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Production and Characterization of GABA Rice Yogurt

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Abstract Yogurt containing high γ-aminobutyric acid (GABA) was developed using lactic acid bacteria and germinated brown rice. *Lactobacillus acidophilus, L. plantarum, and L. brevis* OPY-1 strains were inoculated into *Lactobacillus* MRS broth for use as yogurt starter. After treatment with 5% monosodium glutamate in MRS broth, *L. brevis* OPY-1 strain isolated from *Kimchi* produced GABA concentration of 8,003.28 nmol/mL. Starter was inoculated into fermentation substrate mixture containing germinated brown rice extract and blend of powdered whole milk and skim milk. Samples were incubated, and viable cell colonies were counted. Highest number of lactic acid bacteria was reached between 16 and 20 hr. Concentrated rice milk fermented with high GABA-producing strain contained GABA concentrations of 137.17 μg/g D.W., whereas concentrated fermented milk prepared by conventional method contained GABA of 1.29 μg/g D.W. Sensory evaluation panelists gave favorable ratings to fermented rice milk containing high GABA concentration.

Keywords: GABA, germinated brown rice, yogurt, *Lactobacillus brevis*

Introduction

Yogurt, made from milk fermented by bacteria of the *Lactobacillus* genus and other lactic acid bacteria, including *Bifidobacterium* (1, 2), is a nutrient dense food, because it is made from milk; however, fermentation also increases contents of various organic acids, peptones, peptides, other trace activators, and lactic acid bacteria. Yogurt has an intestine-cleaning function through the proliferation of intestinal lactic acid bacteria. Korea's dairy product producers fortify yogurt with 3 or 4% skim milk to increase the content of non-fat milk solids (MSNF). Other ingredients including non-fat dry milk, soy protein (3, 4), vegetables (5), sweet potato (6, 7), pumpkin (8), and plum (9) are sometimes added into Korean yogurts as well. In addition, pectin or sarcocarp is also added to improve the texture and flavor, and additional fermentation substrates such as mugwort (10) and sea tangle (11) are used to develop new lactic acid drinks.

Brown rice under optimal moisture, temperature, and oxygen condition will grow into 1 to 5 mm sprouts called 'germinated brown rice'. The genetic material of rice is tightly covered with chaff and endoderm to facilitate the production of new life. Although brown rice is highly nutritional, it is indigestible, not due to its hard hull but rather due to the presence of phytic acid, which protects the life materials. However, when brown rice is sprouted, phytic acid is degraded into phosphorous and inositol, and does not interfere with digestion. In addition, new ingredients, such as γ-aminobutyric acid (GABA), useful amino acids, enzymes, super oxide dismutase (SOD), arabinoxylan, γ-oryzanol, are generated during germination. Therefore, the nutritive value, bioactive materials, and activated dietary fiber of germinated brown rice, among others are of great interests (12, 13).

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GABA is a ubiquitous non-protein amino acid produced primarily by the α-decarboxylation of L-glutamic acid (Glu), which is catalyzed by the enzyme glutamate decarboxylase (GAD) (14). GABA functions in animals as a major inhibitory neurotransmitter (15,16) and 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 (16). Many neurological disorders, such as seizures, Parkinson's disease, stiff-man syndrome, and schizophrenia are known to be related to alterations in GABA and GAD levels in the brain (16-18). Alcoholics have a remarkably low plasma GABA concentration and reduced expression of GABA receptors in the brain (15, 19). Consumption of GABA-enriched foods such as milk (20), soybean (21), gabaron tea (22), red-mold rice (23), and Chlorella (24) has been reported to depress the elevation of systolic blood pressure in spontaneously hypertensive rats (SHRs).

Our research team has recently reported that addition of chitosan in the medium for the germination of brown rice increases the GAD activity. This report brought about the production of germinated brown rice with increased GABA concentration (25). Furthermore, the finding that chitosan can inhibit the growth of many bacteria, which are parasitic on brown rice, suggested that chitosan may improve the quality of the germinated brown rice (25). Likewise, it was reported that GABA and free amino acid in germinated brown rice may be remarkably increased by joint treatment with chitosan and glutamic acid (26).

