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Isolation and Characterization of *Lactobacillus buchneri* Strains with High γ-Aminobutyric Acid Producing Capacity from Naturally Aged Cheese

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Abstract Two lactic acid bacteria (LAB) with high γ-aminobutyric acid (GABA)-producing capacity were isolated from naturally aged cheese. Examination of the biochemical features using an API kit indicated that the two strains belonged to *Lactobacillus*. They were gram positive, rod-type bacteria, and fermented arabinose, melezitose, melibiose and xylose, but did not utilize cellobiose or trehalose. 16S rDNA sequencing analysis confirmed that they were *Lactobacillus buchneri* and *Lactobacillus* sp. They were accordingly named as *Lactobacillus buchneri* OPM-1 and *Lactobacillus* sp. OPM-2, and could produce GABA from MRS broth supplemented with 10 g/L of monosodium glutamate (MSG) at a productivity of 91.7 and 116.7 mg/L/hr, respectively. Cell extracts of *L. buchneri* OPM-1 and *Lactobacillus* sp. OPM-2 showed glutamate decarboxylase (GAD) activity, for which the optimum pH and temperature were 5.5 and 30°C, respectively.

Keywords: cheese, γ-aminobutyric acid (GABA), *Lactobacillus* species

Introduction

Gamma-aminobutyric acid (GABA) is a ubiquitous, non-protein amino acid produced primarily by the α-decarboxylation of L-glutamic acid, which is catalyzed by the enzyme glutamate decarboxylase (GAD) (1, 2). GABA functions in animals as a major inhibitory neuro-transmitter, is involved in the regulation of cardiovascular functions such as blood pressure and heart rate, and plays a role in the sensations of pain and anxiety (1, 3). Therefore, GABA has potential as a functional bioactive component of foods and pharmaceuticals. Consumption of GABA-enriched foods such as milk (4), soybean (5), gabaron tea (6), red-mold rice (7), and *Chlorella* (8) has been reported to depress the elevation of systolic blood pressure in spontaneously hypertensive rats.

Lactic acid bacteria (LAB) exist not only in dairy products, such as yogurt, cheese and butter, and in meat products, such as fermented sausage, but also in traditional Korean fermented foods such as *kimchi*, salted preserves, and soybean pastes (9-11). They are beneficial inhabitants of the intestines of human and animals (12). Up to now, isolation of LAB has been attempted with the intention of producing GABA using methods compatible with industrial and functional materials (11, 13, 14).

This study was carried out to isolate and characterize *Lactobacillus* stains having high GABA-generating capacity. Here, we report the isolation and characterization of *Lactobacillus buchneri* strains from naturally aged cheese as high GABA producing LAB strains.

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Materials and Methods

Isolation of GABA producing *Lactobacillus* strains Naturally aged, laboratory-made cheese was used for isolation of LAB with high GABA producing ability. Peptone solution (1%) was used for dilution of cheese sample. Subsequently, 1 mL diluted solution was spread on the plate count agar (PCA) for 48 hr, and the bacterial colonies were counted as total bacterial count. In the same manner, the diluted solution was plated on MRS agar (Difco, Detroit, MI, USA) with 0.002% bromophenol blue, at 25°C for 72 hr. Among the colonies, those with a ring and light blue or white color were classified as *Lactobacillus*. Among the isolates, those classified as *Lactobacillus* strains were further characterized for their GABA-generating capacity as previously described (11).

