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Detection of 1,4-Dihydroxy-2-Naphthoic Acid from Commercial *Makgeolli* Products

－ Research Note －

Ji-Eun Eom[1], Sang-Chul Kwon[2], and Gi-Seong Moon[1†]

[1]*Department of Biotechnology, Chungju National University, Chungbuk 368-701, Korea*
[2]*Food Safety Support Organization, Korea Food Industry Association, Seoul 137-867, Korea*

Abstract

To support beneficial effects of *makgeolli* for human health, we investigated for the presence of 1,4-dihydroxy-2-naphthoic acid (DHNA), a bifidogenic growth stimulator (BGS), from commercial *makgeolli* products. Among eleven *makgeolli* products (A～K), four showed positive peaks for DHNA in high performance liquid chromatography analysis. *Makgeolli* product A in particular contained the highest concentration of DHNA (0.44 ppm), as confirmed by liquid chromatography-mass spectrometry. Furthermore, BGS activity of the *makgeolli* product A was higher than those of products in which DHNA was not detected. These results indicate that *makgeolli* can be a good source for DHNA and that DHNA-enriched *makgeolli* could be developed by modifying manufacturing procedures and controlling its microbiota.

Key words: *makgeolli*, 1,4-dihydroxy-2-naphthoic acid, bifidogenic growth stimulator

INTRODUCTION

Makgeolli is a Korean traditional rice wine with a low content of alcohol (6～8%), which is relatively less toxic to the liver and stomach. Unlike other alcoholic beverages, *makgeolli* contains abundant nutrients such as sugars, proteins, vitamins, and amino acids including valine, leucine, serine, proline, and glycine (1). Yeasts are mainly involved in the fermentation that occurs in *makgeolli* production, as well as many species of lactic acid bacteria (LAB) (2). *Makgeolli* is recognized as functional alcoholic beverage and its consumption has markedly increased in Korea. Also, consumption in other countries such as Japan has increased, resulting in increased export of *makgeolli* (3); however, the shelf life of non-sterilized *makgeolli* containing yeast and LAB can be as short as 10 days, even under refrigeration conditions. Studies have sought to increase the shelf life of non-sterilized *makgeolli* (4,5). One study reported an extended shelf-life of up to 30 days using heat-inactivation of saccharolytic enzymes and re-inoculation of yeast (6). Studies aimed at developing functional *makgeolli* have assessed the effect of adding biologically active materials such as pears, *Gugija-Liriope tuber*, and black garlic extract, among other substances (7-9).

1,4-Dihydroxy-2-naphthoic acid (DHNA) was recently identified as a bifidogenic growth stimulator (BGS) (10). DHNA also displays inhibitory activity against clinical isolates of *Helicobacter pylori* that are resistant to clarithromycin (11) and suppresses bone resorption (12). DHNA is an intermediate in the biosynthetic pathway of vitamin K_2 (menaquinone), which plays a role in respiratory chain of bacteria as an electron carrier (13). Therefore, most LAB produce DHNA as an intermediate compound, with a small amount exported from the bacteria. It would be helpful to isolate DHNA-producing LAB for commercial use as a starter strain in the manufacturing of functional *makgeolli* products.

Before this goal can be achieved, however, it is necessary to ascertain whether current commercial *makgeolli* products contain DHNA. Therefore, we undertook this effort in this study, using high performance liquid chromatography (HPLC) to determine if DHNA was present in each sample of *makgeolli*. Additionally, we compared the BGS activity of DHNA-containing *makgeolli* products with those of *makgeolli* products where DHNA was not detected.

MATERIALS AND METHODS

Makgeolli products

To analyze DHNA from commercial *makgeolli* products, 11 *makgeolli* products (A～K) from several companies were purchased from a local market (Table 1).

