J Ind Microbiol Biotechnol (2013) 40:927–933
DOI 10.1007/s10295-013-1289-z

METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY

Efficient gamma-aminobutyric acid bioconversion by employing synthetic complex between glutamate decarboxylase and glutamate/GABA antiporter in engineered *Escherichia coli*

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Received: 7 March 2013 / Accepted: 10 May 2013 / Published online: 2 June 2013

Abstract Gamma-aminobutyric acid (GABA) is a precursor of one of the most promising heat-resistant biopolymers, Nylon-4, and can be produced by the decarboxylation of monosodium glutamate (MSG). In this study, a synthetic protein complex was applied to improve the GABA conversion in engineered *Escherichia coli*. Complexes were constructed by assembling a single protein–protein interaction domain SH3 to the glutamate decarboxylase (GadA and GadB) and attaching a cognate peptide ligand to the glutamate/GABA antiporter (GadC) at the N-terminus, C-terminus, and the 233rd amino acid residue. When GadA and GadC were co-overexpressed via the C-terminus complex, a GABA concentration of 5.65 g/l was obtained from 10 g/l MSG, which corresponds to a GABA yield of 93 %. A significant increase of the GABA productivity was also observed where the GABA productivity increased 2.5-fold in the early culture period due to the introduction of the synthetic protein complex. The GABA pathway efficiency and GABA productivity were enhanced by the introduction of the complex between Gad and glutamate/GABA antiporter.

Keywords Gamma-aminobutyric acid · Glutamate decarboxylase · Glutamate/GABA antiporter · Synthetic protein complex

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Introduction

Considering the limited supply and elevating costs of fossil resources, biomass-based production of energy carriers, chemicals, and polymers is becoming a new paradigm in biotechnology research. To replace the need for polystyrene or polyethylene-based plastics, several biomass-based polymers such as polylactic acid, poly-3-hydroxybutyrate, and poly(butylenes succinate) have been intensively studied. Another promising bio-based biodegradable polymer is polyamide 4, also known as Nylon-4, which is composed of a repeating gamma-aminobutyric acid (GABA) unit [9].

In *Escherichia coli*, the GABA pathway, which is constituted with glutamate decarboxylase (Gad) and glutamate/GABA antiporter, controls the acidification in the cytosolic environment through decarboxylating an acidic substrate (glutamate) into a neutral compound (GABA) [3, 8]. Glutamate decarboxylase (GadA and GadB) catalyzes the irreversible decarboxylation of L-glutamate to GABA. GABA is exported into the extracellular medium and glutamate is brought into the cell by glutamate/GABA antiporter (GadC), contributing to local alkalization of the extracellular environment [1].

Considering the industrial importance of GABA, various studies have been conducted to improve GABA production in recombinant bacteria. By the overexpression of rice Gad in *Bifidobacterium longum*, 0.1 g/l GABA was produced

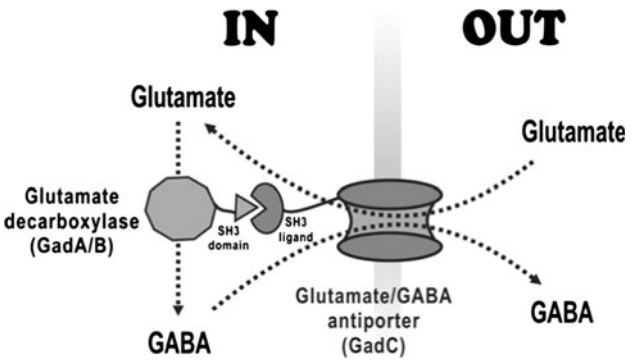

Fig. 1 Schematic diagram of GABA conversion in recombinant *E. coli*. Glutamate decarboxylase (GadA/GadB) and the glutamate/GABA antiporter (GadC) were overexpressed

from 30 g/l monosodium glutamate (MSG) [7]. When *Lactobacillus brevis* Gad was overexpressed in *Bacillus subtilis*, 0.4 g/l GABA was produced from 30 g/l MSG [8]. It was also reported that overexpression of Gad from *Lactobacillus plantarum* ATCC 14917 in *Lactobacillus sakei* B2-16 elevated the final GABA concentration 1.4-fold [4]. When *E. coli* Gad and glutamate/GABA antiporter were co-overexpressed in a GABA aminotransferase knockout *E. coli* strain, a final GABA concentration of 5.46 g/l was obtained from 10 g/l MSG, which corresponds to a GABA yield of 89.5 % [10].

