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Short Communication

Enhancement of γ-Amminobutyric Acid Production by *Lactobacillus sakei* B2-16 Expressing Glutamate Decarboxylase from *Lactobacillus plantarum* ATCC 14917

Moo-Chang Kook[1,2†], Myung-Ji Seo[3†], Chan-Ick Cheigh[4], Sang-Jae Lee[5], Yu-Ryang Pyun[2,5], and Hoon Park[6*]

[1]*Department of Marine Biotechnology, Anyang University, Incheon 417-833, Republic of Korea*
[2]*R&D Division, Biovan Co. Ltd., Yonsei University, Seoul 120-749, Republic of Korea*
[3]*Department of Chemistry, Box H, Brown University, Providence, RI 02912-9108, U.S.A.*
[4]*Department of Food Science and Engineering, Ewha Womans University, Seoul 120-750, Republic of Korea*
[5]*Department of Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea*
[6]*Department of Food Science, Sunmoon University, Asan 336-708, Republic of Korea*

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To enhance γ-amminobutyric acid (GABA) production using genetically engineered lactic acid bacteria, glutamate decarboxylase gene was isolated from *Lactobacillus plantarum* ATCC 14917 and cloned into *Lactobacilli-E. coli* shuttle-expression vector (pTRKH2) to yield pTRKH2GAD, which was expressed in *L. sakei* B2-16 isolated from *Kimchi*. The recombinant *L. sakei* B2-16 showed 1.35- and 1.42-fold higher GABA production compared to the untransformed strain in 500-mL flask and 5-L fermentor scale, respectively. The maximum GABA concentration was 265.3 mM.

Key words: γ-amminobutyric acid, glutamate decarboxylase, *Lactobacillus plantarum*, *Lactobacillus sakei*

γ-Amminobutyric acid (GABA) is a four carbon non-protein amino acid widely distributed in nature, playing a major role as inhibitory neurotransmitter in central nervous system [Manyam *et al*., 1981]. Due to the physiological functions of GABA such as the induction of hypotensive, diuretic, tranquilizing effects, and the regulation of neurological disorders, development of nutraceuticals and pharmaceuticals containing highly concentrated GABA is in demand [Jakobs *et al*., 1993; Inoue *et al*., 2003]. Glutamate decarboxylase (GAD) catalyzes the α-decarboxylation of acidic glutamate to generate neutral GABA, which could survive under strong acidic condition, via incorporation of H^+ involved in conferring acid resistance to bacteria [Sanders *et al*., 1998]. So far, the isolation and characterization of GAD in microorganisms have been reported in various studies [Smith *et al*., 1992; Kato *et al*., 2002; Komatsuzaki *et al*., 2008]. Focusing on the lactic acid bacteria (LAB), GAD from *Lactococcus lactis*, *Lactobacillus brevis*, and *L. paracasei* have been recently characterized [Nomura *et al*., 1999; Kim *et al*., 2007; Komatsuzaki *et al*., 2008].

Here, we focused on the LAB as GABA and GAD producers, because LAB have a high potential commercially as the starters for functional fermented food production. In addition, some LAB have been considered as probiotics, whose products have been of interest in the nutraceutical fields [Shortt, 1999]. Recently, we isolated a strain, *L. sakei* B2-16, capable of producing high content of GABA from the Korean traditional fermented food, *kimchi*, with 100% conversion yield from the supplied monosodium glutamate (MSG) to GABA and developed the fermentation process using by-products of agricultural crop, rice bran, for cost-effective GABA production [Kook *et al*., 2010]. *L. sakei* has been reported as one of various LAB for *kimchi* starters [Jin *et al*., 2008]. Previous studies have focused on development of the genetically engineered *L. sakei* B2-16 as recombinant GABA producer expressing the foreign GAD to overcome the limits of fermentation process for the highest GABA

†These authors equally contributed to this work.

*Corresponding author
Phone: +82-41-530-2262; Fax: +82-41-530-2917
E-mail: hpark@sunmoon.ac.kr

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production. Recently, the isolation of GAD gene from *L. plantarum* and its expression in *Escherichia coli* were reported [Park and Oh, 2004]. However, the previous studies including *L. plantarum*-derived GAD have mostly focused on the expression of foreign GAD in *E. coli* and its biochemical characterizations. Therefore, the enhanced GABA production by a foreign GAD in GABA-producing microorganism, especially LAB, could be worth investigation for development of effective GABA-producing strain and to apply it to industrial fields. The present study describes the expression of *L. plantarum*-derived GAD in *L. sakei* B2-16, which could be a good GABA-producer, to investigate the applicability of genetically engineered *L. sakei* B2-16 for the enhancement of GABA production.