Bacterial strains and culture conditions

For BGS activity test, *Bifidobacterium longum* FI10564 and *Bifidobacterium lactis* BL 750 (Culture Systems, Mishawaka, IN, USA) were cultivated in reinforced clos-

†Corresponding author. E-mail: gsmoon@cjnu.ac.kr
Phone: +82-43-820-5251, Fax: +82-43-820-5272

Table 1. *Makgeolli* products analyzed in this study

Products	Starch source	Additives (sweetener)	Sterilization
A	Rice (100%)	Aspartame	No
B	Rice (100%)	Aspartame, Acesulfame K	Yes
C	Rice (100%)	Aspartame	No
D	Rice (80%), flour (20%)	Aspartame	Yes
E	Rice (50%), flour (30%), corn starch (20%)	Aspartame, Acesulfame K	Yes
F	Rice (100%)	Aspartame	Yes
G	Rice (40%), flour (40%), starch sugar (20%)	Aspartame, Acesulfame K	Yes
H	Rice (100%)	Aspartame	No
I	Rice (100%)	Aspartame	No
J	Rice (80%), flour (10%), starch sugar (10%)	Aspartame	No
K	Rice (100%)	Aspartame, Acesulfame K	Yes

tridial medium (RCM) broth (Difco, Detroit, MI, USA) at 37°C in an anaerobic jar (Oxoid, Cambridge, UK).

HPLC analysis for DHNA

For HPLC detection of DHNA from *makgeolli* samples, 10 mL of each *makgeolli* sample was freeze-dried. The freeze-dried samples were each diluted with 150 μL of water and mixed with 300 μL of methanol. The mixtures were centrifuged at 5,000×*g* for 10 min and the supernatants were filtered with 0.45 μm pore size syringe filters (Millipore, Billerica, MA, USA). The filtrates were used as injection samples for HPLC using an ACE 5 C_{18} column (4.6×150 mm; Advanced Chromatography Technologies, Aberdeen, Scotland). Column temperature was maintained at 45°C during analysis. The flow rate was 1 mL/min, injection volume was 20 μL, and detection wavelength was 254 nm. For construction of standard curve, reagent grade DHNA (Sigma-Aldrich, St. Louis, MO, USA) was used. The mobile phase was composed of acetonitrile : methanol : water : acetic acid (15:25:225:0.1) and the pH was adjusted to 5.5 with 5% (w/v) ammonium hydroxide.

Liquid chromatography-mass spectrometry (LC-MS) analysis

For confirmation of DHNA from one of the *makgeolli* products (A), DHNA fractions evident on HPLC were collected and subjected to LC-MS. LC utilized a Prominance 20A apparatus (Shimadzu, Kyoto, Japan). MS utilized a LCMS-IT-TOF system (Shimadzu). An XR-ODS LC column (3×75 mm, Shimadzu) was used. The column temperature was equilibrated at 45°C. Sample injection volume was 20 μL and flow rate was 0.2 mL/min. Mobile phase composition was the same as the aforementioned HPLC condition.

BGS activity

For the BGS activity test of *makgeolli* product samples, *B. longum* FI10564 and *B. lactis* BL 750 were used as the target bifidobacteria. Briefly, *makgeolli* product samples were centrifuged at 6,000×*g* for 10 min. The resulting supernatants were syringe-filtered as described above and the filtrates were used as the test samples. One hundred microliters of each filtrate was added to RCM broth (Difco) that had been previously inoculated with a bifidobacterial strain (2% of final concentration). These inocula were incubated at 37°C for 12 hr in an anaerobic jar supplemented with a GasPak EZ Anaerobe Container System (BD, Sparks, MD, USA) and the optical density at 600 nm (OD_{600}) was measured with time.

RESULTS AND DISCUSSION

To investigate whether commercial *makgeolli* products contain DHNA, eleven commercial *makgeolli* products were purchased from a local market and analyzed by HPLC. Four of the product samples (A, E, F, and J) showed positive peaks for DHNA. Two products, A and F, could be quantified using a standard curve which ranged 0.0～1.0 μg/mL (ppm) (data not shown). Product A, which was produced from a local brewing company located in Chungbuk province, displayed the most content of DHNA (0.44 ppm) as shown in Fig. 1B. The *makgeolli* product F contained 0.089 ppm of DHNA. Although the study involved a relatively small sampling of *makgeolli* products, the fact that four of the eleven products contained detectable levels of DHNA is promising, and indicates the potential of commercial *makgeolli* products as reliable sources of DHNA.

