Microbiol. Biotechnol. Lett. (2015), 43(3), 300–305
http://dx.doi.org/10.4014/mbl.1509.09003
pISSN 1598-642X eISSN 2234-7305

MBL
Microbiology and Biotechnology Letters

Production of γ-Aminobutyric Acid Using Immobilized Glutamate Decarboxylase from *Lactobacillus plantarum*

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Received: September 3, 2015 / Revised: September 5, 2015 / Accepted: September 6, 2015

The glutamate decarboxylase gene (*gadB*) from *Lactobacillus plantarum* WCFS1 was cloned and expressed as an N-terminal hexa-histidine-tagged fusion protein in *Escherichia coli* BL21 (DE3) as the host strain. Purified glutamate decarboxylase (GAD) was immobilized onto porous silica beads by covalent coupling. The pH dependence of activity and stability of the immobilized GAD was significantly altered, when compared to those of the free enzyme. Immobilized GAD was stable in the range of pH 3.5 to 6.0. The resulting packed-bed reactor produced 41.7 g of γ-aminobutyric acid/l·h at 45°C.

Keywords: Glutamate decarboxylase, immobilization, γ-aminobutyric acid, *Lactobacillus plantarum*, packed-bed reactor

Recently, γ-aminobutyric acid (GABA), which is a suppressive neurotransmitter present in the mammalian brain and spinal cord, has attracted attention as a good natural functional substance, widely distributed in animals and plants [10]. It shows excellent biological activity such as decrease in blood pressure, mental stability, brain function, promotion of diuresis, alcohol metabolism, prevention of obesity, and improved liver function [7, 8, 13]. In food, GABA is widely distributed in various vegetables, rice, beans, fruit, tea, etc. However, since its content is very low by 1–200 mg per 100 g food, commercial levels of GABA production should be required in the food and pharmaceutical markets. Towards this aim, it is reported that increased GABA content (250–700 mg/100 g) was achieved by lactic acid bacteria fermentation [9, 12, 17]. Nevertheless, microbial fermentation for the production of GABA still has some practical limitations due to low yields of productivity, high facility investment, the need for space, the culture time, and changes in the food tastes and smell.

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Glutamate decarboxylase (GAD) is a pyridoxal 5'-phosphate (PLP) dependent intracellular enzyme, which is responsible for neutralization of an acidic cytosolic environment by decarboxylating an acidic substrate (glutamate) into a neutral compound (GABA) via incorporation of H^+ [6]. Bacterial GADs exhibit acidic pH optimum of 3.8 to 4.6 and are expressed in response to environmental stresses [4, 6]. Lactic acid bacteria are well-known sources for GADs because they thrive on acidic environments [14].

Biological production of highly concentrated GABA has been performed through immobilization of GABA converting enzymes or strains [11, 20]. Several GADs derived from *Lactobacillus* spp. have been exploited for the conversion of GABA from monosodium glutamate (MSG) as a cheap starting material [3]. When acidophilic GADs were immobilized using a cell-alginate bead complex, the activity of acidophilic enzymes are likely to be decreased due to increase in pH by decarboxylation reaction and addition of pyridoxal 5-phosphate, resulting in loss of hardness stability and enzyme activity [1].

Previously, we have cloned and heterologously expressed the *Lactobacillus plantarum gadB* gene encoding GAD, which catalyses the irreversible α-decarboxylation of L-

