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Pyrroloquinoline quinone from *Gluconobacter oxydans* fermentation broth enhances superoxide anion-scavenging capacity of Cu/Zn-SOD

Ke Ma, Jun-Zhu Cui, Jian-Bin Ye, Xian-Mei Hu, Ge-Li Ma, Xue-Peng Yang *

School of Food and Biological Engineering, Henan Provincial Collaborative Innovation Center for Food Production and Safety, Zhengzhou University of Light Industry, Dongfeng Road, Zhengzhou 450002, People's Republic of China

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ABSTRACT

A bioassay-guided fractionation of extract from *Gluconobacter oxydans* fermentation broth afforded Compound **1**, which was identified as pyrroloquinoline quinone (PQQ) by spectroscopic methods. PQQ has been shown to enhance the superoxide anion-scavenging capacity significantly for Cu/Zn-SOD. To illustrate the mechanism, the interaction between PQQ and Cu/Zn-SOD was investigated. The multiple binding sites involving hydrogen bonds and van der Waals force between PQQ and Cu/Zn-SOD were revealed by isothermal titration calorimetry. The α-helix content was increased in the Cu/Zn-SOD structure with the addition of PQQ into the solution through ultraviolet (UV) spectroscopy. These results indicated that PQQ could change the conformation of Cu/Zn-SOD through interaction, which could enhance its superoxide anion-scavenging capacity. Therefore, PQQ is a potential natural antioxidant.

1. Introduction

Reactive oxygen species (ROS), including superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$), and singlet oxygen ($^1O_2$), are continuously produced by living organisms. A normal amount of ROS is essential for the biological oxidation of cells (Fridovich, 2004; Muñoz et al., 2000), but excess ROS results in the oxidation and loss of cellular macromolecules, which is related to diseases such as cancer, diabetes, and atherosclerosis (Sun, Zhu, Jiang, Li, & Ge, 2014; Umasuthan et al., 2013; Valko et al., 2007). Therefore, to help retard oxidative damage to humans, synthetic antioxidants are widely applied in food-related production. However, the use of synthetic antioxidants has been restricted, as they possibly have toxic and carcinogenic effects (Ito et al., 1986). Consequently, there has been increased interest to find natural antioxidants to protect humans from oxidative damage.

Gluconobacter oxydans is known for its rapid and incomplete oxidation of numerous sugars, sugar acids, polyols, and alcohols, and is currently used for industrial food-related production, such as acetic acid (vinegar), L-(-)-sorbose (used in vitamin C synthesis), and 6-amino-L-sorbose (a miglitol precursor) (Deppenmeier, Hoffmeister, & Prust, 2002; Gupta, Singh, Qazi, & Kumar, 2001; Prust et al., 2005). To the best of our knowledge, the metabolites of *G. oxydans* have not been investigated previously. Hence, screening for compounds with antioxidant activity from the metabolites of *G. oxydans* is an interesting and practical proposal, owing to their important role in oxidation reactions.

The aim of the current study was to determine the chemical constituents of the *G. oxydans* fermentation broth, and their possible antioxidant activities. The chemical compounds were analyzed using a HP-20 macroporous resin. Bioassay-guided fractionation was subsequently carried out by the SOD Assay Kit-WST test, and purified by ODS column chromatography to yield Compound **1**, elucidated as pyrroloquinoline quinone (PQQ). The SOD Assay Kit-WST test suggested that PQQ could significantly enhance the superoxide anion-scavenging capacity of Cu/Zn-SOD. The interaction between PQQ and Cu/Zn-SOD was researched by isothermal titration calorimetry to explain this phenomenon. Binding at multiple sites between PQQ and Cu/Zn-SOD occurred mainly through hydrogen bonds and van der Waals force. UV-spectroscopy indicated that the conformation of Cu/Zn-SOD was changed by adding PQQ to the solution.

2. Materials and methods

2.1. Materials

Cu/Zn-SOD was obtained from the Sigma-Aldrich Chemicals Company (USA). The SOD Assay Kit-WST was obtained from Dojindo, Japan. The stock solution of Cu/Zn-SOD was prepared in

* Corresponding author.
E-mail address: yangxuepeng@zzuli.edu.cn (X.-P. Yang).

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double-distilled water at concentrations of 4 and 0.02 mg/mL. All samples were prepared by dissolving an appropriate amount in double-distilled water and kept in the dark at 4 °C. All other reagents were of analytical purity or higher. The pH was measured using a digital pH-meter with a combined glass-calomel electrode. All solutions were thoroughly degassed before use.

2.2. SOD Assay Kit-WST test

To screen for the superoxide anion-scavenging, an SOD Assay Kit-WST was used. Reagents No. 1, 2, 3, and 4 were pretreated following the manufacturer's instructions. The sample and reagents were thoroughly mixed and measured after 40 min at 37 °C. Then, the mixture was allowed to stand for 10 min at 25 °C, and analyzed using a TU-1810SPC spectrophotometer at 550 nm.

Herein, 1 U (the unit of activity of SODs) is the quality of SODs when the superoxide anion free radical content in 1 ml PCR mixture is 50%.

$$\text{superoxide anion scavenging capacity} = \frac{\text{Control group OD} - \text{Assay Group OD}}{\text{Control group OD}} \times \text{Dilution ratio of reaction system} \times \text{Dilution ratio of sample} \quad (1)$$

2.3. Microorganisms and fermentation conditions

The strain *G. oxydans* ATCC 621H was used in the experiments. The fermentation medium was composed of 80 g/L sorbitol, 20 g/L yeast extract, 5 g/L $(NH4)_2SO_4$, 2 g/L KH_2PO_4, and 5 g/L $MgSO_4{\cdot}7H_2O$. All media were sterilized by autoclaving at 115 °C for 15 min. The pre-culture was inoculated with a single-celled colony from an agar plate and incubated in a shaker at 250 rpm for 24 h at 28 °C. A 10% (v/v) solution of the cells was added to 100 mL medium in a flask and grown at 28 °C for 24 h.

