Characterization of Three GADs from Bacillus spp. for Efficient γ-Aminobutyric Acid Production

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Research

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Abstract

Background

Gamma-aminobutyric acid (GABA) is an important bio-product used in pharmaceuticals and functional foods and as a precursor of the biodegradable plastic polyamide 4. Glutamate decarboxylase (GAD) converts l-glutamate (l-Glu) into GABA via decarboxylation. Compared with other methods, develop a bioconversion platform to produce GABA is of considerable interest for industrial use.

Results

Three GAD genes were identified from three *Bacillus* strains and heterologously expressed in *Escherichia coli* BL21 (DE3). The optimal reaction temperature and pH values for three enzymes were 40 °C and 5.0, respectively. Of the GADs, GADZ11 had the highest catalytic efficiency towards l-Glu (2.19 $mM^{-1} \cdot s^{-1}$). The engineered *E. coli* strain that expressed GADZ11 was used as a whole-cell biocatalyst for the production of GABA. After repeated use 8 times, the cells produced GABA with an average molar conversion rate of 97% within 8 h.

Conclusions

Three recombinant GADs from *Bacillus* strains have been conducted functional identification. And engineered *E. coli* strain heterologous expressing GADZ1, GADZ11, and GADZ20 could accomplish the biosynthesis of l-Glu to GABA in a buffer-free reaction at a high l-Glu concentration. The novel engineered *E. coli* strain has the potential to be a cost-effective biotransformation platform for the industrial production of GABA.

Background

As a four-carbon and water-soluble non-protein amino acid, γ-aminobutyric acid (GABA) plays an important role as an inhibitory neurotransmitter in mammals and plants [1]. Owing to its many biological activities, including roles in hypotension, sedation, diuresis, sleep enhancement and memory improvement, and hormone regulation, GABA has generally been employed in functional foods and pharmaceuticals [2-6]. Furthermore, GABA can be converted into polyamide 4, also known as nylon 4, which is a linear polymer of 2-pyrrolidone that can be chemically synthesized from GABA [7]. Nylon 4 has excellent physical properties and is environmentally safe owing to its heat-resistance (melting point at 260 °C) and biodegradability [8]. Therefore, GABA also has promising applications in the chemical industry and in protecting the environment [9]. Developing a process for enabling highly efficient GABA production subsequently is a key focus for its industrial application.

GABA can be produced through the biocatalytic α-decarboxylation of l-Glu using glutamate decarboxylase (GAD, EC4.1.1.15) (Fig. 1) [10]. A variety of GADs are found in bacteria, actinomycetes, fungi, and plant, and they play a central role in the synthesis of GABA using pyridoxal 5’-phosphate (PLP)

as a cofactor. Generally, GABA biosynthesis is performed in the laboratory or commercially using isolated enzymes or whole cells [11]. Compared to the purified enzyme method, several advantages have been observed in whole-cell bioconversion owing to its high efficiency, simple preparation, and lower cost, which are of particular interest for large-scale, high-speed, industrialization GABA production processes [12]. The GADs isolated from microbial sources, including *Escherichia coli*, *Bacillus megaterium*, lactic acid bacteria, and *Aspergillus oryzae*, are valuable for industrial production. Among these, the most common whole-cell bacterium [13] is *E. coli*, which has been used extensively to produce numerous valuable compounds [12].

In whole-cell biosynthesis for industrial applications, it is important to explore strains expressing GADs with high catalytic capacity. The genus *Bacillus* comprises gram-positive bacteria that are widely distributed on plant surfaces and in the soil and air. It produces many bioactive substances and various kinds of enzymes, such as amylases, proteases, and lipases [14]. As a result, the genomes of three *Bacillus* species, *Bacillus* sp. Z1, *Bacillus* sp. Z11, and *Bacillus* sp. Z20, were sequenced and analyzed. Each strain contains a putative *gad* gene, namely, *gadz1* in *Bacillus* sp. Z1, *gadz11* in *Bacillus* sp. Z11, and *gadz20* in *Bacillus* sp. Z20. The three relevant *gad* genes were cloned and expressed in *E. coli* BL21 (DE3). The target protein was purified for biochemical characterization using Ni-NTA chromatography. In addition, the capability of the engineered *E. coli* BL21 (DE3) cells expressing GADZ1, GADZ11, and GADZ20 for GABA production was evaluated. Altogether, we have presented here an efficient biosynthetic pathway for the industrial production of GABA.

Results

Gene cloning and expression of putative GADs

The three GAD genes, *gadz1*, *gadz11*, and *gadz20*, were isolated from the corresponding *Bacillus* strains. The sequence length of the gad fragment was 1470 bp in all strains, and it encoded a 489-amino acids long polypeptide. No putative signal peptide was predicted by using the SignalP-5.0 server. These results indicate that the three GADs are intracellular enzymes. The calculated molecular masses were 55.5 kDa for GADZ1, 55.4 kDa for GADZ11, and 55.6 kDa for GADZ20. Based on the sequence analysis, the three deduced GADs belong to the AAT–I superfamily and shared 96–97% sequence identities. Several conserved motifs were found; GETYTG (235–240) is probably the primary catalytic site or substrate-binding site, and the HVDAASGG (266–273) motif is highly conserved in PLP-dependent decarboxylases [15,16]. Further, the motif INVSGHKYGLVYPGLGWIIWR (295–315) is a PLP-binding domain, in which the ε-NH_2 of Lys301 forms Schiff bases with the PLP cofactor through an imine linkage. This step plays a key role in catalysis by the PLP-dependent decarboxylases [17,18].

Expression and purification of recombinant GADs

The resulting plasmids, including pET28a-*gadz1*, pET28a-*gadz11*, and pET28a-*gadz20*, were successfully expressed in *E. coli* BL21 (DE3). Substantial GAD activity was detected after induction at 16 °C for 16 h.