glutamate to GABA [18]. Briefly, we amplified the *gadB* gene by PCR from *L. plantarum* genomic DNA using the forward/reverse primer pair 5'-CATATGGCAATGTTATAC-GGTAAACAC-3' (*Nde*I site underlined) and 5'-GAATTCT-CAGTGTGTGAATCCGTATTTC-3' (*EcoR*I site undelined), and finally ligated the amplified *gadB* gene into the pET-28a vector (Novagen, Madison, WI), which directs the formation of fusion proteins with N-terminus polyhistidine (6 × His) to generate pET-28a-GAD. For expression of the recombinant enzyme, *E. coli* BL21 (DE3) cells transformed with pET-28a-GAD were grown in 1 liter of LB medium containing 50 μg of kanamycin per ml at 25°C induced at mid-exponential phase (A_{600} ~0.6) with 1 mM of IPTG, grown for an additional 12 h, and harvested by centrifugation (10,000 × *g*, 20 min, 4°C). Bacterial pellets were stored at -70°C. Then bacterial pellets were thawed and resuspended in 50 ml of 1× His-binding buffer (0.5 M NaCl, 20 mM Tris, 5 mM imidazole; pH 7.9), and disrupted by sonication. The lysate was centrifuged at 14,000 × *g* for 20 min to remove cell debris, and the supernatant was loaded on a His-bind resin (Novagen, Madison, WI) column (10 ml) equilibrated with the 1× His-binding buffer. The column was washed with 10 volumes of wash buffer (0.5 M NaCl, 20 mM Tris, 60 mM imidazole, pH 7.9), and a gradient of imidazole (from 5 mM to 1 M) was applied to elute the recombinant protein. The fractions containing enzyme activity were pooled and dialyzed against 50 mM sodium acetate buffer (pH 5.0), and the dialyzed enzyme preparation was stored at 4°C until use. Protein concentrations were determined by the bicinchoninic acid method, with bovine serum albumin as the standard. Enzyme fractions were analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Coomassie brilliant blue R-250. The purity of the enzyme was estimated by SDS-PAGE, consistent with the M_r (53,000 Da) calculated from the presumptive amino acid sequence [18].

To immobilize the recombinant *L. plantarum* GAD, silica beads were activated by silanization with 3-aminopropyltrimethoxysilane (Sigma Chemical Co., USA). One gram of silica beads was treated with 50 ml of 2.5% glutaraldehyde in 50 mM potassium phosphate buffer pH 8.0 for 1.5 to 2 h at room temperature under mild agitation. With distilled water to remove unreacted glutaraldehyde was washed the activated support, to which 10 ml of enzyme solution was then added. After 12 h at room temperature, the support was recovered by sedimentation and washed by distilled water. Unbounded proteins in the supernatant were pooled to determine the amount of protein and activity in order to indirectly measure the amount of the proteins bound to the support.

The effect of pH on the activities of immobilized enzyme was studied at various pHs (3.5–6.0) and compared to that of free enzyme. The temperature and pH dependence of free and immobilized GAD enzyme was determined by using HPLC after a 20-min incubation at various temperatures and pHs. For enzyme activity assay, 3 μl of 0.5 M MSG and 11 μl of 0.2 mM PLP were added to 100 μl of purified *L. plantarum* GAD solution (0.005–0.02 μg GAD/μl) in 200 mM sodium acetate/acetic acid, pH 5.0. After incubation for 20 min at 37°C, 600 μl of absolute ethanol (-20°C) was added to terminate the reaction. The suspension was centrifuged (15,000 × *g*, 10 min, 4°C) and filtered through a 0.22 μm pore size filter. The GABA products were separated by HPLC with an XTerra column (Waters; RP 185 m, 4.6 mm × 150 mm). To prepare *o*-phthalaldehyde (OPA) solution (pH 9.3), 1.0 ml methanolic OPA, 250 μl borate buffer (pH 9.9), and 25 μl 2-mercaptoethanol were mixed. Methanolic OPA contained 2.56 g OPA in 50% methanol, and borate buffer was mixed in 50:50 volume ratios of 0.2 M boric acid (dissolved in 0.2 M KCl) and 0.2 M NaOH, pH 9.9. Derivatization was performed with reagents prepared at least 90 min prior to use and saved no longer than nine days. The resulting supernatant (120 μl) of the reaction mixtures by GAD was mixed with 380 μl of OPA solutions one by one and left to react for 8 min before injection. The amount of GABA were then determined based on the HPLC data as described previously [18]. One unit of GAD activity is defined as the amount of enzyme that produces 1 μmol of product per minute under the assay conditions.

