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Research article

Molecular cloning, expression, and immobilization of glutamate decarboxylase from *Lactobacillus fermentum* YS2

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ABSTRACT

Background: GABA (γ-aminobutyric acid) is a four-carbon nonprotein amino acid that has hypotensive, diuretic, and tranquilizing properties. Glutamate decarboxylase (GAD) is the key enzyme to generate GABA. A simple and economical method of preparing and immobilizing GAD would be helpful for GABA production. In this study, the GAD from *Lactobacillus fermentum* YS2 was expressed under the control of a stress-inducible promoter and was purified and immobilized in a fusion form, and its reusability was investigated.
Results: The fusion protein CBM-GAD was expressed in *Escherichia coli* DH5α carrying pCROCB-*gadB*, which contained promoter P_{rpoS}, *cbm*3 (family 3 carbohydrate-binding module from *Clostridium thermocellum*) coding sequence, the *gadB* gene from *L. fermentum* YS2 coding for GAD, and the T7 terminator. After a one-step purification of CBM-GAD using regenerated amorphous cellulose (RAC) as an adsorbent, SDS-PAGE analysis revealed a clear band of 71 kDa; the specific activity of the purified fusion protein CBM-GAD reached 83.6 ± 0.7 $U \cdot mg^{-1}$. After adsorption onto RAC, the immobilized GAD with CBM3 tag was repeatedly used for GABA synthesis. The protein-binding capacity of RAC was 174 ± 8 $mg \cdot g^{-1}$. The immobilized CBM-GAD could repeatedly catalyze GABA synthesis, and 8% of the initial activities was retained after 10 uses. We tested the conversion of monosodium glutamate to GABA by the immobilized enzyme; the yield reached 5.15 g/L and the productivity reached 3.09 g/L·h.
Conclusions: RAC could be used as an adsorbent in one-step purification and immobilization of CBM-GAD, and the immobilized enzyme could be repeatedly used to catalyze the conversion of glutamate to GABA.

1. Introduction

GABA (γ-aminobutyric acid) is a four-carbon nonprotein amino acid that acts as the major inhibitory neurotransmitter in the mammalian central nervous system. GABA has been reported to have hypotensive, diuretic, and tranquilizing effects [1,2]. Because of these beneficial functions, there have been many attempts to synthesize GABA using biological or chemical methods for use in foods, pharmaceuticals, and other industries.

Glutamate decarboxylase (GAD, EC 4.1.1.15) is the key enzyme that catalyzes the α-decarboxylation of glutamate, which in turn generates GABA. Various microorganisms with GAD activity have been identified. In particular, many researchers focused on lactic acid bacteria (LAB), such as *Lactobacillus brevis* [3], *Lactobacillus paracasei* [4], *Lactobacillus rhamnosus* [5], and *Lactobacillus sakei* [6], as they have been used in the fermentation of foods for a long time and possess probiotic potential. The growth of LAB is often slow, and the yield of purified GAD is not adequate for large-scale production. To acquire adequate enzyme yield for GABA production, many researchers exploited *Escherichia coli* to express LAB-derived GADs, in which IPTG-inducible promoters are often widely used. However, high cost and toxicity of IPTG restrict its usages, and alternative strong promoters greatly gained the attention of researchers. However, fully take advantage of the enzyme and decrease the cost of GABA production, immobilization of GAD has been investigated in several studies, in which metal affinity resin was used for the immobilization of *L. brevis* GAD [7] and *E. coli* GAD [8]. Another similar method involved the use of metal affinity resin in GAD purification and crystalline cellulose (Avicel) in GAD immobilization [9]. When purifying or immobilizing enzymes intended for the food and drug

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industry, the use of metal affinity resins, especially nickel-chelating resin, raised safety concern because of heavy metal toxicity. Although other immobilization methods, such as using bacterial cellulose membrane for *E. coli* GAD [10] and porous silica beads for *Lactobacillus plantarum* GAD [11] were proposed, few economical, highly effective, and safe immobilization methods were available. Regenerated amorphous cellulose (RAC) has a high adsorption capacity, and a simple, low-cost protein purification method using RAC has been developed and successfully applied for several enzymes' production [12]. However, there have been few reports on the use of this protein purification method for enzyme immobilization purpose.