Identification of GABA producing *Lactobacillus* strains Fermentation characteristics and biochemical features of the isolated strains were tested using an API 50 CHL kit (BioMerieux, Marcy-L'Etoile, France). For PCR, genomic DNA was isolated from the selected strains using a Wizard DNA kit (Promega, Madison, WI, USA), and the process of purifying DNA followed the manufacturer's instructions. The PCR instrument used was a Biometra thermocycler (Tampa, FL, USA), and a TA cloning kit (Promega, Madison, WI, USA) was used to clone the PCR fragment. DNA polymerase and MRS broth were purchased from Takara (Shiga, Japan) and Difco (Detroit, MI, USA), respectively. Other reagents used were of analytical grade. To clone 16S rDNA of the isolated strains, PCR amplification was performed using the primers 5'-AGAGTTTGATCMTGGCTCAG-3' (forward) and 5'-ACGGGCGGTGTGTRC-3' (reverse). For the PCR amplification, 100 ng of template DNA, 200 ng of each primer, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl (pH 9.0) containing 0.1% Triton X-100

were used with 30 cycles of denaturation, annealing, and extension at 95°C for 30 sec, 43°C for 30 sec, and 72°C for 90 sec, respectively, as previously described (11). PCR product was separated by 1% agarose gel electrophoresis, and the amplified DNA scrap was ligated into pGEM T-Easy vector using T4 ligase. The cloned 16S rDNA was sequenced using the dideoxynucleotide termination procedures based on synthetic oligonucleotide primers and dsDNA Cycle System (Perkin Elmer, Boston, MA, USA). The nucleotide sequences were analyzed using Clustal W (1.81) program. The partial 16S rDNA sequences of the isolated strains were compared with those of several strains in the Entrez PubMed database (www.ncbi.nlm.nih.gov) for determination of similarity index.

Cultivation of GABA producing *Lactobacillus* OPM strains The seed culture was performed, with a colony taken from a solid agar plate on which *L. buchneri* OPM-1 or *Lactobacillus* sp. OPM-2 had been grown, in a test tube with 10 mL working MRS broth volume at 30°C, for 24 hr. A 4% volume of seed culture was used as its inoculum for media bottle culture. In order to investigate the GABA production capacity of *L. buchneri* OPM-1 and *Lactobacillus* sp. OPM-2, the culturing was performed in a 1 L media bottle with 950 mL of working MRS broth volume with 1% of monosodium glutamate (MSG) at 30°C for 48 hr.

Glutamate decarboxylase assay Cells were harvested by centrifugation at 3,500×g at 4°C for 15 min and resuspended in the cell lysis buffer with 20 mg/mL of lysozyme [50 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 2 mM β-mercaptoethanol, 10% glycerol and 1 mM phenyl-methylsulphonylfluoride (PMSF)]. This suspension was incubated for 1 hr at 4°C and disrupted by ultrasonic treatment. The cell debris was removed by centrifugation at 13,000×g at 4°C for 15 min. After adding PMSF (to a final concentration of 1 mM) to the supernatant, the protein concentration was measured by Bradford method (15). The remainder of the solution was preserved and stored at -20°C. GAD activity was estimated according to the procedure of Snedden *et al.* (16), which measures the production of $^{14}CO_2$ from L-[1-^{14}C] glutamic acid. Crude enzyme extracts were added to a reaction mixture consisting of 200 mM pyridine-HCl, pH 5.5, 0.5 mM pyridoxal-5'-phosphate, 1 mM DTT, 10%(v/v) glycerol, and 2.5 mM L-glutamate (0.1 μCi/reaction), and the reaction proceeded in a flask equipped with a CO_2 trap (Kontes, USA) submerged in a 30°C water bath. After 40 min, the reaction was ended by adding a stop solution (18 N H_2SO_4 : DW = 1:1, v/v), and enzyme activity was calculated from the amount of $^{14}CO_2$ captured by the CO_2 trap (0.1 N NaOH, 0.4 mL) in a liquid scintillation counter.

