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Production of gamma-aminobutyric acid from glucose by introduction of synthetic scaffolds between isocitrate dehydrogenase, glutamate synthase and glutamate decarboxylase in recombinant *Escherichia coli*

Van Dung Pham[a], Seung Hwan Lee[b], Si Jae Park[c], Soon Ho Hong[a,*]

[a] *Department of Chemical Engineering, University of Ulsan, 93 Daehakro, Nam-gu, Ulsan 680-749, Republic of Korea*
[b] *Department of Biotechnology&Bioengineering, Chonnam National University, 77 Yongbong-ro, Buk-gu, Gwangju 500-757, Republic of Korea*
[c] *Department of Environmental Engineering and Energy, Myongji University, San 38-2, Nam-dong, Cheoin-gu, Gyeonggido, Yongin-si 449-728, Republic of Korea*

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ABSTRACT

Escherichia coli were engineered for the direct production of gamma-aminobutyric acid from glucose by introduction of synthetic protein scaffold. In this study, three enzymes consisting GABA pathway (isocitrate dehydrogenase, glutamate synthase and glutamate decarboxylase) were connected via synthetic protein scaffold. By introduction of scaffold, 0.92 g/L of GABA was produced from 10 g/L of glucose while no GABA was produced in wild type *E. coli*. The optimum pH and temperature for GABA production were 4.5 and 30 °C, respectively. When competing metabolic network was inactivated by knockout mutation, maximum GABA concentration of 1.3 g/L was obtained from 10 g/L glucose. The recombinant *E. coli* strain which produces GABA directly from glucose was successfully constructed by introduction of protein scaffold.

1. Introduction

Gamma-aminobutyric acid (GABA), a four-carbon nonprotein amino acid, is known as the major inhibitory neurotransmitter in mammalian brains (Kim et al., 2007; Park and Oh, 2006). GABA has been used extensively in pharmaceuticals and functional foods considering its several important physiological functions such as blood pressure decreasion, anxiety inhibition and metabolic function acceleration. It was reported that GABA enhances the immune response and represses the cancer cell proliferation (Oh and Oh, 2004). Another potential application of GABA is a building block of nylon 4, which is one of the most promising novel biodegradable polymer (Park et al., 2013).

Generally, GABA is produced by the decarboxylation of glutamate, which produced from sugar by *Corynebacterium* fermentation. Many attempts for the development of efficient GABA producing bacteria have been undertaken due to increasing commercial demand of GABA (Park and Oh, 2006). When rice glutamate decarboxylase was overexpressed in *Bifidobacterium longum*, 1.4 g/L of GABA was produced from 30 g/L of monosodium glutamate (MSG) (Park et al., 2005). By overexpression of glutamate decarboxylase and glutamate/GABA antiporter in the GABA aminotransferase mutant *Escherichia coli* strain, 5.46 g/L of GABA concentration was achieved from 10 g/L of MSG (Vo et al., 2012). By introduction of synthetic protein scaffold between glutamate decarboxylase and glutamate/GABA antiporter, a GABA concentration was further increased to 5.65 g/L, which correspond to a GABA yield of 93% (Vo et al., 2013).

In spite of the successful studies on GABA production from glutamate, in the industrial and economical point of view, the direct production of GABA from sugar looks more attractive than current two step process (glutamate fermentation and glutamate decarboxylation). However, the direct production of GABA or its precursor glutamate from sugar in recombinant *E. coli* is challenging task considering the importance of glutamate in the *E. coli* metabolic network. Unlike *Corynebacterium*, metabolism of *E. coli* is not optimized for the production of glutamate or GABA. Then, to construct direct GABA producer, the novel metabolic engineering strategy is required rather than the simple overexpression of related enzymes. Introduction of the synthetic protein scaffold is considered as one of the most promising strategy for the bacterial metabolism engineering.

The synthetic protein scaffold strategy, which co-localizes functionally related enzymes, has been used for the development

* Corresponding author. Tel.:+82 52 259 1293.
E-mail address: shhong@ulsan.ac.kr (S.H. Hong).