In order to construct the plasmid harboring *L. plantarum*-derived GAD, we isolated the genomic DNA of *L. plantarum* ATCC 14917 by using a genomic DNA extraction kit (Qiagen) according to manufacturer's instructions. Subsequently, to express the GAD under the control of *L. plantarum*-derived promoter, the region covering the full-length open reading frame and additional upstream sequence of GAD gene was amplified from the genomic DNA by PCR using the primers GAD-F (5'-CGGGATCCTACGAATTCTAAAA ATCGGGGTGAG-3') and GAD-R (5'-AACTGCAGTC AGAATCTGGATGGTGCTA-3'), where the underlined sequences are the introduced *Bam*HI and *Pst*I sites, respectively. The amplified gene was ligated into the *Bam*HI/*Pst*I-digested pTRKH2, *Lactobacilli-E. coli* shuttle-expression vector [O'Sullivan and Klaenhammer, 1993], to yield the plasmid designated as pTRKH2GAD. To prepare the *L. sakei* B2-16 competent cells, the pre-cultured *L. sakei* B2-16 in MRS medium was inoculated into the MRS medium containing 3% glycine and 5% sucrose, and grown at 30°C to an OD_{600} of 0.4-0.6. After cultivation, the cells were collected by centrifugation at 4,000×g for 10 min, washed with 0.1 M $MgCl_2$ solution, and were suspended with 0.5 M sucrose solution containing 10% glycerol. For transformation, the prepared competent cells and plasmid pTRKH2GAD were mixed, transferred to a pre-chilled electroporation cuvette, and then exposed to a single electric pulse using a Gene-Pulser (Bio-Rad). The suspensions were immediately mixed with MRS medium containing 3% glycine, 5% sucrose, 20 mM $MgCl_2$, and 2 mM $CaCl_2$, diluted, and then incubated at 30°C for 2 h. The incubated cells were spread on the MRS agar plate containing erythromycin (final concentration of 150 μg/mL) and incubated at 30°C for 48 h.

After transformation of *L. sakei* B2-16 with pTRKH2GAD, the expression of GAD was monitored by SDS-PAGE. The SDS-PAGE analysis of whole cell proteins showed

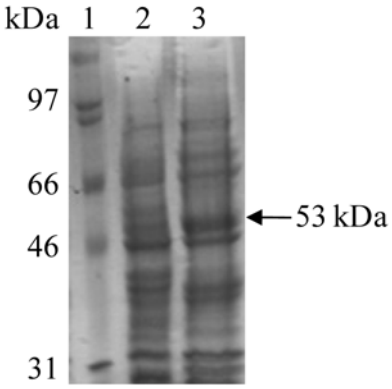

Fig. 1. SDS-PAGE analysis of the untransformed and recombinant *L. sakei* B2-16 harboring pTRKH2GAD. M, molecular weight marker; lane 1, untransformed; lane 2, recombinant.

that one band of approximately 53 kDa was more strongly expressed in recombinant *L. sakei* B2-16 harboring pTRKH2GAD in comparison with the untransformed *L. sakei* B2-16, though the expression levels of other proteins were almost similar (Fig. 1). However, it should be noted that the untransformed *L. sakei* B2-16 also expressed a similar sized-protein (53 kDa), which could be explained by *L. sakei* B2-16 producing GABA, which is also generated from MSG by *L. sakei*-derived GAD. The amino acid sequences of *L. plantarum*-derived GAD expressed in the present study showed similarities of 91.0 and 65.4% with the other GAD proteins from *L. brevis* and *L. paracasei*, from which the expressed GAD sizes were estimated to be 49 kDa and 57 kDa by SDS-PAGE, respectively, though they were expressed in the *E. coli* (Fig. 2) [Kim *et al*., 2007; Komatsuzaki *et al*., 2008].