A synthetic protein complex is a novel synthetic biology strategy which co-localizes pathway enzymes consisting of metabolic pathway. Pathway enzymes are tied up using protein–protein interaction domains and their specific ligands. Because pathway enzymes catalyzing successive reactions are co-localized in a specific space, a reactant can be converted to the product more efficiently with less enzymes. Therefore, the metabolic flux can be enhanced with less metabolic burden via the introduction of a synthetic protein complex [2]. When three mevalonate biosynthetic enzymes were tied up by a synthetic protein complex, the final product concentration increased 77-fold with a low enzyme expression level [2]. In the case of D-glucaric acid, the introduction of a synthetic complex led to a five-fold improvement of the product concentration [6]. In this study, a complex strategy was employed to enhance the GABA production in recombinant *E. coli*. Glutamate decarboxylase and glutamate/GABA antiporter were co-overexpressed with the complex device in engineered *E. coli* (Fig. 1).

Materials and methods

Bacterial strains, plasmids, and medium

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* XL1-Blue (XB) was used as the host strain. pGEMT vector (Promega, Madison, WI, USA) was used for DNA cloning and pMAL-p4X (New England Biolabs, Ipswich, MA, USA) was used to construct the expression plasmids. Chemically competent cells were prepared using a standard procedure and were stored at −80 °C until needed.

E. coli strains were cultured at 37 °C and 250 rpm in Luria–Bertani (LB) broth (10 g/l bacto-tryptone, 5 g/l bacto-yeast extract, and 5 g/l NaCl) supplemented with 100 μg/ml ampicillin sodium salt for the selection of transformants harboring recombinant plasmids.

Cloning of gad genes

gadA, *gadB*, and *gadC* genes were amplified using the Expand high-fidelity polymerase chain reaction (PCR) system (Roche Molecular Biochemicals, Mannheim, Germany) from XB chromosomal DNA. SH3 domain and SH3 ligand genes were PCR amplified from the pJD758 plasmid, which was kindly provided by Professor John E. Dueber [2, 6]. Then, the SH3 domain gene was fused to the C-terminus of *gadA* and *gadB* by overlap PCR to make *gadA-SH3D* and *gadB-SH3D*, respectively. The SH3 ligand was attached to the N-terminus, C-terminus, and 233rd amino acid residue of the *gadC* gene to make *gadCN-SH3L*, *gadCC-SH3L*, and *gadCS-SH3L*, respectively (Fig. 2). The PCR primers used in this study are listed in Table 2. The PCR products were purified using a GENE-ALL PCR purification kit (General Biosystem, Seoul, Korea) and cloned into the pGEMT vector. The *gadA-SH3D* and *gadB-SH3D* genes were cloned into pMAL-p4X using NdeI and BamHI restriction sites to construct the pHAD and pHBD plasmids, respectively. The *gadCN-SH3L* gene was cloned downstream of *gadA-SH3D* and *gadB-SH3D* genes using BamHI and XbaI restriction sites to construct the pH1AC and pH1BC expression plasmids, respectively. The *gadCS-SH3L* gene was cloned into pHAD and pHBD to construct the pH2AC and pH3BC expression plasmids, respectively. The *gadCC-SH3L* gene was cloned downstream of *gadA-SH3D* and *gadB-SH3D* genes using BamHI and XbaI restriction sites to construct the pH3AC and pH3BC expression plasmids, respectively.

GABA bioconversion and analysis

Recombinant strains were cultivated in 250 ml flasks in 100 ml LB (100 μg/ml ampicillin added) containing 10 g/l MSG at 37 °C and 250 rpm. When the OD_{600} reached 1.2, gene expression was induced with 0.5 mM IPTG. Then, the pH was adjusted to 3.5 and the strain was incubated at 30 °C and 250 rpm for 48 h.

