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Characterization of a Glutamate Decarboxylase (GAD) from *Enterococcus avium* M5 Isolated from Jeotgal, a Korean Fermented Seafood S

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To develop starters for the production of functional foods or materials, lactic acid bacteria producing γ-aminobutyric acid (GABA) were screened from jeotgals, Korean fermented seafoods. One isolate producing a high amount of GABA from monosodium L-glutamate (MSG) was identified as *Enterococcus avium* by 16S rRNA gene sequencing. *E. avium* M5 produced 18.47 ± 1.26 mg/ml GABA when incubated for 48 h at 37°C in MRS broth with MSG (3% (w/v)). A *gadB* gene encoding glutamate decarboxylase (GAD) was cloned and overexpressed in *E. coli* BL21 (DE3) using the pET26b (+) expression vector. Recombinant GAD was purified through a Ni-NTA column and the size was estimated to be 53 kDa by SDS-PAGE. Maximum GAD activity was observed at pH 4.5 and 55°C and the activity was dependent on pyridoxal 5′-phosphate. The K_m and V_{max} values of GAD were 3.26 ± 0.21 mM and 0.0120 ± 0.0001 mM/min, respectively, when MSG was used as a substrate. *Enterococcus avium* M5 secretes a lot of GABA when grown on MRS with MSG, and the strain is useful for the production of fermented foods containing a high amount of GABA.

Keywords: Gamma-aminobutyric acid, glutamate decarboxylase, *gad* gene cloning, *Enterococcus avium*, jeotgal

Introduction

γ-Aminobutyric acid (GABA), a major inhibitory neurotransmitter in the mammalian central nervous system, is known to possess several important physiological functions such as anti-diabetic, diuretic, anti-anxiety, and anti-hypertensive effects in humans [1–4]. Glutamate decarboxylase (GAD, EC 4.1.1.15) is a pyridoxal 5′-phosphate (PLP)-dependent enzyme, and plays an important role catalyzing the irreversible α-decarboxylation of L-glutamate to GABA [5]. GAD activities are widely distributed among plants, animals, and bacteria [6, 7]. Recently, efforts have been reported for the production of GABA by food-grade, safe microorganisms such as lactic acid bacteria (LAB). LAB, the most well-known GRAS (generally recognized as safe) organisms, have been widely used as starters for various fermented foods [8]. Some LAB produce GABA, and the GABA-producing ability varies among the species of LAB. Strains of *Lactobacillus brevis* are so far the most outstanding GABA producers [9]. Most studies have been done on the genera *Lactobacillus* and *Lactococcus*, but relatively few studies have been reported on other genera such as *Enterococcus*. Isolation of novel GABA-producing LAB is desirable for the enlargement of the list of LAB that could be used for the production of GABA.

In this study, a GABA-producing *Enterococcus avium* strain was isolated from jeotgals, Korean traditional fermented and salted seafoods. A *gadB* gene from *E. avium* M5 was cloned and overexpressed in *Escherichia coli* BL21(DE3). The recombinant GAD was purified and its properties were studied at detail.

Materials and Methods

Isolation of GABA-Producing LAB

GABA-producing LAB were isolated from jeotgals purchased at local markets in Jinju, Gyeongnam, Republic of Korea during the winter season in 2015. Jeotgals were homogenized with 0.1%

peptone water by using a stomacher 80 (Seward, UK). Homogenized samples were serially diluted with 0.1% peptone water, and spread on de Man–Rogosa–Sharpe (MRS; Difco, Becton Dickinson Co., USA) agar plates with 1% $CaCO_3$ and 0.006% bromocresol purple. After 48 h incubation at 30°C, yellow colonies with clear zones were picked up as putative LAB. GABA-producing strains were screened by thin-layer chromatography (TLC) [10]. Each isolate was inoculated into 1 ml of MRS broth with 3% (w/v) monosodium glutamate (MSG), and incubated at 30°C for 48 h. Cultures were centrifuged (12,000 ×*g*, 4°C, 10 min), and 1 µl of culture supernatant was spotted onto a TLC plate (Silica gel 60 F254; Merck Co., Germany). Separation was done with *n*-butanol/acetic acid/water (4:1:1 (v/v)). The plate was treated with 0.2% ninhydrin solution (w/v) and heated at 65°C for 15 min to visualize the spots.