To determine whether the expression of *L. plantarum*-derived GAD in *L. sakei* B2-16 identified by SDS-PAGE is associated with the enhanced GABA production in recombinant *L. sakei* B2-16, the GABA concentration after cultivation was monitored by high performance liquid chromatography (HPLC) analysis. Recombinant *L. sakei* B2-16 harboring pTRKH2GAD was cultured in the MRS medium (Difco, Sparks, MD) containing 7% food grade MSG (purity >99%, Daesang, Siheung-city, Gyonggi-do, Korea) as a fermentation medium (MRS-MSG medium). For flask scale culture, the culture broth of 2.5 mL was inoculated into 250 mL MRS-MSG medium in a 500-mL Erlenmeyer flask and cultured at 30°C for 48 h. For fermentor scale culture, the pre-cultured broth was used to inoculate the cells in a 5-L jar fermentor (KF-5, Ko-biotech, Incheon-city, Korea) containing a working volume of 3 L MRS-MSG medium at 1% level. The fermentor was operated with slow agitation speed (50 rpm) at 30°C for 48 h. The pH was initially adjusted to 6.0

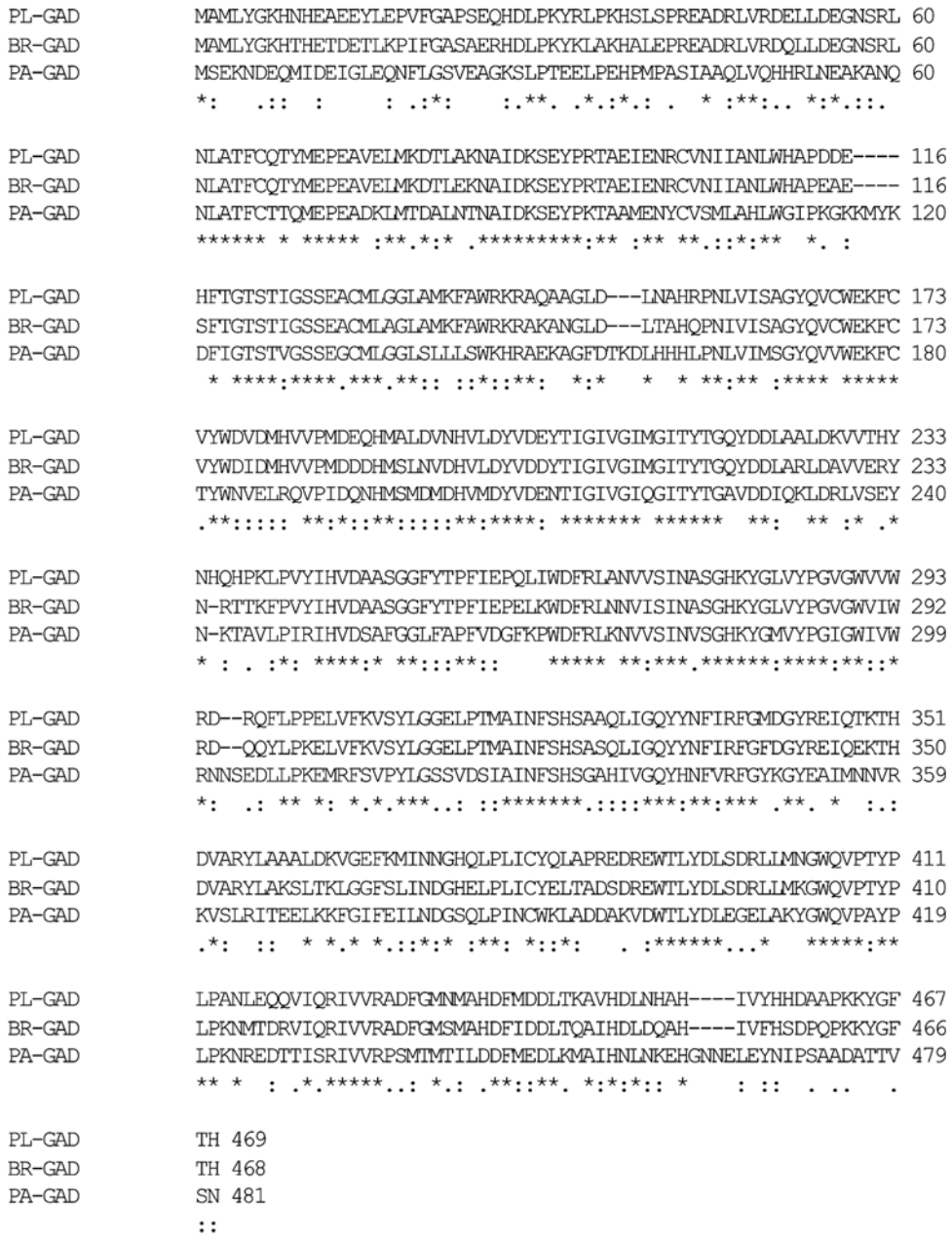

Fig. 2. Multiple alignment of *L. plantarum*-derived GAD with other GAD proteins. Amino acid sequences of GAD from *L. plantarum* (PL-GAD, GenBank accession no. ZP_04014981), *L. brevis* (BR-GAD, ADG02973), and *L. paracasei* (PA-GAD, BAG12190) were aligned by using ClustalW2.