The GABA bioconversions were quantitatively analyzed by HPLC using an OptimaPak C18 column (4.6 × 150 mm)

Table 1 List of bacterial strains and plasmids used in this study

Strain, plasmid	Genotype and/or property	Reference
Escherichia coli strains		
XL1-Blue	SupE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lacF'[proAB^{+}lacI^{q}lacZΔM15 Tn10 (tet^{R})]	Laboratory stock
Plasmid		
pGEMT	Ap^{R}	Promega (USA)
pMAL-p4X	Ap^{R}	New England Biolabs (USA)
pJD758		Moon et al. [6]
pHAC	pMAL-p4X containing *gadA* and *gadC*	Vo et al. [10]
pHBC	pMAL-p4X containing *gadB* and *gadC*	Vo et al. [10]
pHAD	pMAL-p4X containing *gadA-SH3D*	This work
pHBD	pMAL-p4X containing *gadB-SH3D*	This work
pH1AC	pMAL-p4X containing *gadA-SH3D* and *gadC-SH3L*, N-terminal model	This work
pH1BC	pMAL-p4X containing *gadB-SH3D* and *gadC-SH3L*, N-terminal model	This work
pH2AC	pMAL-p4X containing gadA-SH3D and gadC-SH3L, sandwich model	This work
pH2BC	pMAL-p4X containing gadB-SH3D and gadC-SH3L, sandwich model	This work
pH3AC	pMAL-p4X containing gadA-SH3D and gadC-SH3L, C-terminal model	This work
pH3BC	pMAL-p4X containing gadB-SH3D and gadC-SH3L, C-terminal model	This work

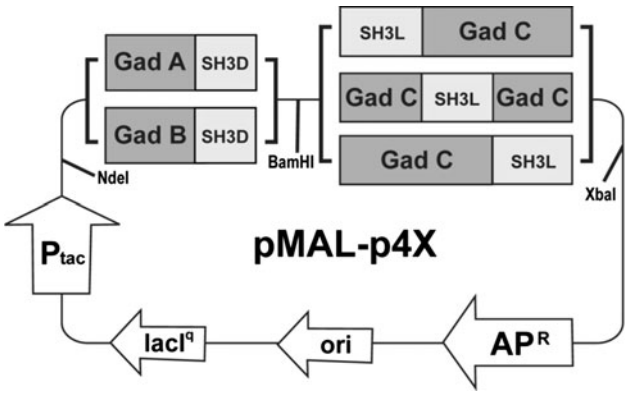

Fig. 2 Schematic maps of *gadABC* complex plasmids. Two glutamate decarboxylase (GadA and GadB) and three glutamate/GABA antiporter-SH3 ligand models were tested in combination

(RS tech Corporation, Daejeon, Korea). The sample preparation was performed as follows. Samples were centrifuged at 12,000 rpm for 5 min and then 100 μl of the supernatant was added to an Eppendorf tube. Next, 200 μl of a 1 M sodium bicarbonate buffer at a pH of 9.8, 100 μl of 80 g/l dansyl chloride in acetonitrile, and 600 μl of double-distilled water were added to make a 1 ml reaction mixture. The mixture was then incubated at 80 °C for 40 min. Subsequently, 100 μl of 20 μl/ml acetic acid was added to stop the reaction. The mixture was then centrifuged at 12,000 rpm for 5 min. Next, the supernatant was filtered through a 0.22 μm Millipore filter and analyzed by HPLC on an Agilent system using UV detection. Separation of the derivatized samples was performed using a binary non-linear gradient with eluant A [tetrahydrofuran/methanol/50 mM sodium acetate with a pH of 6.2 (5:75:420, by volume)] and eluant B (methanol). The column temperature was set at 30 °C and the elution conditions were as follows: equilibration (6 min, 20 % B), gradient (20 min, 20–80 % B), and cleaning (3 min, 100 % B). The flow rate of the mobile phase was 1 ml/min and the samples were detected at a wavelength of 286 nm in the UV region. The standard curve for GABA was generated from 10 standard solutions (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 g/l GABA) (Sigma, St. Louis, MO, USA) using the same procedure.