Bacterial Strains and Culture Conditions

E. avium M5 was cultivated in MRS broth containing 3% (w/v) MSG at 37°C without aeration. *E. coli* DH5α and BL21 (DE3) were cultivated with aeration in Luria-Bertani (LB; peptone 10 g, yeast extract 5 g, sodium chloride 5 g/l, pH 7.0) broth at 37°C. pET 26b (+) (Novagen, USA) was used for overexpression of the *gadB* gene from *E. avium* M5 in *E. coli* BL21(DE3). For cultivation of cells harboring pET26b(+) or its derivative, kanamycin was included in the medium (60 µg/ml).

Identification of GABA-Producing LAB

A GABA-producing isolate was identified using the API 50CHL kit (bioMerieux, France) and 16S rRNA gene sequencing. 16S rRNA genes were amplified by PCR using an universal primer set: 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The PCR was done using a MJ Mini thermal cycler (BioRad, USA) as follows: denaturation at 94°C for 5 min, 30 cycles of 30 sec at 94°C, 30 sec at 55°C, and 2 min at 72°C. PCR products were examined by agarose gel electrophoresis and purified using a PCR purification kit (Favorgen, Taiwan). Nucleotide sequences were determined at Cosmogenetech (Korea) and homology search was done using the Basic Local Alignment Search Tool (BLAST) and GenBank database (http://blast.ncbi.nlm.nih.gov). Phylogenetic analyses were performed using MEGA 5.05 [11] and a phylogenetic tree was inferred based on the neighbor-joining method [12].

Growth Properties of *E. avium* M5

E. avium M5 was grown in MRS broth for 18 h at 30°C without shaking and inoculated (1% (v/v)) into 50 ml of fresh MRS broth with different growth conditions: initial pH (pH 2–10), temperature (4°C, 10°C, 20°C, 30°C, 37°C, and 45°C), and NaCl concentration (3%, 5%, 7%, 10%, and 15% (w/v)). Cultures were incubated for 72 h and the OD_{600} values were measured during cultivation.

Cloning of *gadB* and *gadC* Genes from *E. avium* M5

A *gadB* gene was amplified from the *E. avium* M5 genome by using a primer set based on the *gadB* sequence from *E. avium* G-15 [13]: gadB-F (5′-GGGCATATGTTATATGGAAA AGAA-3′, *Nde*I site underlined) and gadB-R (5′-GGGCTCGAGATGCGTAAATCC GTA-3′, *Xho*I site underlined). A *gadC* gene was amplified by using a primer set based on *gadC* from *E. avium* G-15 [13]: gadC-F (5′-GGGCATATGAATCAGAAAAAATTATC–3′, *Nde*I site underlined) and gadC-R (5′-GGGCTCGAGATGTTTCAATGT GTG–3′, *Xho*I site underlined). Amplified PCR products were purified and ligated with the pGEM T-easy vector (Promega, USA). *E. coli* DH5α cells were transformed with the ligation mixture by electroporation.

Overexpression of *gadB* in *E. coli* BL21 (DE3) and Purification of GAD

A *gadB* gene was cloned into a pET26b(+) expression vector after being digested with *Nde*I and *Xho*I. The ligation mixture was introduced into *E. coli* BL21(DE3) competent cells. An *E. coli* BL21 (DE3) transformant harboring the recombinant plasmid was grown in 100 ml of LB broth containing kanamycin at 37°C until the OD_{600} value reached 0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to 1 mM concentration and growth continued for 14 h at 20°C. Cells were harvested by centrifugation (12,000 ×*g*, 20 min, 4°C), washed three times with phosphate-buffered saline (PBS, pH 7.4), and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 7.0). Cells were disrupted by using an ultrasonicator (Bandelin Electronic, Germany). The disrupted cells were centrifuged at 12,000 ×*g* for 20 min, and the pellet (insoluble fraction) and supernatant (soluble fraction) were obtained. The insoluble fraction was loaded onto a Ni-NTA column (GE Healthcare, Sweden) after being resuspended in lysis buffer. GAD was eluted by stepwise increase of the imidazole concentration (10–500 mM) of elution buffer. SDS-PAGE was carried out using a 12% (w/v) acrylamide gel. Protein concentration was determined by the Bradford method with bovine serum albumin as a standard.

