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Short communication

The inhibition of *Saccharomyces cerevisiae* cells by acetic acid quantified by electrochemistry and fluorescence

Jinsheng Zhao [a,*], Zhong Wang [b], Min Wang [b], Qingpeng He [a], He Zhang [a]

[a] *Department of Chemistry, Liaocheng University, 252059, Liaocheng, PR China*
[b] *School of Medicine, Ehime University, 791-0295, Toon, Japan*

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Abstract

In this paper, the effect of acetic acid on *S. cerevisiae* metabolism was studied for the first time with the combinational utilization of the mediated electrochemical method and intrinsic fluorescence method, and the results from both methods were compared with each other. It was confirmed in vivo that not only the glycolysis enzymes but also the NADH dehydrogenase were sensitive to acetic acid. The established technique might also be applicable in studying the toxicity of other environmental pollutants, such as phenol and its derivatives.
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Keywords: Electrochemical methods; Menadione; Double mediator system; Intrinsic fluorescence

1. Introduction

Metabolic pathways in living cells are tightly regulated and highly sensitive to cellular needs. All of the major metabolic pathways in cells involve redox reactions and specific redox couples that cycle between oxidized and reduced states. The concentrations of the redox couples help regulate the flow of metabolites through these pathways [1]. It has been shown by numerous studies that cellualr redox state especially NADH/NAD level may be a primary control site in numerous biological process linked to rhythm, senescence, cancer and death. The celluar NADH/NAD redox state is considered as a control factor in cell metabolism [2]. A double mediator system comprising of lipophilic and hydrophilic mediators was used for the electrochemical detection of intracellular redox activity in mammalian cells. The function of the lipophilic mediator menadione was to shuttle electrons from intracellular redox sites to the cell surface for reaction with the hydrophilic mediator ferricyanide (Fig. 1) [1]. The magnitude of the signal from the electrochemical measurment is an indication of the dynamic reoxidation rate of NADH to NAD [3]. In recent years, the method has been applied to toxicity assessment. Injury sustained by a cell in response to a toxicant changes the capacity of some central function of cellular metabolism, compared to unaffected cells. These toxic effects can be easily measured as a deviation away from the electrochemical signal produced by health cells [4,5].

Intrinsic fluorescence, in particular, has the advantage over the extrinsic fluorescence of an unperturbed enviroment during investigation, especially in complex systems such as biological cells and tissues [6,7]. Cellular fluorescence provides a sensitive index of the functional state of living cells. NAD has no fluoresence and plays a central role in cellular metabolism by functioning as a cofactor in over 300 red-ox reactions. NADH is an important fluorescent substance in living cells that participate in the process of transfer electron in the multienzyme redox system, acting as electron donor in electron transport chain and it is an important product of tricarboxylic acid cycle (TCAC). Therefore, the intrinsic fluorescence method may be very useful in studying cell metabolism, and even toxicity assessment. The result from intrinsic fluorescence is an indication of the static intracelluar NADH level of living cultures [8]. The combinational utilization of the mediated electrochemical method and intrinsic method could give more information about the physiological condition of bacterium than either method alone does.

* Corresponding author. Tel.: +86 635 8537064; fax: +86 635 8239227.
E-mail address: zhaojinsheng@lcu.edu.cn (J. Zhao).

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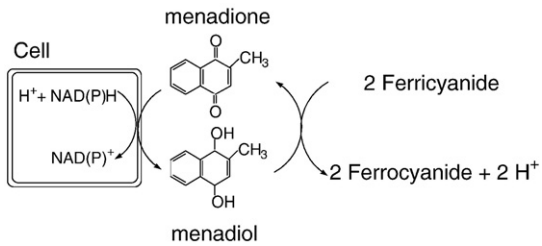

Fig. 1. Detection of intracellular redox activities with use of menadione to link intra-and extracellular redox pairs. Hypothetical menadione reaction cycle. Menadione diffuses into a yeast cell, where it is reduced to menadiol, an then diffuses out of the cell and reacts with ferricyanide, converting it into ferrocyanide, and regenerating menadione to repeat the cycle.

In nature, weak acids are found in fruits and vegetables in conjunction with a pH of 5.0 or less as a deterrent to bacterial growth. Moreover, it has been shown by several groups that high concentrations of acetic, propionic and butyric acids are produced in the large intestine, and presumably these can inhibit the growth of pathogens that encounter them in the intestinal environment [9]. Weak acids have been used as a means for preserving foods for generations, and in modern times, weak acids such as acetic, propionic, lactic, and sorbic acid have been widely used as food preservatives as well is in the pre-harvest and post harvest phases of agriculture [10]. These are among many reasons why the study of weak acids and their influence on bacterial cells is warranted.