The three His6-tagged GADs were isolated using ultrasonic waves. A single Ni-NTA affinity chromatography step was used to purify the enzymes to >95% purity, with an apparent molecular weight of 55 kDa (Fig. 2a). HPLC analysis revealed that GADZ11 showed the best catalytic properties at pH 5.0 and 40 °C. The specific activity of the purified recombinant l-Glu enzymes towards l-Glu was 48.2 ± 1.5 U/mg for GADZ1, 98.9 ± 6.5 U/mg for GADZ11, and 13.5 ± 0.2 U/mg for GADZ20 (Fig. 2b).

Functional identification of the three GADs

The properties of the three purified GADs were compared with those of l-Glu. The three enzymes showed differences in catalytic activities. However, all the enzymes exhibited the highest catalytic activity at pH 5.0 and 40 °C and retained 60% of the maximum activity in the pH range of 4.5–5.5 and more than 50% of the maximum activity in the temperature range of 25–50 °C. GADZ11 exhibited the best properties, and GADZ1 and GADZ20 exhibited only half and one-tenth of the activity of GADZ11, respectively (Fig. 3a, 3b). The results indicated the potential value of GADZ11 in the bioconversion of l-Glu.

The kinetic parameters of the three GADs

The kinetic parameters of the three GADs with reference to l-Glu were obtained and are summarized in Table 1. The purified GADZ11 enzyme showed a k_{cat} value that was ~2.1 and 8.4 times higher than that of purified GADZ1 and GADZ20, respectively, suggesting the high conversion efficiency of GADZ11. However, GADZ1 and GADZ20 exhibited low K_m values of ~13% and 75%, respectively, compared with GADZ11, suggesting the highest substrate-binding affinity of GADZ20. Based on these two aspects above, GADZ11 showed the highest catalytic efficiency (k_{cat}/K_m values) of 2.19 $mM^{-1}{\cdot}s^{-1}$, nearly 2-fold higher than that of GADZ1 and GADZ20. These results indicate that GADZ11 exhibited higher catalytic performance under the same reaction conditions compared with the other two GADs.

Whole cells bioconversion for GABA synthesis

In general, GADs can utilize both monosodium glutamate (MSG) and l-Glu as substrates [19]. To investigate the application value of the engineered *E. coli* strains expressing GADs in the context of GABA synthesis, whole-cell biosynthesis of GABA via l-Glu and MSG was performed. As shown in Fig. 4a, in both sodium acetate solution and water, l-Glu rather than MSG was identified as a better substrate for producing GABA irrespective of the GAD-harboring *E. coli* strain. However, the *E. coli* BL21(DE3)/GADZ11 strain was the best biocatalyst; the GABA yield using this strain with l-Glu was 343 ± 11 mM in sodium acetate buffer and 920 ± 33 mM in water. However, only 115 ± 8 mM GABA was produced in the sodium acetate buffer using the same strain with MSG. Thus, GABA production in the buffer was much lower than that in water, even when l-Glu was used as the substrate. The pH value was 4.6 at the beginning of the reaction mixture in the sodium acetate buffer, but the pH changed to 6.5 after a 1-hour reaction. It is worth noting that no GABA was detected in the MSG reaction when water was used. This proves that the reaction mixture was alkaline, and an acidic environment was necessary for the biotransformation. Therefore, the following whole-cell bioconversion studies were evaluated in the water reaction system towards l-Glu.

To optimize GABA production, we explored the initial concentration of substrate in the reaction mix. The conversion rate was 99–100% when the concentration of l-Glu was lower than 1 M in the reaction mixture (Fig. 4b). The conversion rate decreased substantially, although the yields of GABA were higher when the l-Glu concentration was higher than 1 M. We assumed that the higher the GABA concentration, the stronger the substrate inhibition. In addition, high concentrations of GABA may cause an osmotic pressure imbalance between the intracellular and extracellular environment; high extracellular osmotic pressure may negatively affect the conversion rate [20]. Thus, for the subsequent whole-cell bioconversion studies, 1 M l-Glu was chosen.

The effect of cell concentration and PLP concentration on the conversion rate

The effect of different cell concentrations in the reaction system was examined at 37 °C for 2 h. To ensure that l-Glu was sufficient for a high cell concentration, we used 6 M l-Glu for GABA production. When the cell concentration was lower than OD_{600} 20, the conversion rate increased with increasing cell concentration. When the cell concentration was higher than OD_{600} 20, the highest conversion rate was observed, with a GABA yield of 1.8 M (Fig. 5a). Thus, the cell concentration for subsequent studies was set at OD_{600} 20.

PLP was involved in the regulation of proton translocation to catalyze the decarboxylation of l-Glu, and the effect of different concentrations of PLP on GABA production was also evaluated [21]. The yield of GABA was 580 ± 18 mM without added PLP, and it did not change with increasing amounts of PLP supplementation beyond 0.1 mM (Fig. 5b). Thus, under the condition of 1 M l-Glu, the appropriate concentration for PLP addition was 0.1 mM.

The upper bound estimation of *E. coli* BL21(DE3)/GADZ11 to convert GABA from l-Glu

Next, we tested the time profiles of GABA production using different concentrations of l-Glu (Fig. 6a). *E. coli* BL21/GADZ11 could convert 94% l-Glu in 1 h, while for the 2 M l-Glu, the complete conversion was achieved in 2 h. The highest efficiency of GABA was observed in the first hour, and dropping thereafter. In the first hour of the reaction, 941 ± 25 mM (94.1%), 1682 ± 58 mM (84.1%), 2226 ± 221 mM (74.2%), and 2371 ± 269 mM (59.3%) of GABA was produced from 1–4 M l-Glu, respectively. Based on the conversion rate, the addition rate of l-Glu was 1 M for the batch reactions.

To investigate whether or not the cells are capable of efficient recovery and reuse is of great significance to reduce the production cost (Fig. 6b). The conversion ratio was 94 mol% in the first hour, and almost complete conversion was achieved in all the subsequent reaction batches. Each reaction could reach this rate with 1 M l-Glu and 0.1 mM PLP. Finally, *E. coli* BL21/GADZ11 could be used for eight batches with a conversion rate of 94–99%. Within 8 h, 8 M l-Glu was converted to 7.8 M GABA. Thus, *E. coli* BL21 (DE3)/GADZ11 has the potential to become a strain with excellent conversion properties.