Enzyme Assay of Recombinant GAD

The activity of recombinant GAD was measured by a method previously reported [14]. The enzyme solution (1 µg GAD in 0.1 ml of lysis buffer) and 0.1 ml of 4 M ammonium sulfate were mixed. After 30 min of pre-incubation, the enzyme solution was mixed with 1.3 ml of substrate (20 mM MSG, 0.2 mM PLP, 0.2 M pyridine/HCl, pH 4.5), and incubated for 1 h. The reaction was stopped by boiling for 5 min, and the amount of GABA was analyzed by GABase assay as described previously [10]. One unit of GAD activity was defined as the amount of enzyme producing 1 µmol GABA per minute under the experimental conditions.

Properties of Recombinant GAD

The effect of pH on the GAD activity was examined. Purified GAD was incubated for 1 h at 45°C at different pH values (pH 3–10) and then the remaining activities were measured by GABase assay. Buffers of 100 mM concentration were used: pyridine-HCl (pH 3–5.5), sodium phosphate (pH 6–8), and glycine–NaOH (pH 9-10).

The effect of temperature was determined after a 30 min incubation at different temperatures (20–60°C, pH 5.5). The effects of PLP concentration (0–1.8 mM) and chemicals (2 mM) were determined after a 30 min incubation at 45°C (pH 5.5). The effect of MSG concentration (0.2–10 mM) was investigated after a 30 min incubation at 45°C (pH 5.5). The kinetic parameters of recombinant GAD were determined at 45°C and pH 5.5 (0.5 M potassium pyrophosphate buffer) with MSG (1–100 mM) as the substrate.

Results and Discussion

Isolation and Identification of GABA-Producing LAB

A total of 1,000 putative LAB were isolated from jeotgal samples (anchovy and shrimp jeotgals) and screened for GABA production by TLC (Fig. 1). An isolate, M5, produced GABA profusely, as comparable to *Lb. zymae* GU240, a strain isolated from kimchi and producing GABA in a large quantity [10]. M5 was a non-spore-forming, gram-positive, and non-motile coccus. M5 was identified by API 50 CHL kit and 16S rRNA gene sequencing. The carbohydrate utilization pattern of M5 was similar to that of *Lactobacillus plantarum* (98.6%) but 16S rRNA gene sequence (1,458 nucleotides, KX789690) showed 99% identity with those of *Enterococcus avium* strains such as JCM 8722, NBRC 100477, and ATCC14028. When a phylogenetic tree was constructed from 16S rRNA genes from M5 and other *E. avium* strains, M5 belonged to *E. avium* (Fig. 2). From these results, M5 was identified as *Enterococcus avium*.

E. avium M5 produced GABA at the concentration of 13.85 ± 1.13 mg/ml culture supernatant when grown on MRS with MSG (3% (w/v)) for 48 h as determined by GABase assay. The conversion yield of MSG into GABA was 75.12%. The GABA yield of M5 was much higher than that of *Leuconostoc mesenteroides* ATCC10830 (0.08 ± 0.02 mg/ml), a negative control, but lower than that of *Lb. zymae* GU240 (16.94 ± 1.24 mg/ml).

GABA-producing LAB have been isolated from various fermented foods such as *Lactococcus lactis* from cheeses [15], *Lb. brevis* IFO-12005 from alcohol distillery lees [16], *Lb. paracasei* and *Lb. plantarum* from cheeses [17], *Lb. sakei* A156 from jeotgal [18], *Lb sakei* B2-16 from kimchi [19], and *Lb. zymae* GU240 from kimchi [10]. Most GABA producers belong to the genus *Lactobacillus*, and a few isolates belong to other genera such as *Enterococcus* and *Streptococcus*. This might be due to naturally higher GABA production abilities of *Lactobacillus* species compared with other members of LAB.

