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Synergistic reduction of toluylene blue induced by acetaldehyde and menadione in yeast cell suspension: Application to determination of yeast cell activity

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ABSTRACT

Membrane permeant acetaldehyde and menadione induced the synergistic reduction of toluylene blue (TB) acting as non-membrane permeant redox indicator in yeast cell suspension. NADH and acetaldehyde also induced the synergistic TB reduction in permeabilized yeast cells and phosphate buffer, but menadione had no ability to promote TB reduction. The pre-incubation of acetaldehyde inhibited the above synergistic reduction of TB in intact and permeabilized yeast cell suspension. The pre-incubation of acetaldehyde might promote NADH oxidation by alcohol dehydrogenase, because acetaldehyde decreased the intracellular NAD(P)H concentration. The above facts indicate that the synergistic reduction of TB is controlled by the order of addition of menadione and acetaldehyde. The synergistic reduction of TB by menadione and acetaldehyde was proportional to viable yeast cell number from 10^4 to 2×10^6 cells/ml, and this assay was applicable to cytotoxicity test. The time required for the above assay was only 2 min.

1. Introduction

The various assays of cell activity have been developed for the evaluation of biological, chemical, and physical effects on cell growth or viability. For example, antimicrobial susceptibility test [1] and drug discovery screens [2] are performed by the determination of viability of bacteria, fungi or animal cells exposed to antibiotics or drugs. The physical growth conditions including temperature, aeration, and agitation are also investigated by the various assays.

The determination of yeast cell activity has been performed with flow cytometric methods [3,4], fluorescent assays [5,6], biological [7] or chemical [8–10] luminescent assays, colorimetric assays [11,12], and electrochemical assays [13,14]. In general, the colorimetric WST-1 (Water Soluble Tetrazolium) assay [15] and MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay [16] have been used as the conventional methods among the above assays.

WST-1 is reduced by NAD(P)H in viable cells through redox mediator such as 1-methoxy PMS (1-Methoxy-5-methylphenazinium methylsulfate) and is changed into water-soluble colored formazan. MTT reduced by various dehydrogenases in cells also changed into water-insoluble colored formazan. However, these colorimetric assays are inferior to biological or chemical luminescent assays in the measuring time or the detection limit of viable cell activity. Quinone-mediated WST-1 assay [17] was improved in order to shorten the amount of measuring time for the determination of viable yeast cell activity. However, this assay required at least 10 min and the additional treatment such as the filtration with membrane filter and the neutralization of filtrate.

This study proposed the simple, rapid, and sensitive assay for determination of yeast cell activity compared to the above colorimetric assays. The colorimetric assay developed by this study was dependent on the synergistic reduction of toluylene blue (TB) by the combination of menadione and acetaldehyde. The mechanism about the synergistic reduction of TB was discussed, and the application to the assay of yeast cell activity was demonstrated in this study.

2. Materials and methods

2.1. Yeast strain and growth conditions

Baker yeast cells *Saccharomyces cerevisiae* IFO2044 were supplied from National Institute of Technology and Evaluation in Japan. The cells were grown in test tube filled with YPD medium (2% glucose, 1% peptone, and 0.5% yeast extract) at 30 °C for 18 h. The test tube was degassed and sealed during the cultivation. The cells were washed two times by centrifugation, and the cell density was adjusted to the desirable density with 0.1 M potassium phosphate buffer (pH 7.0) on the basis of the analytical curve between the turbidity (absorbance at

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600 nm) and CFU (colony forming unit) of yeast cell suspension.

CFU was counted after incubating the portion of yeast cell suspension on agar plate at 30 °C for 2 days. Agar plate was composed of 2% glucose, 1% peptone, 0.5% yeast extract, and 1.4% agar.

2.2. Permeabilization of yeast cells [18]

Yeast cells were collected by centrifugation, and 1g of pellet was suspended in 4 ml of permeabilization buffer (0.4 M sorbitol/0.1 M potassium phosphate buffer; pH 7.0). The suspension was mixed with 9 ml of 99.5% toluene and agitated at 42 °C for 5 min. The mixture was cooled in ice and washed two times by centrifugation at 10,000g for 10 min. The pellet was suspended in the above permeabilization buffer and adjusted to the desirable cell density.

2.3. TB reduction mediated by menadione and acetaldehyde in yeast cell suspension

The decrease in the absorbance at 645 nm due to TB reduction was followed after the addition of TB, menadione, and/or acetaldehyde to yeast cell suspension (4×10^6 cells/ml). The final concentration of TB, menadione, and acetaldehyde was 200 μM, 50 μM, and 50 mM, respectively. The final concentration of ethanol used as solvent of menadione was 70 mM and had little effect on TB reduction due to yeast cell activity. The reaction temperature was 25 °C.