Purification and crystallization of GABA

In the previous eight batch studies, 8 M l-Glu was completely totality converted within 8 h to produce 7.8 M GABA in a 160 mL water system, with an average molar conversion rate of 97%. The reaction mixture was collected by centrifugation and concentrated by rotary evaporation. The produced GABA was dried to a white powder at 65 °C. Furthermore, the GABA powder obtained was highly pure, as shown in the HPLC chromatogram (Fig. 7). There was no difference between the sample and the reference standard with respect to purity.

Discussion

In recent years, GABA has become hugely popular as a bioactive component in the pharmaceutical and food industries. Thus, development of a reliable and stable platform for the cheap production of GABA is of great economic significance. With the intent of developing a novel biotransformation system, we identified three GADs from three *Bacillus* spp., which had similar optimum pH and temperature as those of other strains. Of note, the catalytic efficiency of the purified GADZ11 was 2.19 $mM^{-1} \cdot s^{-1}$, higher than previous reports on GAD (Table 2). Furthermore, the *E. coli* cells heterologous expressing GADZ11 could produce GABA directly using high-concentration l-Glu in a buffer-free reaction, and its conversion rate was over 95%. The excellent transformation properties were more suitable for commercial production and post-purification of GABA.

To date, most reported GADs are more active and stable within the temperature and pH ranges of 30–50°C and pH 4.0–5.0, respectively, and their activities dropped rapidly at pH >6 [30]. In microorganisms, GAD activity is one of the important mechanisms of resistance to low-acid environments, which is the reason why most of GADs only work at low pH, including GADZ11 in our study [31]. The low pH range (pH 4–5) of the reaction buffer is a critical limitation for the efficient production of GABA [32]. GABA conversion by *E. coli* BL21/GADZ11 reached 34.3% in sodium acetate solution and 92.0% in water. For MSG, the conversion rate was 11.1% in sodium acetate buffer and almost nil in the water. These results explain why MSG is not the best choice for GABA production [33,34]. The pH of a solution of 1 M MSG in 20 mL water is pH 7.0, while that of a solution containing the same concentration of l-Glu in water is 2.3. Moreover, l-Glu is less soluble in water than MSG. The solubility of l-Glu in water is 15.1 g/L at 40 °C, while the solubility of MSG in water is 717 g/L. Therefore, most of the l-Glu exists in the reaction system in solid form. In other words, the osmotic pressure of the mixture with l-Glu is much lower than that of MSG, a feature that favors the biosynthesis reaction. Furthermore, when the concentrations of l-Glu and MSG were equal in the reaction system, l-Glu could maintain an acidic pH while being consumed [12,35]. Thus, l-Glu is a better choice for the synthesis of GABA.

It is known that GAD is a typical PLP-dependent enzyme [19]. PLP is a cofactor for a variety of enzymes involved in the metabolism of amino compounds and in the synthesis of biomolecules, such as dopamine, epinephrine, norepinephrine, and histamine [36]. In our study, PLP did not seem to be necessary for the biosynthesis of GABA, and the production of GABA was 583 ± 23 mM without PLP added to 1 M of l-Glu. However, it is difficult to determine whether PLP exists naturally in *E. coli*. Although not necessary in our system, the addition of PLP has previously demonstrated substantial positive

effects on GABA production [37]. Our results are consistent with those of other studies. The rate of conversion increased with an increase in PLP concentration and the maximum production achieved was 985 ± 15 mM (98.5%).

Our study reveals that the reaction of 4 M l-Glu required 2 h to reach 95 mol% with a cell concentration of OD_{600} 20 and PLP concentration of 0.4 mM. Therefore, without considering the time cost, with the proper concentration of cells and PLP, l-Glu can be completely converted in a short period. Furthermore, high-purity of GABA can be obtained after simple purification and concentration from the reaction mixture. To summarize, the platform that we developed has the following merits: this engineered *E. coli* strain may be used in industry for the commercial-scale production of GABA, and it provides the preliminary date for the separation and purification of GABA from the fermentation broth.

Conclusions

Functional identification was conducted for three recombinant GADs from *Bacillus* strains. Among them, GADZ11 possessed the best bioconversion properties. An engineered *E. coli* strain heterologous expressing GADZ1, GADZ11, and GADZ20 could accomplish the biosynthesis of l-Glu to GABA in a buffer-free reaction at a high l-Glu concentration. The engineered *E. coli* BL21 (DE3)/GADZ11 strain was able to achieve the complete conversion of 1 M l-Glu in 1h. We believe that the novel engineered *E. coli* strain has the potential to be a cost-effective biotransformation platform for the industrial production of GABA.

Methods

Strains, media, plasmids and chemicals

The three *Bacillus* spp. were isolated from desert sand samples obtained from Ningxia Province, China, and deposited in the Agricultural Culture Collection of China under registration numbers ACCC 61750, 61747, and 61748, respectively. These strains were cultured in Luria–Bertani medium at 30 °C. The *E. coli* XL10 was used for gene cloning. *E. coli* BL21 and plasmid pET-28a were used as the expression host and vector, respectively. The DNA purification kit, LA Taq DNA polymerase, and restriction endonucleases were purchased from TaKaRa (Tsu, Japan). T4 DNA ligase was purchased from New England Biolabs (Hitchin, UK). All chemicals were of analytical grade and were commercially available.

Cloning of *gad* genes from *Bacillus* strains and plasmid construction

Genomic DNA from *Bacillus* strains grown in LB medium was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The GAD genes, *gadz1*, *gadz11*, and *gadz20* (GeneBank accession number: MW703457, MW703456 and MW703455) were amplified by PCR from the genomic DNA of *Bacillus* strains using suitable primer pairs (GADF: 5’-CTGAATTCATGTCCAAGGATCGAAAAGCAG-3’ and GADR: 5’-TTCGCCGGCGAAGCGGCCGCCTAATGATGAAACCCATT-3’). The amplified DNA fragment was purified from a 1.0% agarose gel using the Wizard SV Gel and PCR Clean-Up System (Promega) after

gel electrophoresis. The purified 1470 bp gad fragment was digested with *EcoR*I and *Not*I and ligated (T4 DNA ligase) into pET-28a (+) to generate pET-28a-*gadz1*, pET-28a-*gadz11*, and pET-28a-*gadz20*. The constructed plasmids were used for expression in *E. coli* BL21 (DE3).