As expected, the pH-activity profile of the immobilized GAD was much broader than that of the free enzyme (Fig. 1A). On the other hand, the pH optimum of the immobilized GAD was pH 4.0–5.5, which is very similar to that of free enzyme. This is understandable since there is no obvious reason why entrapment of an enzyme in neutral polymers should alter its optimum pH [5]. The optimum temperature for recombinant free GAD activity was 40–45°C. As shown in Fig. 1B, the temperature optimum of the immobilized enzymes was slightly higher (by 5°C) than that of the free enzyme under the standard assay conditions, probably because of protection from heat denaturation by the silica beads [15]. Next, the pH stability of the immobilized

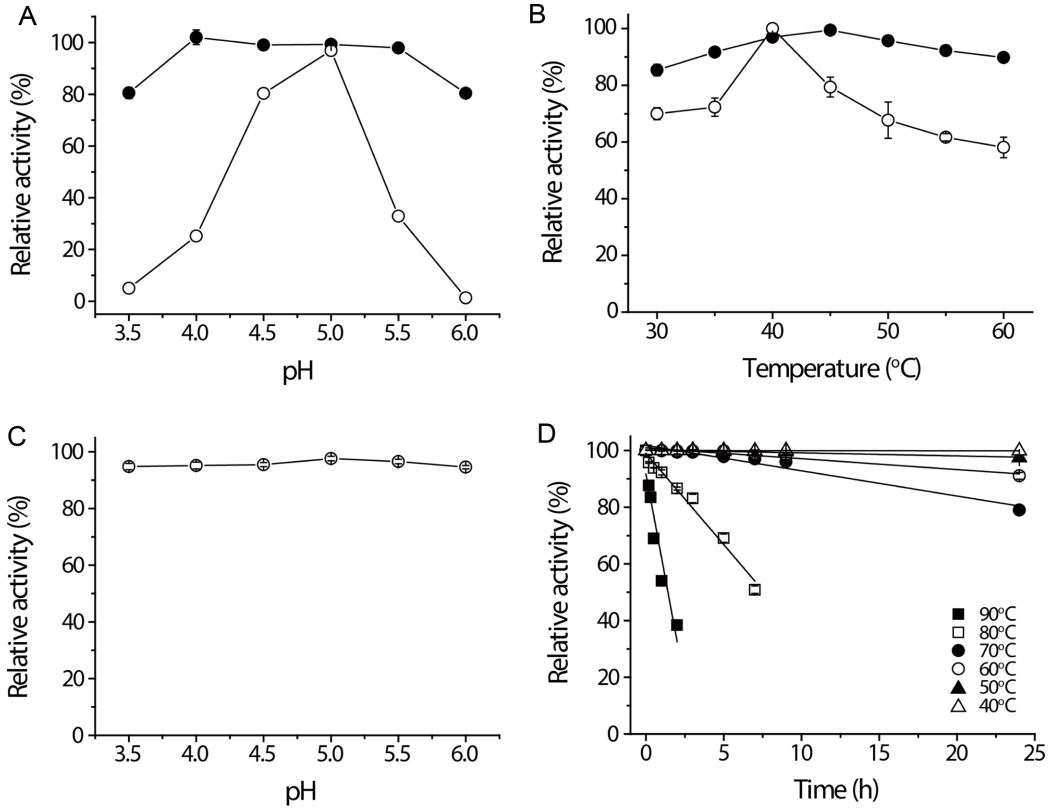

Fig. 1. Effects of pH and temperature on the activities of the free and immobilized GAD activity. (A) Optimum pH of the free (open cicle) and immobilized (closed circle) GAD was determined in 100 mM sodium-acetate buffer at pH 3.5 to 6.0. (B) Optimum temperature of the free (open circle) and immobilized (closed circle) GAD was checked in 100 mM sodium-acetate buffer at pH 5.0 for 20 min. (C) For pH stability, residual enzyme activity in each buffer was determined after 7 days pre-incubation at room temperature. (D) For thermostability, the enzyme was incubated for different periods of incubation (0–24 h) at various temperatures (40 to 90°C) in the presence of 100 mM sodium acetate (pH 5.0).

