation of an actual or more realistic technique for antioxidant measurement has become important.

Recently, cell-based biosensors have been widely used in many fields, including drug screening, environmental testing, medical diagnosis, and national security (Jiang et al., 2014). Using the natural ability of macrophage cells to detect and determine oxidative stress provides a stable and accurate strategy mimicking physiological conditions. For instance when RAW264.7 cells are activated with phorbol 12-myristate 13-acetate (PMA) within a short time, it triggers the generation of ROS in the cytoplasm causing the release of H_2O_2 (Wang et al., 2016). Hence, RAW 264.7 cells have an excellent potential for the application of electrochemical biosensors as a result of their sensitivity to high oxidative stress. The cells can be used to evaluate the antioxidant activity of specific antioxidants and convert biological recognition into a signal that can be readily recorded and quantified (Rider et al., 2003). The H_2O_2 from RAW264.7 cells which is immobilized on an electrode can be detected rapidly and accurately converted to a sensitive and specific electrochemical signal.

For producing cell-based electrochemical biosensors, the immobilization of cells on an appropriate transducer surface is a crucial step in the electrochemical process, in order to achieve the signal sensitively, stably and reproducibly (Taniguchi, 2010). To achieve optimal cell adhesion and to maintain cell viability, alginate (NaAlg) and Graphene oxide (GO) hydrogels can be used to encapsulate RAW264.7 cells and provide a three dimensional (3D) culture system for maintaining cell activity as well as preventing environmental interference (Lee et al., 2016). This technique provides excellent biocompatibility and electrical conductivity and hydrophilicity, and has strong mechanical strength.

The recent fast development of nano-science and nano-technology has played an important role in improving electro-analytical performance leading to their excellent electrical conductivity, large specific surface areas and biocompatibilities (Zhao et al., 2017). Because of the excellent catalytic oxidation properties, nano-materials have been widely used as catalysts and electrode materials for ROS species detection (Feng et al., 2016). However, the complex fabrication step and the mechanism of action are always the shortcomings of these MnO_2 nano-particle-based sensors. Hence, a one-step modification has become essential for fast detection. Recently, acidified MnO_2 (a-MnO_2) has attracted much attention due to its easier method of synthesis, improved catalytic properties and lower charge-transfer resistance, making it become an excellent modified material for the detection of H_2O_2 released from living cells (Bai et al., 2017). Thus, with the assistance of these nano and biocompatible materials, the 3D cell culture systems developed for evaluating antioxidant capacity have achieved fast, direct electron transfer between immobilized macrophage cells and the fabricated electrode surface, resulting in improved sensitivity and selectivity (Das et al., 2016).

In this study, a simple and novel electrochemical RAW264.7 cell-based sensor was explored for the electrochemical assessment of the antioxidant capacities of extracts from *L. plantarum*. The synthesis of A-MnO_2 was achieved using a mixture of sulfuric acid and nitric acid and was modified on GE by physical adsorption. The NaAlg/GO hydrogel was prepared for the capsulation of RAW264.7 in order to create an *in vitro* 3D culture system. The encapsulation of the living cell, mimicking culture conditions, is thus unaffected by external interferences, and can extend the actual working life of macrophage cells. Then the a-MnO_2 "one-step" modified gold electrode was used to immobilize RAW264.7 cells capsulated in NaAlg/GO hydrogel. The antioxidant and the redox profile of *L. plantarum* extracts were characterized by various electrochemical responses caused by oxidative stress changes in cells. Biological assays including CCK-8, flow cytometry and ROS were carried out to verify the cell electrochemical results.

2. Experimental procedure

2.1. Materials and apparatus

Graphite was obtained from Xian Feng Nano-materials Technology Co. Ltd (Nanjing, China). MnO_2 was purchased from DK Nano-technology Co. Ltd (Beijing, China).

RAW 264.7 macrophage cells were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Phorbol 12-myristate 13-acetate (PMA, ≥99%) and alginate sodium were obtained from Sigma-Aldrich Inc (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum were obtained from Gibco Laboratories (Gaithersburg, MD, USA). All cell assay kits were purchased from Beyotime Biotechnology Co. Ltd (Shanghai, China), all solutions were prepared with deionized water and all reagents were of analytical grade.

All electrochemical experiments were performed on a CHI660E electrochemical workstation (Chenhua Technology Co., Ltd., Shanghai, China) using a conventional three-electrode system in a solution containing 1.0 mM of $Fe(CN)_6^{3-/4-}$ or in 0.1 M PBS solution (pH 7.4). The structure of nano-materials and the morphologies of cells were characterized using a transmission electron microscope (Tecnai 12, Philips, Netherlands) and a scanning electron microscope (S-4800 II, Hitachi, Japan). The FT-IR spectrum was recorded on a Fourier transform infrared (FT-IR) spectrometer (FI-IR, NICOLET MEXUS 470, Thermo Electron Corporation, Ramsey, Minnesota, USA) using a KBr disk at a resolution of 4 cm^{-1}. RAW264.7 cells were incubated in a CO_2 incubator (Thermo Scientific Forma Series II Water Jacket, Thermo Fisher Scientific Inc., Rockford, Illinois, USA).

2.2. Preparation of cell-free extracts of Lactobacillus plantarum strains

Lactobacillus plantarum NJAU-01, previously isolated from traditional Chinese dry-cured ham, was used for the inoculation of fermented sausage as starter cultures. It was identified by API 50 CHL kit (BioMérieux Inc., France) and 16S rDNA sequencing analysis. The strains were grown in MRS broth at 37 °C for 18 h.

The intracellular cell-free extracts were prepared by the method of Li et al. (2012) with minor modifications. Firstly, the bacterial cells were harvested, washed and resuspended in de-ionized water. The bacterial numbers were adjusted to 10^5, 10^6 10^7, 10^8, 10^9 and 10^{10} CFU/mL. For each dilution, the cells were incubated with 1 mg/