S. cerevisiae was used in previous studies as a model system for toxicity [11]. In this paper, the influence of acetic acid on the *S. cerevisiae* metabolism was studied for the first time with the joint utilization of the mediated electrochemical method and intrinsic fluorescence method, and the results from both methods were compared with each other. It was confirmed in vivo that not only the glycolysis enzymes but also the NADH dehydrogenase were sensitive to acetic acid. The established technique might also be applicable in studying the toxicity of other environmental pollutants, such as phenol and its derivatives.

2. Experimental parts

2.1. Microorganism and maintenance conditions

Saccharomyces cerevisiae was obtained from the strain collection center of the Institute of Microbiology, Chinese Academy of Science. The yeast were maintained on agar slants containing (g L^{-1}): glucose, 30; yeast extracts, 3; peptone, 5; and agar, 15; pH 6.0, at 4 °C.

2.2. Solution preparations

Phosphate buffer (pH 6.0), containing 0.1 M KH_2PO_4 and 0.08 M Na_2HPO_4, was used for washing of cell pellets. Phosphate buffered saline (pH 6.0) prepared from 0.1 M KH_2PO_4, 0.08 M Na_2HPO_4 and 0.1 M KCl. Potassium ferricyanide solutions were prepared in phosphate buffered saline to give a concentration of 0.30 M. Glucose solution was prepared in phosphate buffered saline to give a concentration of 50 mM. Menadione was dissolved in 96% ethanol to give a 20 mM solution, filter-sterilized and stored in a light-proof container at 4 °C [12].

2.3. Cultivation of microorganisms

S. cerevisiae was prepared in the medium containing the following compounds (g L^{-1}): D-glucose, 25; yeast extracts, 1; peptone, 1; KH_2PO_4, 4; Na_2HPO_4, 4; pH 5.5. The media were sterilized by autoclaving at 120 °C for 15 min, and D-glucose was autoclaved separately at 110 °C for 10 min. After sterilization and cooling, solutions were mixed to form a complete medium prior to inoculation. A 250 mL Erlenmeyer flask containing 50 mL medium was inoculated from a fresh agar plant, and inoculated at 30 °C on a rotary shaker at 250 rpm. Growth proceeded overnight for 9 h to allow the yeast growth to its exponential phase, after which the broth was centrifuged at 10,000 rpm for 10 min the cell pellet was washed twice in phosphate buffer and suspended in phosphate buffered saline solution. The cells were adjusted to the desired optical density measured at 600 nm (OD_{600} = 9.0) [13].

2.4. Incubation of cells with mediator(s) and substrates

A total volume of 20.0 mL incubation suspension was prepared for each trial. The standard incubation suspension comprised: 12 mL of cell suspension, 3.0 mL of ferricyanide solution, 100 μL of menadione solution, 3.0 mL of glucose solution, required amounts of acetic acid or not. Cell suspensions were adjusted to the desired pH with 2 M HCl or 2 M NaOH, the final volume of cell suspension was fulfilled with sterile distilled water to 20 mL. Incubation of cells with mediators and substrate was at 30 °C under oxygen-free nitrogen sparging conditionon a rotary shaker at 250 rpm. After the completion of incubation, the cells were pelleted by centrifugation (10,000 rpm, 4 °C, 15 min) and the supernatants were taken for analysis [3].

2.5. Determination of optical density

A measured aliquot of the cell suspension was centrifuged at 10,000 rpm for 10 min. The supernatant was removed, and the pellet was reconstituted by the addition of 1.5 mL of distilled water. Sample dilution factors were chosen such that the measured optical density (600 nm) was between 0.1 and 0.6 au, using water as a reference.

2.6. The effect of acetic acid on the fluorescence of NADH in S. cerevisiae

The exponential cell suspensions obtained at part 2.3 (OD_{600} = 9.0) were diluted to the concentration of OD_{600} = 1.0. A total volume of 20.0 mL incubation suspension was prepared for each experiment. The incubation suspension comprised: 10 mL of cell suspension, 3.0 mL of glucose solution, required amounts of acetic acid or not. Cell suspensions were adjusted to the pH 5.0 with 2 M HCl or 2 M NaOH, the final volume of cell suspension was fulfilled with sterile distilled water to 20 mL. The cultivation of the suspensions were conducted at 30 °C for 2.5 h, on a rotary shaker at 250 rpm.

2.7. Electroanalysis of the supernatant

Steady-state voltammetry was conducted using an electrochemical working station (CHI 900, USA) controlled by CHI 900 software. Steady-state voltammograms were obtained at a scan rate of 10 mV s^{-1} scanning from 500 to 100 mV versus saturated Ag/AgCl reference electrode. A 15 μm diameter Pt disk working electrode was used as the working electrode and a Pt gauze auxiliary electrode was used to complete the three-electrode electrochemical cell. The microelectrode was pretreated by polishing with 0.05 μm alumina/water slurry on a flocked twill polishing cloth. The steady-state current at 500 mV was measured and the mean current from three repeat voltammograms was calculated.

