Isolation and Antioxidative Activity of Amino Acid Derivatives Produced by *Leuconostoc mesenteroides*

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Abstract One new phenyl lactic acid derivative, *N*-(5-amino-2-hydroxy-1-oxopentyl)-tyrosine (**2**), and three known metabolites [(*S*)-8-hydroxy-4-hydroxy-phenylpropanoic acid (**1**), (*S*)-8-hydroxy-phenylpropanoic acid (**3**), and indole-(*S*)-3-lactic acid (**4**)] were isolated from the culture medium of *Leuconostoc mesenteroides* guided with DPPH radical-scavenging assay. The chemical structures of the isolated compounds were determined by NMR and electrospray ionization mass spectroscopy. The isolated four compounds were assumed to be metabolites formed by the transformation of amino acids. The DPPH radical-scavenging activities of **1** and **2** were significantly higher than those of **3** and **4**. This result may provide meaningful information of antioxidative metabolites from lactic acid bacteria.

Keywords: *Leuconostoc mesenteroides*, amino acid derivatives, antioxidants, metabolites, transformation

Introduction

Free radicals cause oxidative damage to biological molecules such as lipids, nucleic acids, and proteins (1). Oxidative damage is the main cause of various diseases including cancer and cardiovascular diseases (2). Therefore, effective free radical scavengers, called antioxidants, are necessary for preventing diseases induced by free radicals (1,2). In recent years, many studies have been conducted to identify effective natural antioxidants from several supplement sources. In particular, attempts to take advantage of fermentation as an efficient way to enhance the antioxidative capacity of raw materials have increased (3,4). In addition, many reports have indicated that fermented food have higher antioxidative capacity than their raw materials (3,5,6).

Lactic acid bacteria (LAB) have attracted much attention for decades because they are mainly involved in the fermentation process of various fermented foods including kimchi, yogurt, and cheese (7,8). LAB produce secondary metabolites that improve food quality including taste, flavor, and shelf life (9,10). In addition, many studies have indicated that LAB species such as *Lactobacillus* sp. and *Pediococcus* sp. produce various secondary metabolic compounds such as 3-hydroxy fatty acid (11), phenyllactic acid (12), benzoic acid (13), mevalonolactone (13), and peptides (14), which exert antimicrobial activity. However, though the antimicrobial effects of the secondary metabolites produced by LAB have been largely understood, the information on antioxidants from LAB is very limited.

Leuconostoc mesenteroides is a typical heterofermentative LAB and produces metabolites such as mannitol, carbon dioxide, and acetic acid from fructose, which are relevant to carbohydrate metabolism (7,10). In addition, *L. mesenteroides* is a dominant species at early fermentation stages in the kimchi preparation process, and is therefore considered a key player in kimchi fermentation (15,16). Therefore, a lot of attempts have been made to improve the quality of kimchi products using *L. mesenteroides* in the Korean food industry (10,15,17). Nevertheless, the secondary metabolites produced by *L. mesenteroides*, with the exception of compounds related to carbohydrate metabolism, have not yet been fully studied (8,10,15). In the present study, the antioxidative activity was observed in culture fermented with *L. mesenteroides*. We subsequently performed isolation and identification of the secondary metabolites from *L. mesenteroides* having antioxidative activity using an *in vitro* system.

Materials and Methods

Chemicals Methanol (MeOH), ethanol (EtOH), and ethyl acetate (EtOAc) were obtained from Duksan (Ansan, Korea). HPLC grade trifluoroacetic acid (TFA) and 2,2-diphenyl-1-picrylhydrazyl were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Methanol-*d*₄ (CD₃OD) was obtained from Acros Organics (Fair Lawn, NJ, USA). Solvents used for analyses were purchased from Fisher Scientific Korea Ltd. (Seoul, Korea).