2.4. TB reduction mediated by menadione and acetaldehyde in permeabilized yeast cell suspension

The decrease in the absorbance at 645 nm was followed after the addition of TB, NADH, menadione, and/or acetaldehyde to permeabilized yeast cell suspension (7.5×10^5 cells/ml). The final concentration of TB, NADH, menadione, and acetaldehyde was 200 μM, 500 μM, 50 μM and 50 mM, respectively. The final concentration of ethanol used as solvent of menadione was 70 mM. The reaction temperature was 25 °C.

2.5. TB reduction mediated by menadione and acetaldehyde in phosphate buffer

The decrease in the absorbance at 645 nm due to TB reduction was followed in the absence of permeabilized yeast cells under the conditions as described in Section 2.4.

2.6. Determination of electron flow from yeast cells to ferricyanide

Disposition of reaction container, electrode, salt bridge, and amperemeter was the same as described in the previous paper [19]. The cathodic reaction mixture was composed of 0.1 M potassium phosphate buffer (pH 7.0) and 1 mM potassium ferricyanide, and the anodic reaction mixture was composed of 0.1 M potassium phosphate buffer (pH 7.0), 200 μM TB, and yeast cells (4×10^6 cells/ml). The volume of each reaction mixture was 5 ml, and the both mixtures were shaken at 25 °C. The change in current was followed after the addition of menadione and/or acetaldehyde to the anodic reaction mixture. The final concentration of menadione and acetaldehyde was 50 μM and 50 mM, respectively. The final concentration of ethanol used as solvent of menadione was 70 mM.

2.7. NAD(P)H oxidation by menadione and acetaldehyde in permeabilized yeast cells suspension

The change in the absorbance at 340 nm was followed after the addition of NAD(P)H, menadione, and/or acetaldehyde to permeabilized yeast cell suspension (7.5×10^5 cells/ml). The final concentration of NAD(P)H, menadione, and acetaldehyde was 250 μM, 50 μM, and 50 mM, respectively. The final concentration of ethanol used as solvent of menadione was 70 mM. The oxidation rate of NAD(P)H was calculated on the basis of molar extinction coefficient of 6270 at 340 nm. The reaction temperature was 25 °C.

2.8. Fluorescence intensity due to NAD(P)H in yeast cell suspension

The concentration of NAD(P)H in yeast cells was determined by fluorescence (ex. 365 nm, em. > 430 nm). The change in fluorescence intensity was followed after the addition of menadione and/or acetaldehyde to yeast cell suspension (7.5×10^6 cells/ml). The final concentration of menadione, acetaldehyde, and ethanol was 50 μM, 50 mM, and 70 mM, respectively. In the case of the excessive addition of menadione solution, the final concentration of menadione and ethanol was 300 μM and 420 mM, respectively.

2.9. Determination of viable yeast cell number by TB reduction mediated by menadione and acetaldehyde

The yeast cell suspension was diluted to the desirable cell density with 0.1 M phosphate buffer (pH 7.0). The cell density was determined on the basis of CFU and the absorbance at 600 nm due to yeast cell suspension described in Section 2.1. The decrease in the absorbance at 645 nm due to TB reduction was determined after the addition of menadione for 1 min and then after the subsequent addition of acetaldehyde for 1 min at 25 °C. The final concentration of TB, menadione, acetaldehyde, and ethanol was the same as described in Section 2.3. The initial reduction rate of TB was calculated on the basis of the difference between the absorbance before and after the addition of menadione and/or acetaldehyde.

2.10. Cytotoxic effect of triton X-100

Yeast cells (4×10^6 cells/ml) was incubated with Triton X-100 at 25 °C for 90 min, and the initial reduction rate of TB was determined under the conditions described in 2.9. Yeast cells (2.5×10^5 cells/ml) was incubated in the YPB broth (2% glucose, 1% peptone, and 0.5% yeast extract) including Triton X-100 at 30 °C for 15 h. The absorbance due to the turbidity of yeast cell suspension was determined at 600 nm as described in Section 2.1.

2.11. Chemicals

Chemicals were obtained from Wako Pure Chemical Industries, Ltd.

2.12. Statistical analysis

The mean and the standard deviation were calculated by using Office Excel.

3. Results

3.1. TB reduction by menadione and acetaldehyde in yeast cell suspension

TB reduction in yeast cell suspension was promoted by the addition of menadione (•, ▲ at 2 min in Fig. 1), and TB reduction promoted by menadione was faster than that by acetaldehyde (■ at 2 min in Fig. 1). The combination of menadione and acetaldehyde (▲, ■ at 4 min in Fig. 1) promoted TB reduction faster than menadione-mediated TB reduction (• at 2 min in Fig. 1) and induced the synergistic reduction of TB. The addition of acetaldehyde to menadione-mediated TB reduction (▲ at 4 min in Fig. 1) induced greater synergistic TB reduction than the addition of menadione to acetaldehyde-mediated TB reduction (■ at 4 min Fig. 1). The above facts indicate that the order of addition of acetaldehyde and menadione influences the synergistic reduction of TB.