2.8. Culture fluoromeasure measurement

Fluorescence spectra of yeast samples were obtained using a Hitachi F-4500 fluorescence spectrophotometer. Yeast samples (2 mL) were placed in a quartz cuvette. Fluorescence emission spectra (380.0–600.0 nm, resolution: 1 nm) were recorded with excitation wavelength set at 360 nm. The scan speed was 240 nm/min, the slit was (EX/EM) 2.5 nm/5.0 nm, the PMT voltage was 700 V and the response was auto. The shutter control was off [6,7].

3. Results and discussion

The use of voltammetric microelectrodes has been shown to be a simple, reliable and rapid method for determing the concentration of electroactive species such as ferrocyanide [12], diffusion limiting currents (quantitatively related to concentration) can be established in second time scale with virtually zero destruction of analyte. Fig. 2 shows the steady-state voltammogram of ferrocyanide accumulations before or after a 2.5 h incubation of the yeast suspensions. The steady-state anodic plateau current measured at $E = 500$ mV was used as a relative measure of the amount of ferrocyanide produced. Before incubation, the mediator is fully in the oxidized form, and the steady-state anodic plateau current measured at $E = 500$ mV was about zero (Fig. 2, curve 1). After incubation for 2.5 h with the yeast, the voltammogram has shifted down the current axis and there is both anodic current (arising from oxidation of ferrocyanide) and cathodic current (arising from reduction of ferricyanide). The limiting oxidative currents of ferrocyanide arising from menadione mediated reduction of ferricyanide was 72.01 nA (Fig. 2, curve 2). It has been well known that reduced nicotinamide adenine dinucleotide (NADH) is the intracellular reductant for ferricyanide reduction catalyzed by menadione [14]. NADH is a ubiquitous biological molecule that participates in the process of anti-oxidate injury and respirotory chain. As a necessary coenzyme in cell metabolism, NADH acts as electron transporter and presides the transportation of proton. The electrons produced in the reactions of metabolism are usually transferred to NAD and reduced NAD to NADH. In the following succession, NADH transfers the electrons to oxidate, reacts with molecular O_2 and release energy. It is crucially important for continued cell growth that NADH be oxidized to NAD and a redox balance be achieved [15].

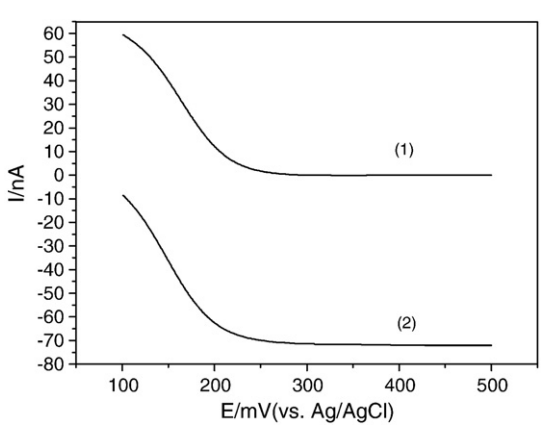

Fig. 2. Steady-state voltammograms for the oxidation of ferrocyanide accumulations before (curve 1), and after 2.5 h mediated reactions (curve 2). Yeast final OD_{600}, 5.4; ferricyanide, 45 mM; glucose, 7.5 mM, menadione, 100 μM, initial pH, 6.0, Scan rate, 10 mV S^{-1}.

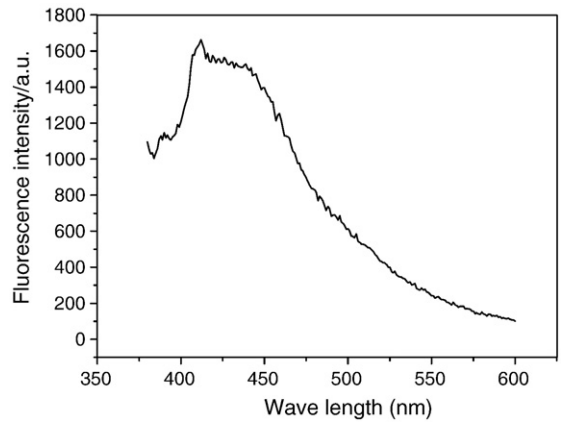

Fig. 3. Fluorescence spectra recorded following excitation at 360 nm on dilute suspensions of *S. cerevisiae*.

