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Investigations of riboflavin photolysis via coloured light in the nitro blue tetrazolium assay for superoxide dismutase activity

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ABSTRACT

Determination of the superoxide dismutase activity is an important issue in the fields of biochemistry and the medical sciences. In the riboflavin/nitro blue tetrazolium (B_2/NBT) method, the light sources used for generating superoxide anion radicals from light-excited riboflavin are normally fluorescent lamps. However, the conditions of B_2/NBT experiments vary. This study investigated the effect of the light source on the light-excitation of riboflavin. The effectiveness of the photolysis was controlled by the wavelength of the light source. The spectra of fluorescent lamps are composed of multiple colour lights, and the emission spectra of fluorescent lamps made by different manufacturers may vary. Blue light was determined to be the most efficient for the photochemical reaction of riboflavin in visible region. The quality of the blue light in fluorescent lamps is critical to the photo-decomposition of riboflavin. A blue light is better than a fluorescent lamp for the photo-decomposition of riboflavin. The performance of the B_2/NBT method is thereby optimized.

1. Introduction

Superoxide dismutase (SOD) is essential to many living organisms. This enzyme is one of the most important antioxidant defence mechanisms in microorganisms and plant cells that are exposed to oxygen [1]. The antioxidant capacity of natural ingredients is also a significant issue for foods with special dietary purposes [2]. SOD, as a free radical scavenger, can convert superoxide anion radicals ($O_2^{\cdot-}$) into H_2O_2 and O_2 in living cells. By scavenging $O_2^{\cdot-}$, the oxidation of lipid membranes can be prevented [3]. Superoxide anion radicals are intermediate products generated during oxidation or reduction. Formed from hydroxyl radicals or hydroxyl peroxide compounds, $O_2^{\cdot-}$ causes damage, inflammation, atherosclerosis and aging of cells [4,5]. Hence, the determination of SOD activities, either *in vivo* or *in vitro*, is an important topic in the fields of biochemistry and the medical sciences.

Many methods, both direct and indirect, have been developed for the determination of SOD activity. However, direct assays for SOD determination are scarce because of their need for special apparatus, such as an electron paramagnetic resonance spectrometer (EPR). Indirect assays relying on the ability of SOD to inhibit $O_2^{\cdot-}$-driven reactions are more widely applied in biochemical laboratories [6]. Beyer and Fridovich [7] investigated the effects of experimental variables on two indirect assays, the xanthine oxidase and cytochrome C method and the riboflavin/nitro blue tetrazolium method (B_2/NBT). The B_2/NBT method is considered simpler and is preferred for the quantification of SOD activity in crude extracts [7].

Riboflavin, also known as vitamin B_2, is very sensitive to light. It decomposes after being irradiated by ultraviolet (UV) or visible light (420–560 nm) for a very short time [8], generating free radicals of reactive oxygen species (ROS), such as $O_2^{\cdot-}$ and singlet oxygen [9]. The riboflavin photochemical treatment with blue light can be employed to inactivate *E. coli* with generated ROS [10,11].

Superoxide anion radicals generated from light-excited riboflavin can be utilized to examine the effect of luminance on light reactions of nitro blue tetrazolium (NBT) [5]. In this study, NBT is used as an indicating scavenger to be reduced by $O_2^{\cdot-}$. NBT reduction causes an increase in the absorbance at 560 nm in a process that may be inhibited by SOD. In addition, the B_2/NBT method can be employed to evaluate the contents of phenolic compounds in functional foods through ROS scavenging.

In the B_2/NBT method, the light sources used for generating $O_2^{\cdot-}$ from light-excited riboflavin are normally fluorescent lamps. A fluorescent lamp is excited to generate ultraviolet rays by a low-pressure mercury vapour and argon. The inner wall of a glass tube is coated with a fluorescent substance and stimulated by ultraviolet rays to generate a hybridized visible light. However,

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the conditions and apparatuses for the B_2/NBT experiments varied in the studies reviewed. The fluorescent lamps used were of different specifications, including 13 W [1], 15 W [12,13], 20 W [7,14], 25 W [15], 30 W [16] and 40 W [17], and were illuminated over different distances and for different durations. The reactions were initiated by adding riboflavin at 2000 [18], 3000 [19], 4000 [20] and 5000 lux [21] for the luminance of fluorescent lamps.

The effects of light source properties, such as colour and wavelength, on the light-excitation of riboflavin have been investigated using the B_2/NBT assay [22]. The fluorescent lamp is excited to generate a hybridized visible light comprised of multiple colour lights. Thus, the properties of the light source could affect the riboflavin photochemistry, leading to incorrect conclusions from the B_2/NBT assay. To ensure high accuracy, the widely accepted B_2/NBT assay for the quantification of SOD activity has to be validated.

The current study developed an effective SOD assay from the B_2/NBT method by applying a well-defined light source to riboflavin photochemical reactions. The goal was to investigate the effects of light quality on the light-excitation of riboflavin as assayed by the B_2/NBT method. The results thus obtained would promote the consistency of enzymatic measurements using photolysis reactions.