Results

Plasmid construction, expression of gad genes, and complex

In this study, for more efficient production of GABA, a synthetic protein complex strategy was applied to the GABA pathway. The SH3 protein–protein interaction domain was attached to the C-terminus of Gads (GadA and GadB) via a nine-residue glycine-serine linker [6]. Its cognate SH3 peptide ligand was attached to the N-terminus, C-terminus, and 233rd amino acid residue of glutamate/GABA antiporter (GadC) via an 11-amino-acid linker (Fig. 2) [6]. The GadC-SH3 ligand fusion protein was designed considering the GadC structure (Fig. 3) [5]. Since the N- and C-terminuses and the 233rd amino acid residue of GadC are in the cytoplasm of *E. coli* cells, the SH3 ligand that attaches those three positions is located in the cytoplasm. Via binding of the SH3 domain and SH3 ligand,

Table 2 Primers used for PCR in this study

Primer	Sequence	Gene
Forward	5′-GGAATTCCATATGATGGACCAGAAGCTGTTAA-3′	*gadA*
Reverse	5′-GGGCCCGCACATACTCTGGCTGCCGCCGCTGCCGCTGCCGCTGCCGGTGTGTTTAAAGCTGTTCTG-3′	
Forward	5′-GGAATTCCATATGATGGATAAGAAGCAAGTAACG-3′	*gadB*
Reverse	5′-GGGCCCGCACATACTCTGGCTGCCGCCGCTGCCGCTGCCGCTGCCGGTATGTTTAAAGCTGTTCT-3′	
Forward	5′-ATGGCTACATCAGTACAGA-3′	*gadC*
Reverse	5′-GCTCTAGATTAGTGTTTCTTGTCATTCATC-3′	
Forward	5′-CAGAGTATGTGCGGGCCC-3′	SH3 domain
Reverse	5′-CGCGGATCCTTAATACTTCTCCACGTAAGG-3′	
Forward	5′-CGGGATCCTTCACCAACAAGGACCATAGCATGCTGGAAGGCTCTGGCT-3′	SH3 ligand
Reverse	5′-TCTGTACTGATGTAGCCATGCCGCTGCCGCTGCCGCCGCCGCTGCCTTCCAGGCCCGGACGACGACGT-3′	