In this study, a stress-inducible promoter and ultra-high-binding capacity adsorbent RAC were used in combination. A LAB-derived GAD fused with a carbohydrate-binding module was purified and immobilized simultaneously, and the fusion enzyme was used for the conversion of glutamate to GABA.

2. Materials and methods

2.1. Strains and medium

Strain YS2, isolated from Chinese traditional pickled vegetable and identified as *Lactobacillus fermentum* by 16S rRNA gene sequencing (DDBJ accession no. AB856983), was used as the source of the GAD gene *gadB*. *E. coli* DH5α was used for gene cloning and expression. MRS medium was used for growing *L. fermentum* YS2 and LB medium for *E. coli*.

2.2. Construction of expression vector for GAD immobilization

The genomic DNA of *L. fermentum* YS2 was extracted using Bacterial Genomic DNA Extraction Kit (CoWin Biosciences, Beijing, China). By searching GenBank data, two primers (Ferm-1: 5′-ATGTCACTTTACGG AAAGTACGACCAAG-3′; Ferm-2: 5′-TTAGTGGGTAAAGCCGTACTTTTTCA GG-3′) were designed to clone the *gadB* gene from the *L. fermentum* YS2 genome. The full-length *gadB* gene of *L. fermentum* YS2 was amplified using *Pfu* DNA polymerase (Sangon Biotech Co. Ltd., Shanghai, China). The PCR product of 1404 bp was treated using DNA A-tailing Kit (Takara Biotech CO. Ltd., Dalian, China), purified, and then inserted into pEASY-E1 vector (Transgen Biotech Co. Ltd., Beijing, China). The cloned gene sequence was verified by sequencing (Sangon Biotech Co. Ltd., Shanghai, China).

To construct the expression vector, a fragment containing promoter P_{rpoS}, *cbm*3 (family 3 carbohydrate-binding module from *Clostridium thermocellum*) coding sequence, and T7 terminator chemically synthesized by GenScript (Nanjing, China) was inserted into pUC57 previously digested with *Nde*I/*Hin*dIII. The resulting construct was named pRPOCB. Two primers (5′-GAATTCCTCGAGGGCTCTTCCAG-3′, 5′-GGATCCCGGTTCTTTACCCCAAACC-3′) were used for PCR to generate the linearized vector sequence, and two other primers (5′-GCCCTCGAG GAATTCTTAGTGGGTAAAGCCGTACTTTTTCAGG-3′, 5′-AAAGAACCGGG ATCCATGTCACTTTACGGAAAGTACGACCAAG-3′) were used to amplify *gadB* gene. The two PCR products were assembled into a recombinant plasmid, generating pRPOCB-*gadB*, using In-Fusion HD Cloning Kit (Takara Biomedical Technology Co., Beijing, China); finally, the vector was transformed into *E. coli* DH5α competent cells.

2.3. Expression and characterization of recombinant GAD

RAC was prepared as previously described [12]. A single colony of *E. coli* DH5α transformed by pRPOCB-*gadB* was inoculated into 20 mL LB medium containing 100 $\mu g \cdot mL^{-1}$ ampicillin and incubated at 37°C, 200 rpm. After 12 h of cultivation, the cells were reinoculated into 100 mL LB and grown overnight at 37°C, 200 rpm. The cells were harvested by centrifugation and washed, and sonication was performed at 4°C to lyse the cells. The supernatant of the cell lysate after centrifugation was mixed with RAC at room temperature for 30 min. The RAC with CBM-GAD was precipitated by centrifugation at 6000 × g, 4°C, for 20 min and then transferred to a chromatography column. After washing twice with 60 mL of 50 mM Tris–HCl buffer (pH 8.5), the RAC matrix in the chromatography column was washed with ethylene glycol, and CBM-GAD on the surface of RAC was eluted. After centrifugation, the supernatant containing CBM-GAD was dialyzed against 0.1 M sodium acetate buffer (pH 5.0) to remove ethylene glycol. Then the dialysis bag was embedded in sucrose overnight to re-concentrate the protein. GAD activity assay was performed in a volume of 0.2 mL sodium acetate buffer (0.2 M, pH 4.5) containing 10 mM L-glutamate and 0.2 mM pyridoxal 5′-phosphate (PLP), mixed with 100 μL of purified GAD solution, incubated at 40°C for 60 min, and then inactivated by boiling for 5 min. The reaction mixture was centrifuged at 12000 × g, and the GABA content in the supernatant was estimated by Berthelot reaction [13]. One unit of enzyme activity (U) was defined as the amount of enzyme that produced 1 μmol GABA in 1 h. The purity of GAD was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 12% in a Tris-glycine buffer.