Culture condition *L. mesenteroides* KCTC 13374 was obtained from Korean Collection for Type Cultures (KCTC, Daejeon, Korea), and incubated on MRS agar (Difco Laboratories, Detroit, MI, USA) at 30°C for 48 h. A single colony selected from the agar plate was preserved in glycerol/H₂O (25:75, v/v) at –80°C until used.

Fermentation Three liters of modified MRS broth (5 g of proteose peptone, 5 g of beef extract, 2.5 g of yeast extract, 10 g of dextrose, 0.5 g of polysorbate 80, 1 g of ammonium citrate, 2.5 g of sodium acetate, 0.05 g of magnesium sulfate, and 1 g of dipotassium phosphate, per L, pH 6.2) was divided into 20 portions (each 150 mL) in 200 mL erlenmeyer flasks. The *L. mesenteroides* cells (10⁸ cfu/mL) were then inoculated at 30°C for 48 h without shaking.

Assay for purification of antioxidants The isolation of antioxidative active compounds was guided by thin TLC-DPPH assay, as described previously (18). Briefly, each fraction obtained in the purification steps was spotted on silica gel TLC plates (silica gel 60 F254, 0.25 mm thickness; Merck, Darmstadt, Germany) and developed with EtOAc: AcOH:H₂O=6:1:1 (v/v). Then, DPPH EtOH solution (200 µM) was sprayed on the dried TLC plate. The antioxidative activity was measured by visualization of the decolorized spot after spraying with DPPH EtOH solution.

Purification and isolation procedure The cells in the cultured broth were removed by centrifugation (8,000×g for 15 min at 4°C), and the supernatants were combined. The combined medium was added to an equal volume of H₂O (3 L, total 6 L) and acidified to pH 2.65 using 1 N HCl. The culture medium was extracted with *n*-hexane (3 L, twice) to remove the lipophilic component, and the aqueous fraction was extracted with EtOAc (3 L, 3 times). The EtOAc fraction was combined and concentrated *in vacuo* at 35°C. The concentrates of the EtOAc fraction was fractionated using a solid phase extraction (SPE) column (C₁₈, 10 g, Sep-pak; Waters, Milford, MA, USA). Briefly, the SPE column was activated by 100 mL of MeOH and equilibrated by H₂O (100 mL, pH 3.0 by TFA). A portion (500 mg) of concentrates was eluted with a step-wise system of H₂O/MeOH (10:0, v/v, pH 2.6, 50 mL) and H₂O/MeOH (9:1, 8:2, 7:3, 5:5, 0:10, v/v, each 50 mL). Fraction B (H₂O/MeOH, 8:2, 251 mg) was subjected to a semi-preparative ODS-HPLC (µBondapak™, 7.8×300 mm, Waters) using a linear gradient system of H₂O (eluent A) and 15% acetonitrile (eluent B), starting with 100% A, increasing to 100% B for 25 min and holding at 100% B for 30 min, to yield **1** (*t_R* 8.9 min, 52 mg) and **2** (*t_R* 15.1 min, 41 mg). Fraction D (H₂O/MeOH, 5:5, 70.4 mg) was purified using a linear gradient of H₂O (eluent C) and 50% acetonitrile (eluent D), from 100% C to 30% D for 15 min, holding for 25 min, and increasing to 50% D for 25 min, to yield **3** (*t_R* 11.4 min, 2 mg) and **4** (*t_R* 15.6 min, 8 mg). The flow rate was 5.5 mL/min and the compounds were monitored at 254 nm.

Structural analysis ¹H- and ¹³C-NMR spectra were obtained from

an unitINOVA 500 and 600 spectrometer (Varian, Walnut Creek, CA, USA). The chemical shift was measured with tetramethylsilane serving as an internal standard in CD₃OD. High resolution electrospray ionization MS (HR-ESI-MS) data was acquired on a SYNAPT G2 hybrid (Waters). Low resolution ESI-MS spectra were acquired on an MS spectrometer (LCMS-8030; Shimadzu, Kyoto, Japan) equipped with an LC-20AD pump system (Shimadzu). Specific rotations ([α]_D²⁵) of compounds were measured by a P-8000 polarimeter (A. Krüss Optronic GmbH, Hamburg, Germany).