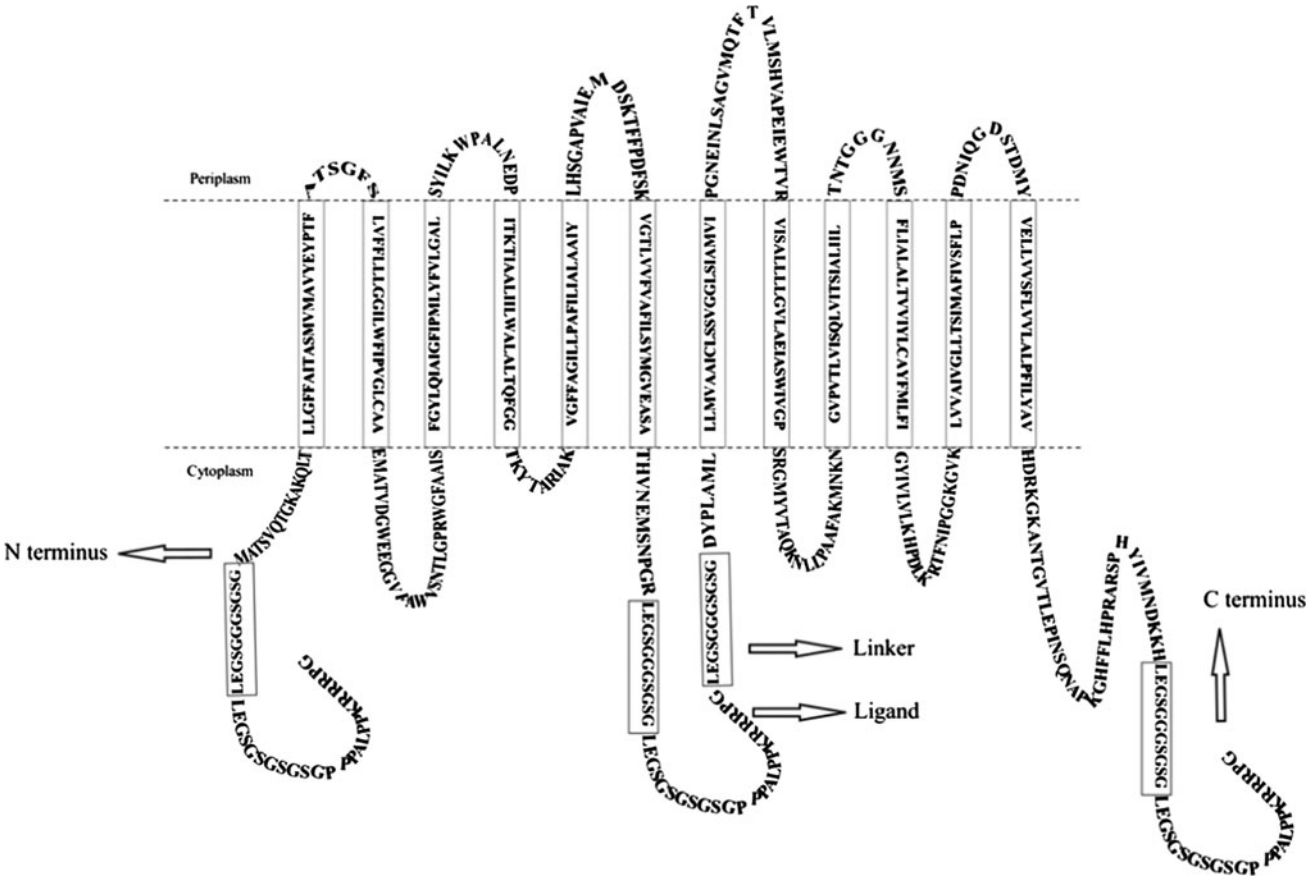

Fig. 3 Fusion models of the glutamate/GABA antiporter (GadC) with a SH3 ligand via an 11-amino-acid linker (*in box*) at three positions: N-terminus, the 233rd amino acid residue, and C-terminus

GadA/B and GadC will be located closely and more efficient GABA production is expected.

To evaluate the effects of Gad and the location of the SH3 ligand, two Gads and three GadC-SH3 ligand fusion proteins were tested in combination. For this, six expression plasmids were constructed, as mentioned in the Materials and methods section. The overexpression of the GadA-SH3 domain, GadB-SH3 domain, and GadC-SH3 ligand were analyzed by SDS-PAGE (Fig. 4).

GABA bioconversion

The results of the GABA conversion from MSG by various recombinant strains containing GadABC synthetic complex