Analysis of GABA GABA was extracted essentially as described by Baum *et al.* (17) with minor modifications. Briefly, 800 μl of mixed organic solvent solution (methanol: chloroform: water = 12:5:3) was added to 200 μl of cell suspension sample. The aqueous solution layer containing GABA was obtained by centrifugation (13,000 ×g, 4°C, 15 min), and the obtained supernatant was recentrifuged to remove the remnant impurities. The supernatant was then freeze-dried, resuspended in water, filtered through a 0.45-μm PVDF membrane, and analyzed by HPLC (Waters, Milford, MA, USA) after 6-aminoquioly-N-hydroxysuccinimidyl carbonate (AQC) derivatization. To separate the derivatives, a 3.9×150 mm AccQ·Tag™ (Nova-Pak™ C_{18}, Waters) column at 37°C was used, along with mobile phases (AccQ·Tag Eluent A and 60% acetonitrile) with a 1.0 mL/min flow rate and a fluorescence detector (Waters). The GABA content was calculated using a commercial GABA standard based on a standard curve.

Results and Discussion

Isolation of lactic acid bacteria LAB with high GABA-generating ability were isolated from naturally aged, laboratory-made cheese. Colonies with a ring and light blue or white color were isolated from MRS plates with bromophenol blue and classified as *L.* species on the basis of API test results (Table 1). The strains were gram-positive and rod-type bacteria with negative catalase

Table 1. Characteristics of *Lactobacillus buchneri* OPM-1 and *Lactobacillus* sp. OPM-2

Characteristics	OPM-1 & OPM-2
Gram staining	+
Form	Rod type
Spore production	-
Gas production ability in glucose broth	CO_2 production
Catalase production	-
Glucose	+
Ribose	+
Galactose	+
Fructose	+
Maltose	+
Sucrose	+
Glycerol	+
Starch	-
Acetic acid	-
Arabinose	+
Melezitose	+
Melibiose	+
Xylose	+
Cellobiose	-
Trehalose	-
Viability at 15°C	+
Viability at 45°C	-
Viability at 50°C	-
Size	1.0-1.5 μm

+: Positive, -: Negative.

activity, and grew well at 25-37°C, but not over 45°C. The strains were able to grow anaerobically with CO_2 production. The heterofermentative isolates grew rapidly on MRS broth and fermented arabinose, melezitose, melibiose and xylose, but not cellobiose or trehalose. These characteristics suggested that the isolates belong to *L. buchneri* species (18).

Identification of isolates by 16S rDNA sequence 16S rDNA sequences analysis was used to confirm their identification. The 16S rDNA sequence of *Lactobacillus* sp. OPM-1 showed 99% homology with the *L. buchneri* strain 16S ribosomal RNA (Access No. AB205055) and 97% homology with the *L. parabuchneri* strain 16S ribosomal RNA (Access No. AB205056). The 16S rDNA sequence of *Lactobacillus* sp. OPM-2 showed 99% homology with the *L. buchneri* strain 16S ribosomal RNA (Access No. AB205055), the *L. parabuchneri* strain 16S ribosomal RNA (Access No. AB205056), and *Lactobacillus* sp. rennanqilfy19 (AY363377). The strains were therefore named as *L. buchneri* OPM-1 and *Lactobacillus* sp. OPM-2, respectively. Based on these results and according to the scheme of Sharpe *et al.* (18), these characteristics most closely fit an identification of *L. buchneri.* The strain *Lactobacillus* sp. OPM-2 was deposited at the Korea Culture Center of Microorganisms with the identification number KFCC 11353P.

Growth profiles and GABA production of *Lactobacillus* OPM strains To characterize cell growth rates and GABA production capacities of the selected LAB strains, *L. buchneri* OPM-1, *Lactobacillus* sp. OPM-2, and *L. plantarum* KCTC 3103 were cultured in MRS broth supplemented with 10 g/L of MSG. *L. plantarum* was selected as a control because the existence of GAD gene in the strain was reported previously (19). The growth rates of the 3 LAB strain cultures were almost same (Fig. 1). GABA production of *L. buchneri* OPM-1 and *Lactobacillus* sp. OPM-2, but not of the control *L. plantarum* cells, was increased significantly in a cultivation time-dependent manner (Fig. 1). *L. buchneri* OPM-1 almost completely metabolized 10 g/L of MSG after 48 hr of fermentation (Fig. 2) and *Lactobacillus* sp. OPM-2 also metabolized 10 g/L of MSG completely (data not shown). After 48 hr of fermentation, *L. buchneri* OPM-1 and *Lactobacillus* sp. OPM-2 produced about 4.4 g/L and 5.6 g/L of GABA, respectively, giving hourly GABA production rates of 91.7 and 116.7 mg/L/hr, respectively. However, *L. plantarum* KCTC 3103 and *L. brevis* KCTC 41028 tested in this study as control strains were not able to produce GABA efficiently from MSG (Table 2).