Compound 1: Amorphous white powder; [α]_D²⁵ +0.15 (c 0.025, CH₃OH); UV (CH₃OH): λ_{max} (log ε) 278 (3.33), 285 (3.26) nm; ¹H-NMR spectrum (500 MHz, CD₃OD, TMS) δ 7.07 (2H, d, *J*=8.5 Hz, H-2, 6), 6.69 (2H, d, *J*=8.5 Hz, H-3, 5), 4.27 (1H, dd, *J*=7.8, 4.5 Hz, H-8), 2.99 (1H, dd, *J*=14.0, 4.5 Hz, H-7a), 2.81 (1H, dd, *J*=14.0, 7.8 Hz, H-7b); ¹³C-NMR spectrum (125 MHz, CD₃OD, TMS) δ 180.5 (C-9), 156.8 (C-4), 131.7 (C-2, 6), 131.1 (C-1) 131.7 (C-3, 5), 74.8 (C-8), 41.6 (C-7); ESI-MS (negative) *m/z* 180.90 [M-H][–].

Compound 2: Amorphous white powder; [α]_D²⁵ –0.45 (c 0.025, CH₃OH); UV (CH₃OH): λ_{max} (log ε) 278 (3.36) nm; ¹H-NMR spectrum (500 MHz, CD₃OD, TMS) δ 7.04 (2H, d, *J*=8.5 Hz, H-2, 6), 6.70 (2H, d, *J*=8.5 Hz, H-3, 5), 4.36 (1H, br. t, *J*=5.0 Hz, H-8), 4.04 (1H, dd, *J*=11.0, 6.0 Hz, H-2'), 3.55 (1H, dd, *J*=17.0, 12.0 Hz, H-5'a), 3.35 (1H, dd, *J*=17.0, 7.0 Hz, H-5'b), 3.09 (1H, dd, *J*=14.0, 5.0 Hz, H-7a), 3.03 (1H, dd, *J*=14.0, 5.0, H-7b), 2.09 (1H, m, H-3'a), 1.80 (2H, m, H-4'), 1.21 (1H, m, H-3'b); ¹³C-NMR spectrum (125 MHz, CD₃OD, TMS) δ 170.9 (C-1'), 167.1 (C-9), 157.8 (C-4), 132.3 (C-2, 6), 127.7 (C-1), 116.3 (C-3, 5), 60.2 (C-2'), 58.0 (C-8), 46.0 (C-5'), 37.8 (C-7), 29.5 (C-3'), 22.9 (C-4'); ESI-MS (negative) *m/z* 295.10 [M-H][–]; HR-ESI-MS *m/z* 295.1287 [M-H][–] (calculated for C₁₄H₁₉N₂O₅, *m/z* 295.1294, 0.7 mDa).

Compound 3: Amorphous white powder; [α]_D²⁵ +0.5 (c 0.025, CH₃OH); UV (CH₃OH): λ_{max} (log ε) 258 (2.66) nm; ¹H-NMR spectrum (600 MHz, CD₃OD, TMS) δ 7.28 (2H, d, *J*=7.8 Hz, H-2, 6), 7.24 (2H, t, *J*=7.8 Hz, H-3, 5), 7.16 (1H, t, *J*=7.8 Hz, H-4), 4.15 (1H, dd, *J*=8.0, 3.0 Hz, H-8), 3.11 (1H, dd, *J*=13.8, 3.0, Hz, H-7a), 2.80 (1H, dd, *J*=13.8, 8.0 Hz, H-7b); ¹³C-NMR spectrum (150 MHz, CD₃OD, TMS) δ 180.2 (C-9), 140.3 (C-1), 130.8 (C-2, 6), 129.2 (C-3, 5), 127.2 (C-4), 74.5 (C-8), 42.5 (C-7); ESI-MS (negative) *m/z* 165.05 [M-H][–].