Expression and enzyme purification

E. coli BL21(DE3) transformed with the plasmids pET-28a-*gadz1*, pET-28a-*gadz11*, and pET-28a-*gadz20* were cultured in LB medium containing kanamycin (50 μg/mL) at 37 °C for 12 h. Then, the culture was transferred to 400 mL LB broth at 37 °C (1% by volume of inoculant). When suitable bacterial concentration was achieved (OD_{600} of 0.6–0.8), protein expression was induced by adding 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) and shaking at 200 rpm (16 °C, 16 h). The cells were collected at 8000 *×g* for 10 min and resuspended in lysis buffer (20 mM Tris-HCl buffer, 500 mM NaCl, pH 7.6). Then, cells were disrupted by ultrasonic waves. After centrifugation, the protein was separated by Ni-NTA affinity chromatography using elution buffer (20 mM Tris-HCl buffer, 500 mM NaCl, 200 mM imidazole, pH 7.6), and then, the proteins in the supernatant and pellet were resolved SDS-PAGE [38]. The final protein concentrations were determined using the Bradford assay (BSA was used as a standard) [39].

Determination of enzyme activity and GABA formation

Enzyme activity was determined by measuring GABA production using HPLC analysis, with some modifications [40,41]. The reaction mixture comprised 400 μL Na_2HPO_4-citric acid buffer (80 mM, pH 6.0), 500 μL l-Glu (50 mM), 50 μL PLP (0.02 mM), and 50 μL purified enzyme. Ice-chilled 80% ethanol was added at an equal amount (1 mL) to stop the reaction after 30 min at 40 °C. The reaction supernatant (500 μL) was mixed with 100 μL of $NaHCO_3$ (2.5 g/L) and 200 μL of 4-N, N-dimethylaminoazobenzene-4'-sulfonyl chloride (DABS-Cl) (0.25 g/L, dissolved in acetonitrile), and incubated it at 70 °C for 20 min. This was followed by analysis on a SHIMADZU 20A series instrument (Shimadzu, Kyoto, Japan) and Agilent ZORBAX SB-C18 column (5 μm, 4.6 × 150 mm) (Agilent, Santa Clara, CA, USA). The mobile phase was a solution of 35% (v/v) acetonitrile solution and 65% 50 mM sodium acetate. The flow rate and column temperature were 1 mL/min and 30 °C, respectively; the injection volume was 10 μL; and the detection wavelength was 436 nm. The GABA content in the test solutions was calculated by taking into consideration of the peak areas with the standard. The definition of one enzyme activity unit is the amount of enzyme required for the release of 1 mM free GABA in 1 min. Three parallel wells were set per group.

Reaction conditions and kinetic parameters of enzymes

Optimum pH and temperature assay

The optimum pH properties of the GADs were determined at reaction pH values from 3.0 to 7.0. The pH-activity profiles were examined for 30 min at 37 °C. The optimum temperature was examined at pH 5.0 over a temperature range of 25–55 °C. Each experiment was performed in triplicate.

Enzyme kinetic assays

Enzyme kinetic assays were performed in Na_2HPO_4-citric acid buffer containing 5–150 mM l-Glu at 40 °C for 15 min. The pH of the mixture containing l-Glu and Na_2HPO_4-citric acid buffer was 5.0. Each experiment was performed in triplicate. The K_m and V_{max} values were nonlinearly fitted using the GraphPad Prism 5 software.

Whole-cell bioconversion process

Recombinant *E. coli* cells harboring GADZ1, GADZ11, and GADZ20 were cultured at 37 °C. Then, protein expression in the engineered bacteria was induced at 16 °C for 16 h. After this, the cells were centrifuged at 8000 *×g* for 10 min, washed, and resuspended it in water containing MSG or l-Glu at appropriate concentrations. The OD_{600} was measured to indicate the cell concentration. The reaction of the 20 mL mixture in 100 mL flasks was performed at 37 °C at 120 rpm. GABA production of the reaction was analyzed and calculated by HPLC.

To optimize the reaction conditions, the effects of substrate specificities, substrate concentration, cell concentration, PLP concentration, time-course analysis of single-batch reactions, and the recycling ratio of batch reactions were analyzed simultaneously in this study.

Substrate specificity and optimum substrate concentration

The substrate specificities of whole-cell biosynthesis were investigated in an assay system containing the following substrates: MSG or l-Glu. The whole-cell biotransformation reactions were conducted in 0.1 M sodium acetate buffer (pH 4.6) or water by single factor and orthogonal experiments. The reaction condition was obtained as follows: reaction time, 1 h; OD_{600}, 20; and l-Glu or MSG concentration, 1 M.

To determine the effects of the l-Glu concentration, the following procedure was performed: after 16 h of preculture of *E. coli* GADZ11, the cells were centrifuged and suspended in water. The reaction system consisted of 20 mL water containing resuspended cells in a 100 mL Erlenmeyer flask. To this, different amounts of l-Glu (0.5, 1, 2, 3, and 4 M) were added. The reaction mixture contained 0.02 mM PLP. The reaction was stopped by adding 30 mL ethanol after 1 h, and the volume was made up to 100 mL with water. The reaction supernatants were collected to measure the GABA content.

Optimum cell and PLP concentration

To determine of the optimal cell concentration, the following procedure was performed. The 20 mL reaction system was contained 6 M l-Glu, 0.02 mM PLP, and a predetermined quantity of cells (OD_{600} 2, 5, 10, 15, 20, 30). The reaction mixture was incubated in a shaker at a specified shaking rate (120 rpm) at 37 °C for 2 h. Aliquots (500 μL) were withdrawn into an equal volume of ice-chilled 80% ethanol after 2 h to stop the reaction. The reaction supernatants were collected to measure the content of GABA via HPLC.