LAB having high GABA producing capacity such as *L. buchneri* strains, *L. brevis* strains (11), and *L. lactis* ssp. *lactis* (13) will be useful in the food industry for the production of GABA-enriched health foods such as yogurt and cheese. A recent report described the production of GABA-enriched yogurt utilizing the high GABA producing capacity of *L. brevis* OPY-1 (11). *L. brevis* OPY-1 was able to produce GABA from MSG at a productivity of about 100 mg/L/hr. Therefore, the 2 strains used in this study, *L. buchneri* OPM-1 and *Lactobacillus* sp. OPM-2, seem to be efficient for the production of GABA because they demonstrated a high GABA productivity (Fig. 1 and Table 2), similarly to that previously reported (11).

GAD activity of *Lactobacillus* OPM strains GAD catalyzes the irreversible conversion of L-glutamate to GABA. GAD has been found in bacteria (20), animals (21), and higher plants (22). The existence of GAD in LAB has been reported by several investigators (13, 14, 19, 23), however the activity of GAD from *L. buchneri* strains has not been reported. In the present study, GAD activity was detected in the cell extracts of *L. buchneri* OPM-1 and *Lactobacillus* sp. OPM-2. The optimum pH and temperature for both strains was 5.5 and 30°C (Fig. 3), respectively, and the production of GABA depended on the concentration of cell extracts (data not shown). Therefore, the conversion of MSG to GABA by the cell extracts of *L. buchneri* OPM-1 and *Lactobacillus* sp.

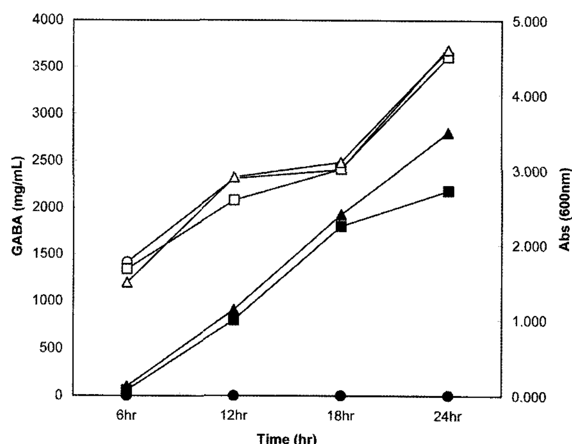

Fig 1. Growth and GABA production of selected *Lactobacillus* strains. Circles (○), squares (□), and triangles (△) show the growth profiles of *L. plantarum* KCTC 3103, *L. buchneri* OPM-1, and *L. sp.* OPM-2, respectively. Circles (●), squares (■), and triangles (▲) show the profiles of GABA production of *L. plantarum* KCTC 3103, *L. buchneri* OPM-1, and *Lactobacillus* sp. OPM-2, respectively.