Compound 4: Amorphous white powder; [α]_D²⁵ +0.924 (c 0.023, CH₃OH); UV (CH₃OH): λ_{max} (log ε) 281 (3.63), 290 (3.57) nm; ¹H-NMR spectrum (500 MHz, CD₃OD, TMS) δ 7.59 (1H, d, *J*=8.0 Hz, H-4), 7.32 (1H, d, *J*=8.0 Hz, H-7), 7.13 (1H, s, H-2), 7.07 (1H, t, *J*=8.0 Hz, H-6), 7.00 (1H, t, *J*=8.0 Hz, H-5), 4.42 (1H, dd, *J*=7.5, 4.5 Hz, H-2'), 3.16 (1H, dd, *J*=15.0, 4.5 Hz, H-3'a), 3.09 (1H, dd, *J*=15.0, 7.5 Hz, H-3'b); ¹³C-NMR spectrum (125 MHz, CD₃OD, TMS) δ 177.8 (C-1'), 138.1 (C-8), 129.1 (C-9), 124.7 (C-2), 122.3 (C-6), 119.7 (C-4), 119.6 (C-5), 112.2 (C-7), 111.4 (C-3), 72.5 (C-2'), 31.7 (C-3'); ESI-MS (positive) *m/z* 206.00 [M + H]⁺; ESI-MS (negative) *m/z* 203.90 [M-H][–].

DPPH radical-scavenging activity of isolated compounds The DPPH radical-scavenging activity of the isolated compounds (**1–4**) was evaluated according to a previous study (19), with slight

modifications. Briefly, each compound (final concentration, 10 mM) was diluted with MeOH (200 μ L) and added to a DPPH solution (final conc. 250 μ M). The absorbance was recorded at 517 nm using a spectrophotometer (V-550; Jasco, Tokyo, Japan). The DPPH radical-scavenging activity of each compound was determined as the percentage decrease when compared to the absorbance of a blank sample.

Statistical analysis The data for the DPPH radical-scavenging activities of the isolated compounds were expressed as the mean $\pm$ standard deviation ($n=3$) using the Statistical package for Social Sciences (IBM, Armonk, NY, USA) version 18.0. The significant differences were evaluated with one-way ANOVA, followed by the Tukey-Kramer test ($p<0.05$).

Results and Discussion

L. mesenteroides in MRS broth was incubated for 2 days at 30°C. The cultured broth was centrifuged and the supernatant was solvent-fractionated to yield *n*-hexane, EtOAc, and water fractions. The free radical-scavenging activity of these fractions was determined using DPPH radicals (19). The EtOAc fraction showed the highest DPPH radical-scavenging activity (Fig. 1). In addition, this activity was significantly higher than the EtOAc fraction obtained after solvent-fractionation of the MRS broth incubated without *L. mesenteroides*. These results suggest that antioxidative compound(s) might be produced by *L. mesenteroides*. Therefore, we attempted to purify and isolate antioxidative compounds from the culture medium of *L. mesenteroides*. *L. mesenteroides* was cultured in MRS broth (3 L) on a large scale to produce the antioxidative compounds. The EtOAc fraction (5.8 g) obtained after solvent fractionation was subjected to C₁₈-SPE and five fractions (A-E) were purified. Of these, fractions B and D contained higher antioxidative compounds than other fractions on the TLC plate. Therefore, fraction B (20% MeOH eluate, 251 mg) was purified by ODS-HPLC to yield **1** (52 mg) and **2** (41 mg). In addition, compounds **3** (1 mg) and **4** (8 mg) were isolated from fraction D (50% MeOH eluate, 71 mg) by ODS-HPLC purification. Purification and isolation procedures for compounds **1-4** are shown in Fig. 2. Based on NMR and MS analyses, compounds **1**, **3**, and **4** were identified to be (S)-8-hydroxy-4-hydroxy-phenylpropanoic acid (**1**), (S)-8-hydroxy-phenylpropanoic acid (**3**), and indole-(S)-3-lactic acid (**4**), respectively. These compounds have been identified as antifungal compounds from LAB species (*Leuconostoc* sp. and *Lactobacillus* sp.) (12,20,21).