2. Materials and methods

2.1. Setup of illumination units

The photo-induced reactions were performed in a plastic box (104 cm × 74 cm × 55 cm) with a light source. The box was made of white cardboard, and its outer surface was covered with black cloth. Three light-emitting diode (LED) tube lights (580 mm length) in red, green and blue (VITALUX T8HO LED tube lights, Vita LED Technologies Co., Tainan, Taiwan) and two fluorescent lamps, Fluor-A (38 W, FHF38WEX, Taiwan Fluorescent Lamp Co., Taipei, Taiwan) and Fluor-B (30 W, FCL30D/28, China Electric MGF. Co., Taipei, Taiwan), were used as light sources. Irradiance was measured by the power of the electromagnetic radiation per unit area (mW/cm^2) with radiometry and validated by a solar power meter (TM-207, Tenmars Electronics Co., Taipei, Taiwan). Luminance, a term used in photometry, was measured in lux (lx) or lm/m^2 by a digital light meter (YF-170, Tenmars Electronics Co., Taipei, Taiwan).

2.2. Chemicals

Gallic acid, l-methionine, monopotassium phosphate, potassium dihydrogen phosphate, riboflavin and SOD (S9697-15KU) were purchased from Sigma–Aldrich (St. Louis, MO). The SOD was assayed by Sigma–Aldrich using the xanthine oxidase/cytochrome C method [23]. Nitro blue tetrazolium (NBT) was purchased from Bio Basic, Inc. (Markham, Ontario, Canada). Ultra-pure deionized water from a Milli-Q system was used as a solvent in this study.

2.3. Spectrometry of light sources and riboflavin

The emission spectra of the fluorescent lamps and LED tube lights were measured using a UV–vis miniature fibre optic spectrometer (USB4000 UV/Vis, Ocean Optics, USA) and were normalized, as shown in Fig. 1. The wavelengths of the emitted maxima of the blue, green, yellow and red lights were 463, 529, 589 and 632 nm, respectively, and the spectral widths at half height ($W_{1/2}$) were 23, 31, 16 and 14 nm, respectively. The spectra of the fluorescent lamps are usually comprised of several peaks, as shown in Fig. 1.

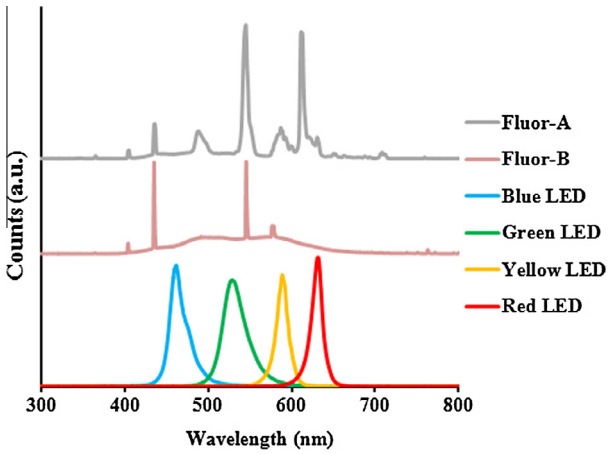

Fig. 1. Visible spectra of fluorescent and LED lamps used in this study.

The riboflavin (2.4 μM in 50 mM, pH 7.8 phosphate buffer) was irradiated by the fluorescent lamps and LED tube lights at 1.0 mW/cm^2 for 20 min. The absorbance of the illuminated riboflavin was detected at 200–800 nm by a UV/vis spectrometer (Lambda35, Perkin-Elmer).

2.4. Effects of light sources on the generation of $O_2^{\cdot-}$ with the B_2/NBT method

The reduction in NBT was determined using the method developed by Beauchamp and Fridovich [24]. All solutions were 50 mM in phosphate buffer (pH 7.8). 3 mL of reactant was used, and the concentrations of riboflavin, methionine and NBT were 2.4×10^{-6} M, 0.01 M and 1.6×10^{-4} M, respectively. The distance between the reactant and the lamps was fixed, and the irradiance was controlled. The reactant was illuminated by blue, green, yellow or red LED irradiation at 1.0 mW/cm^2, by the fluorescent lamps at 1.0 mW/cm^2, and by the blue light irradiation at 0.1 mW/cm^2 for 10, 20 or 30 min. For the control treatment, the reactant was kept in the dark. The photo-chemically reduction of riboflavin generated $O_2^{\cdot-}$, which reduced NBT to form blue formazan, which can be detected at 560 nm (Lambda35, Perkin-Elmer).

2.5. Effects of light source on $O_2^{\cdot-}$ scavenging activity using gallic acid

Gallic acid was employed to determine the effects of the light source on the $O_2^{\cdot-}$ scavenging activity using the B_2/NBT method described in Section 2.4. In brief, gallic acid (50 μL) was added to 3 mL reactant to final concentrations of 0, 10, 20, 40, 60, 80 and 100 μg/mL. Then, the mixed solutions were subjected to Fluor-A or Fluor-B irradiation at 1.0 mW/cm^2 or blue-light irradiation at 0.1 mW/cm^2 for 20 min. Gallic acid can inhibit NBT reduction, and the scavenging capacity of the $O_2^{\cdot-}$ generated was calculated using the following equation, where A denotes the absorbance of the blue formazan measured at 560 nm.

$$O_2^{\cdot-}\text{scavenging activity}(\%) = \left[(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}\right] \times 100\% \quad (1)$$

2.6. Effects of blue light on SOD activity

The effects of blue light on $O_2^{\cdot-}$ scavenging activity were examined with the B_2/NBT method using SOD, as described in Section 2.4. In brief, (A) 50 μL SOD was added to 3 mL reactant, and the final activity of SOD (1.0 unit/g) was used as a standard. Then, the mixed solutions were subjected to blue light irradiation