enzymes was examined in the range of pH 3.5 to 6.0 (Fig. 1C). To examine pH stability, we measured the residual activity of immobilized enzyme at 37°C after 7 day of incubation at pHs in the range of 3.5 to 6.0 and room temperature. As shown in Fig. 1C, more than 90% of the immobilized enzymes activity was retained in the range of pH 3.5 to 6.0. The above results thus demonstrate that immobilized GAD enzymes not only retain the biochemical properties of free GAD, but also have greater temperature and pH stability than free enzyme [18]. The covalent bond formation via amino groups of the immobilized GAD might reduce the conformational flexibility. One of the main reasons for enzyme immobilization is the anticipated increase in its stability to various deactivation forces, due to restricted conformational mobility of the molecules following immobilization [2]. The internal diffusion effect, as well as the conformation effect, may be responsible for the increase of the stability on the acid side [16]. The thermostability of free enzyme was examined in the range of 40 to 90°C [18]. More than 95% of initial activity was retained in the temperature range of 40 to 60°C, and 70% was retained at 70°C for 9 h. Its half-life was estimated to be 46 min at 90°C and the enzyme was rapidly inactivated at 100°C. Effect of temperature on the stability of immobilized GAD was also examined at different temperatures (40–90°C). More than 95% of original activity was retained in the temperature range of 40 to 60°C, and about 80% was retained at 70°C after incubation for 24 h (Fig. 1D). Its half-life was estimated to be 60 min at 90°C. These results suggested that the thermostability of immobilized enzyme increased considerably as a result of covalent immobilization onto porous silica beads. It was reported that the activity of the immobilized enzyme, especially in a covalently bound system, was more resistant than that of the soluble

http://dx.doi.org/10.4014/mbl.1509.09003

form against heat and denaturing agents [19].

To examine the operation of a packed-bed reactor for production of GABA, jacketed and fritted glass reactor (1.0 × 12 cm) was used to contain the immobilized enzymes. Temperature was maintained by submerging the reactor in the water bath. The substrate (MSG) was brought to reaction temperature (45°C) and pumped downward through the peristaltic pump. The flow rate was varied between 0.2 and 2.0 ml/min using a peristaltic pump. The 55 mg of immobilized enzymes were added to the reactor. The reactor was fed with various concentrations of MSG solution (10 to 150 mM) in 50 mM sodium acetate buffer. Space velocity (SV) was calculated using the total volume of reactor. Samples of the product were collected and analyzed for GABA content. The amount of MSG and GABA were determined using HPLC.

Prior to the operational studies of GAD production, we first optimized SV and substrate concentration in a 38 ml-packed-bed reactor at 45°C. The flow rate of this column was varied from 0.2 (0.32 SV) to 2 ml/min (3.2 SV), and displayed maximum volumetric productivity at 2 ml/min (3.2 SV). Therefore, to assess the effect of initial MSG concentration (10 to 150 mM) on the production of GABA by the immobilized GAD, the reactor was operated at 2.0 ml/min, pH 5.0 and 45°C. The maximal volumetric productivity of GABA by immobilized enzyme was obtained with 80 mM MSG at 45°C after a 12-min reaction. However, higher MSG concentrations (150 mM) did not increase significantly the volumetric productivity (26.5 g/l·h) even though overall productivity was increased. On the basis of these preliminary data described above, we operated the immobilized enzyme reactor at 2 ml/min as described above except that the concentrations of MSG tested were not changed until the conversion of MSG to GABA had reached 100% conversion. With initial MSG concentrations of 10, 80 and 150 mM, the reactor (57 U in 38 ml) gave 100% conversion in 10, 12, and 35 min with volumetric productivities of 6.3, 41.7, and 26.5 g GABA/l·h at 45°C, respectively (Table 1). Consequently, 100% of conversion of 80 and 150 mM GABA was obtained after 12 and 35 min of operation (Fig. 2). Thus, these results suggest that bioconversion of MSG to GABA can be successfully performed using this immobilized GAD enzyme reactor.