The effect of pH on GAD activity was determined within a range of pH from 3.0 to 7.0. For pH stability investigation, the fusion enzyme CBM-GAD solution was incubated at 40°C at various pH values; then the remaining enzyme activity was measured as described above. The effect of temperature on GAD activity was determined at pH 4.5 and various reaction temperatures. To investigate the enzyme thermostability, the enzyme solution was pre-incubated at 30–55°C, and then the remaining enzyme activity was measured as described above. To evaluate the enzyme's dependence on PLP, 0–0.15 mM PLP was added to the reaction mixture. Moreover, 1 mM of various metal reagents was added to the reaction mixture and incubated at 40°C and pH 4.5 to investigate the effect of chemical reagents on GAD activity. The activity of the control in the absence of chemical reagents was taken as 100%. The K_m value was determined for the reaction using glutamate as the substrate under the optimal temperature and pH conditions. Each value is expressed as the mean ± the standard deviation of three independent experiments.

2.4. Reusability assay of immobilized CBM-GAD

To evaluate the reusability of immobilized CBM-GAD, approximately 2.4 mg of the enzyme adsorbed on RAC was resuspended in 5 mL of substrate solution [40 mM monosodium glutamate (MSG), 0.2 mM PLP, 0.2 M sodium acetate buffer, pH 4.5]. The reaction mixture was incubated at 40°C for 2 h and centrifuged at 6000 × g, 4°C, for 20 min to separate the product solution from the immobilized enzyme. After removing the product solution and washing with 0.2 M sodium acetate buffer (pH 4.5), the RAC matrix was repeatedly mixed with the substrate solution and incubated at 40°C for 2 h. The reaction using the immobilized enzyme was repeated 10 times. The supernatants of the reaction product and MSG and GABA standard solution were analyzed by pre-staining paper chromatography [14].

2.5. Adsorption isotherm measurements

To evaluate the binding capacity of CBM-GAD adsorbed on the RAC, the adsorption of CBM-GAD on RAC was conducted in 1.2 mL of 0.1 M sodium acetate buffer (pH 6.0) containing 0–100 μg of purified CBM-GAD and 0.2 mg of RAC for 30 min at room temperature. After centrifugation, the protein mass concentration in the supernatants was determined using Super-Bradford Protein Assay Kit (CoWin Biosciences, Beijing, China) with bovine serum albumin as the standard protein. The difference between the initial amount of protein used and the amount of supernatant protein was taken as the amount of CBM-GAD adsorbed onto RAC. Each measurement was performed in triplicate.

2.6. Production of GABA using the immobilized enzyme

To examine the usefulness of the immobilized enzyme for GABA formation, approximately 0.2 g of RAC carrying CBM-GAD was placed at the bottom of a 250-mL flask. Then, 100 mL substrate solution (80 mM MSG, 0.2 mM PLP, 0.2 M sodium acetate buffer, pH 4.5) was added to the container and incubated at 40°C for 100 min. The time at the addition of substrate was taken as *t*0 in all cycles. A sample of 1 mL was collected from the container for GABA analysis every 10 min. After one cycle ended, all reaction solution was centrifuged to recover RAC; this recovered RAC was placed in flask, and new substrate solution was added for the next cycle of GABA bioconversion.

3. Results and discussion

3.1. Cloning of L. fermentum gadB gene

The recombinant vector pEASY-*gadB* was transformed into *E. coli* DH5α competent cells. The sequence of gene *gadB* was determined and deposited in DDBJ under the accession no. AB856984. The gene *gadB* encoded a protein of 467 amino acid residues with a calculated molecular weight of 54.1 kDa. Multiple alignment of this GAD amino acid sequence with other bacterial GADs indicated that it has high identity with the GAD of *Lactobacillus gastricus* (84%), *Lactobacillus oris* (78%), *Lactobacillus antri* (78%), *Enterococcus faecium* DO (77%), *Lactobacillus reuteri* (77%), *Lactobacillus suebicus* (76%), and *Enterococcus avium* G-15 (76%). The amino acid sequence contained a highly conserved lysine residue (K278), which is essential for pyridoxal 5′-phosphate binding, and the active site residues T214 and D245, both of which promote decarboxylation [15].

