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Research Note

Production of γ-Aminobutyric Acid by *Lactobacillus brevis* 340G Isolated from *Kimchi* and Its Application to Skim Milk

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Abstract

The *Lactobacillus brevis* 340G strain isolated from traditional Korean fermented food (*kimchi*) produced 15.50 mM of γ-aminobutyric acid (GABA) after 48 h of cultivation in MRS media containing 1% L-monosodium glutamate (MSG). The culture conditions of *Lb. brevis* 340G were optimized for GABA production. *Lb. brevis* 340G was cultivated at 30°C in optimized MRS media containing 3% sucrose and 2% yeast extract with 3% MSG, resulting in maximum GABA production (68.77 mM) after 54 h of cultivation. Skim milk fermented with *Lb. brevis* 340G produced 4.64 mM of GABA in the presence of 1% MSG. These results suggest that *Lb. brevis* 340G could be used as a starter for functional fermented foods and skim milk fermented with *Lb. brevis* 340G could be further developed to become functional dairy food fortified with GABA.

Key words: γ-aminobutyric acid, *kimchi*, *Lactobacillus brevis*, skim milk

Introduction

γ-Aminobutyric acid (GABA) is a non-protein amino acid that is widely distributed in nature (Manyam et al., 1981). GABA is produced primarily by the irreversible α-decarboxylation of acidic glutamate in the reaction catalyzed by the glutamate decarboxylase (GAD; EC 4.1.1.15), where an incorporation of hydrogen ion (H^+) ions might confer resistance to acidic pH (Castanie-Cornet et al., 1999). GABA plays an important role as the major inhibitory neurotransmitter in the mammalian central nervous system. It has a variety of physiological functions such as the induction of hypertensive, diuretic, anti-depression, and tranquilizing effects. In addition, it has been implicated in the regulation of several neurological disorders including epilepsy, convulsions, Parkinson's disease, Alzheimer's disease, and Huntington's chorea (Manyam et al., 1981; Jakobs et al., 1993; Inoue et al., 2003). Due to its physiological functions, the interest of GABA has been recently increased as bioactive compound. This increasing commercial demand of GABA has prompted the studies on the development of GABA-fortified foods such as soybean, yogurt, and black raspberry (Park & Oh, 2007; Kim et al., 2009).

However, because the plant-derived GABA is not always present in high quantities, there have been reported the studies on GABA production from various microorganisms including yeast and fungi as well as bacteria (Hao & Schmit, 1993; Masuda et al., 2008). In this study, we focused on the lactic acid bacteria (LAB) as GABA-producer due to its commercial potential as a starter for fermented foods. LAB has been widely used not only for fermented milk products such as yogurt, butter and cheese, but also for traditional fermented foods such as *kimchi* and *tarhana* (Erdoğrul & Erbilir, 2006; Park & Oh, 2006). In addition, LAB has been known to improve the stability of fermented foods during their storage by its probiotic potential, which may contribute to produce more natural safe foods without using chemical additives (Naidu et al., 1999). Due to these potent activities of LAB, a variety of LAB have been recently screened to isolate GABA-producing LAB, which have been then reported, including *Lactobacillus brevis* isolated from alcohol distillery lees (Yokoyama et al., 2002) and *Lactococcus lactis* from cheese starters (Nomura et al., 1998). Especially, focusing on the traditional Korean fermented food, *kimchi*, the isolation of

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GABA-producing LAB have been more recently reported in Korea, including *Lb. buchneri* and *Lb. brevis* (Cho et al., 2007; Kim et al., 2009). However, it is still needed to study on the production and activity of bioactive compounds from *kimchi*-derived LAB, since *kimchi* has various kinds and diverse LAB depending on the materials and methods for its production.

Recently, we isolated *Lb. brevis* 340G as a GABA-producing LAB from the traditional Korean fermented food, *kimchi*. This study describes a set of optimal culture conditions of *Lb. brevis* 340G for high GABA production by determining the optimal carbon and nitrogen sources, culture temperature, and exogenous L-monosodium glutamate (MSG) concentration. Additionally, the application of *Lb. brevis* 340G as a starter in skim milk fermentation was investigated for the development of functional diary food.