E. avium G-15 produced 77.1 ± 6.4 g/l GABA (conversion ratio, 86.0 ± 5.0%) from L-MSG at 70 h cultivation in a jar fermentor, where L-MSG (200 g/l in total) was continuously fed between 22 and 52 h [13]. Although this GABA yield is higher than other reports, including ours, the production

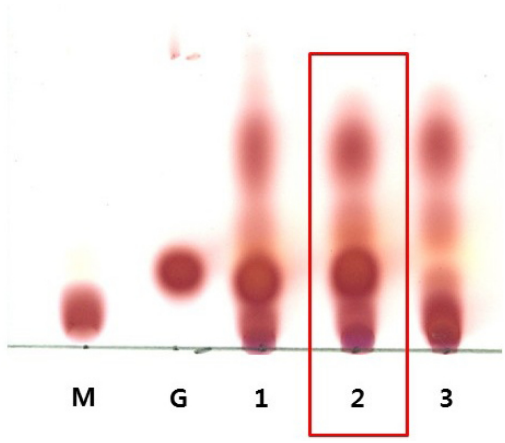

Fig. 1. Thin-layer chromatogram showing GABA production of *E. avium* M5.

M, 1 µl of 0.1 mM monosodium glutamate; G, 1 µl of 0.1 mM γ-aminobutyric acid; 1, *Lb. zymae* GU240 (positive control); 2, *E. avium* M5; 3, *Leu. mesenteroides* ATCC10830 (negative control).

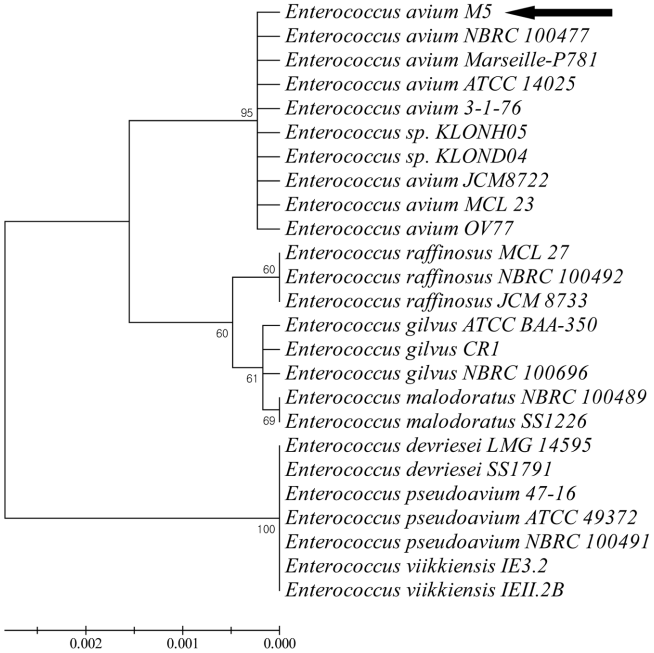

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences from strain M5 and related species.

Numbers at nodes indicate bootstrap values (greater than 70%) expressed as a percentage of 1,000 replicates. Scale bar, 0.0005, represents base substitutions per site.

conditions used can not be applied for food fermentations. *E. faecium* JK29 produced 1.56 mM GABA at 48 h cultivation in MRS broth with 1% L-MSG, and the amount of GABA increased to 14.86 mM (conversion ratio, 50.26%) when modified MRS containing 0.5% (w/v) sucrose, 2% (w/v) yeast extract, and 0.5% (w/v) MSG was used [20]. The GABA production yield of *E. avium* M5 (134.3 mM) was much higher than that of *E. faecium* JK29, and could be increased further if optimization efforts are made. In this respect, *E. avium* M5 deserves to be used as a production host for GABA.