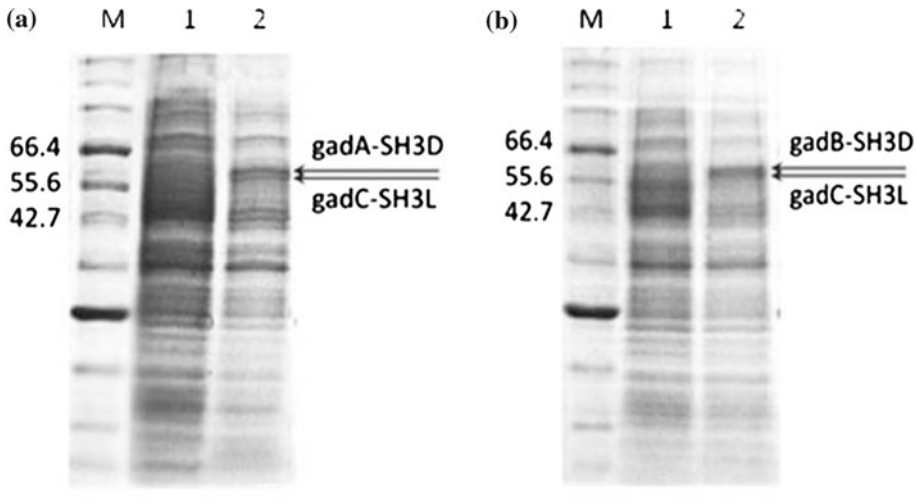

Fig. 4 Results of the SDS-PAGE analysis of the overexpression of gad genes and complex device where *M* is the marker protein, *lane 1* is the cell extract of the untransformed strain, and *lane 2* is the cell extract of strains harboring **a** pH1AC (GadA-SH3D and GadC-SH3L) and **b** pH1BC (GadB-SH3D and GadC-SH3L). (GadA-SH3D: 60.12 kDa, GadB-SH3D: 60.1 kDa, and GadC-SH3L: 58.36 kDa)

Table 3 Final GABA concentrations (g/l) obtained from the conversion

Enzymes	Complex type			
	w/o complex	N-terminus	Sandwich	C-terminus
GadA and GadC	4.34 ± 0.17	4.94 ± 0.37	5.06 ± 0.76	5.65 ± 0.04
GadB and GadC	4.51 ± 0.61	4.84 ± 0.38	4.74 ± 0.25	4.65 ± 0.75

are summarized in Table 3. When GadA/B and GadC were not overexpressed, GABA was not produced (data not shown). When normal Gad and glutamate/GABA antiporter were overexpressed without a synthetic complex, about 4.5 g/l GABA was produced from 10 g/l MSG. An increase of the final GABA concentration was observed regardless of the type of complex when a synthetic complex was introduced to GadB and GadC. By introduction of the N-terminus synthetic complex to GadA and GadC, the final GABA concentration increased to 4.9 g/l. The maximum GABA concentration of 5.65 g/l was obtained with the C-terminus synthetic complex of GadA and GadC, which is 30 % higher than that obtained with normal GadA and GadC (Table 3). Based on the higher GABA concentration, it can be deduced that more glutamate, which can be used for other metabolites, was converted to GABA by complex of Gad and glutamate/GABA antiporter.

To investigate the effects of the synthetic protein complex on the GABA production in detail, the time profiles of the GABA production were analyzed. First, the complexes of GadA and GadC were tested (Fig. 5). All three types of complexes provided higher GABA concentrations than that obtained without a complex during the overall culture period. This result suggests that more carbon flux was directed into the GABA pathway than other pathways. Besides the higher final GABA concentration, the other notable result was the higher GABA productivity. During the first 12 h of the culture time, about 3 g/l GABA was produced by the GadA-GadC complex-employed recombinant strains while less than 2 g/l GABA was produced by *E. coli* (pHAC) in which GadA and GadC were simply overexpressed. With *E. coli* (pHAC), a GABA concentration of about 3 g/l was obtained after a culture time of 30 h (Fig. 5). Based on this result, it can be deduced that the GABA productivity increased 2.5-fold in the early culture period due to the introduction of the synthetic protein complex between GadA and GadC. When GadA and GadC were bound by the C-terminus complex, the highest GABA concentration of 5.7 g/l was obtained after a culture time of 48 h along with a GABA yield of 93 %.

Secondly, the effect of the GadB-GadC complex on GABA production was investigated (Fig. 6). Similar to the GadA-GadC complex results, a higher GABA concentration was obtained with the introduction of the GadB-GadC complex in the early stage of the culture. *E. coli* (pH1BC) containing the C-terminus GadB-GadC complex produced 3.2 g/l GABA during the first 12 h of the culture while about 1.5 g/l GABA was produced by complex-unincorporated *E. coli* (pHBC). To achieve a GABA concentration of 3.0 g/l with *E. coli* (pHBC), a culture time of more than 30 h was required. This result suggests a higher GABA productivity by using complex-introduced recombinant strains than the simple overexpression strain.