3.2. Construction of expression vector

The expression vector pRPOCB contained a stress-inducible promoter P_{rpoS}, CBM3 coding sequence, and T7 terminator. Promoter P_{rpoS} (743 bp) including the 5′-untranslated re-regulation region belongs to the transcription and regulation system RpoS, which played an important role in the bacterial response to stress conditions [16]. Unlike other inducible promoters such as the widely used IPTG-inducible promoters, promoter P_{rpoS} is independent of any additional inducer, and it could efficiently drive the expression of heterologous proteins during the exponential growth phase. Given the high cost of the widely used inducer IPTG, the use of a promoter independent of inducer for protein expression was thought to lower the cost. CBM3, a powerful protein separation tag, was used for one-step purification and immobilization in this study. Its coding sequence was placed immediately downstream of promoter P_{rpoS}. *L. fermentum* YS2 *gadB* gene was inserted into pRPOCB, between the CBM3 coding sequence and T7 terminator (Fig. 1).

3.3. Expression and immobilization of GAD

The fusion protein CBM-GAD was expressed under the control of P_{rpoS} in *E. coli* DH5α and adsorbed on RAC. After adsorption and elution, a clear band of the fusion enzyme CBM-GAD was visualized on SDS-PAGE (Fig. 2). The molecular weight of the fusion enzyme was approximately 71 kDa, which was consistent with the calculated total molecular weight of CBM3 (17.7 kDa) and GAD (54 kDa). Almost no other bands were observed on SDS-PAGE. In some cases, when the recombinant protein is expressed in a fusion form, an appropriate linker sequence between the tag and the target protein is necessary to prevent proteolytic cleavage so that the fusion protein remains intact. For instance, a S_3N_{10} peptide prevents the proteolysis of *E. coli* GAD fused with CBD from *Trichoderma harzianum* [9]. In this study, although the CBM3 coding sequence and *gadB* gene were linked directly without any additional linker sequence, it seemed that the fusion protein was not affected by proteolysis, as seen on SDS-PAGE.

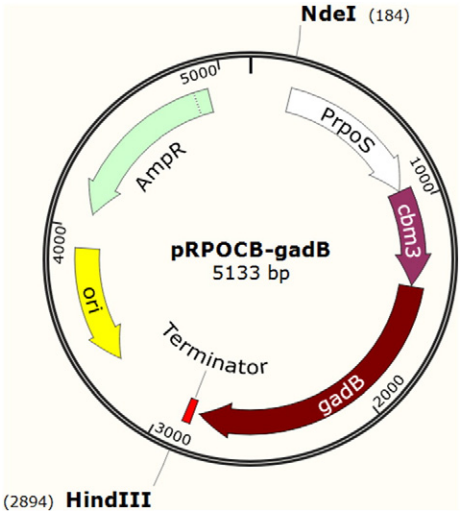

Fig. 1. Map of the expression vector pRPOCB-*gadB*. P_{rpoS}: a stress-inducible promoter including the 5′-untranslated re-regulation region in *E. coli*; *cbm3*: coding sequence of family 3 carbohydrate-binding module from *C. thermocellum*; *gadB*: *gadB* gene from *L. fermentum* YS2.

After elution, the specific activity of the fusion protein CBM-GAD reached 83.6 ± 0.7 U mg^{-1}. From 200 mL of *E. coli* broth, the yield of purified CBM-GAD was 3.3 ± 0.6 mg. The result was similar to that of a previous study, in which 13 mg of recombinant GAD was purified from 1000 mL of *E. coli* culture [17]. The apparent K_m was 10.6 mM when glutamate was used as the substrate. Like many other GADs from microorganisms, the maximum activity of the GAD of *L. fermentum* YS2 was observed in acidic condition at pH 4.5 (Fig. 3). Among the tested pH ranges, the enzyme was also most stable at pH 4.5. The major reason may be closely related to the functional role of bacterial GADs: maintenance of intracellular neutral pH against external acidic environment. The enzyme showed highest activity at 40°C (Fig. 5), which is higher than that of GAD from *L. brevis* IFO 12005 [18] but lower than those of other GADs from LAB strains such as *L. brevis* 877G [19], *L. brevis* CGMCC 1306 [17], *L. paracasei* NFRI 7415 [20], and *Streptococcus salivarius* ssp. *thermophilus* Y2 [21]. As Fig. 4 indicates, although the optimal reaction temperature was 40°C, the enzyme retained approximately 78% of its initial activity after 6 h at 30°C; this implies that under lower temperature, the enzyme was more stable. In addition, PLP was essential for enzyme activity (Fig. 5). Like many other bacterial GADs, without PLP, the enzyme exhibited almost no activity.