To determine the effects of the coenzyme PLP, 0 (control), 0.02, 0.05, 0.1, 0.2, 0.5, and 1 mM PLP was added to the 20 mL mixture. The l-Glu and cell concentrations were 1 M and OD_{600} 20, respectively. After 2 h of culture at 37 °C, 500 μL of the reaction mixtures were taken and measured with HPLC.

The upper bound estimation of *E. coli* BL21 (DE3)/GADZ11 to convert GABA from l-Glu

To optimize the reaction conditions and decrease the manufacturing cost, reactions with different concentrations of l-Glu were conducted for the time-course assays. PLP at a final concentration of 0.4 mM and cells at a final concentration of OD_{600} 20 were added to the reaction system, and the l-Glu concentrations tested were 1, 2, 3, and 4 M. To stop the reaction, 30 mL ethanol was added to the mixtures (when the solid l-Glu was fully consumed), and water was added to make up the volume to 100 mL. The reaction mixtures were subjected to HPLC.

The concentrations of l-Glu and PLP were 1 M and 0.1 mM, respectively, and the cell concentration was OD_{600} 20 in the batch reaction. The reaction was conducted at 37 °C for 1 h, and the cells were collected by centrifugation for the next batch when the solid l-Glu was fully consumed. There were eight batches in total. The samples were tested when the reactions were complete.

Conversion of high concentrations of l-Glu and the purification and crystallization of GABA

To prepare GABA crystals, the mixture from the previous eight batches was separated and concentrated by rotary evaporation. GABA crystals were collected from the concentrated solution. The crystals were oven-dried at 65 °C until they were converted into a white powder [42]. The purity of the powder was determined by HPLC.

Abbreviations

l-Glu: l-glutamate; MSG: monosodium glutamate; GABA: Gamma-aminobutyric acid; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GAD: Glutamate decarboxylase; K_m: Michaelis constant; V_{max}: maximum reaction rate; k_{cat}: turnover number; PLP: pyridoxal 5'-phosphate; DABS-Cl: 4-N, N-dimethylaminoazobenzene-4'-sulfonyl chloride

Declarations

Authors' contributions

LS and TT contributed to the conceptualization. TT contributed to the methodology and investigation. YB, XS and JZ performed the formal analysis. XZ and CZ contributed to the resources. YB and YW analyzed and interpreted the data. LS curated the data and wrote the manuscript. YW and TT reviewed and edited the manuscript critically for the manuscript. HL and BY gave the project administration and funding acquisition. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files.

Consent for publication

All authors have read and approved this manuscript to publish.

Ethics approval and consent to participate

Not applicable.

Ethical statement

This article does not contain any studies with human participants or animal performed by any of the authors.

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Tables

Table 1. Kinetic parameters of three GADs.

Substrate	V_{max} (μM·min^{-1}·mg^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1}·s^{-1})
GADZ1	58.02 ± 3.90	55.6 ± 5.88	45.40 ± 8.63	1.23
GADZ11	124.20 ± 4.83	114.86 ± 4.18	52.37 ± 5.44	2.19
GADZ20	14.75 ± 0.48	13.67 ± 0.33	13.26 ± 1.95	1.03

Table 2. Essential properties and kinetic parameters of GADs in this study and other microbial GADs.

Microorganism	pH optimum	Temperature optimum (°C)	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)	Ref
Bacillus sp. Z11	5.0	40	2.19	This study
Bacillus sp. Z1	5.0	40	1.23	This study
Bacillus sp. Z20	5.0	40	1.03	This study
Lactiplantibacillus plantarum	5.0	40	0.00012	[25]
Lactobacillus brevis 877G	4.2	45	0.7	[26]
Lactobacillus paracasei	5.0	50	1.43	[27]
Lactococcus lactis	4.7	30	ND	[28]
Streptomyces chromofuscus	4.2	37	1.21	[29]

[a]Catalytic efficiency (k_{cat}/K_m value) was determined using l-Glu as the substrate.

[b]ND, not determined.

Figures

Fig. 1

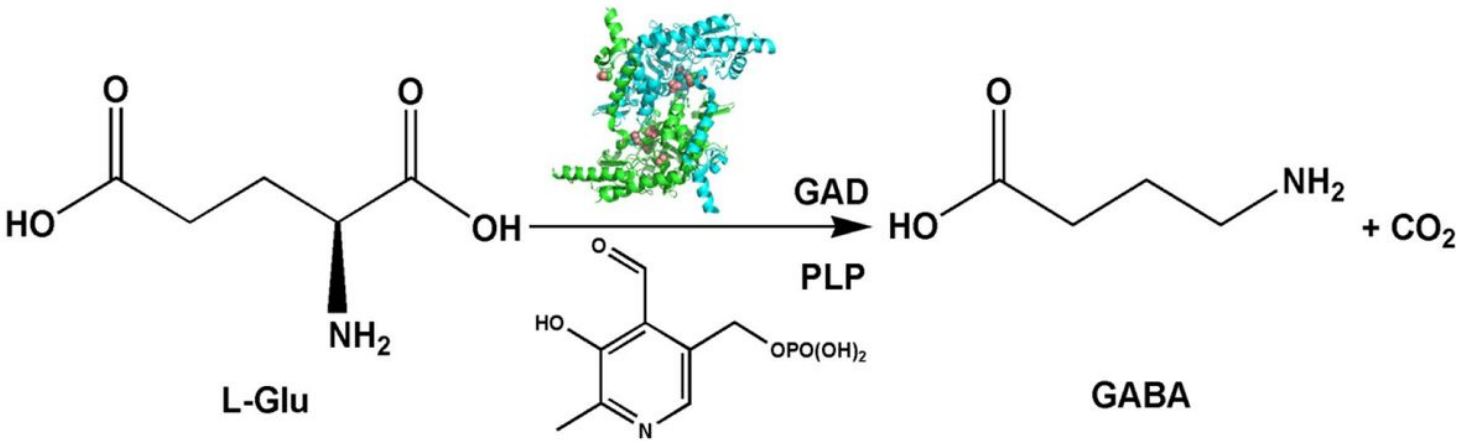

Figure 1

GABA production by GAD with PLP as a cofactor.

