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Antioxidant activity in vitro of the selenium-contained protein from the Se-enriched *Bifidobacterium animalis* 01

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ABSTRACT

Several studies indicated that bifidobacteria possessed strong antioxidant activity. In present study, the antioxidant activities of *Bifidobacterium animalis* 01 proteins were evaluated using six assays, namely, linoleic acid preoxidation assay, erythrocyte hemolysis assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, reducing power assay, hydroxyl (•OH) and superoxide radicals ($\bullet O_2^-$) assays, in which the last two assays were measured by electron spin resonance (ESR). There were two kinds of *B. animalis* 01 proteins in this study, the regular *B. animalis* 01 protein (Pro-CK) and the *B. animalis* 01 selenium-contained protein (Pro-Se). Both Pro-CK and Pro-Se showed concentration dependent antioxidant activity in DPPH assay, reducing power assay and erythrocyte hemolysis assay. All results of six assays indicated that the antioxidant activity of the *B. animalis* 01 protein was improved remarkably after selenium was incorporated. The antioxidant activity of Pro-Se increased with the increase of selenium content in Pro-Se suggesting selenium played a positive role in enhancing the antioxidant activity of *B. animalis* 01 protein. Moreover, organic selenium was more effective than inorganic selenium on enhancing the hydroxyl radical scavenging ability of *B. animalis* 01 protein.

1. Introduction

Oxidative modification of DNA, proteins, lipids and small cellular molecules caused by reactive oxygen species (ROS) played a role in a variety of common diseases and age-related degenerative conditions [1]. Under normal circumstances, 1–5% of the molecular oxygen in the cells creates active Oxy-free radicals in many different ways, but the human bodies possess enzymatic and non-enzymatic antioxidative mechanisms and minimize the generation of reactive oxygen species to levels that are not harmful to the cells. When the generation of the active Oxy-free radical is overgrown or the free radical scavenging capability is weakened for some reason, many degenerative diseases, such as brain dysfunction, cancer, heart diseases and declination of the immune system, could be caused by the excessive of free radicals [2]. With the aging of the human body, the biosynthesis of enzymes which scavenge free radicals is decreasing. Thus, excessive free radicals react with biologically active substances such as lipids, protein and DNA to cause cell membrane injury, protein denaturation and wrong DNA replications.

Selenium, a kind of essential minor elements for human body, has biological effect in many ways. In the first half of the 20th century, selenium was considered an undesirable element in human body due to its toxicity. In the second half of the 20th century, Schwarz and Foltz reported that selenium at very low dietary concentrations is an essential nutrient [3]. Recently, selenium has been reported to possess strong free radical scavenging ability and can protect the cell membrane, preventing cells from malignant transformation [4]. Furthermore, the antioxidant activity of the scavenger was enhanced when it contained selenium. It is reported that selenium enhanced the antioxidant activity of the protein extracted from the Se-enriched mushroom [5]. On the other hand, the carcinostatic activities of selenium compounds have been shown concentration dependent and several studies have indicated that the concentration of selenium may play an important role in selenium catalysis and toxicity [6]. Another study reported that selenium compounds at low concentration may have protective anticarcinogenic properties, whereas at higher concentration they could be possibly carcinogenic [7]. Hence, whether selenium is beneficial or harmful to human body is determined by its concentration.

Since bifidobacteria was isolated by Doctor Tissier, the research on it has never stopped, especially on its physiological function [8]. *Bifidobacterium longum* ATCC 15708 and *Lactobacillus acidophilus* ATCC 4356 has been reported to have strong inhibitory ability on the plasma lipid peroxidation [9]. Lin and Yen found out that the

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cell-free extraction of the *L. acidophilus* and *B. longum* showed strong inhibitory effect on linoleic acid peroxidation [10]. In addition, a kind of soymilk fermented with bifidobacteria was reported that it could scavenge superoxide anion and possessed hydrogen peroxide scavenging ability [11]. However, few reports dealing with which substance in bifidobateria was responsible for the antioxidant activity could be found to date. In present study, we isolated Pro-CK, trying to evaluate its antioxidant activity. What is more, as a follow-up to our previous characterization on the distribution of selenium in *Bifidobacterium animalis* 01 which indicated most of the organic selenium was found in the protein fraction [12], we also isolated Pro-Se and comspared the difference of the antioxidant activity between Pro-CK and Pro-Se, trying to disclose the importance of the selenium in the antioxidant process.

2. Materials and methods

2.1. Chemicals and reagents

Xanthine, xanthine oxidase, linoleic acid, Tween 20, trichloroacetic acid (TCA), butylated hydroxytoluene (BHT), DPPH (1,1-diphenyl-2-picrylhydrazyl), thiobarbituric acid (TBA) and DMPO (dimethyl pyridine N-oxide) were purchased from Sigma-Aldrich (Shanghai, China). Tris was obtained from Roche (China). DETAPAC (diethylenetriamine penta-acetic acid), ascorbic acid, ferrous sulfate, hydrogen peroxide, sodium hydroxide and ammonium sulfate which were purchased from Beijing Chemicals Co. Ltd. (China) were of research purity grade.