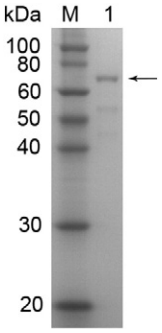

Fig. 2. SDS-PAGE analysis of purified fusion protein CBM-GAD. Lane M: Protein Marker. Lane 1: GAD. The CBM-GAD band is indicated by an arrow. The gel was stained with Coomassie Brilliant Blue.

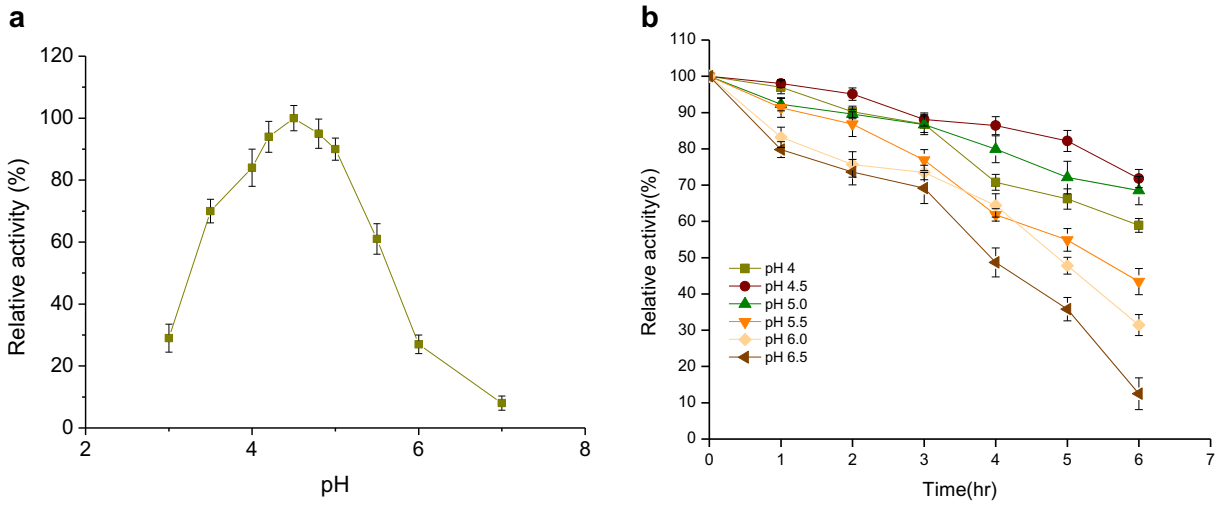

Fig. 3. Effect of pH on (a) enzyme activity and (b) stability. The initial activity before pre-incubation was taken to be 100%.