To confirm the presence of DHNA in the HPLC peak from *makgeolli* product A, DHNA fractions were collected and subjected to LC-MS. The mass spectra of DHNA from a standard solution and the *makgeolli* product sample revealed an *m/z* 203.03 (Fig. 1C and D), which corresponded to a de-protonated DHNA ion $[M-H]^-$. The result confirmed the presence of DHNA in *makgeolli* product A. Furthermore, we investigated whether the DHNA-containing *makgeolli* product A displayed BGS activity, which is a biological function of DHNA. For the test, the bifidobacterial strains *B. longum* FI10564 and *B. lactis* BL 750 were used. *B. longum* FI10564 was

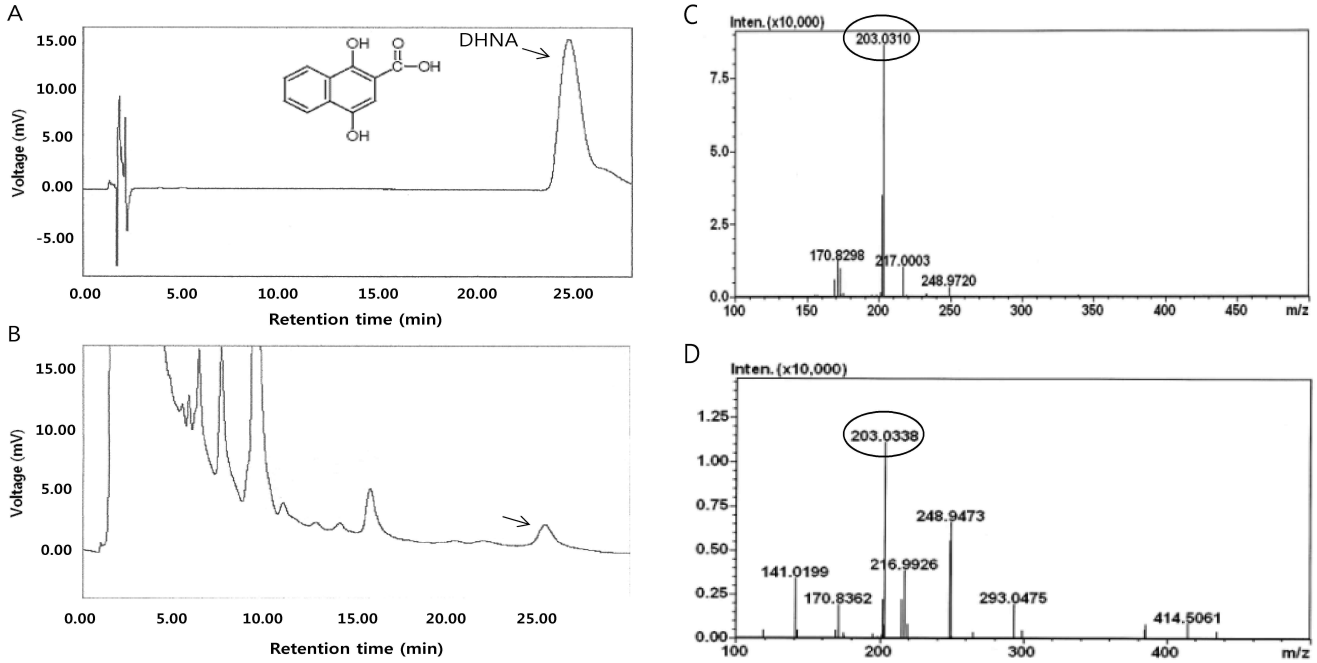

Fig. 1. HPLC chromatograms (A, B) and LC-MS spectra (C, D) for DHNA standard and commercial *makgeolli* product A sample. The arrows indicate peaks for DHNA and the circles indicate spectra of molecular masses (*m/z*) for a de-protonated DHNA ion $[M-H]^-$.

previously isolated from a stool sample of healthy human volunteer (14) and *B. lactis* BL 750 is commercially available and used in the dairy industry as a probiotic. For *B. longum* FI10564, BGS activity of *makgeolli* products B and H, in which DHNA was not detected, was not evident. The OD_{600} (0.76 and 0.75) were not significantly different with that of a control where *B. longum* FI10564 strain was inoculated alone (0.70). However, *makgeolli* product A, which had been confirmed to contain DHNA, did present BGS activity, evident as an OD_{600} of 0.94 (Fig. 2A). For *B. lactis* BL 750, the BGS activity of *makgeolli* product A was higher than *makgeolli* products B and H. After 12 hr culture, the OD_{600} of the control was 0.76 and those of test samples A, B, and H were 1.35, 0.94, and 0.79, respectively (Fig. 2B). These results indicate that DHNA-enriched *makgeolli* products can improve BGS activity, although other factors can also influence the activity. In case of *makgeolli* product B, other factors including fibers might have affected the activity; if so, their effect was not significant.