Materials and Methods

Culture conditions and fermentation of skim milk

The seed culture of *Lb. brevis* 340G cultivated in the basal MRS media (1% proteose peptone No.3, 1% beef extract, 0.5% yeast extract, 2% dextrose, 0.1% polysorbate 80, 0.2% ammonium citrate, 0.5 sodium acetate, 0.01% magnesium sulfate, 0.005% manganese sulfate, 0.2% dipotassium phosphate, w/v) without shaking for 18 h at 30°C was transferred with 1% inoculums to a 250 ml of main flask containing 100 ml working volume of MRS medium with 1% MSG and then cultured without shaking for 60 h at 30°C. To investigate the effects of carbon sources on GABA production by *Lb. brevis* 340G, various carbon sources (glucose, lactose, sucrose, xylose, fructose, maltose, galactose, and arabinose) were added at 2% to the modified MRS media eliminated with 2% dextrose. Additionally, various nitrogen sources (beef extract, tryptone, soytone, yeast extract, peptone, casitone, casamino acid, and proteose peptone No. 3) were added at 2.5% to the modified MRS media that all nitrogen sources in the basal MRS media (proteose peptone No. 3, beef extract and yeast extract) are eliminated.

To prepare the fermented skim milk, the *Lb. brevis* 340G cultivated in the MRS media for 24 h was transferred with 1% inoculums to a 250 ml flask containing 100 mL of 10% skim milk (Seoul milk, Republic of Korea) and 1% MSG and then fermented for 48 h at 30°C. The culture broth was centrifuged at 13,000 rpm for 10 min to prepare the supernatant for the identification and quantification of GABA.

Identifications of GABA

The produced GABA was identified by ultra performance liquid chromatography-electronspray ionization-quadrupole-time of flight-mass spectrometry (UPLC-ESI-Q-TOF-MS), as described in a previous study with slight modifications (Buck et al., 2009). An Agilent 1290 Infinity UPLC system was employed with a hydrophilic interaction chromatography (HILIC) column (ZORBAX HILIC Plus, 2.1×100 mm, 3.5 μm, Agilent Technologies, USA). The elution was carried out at a flow rate of 0.3 ml/min using the following solvent A (5 mM ammonium acetate in water)/solvent B (acetonitrile with 0.1% formic acid) gradient: 0 min (90% B), 0.1 min (90% B), 9.9 min (60% B), 1 min (100% B), 2 min (100% B), 1 min (90% B), and 6 min (90% B). The elutes were detected using an Agilent 6250 Q-TOF mass spectrophotometer (Agilent Technologies, USA) with a gas (N_2) flow rate of 8 L/min, gas temperature of 325°C, fragmentor voltage of 70 V, and capillary voltage of 4,000 V.

Quantitative determination of GABA

To determine GABA concentration, a spectrophotometric assay was employed using GABase enzyme (Sigma, USA) (Zhang & Brown, 1997) with a microplate reader (BMG Labtech, Germany). Each of the individual wells of the microplate contained 100 mM $K_4P_2O_7$ buffer (pH 8.6, 234 μL), 10 mM $NADP^+$ (42 μL), and 5 unit/mL-GABase (5 μL). The culture supernatant (12 μL) was then loaded into the individual wells and the initial absorbance was read at 340 nm. After adding 100 mM α-ketoglutarate (6 μL), the microplate was incubated for 60 min at room temperature and the final absorbance was then read again. Finally, the produced GABA concentration was determined by the difference in the two A_{340} values.