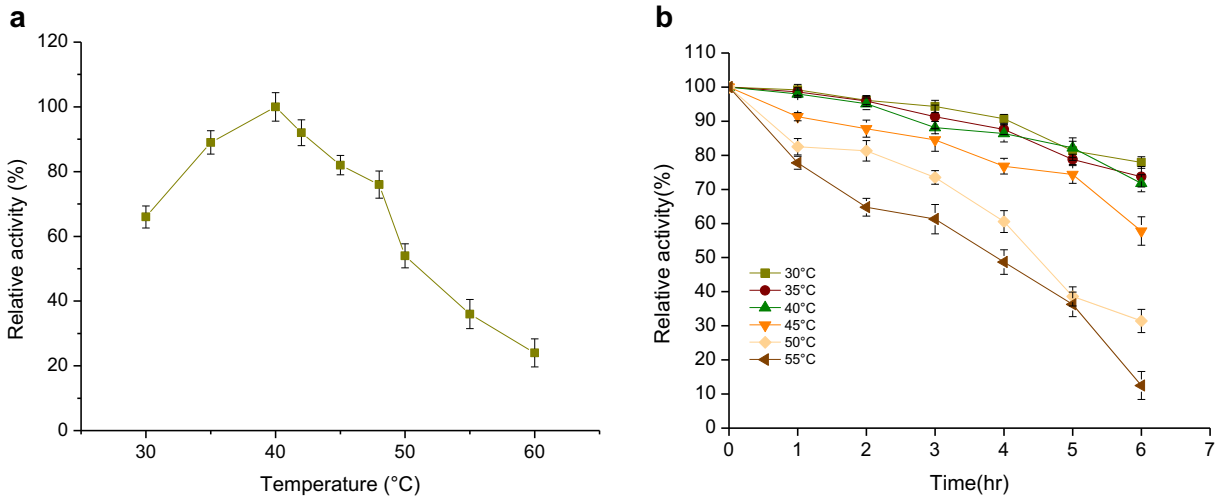

Fig. 4. Effect of temperature and thermostability. (a) Effect of temperature on enzyme activity. (b) Thermostability. The initial activity before pre-incubation was taken to be 100%.

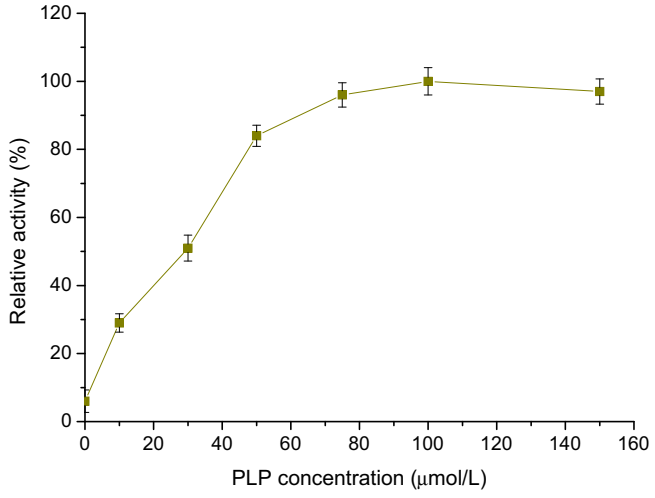

Fig. 5. Effect of PLP on enzyme activity. The enzyme activity with 100 μM PLP was taken to be 100%.

Different metal ions displayed different effects on GAD activity (Fig. 6). Among the tested ions, Cu^{2+}, Fe^{2+}, Fe^{3+}, and Ag^{+} could strongly inhibit the enzyme activity, whereas Ca^{2+} and Mg^{2+} showed positive effect. Similarly, Ca^{2+} has been reported to stimulate GAD from *L. brevis* 877G [19] and *L. paracasei* NFRI 7415 [20]. Moreover, it has been reported that GAD from plants could bind calmodulin, and the activity could be strongly increased with the addition of Ca^{2+} [22]. In contrast, Ca^{2+} had little effect on GAD from *S. salivarius* ssp. *thermophilus* Y2 [21], which might be explained by differences in the gene sequence and enzyme structure. Further investigation indicated that the optimal concentrations of Ca^{2+} and Mg^{2+} were 2.5 and 4.0 mM, respectively. The positive effect of metal ions could be used to enhance GAD activity and GABA productivity.

3.4. Reusability and adsorption isotherm of immobilized enzyme

The RAC matrix carrying the fusion protein was used as an immobilized enzyme to convert MSG to GABA, and this was repeated 10 times (Fig. 7). In the first and second rounds of reaction, the immobilized enzyme could convert almost all the substrate to GABA.