Fig. 2

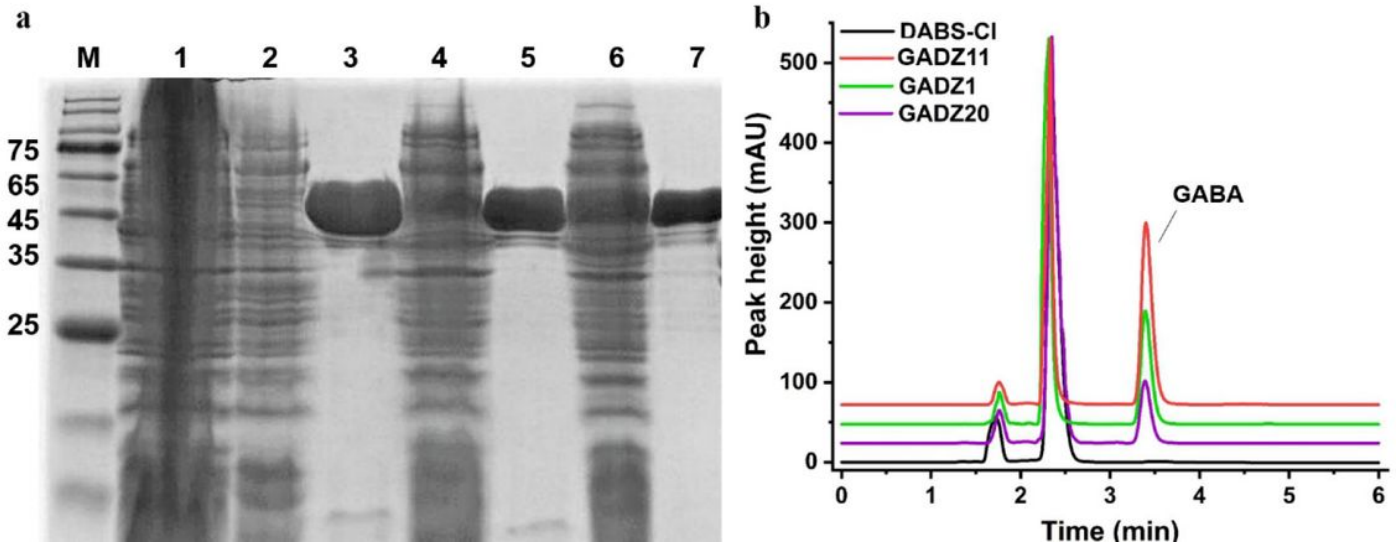

Figure 2

(a) SDS-PAGE analysis of purified recombinant GADs. M: protein ladder; 1: intracellular proteins of E. coli BL21 (DE3)/pET28a (+) (control); whole-cell intracellular proteins and affinity-purified proteins of E. coli

BL21 (DE3)/GADZ1 (lanes 2 and 3), E. coli BL21 (DE3)/GADZ11 (lanes 4 and 5), and E. coli BL21 (DE3)/GADZ20 (lanes 6 and 7). (b) After incubation of the purified GADZ1, GADZ11, and GADZ20 proteins with L-Glu at pH 5.0, the reaction mixtures were analyzed using HPLC.

Fig. 3

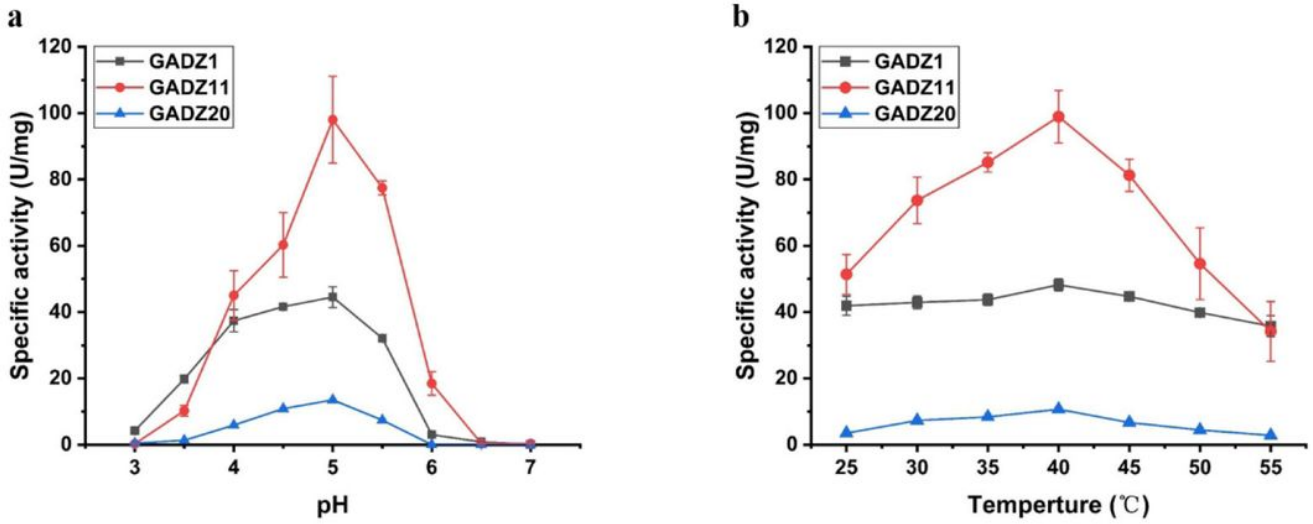

Figure 3

Enzymatic properties of GADZ1, GADZ11 and GADZ20. (a) The pH optima of GAD; (b) the temperature optima of GAD.