Identification of compound 2 The ESI-MS (negative) spectrum for **2** revealed a pseudomolecular ion peak at m/z 295.1 [M-H]⁻ and fragment ion peak at m/z 165.0 [M-C₅H₁₀N₂O₂]⁻, indicating that the molecular weight of **2** was 296. The molecular formula was determined to be C₁₄H₂₀N₂O₅ from the HR-ESIMS data (calculated for

Fig. 1. DPPH radical-scavenging activity of fractions obtained after solvent fractionation of the cultured media with or without inoculation of *L. mesenteroides*. The medium was incubated for 2 days at 37°C with (white bars) or without (black bars) *L. mesenteroides*. The final concentration of the DPPH radical was 250 μ M. The concentration of each fraction was 1 mg/mL. Data represent mean $\pm$ standard deviation ($n=3$). ^{a-d}Different letters indicate significant difference ($p<0.05$).

C₁₄H₂₀N₂O₅, m/z 295.1294 [M-H]⁻, 0.7 mDa). The ¹H-NMR spectrum (500 MHz, CD₃OD) for **2** showed the presence of *para*-substituted benzene ring proton signals at δ 7.04 (2H, d, $J=8.5$ Hz, H-2 and 6) and 6.70 (2H, d, $J=8.5$ Hz, H-3 and 5). In addition, proton signals of the ethyl (-CH₂CH-) moiety at δ 4.36 (1H, br. t, $J=5.0$ Hz, H-8), 3.09 (1H, dd, $J=14.0, 5.0$ Hz, H-9a), and 3.03 (1H, dd, $J=14.0, 5.0$ Hz, H-9b) and proton signals of the butyl (-CHCH₂CH₂CH₂-) moiety at δ 4.04 (1H, dd, $J=11.0, 6.0$ Hz, H-2'), 3.55 (1H, dd, $J=17.0, 12.0$ Hz, H-5'a), 3.35 (1H, dd, $J=17.0, 7.0$ Hz, H-5'b), 2.09 (1H, m, H-3'a), 1.80 (2H, m, H-4'), and 1.21 (1H, m, H-3'b) were observed, which were confirmed by the proton-proton correlation in the ¹H-¹H COSY spectrum (Fig. 3). Considering the molecular formulation and the chemical shifts of 14 carbon signals observed in the ¹³C-NMR spectrum for **2**, the *para*-substituted benzene ring moiety was assigned from 6 carbons at δ 157.8-116.3. Two carbonyl carbons at δ 167.1 (C-9) and 170.9 (C-1'), and six *sp*³ carbons at δ 58.0-22.9 were observed. Three of them were nitrogen-bearing carbons at δ 170.9 (C-1'), 58.0 (C-8), and 46.0 (C-5'). Therefore, the skeletal structures for **2** were presumed to be amino-(4-hydroxyphenyl) propanoic acid and amino-2-hydroxypentanoic acid from the HR-ESI-MS and 1D NMR spectra. In addition, the ¹H- and ¹³C-NMR spectra for amino-(4-hydroxyphenyl) propanoic acid were similar to those for tyrosine [8-amino-(4-hydroxyphenyl) propanoic acid] (22). The complete NMR assignment and connectivity for **2** were determined by heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments. The important correlations observed in the ¹H-¹H COSY (bold lines) and the HMBC (arrows) spectra are shown in Fig. 3. The partial structures of **2** were confirmed to be tyrosine and 5-amino-2-hydroxypentanoic acid. Particularly, the HMBC correlation of the H-8 with C-1' indicated that the amino acid group of tyrosine was linked

Fig. 2. Isolation procedure for antioxidative metabolites from the culture medium of *L. mesenteroides*.