Growth Properties of *E. avium* M5

E. avium M5 grew rapidly at 30°C and 37°C, reaching an OD_{600} value of 1.11 and 1.16, respectively, in 24 h, and 1.45 and 1.48, respectively, in 72 h (Fig. 3A). *E. avium* M5 grew at 45°C, and reached an OD_{600} value of 1.00 and 1.45 in 24 h and 72 h, respectively, but did not grow at 4°C and 10°C in 96 h. *E. avium* M5 grew rapidly at initial pH of 4–8 and reached OD_{600} values of 0.9–1.2 in 24 h (Fig. 3B). *E. avium* M5 grew slowly at initial pH of 3.0 and 9.0, but did not grow at initial pH of 2.0 and 10.0. In MRS broth with 3% (w/v) NaCl, *E. avium* M5 grew to an OD_{600} value of 0.63 in 12 h, and 1.22 in 72 h. It grew slowly at 5% NaCl, but did not grow at 10% and 15% NaCl (Fig. 3C). *E. avium* M5 has a moderate degree of NaCl tolerance. Some types of jeotgals have relatively low NaCl contents (around 5%) whereas other types have more than 25% NaCl contents. Considering these results, *E. avium* M5 might be used as a starter for low-salt jeotgals.

Cloning of *gadB* and *gadC* Genes from *E. avium* M5

A *gadB* gene was amplified by PCR and sequenced (Fig. S1). An ORF of 1,401 nucleotides was located, which could encode a protein of 466 amino acids with calculated size of 53,755.34 Da and isoelectric point (pI) of 5.00. The nucleotide sequence of *gadB* from *E. avium* M5 showed 99% similarity to that of *gadB* from *E. avium* G-15. Among the 1,401 nucleotides, six were different from those of *E. avium* G-15: G142A, C456G, C468T, G897A, G1209A, and C1347T (nucleotide of *E. avium* M5 was shown first). When the translated amino acid sequence of GAD was aligned with those of other GADs, GAD from *E. avium* M5 showed the highest similarity to GAD from *E. avium* G-15 (AB548685.1) (100%), followed by GAD from *E. malodoratus* ATCC43197 (WP_010743125) (99%), *E. hirae* 877_EHIR (WP_048720427) (98%), *E. raffinosus* ATCC49464 (WP_010746838) (96%), *E. avium* ATCC14025 (AB548685.1) (95%), and *Lactococcus lactis* subsp. *lactis* M20 (WP_058211962) (91%).

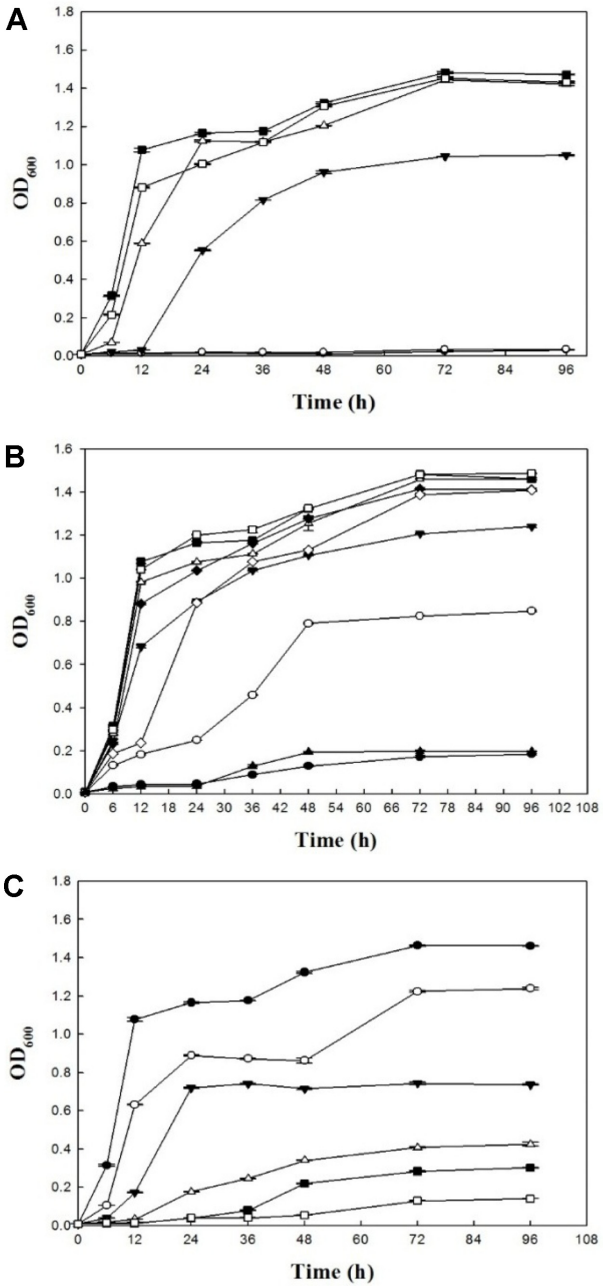

Fig. 3. Growth of *E. avium* M5 on MRS broth under different conditions.