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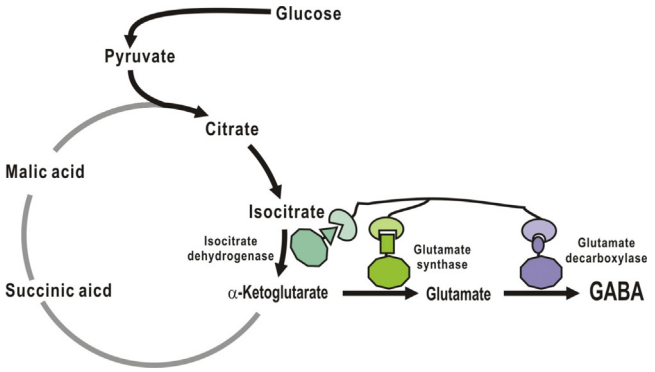

Fig. 1. Model of synthetic scaffold system between isocitrate dehydrogenase, glutamate synthase and glutamate decarboxylase in recombinant *Escherichia coli*.

of various metabolite production systems (Dueber et al., 2009; Ignacio Tinoco et al., 2002). Particular enzymes can be co-localized using protein–protein interaction domains and their specific ligands, and locally increased enzyme concentration leads more efficient enzymatic reactions. By introduction of synthetic scaffold, concentrations of resveratrol, 1,2-propandiol, and mevalonate significantly enhanced (Conrado et al., 2012; Delebecque et al., 2011). It was also reported that the concentration of D-glucaric acid increased by 5 fold by introduction of modular synthetic scaffolds (Good et al., 2011; Moon et al., 2010).

By isocitrate dehydrogenase, isocitrate is converted to alpha-ketoglutarate, which further converted to glutamate by glutamate synthase. Then glutamate is converted by glutamate decarboxylase (Burke et al., 1974; Goss et al., 2001; Hurley et al., 1989; Smith et al., 1992). In this study, novel synthetic protein scaffold was introduced between three enzymes consisting GABA pathway for the production of GABA from glucose (Fig. 1). It was showed that the redirection of carbon flux from TCA cycle to GABA could be achieved by co-localization of those three enzymes via synthetic protein scaffold.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. The strains were cultivated in Luria–Bertani medium (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract and 5 g/L NaCl) supplemented with antibiotics (50 μg/mL ampicillin or/and 20 μg/mL chloramphenicol) (Sambrook and Russell, 2001).

100 ml of LB media supplemented D-glucose was incubated at 30 °C under vigorous shaking until the optical density at 600 nm (OD_{600}) reached 1.2, and gene expression was induced with 1% L-arabinose and 108 nM anhydrotetracycline (aTc). For the optimization of the culture conditions various temperature, pH and glucose concentrations were tested.

2.2. Construction of recombinant plasmids

Plasmids were used and/or constructed in this study are listed in Table 1, and oligonucleotides used in this study are listed in Table 2. To construct synthetic scaffold, three ligands were attached to corresponding GABA pathway enzymes. Gene encoding GBD protein ligand was synthesized by polymerase chain reaction (PCR). The *icd*, *gltB*, *gadA* and *gadB* genes were amplified from *E. coli* X1_Blue chromosomal DNA using PCR. PCR was performed with the MJ mini Personal Thermal Cycler (BioRad Laboratories, USA) using the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany). Amplified *GBD* gene was attached to *icd* gene by overlap PCR. *SH3* and *PZD* genes were incorporated in the reverse primers, and they integrated to *gltB* and *gadA*/*gadB* genes during PCR (Table 2). The *icd-GBD* gene was cloned into pBAD30C plasmid, in which ampicillin resistance gene was replaced with chloramphenicol resistance gene, using *Sac*I and *Bam*HI restriction enzymes. The *gltB-SH3* gene was cloned downstream of *icd-GBD* using *Bam*HI and *Xma*I. Then *gadA-PDZ* and *gadB-PDZ* genes were cloned using *Xma*I and *Xba*I to construct pBIGA and pBIGB expression plasmids, respectively. Under the control of Ara promotor, Icd-GDB, GltB-SH3 and GadA/GadB-PDZ are expressed. Nine plasmids containing GBD, SH3 and PDZ protein interaction domains were kindly provided by Prof. Dueber (Table 1).