Currently, red rice yeast is actively being studied for applications in GABA production; particular interests include ways to maximize GABA production by improving the existing strains (27, 28). Moreover, strain isolation and its mass culturing have already been attempted with the intention of producing GABA using methods compatible with industrial and functional materials (29). However, attempts at producing high-GABA dairy products using high GABA-generating strains have mostly been unsuccessful.

This study was carried out to develop a yogurt with high nutritive value and functional properties. Here, we report the methods and materials applied for the production of yogurt, which contains high levels of GABA and amino acids.

Materials and Methods

Isolation of starter strain Samples from commercially produced Korean fermented foods (*Kimchi*, pickles, etc) were diluted to between 10^{-1} and 10^{-8} using 1% peptone solution. Subsequently, 1 mL solution was incubated 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 incubated on agar, with 0.002% bromophenol blue added into the MRS broth, at 25°C for 72 hr to observe the colonies. Among the colonies, those which had no ring and a dark blue color were classified as *Leuconostoc*, and those with a ring and light blue or white color were classified as *Lactobacillus*. These strains were further characterized for their GABA-generating capacity using TLC. TLC was performed with a solvent mix of 4:1:1 butanol:acetic acid:water for 30 min, followed by ninhydrin staining. The bacterial strains exhibiting the highest GABA production were chosen for additional experiments.

Identification of strain For use as the PCR template DNA, genomic DNA was isolated from the selected strains using a Wizard DNA kit (Promega, Madison, WI, USA), and the process of refining DNA followed the manual provided by the manufacturer. The PCR instrument used was a Biometra thermocycler (Tampa, Florida, USA), and a TA cloning kit (Promega). 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 rDNAs of the isolated strains, PCR amplification was performed using the primers 5'-AGAGTTTGATCMTGG CTCAG-3' (forward) and 5'-ACGGGCGGTGTGTRC-3' (reverse). For the PCR amplification, 100 ng template DNA, 200 ng 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 a total 30 cycles of denaturation, annealing, and extension at 95°C for 30 sec, 43°C for 30 sec, and 72°C for 1 min 30 sec, respectively. 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 dideoxy-nucleotide termination procedures based on synthetic oligonucleotide primers and dsDNA Cycle System (Perkin Elmer, Boston, MA, USA), and the nucleotide sequences were analyzed using Clustal W (1.81) program.

Producing germinated brown rice The production of germinated brown rice followed the method of Oh and Oh (2004). In brief, 50 g commercial brown rice was germinated in an incubator with germination solution (500 mL; 50 ppm chitosan dissolved in 5 mM glutamic acid) at 25°C. The germination solution was exchanged with a fresh solution at 12-hr intervals until germination was complete at 72 hr. The germinated brown rice was then removed from the solution and dried on a filter paper.

Fermentation substrate The germinated brown rice was frozen in liquid nitrogen, and ground with a mortar and pestle. Four volumes of water was added to the brown rice powder, and the mixture was sterilized in an autoclave at 121°C and 15 psi for 20 min. The sterilized sample was filtered and treated with α-amylase to use as a fermentation substrate.

Producing starter Laboratory-grown *Lactobacillus acidophilus* (KCCM 40265), *L. plantarum* (KCTC 3105), and *L. brevis* OPY-1 (KFCC 11337) strains were inoculated into the *Lactobacillus* MRS broth (4%, v/v), and the inoculum was activated three times at 37°C for 24 hr to use as a starter for the production of yogurt.

Producing yogurt Powdered whole milk (18%) and skim milk (2%) were added to the prepared fermentation substrate, homogenized in a Warning blender for 5 min, and sterilized in an autoclave for 20 min at 121°C. After the sterilized substrate was warmed to 37°C, the substrate was inoculated with the mixed strain starter (*L. acidophilus* + *L. plantarum* + *L. brevis* OPY-1 strain, 1:1: 3, v/v) and fermented at 37°C.