2.2. Microbial strains and culture conditions

B. animalis 01 was obtained from College of Food Science & Nutritional Engineering, China Agriculture University, which was isolated from a healthy centenarian in GuangXi of China. *B. animalis* 01 was cultured on the improved MRS broth medium which was composed of peptone (10.0 g/L), beef extract (10.0 g/L), yeast extract (5.0 g/L), K_2HPO_4 (2.0 g/L), triammonium cetrate (2.0 g/L), sodium acetate (5.0 g/L), glucose, tween80 (1.0 ml/L), $MgSO_4 \cdot 7H_2O$ (0.58 g/L), $MnSO_4 \cdot H_2O$ (0.25 g/L), corn steep liquor (3.0 g/L) and cysteine hydrochloride (0.3 g/L). After 6 h, sodium selenite of different concentration (0, 2.5, 5.0, 8.0, 10.0 μg/ml) was added to the medium [12]. The Se-enriched bifidobacteria were marked with CK, Se2.5, Se5.0, Se8.0 and Se10.0 respectively, according to the sodium selenite concentration. The Se-enriched bifidobacteria were removed by centrifugation after cultured with sodium selenite for another 12 h, and then it was preserved in the dark at room temperature after freeze drying (−50 °C, under vacuum) by vacuum freeze drier (FD-1B-50, Beijing Boyikang Instruments Co. Ltd).

2.3. Preparation of B. animalis 01 proteins

The protein was isolated according to the method of Zhang [12]. 3.0 g of each kind of dry bacteria sample (CK, Se2.5, Se5.0, Se8.0, Se10.0) was dissolved in cold sodium hydroxide solution (0.25 M), the bacteria samples were broken by ultrasonication and incubated at 50 °C in DK-8B water bath (Shanghai Jinghong Instruments Co. Ltd, China) for 2 h. The supernatant was obtained by filtration and the residue was repeated twice with 50 ml of NaOH (0.25 M). Then ammonium sulfate was added to the supernatant to make 95% saturated solution which was followed by keeping it overnight at 4 °C. Protein was precipitated using Thermo Scientific CL 10 centrifuge (Thermo Fisher Scientific, US) at 6000 rpm for 30 min at 4 °C. The resulting precipitate was then dissolved in 10.0 ml of Tris-HCl (pH = 8.0, 50 mM). This solution was passed through 0.22 μm syringe filter (13 mm, Millipore US) and dialysed against 1.0 L of Tris-HCl (pH = 8.0, 50 mM) using a membrane (Shanghai OuweiDa Instruments Scientific Co. Ltd, China) with 3500 molecular weight cutoff at 4 °C three times to remove ammonium sulfate. Finally, the solution in dialysis bag was lyophilised using vacuum freeze drier (FD-1B-50, Beijing Boyikang Instruments Co. Ltd). Each kind of protein was marked with Pro-CK, Pro-Se2.5, Pro-Se5.0, Pro-Se8.0 and Pro-Se10.0. The selenium content in each protein sample has been determined to be 0, 0.269, 0.325, 0.498 and 0.641 mg/L respectively by hydride generation-atomic absorption spectrometry (HG-AAS, haiguang analytical Co., China) in our previous study [12].

2.4. Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was measured by the modified method of Liu et al. [13]. Hydroxyl radical was generated by the addition of ferrous ion to a reaction mixture which contains phosphate buffer. The adduction activity of the hydroxyl radical caused the decrease of absorbance at 510 nm measured by UV-2100 spectrophotometer (UNICO, US). If hydroxyl radical scavenger was added to the mixture, part of the hydroxyl radical can be scavenged and the absorbance decrease at 510 nm will reduce. In order to conduct the rest of assays with a proper sample concentration, two concentration groups were involved in this assay-the high concentration group (5.00 mg/L) and the low concentration group (1.00 mg/L). 1.0 ml sample was added to tubes containing 1.0 ml phosphate buffer (pH = 7.4, 0.75 M), 0.5 ml phenanthroline (1.0 mM), 3.5 ml deionized water, 1.0 ml ferrous sulfate (7.5 mM) and 1.0 ml H_2O_2 (0.01%, v/v). The tube was incubated at 37 °C for 90 min. In the H_2O_2 control, the sample was substituted with deionized water. In the normal control, both the sample and the H_2O_2 were substituted with deionized water. In the positive control, the sample was substituted with ascorbic acid. The capability of scavenging hydroxyl radical was calculated using the following equation:

$$\text{Scavenging effect}(\%) = \left[\left(A_{\text{sample}} - A_{\text{normal}}\right) \Big/ \left(A_{H_2O_2} - A_{\text{normal}}\right)\right] \times 100$$

2.5. Inhibition of linoleic acid peroxidation

The inhibitory effect of the *B. animalis* 01 proteins was determined according to the thiobarbituric acid method [14]. Linoleic acid was prepared in Tween 20, a slight modification according to the method of Grossman and Zakut [15]. In brief, the linoleic acid emulsion was prepared by addition of 0.1 ml linoleic acid to 19.7 ml deionized water containing 0.2 ml Tween 20. In 1.0 ml linoleic acid emulsion, 0.2 ml ferrous sulfate (0.01%, w/v), 0.2 ml H_2O_2 (0.02%, v/v) and 1.0 ml sample (1.00 mg/L) was dissolved followed by incubation at 37 °C for 12 h. After that, 0.2 ml trichloroacetic acid (4.0%, w/v) was added to terminate the reaction, and then the solution was heated at 100 °C with 2.0 ml TBA (0.8%, w/v) and 0.2 ml BHT (0.4%, w/v) for 30 min. The absorbance was measured by UV-2100 spectrophotometer (UNICO, US) at 532 nm. In the control, the samples were substituted with phosphate buffered saline (PBS) buffer (pH = 7.4, 0.02 M) which was composed of Na_2HPO_4 (0.02 M) and NaH_2PO_4 (0.02 M). The capability of inhibitory effect was calculated using the following equation:

$$\text{Inhibitory effect}(\%) = \left(1 - A_{\text{sample532}}/A_{\text{control532}}\right) \times 100$$

2.6. DPPH radical scavenging activity assay

DPPH radical scavenging activity of the *B. animalis* 01 proteins was evaluated as described by Blois with some modifications [16].