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A novel method for evaluating free radical scavenging abilities of antioxidants using ultraviolet induction of bacteriophage λ

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Abstract

A novel biological method used to evaluate free radical scavenging abilities of antioxidants using ultraviolet (UV) induction of bacteriophage λ in lysogenic *Escherichia coli* κ12 (λ^+) has been developed. This method is based on the induction of bacteriophage λ from lysogenic cycle to lytic cycle by ultraviolet irradiation, and formation of free radicals during the course of induction. In the experiments, 10^8 cells/ml and 30 s (39 J/m^2) were determined as the cell density of the lysogenic bacterium and UV irradiation time, respectively. The reliability of this method was demonstrated by electron paramagnetic resonance (EPR) spectroscopy and spin trapping with DMPO. This method had good reproducibility with intra-day variations (RSD, %) of less than 4% and inter-day variations (RSD, %) of less than 5%, respectively. By this method, the free radical scavenging abilities (ID_{50}) of well-known antioxidants such as glutathione, superoxide dismutase (SOD), catalase (CAT) and carotenoids were determined quantitatively. The results were consistent with the ones obtained by conventional methods for evaluating free radical scavenging abilities. This developed method is reliable and uses common instruments and inexpensive, stable reagents, thus it could be utilized as a routine laboratory quantitative assay to screen a large number of substances that have potential to scavenge the free radical.

Keywords: Bacteriophage λ; Ultraviolet induction; EPR; Free radical; *Escherichia coli*; Antioxidants

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1. Introduction

The technology available for detecting free radical reactions has improved over the past 30 years and now includes a variety of methods including spectrophotometric method with ABTS [1], chemiluminescence [2], high-performance liquid chromatography (HPLC) with DPPH [3], gas chromatography mass spectrometry (GC-MS) [4], proton-electron-double-resonance imaging (PEDRI) [5], cytochrome *c* reduction, fluorescence probes detection [6], immunochemical detection [7] and electron paramagnetic resonance (EPR) [8–10]. These methods have been applied to evaluate the free radical scavenging abilities of antioxidants. However, the methods such as spectrophotometric method with ABTS and HPLC with DPPH are single radical generating systems and other methods either require special and expensive instruments or cost-consuming.

Escherichia coli κ12 (λ^+) is a lysogenic bacterium and bacteriophage λ is a temperate phage which is capable of both lytic and lysogenic cycles. Lysogenic cycle occurs when the injected DNA (prophage) of the bacteriophage is integrated into the bacterial chromosome, and specific phage repression proteins (repressors) act to prevent the autonomous replication of the phage DNA and the expression of phage functional proteins which are required for the lytic cycle. Prophage enters the lytic cycle following the inactivation of the repressors. This induction process can be initiated by experimental means such as exposing lysogenic bacterium to ultraviolet (UV) irradiation. Accumulative evidences indicate that UV irradiation can produce various types of free radicals, including superoxide and hydroxyl radical [11–13]. These free radicals attack biological macromolecules such as nucleic acids, proteins, lipids, leading to cell damage and pathological changes in organisms [14–16]. After UV irradiation, the SOS response in lysogenic bacterium is induced in response to the DNA damage [17]. It is possible that the repair of UV damage leads to the production of single-stranded DNA regions near DNA lesions, stimulates the proteolytic activity of the RecA protein (product of *recA* gene), and then RecA protein promotes auto-proteolytic cleavage of certain prophage repressors, resulting in activation of the prophage to produce a full yield of phage particles, assembly of virus particles and lysis of the cell wall [18–20].

To the author's knowledge, there is little information available in the literature about the methods for evaluating the free radical scavenging abilities of antioxidants based on a biological method. In this study, we develop such a biological method using the lysogenic bacterium of *E. coli* κ12 (λ^+) for the first time. This method is based on the induction of bacteriophage λ from lysogenic cycle to lytic cycle by ultraviolet (UV) irradiation, and formation of free radicals during the course of induction. The free radical scavenging abilities of antioxidants were quantitatively evaluated by the inhibitory rates of antioxidants on UV induction of bacteriophage λ. The inhibitory rates (%) were determined by measuring the cell viability by counting the colony forming units (CFU) of *E. coli* κ12 (λ^+) after definite dose of UV irradiation. This newly developed method is reliable and uses common instruments and stable, inexpensive reagents, thus it could be utilized as a routine laboratory quantitative assay to screen a large number of substances that have potential to scavenge the free radical.