and not controlled during fermentation process. The GABA production was analyzed by HPLC after derivatization with o-phthaldialdehyde (OPA). The culture broth was separated by centrifugation (8,000×*g* for 15 min), and the supernatant was treated with OPA reagent, which was previously prepared in a mixture of methanolic OPA, borate buffer, and 2-merchaptoethanol (100:4,000:1, v/v/v), to yield the sample. For the HPLC analysis, the derivatized sample was analyzed using Xterra column (RP18, 4.6×150 mm, 5 μm, Waters, Milford, MA). The chromatogram was developed with the solvent A (0.1 M sodium acetate, pH 7.2) over a convex gradient (curve 6) to the solvent B (0.1 M sodium acetate-acetonitrile-methanol, 46:44:10, v/v/v, pH 7.2) at a flow rate of 1.0 mL/min for 60 min and monitored at 338 nm. To determine the GAD activity, the cells collected by centrifugation were suspended in the PBS buffer and then disrupted by sonication. After centrifugation at 11,000×*g* for 15 min, the supernatants were used as crude extracts for the GAD activity assay. The crude extracts were then incubated with 1.32 mM MSG and 0.02 mM pyridoxal 5-phosphate in 200 mM sodium acetate/acetic acid (pH

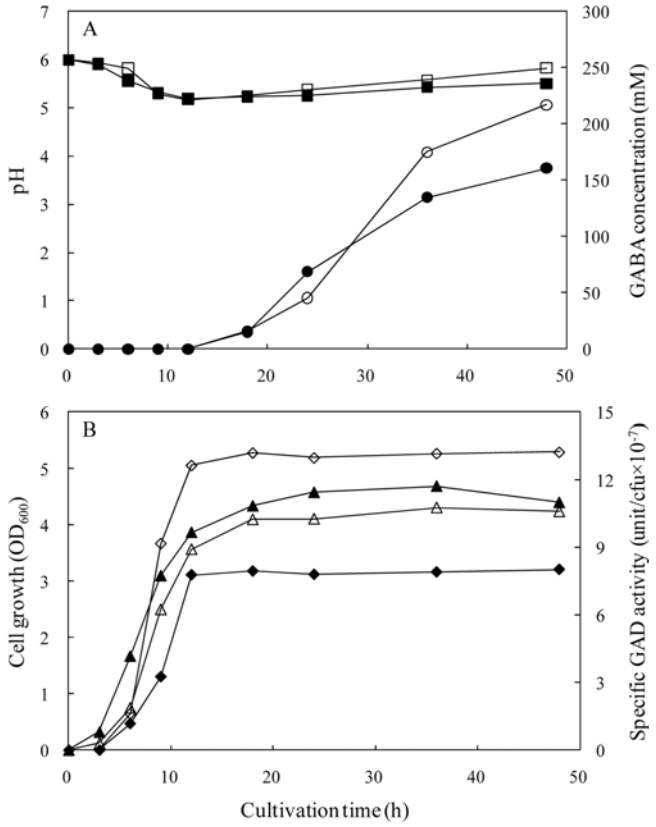

Fig. 3. Time profiles of pH and GABA concentration (A), and cell growth and specific GAD activity (B) for the untransformed and recombinant *L. sakei* B2-16 harboring pTRKH2GAD in 500-mL flask scale culture. pH for untransformed (■) and recombinant (□); GABA concentration for untransformed (●) and recombinant (○); cell growth for untransformed (▲) and recombinant (△); specific GAD activity for untransformed (◆) and recombinant (◇).

