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# Thermostabilization of glutamate decarboxylase B from *Escherichia coli* by structure-guided design of its pH-responsive N-terminal interdomain

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ABSTRACT

Glutamate decarboxylase B (GadB) from *Escherichia coli* is a highly active biocatalyst that can convert L-glutamate to γ-aminobutyrate (GABA), a precursor of 2-pyrrolidone (a monomer of Nylon 4). In contrast to vigorous studies of pH shifting of GadB, mesophilic GadB has not been stabilized by protein engineering. In this study, we improved the thermostability of GadB through structural optimization of its N-terminal interdomain. According to structural analysis, the N-terminal fourteen residues (1–14) of homo-hexameric GadB formed a triple-helix bundle interdomain at acidic pH and contributed to the thermostability of GadB in preliminary tests as the pH shifted from 7.6 to 4.6. GadB thermostabilization was achieved by optimization of hydrophobic and electrostatic interactions at the N-terminal interdomain. A triple mutant (GadB-TM: Gln5Asp/Val6Ile/Thr7Glu) showed higher thermostability than the wild-type (GadB-WT), i.e., 7.9 and 7.7 °C increases in the melting temperature ($T_m$) and the temperature at which 50% of the initial activity remained after 10 min incubation ($T_{50}{}^{10}$), respectively. The triple mutant showed no reduction of catalytic activity in enzyme kinetics. Molecular dynamics (MD) simulation at high temperature showed that reinforced interactions of the triple mutant rigidified the N-terminal interdomain compared to the wild-type, leading to GadB thermostabilization.

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## 1. Introduction

Glutamate decarboxylase (GAD, EC 4.1.1.15) is an enzyme capable of converting L-glutamate to GABA through α-decarboxylation using pyridoxal 5′-phosphate (PLP) as a cofactor. $CO_2$, a byproduct of the decarboxylation, affects the local pH environment (Pennacchietti et al., 2009) and is important in the acid resistance system of *Escherichia coli*. The glutamate-dependent acid resistance system uses GAD and is effective in allowing enterohemorrhagic *E. coli* to pass through the acidic barrier of the stomach (Lin et al., 1996).

GABA synthesized by GAD exhibits various physiological functions. It is a major inhibitory neurotransmitter in the central nervous system, and it can regulate the cardiovascular system (Gomes Da Silva et al., 2012). It has been used in pharmaceuticals such as aminalone, gammalone, pycamilone, and pantogam for the treatment of disturbances of brain activity (Adeghate and Ponery, 2002). In addition to this biological importance, GABA can be converted into 2-pyrrolidone, an industrial solvent and a precursor of Nylon 4. Nylon 4 polymer has drawn much attention in industrial fields due to excellent thermal and mechanical properties and its biodegradability (Kawasaki et al., 2005).

GadB from *E. coli* has been used in GABA production due to its high activity. Its rate of reaction increases at high temperature, but GadB cannot be used for GABA production at high temperature due to its low thermostability (Lammens et al., 2009). Therefore, it is important to find thermostable GADs. Recently, a few GADs were cloned from hyperthermophiles (Kim et al., 2009; Lee et al., 2013). However, these hyperthermophilic GADs are not suitable for enzymatic GABA production due to their poor biochemical properties, such as low expression level in *E. coli* or narrow temperature-activity profile at high temperature. Mesophilic GadB has been stabilized slightly by immobilizing it on Eupergit or in calcium alginate (Lammens et al., 2009), but no successful thermostabilization of GadB by protein engineering has been reported.

Previous mutational studies of GadB have focused on elucidating the pH-dependent cooperativity of its activity. Although GadB retains a quaternary structure at neutral and acidic pH, large pH-dependent conformational changes occur at both

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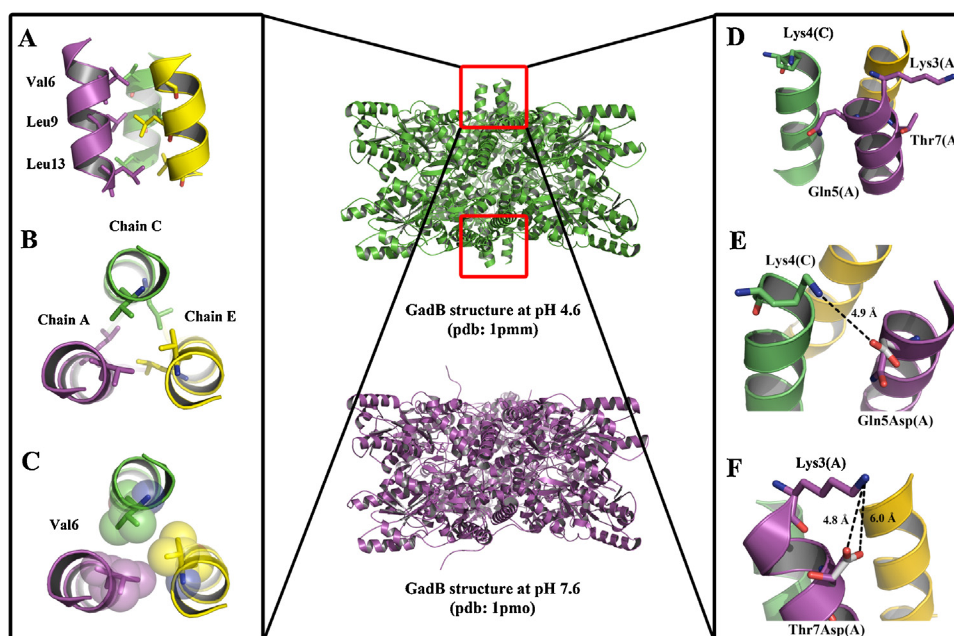