Results and Discussion

Cell growth and GABA production of *Lb. brevis* 340G

The cell growth of *Lb. brevis* 340G reached stationary phase after 18 h of cultivation in MRS media containing 1% MSG and the GABA production significantly increased from 9 h of cultivation, resulting in the highest GABA production of 15.50 mM after 48 h of cultivation (Fig. 1). The GABA production by *Lb. brevis* 340G was identified by UPLC-ESI-Q-TOF-MS analysis where the culture supernatant showed a peak with *m/z* for $[M+H]^+$ of 104.07 with positive ion mode in an extracted ion chromatogram at a retention time of 5.1 min,

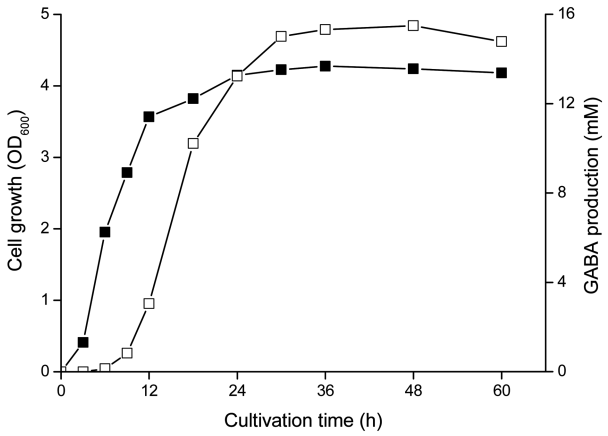

Fig. 1. Time profiles of cell growth (■) and GABA production (□) by *Lb. brevis* 340G with 1% MSG.

which was identical to the authentic GABA standard ($C_4H_9NO_2$, molecular weight 103.12) (Fig. 2).

Determination of carbon and nitrogen sources for GABA production by *Lb. brevis* 340G

The effect of various carbon sources on GABA production by *Lb. brevis* 340G was tested in the modified MRS media containing 2% carbon source and 1% MSG. Sucrose, fructose, and glucose were suitable carbon sources for the cell growth. However, the sucrose showed the highest effect on GABA production (23.64 mM), followed by fructose and lactose (Fig. 3A). After using different sucrose concentrations, the highest GABA production (25.23 mM) was found with 3% sucrose (data not shown).

To determine the effect of the nitrogen sources on the GABA production, all nitrogen sources (proteose peptone No. 3, beef extract, and yeast extract) were eliminated in the basal MRS media, where 3% sucrose replaced 2% glucose, and various 2.5% nitrogen sources were then added. Fairly low levels of GABA were detected with all of each nitrogen source except the yeast extract which had the similar effect on GABA production with the control mixture of three nitrogen sources (Fig. 3B). After the investigation of yeast extract concentration on GABA production, its optimized concentration was determined to be 2%, producing the 25.67 mM GABA (data not shown).

Effect of culture temperature on GABA production by *Lb. brevis* 340G

The investigation of effect of culture temperature on the GABA production exhibited that the GABA production was

