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RESEARCH NOTE

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Antioxidant Activity of Partially Purified Extracts Isolated from *Bacillus polyfermenticus* SCD Culture

Tae-Hoon Kim, Na-Kyoung Lee, Kyung-Hoon Chang, Eunju Park[1], Shin-Yang Choi[2], and Hyun-Dong Paik*

Division of Animal Life Science, Konkuk University, Seoul 143-701, Korea

[1]Department of Food Nutrition, Kyungnam University, Masan, Gyeongnam 631-701, Korea

[2]Korea Food Research Institute, Seongnam, Kyonggi 463-420, Korea

Abstract The antioxidant activity of *Bacillus polyfermenticus* SCD was studied by partially purified culture extracts using various methods: ammonium sulfate precipitation, adsorption to Diaion HP-20 columns using polar solvents, and extraction using non-polar solvents. The 1,1-diphenyl-2-picyrylhydrazyl (DPPH) radical scavenging activity of these partially purified fractions was then investigated. The precipitate isolated using 75%-saturated ammonium sulfate was shown to contain about 77.2% DPPH radical scavenging activity. Using the Diaion HP-20 resin adsorption method, the fraction obtained using 60% ethanol and 60% methanol possessed 76.7 and 89.5% DPPH radical scavenging activity, respectively. Fractions obtained by extracting with the non-polar solvents 80 mg/mL chloroform, 80 mg/mL n-hexane, 80 mg/mL ethyl acetate, and 80 mg/mL butanol contained 68.4, 75.0, 70.7, and 87.5% DPPH radical scavenging activity, respectively. Further study is needed to characterize the antioxidant substance(s) released by *B. polyfermenticus* SCD cultures.

Key words: *Bacillus polyfermenticus* SCD, antioxidant activity, DPPH, Diaion HP-20

Introduction

Free radicals can be generated in biological systems in the form of reactive oxygen species (ROS): superoxide anion radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$), and singlet oxygen ($^1O_2$) (1). These reactive ROS cause destructive and irreversible damage to cellular components such as lipids, proteins, and DNA (2). The continuous accumulation of damage to the cells contributes to diseases such as cancer, atherosclerosis, hypertension, and aging (3-5). Antioxidants play a preventive role against these diseases by removing the ROS in biological systems (6). Antioxidants are often added to various foods to prevent free radical-induced lipid oxidation, off-flavors, and production of undesirable chemical compounds in food.

In the food industry, synthetic antioxidants such as BHA and BHT are often used because they are more effective and cheaper than natural antioxidants. However, there is an increasing interest in finding natural antioxidants. Most natural antioxidants are phenolic compounds such as flavonoids (quercetin, kampferol, mycetin, etc.) and vitamins C, E (7). These compounds are found in many plant raw materials, particularly in fruits, seeds, and herbs (8-12), and in many microorganisms (13-15).

Strains of *Bacillus polyfermenticus* SCD, known as Bispan strains commercially, have been used in the treatment of long-term intestinal disorders, as these live strains are able to facilitate their resolution. Live Bispan strains produce a variety of enzymes which lyse pathogenic strains such as typhoid bacillus, paratyphoid bacillus, shigella, and cholera. The ingestion of Bispan strains can enhance the appetite and promote digestion in humans, serve as vitamin B_1 and B_2 sources, as well as strengthening protection against non-oral infection and oral immunization (16, 17). Also, we have determined that *in vitro* and *in vivo* experiments, *B. polyfermenticus* SCD has cholesterol-reducing activity, and exerts antioxidant effects (18-20).

In this study, the antioxidant effects of the various partially purified fractions obtained from *B. polyfermenticus* SCD culture were investigated by testing their scavenging effects on DPPH radicals.

*Corresponding author: Tel: 82-2-2049-6011; Fax: 82-2-455-1044
E-mail: hdpaik@konkuk.ac.kr
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Materials and Methods

Bacterial strain and culture media *B. polyfermenticus* SCD was maintained at -70°C in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI, USA) containing 20%(v/v) glycerol. This strain was cultivated in TSB with shaking at 37°C.

Production of antioxidant from *B. polyfermenticus* SCD Cultures were grown in 500 mL of TSB medium as a working volume in 1,000 mL flasks. The temperature was maintained at 37°C and the agitation speed was 150 rpm. The culture broth was then centrifuged at 15,000×g for 15 min at 4°C.