Fig. 4

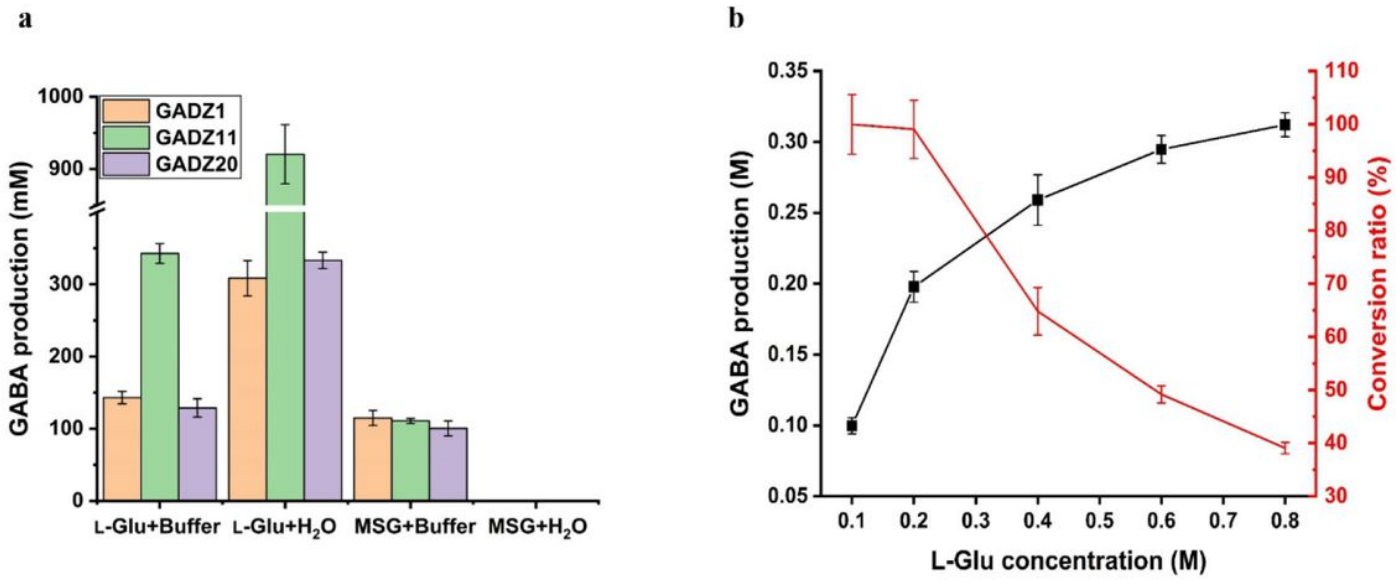

Figure 4

Biotransformation of E. coli to produce GABA. (a) Determining the suitable substrate (MSG/L-Glu) and reaction environment (buffer/water) for the conversion. (b) Conversion rate with different concentrations

of L-Glu. The operating conditions chosen were as follows: reaction temperature, 37 °C; reaction time, 1 h; and PLP concentration, 0.02 mM. Data are shown as the mean ± standard deviation (n = 3).

Fig. 5

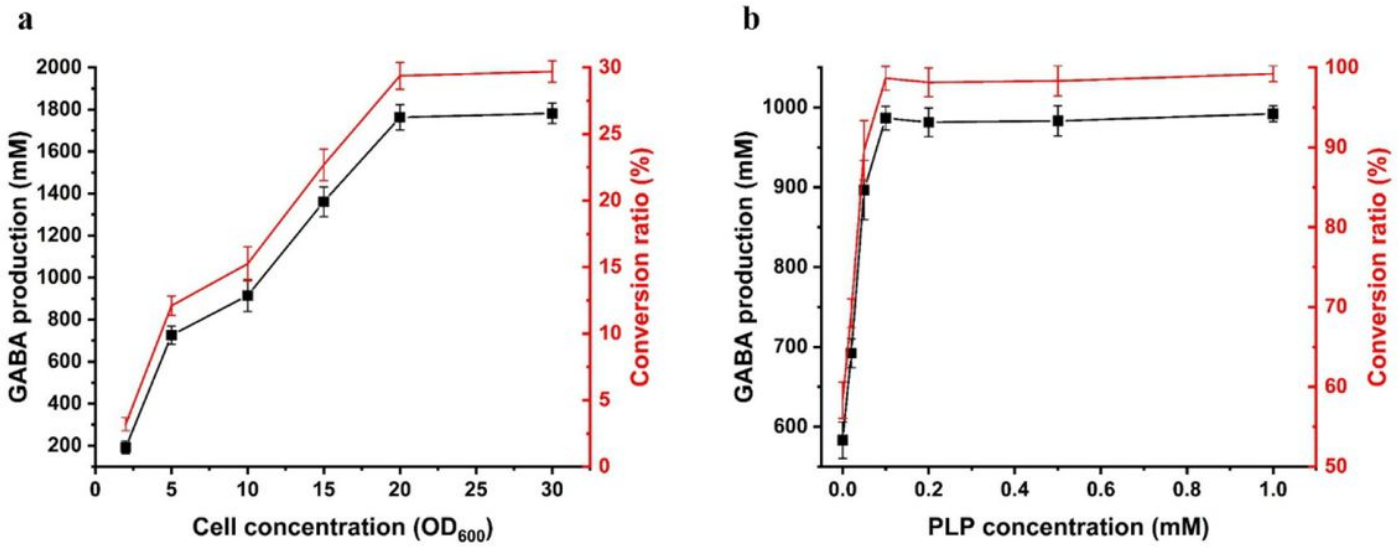

Figure 5

The optimum cell (a) and PLP (b) concentration for GABA production by E. coli BL21 (DE3)/GADZ11 in presence of L-Glu. The black line represents the yield of GABA and the red line indicates the conversion rate.