2. Materials and methods

2.1. Bacteria strains and media

The strain of *E. coli* κ12 (λ^+) from CCTCC (China Center for Type Culture Collection, Wuhan, China) was used as the lysogenic bacterium. It was grown in LB (Luria-Bertani) medium (1% tryptone, 0.5 % yeast extract, 1% NaCl, pH 7.0) at 37 °C or LB agar plates supplemented with 1.5% agar.

2.2. Preparation of antioxidants

The lipid- and water-soluble antioxidants were dissolved in appropriate solvents according to their respective solubilities. For evaluating the free radical scavenging abilities, the antioxidants were diluted to different concentration solutions with corresponding solvents.

2.3. Bacteria culture and UV irradiation procedure

An overnight culture of *E. coli* κ12 (λ^+) was grown at 37 °C in LB medium with continuous agitation (200 rpm). Cells in the late exponential phase were taken. The culture was centrifuged (4000 rpm for 10 min at 4 °C) and the sediment was resuspended in 2 ml phosphate buffer (pH 7.0). Afterward the cell suspension was spread onto the glass Petri dishes (6 cm in diameter). Care was taken to ensure that the desired cell concentration of the cell suspension to be irradiated was not higher than 10^8 cells/ml ($OD_{600}=0.4$). The influence of cell densities of lysogenic *E. coli* κ12 (λ^+) on the phage induction frequency were determined by measuring the phage forming units (PFU) according to the method reported previously [19].

The UV irradiation apparatus in a fume hood was used in this study. The 15 W low-pressure TUV mercury germicidal lamp (Philips Corp., Holland) was suspended horizontally in a reflecting hood. The lamp had a peak maximum emission at 254 nm with an intensity of 1.3 J/m^2 s determined by the DRC-100X digital radiometer (Spectronics Corp., New York, USA). The lamp was set at a constant distance of 30 cm from the samples irradiated. It was turned on for calibration at least 30 min prior to sample irradiation to ensure a uniform irradiation dose output. The samples were irradiated in the above sterile glass Petri dishes and the dishes were always placed on the center of the plate perpendicularly to the lamp. During sample irradiation, the dishes were opened and the cell suspension was stirred continuously. After irradiation, samples were kept in the dark and all operations were done in the subdued light. Irradiated cells were diluted and spread on the LB agar plates to determine the cell viability.

2.4. Determination of the cell viability by colony forming units (CFU)

To determine CFU of *E. coli* κ12 (λ^+), the bacterial dilutions were spread on the LB agar plates operated in subdued light. Then all the plates were incubated in the dark at 37 °C to avoid photoreactivation repair mechanisms. Colonies were counted after 48 h incubation at 37 °C and LB agar plates with 50–200 colonies were counted.

2.5. Evaluation of free radical scavenging abilities of antioxidants

At the same time when the samples were irradiated, the same bacterial suspension without adding any antioxidant and not irradiated was used as a negative control, and the same bacterial suspension irradiated and added the same concentration of corresponding solvent was used as a positive control. The free radical scavenging abilities of antioxidants were determined by measuring the inhibitory rates (%) on UV induction of bacteriophage λ. The inhibitory rates were calculated according to CFU on LB agar plates using the following formula:

$$\text{Inhibitory rate } (\%) = 100 \times (\text{CFU sample} - \text{CFU positive control}) / (\text{CFU negative control} - \text{CFU positive control}).$$

2.6. EPR measurements

To evaluate the reliability of this method, electron paramagnetic resonance (EPR) spectroscopy and the spin-trapping technique were used. All EPR measurements were performed using a Bruker ESP 300 EPR spectrometer (Bruker Corporation, Germany) operating at X-band with a TM_{110} cavity. EPR spin-trapping studies were performed with DMPO (Sigma, St. Louis, MO) at 0.8 mM. EPR spectra were recorded at room temperature in a flat quartz capillary. The instrument settings used in the spin-trapping experiments were as follows: modulation amplitude, 0.95 G; time constant 0.2 s; scan time 90 s; modulation frequency, 25 kHz; microwave power, 10 mW; and microwave frequency, 9.7 GHz.

For the measurements of the inhibitory effects of antioxidants on the free radical signals of UV induction of bacteriophage λ, the EPR signals were recorded when the mixtures of antioxidants and the cell suspension were put into the quartz cell and subjected to UV irradiation for 60 s. The 50 μl reaction mixture was 28 μl of lysogenic cells (10^8 cells/ml), 5 μl DMPO (0.8 mM), 5 μl DETAPAC (1 mM) and 12 μl NaCl (0.85%). The free radical scavenging abilities of antioxidants were investigated by comparing EPR spectra and quantified by comparing the relative height of the EPR signals in the presence of individual antioxidant to the control. Conditions of EPR measurements were the same in the experiments.