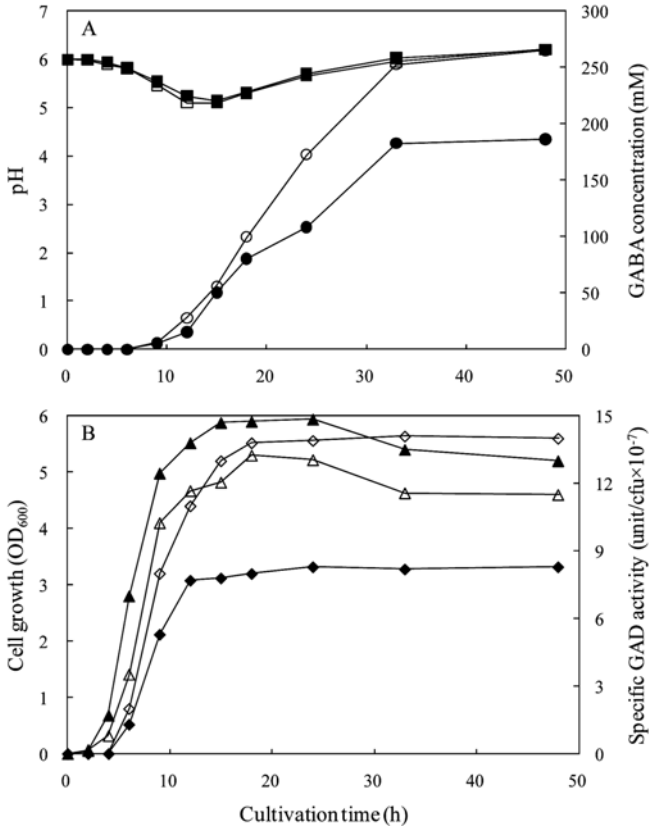

Fig. 4. Time profiles of pH and GABA concentration (A), and cell growth and specific GAD activity (B) for the untransformed and recombinant *L. sakei* B2-16 harboring pTRKH2GAD in 5-L fermentor scale culture. pH for untransformed (■) and recombinant (□); GABA concentration for untransformed (●) and recombinant (○); cell growth for untransformed (▲) and recombinant (△); specific GAD activity for untransformed (◆) and recombinant (◇).

5.0). After incubation at 37°C for 20 min, the enzyme reaction was quenched by adding the ethanol, centrifuged at 1,500×*g* for 10 min, and then assayed by HPLC. One unit of GAD activity is defined as the amount of enzyme producing 1 μmol GABA per min under the enzyme assay condition.

In 500 mL flask scale, the GABA production (217.0 mM) from *L. sakei* B2-16 harboring pTRKH2GAD was about 1.35-times higher than the corresponding value from untransformed *L. sakei* B2-16 (Fig. 3A). The enhanced GABA production from recombinant *L. sakei* B2-16 indicates that the *L. plantarum*-derived GAD is well expressed in *L. sakei* B2-16. In addition, it could be suggested that the expression of *L. plantarum*-derived GAD possibly results in the enhancement of GABA production. The GABA production (160.8 mM) from untransformed *L. sakei* B2-16 could have resulted from the activity of *L. sakei*-derived GAD, because the SDS-PAGE analysis showed that the *L. sakei* B2-16 also expressed its own GAD gene. The specific GAD activity of *L. sakei* B2-16 harboring pTRKH2GAD was also higher than that of the untransformed *L. sakei* B2-16, strongly supporting the expression of *L. plantarum*-derived GAD (Fig. 3B). The similar studies were recently reported using rice-derived GAD and *Bifidobacterium longum* as the host strain [Park *et al*., 2005]. The same group developed the recombinant strain of *Bacillus subtilis* harboring *L. brevis*-derived GAD, which significantly enhanced the GABA production according to the increases of supplied MSG concentration [Park and Oh, 2006]. They also applied the recombinant *B. subtilis* to prepare Korean traditional fermented soybean product, *Chungkukjang*, with the enhanced level of GABA.

To investigate the possibility of scale-up application, the GABA production from recombinant *L. sakei* B2-16 was also attempted in a 5-L jar fermentor scale. The

enhancement rate of GABA production in fermentor scale was similar with that in flask scale, showing that the GABA production from recombinant *L. sakei* B2-16 was 265.3 mM, which was about 1.42-fold higher than that from untransformed *L. sakei* B2-16 (Fig. 4).

In this study, we genetically engineered the *L. sakei* B2-16 by introducing the *L. plantarum*-derived GAD gene to enhance GABA production. The expression of *L. plantarum*-derived GAD in *L. sakei* B2-16 contributed to the enhancement of GABA production. In addition, considering the fact that the LAB can serve as the starter for fermented foods, the recombinant *L. sakei* B2-16 harboring pTRKH2GAD appears to have a promising application in functional fermented food production as highly effective starter as well as enhanced-GABA producer, though the further study on the development and use of food-grade selectable markers such as aspartate aminotransferase of *L. lactis* and α-galactosidase of *B. longum* is need for the fermented food production using genetically engineered LAB system [Sridhar *et al*., 2006].

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