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

Strain or plasmid	Description[a]	Reference
Strains		
XL1_Blue	F′ (proAB⁺lacI^q lacZΔM15 Tn10 (Tet^r)	Laboratory stock
XBT	*E. coli* XL1_Blue (Δ*gabT*)	Vo et al. (2012)
XBM1	*E. coli* XL1_Blue (Δ*pflB, ldhA, gabT*)	This work
XBM3	*E. coli* XL1_Blue (Δ*ackA, gabT*)	This work
XBM4	*E. coli* XL1_Blue (Δ*frdB, gabT*)	This work
XBM8	*E. coli* XL1_Blue (Δ*pflB, adhE, poxB, frdB, ldhA*)	This work
Plasmids		
pBAD30	pBAD30, araC promoter, Amp^r	NEB[b]
pBAD30C	pBAD30, (Amp^r was replaced with Cm^r)	This work
pBIGA	pBAD30C, (*icd-GBD*, *gltB-SH3*, *gadA-PDZ*)	This work
pBIGB	pBAD30C, (*icd-GBD*, *gltB-SH3*, *gadB-PDZ*)	This work
pJD757	P_{tet}, $GBD_1SH3_1PDZ_1$, Amp^r	Moon et al. (2010)
pJD758	P_{tet}, $GBD_1SH3_1PDZ_2$, Amp^r	Moon et al. (2010)
pJD759	P_{tet}, $GBD_1SH3_1PDZ_4$, Amp^r	Moon et al. (2010)
pJD760	P_{tet}, $GBD_1SH3_2PDZ_1$, Amp^r	Moon et al. (2010)
pJD761	P_{tet}, $GBD_1SH3_2PDZ_2$, Amp^r	Moon et al. (2010)
pJD762	P_{tet}, $GBD_1SH3_4PDZ_1$, Amp^r	Moon et al. (2010)
pJD763	P_{tet}, $GBD_1SH3_4PDZ_2$, Amp^r	Moon et al. (2010)
pJD764	P_{tet}, $GBD_1SH3_4PDZ_2$, Amp^r	Moon et al. (2010)
pJD765	P_{tet}, $GBD_1SH3_4PDZ_2$, Amp^r	Moon et al. (2010)

[a] Amp^r, ampicillin; Cm^r, chloramphenicol; Tet^r, tetracyclin.
[b] New England Biolabs, Beverly, MA, U.S.A.

2.3. GABA analysis

GABA concentration was measured by HPLC using an OptimaPak C18 column (4.6 × 150 mm, RS tech Corporation, Daejeon, Korea). Samples were centrifuged at 12,000 rpm for 5 min and then 100 μL of the supernatant was added to an Eppendorf tube. 200 μL of 1 M sodium bicarbonate buffer pH 9.8, 100 μL of 80 g/L dansylchloride in acetonitrile and 600 μL of double-distilled water was added to make a 1 mL reaction mixture. The mixture was incubated at 80 °C for 40 min, and 100 μL of 20 μL/mL acetic acid was added to stop the reaction. The mixture was centrifuged at 12,000 rpm for 5 min. The supernatant was then filtered through a 0.2 μm Millipore filter and analyzed by HPLC on an Agilent system using UV detection. Separation of the derivatized samples was attained using a binary nonlinear gradient with eluant A [tetrahydrofuran/methanol/50 mM sodium acetate pH 6.2 (5:75:420, by vol.)] and eluant B (methanol). The column temperature was set at 30 °C. 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 UV 286 nm. The standard curve for GABA was determined using the same procedure for eight standard solutions: 0.1, 0.2, 0.3, 1, 2, 3, 5, and 10 g/L of GABA (Sigma, Missouri, USA).

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