Measuring viable count One milliliter sample was collected every 4 hr after inoculation and diluted 10-fold with sterilized physiological saline. Subsequently, a 0.1-mL aliquot was smeared on the MRS plate count agar using a micropipette and incubated for 48 hr at 37°C. Visible colonies were then counted, and the units expressed as CFU (colony forming unit)/mL.

Content of GABA in yogurt The GABA content was measured according to the method of Oh and Oh (2004). Briefly, 800 μL mixed organic solvent solution (methanol: chloroform:water = 12:5:3) was added to 200 mg freeze-dried sample. The aqueous solution layer containing GABA was obtained through microcentrifugation (15,000 rpm, 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. AccQ Tag Reagent was used to implement the fluorescence derivatization of GABA. To separate the derivatives, 3.9 × 150 mm AccQ·Tag™ (Nova-Pak™ C18, Waters) column was used. The GABA content was calculated using a commercial GABA standard based on a standard curve.

Sensory evaluation of yogurt To evaluate the sensory properties of the product, the curd of yogurt, incubated at 20°C for 20 hr, was broken and kept in a refrigerator at 4 °C for 5 hr. Subsequently, 20 panelists evaluated overall acceptability, taste, odor, texture, etc., each item scored between 1 and 5 points. Differences in preferences between the conventional yogurt and GABA rice yogurt were compared using Student's T-test to evaluate the significance of the differences. Statistical analyses were performed using SAS software version 8 (SAS Institute, Cary, NC, USA).

Results and Discussion

To produce yogurt with improved nutritive value and functionality, germinated brown rice having a high GABA content was fermented with high GABA-producing lactic acid bacteria. GABA production in the germinated brown rice was stimulated by adding chitosan and glutamic acid to the germination solution (26). An extract of the germinated brown rice produced was used to make the rice yogurt. In addition, to further increase the GABA content of the yogurt, starter strains having strong GABA-generating power were selected for fermentation use. Characterization of the biochemical features of the strains through an API test resulted in the selection of *Lactobacillus* strain as a high GABA-producing strain (Table 1). The 16S rDNA sequence of the strain was analyzed by separating its genomic DNA (Fig. 1). The 16s rDNA sequence of the selected strain showed 99% homology with the *L. brevis* strain RO97 16S ribosomal RNA (Access No. AF515219) and the *L. brevis* strain RO66 16S ribosomal RNA (Access No. AF515220) (Fig. 1). The strain was therefore named as *L. brevis* OPY-1. The GABA-generating ability of the selected strain was compared with those of other strains under the condition of 5% monosodium glutamate (MSG). Results showed a GABA productivity of 8,003.28 nmol/mL, which was remarkably higher than those of other strains (Table 2).

For use as a fermentation substrate, the germinated brown rice was added to water (1:4), sterilized in an autoclave at 121°C for 20 min, filtered through a mesh, and treated with α-amylase. Milk was prepared from powdered whole and skim milk at 18 and 2%, respectively, and homogenized in a Warning blender for 5 min. The live bacterial strain having high GABA productivity was then inoculated into the substrate and incubated for 24 hr as the final step of the yogurt production. The initial viable bacterial count in the yogurt was low due to short incubation time, but increased in a time-dependent manner with remarkable increases between 16 and 20 hr incubation (Table 3). The GABA content of the rice yogurt, measured in the dry powder after lyophilization, was 137.17 μg/g D.W., which was about centuple compared with that of the yogurt prepared by conventional laboratory methods. Likewise, sensory scores were higher in the high-GABA rice yogurt than the conventional yogurt. In addition, GABA rice yogurt had higher scores in color, odor, and acceptability compared with conventional yogurt (Table 4).