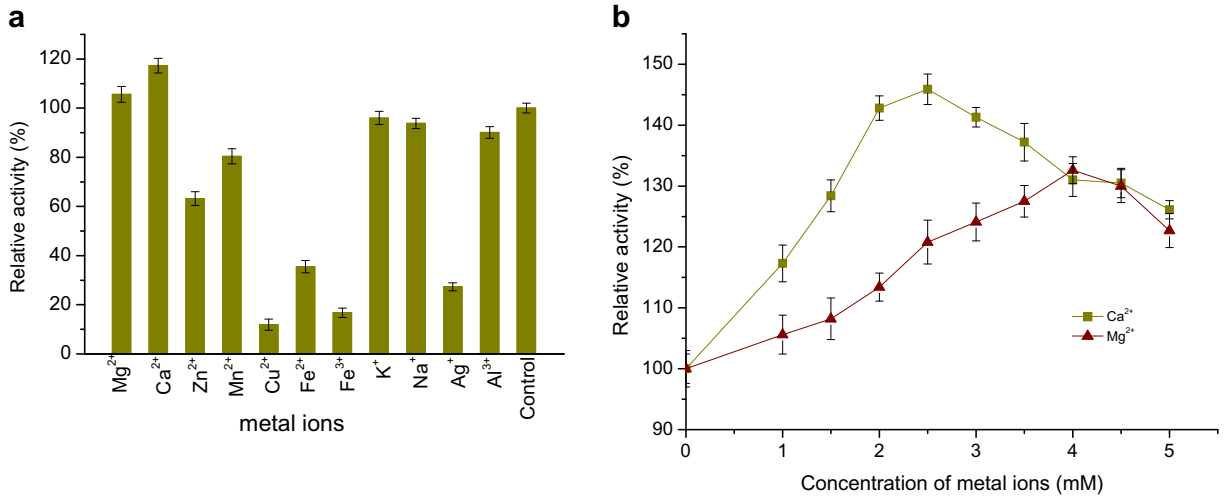

Fig. 6. Effect of (a) different metals on enzyme activity and (b) different concentrations of Ca^{2+} and Mg^{2+} on GAD activity.

Nevertheless, the enzyme activity significantly decreased after several cycles, and in the 10th cycle, the immobilized enzyme retained only 8% of its initial activity, far below that of *E. coli*-derived immobilized GAD [9].

After purification, the protein was used to measure the adsorption isotherm of CBM-GAD on RAC (Fig. 8). Although the stability of GAD from *L. fermentum* was relatively low, RAC was shown to adsorb target protein efficiently. The binding capacity of 174 ± 8 mg·g^{-1}, *i.e.*, 2.4 ± 0.1 μmol·g^{-1}, observed in this study was much higher than that in a previous report, in which the cellulose-binding capacity was 33 ± 2 nmol·g^{-1} when *E. coli* GAD was immobilized onto Avicel [9]. The difference might be explained as follows: RAC has a much greater surface area, and its binding surface was external, whereas most of the binding surface of commercial microcrystalline cellulose was internal [12].

3.5. Production of GABA by the immobilized enzyme

GABA production by *in vitro* enzymatic conversion was carried out, and GABA yield of 4 cycles is demonstrated in Fig. 9. The maximum yield of GABA reached 5.15 g/L (62.4% molar conversion) after the first run, and the highest productivity of 3.09 g/L·h was achieved at 100 min in the first run. The productivity was lower than that of other reported immobilized GADs, *e.g.*, 6.03 g/L·h of immobilized GAD from *E. coli* with bacterial cellulose membrane [10] and 6.3, 41.7, and 26.5 g/L·h of immobilized GAD from *L. plantarum*, which corresponds to 10, 80, and 150 mM of initial MSG concentrations [11]. After the fourth run, because of the loss of enzyme activity, the yield of GABA fell by 21.6%, which is consistent with the results of the thermostability test. Nevertheless, these results indicate that the GAD