Fig. 3. Chemical structures of the isolated compounds (1-4) and important ^1H - ^1H COSY (bold lines) and HMBC (arrows) correlations for 2.

with the carbonyl group of 5-amino-2-hydroxypentanoic acid as an amide bond. Consequently, 2 was determined to be *N*-(5-amino-2-hydroxy-1-oxopentyl)-tyrosine. In addition, planar structure of 2 was identified to be a new compound. The absolute stereochemical assignment for the amine group at C-8 and the hydroxyl group at C-2' in 2 is further required after isolation in large amount from the culture medium of *L. mesenteroides*.

DPPH radical-scavenging activity of the isolated compounds The antioxidative activities of the isolated compounds (1-4) were determined by a DPPH radical-scavenging assay (Fig. 4). Compounds 1 and 2, which have a *p*-hydroxy benzyl moiety, showed significantly higher DPPH radical-scavenging activity than the other two compounds (3 and 4). Compounds 1 and 2 showed similar radical-scavenging activities to *p*-coumaric acid, which was used as a positive control. In

addition, compounds 3 and 4, which did not contain a *p*-hydroxy benzene moiety, showed significantly lower DPPH radical-scavenging activity than *p*-coumaric acid. This result indicated that the *para*-hydroxyl group of the benzene ring is an important active site for the free radical-scavenging activity of 1 and 2.

In the present study, four antioxidative active compounds (1-4) were isolated from the culture medium of *L. mesenteroides*, and identified as (S)-8-hydroxy-4-hydroxy-phenylpropanoic acid (1), (S)-8-hydroxy-phenylpropanoic acid (2), and indole-(S)-3-lactic acid (4). Among these, compounds 1, 3, and 4 have been identified previously as antimicrobial active compounds from *Lactobacillus* sp. (12,21) and *Leuconostoc* sp. (20). However, compound 2 was determined to be a new compound. Meanwhile, previous reports (23,24) have shown that 1, 3, and 4 are conversion compounds from amino acids (tyrosine, phenyl alanine, and tryptophan, respectively) followed by

Fig. 4. DPPH radical-scavenging activities of the metabolites isolated from the culture medium of *L. mesenteroides*. The final concentrations of the DPPH radical and isolated compounds (**1-4**) were 200 μ M and 10 mM, respectively. **1**, (S)-8-hydroxy-4-hydroxy-phenylpropanoic acid; **2**, N-(5-amino-2-hydroxy-1-oxopentyl)-tyrosine; **3**, (S)-8-hydroxy-phenylpropanoic acid, **4**, indole-(S)-3-lactic acid. CmA (*p*-coumaric acid, 10 mM) was used as a positive control. Data represent mean $\pm$ standard deviation ($n=3$). ^{a,b}Different letters indicate significant difference ($p<0.05$).

a transamination reaction. This reaction was mainly catalyzed by glutamate dehydrogenase, which converts glutamine to glutamate to provide an energy advantage through NAD(P)H regeneration (25). In addition, it was reported that *Lactobacillus* sp. transforms phenylalanine into phenylpyruvic acid, phenyllactic acid, and phenylacetic acid (26). Therefore, it was speculated that compound **2** might be derived from a dipeptide of tyrosine and ornithine through a transaminase reaction. That is, the 2-amino group of ornithine (2,5-diaminopentanoic acid), which is a well-known metabolite of arginine by LAB, may be transformed into a hydroxyl group by the transaminase produced by *L. mesenteroides* and the ornithine may combine with tyrosine (23). However, it is not clear which reaction is preferred in the structural conversion of ornithine and the binding of tyrosine and ornithine. Therefore, further investigation is needed to elucidate the precise mechanism for the transformation of **2** during the fermentation process.

It has been reported that amino acid transamination contributes to the flavor and functional features of fermented foods (25,27). Therefore, all of the compounds (**1-4**) isolated in this study may improve the quality of fermented foods. In addition, the results of the present study may provide better understanding of the metabolites from LAB, and provide very useful information on the antioxidants from LAB.

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Disclosure The authors declare no conflict of interest.

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