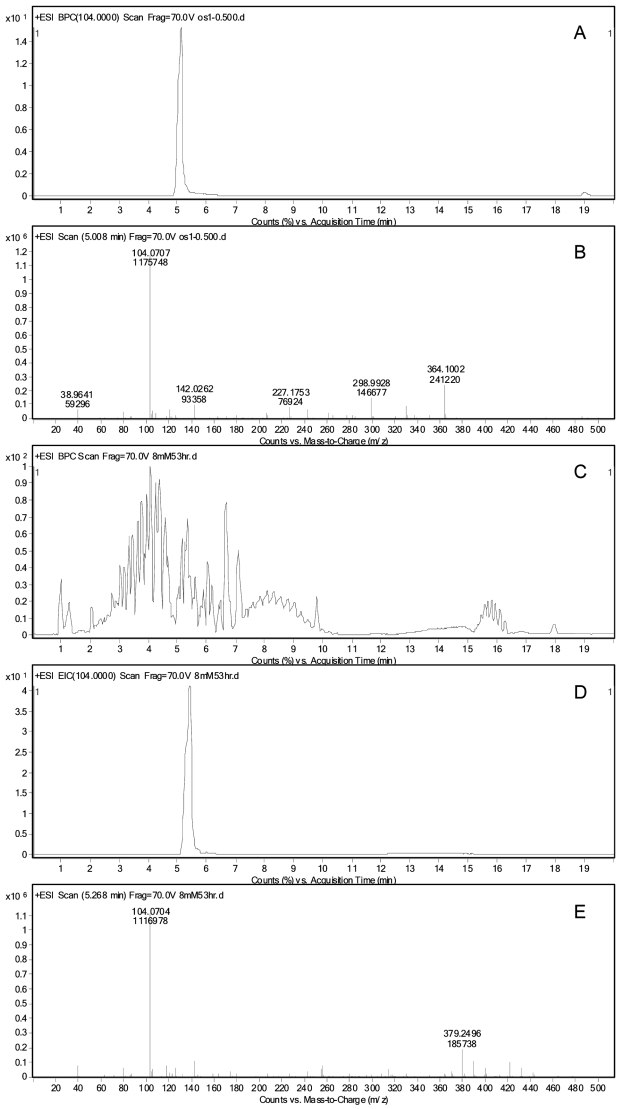

Fig. 2. UPLC-ESI-MS-Q-TOF-MS analysis of the authentic GABA standard (A, B) and the culture supernatant of *Lb. brevis* 340G (C, D, and E). Panels: A and C, total ion chromatogram; D, extracted ion chromatogram with *m/z* for $[M+H]^+$ of 104; B and E, MS spectra at the corresponding peak.

enhanced as the temperature was increased from 25°C to 30°C, showing that the maximum GABA production of 26.12 mM was obtained after 48 h of cultivation at 30°C (Fig. 4). However, both of cell growth and GABA production were lowest at 35°C. Here, it should be mentioned that the GABA production increased drastically from 9 h to 30 h and reached the maximum level at 48 h, while the cell growth reached the stationary phase after 18 h. The increase of GABA production at the stationary phase of cell growth could be explained by the activation of GAD for the generation of GABA from MSG, with consuming

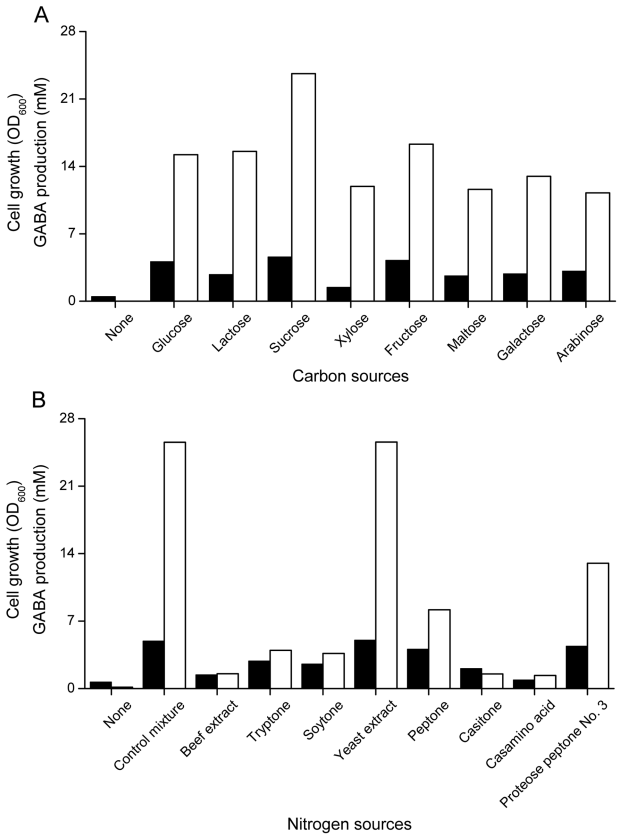

Fig. 3. Effects of various carbon (A) and nitrogen (B) sources on cell growth (closed bars) and GABA production (opened bars) of *Lb. brevis* 340G. Control mixture contains 1% proteose peptone No. 3, 1% beef extract and 0.5% yeast extract.