Absorbance of each culture was measured at 600 nm and each value represents the mean value from three independent measurements. (**A**) temperature: ●, 4°C; ○, 10°C; ▼, 20°C; △, 30°C; ■, 37°C; □, 45°C. (**B**) pH: ●, pH 2; ○, pH 3; ▼, pH 4; △, pH 5; ■, pH 6; □, pH 7; ◆, pH 8; ◇, pH 9; ▲, pH 10. (**C**) NaCl concentration: ●, 0%; ○, 3%; ▼, 5%; △, 7%; ■, 10%; □, 15%.

A *gadC* gene was amplified and sequenced (Fig. S1). An ORF of 1,512 nucleotides was located that could encode a

protein of 502 amino acids. The nucleotide sequence of *gadC* from *E. avium* M5 showed 99% similarity to *gadC* from *E. avium* G-15. Among the 1,512 nucleotides, eight were different from those of *E. avium* G-15: C81A, C87T, A447T, C828T, A1044G, G1182A, C1188T, and G1356A (nucleotide of *E. avium* M5 was shown first). When the translated amino acid sequence of GadC was aligned with those of homologous proteins, GadC from *E. avium* M5 showed the highest similarity to GadC from *E. avium* G-15 (AB548685.1) (100%) followed by GadCs from *E. malodoratus* ATCC43197 (WP_010743125) (99%), *E. hirae* 877_EHIR (WP_048720427), *E. avium* ATCC14025 (AB548685.1) (95%), *E. raffinosus* ATCC49464 (WP_010746838) (94%), and *Lactococcus lactis* subsp. *lactis* M20 (WP_058211962) (88%). Putative promoter sequences consisting of TTCCCG (-35 region) and AATAAT (-10 region) were located upstream of *gadC*. A putative ribosome-binding site was present upstream of the *gadC* start codon. The intervening sequence between *gadC* and *gadB* is just 29 nucleotides in length, which is indirect evidence for an operon structure of *gadCB*. The same operon structure was observed in *Lb. zymae* GU240 [10] and *Lb. sakei* A156 [18].

GAD requires PLP for its activity [21]. The GAD of *E. avium* M5 contains a lysine residue essential for the PLP binding site (S124, S125, C128, I209, D244, S247, S274, K277), designated as PLP lysine. The nucleotide sequences of *gadB* and *gadC* from *E. avium* M5 were deposited to GenBank under the accession numbers KX553898 and KX553899, respectively.

Overexpression of *gadB* in *E. coli* BL21(DE3) and Purification of GAD

The *gadB* gene from *E. avium* M5 was overexpressed in *E. coli* BL21(DE3) and a 55 kDa protein was overproduced from IPTG-induced cells (Fig. 4). A control, *E. coli* BL21 (DE3) harboring an intact pET26b(+), did not produce the protein (results not shown). Recombinant GAD was observed from both soluble and insoluble fractions of cell extracts. The insoluble fraction was used as the sample for GAD purification because it showed stronger band intensity (results not shown). Recombinant GAD was produced as a His-tagged fusion protein to facilitate its purification, and eluted from a Ni-NTA column at the imidazole concentration of 100 mM and more. Sufficiently purified GAD was obtained, and the size was 55 kDa on a SDS gel, matching well with the expected size of a fusion protein (Fig. 4).

Properties of Recombinant GAD

The optimal pH for recombinant GAD was 5.5 (Fig. 5).

