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ORIGINAL PAPER

Enhancement of γ-aminobutyric acid production in Chungkukjang by applying a *Bacillus subtilis* strain expressing glutamate decarboxylase from *Lactobacillus brevis*

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Abstract For a foreign glutamate decarboxylase (GAD) to be expressed in *Bacillus* host system, a recombinant DNA (pLip/*LbGAD*) was constructed by ligating an *LbGAD* gene from *Lactobacillus brevis* OPK-3 into *Escherichia coli–Bacillus* shuttle vector, pLip. The pLip/*LbGAD* construct was then transformed into *Bacillus subtilis*. The culture of the transformed *Bacillus* strain with the pLip/*LbGAD* construct had higher GAD activity and γ-aminobutyric acid (GABA) concentration than those of untransformed *Bacillus* counterpart. In addition, Chungkukjang, a traditional Korean fermented soybean product prepared by the transformed *Bacillus subtilis*, contained a significantly higher level of GABA than conventional ones. Thus, by introducing a foreign GAD gene, *Bacillus* strains have been genetically engineered to produce high levels of GAD and GABA.

Keywords *Bacillus subtilis* · γ-aminobutyric acid · Glutamate decarboxylase · *Lactobacillus brevis*

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Introduction

Glutamate decarboxylase (GAD) catalyzes the conversion of L-glutamate to γ-aminobutyric acid (GABA). GAD has been found in bacteria, animals, and higher plants (Ueno 2000). GABA in animals has been well understood as an inhibitory neurotransmitter with hypotensive and analgesic properties (Ueno 2000). It has recently been reported that GABA has an improving effect of visual function on old animals (Leventhal et al. 2003). Therefore, GABA has potential as a functional bioactive component of foods and pharmaceuticals.

Bacillus subtilis is one of the major microorganisms found in Chungkukjang, a traditional Korean fermented soybean product (Lee et al. 2003). While devising strategies to optimize diverse biotechnological capabilities in *Bacillus* strains, we previously observed that its capacity to produce GABA is relatively weak compared with other microorganisms such as *Lactobacillus* strains (Ueno et al. 1997; Park and Oh 2006) and *Lactococcus* strains (Nomura et al. 1998, 1999).

Recently, we have cloned GAD genes from *Lactobacillus* strains to study the mechanism of GAD regulation at the molecular level (Park and Oh 2006). To enhance GABA production, in the present study, in *Bacillus subtilis* using *GAD* from the lactic acid bacteria, we developed a *Bacillus subtilis* strain with enhanced GABA productivity

by introducing *LbGAD* (GenBank Accession No. DQ168031) cloned from *Lactobacillus brevis* OPK-3 (Park and Oh 2006). We report here that GAD and GABA levels in *Bacillus subtilis* were greatly enhanced by expressing *LbGAD*. In addition, we report that Chungkukjang with an enhanced level of GABA was produced by applying transformed *Bacillus subtilis*.

Materials and methods

Bacterial strains, media and plasmids

Escherichia coli DH5α was cultured at 37°C in LB medium with an additional 50 μg ampicillin ml^{-1} to select transformed bacteria harboring pLip (Lee et al. 2003) and pLip/*LbGAD*. *B. sbutilis* 168 (ATCC 23857) was grown in LB medium at 37°C. *B. sbutilis* (pLip/*LbGAD*) was selected on LB agar plates containing 30 μg kanamycin ml^{-1} and incubated at 37°C. *Lactobacillus brevis* OPK-3 (KFCC 11330, Park and Oh 2006) was grown in MRS media at 37°C.

Cloning of *LbGAD*

LbGAD was PCR-amplified from the pGEM T-easy vector (Promega, Madison, WI) containing the *LbGAD* gene (Park and Oh 2006). The primers for *LbGAD* primers were 5′-GGTACCAT-GGAAAACACACGCATGAAAC-3′ (*Kpn*I site underlined) and 5′-AAGCTTTTAGTGCGTG AACCCGTATTTTT-3′ (*Hin*dIII site underlined). After 30 PCR cycles, the reaction mixture was digested with *Kpn*I and *Hin*dIII and electrophoresed. A 1.4-kb DNA fragment was also eluted from the gel. This fragment was ligated with pLip, which was cut by the same restriction enzymes, to construct pLip/*LbGAD*.

Transformation of *Bacillus subtilis* 168

Electrocompetent *B. subtilis* cells were prepared depending upon the method of Ito and Nagane (2001) with a slight modification. Briefly, overnight-cultured *B. subtilis* was diluted 10-fold in growth medium (neutral complex medium) (Ito and Nagane 2001), and was grown at 37°C to an OD_{600} of 0.5. After adding glycine to the culture to give 1%, the cultivation was continued for a further 1 h. The cells were collected and resuspended in an electroporation medium (0.5 M sorbitol, 0.5 M mannitol, 10% (w/v) glycerol) yielding 10^{10} c.f.u. ml^{-1}. For electroporation, 2 μg DNA (pLip/*LbGAD*) was mixed with 60 μl cell suspension in a pre-chilled Gene-Pulser disposable cuvette, and the cells were exposed to a single electric pulse using a Gene-Pulser (Bio-Rad). For the selection of transformants, LB agar plate supplemented with 30 μg kanamycin ml^{-1} was used.

