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Mediated electrochemical method for the analysis of membrane damage effects of phenolic compounds to *Staphylococcus aureus*

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ABSTRACT

In this study, the mediated electrochemical method and conventional methods, including the atomic force microscopy, respiration inhibition, and potassium efflux methods were integrated for the analysis of the membrane damage effects of phenolic compounds to the food contamination bacteria *Staphylococcus aureus* (*S. aureus*). Hydrophilic ferricyanide, acting as both the permeability indicator and intracellular redox activity indicator, was employed as the mediator. Microelectrode voltammetric method was used to assay the ferrocyanide accumulations arising from the reduction of ferricyanide by the NADH: Fe^{3+} reductase embedded in the cell membrane. When the samples with *S. aureus* suspensions were incubated for 2 h, the mediated electrochemical response increased by 71.9% due to the presence of 3 mM carvacrol, which was consistent with the significant potassium efflux attributed to the presence of the carvacrol, indicating the outer membrane damage of the bacteria. However, the relative respiration activities of the *S. aureus* suspensions remained 32.5% of the controlled sample, which suggested the damage of the respiration chain due to the presence of 3 mM carvacrol. The mediated electrochemical method was also applicable to the other three phenolic compounds including eugenol, thymol, and 3-isopropylphenol, exhibiting the applicability of the mediated electrochemical method for the membrane damage effect of the phenolic compounds to bacteria.

1. Introduction

The antimicrobial activity of essential oils (EOs) and their components has long been recognized, and interest has significantly increased in recent years. The food industry encounters a serious problem related to the progressively increasing number of food borne diseases outbreaks. The main cause is determined to be microorganisms. Thus, the control of pathogens may significantly reduce these outbreaks. EOs have promising potential as new natural food preservatives that can also meet the increasing desire for green products [1–3]. EOs have shown significant antimicrobial functions against food borne pathogens and they have been used for preventing food spoilage and deterioration, thus extending the shelf life of food. Systematic explorations and understanding the mode of action of EOs may facilitate their applications as natural food preservatives, in particular, for their potential use in preservation systems employing multiple hurdles [4,5]. Recent studies involving the mode of action using EO compounds (carvacrol, eugenol, and thymol) against several pathogenic bacteria and yeasts have shown that their activity resides in their ability to perturb the cell membrane, which results in the loss of cellular chemiosmotic control [6,7]. Therefore, membrane damage measurement is of significant importance to evaluate the antimicrobial activities of natural phenolic compounds. Traditional detection methods of cell damage are based on potassium release, pH homeostasis, adenosine triphosphate (ATP) release, cellular morphology changes, and uptake of hydrophobic fluorescent probe [6–8]. The combined utilization of the above mentioned methods is often required because none of them can provide enough information to evaluate the degree of cell damage that reflects the actual physiological status of the cell. Moreover, conventional techniques are disadvantageous because they are time-consuming and require high cost of specialized equipment and trained personnel. Therefore, development of other methods for cell damage detection is highly desirable, and it has also received progressive attention [9–12].

Monitoring the electrochemical behavior of redox mediators in solutions containing viable bacterial cells has been studied and proposed as a simple method for biological toxicity assay [9,13]. The changes in the redox state of the cells indicate the occurrence of electron transport in the biological system and modification in the physiological state of the living cells [14–16]. The electrochemical studies of redox state of living cells might be useful in monitoring different pathophysiological states of the cells, such as oxidative and nutrient stresses, effect of cytotoxic, mutagenic, and carcinogenic preparations [17–19]. Intracellular redox activity has been evaluated in both prokaryotic and eukaryotic cells, using a variety of non-native oxidants such as ferricyanide, 2,3,5,6-tetramethyl-1,4-phenylenediamine, 2,6-dichlorophenolindophenol

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(DCPIP), and benzoquinone and quinone derivatives [20–22]. These compounds possess appropriate reduction potentials and are readily quantitated in their reduced forms. The oxidants are sometimes used in combination, wherein a hydrophobic, membrane-permeable mediator at low concentration is combined with a hydrophilic terminal acceptor present at higher concentration; for e.g., DCPIP and ferricyanide have been used with *Escherichia coli* (*E. coli*) O157 and beer yeasts (Fig. 1) [10,11].

Electrochemical measurements of direct ferricyanide reduction by suspensions of *E. coli* have also been reported [11]. The reduction potential of the ferri–ferrocyanide redox couple (+0.418 V vs NHE) is positive of the terminal oxidases, indicating that any sequence of preceding primary dehydrogenase reactions may contribute to observed ferricyanide reduction rates [11]. Gram-negative bacteria (e.g., *E. coli*) contain porin proteins (channels) in their outer cell wall allowing ferricyanide to interact directly with terminal components of the respiratory pathway. Gram-positive bacteria (e.g., *Staphylococcus aureus* (*S. aureus*)) do not possess porins and are much less reactive towards ferricyanide. Full respiratory activity signals are only observed in the presence of a lipid-soluble redox mediator, such as DCPIP (Fig. 1) [23]. Poor permeability of the plasma membrane of Gram-positive bacteria to ferricyanide provides us with opportunities to use the ferricyanide-mediated microbial reactions for cell membrane damage measurement. By employing scanning electrochemical microscopy (SECM), the amperometric method has been used to study the morphology, metabolic alteration, and membrane permeability changes in the living cells, in which ferricyanide was used as the mediator [24,25]. However, the limitations, including the high cost of SECM, requirement of skilled operators, and complicated sample preparation procedure are obvious for these studies. There are several different ways in which the cell membrane poisons cause the changes in the membrane structures, resulting in different variations in the permeability of potassium ferricyanide, and even in different changes in the enzymatic activity of NADH: Fe^{3+} reductase located on the cell membrane. In this case, different responses would be anticipated for different membrane poisons in the mediated electrochemical method. In this study, four phenolic compounds including carvacrol, eugenol, thymol, and 3-isopropylphenol were studied by the mediated electrochemical method (Fig. 2), and the analysis strategy concerning the method is shown in Fig. 3. Carvacrol is a major component in some