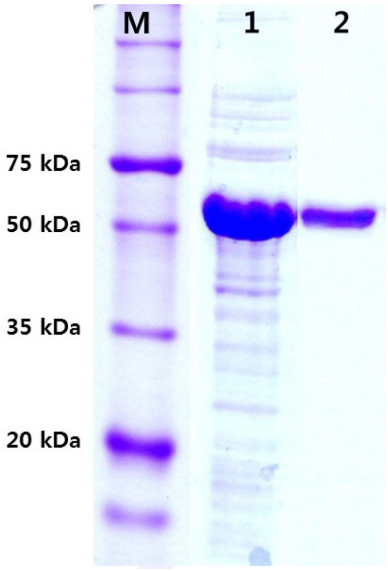

Fig. 4. SDS-PAGE of recombinant glutamate decarboxylase (GAD).

M, size marker (DokDo-Mark; Eplis Biotech, Korea); 1, insoluble fraction from *E. coli* BL21 (DE3) harboring pETM5 cultivated overnight at 20°C after IPTG induction; 2, purified GAD using a Ni-NTA column. A 12% (w/v) acrylamide gel was used and stained with Brilliant Blue R.

Most GADs are active at pH 4–5 as reported for GAD from *E. coli* [22], *Lactococcus lactis* (pH 4.7) [15], *Lb. brevis* CGMCC1306 (pH 4.8) [23], *Lb. paracasei* NFRI7415 (pH 5.0) [24], *Lb. sakei* A156 (pH 5.0) [18], *Lb. zymae* GU240 (pH 4.5) [10], and *S. thermophiles* Y2 (pH 4.2) [25]. The optimal pH was 4.4 for GAD from *E. avium* G-15 [13] and 6.0 for GAD from *E. avium* 9184 [26]. The optimal pH of GAD from *E. avium* M5 was different from those of GADs from other *E. avium* strains. However, both previous reports did not present activity measurement results using purified GADs, but rather showed optimal pH for GABA production by culture. GADs are believed to be involved in maintaining cellular pH under acidic conditions and this role is important for LAB [10]. The optimal temperature for recombinant GAD was 55°C. It was higher than that of GAD from *Lb. zymae* GU240 (41°C), but the same as that of GADs from *Lb. sakei* A156 and *S. thermophiles* Y2.

Recombinant GAD from *E. avium* M5 depends on PLP for its activity (Fig. 5C). It was inactive without PLP, and the activity increased as PLP concentration increased. The highest activity was observed at 0.8 mM PLP, but the degree of increase was small at PLP concentrations above 0.1 mM. The effect of chemicals (2 mM) on GAD activity was examined (Table 1). The activity was increased slightly