Analysis of GAD activity and GABA

GAD activity was estimated by the method of Snedden et al. (1996). *B. subtilis* and transformed *B. subtilis* with pLip/*LbGAD* were cultivated in LB broth supplemented with 1% (w/v) monosodium glutamate (MSG), harvested and then lysed by lysozyme digestion followed by sonication as previously described (Park et al. 2005). After centrifugation at 11,000*g* for 10 min, the supernatants were used for the analyses of GAD activity and total protein. The specific activity of GAD was expressed as nmol CO_2/min mg $protein^{-1}$. GABA was extracted from the supernatants essentially as described by Baum et al. (1996). GABA content was calculated using the Autochro WIN program (Young-Lin, Korea) and was expressed as an average value (μg GABA ml^{-1}) of triplicate analyses.

Preparation of Chungkukjang with the transformed *Bacillus subtilis* strain

To prepare Chungkukjang, soybeans were sterilized at 121°C for 1 h and fermented with transformed *B. subtilis* (168-GAD) or untransformed *B. subtilis* 168 at 42°C for 60 h.

Results and discussion

Transformation of *B. subtilis* with a pLip/*LbGAD* containing a *Lactobacillus* GAD gene

In order to develop *B. subtilis* with enhanced GABA productivity, we cloned lactic acid

bacteria-derived GAD gene (*LbGAD*) into *E. coli-Bacillus* shuttle vector pLip to construct pLip/*LbGAD* (Fig. 1). The pLip/*LbGAD* was transformed into *B. subtilis* 168 to express the *LbGAD* under the control of the *Hpa*II promoter (Lee et al. 2003). The *LbGAD* is a full-length GAD gene originated from a *Lactobacillus brevis* OPK-3 with GenBank accession no. DQ168031 (Park and Oh 2006). The *LbGAD* (1.4 kb) was detected from the transformed *B. subtilis* (168-GAD) by a colony PCR analysis (Fig. 2).

GAD activity and GABA in transformed *Bacillus subtilis*

To test that the *LbGAD* was expressed in *B. subtilis*, the specific activities of GAD in the extracts of *B. subtilis* (168-GAD) were analyzed by the radiometric method (Snedden et al. 1996). The GAD activity (85 nmol CO_2/min mg protein^{-1}) of transformed *B. subtilis* (168-GAD) extract was significantly increased compared with that (13.2 nmol CO_2/min mg protein^{-1}) of untransformed *B. subtilis* 168 extract. The data suggests that the *LbGAD* is over-expressed in *B. subtilis*. The over-expression of *LbGAD* in *B. subtilis* possibly resulted in the enhanced efficiency in converting MSG to GABA (Fig. 3). When the 168 and 168-GAD were cultured in LB media containing 1% MSG, 168 produced 46 μg GABA ml^{-1}, whereas 168-GAD produced 219 μg GABA ml^{-1}. The production of GABA in 168-GAD was further enhanced (401 μg GABA ml^{-1}) by increasing MSG concentration up to 3%(w/v). However, the 168 strain showed little response to increased concentration of MSG (Fig. 3). According to our previous study with the GAD gene from rice, *OsGADC*$^-$, the introduction of *OsGADC*$^-$ enhanced the specific activity of GAD and GABA concentration in transformed *Bifidobacterium longum* cells up to 55 nmol CO_2/min mg protein^{-1} and 104 μg GABA ml^{-1} in the presence of 3%(w/v) MSG, respectively (Park et al. 2005). By comparison, the GAD activities and GABA concentration in transformed *B. subtilis* and *Bifidobacterium longum* were significantly higher than those in untransformed *B. subtilis* and *Bifidobacterium longum.* In addition, both untransformed strains showed little response to the increased concentration of MSG (Park et al. 2005, Fig. 3 in this study). The data suggests that the introduction of