2.4. Isolation and purification

The fermentation broth (2000 mL) was centrifuged at 6000*g* for 15 min to separate the cells. The supernatant was applied to a 700-mL HP-20 resin column, and eluted with a gradient of MeOH/H_2O (2100 mL for 0:1, v/v; 4200 ml for 1:1, v/v; 3500 mL for 7:3, v/v) to afford four fractions (Fr. 1–4). The four fractions were evaluated by the SOD Assay Kit-WST test. Fraction 2 was subjected to a Sephadex LH-20 column, and eluted with H_2O, to yield four sub-fractions (sub-Fr. 1–4). The four sub-fractions were evaluated by the SOD Assay Kit-WST test. Sub-fraction 2 was subjected to ODS column chromatography, eluting with a gradient of ACN/H_2O (5:95 to 99:1, v/v, containing 0.1% TFA), to yield Compound **1** (8 mg, 4 mg/L in the fermentation broth).

2.5. Isothermal titration calorimetry

Calorimetry was carried out in an isothermal calorimeter for VP-ITC titrations (MicroCal-USA) at a temperature of 25 °C. A measurement cell contained 1.4275 mL of degassed solution of PQQ in water. The aqueous solution of PQQ with a concentration of 0.26 mM was titrated with an aqueous solution of Cu/Zn-SOD with a concentration of 0.013 mM from a syringe.

All the measurements were carried out at a temperature of 25 °C. The titrant solutions in all the mentioned measurements were injected from a syringe with 50 ratios, 5 μl each. The injection duration was 30 s, and was carried out at 85 min intervals, and the stirrer revolution rate was 180 rpm.

The relationships between the thermodynamic parameters and binding force have been summarized by Ross and Subramanian (1981). The values of molar enthalpy (ΔH) and free enthalpy (ΔG) indicated the titration of SOD with Compound **1** solutions is spontaneous and exothermic. When the molar enthalpy (ΔH) and entropy (ΔS) is less than zero, the binding force consists of a hydrogen bond and van der Waals force.

2.6. Ultraviolet (UV)-visible (Vis) absorption spectroscopy

The UV–Vis absorption measurements were carried out using a TU-1810SPC spectrophotometer (Puxi Analytic Instrument Ltd. of Beijing, China) in a 1.0-cm quartz cuvette at 25 °C. All samples were incubated for 30 min and the spectra were recorded in the range of 200–500 nm.

3. Results

3.1. Bioassay-guided fractionation of fermentation broth

The SOD Assay Kit-WST was developed by Dojindo, Japan. Owing to its high sensitivity and accuracy, this method was widely applied for the superoxide anion-scavenging activity test. In the present study, this method was applied for screening the antioxidant activity from the fermentation broth of *G. oxydans*. Bioassay-guided fractionation through HP-20 macroporous resin, polyamide, and ODS column chromatography resulted in the isolation of Compound **1**.

Compound **1** was obtained as a red brown powder, and dissolved in water. Its molecular formula was established as $C_{14}H_6N_2O_8$, using ESIMS (*m*/*z* 329 $[M\text{-}H]^-$), ^{13}C NMR, and ^1H NMR. Its NMR data (600 MHz, DMSO): ^1H NMR 7.2 (s, 1H, H-C_3), 8.6 (s, 1H, H-C_8), 13.3 (br, 1H, NH); ^{13}C NMR 113.4, 124.5, 126.4, 127.6, 128.9, 134.0, 135.5, 178.0. The structure was elucidated as PQQ by comparison with data reported in the literature (Kong et al., 2011).

The details of the bioassay are shown in Fig. 1, which showed Compound **1** has little superoxide anion scavenging activity. However, the effect of Compound **1** on the superoxide anion scavenging

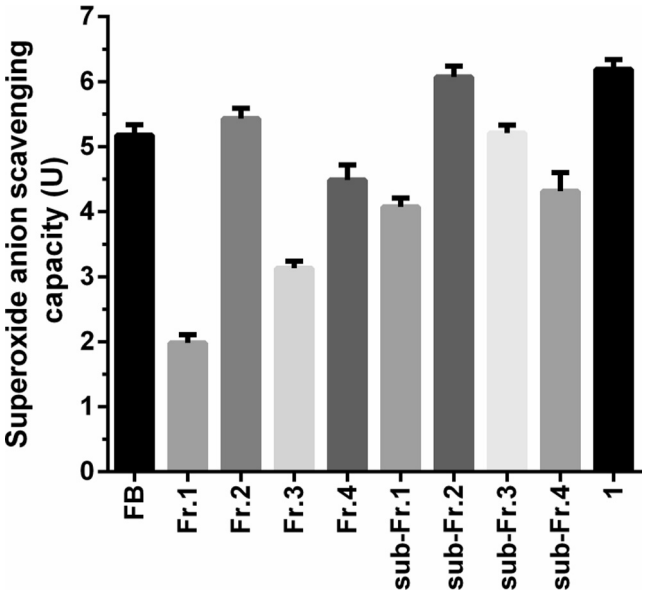

Fig. 1. Bioassay-guided fractionation of fermentation broth via superoxide anion scavenging capacity test (The concentration of all sample is 1 mg/ml, and FB means fermentation broth.)