Initially, we had expected that most of the *makgeolli* products would contain DHNA as a functional ingredient. In reality, four of the eleven products contained detectable levels of the functional material. Nevertheless, *mak-*

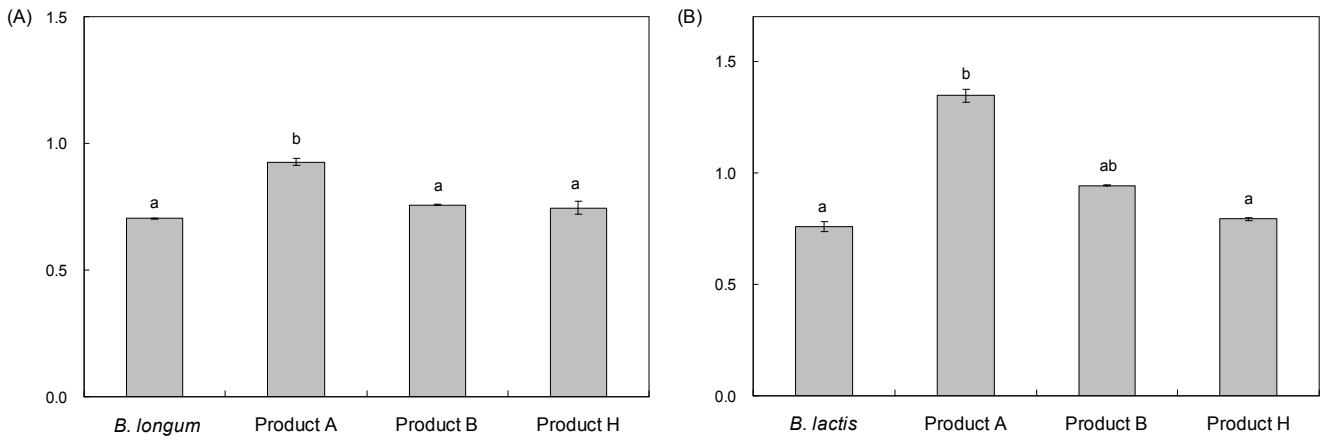

Fig. 2. BGS activity of commercial *makgeolli* samples for *B. longum* (A) and *B. lactis* (B). *B. longum* or *B. lactis*: *B. longum* FI10564 or *B. lactis* BL 750 strain was inoculated alone; Product A, B, or H: *B. longum* FI10564 or *B. lactis* BL 750 strain was inoculated with commercial *makgeolli* product A, B, or H sample. Values are mean±SD (n=3). Means having different letters are significantly different by Duncan's multiple range tests ($p<0.05$).

geolli products may be good sources for DHNA, pending modification of the manufacturing procedure, including use of starter strains that produce DHNA. To achieve this goal, it will be necessary to monitor the microbiota and DHNA content of *makgeolli* during fermentation.

Most of the previous research and patents in this field have related to manufacturing procedures, quality characterization, and biological functions. More recently, *makgeolli* has been recognized as a functional food (1, 15,16). In particular, *makgeolli* contains farnesol, an anti-cancer or anti-tumor agent (17), which has encouraged consumption of *makgeolli* products in Korea and exportation to other countries, including Japan (18); therefore, it is very important to enlarge the capacity of functional properties of *makgeolli* for commercial aspects. For that reason, DHNA could also be a functional material candidate linked to *makgeolli* products.

The present data indicate the potential of *makgeolli* as a good source for DHNA. DHNA-enriched *makgeolli* products may be developed by modifying the manufacturing procedures. Planned studies will focus on the development for DHNA-enriched *makgeolli* product.

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