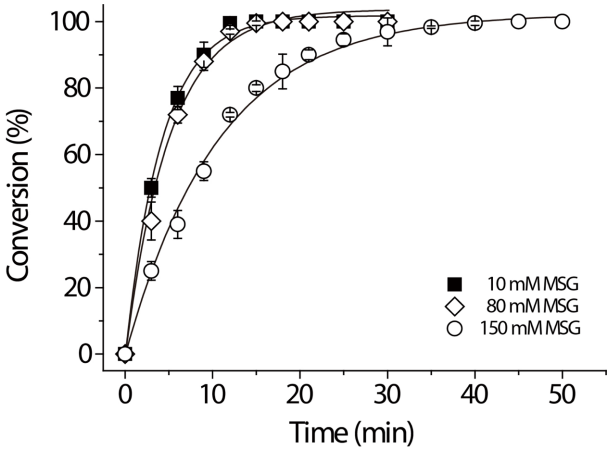

Fig. 2. Bioconversion of MSG to GABA by immobilized *L. plantarum* GAD reactor at 45°C.

Although a few microbial GADs had been isolated and immobilized to produce GABA using a bioreactor prior to the present work, the biological production of GABA needed to overcome practical limitations due to expense of purification, low conversion yield, slow reaction rate, metal uses, and low GAD pH stability [11, 20]. In light of this, *L. plantarum* GAD has several prominent properties that it is very active and stable at high temperatures and low pH in comparison with other microbial GADs, suggesting that this enzyme appears to be optimally adopted to work under acidic conditions and at high temperatures.

Such properties led us to immobilize *L. plantarum* GAD on porous silica beads and develop the enzymatic process for the production of GABA. Remarkably, the immobilized GAD obtained significant tolerance against acidic and alka-

Table 1. Kinetic parameters of the immobilized *L. plantarum* GAD.

MSG (mM)	Running time (min)	$Y_{P/S}$ (g GABA/g MSG)	$Y_{P/X}$ (g GABA/g GAD)	Volumetric productivity (g GABA/l·h)
10	10	1	0.4	6.3
80	12	1	3.6	41.7
150	35	1	19.6	26.5

The reactor was operated with a flow rate of 2 ml/min at 45°C. The amounts of glutamate and GABA were determined by HPLC as described in the text.

line pHs, together with improved thermostability. These features are very advantageous for industrial applications. We have now developed a feasible and simple biological process using a *L. plantarum* GAD that gives a high conversion yield of GABA from MSG.

Acknowledgments

We thank Young-Ho Hong, Hana Kim, Yu-Ryang Pyun for technical assistance with enzyme immobilization and critical reading. This research was supported by Kyungpook National University Research Fund, 2012.

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국문초록

Lactobacillus plantarum 유래 글루탐산 탈탄산효소의 고정화를 이용한 γ-aminobutyric acid의 생산

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효율적인 γ-aminobutyric acid (GABA)의 생산을 위해 *Lactobacillus plantarum* WCFS1로부터 글루탐산 탈탄산효소(glutamate decarboxylase, GAD)를 대장균에 발현, 정제 후 silica beads에 covalent coupling 방법을 이용하여 고정화하였다. 고정화된 효소의 특성을 고정화하지 않은 효소와 비교한 결과, 모든 pH의 범위(pH 3.5–6.0)에서 80% 이상의 활성을 나타내었으며 pH 안정성과 열 안정성 모두 증대되었다. 이 고정화 효소를 packed-bed reactor에 충진하여 GABA의 생산성을 확인한 결과 1리터당 1시간에 최대 41.7 g의 GABA 생산이 가능한 것으로 확인되었다.