2.7. Reproducibility of the method

The measurements of intra- and inter-assay variability were utilized to determine the reproducibility of the developed method. To examine the intra-assay precision, which defined the variability within the same run, six replicates of each concentration of the antioxidant were performed simultaneously. The inter-assay precision representing the variability of different runs under the same condition was evaluated by the same concentrations of antioxidant. The inter-day reproducibility was examined in six separated days. The relative standard deviations (RSD, %) of intra- and inter-day were taken as the measurement of the reproducibility of the method.

2.8. Samples analysis

In this study, the free radical scavenging abilities expressed as 50% inhibitory dose (ID_{50}) of some well-known antioxidants such as glutathione, superoxide dismutase (SOD), catalase (CAT) and the carotenoids extracted from the extremely radioresistant bacterium, *Deinococcus radiodurans*, were determined quantitatively by this established method. The extraction, purification and HPLC analysis for the carotenoids isolated from *D. radiodurans* were performed according to the method reported previously [21].

2.9. Statistical analysis

All the results were presented as the mean ± S.D. One-way analysis on variance (ANOVA) was used to test for differences between samples and control groups. Pairwise comparisons were performed using the Student–Newmans–Keuls multiple-range test. Analysis was performed using ANOVA with the Student–Newmans–Keuls (SNK) options for multiple-range comparisons with the SAS system 8.1 software packages. p values less than 0.05 were considered statistically significant.

3. Results and discussion

Fig. 1 showed the influence of cell densities of lysogenic *E. coli* κ12 (λ$^+$) on the phage induction frequency induced by UV irradiation. The phage induction frequency was determined by measuring the phage forming units (PFU). Since ultraviolet light was not an ionizing irradiation and could penetrate biological tissue poorly, and in a dense suspension of bacteria some cells were shielded by others then the survival curves would have been distorted. From the results, when the cell density to be irradiated was 10^8 cells/ml, the PFU reached the maximum. So 10^8 cells/ml was fixed as the cell density of *E. coli* κ12 (λ$^+$) induced by UV irradiation in this experiment.

Fig. 2 showed the survival curve of the lysogenic *E. coli* κ12 (λ$^+$) with increasing periods of time of UV irradiation. As the source of UV light, the low pressure germicidal lamp used in this study should be calibrated before the experiment. Therefore, a killing curve was required before optimal conditions for the UV irradiation dose could be determined. Previous studies on UV radiation only showed the dose–response curves. In this study, we tried to assess the UV radiation effect by using additional regression analysis to avoid the inaccuracy of the graph representation method. We plotted the survival rate under UV irradiation exposure in dose rate (J/m^2 s). Dose rate was calculated as the product of the UV irradiation intensity (J/m^2) and the exposure time (s). The results demonstrated how the UV irradiation time could influence the survival curve of the lysogenic bacterium of *E. coli* κ12 (λ$^+$). *E. coli* κ12 (λ$^+$) was irradiated with the dose which reduce the cell survival rate to the level of 1–5% [19]. In this experiment, 30 s (39 J/m^2) was used as the irradiation time for all subsequent works.

Fig. 3 showed the inhibition rates (%) of the crude extract and main compound (deinoxanthin) of the carotenoids extracted from the extremely radioresistant bacterium *D. radiodurans* on UV induction of bacteriophage λ. The two carotenoids showed obvious protective effects on the lysogenic bacterium of *E. coli* κ12 (λ$^+$) irradiated by UV with the mean inhibitory rates of 75.3% and 39.5%, respectively.

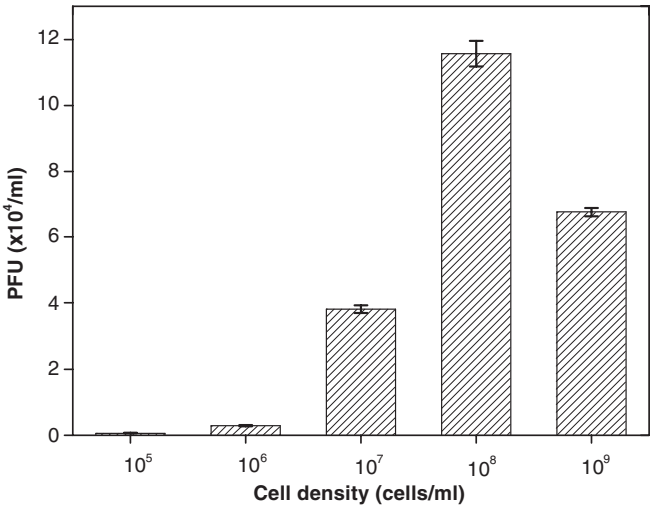

Fig. 1. The influence of cell densities of the lysogenic bacterium, *E. coli* κ12(λ$^+$), on phage induction frequency inducted by UV irradiation measured by phage forming unit (PFU). The values represent the mean±S.D. for at least triplicate determinations; $p<0.05$. The experimental conditions were described in Materials and methods.