Produced GABA has two fates as it can either be directed to the TCA cycle by the function of the GABA aminotransferase and succinate-semialdehyde dehydrogenase or secreted to the media via GadC. To see if GABA was directed back to the TCA cycle by GABA aminotransferase (GabT), GabT knock-out *E. coli* strains (XBT) harboring pH1AC and pH1BC were tested. The knock-out *E. coli* XBT (pH1AC) and XBT (pH1BC) strains produced lower

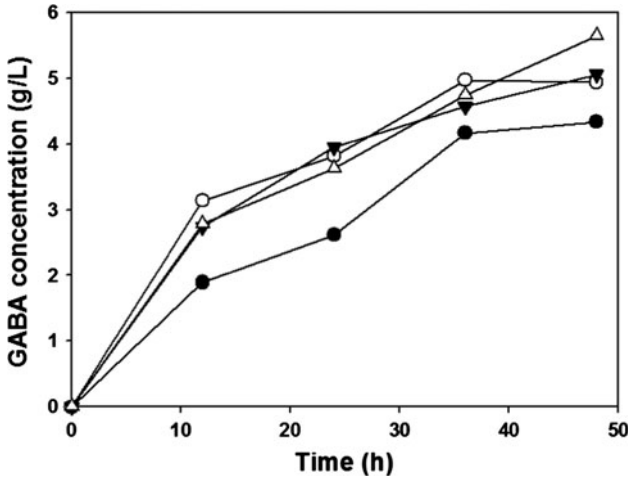

Fig. 5 Time profiles of the GABA concentration obtained with and without GadA and GadC complexes: XB (pHAC) (*filled circle*, without complex), XB (pH1AC) (*unfilled circle*, N-terminus complex), XB (pH2AC) (*inverted triangle*, Sandwich complex), and XB (pH3AC) (*unfilled square*, C-terminus complex)

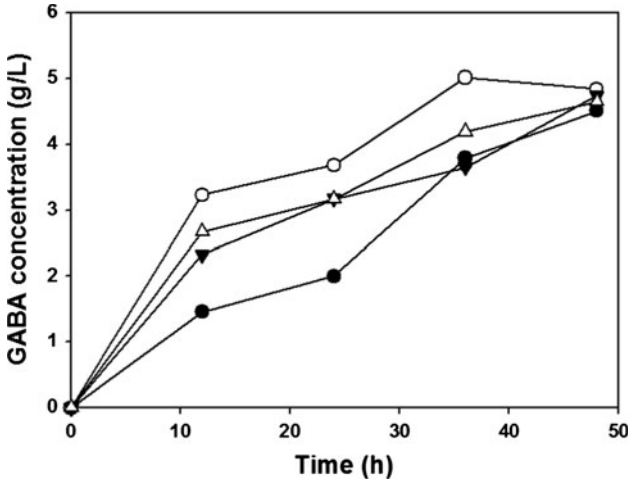

Fig. 6 Time profiles of the GABA concentration obtained with and without GadB and GadC complexes: XB (pHBC) (*filled circle*, without complex), XB (pH1BC) (*unfilled circle*, N-terminus complex), XB (pH2BC) (*inverted triangle*, Sandwich complex), and XB (pH3BC) (*unfilled square*, C-terminus complex)

amounts of GABA when compared to normal *E. coli* strains harboring the same plasmids (data not shown). This result suggests that produced GABA is not redirected to TCA cycle by GabT and knock-out of GabT has little effect on GABA production.

Discussion

In this study, glutamate decarboxylase (GadA, GadB) and a glutamate/GABA antiporter (GadC) were co-overexpressed with a synthetic protein complex to improve the GABA production. When GadA and GadC were bound by a C-terminus complex, a GABA concentration of 5.65 g/l was obtained from 10 g/l MSG, which corresponds to a GABA yield of 92.6 %. The GABA productivity increased 2.5-fold in the early culture period by the introduction of a synthetic protein complex between the Gad and glutamate/GABA antiporter.