GABA synthesis in plants is controlled by various external factors, e.g., physical stimulus, temperature, hypoxia, moisture, stress (30, 31), suggesting that plants utilize the GABA-generating system to cope with environ-

Table 1. Characteristics of *Lactobacillus brevis* OPY-1

Characteristics	Selected strain
Gram staining	Positive
Form	Rod type
Spore production	-
Gas production ability in glucose broth	CO_2 production
Catalase production	+
Viability at 15°C	+
Viability at 45°C	-
Size	1.0-1.5 μm

Table 2. Comparison of GABA generating capacity of different *Lactobacillus* strains

Strain[1)]	GABA content (nmol/mL)
Lactobacillus brevis OPY-1	8,003.28
Lactobacillus plantarum KCTC3103	30.09
Lactobacillus brevis KCTC 41028	2.09
Lactobacillus brevis KCTC 41029	5.15

[1)]The strains were cultured with 5% monosodium glutamate.

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AF515219   ------AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCATGCCTAATACATGCAAG
AF515220   ------AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCATGCCTAATACATGCAAG
OPY-1      TCGATTAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCATGCCTAATACATGCAAG
                 ******************************************************

AF515219   TCGAACGAGCTTCCGTTGAATGACGTGCTTGCACTGATTTTAACAATGAAGCGAGTGGCG
AF515220   TCGAACGAGCTTCCGTTGAATGACGTGCTTGCACTGATTTCAACAATGAAGCGAGTGGCG
OPY-1      TCGAACGAGCTTCCGTTGAATGACGTGCTTGCACTGATTTTAACAATGAAGCGAGTGGCG
           **************************************** *******************

AF515219   AACTGGTGAGTAACACGTGGGAAATCTGCCCAGAAGCAGGGGATAACACTTGGAAACAGG
AF515220   AACTGGTGAGTAACACGTGGGAAATCTGCCCAGAAGCAGGGGATAACACTTGGAAACAGG
OPY-1      AACTGGTGAGTAACACGTGGGAAATCTGCCCAGAAGCAGGGGATAACACTTGGAAACAGG
           ************************************************************

AF515219   TGCTAATACCGTATAACAACAAAATCCGCATGGATTTTGTTTGAAAGGTGGCTTCGGCTA
AF515220   TGCTAATACCGTATAACAACAAAATCCGCATGGATTTTGTTTGAAAGGTGGCTTCGGCTA
OPY-1      TGCTAATACCGTATAACAACAAAATCCGCATGGATTTTGTTTGAAAGGTGGCTTCGGCTA
           ************************************************************

AF515219   TCACTTCTGGATGATCCCGCGGCGTATTAGTTAGTTGGTGAGGTAAAGGCCCACCAAGAC
AF515220   TCACTTCTGGATGATCCCGCGGCGTATTAGTTAGTTGGTGAGGTAAAGGCCCACCAAGAC
OPY-1      TCACTTCTGGATGATCCCGCGGCGTATTAGTTAGTTGGTGAGGTAGAGGCCCACCAAGAC
           ********************************************* **************

AF515219   GATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAA
AF515220   GATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAA
OPY-1      GATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAA
           ************************************************************

AF515219   ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAA
AF515220   ACTCC-ACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAA
OPY-1      ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAA
           ***** ******************************************************

AF515219   TGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACACCT
AF515220   TGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACACCT
OPY-1      TGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACACCT
           ************************************************************

AF515219   TTGAGAGTAACTGTTCAAGGGTTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTG
AF515220   TTGAGAGTAACTGTTCAAGGGTTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTG
OPY-1      TTGAGAGTAACTGTTCAAGGGTTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTG
           ************************************************************

AF515219   CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCG
AF515220   CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCG
OPY-1      CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCG
           ************************************************************

AF515219   AGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGAAGTGCATC
AF515220   AGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGAAGTGCATC
OPY-1      AGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGAAGTGCATC
           ************** *********************************************

AF515219   GGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGGAAT
AF515220   GGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGGAAT
OPY-1      GGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGGAAT

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AF515219   GC
AF515220   GC
OPY-1      GC
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Fig. 1. Comparison of nucleotide sequences of *Lactobacillus brevis* strains. AF515219, *Lactobacillus brevis* strain RO97 16S ribosomal RNA; AF515220, *Lactobacillus brevis* strain RO66 16S ribosomal RNA; OPY-1, *Lactobacillus brevis* OPY-1 strain 16S rDNA selected in this study.