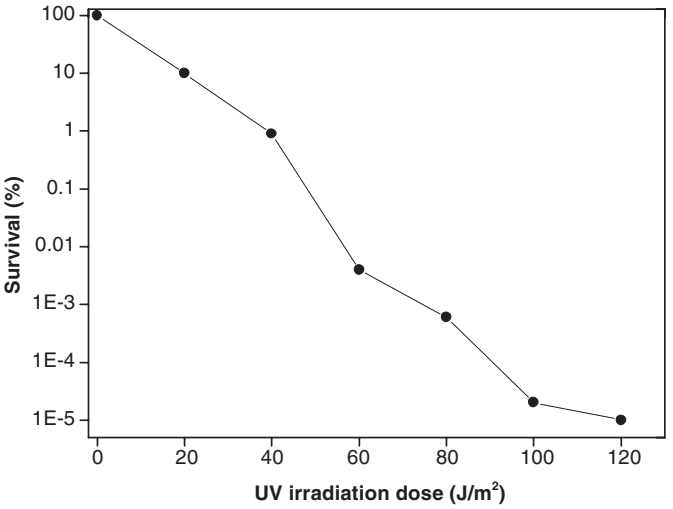

Fig. 2. Survival curve of the lysogenic bacterium of *E. coli* κ12 (λ$^+$) with increasing periods of time of UV irradiation (J/m^2). The cell density of *E. coli* κ12 (λ$^+$) was 10^8 cells/ml. The experimental conditions were described in Materials and methods.

Electron paramagnetic resonance (EPR) spectroscopy and the spin trapping with DMPO were used to directly detect the free radicals formed during the course of UV induction of bacteriophage λ. The results showed that complex types of free radical signals appeared after the *E. coli* κ12 (λ$^+$) was irradiated by UV (Fig. 4B), however, there was no free radical signal if there was no UV irradiation (Fig. 4A). Based on the tests of specific free radical scavengers including superoxide dismutase (SOD) for O_2^-, mannitol and catalase (CAT) for ·OH, β-carotene for $^1O_2$, it can be deduced that the UV induction of bacteriophage λ generated complex types of free radicals, mainly including O_2^- and relative low concentrations of $^1O_2$ and ·OH (data not shown).

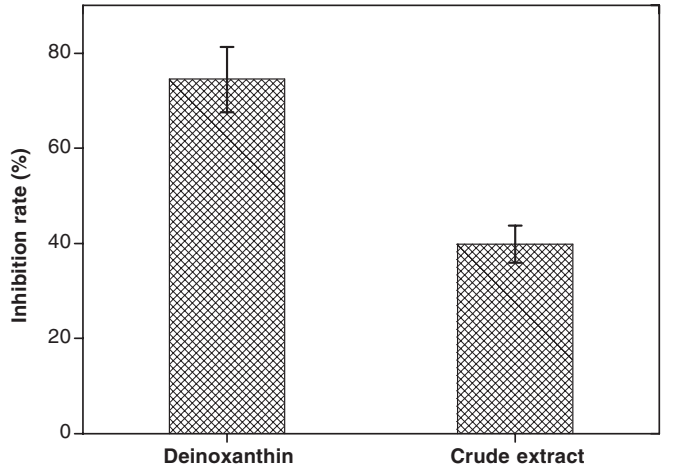

Fig. 3. The inhibitory rates (%) of carotenoids extracted from *D. radiodurans* on UV induction of bacteriophage λ by measuring the colony forming units (CFU). The formula for calculating the inhibitory rate was shown in Materials and methods. The values represent the mean±S.D. for six determinations; $p<0.05$.