Ammonium sulfate precipitation Solid ammonium sulfate was slowly added to the culture supernatant (1,000 mL) up to 75% saturation at 4, with constant stirring for an additional 30 min at 4°C. Precipitated proteins were pelleted by centrifugation at 15,000×g for 30 min at 4°C, re-suspended in a 10 mM phosphate buffer (pH 7.0) and extensively dialyzed in 3 L of 10 mM phosphate buffer (pH 7.0) for 12-18 hr in Spectra-Por No. 3 dialysis tubing (Molecular weight cutoff, 3,500 Da; Spectrum Medical Industries, Gardena, CA, USA). The dialyzed samples

were concentrated using a rotary vacuum evaporator (Eyela, Tokyo, Japan).

Adsorption of Diaion HP-20 column using polar solvent Culture supernatants were injected into 5 cm (diameter) × 1 m (length) Diaion HP-20 columns at a ratio of 1:2 (volume of Diaion HP-20 resin: supernatant). Sixty % ethanol and 60% methanol was used as solvents in the mobile phase at a 15 mL/min flow rate. Each fraction was concentrated using rotary vacuum evaporator (Eyela).

Extraction of non-polar solvent The supernatant of *B. polyfermenticus* SCD cultures was also extracted in various non-polar solvents (chloroform, n-hexane, ethyl acetate, or butanol) under shaking overnight at room temperature. Each fraction was concentrated using a rotary vacuum evaporator (Eyela).

Scavenging effect on DPPH radicals (electron donating activity) The antioxidant activities of unpurified and partially purified *B. polyfermenticus* SCD culture medium extracts were assessed by measuring their ability to scavenge the stable 1,1-diphenyl-2-picyrylhydrazyl (DPPH; Sigma, St. Louis, MO, USA) free radical (13). One milliliter of 100 μM DPPH ethanol solution was added to 200 μL sample solutions of different concentrations and allowed to react at room temperature. After 10 min, the absorbance values were measured at 528 nm using a spectrophotometer and converted into percentage antioxidant activity using the following formula:

Electron donating activity (EDA) = [1-(absorbance of sample at 528 nm)/(absorbance of control at 528 nm)] × 100

Each value is the mean of triplicate measurements.

Results and Discussion

Production of the antioxidant of *B. polyfermenticus* SCD Proton-radical scavenging action is an important anti-oxidation mechanism (21). The scavenging of stable DPPH free radicals can be used to evaluate antioxidant activities in a relatively short time compared to other methods. DPPH radical scavenging by unpurified culture medium extracts of *B. polyfermenticus* SCD is shown in Fig. 1. Antioxidant activity seemed to follow the typical kinetics of primary metabolite synthesis. Antioxidant activity increased up to 9 hr, reaching a maximum of 56.5% DPPH radical scavenging activity and then decreased steadily afterwards.

Ammonium sulfate precipitation The medium containing *B. polyfermenticus* SCD cultures was harvested after incubation for 9 hr and then centrifuged. The culture medium was then partially purified by ammonium sulfate precipitation and the DPPH radical scavenging activity of these precipitates was then measured (Fig. 2A). Figure 2A shows the dose-response curve for the DPPH radical scavenging activity of this precipitate. Thirty mg/mL of this precipitate was shown to contain 77.2% DPPH radical scavenging activity.

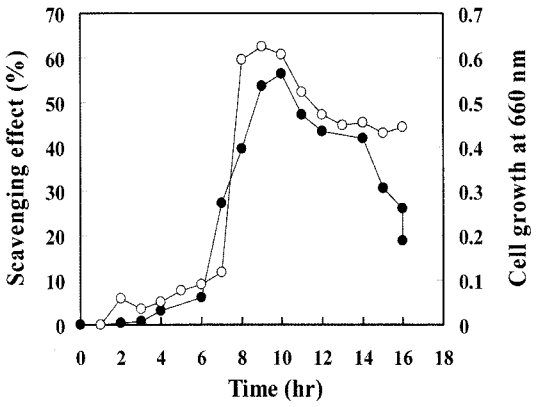

Fig. 1. Scavenging effect on the DPPH radical by *B. polyfermenticus* SCD as incubation time. Cell growth at 660 nm (○) and antioxidant activity (●).

Adsorption of Diaion HP-20 column using polar solvent Figure 2B shows the antioxidant activities of partially purified culture medium fractions obtained using Diaion HP-20 columns with polar solvents. Fractions obtained using 60% ethanol and 60% methanol were shown to contain 76.7 and 89.5% DPPH radical scavenging activity, respectively. The DPPH radical scavenging activity of fractions obtained by 60% methanol adsorption was slightly higher than that obtained using 60% ethanol adsorption. Higher solvent polarity appears to correlates with a higher value of DPPH radical scavenging activity. Fifty percent ethanol extracts of the culture of *B. subtilis* IMR-NK1 demonstrated 86.3% DPPH radical scavenging activity (13). Also, 50% ethanol extracts of Propolis demonstrated higher levels of DPPH radical scavenging activity compared with water extracts (22).