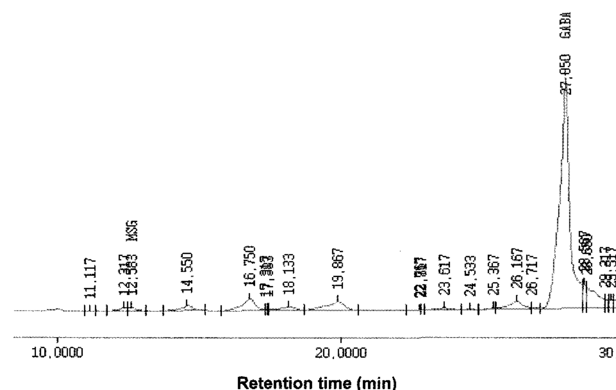

Fig 2. HPLC chromatogram of metabolite from MSG fermentation by *Lactobacillus buchneri* OPM-1. The strain was cultured in MRS broth with 1% monosodium glutamate (MSG). The levels of MSG and GABA were measured by HPLC after 48 hr of fermentation.

Table 2. GABA productivity of *Lactobacillus* strains in MRS broth supplemented with monosodium glutamate

Strain[1)]	GABA productivity (mg/L/hr)[2)]
L. buchneri OPM-1	91.721(±4.424)
Lactobacillus sp. OPM-2	116.727(±6.246)
L. brevis KCTC 41028	0.013(±0.001)
L. plantarum KCTC 3103	0.125(±0.012)

[1)]The strains were cultured in MRS broth with 1% MSG. KCTC stands for Korean Collection for Type Cultures.
[2)]GABA productivity was measured after 48 hr of fermentation.

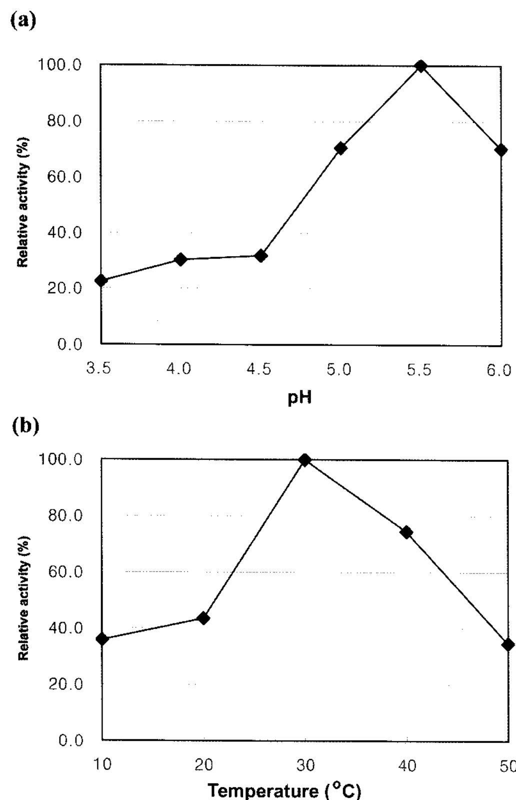

Fig 3. Effects of pH (a) and temperature (b) on GAD activity of *Lactobacillus* sp. OPM-2. For the pH profile, enzyme activity was measured at 30°C in 200 mM pyridine-HCl buffer adjusted to pH 3.5-6.0. For the temperature profile, enzyme activity was assayed in 200 mM pyridine-HCl buffer (pH 5.5) at 10-50°C. Each point is the mean of duplicate determinations of cell extracts.

OPM-2 is suspected to proceed according to an enzymatic reaction of intracellular enzyme.

The GABA-generating system in bacteria contributes to the acid resistance of *Lactococcus lactis* and *E. coli* (24, 25). During GABA production, GAD must use a single molecule of H^+, and it has been postulated that this can help maintain a stable pH inside the cell even in acidic conditions, thereby making cell survival possible (24, 25). Considering its role in pH resistance, bacteria with a high GAD activity can be expected to be characteristically highly resistant to acid, which is a requirement for probiotics to be fully functional since they must pass through the stomach and survive in the intestines. Further studies are needed to test the acid and bile resistance of LAB having high GABA and GAD contents. Future studies with the *L. buchneri* strains may also provide further insights into devising a strategy to optimize the diverse biotechnological capabilities of *Lactobacillus* strains.

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