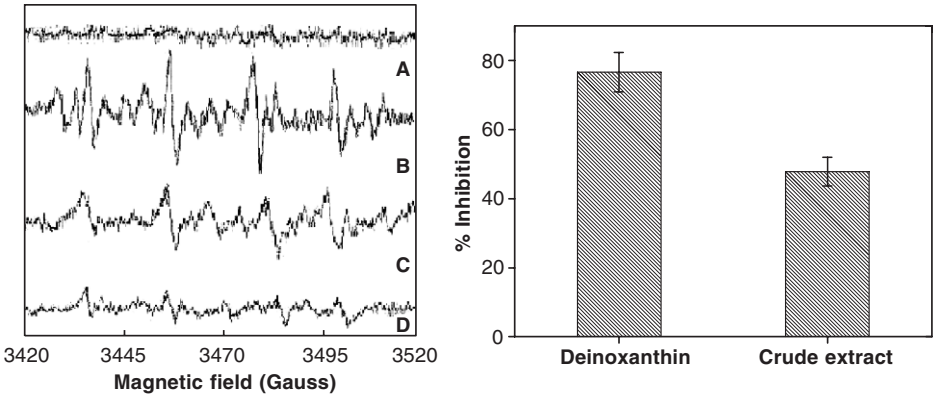

Fig. 4. EPR spectra of free radical signals during the UV induction of bacteriophage λ. (A) Lysogenic bacteria without UV irradiation, (B) lysogenic bacterium by UV irradiation without adding any antioxidant, (C) lysogenic bacterium by UV irradiation after adding the crude extract of carotenoids from *D. radiodurans*, (D) lysogenic bacterium by UV irradiation after adding deinoxanthin from *D. radiodurans*. Mean percent inhibitions of free radical signals were detected by spin trapping with DMPO on the enhanced ESR signals of UV induction of bacteriophage λ by the carotenoids. Each carotenoid was standardized to the control EPR signal detected without adding any compound (set at 0% inhibitory by convention). The values present the mean ± S.D. from at least three independent experiments; $p < 0.05$.

Fig. 4 showed the mean relative scavenging abilities, accompanied by EPR spectra, of the two carotenoids extracted from *D. radiodurans* on UV induction of bacteriophage λ. The EPR signals were significantly decreased in the presence of carotenoids compared with the control. The results obtained by EPR measurements and spin trapping with DMPO were well consistent with the results measured by CFU (Fig. 3). These results demonstrated the obvious free radical scavenging abilities of carotenoids extracted from *D. radiodurans*.

The developed method resulted in good reproducibility with overall intra-day variations (RSD, %) of 4.0% for deinoxanthin and 1.6% for crude extract, and inter-day variations (RSD, %) of 4.5% for deinoxanthin and 1.8% for crude extract, respectively.

By this established method, we have gained the results about the free radical scavenging abilities of some well-known antioxidants such as glutathione, superoxide dismutase (SOD),

Table 1
The free radical scavenging abilities expressed as 50% inhibitory dose (ID_{50}) of some well-known antioxidants and some Traditional Chinese Medicines (TCMs) obtained by the established method

	50% inhibitory dose (ID_{50})
Antioxidants	
SOD	50.60 U/ml
CAT	70 U/ml (to maximum 20% inhibition)
Glutathione (reduced form)	26.01 mM
Vitamin C	1.75 mM
β-Carotene	65.0 μM (to maximum 15.0% inhibition)
Mannitol	20 mM (to maximum 10.2% inhibition)
Melanin	0.44 mg/ml
TCMs	
Gynostemma penaphyllum	33.90 μg/ml
Cordyceps sinensis	4.04 mg/ml

catalase (CAT), vitamin C, carotenoids and some Traditional Chinese Medicines (TCMs). The results shown in Table 1 agreed with the results obtained by other detection methods such as EPR [22–24]. Those results demonstrated that this established method was a reliable method for evaluating the free radical scavenging abilities of antioxidants.

4. Simplified description of the method and its (future) application

This study offers a novel, biological method for evaluating the free radical scavenging ability of different substances. The method is based on the induction of bacteriophage λ in lysogenic *E. coli* κ12 (λ^+) from lysogenic cycle to lytic cycle by UV irradiation and formation of free radicals during the course of induction. The free radical scavenging abilities of antioxidants were quantitatively evaluated by the inhibitory rates of antioxidants on UV induction of bacteriophage λ. The inhibitory rates (%) were determined by measuring the cell viability by counting the colony forming units (CFU) of *E. coli* κ12 (λ^+) after definite dose of UV irradiation. This method is reproducible, reliable and uses common instruments, inexpensive and stable reagents, thus it is well suited for the routine laboratory quantitative assay to screen a large number of substances that have potential to scavenge the free radical.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbbm.2006.02.010.

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