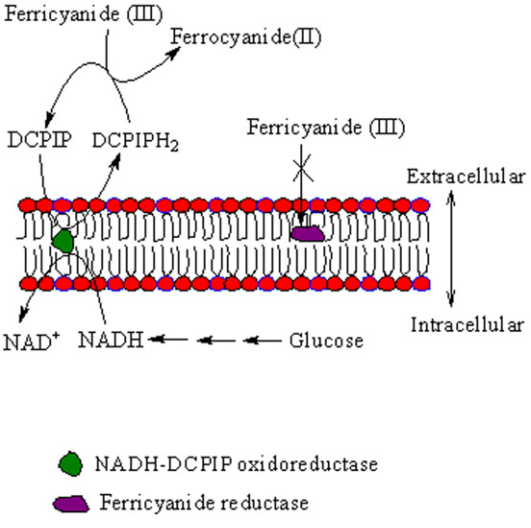

Fig. 1. The schematic diagram of the mediated electrochemical method for the detection of the intracellular redox activities of the gram-positive bacteria. Single ferricyanide mediator cannot penetrate into the intact membrane of the gram-positive bacteria (right). For the DCPIP/ferricyanide mediator system (left), which performed in two separated steps: DCPIP diffuses into a bacterium cell, where it is reduced to $DCPIPH_2$, which then diffuses out of the cell and reacts with ferricyanide and converting it into ferrocyanide, and regenerating DCPIP to repeat the cycle. Ferrocyanide accumulations arising from the above cycle was assayed by microelectrode voltammetric measurements.

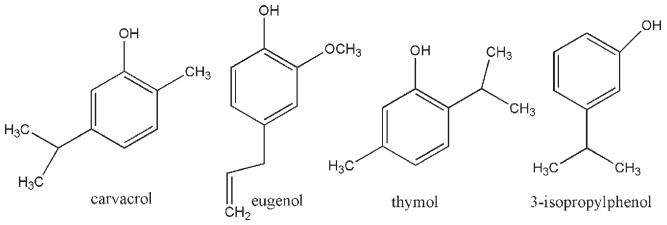

Fig. 2. The molecular structure of four phenolic antibacterial compounds.

EOs and its inhibitory effect on the growth of various microorganisms has been well documented.

For verification and comparison purpose, three other methods including atomic force microscopy (AFM), respiration inhibition, and potassium ion leakage were also employed for evaluation of the membrane damage effects of the phenolic compounds [12,26]. The results of this study indicated that the ferricyanide-mediated electrochemical method has significant potential as a membrane damage assay tool for phenolic EO compounds. The mediated electrochemical method is extremely convenient, efficient, and easy to use; it is less time consuming; and very informative.

2. Experimental methods

2.1. Microorganism cultivation and preparation

S. aureus was obtained from the strain collection center of the institute of Microbiology, Chinese Academy of Science. The strain was maintained on the slant agar (g L^{-1}) D-glucose, 25; yeast extract, 5; peptone, 5; and agar, 15; pH 7.0, at 4 °C. Cell cultures for experimental use were grown in 50 mL of growth media in shake flasks rotated at 200 rpm for 24 h at 30 °C [27]. The growth medium contained (g L^{-1}): D-glucose, 30; Na_2HPO_4, 4; KH_2PO_4, 4; yeast extract, 5; peptone, 5; pH 7.0. Cells were harvested by centrifugation at 10,000 *g* for 5 min at 4 °C. The cells were washed twice with phosphate buffer (PB, 0.05 M K_2HPO_4/KH_2PO_4, pH 7) and resuspended in phosphate buffered saline (PBS, 0.05 M K_2HPO_4/KH_2PO_4, pH 7, 0.1 M KCl). Cell density was adjusted to an optical density at 600 nm (OD_{600}) of 2.5 using a 752-E spectrophotometer [10].

2.2. Preparation of the solutions

Potassium ferricyanide solutions (0.30 M) were prepared in PBS. Glucose solution (50 mM) was also prepared in PBS. DCPIP was

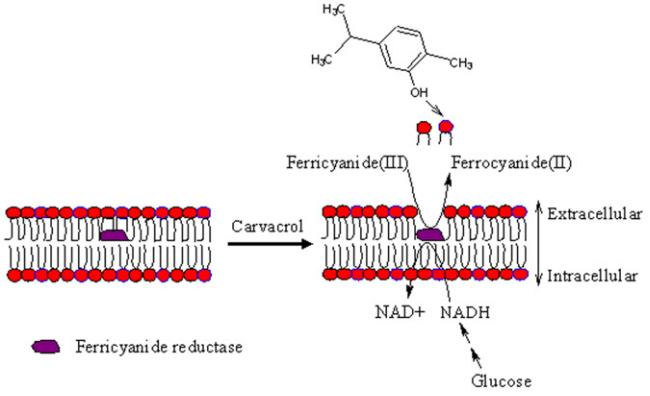

Fig. 3. The use of the single ferricyanide mediator method for the detection of the membrane damage effect of phenolic compounds to *Staphylococcus aureus*: ferricyanide diffuses through the perturbed membrane and get into a cell, where it is reduced to ferrocyanide under the catalysis of ferricyanide reductase embedded in the membrane, and then diffuses out of the membrane. Ferricyanide accumulations were measured by steady-state voltammetry method after the mediated reactions.