Fig. 6

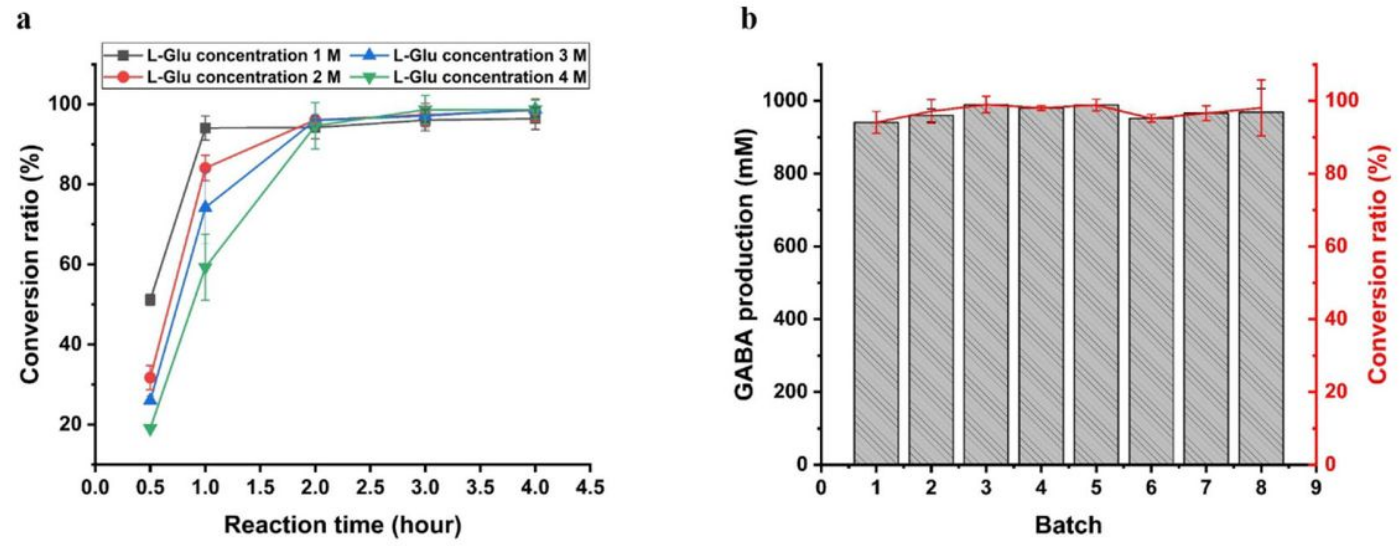

Figure 6

Upper bound estimation of conversion by E. coli BL21(DE3)/GADZ11 from L-Glu. (a) Time-history analysis of GABA formation in single-batch reactions with different molar concentrations of L-Glu. (b)

Reused of E. coli BL21(DE3)/GADZ11. Conditions for each batch condition were as follows: 1 h, 1 M L-Glu, and 0.1 mM PLP.

Fig. 7

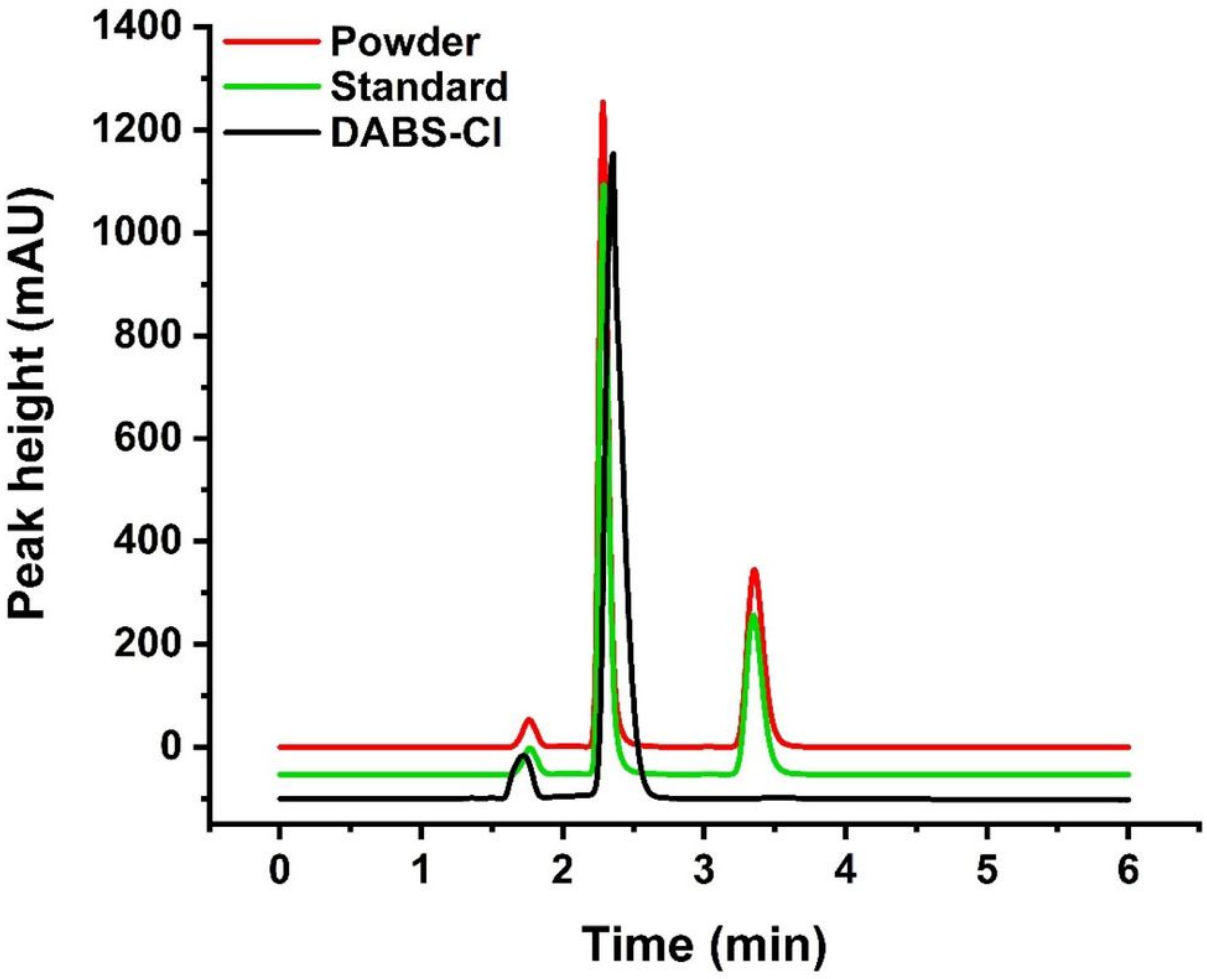

Figure 7

The sample and reference standard were analyzed by HPLC.