The detection of intracellular redox reactions using menadione as electron transfer mediator is based on its diffusion through the cell membrane and reduction by the cytosolic and mitochondrial enzymes catalyzing electron transfer from NADH to quinone substrates. In *S. cerevisiae* cells mitochondrial NADH dehydrogenase (also called NADH:ubiquinone oxidoreductases, EC 1.6.5.3) is involved in the intracellular reduction of quinone mediators [16]. In our recent work, we have shown that the menadione/ferricyanide system was able to act as terminal electron acceptors in the absence of oxygen, and in so doing enable *S. cerevisiae* (a facultative anaerobe) to switch to a nearly complete respiration metabolism (anaerobic respiration), rather than the fermentation pathway normally expected by such an anoxic environment [17]. In this case, the eletrochemical response should has a positive relationship with the complete oxidation of the glucose added in the yeast suspensions. In other words, the magnitude of the electrochemical response within a specific time interval was the function of the specific NADH production rate

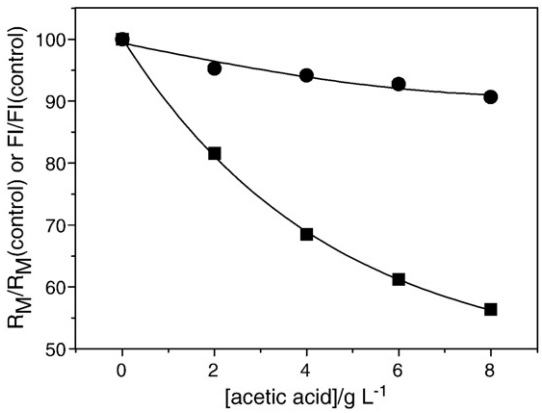

Fig. 4. Effect of acetic acid on the relative R_M (■) and on the relative fluorescence intensity (FI) (●) of *S. cerevisiae*.

(proportional to the catabolic activities) and the enzymatic activity of the NADH dehydrogenases.

Based on the positive relationship between cellular catabolism and cellular redox activity, the inhibitory effects of acetic acid on *S. cerevisiae* was assayed through the variance in relative cellular redox activities of the yeast by the presence of increasing concentrations of acetic acid on in cells suspensions. The oxidative limiting currents of ferrocyanide accumulations were indications of the cellular relative redox activity remained, which was designated R_M. Fig. 4 shows the variation in relative R_M recorded for *S. cerevisiae* by the presence of increasing concentrations of acetic acid. The data presented is the mean of two replicate, each of which is an average of three current measurements. The relative R_M decreased continuously by increasing concentrations of acetic acid, and the IC_{50} value (i.e. the concentration at which the relative R_M was decreased by 50%) was about 8.5 g L^{-1} acetic acid, which was comparable with the value obtained by the traditional turbidity method [18]. According to the above discussions, the continuous decrease in the relative R_M may thus be attributed either to the decrease in intracellular NADH concentrations resulted from the blocked catabolic activity or to the inhibited NADH dehydrogenase by the presence of acetic acid. Previous studies based on in vitro enzymatic assay showed that most of the glycolysis enzymes are sensitive to the inhibitory effects of acetic acid, which blocked catabolic activity of the yeast and be in-part accounting for the continuous decrease in the relative R_M [19].

There is a confusion concerning the problem whether weak acids impose an inhibitory effect on the NADH dehydrogenase [20]. Some reports have noticed that the respiration of some intact cells in acidified media was very sensitive to weak acid, but NADH oxidases in cell extracts were weak acid insensitive[20]. The assay of intracellular NADH concentrations may help us to gain insights into how the NADH dehydrogenase might be affected by the presence of acetic acid in acidified media. If we assume that the NADH dehydrogenase is not sensitive to acetic acid, the variance in the intracellular NADH concentrations might be in line with the variance in the relative R_M values by the presence of increasing concentrations of acetic acid. In contrast, if the NADH dehydrogenase is sensitive to acetic acid, high levels of intracellular NADH would be expected due to the blocked reoxidation of NADH to NAD. The intrinsic fluorescence of the yeast *S. cerevisiae* was used to research the effect of acetic acid on the metabolism of the yeast. Fig. 3 presents the fluorescence spectrum recorded for the yeast *S. cerevisiae*, and the fluorescence spectrum were characterized by a maximum located at 413 nm. With the increment in acetic acid concentrations, the intensity of NADH fluorescence did not change notably and was kept within the range of 100% to 90.67% of the controlled sample which suggested that acetic acid can impose a direct effect on the activity of the NADH dehydrogenase (Fig. 4). In conclusion, the results showed that not only the glycolysis enzymes but also the NADH dehydrogenase were sensitive to acetic acid. The established technique might also be applicable in studying the toxicity of other environmental pollutants, such as phenol and its derivatives.

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