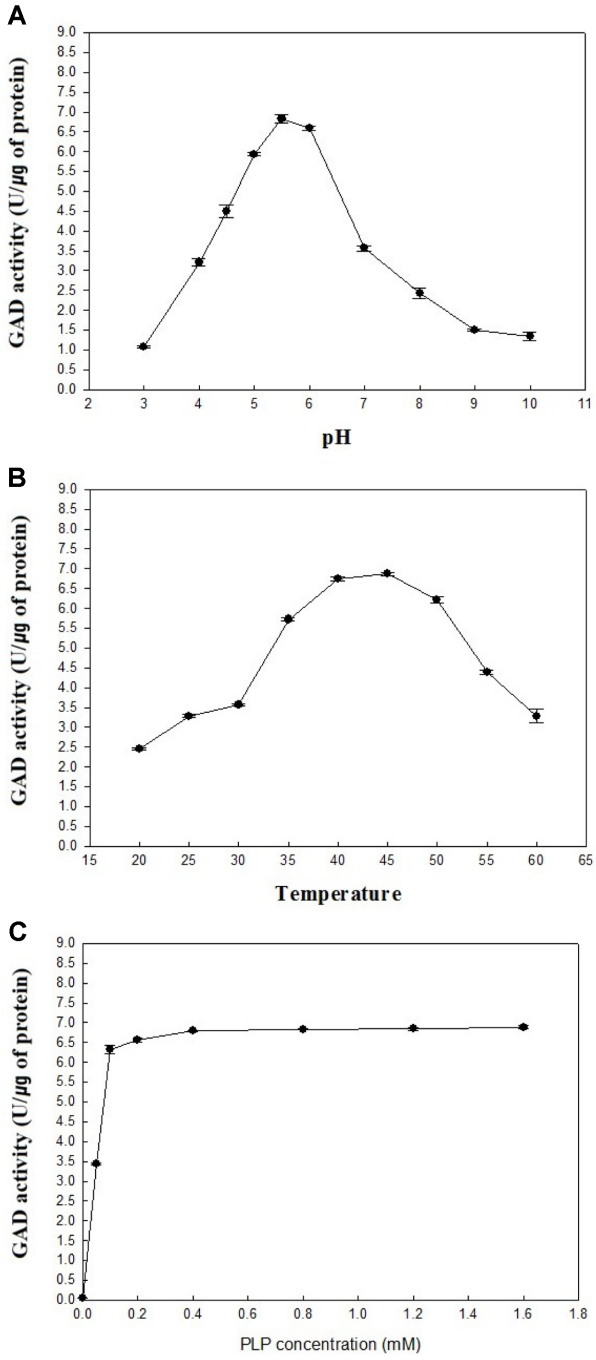

Fig. 5. Optimal pH, temperature, and pyridoxal 5′-phosphate (PLP) concentration effects on glutamate decarboxylase (GAD) activity.

(**A**) pH, (**B**) temperature, and (**C**) PLP concentration. The activity of recombinant GAD was measured at pH 3–10 by GABase assay. To determine the optimal temperature, recombinant GAD was incubated at 20–60°C for 30 min at pH 5.5. The effect of PLP concentration (0–1.8 mM) was determined at 45°C and pH 5.5. One unit of GAD activity was defined as the amount of enzyme producing 1 μmol GABA/min.

Table 1. Effects of chemicals on the activity of glutamate decarboxylase (GAD).

Chemical (2 mM)	Remaining activity (%)
Control	100
$CaCl_2$	124.3 ± 2.1
$CoCl_2$	98.4 ± 3.4
$CuSO_4$	56.8 ± 2.4
$FeCl_3$	99.2 ± 1.6
KCl	97.8 ± 2.1
$MgCl_2$	102.3 ± 1.9
$MnCl_2$	116.4 ± 1.4
$ZnCl_2$	104.1 ± 2.8
$AgNO_3$	44.4 ± 1.2

by $CaCl_2$ (124.3 ± 2.1%) and $MnCl_2$ (116.4 ± 1.4%), but decreased significantly by $CuSO_4$ (56.8 ± 2.4%) and $AgNO_3$ (44.4 ± 1.2%). The results were similar to other reports. In the presence of 2 mM $CaCl_2$, the activity of GAD increased slightly in *Lb. paracasei* NFRI 7415 (114%) [24], *Lb. zymae* GU240 (121%) [10], and *Lb. sakei* A156 (113%) [18].

Kinetic parameters of recombinant GAD were determined by Lineweaver-Burk plot. The K_m of GAD was 3.26 ± 0.21 mM and V_{max} was 0.012 ± 0.0001 mM/min. The K_m of *E. avium* M5 was higher than that of GADs from *L. lactis* [27] and *S. thermophiles* Y2 [25] but lower than that of *Lb. brevis* CGMCC1306 [23] and *Lb. brevis* IFO-12005 [16]. The K_m of *Lb. zymae* GU240 GAD was 1.7 ± 0.05 mM [10] and that of *Lb. sakei* A156 GAD was 16.0 ± 0.05 mM [18]. Therefore, GAD from *E. avium* M5 has higher affinity for MSG than that of *Lb. brevis* strains and *Lb. sakei* A156.

In summary, a GABA-producing *E. avium* M5 was isolated from jeotgals and the strain produced GABA in a large quantity. Since few *Enterococcus* strains have been reported to produce GABA, *E. avium* M5 and its GAD enzyme have the potential for use in food industry and biotechnology applications by enlarging the pool of hosts and GADs used for GABA production. *E. avium* M5 can also be used as a starter for low-salt jeotgal products as long as its safety is confirmed through various tests.

Acknowledgments

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