Table 3. Changes in the number of lactic acid bacteria during yogurt fermentation time (CFU/mL)

	Fermentation time (hr)						
	0	4	8	12	16	20	24
GABA rice yogurt[1)]	-	-	2.2×10^8	3.5×10^8	4.0×10^8	4.2×10^8	2.5×10^8

[1)]GABA rice yogurt was fermented using the fermentation starter prepared as outlined in the Materials and Methods.

Table 4. Sensory evaluation of GABA rice yogurt and conventional yogurt

Yogurt	Color	Taste	Flavor	Acceptability
Conventional	2.60±0.60	2.65±0.99	2.55±0.60	2.80±0.62
GABA rice yogurt	3.20±0.89*	3.20±0.95	3.35±0.93**	3.40±0.94*

Significantly different from the control group (*p<0.05, **p<0.01).

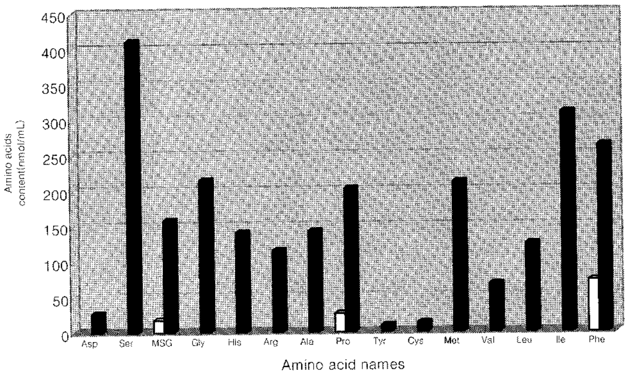

Fig. 2. Comparison of free amino acid levels between GABA rice yogurt and yogurt prepared by conventional laboratory methods. The closed bar (■) and the open bar (□) show the amino acid levels of GABA rice yogurt and conventional yogurt, respectively.

mental stress. Furthermore, GABA-generating system of plants is closely connected to various other factors, such as contents of glutamic acid, GAD, calcium, calmodulin (32-35). By exploiting such systems, germinated brown rice with a high content of GABA was produced (25), and its extract was used for producing yogurt to produce a functional product with high consumer satisfaction. Its functional properties were also enhanced by applying microorganisms having high GABA-producing ability to the production of milk-related products. Studies showed that GABA has a role in microbial growth, specifically in the spore germination of *Bacillus megaterium*. During the spore germination process, the GAD enzyme was remarkably activated with a concomitant increase in GABA concentrations (36). The GABA-generating system also contributes to the acid resistance of *Lactococcus lactis* and *E. coli* (37, 38). Specifically, GAD indispensably spends H^+ in the production of GABA, thereby facilitating the GABA production through acid production, which also helps maintain pH homeostasis in cells. From this viewpoint, the lactic acid bacteria, which has high GAD activity, could survive the high acidity condition of the stomach and remain viable as they pass through the digestive tract. However, features such as acid-and bile-resistances require further study. Furthermore, animal and clinical studies are needed to evaluate the functions, dose responses, and action mechanisms of the germinated brown rice and lactic acid bacteria.

Recently, we found that the germinated brown rice extracts with high GABA content markedly stimulate the immune response, and can repress or block the proliferation of cancer cells (39, 40). Hayakawa et al. (20) showed that GABA-enriched milk (1 nmol/mL) lowered the blood pressure in spontaneously hypertensive and normotensive Wistar-Kyoto rats, suggesting that the amount of GABA incorporated into the yogurt is high enough to have some functional value. The *Lactobacillus* strain and other GABA-producing lactic acid bacteria showed promise for applications in dairy and other health products through exploitation of the functional properties of GABA. The GABA rice yogurt is not only rich in GABA, but also contains various free amino acids (Fig. 2). Previous studies reported that free amino acids and oligopeptides in foods have nutritional advantages due to their rapid absorption (41, 42), muscle protein maintenance (42), and antioxidative activity (43). These physiological effects as well as others such as antihypertensive effects can also be expected for the GABA rice yogurt pending confirmation through further animal and clinical studies.

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