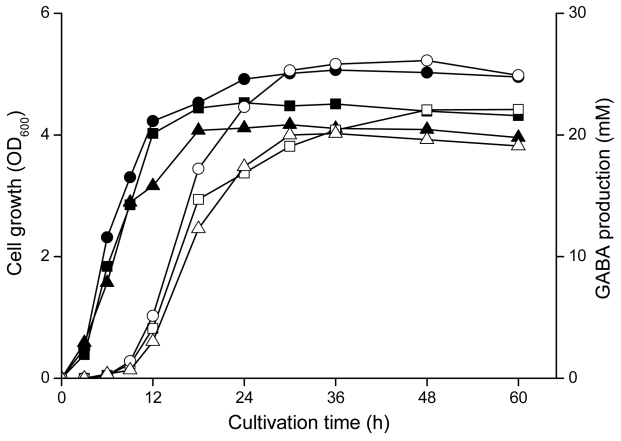

Fig. 4. The cell growth (closed symbols) and GABA production (opened symbols) of *Lb. brevis* 340G cultivated at 25°C (squares), 30°C (circles) and 35°C (triangles). *Lb. brevis* 340G was cultured in the modified MRS media optimized with 3% sucrose and 2% yeast extract supplemented with 1% MSG.

hydrogen ion (H^+) ion mediated by acidic environments at the stationary phase (Castanie-Cornet et al., 1999).

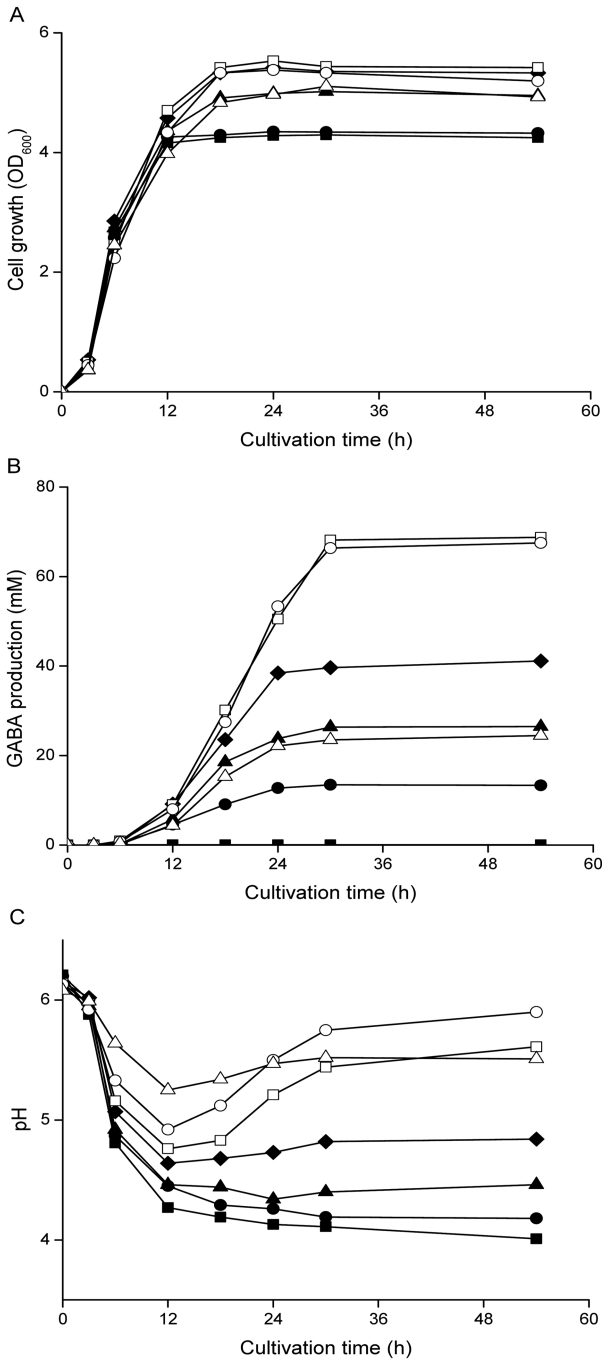

Fig. 5. Time profiles of cell growth (A), GABA production (B) and pH (C) of *Lb.brevis* 340G cultivated with adding various concentrations of exogenous MSG: 0% (■), 0.5% (●), 1% (▲), 2% (◆), 3% (□), 5% (○), and 10% (△).