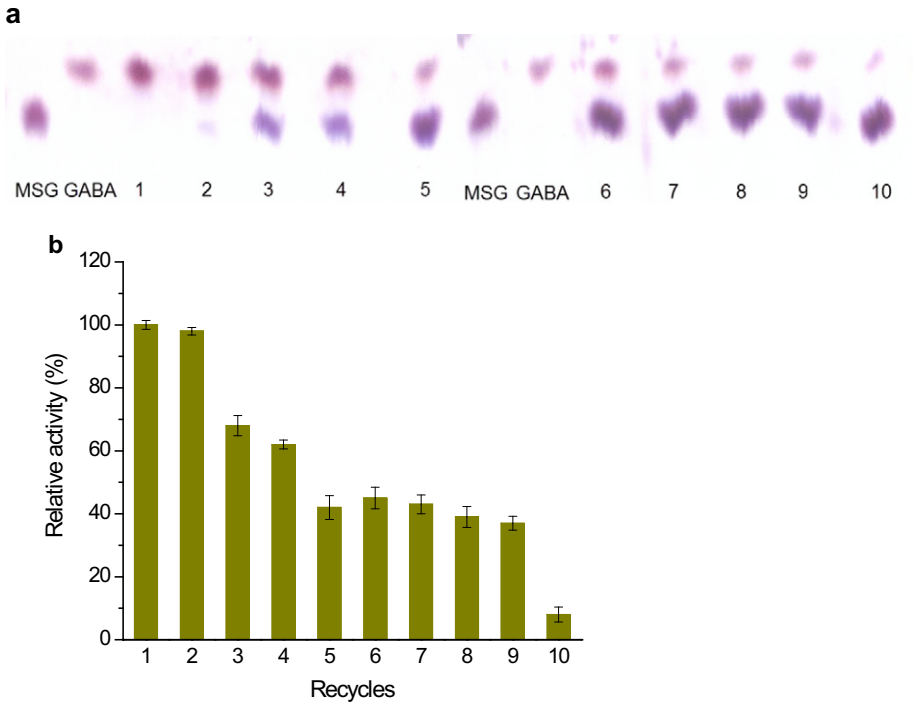

Fig. 7. Pre-staining paper chromatography of immobilized GAD reaction product (a) and relative activity of GAD immobilized on RAC after multiple uses (b). CBM-GAD adsorbed onto RAC was mixed with substrate solution (40 mM MSG, 0.2 mM PLP, 0.2 M sodium acetate buffer, pH 4.5) and was incubated at 40°C for 2 h. Then the product solution was removed, and the RAC matrix was mixed with the substrate solution. The immobilized enzyme reaction was repeated 10 times.

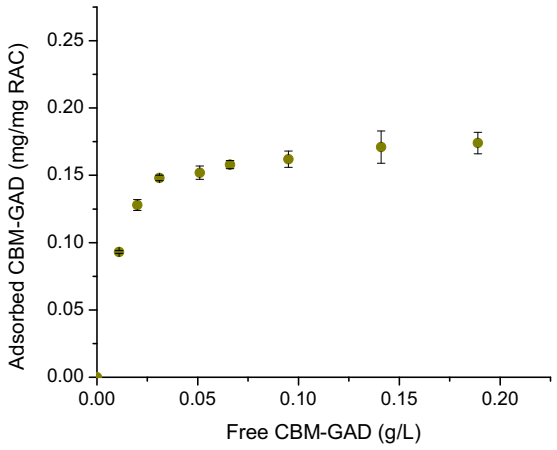

Fig. 8. Adsorption isotherm for the binding of CBM-GAD to RAC. The difference between the initial amount of protein used and the amount of supernatant protein was taken as the amount of CBM-GAD adsorbed onto RAC.

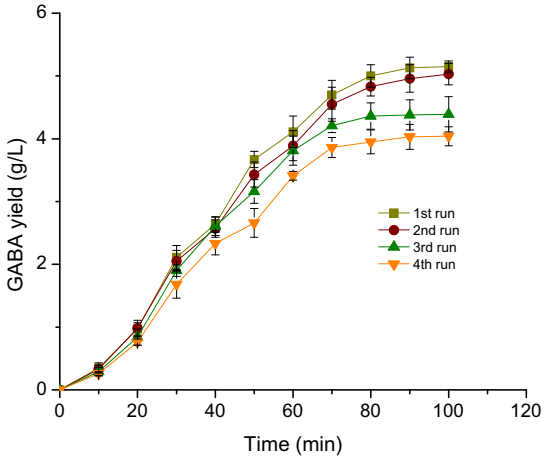

Fig. 9. Biosynthesis of GABA from MSG catalyzed by immobilized GAD at 40°C.

immobilized on RAC can successfully catalyze the conversion of MSG to GABA.

For decades, IPTG-inducible promoters have been widely used in protein expression, but they are not perfectly suitable for food and drug industry because of several disadvantages: IPTG is expensive, leading to high cost of production; IPTG is toxic to cells and may inhibit cell growth; and the timing of inducer addition is not well controlled. Promoter P_{rpoS} used in this study not only avoids the addition of IPTG but also results in a high protein expression level, which is comparable with that of a study using T7 promoter [17]. In addition, the cost of adsorbent RAC is far below that of nickel-chelating resin, which has been widely used in protein purification and causes heavy metal toxicity. In protein purification and immobilization using RAC, there is no toxic chemical involved, and this is favorable for food and drug production purpose. In this study, the fusion enzyme was unstable under experimental conditions, and the stability could be improved by site-directed mutagenesis in further studies; nevertheless, the result provides a possible way to prepare immobilized GAD by a simple and low-cost method with high adsorption capacity for GABA production. Moreover, one-step purification and immobilization of LAB-derived GAD could be useful for industrial applications.

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Conflict of interest

The authors declare no conflict of interest.

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