Extraction with non-polar solvents The fractions prepared by extraction in the non-polar solvents, n-hexane, chloroform, ethyl acetate, and butanol were tested for antioxidant activity as shown in Fig. 2C. The DPPH radical scavenging activities of n-hexane extract (80 mg/mL), chloroform extract (80 mg/mL), ethyl acetate extract (80 mg/mL), and butanol extract (80 mg/mL) were shown to be 75.0, 68.4, 70.7, and 87.5%, respectively. The degree of polarity for each solvent is n-hexane<chloroform<ethyl acetate<butanol<ethanol<methanol. As the extraction solvent polarity increased the DPPH radical scavenging activity of the respective fractions increased correspondingly. Thus, butanol-extracted fractions contained the highest DPPH radical scavenging activity. El-Ghorab *et al.* (23) was reported a similar result where ethanol extracts showed higher antioxidant activity compared to hexane extracts. Ten μg/mL ethyl acetate extracts obtained from onions demonstrated 81% DPPH radical scavenging activity (10). Lee *et al.* (9) reported that the DPPH radical scavenging activity of Propolis extract was related to the concentration of the extraction solvent rather than solvent polarity. Also, the antioxidant activity was higher in roots of *Astragalus membranaceus* bunge extracted in ether versus methanol (24).

In summary, the culture medium fraction obtained by adsorption to Diaion HP-20 columns using methanol contained the highest antioxidant activity as shown by its

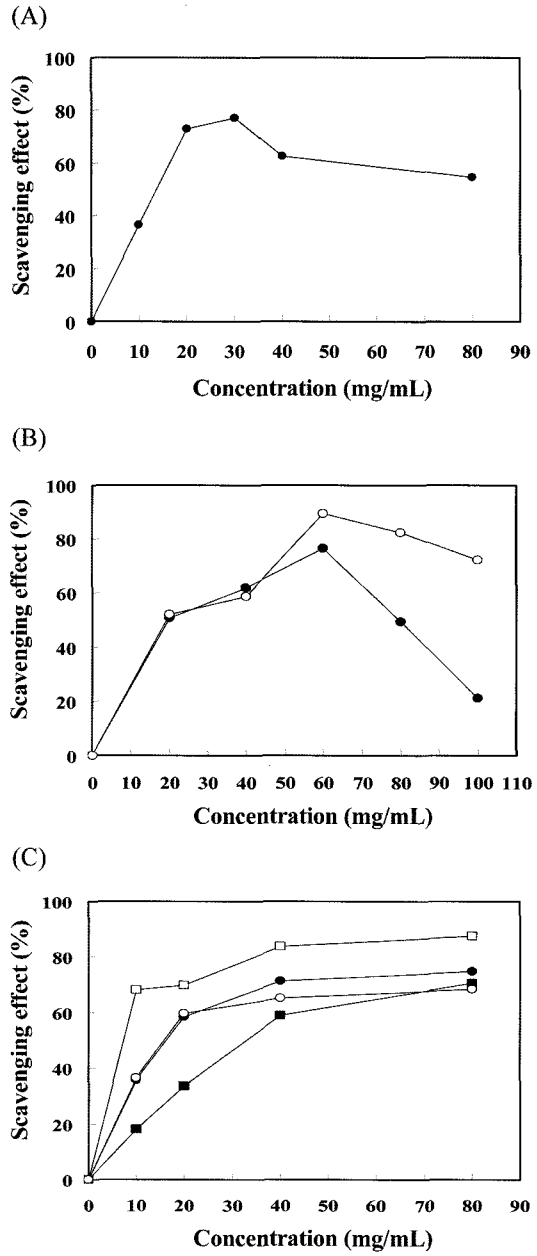

Fig. 2. DPPH radical scavenging by (A) ammonium sulfate precipitates, (B) fractions obtained by adsorption to Diaion HP-20 columns using methanol (○) and ethanol (●), and (C) fractions obtained by chloroform (○), n-hexane (●), butanol (□), and ethyl acetate (■) extraction of *B. polyfermenticus* SCD culture.

89.5% DPPH radical scavenging activity compared with fractions obtained by other methods. Further studies are needed to characterize this antioxidant substance.

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