**Fig. 1.** The target mutational sites of GadB. The N-terminal triple-helix bundle interdomain of GadB only forms at pH 4.6 (pdb: 1pmm), and it becomes unstructured at pH 7.6 (pdb: 1pmo). For clarity of the figure, only the A (purple), C (green), and E (yellow) chains of the hexameric GadB are highlighted, but the B, D, and F chains also form the same triple-helix bundle interdomain. (A) Side view and (B) top view of three hydrophobic residues (Val6, Leu9, and Leu13) in the hydrophobic core. (C) Target hydrophobic residue (Val6) for better packing. (D) Target residues (Gln5 and Thr7) on the surface to introduce new electrostatic interactions. Examples of putative (E) inter-molecular electrostatic interaction (Lys3(C)-Gln5Asp(A)) and (F) intra-molecular electrostatic interaction (Lys4(A)-Thr7Asp(A)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

termini. The unstructured N-terminus observed at neutral pH forms a triple-helix bundle at acidic pH (pdb: 1pmm and Fig. 1), and the well-structured plug of the C-terminus that blocks the active site at neutral pH is unstructured at acidic pH (pdb: 1pmo) (Capitani et al., 2003). The conformational changes of the C-terminus that occur with changing pH directly affect the enzyme's activity, in particular its pH-dependent cooperativity (Pennacchietti et al., 2009; Thu Ho et al., 2013). However, the triple-helix bundle is only known to affect the localization of GadB near the membrane at acidic pH (Capitani et al., 2003).

Enzymes can be stabilized by rigidifying their flexible termini (Kim et al., 2010; Le et al., 2012; Wang et al., 2012), and the thermostability of multimeric enzymes can be improved by optimizing their quaternary structures, particularly the multimeric interface (Choudhury et al., 2010; Guérin et al., 2012). In addition, pH-dependent thermostabilization of enzymes can be achieved by engineering charged residues (Kanaya et al., 1993). These previous results suggest that the pH-responsive N-terminal interdomain could be a good mutation target for GadB thermostabilization.

In this study, we engineered thermostable GadB through structure-guided design of its pH-responsive N-terminus. GadB showed a pH-dependent thermostability profile, and the triple-helix bundle of the N-terminus formed at acidic pH was further optimized by introducing additional hydrophobic and electrostatic interactions. The combination of three single mutations resulted in a kinetically and thermodynamically thermostable mutant without reduction of catalytic efficiency.

## 2. Materials and methods

### 2.1. Materials

*E. coli* strains DH5α and BL21(DE3) were from RBC Bioscience (Taipei, Taiwan). The pET-22b (+) vector was from Novagen (Madison, WI, USA). Ni-NTA agarose was from Qiagen (Valencia, CA, USA). The *gadB* gene was synthesized by GenScript (Piscataway, NJ, USA). PCR primers were synthesized by Cosmo Genetech (Seoul, Korea). A GABase containing the γ-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase was from Sigma–Aldrich (St. Louis, MO, USA). Bradford reagent for enzyme quantification was from Sigma–Aldrich (St. Louis, MO, USA). Restriction enzymes and DNA polymerase were from Takara (Tokyo, Japan). All other chemical reagents were from Sigma–Aldrich (St. Louis, MO, USA).

### 2.2. Site-directed mutagenesis

The *gadB* gene (NCBI Gene Id: 946058) tagged with hexahistidine at the C-terminus was codon-optimized in the pUC57 vector (NCBI GenBank accession number: KF764686). The synthetic plasmid was transformed into *E. coli* DH5α for amplification. The plasmid was digested by *Nde* I and *EcoR* I, and the fragment of *gadB* gene was cloned into pET-22b (+) to produce p22GadB. The p22GadB plasmid was used as a template for site-directed mutagenesis. Site-directed mutagenesis of the GadB gene was conducted by a one-step site-directed mutagenesis protocol (Zheng et al., 2004). The mutational primers are listed in Table S1. The deletion mutant (GadB Δ1–14) was constructed as described previously (Capitani et al., 2003).

### 2.3. Enzyme expression and purification

The plasmid harboring the *gadB* gene was transformed into an expression host, *E. coli* BL21(λDE3). An overnight culture (15 ml) of *E. coli* transformant in Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract, and 10 g sodium chloride per liter) containing ampicillin (100 μg/ml) was inoculated into 300 ml LB medium containing ampicillin (100 μg/ml) and incubated at 37 °C with shaking at 200 rpm. After cultivation until the middle of the logarithmic phase of growth ($OD_{600}$ = 0.6–0.8), GadB expression was induced

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