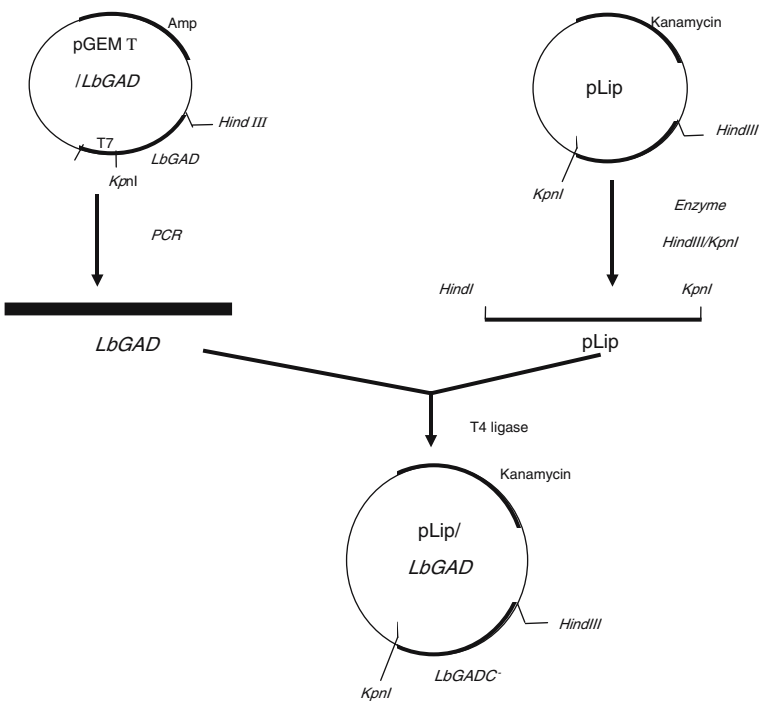

Fig. 1 Cloning of *LbGAD* into pLip shuttle vector

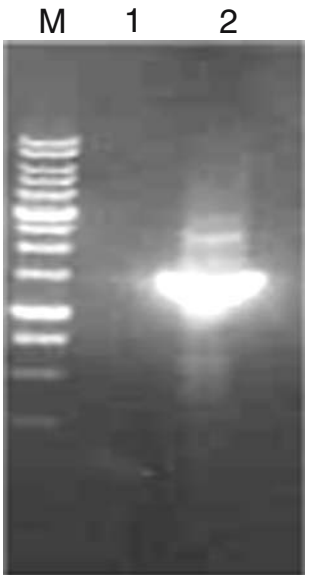

Fig. 2 Agarose gel electrophoresis of PCR product. M: marker DNA; lane 1: negative control (untransformed *B. subtilis*); lane 2: PCR product of *LbGAD* gene amplified from transformed *B. subtilis* 168

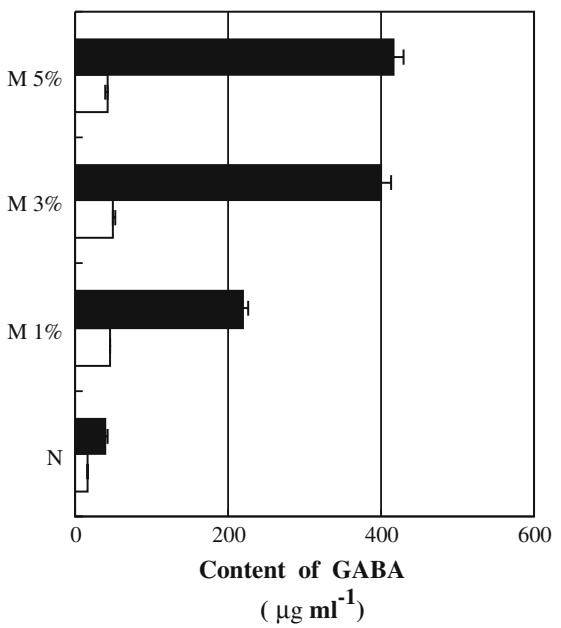

Fig. 3 Comparison of GABA production ability between untransformed 168 and transformed *B. subtilis* 168. The closed and open bars show the GABA contents of transformed *B. subtilis* 168 and untransformed *B. subtilis* 168, respectively. N: no MSG added; M 1%, M 3%, M 5% = 1%, 3%, 5% MSG supplemented, respectively

foreign GADs such as *LbGAD* and *OsGADC*$^{-}$ into microorganisms is an effective way to produce GABA and to develop health foods rich in GABA.

Preparation of Chungkukjang with an enhanced level of GABA

Chungkukjang prepared with transformed *B. subtilis* (168-GAD) contained a significantly higher amount of GABA (149.1 μg/g dry wt) than that (18.9 μg/g dry wt) of Chungkukjang with untransformed *B. subtilis* 168. Glutamic acid (Glu) is one of the most abundant amino acids found in legumes such as soybean (Koh et al. 1997). The data suggests that the LbGAD expressed in the transformed *Bacillus subtilis* strain may be responsible for the conversion of Glu to GABA.

In conclusion, genetic engineering was applied to improve the functional characteristics in *B. subtilis* by introducing lactic acid bacteria derived *LbGAD*. A newly introduced *LbGAD* gene enabled *B. subtilis* to produce significantly enhanced activities levels of GAD and amounts of GABA. Further, transformed *B. subtilis* strain was effective in the production of Chungkukjang with an enhanced level of GABA. This is the first report of microorganism transformation to produce Korean fermented soybean products such as Chungkukjang with a high level of GABA.

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