Effect of MSG concentration on GABA production by *Lb. brevis* 340G

The optimal MSG concentration for GABA production was determined by cultivating *Lb. brevis* 340G in the optimized MRS media with exogenous MSG of various concentrations at

30°C. The detail cell growth was shown to be highest when adding 2-5% MSG, though there were no significant changes in the cell growth (Fig. 5A). The GABA production was enhanced with increasing the MSG concentration up to 3%, showing that the GABA production reached around 68.77 mM after cultivation of 54 h in the optimized MRS media containing 3% MSG (Fig. 5B). However, the 5% MSG had the similar effect on GABA production with 3% MSG and the 10% MSG even inhibited the GABA production, suggesting that the MSG concentration over 3% had negative effect on GABA production in terms of the GABA conversion yield which is determined by the ratio of the produced GABA concentration over the supplied MSG concentration. Therefore, the 3% MSG was determined to be optimal for GABA production by *Lb. brevis* 340G. The pH profiles in the cases of the additions of 3% and 5% MSG were interesting, because the pH increased slightly after cultivation of 12 h when the GABA production started (Fig. 5C). In fact, LAB has a tendency of pH decreases by its acid-producing ability. However, the slight increase of pH in our study could be due to the alkaline GABA, which is synthesized from acidic MSG by GAD in cytoplasm and then exported into the culture medium via an antiporter.

GABA production in skim milk fermented with *Lb. brevis* 340G

To investigate the developmental possibility of functional fermented food, *Lb. brevis* 340G was cultivated in skim milk with 1% MSG. The GABA production of the fermented skim milk was 4.64 mM with adding 1% MSG after 48 of fermentation. However, the fermented skim milk without MSG produced a tiny amount of GABA (0.77 mM) because the amount of free glutamate in skim milk is not enough to significantly produce GABA. It was similar with the previous study showing that the skim milk with *Streptococcus thermophilus* ST110 produced a trace amount of GABA (0.03 mM) without MSG addition and even 0.65 mM GABA with adding 100 mM MSG (Somkuti et al., 2012). The low conversion yield to GABA and limited contents of free glutamate in skim milk could be addressed by employing the high protease or peptidase-producing microorganisms capable to hydrolyze a milk protein to free glutamate. Therefore, the GABA-producing microorganism has been co-cultured with protease-producing microorganism in skim milk to enhance the GABA production in milk products (Inoue et al., 2003).

In summary, the culture conditions of *Lb. brevis* 340G isolated from traditional Korean fermented food, *kimchi* was optimized for the GABA production. The highest GABA production (68.77 mM) was detected after cultivation in the optimized MRS media containing 3% sucrose and 2% yeast extract with 3% exogenous MSG at 30°C. Additionally, *Lb. brevis* 340G was cultured in the skim milk which contained the 4.64 mM GABA under 1% MSG. Recently there have been several studies reporting the production and optimization of GABA from *Lb. brevis* strains. After optimization of GABA production, *Lb. brevis* NCL912 and *Lb. brevis* K203 produced 345.83 mM and 44.4 g/L of GABA from 8.5% and 6% MSG, respectively (Li et al., 2010a; Binh et al., 2013). Compared to these strains, it should be more optimized to produce GABA from *Lb. brevis* 340G with investigating other factors such as growth-stimulating factors (tween-80) for LAB and coenzyme (pyridoxal-5-phosphate) for GAD. Additionally, the further studies on large-scale fermentation of *Lb. brevis* 340G and utilization of crop by-products as fermentation medium will be need for increase of the GABA production and its application in industrial fields (Kook et al., 2009; Kook et al., 2010; Li et al., 2010b).

GABA is regarded to be an interesting compound as a supplement in the functional foods and LAB safe starters in the fermented foods. Accordingly, the GABA-producing *Lb. brevis* 340G itself and the fermented skim milk could be developed as useful starter and functional dairy food. In further study, the co-fermentation of other LAB starter strains together with *Lb. brevis* 340G to enhance the GABA production in the skim milk will be carried out with the optimizations of skim milk fermentation conditions (Inoue et al., 2003; Tung et al., 2011).

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