These results demonstrate the practical benefit of complex which results in improved pathway efficiency. Besides the increase of the final GABA concentration, the introduction of a synthetic protein complex resulted in a huge increase of the GABA productivity, especially in the early culture period. The higher productivity may be due to a shortened GABA reaction path. In the normal strain, once glutamate is imported into the cytoplasm via the glutamate/GABA antiporter, it has to travel all around the cytoplasm until it meets Gad to be converted to GABA. Then, GABA goes to the glutamate/GABA antiporter and is secreted to the media. With the introduction of a protein complex, however, Gad and glutamate/GABA antiporter were closely bound. Imported glutamate is immediately converted to GABA by Gad which is located right next to the glutamate/GABA antiporter via the protein complex. Then, GABA will be secreted via the nearby glutamate/GABA antiporter. Therefore, glutamate can be more effectively converted to GABA by the introduction of a synthetic complex between Gad and the glutamate/GABA antiporter, resulting in a higher GABA productivity.

A decrease of GAGA productivity was observed at the later culture period. At the early culture period, MSG can be efficiently converted to GABA via combined Gad and the glutamate/GABA antiporter, and high GABA productivity can be achieved. At the later culture period, however, MSG concentration became low, and it led to low GABA productivity. Especially, about 93 % of GABA yield was achieved in our system which suggested that only little MSG left at later culture period, and GABA productivity became low.

Considering the efficiency and cost-effectiveness of the GABA production process, productivity is one of the key factors as well as the final GABA concentration, as a high productivity results in a shorter processing time and lower production costs. Our study clearly shows that the pathway efficiency and productivity can be improved by the introduction of a synthetic protein complex. We believe that the synthetic complex strategy can be applied to other metabolite producing systems besides GABA to result in improved productivity and process efficiency. Thus, the results of this study will contribute to the ongoing discovery of new strategies for creating high-yield GABA producing strains.

Acknowledgments This work was supported by a grant from the Next-Generation BioGreen 21 Program (SSAC, Grant Number: PJ00954904), Rural Development Administration, Republic of Korea.

References

1. Capitani G, De Biase D, Aurizi C, Gut H, Bossa F, Gruetter GM (2003) Crystal structure and functional analysis of *Escherichia coli* glutamate decarboxylase. EMBO J 22(16):4027–4037
2. Dueber JE, Wu GC, Malmirchegini GR, Moon TS, Petzold CJ, Ullal AV, Prather KLJ, Keasling JD (2009) Synthetic protein scaffolds provide modular control over metabolic flux. Nat Biotechnol 27(8):753–759
3. Kim SH, Shin BH, Kim YH, Nam SW, Jeon SY (2007) Cloning and expression of a full-length glutamate decarboxylase gene from *Lactobacillus brevis* BH2. Biotechnol Bioprocess Eng 12:707–712
4. Kook MC, Seo MJ, Cheigh CI, Lee SJ, Pyun YR, Park H (2010) Enhancement of γ-aminobutyric acid production by *Lactobacillus sakei* B2–16 expressing glutamate decarboxylase from *Lactobacillus plantarum* ATCC 14917. J. Korean Soc Appl Biol Chem 53(6):816–820
5. Ma D, Lu P, Yan C, Fan C, Yin P, Wang J, Shi Y (2012) Structure and mechanism of a glutamate-GABA antiporter. Nature 483:632–636
6. Moon TS, Dueber JE, Shiue E, Jones P, Kristala L (2010) Use of modular, synthetic scaffolds for improved production of glucaric acid in engineered *E. coli*. Metab Eng 12:298–305
7. Park KB, Ji GE, Park MS, Oh SH (2005) Expression of rice glutamate decarboxylase in *Bifidobacterium longum* enhances c-aminobutyric acid production. Biotechnol Lett 27:1681–1684
8. Park KB, Oh SH (2006) Enhancement of gamma-aminobutyric acid production in Chungkukjang by applying a *Bacillus subtilis* strain expressing glutamate decarboxylase from *Lactobacillus brevis*. Biotechnol Lett 28(18):1459–1463
9. Saskiawan I (2008) Biosynthesis of polyamide 4, a biobased and biodegradable polymer. Microbiol 2(3):119–123
10. Vo TDL, Kim TW, Hong SH (2011) Effects of glutamate decarboxylase and gamma-aminobutyric acid (GABA) transporter on the bioconversion of GABA in engineered *Escherichia coli*. Bioprocess Biosyst Eng 35:645–650