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Effect of Carboxyl-Terminal Truncation on the Catalytic Performance of D-Phenylglycine Aminotransferase

Aiya Chantarasiri [1*], Rachael Patterson[2], Vithaya Meevootisom[2], Suthep Wiyakrutta[2]

[1]Faculty of Science, Energy and Environment, King Mongkut's University of Technology North Bangkok, Rayong Campus, Rayong 21120, Thailand

[2] Department of Microbiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

Corresponding authors E-mail: * aiya.c@sciee.kmutnb.ac.th

Abstract: The D-phenylglycine aminotransferase (D-PhgAT) is a novel enzyme that can be used to synthesize precursors of antibiotics. This research addressed the function of the carboxyl-terminal (C-terminal) of D-PhgAT. Its C-terminal amino acid sequence was compared to other related proteins using bioinformatics tools. The analyzed amino acid sequence was used to produce a genetically modified enzyme having a truncation of the 10 amino acid residues at the C-terminal region. The truncated D-PhgAT was purified and analyzed for catalytic performance. The results revealed that the truncated enzyme had better catalytic performance than the full-length enzyme by 37.49%. This research is a preliminary study for improving the enzymatic performance of D-PhgAT by structure-guided engineering and can be applied in the development of other enzymes.

Keywords: D-phenylglycine aminotransferase**,** Carboxyl-terminus**,** Catalytic performance**,** Bioinformatics tools.

1. Introduction

D-phenylglycine (D-Phg) and its derivative, D-4-hydroxyphenylglycine (D-HPG), are important precursors used in the preparation of semi-synthetic penicillin and cephalosporin antibiotics. These precursors can be prepared by microbial enzymes (Müller et al., 2006). At present, a novel enzyme D-phenylglycine aminotransferase (D-PhgAT) (EC 2.6.1.72), isolated from Thai soil bacterium *Pseudomonas stutzeri* ST-201 (Wiyakrutta & Meevootisom, 1997), has the potential for producing D-Phg and D-HPG using a low-cost and simple process. D-PhgAT is a pyridoxal-5'-phosphate (PLP) dependent enzyme composed of two identical sub-units with a molecular weight of 47,500 Da each (Figure 1). It catalyzes a reversible stereo-inverting transamination of D-Phg or D-HPG with 2-oxoglutaric acid as the amino group acceptor, resulting in the formation of L-glutamic acid and benzoylformic acid (BZF) or 4-hydroxy-BZF (Figure 2). D-PhgAT could be applied in the preparation of D-Phg or D-HPG, the important side chain building blocks of semi-synthetic penicillin and cephalosporin, such as ampicillin and cephalexin, in only a single enzymatic reaction step using a low-cost amino donor as L-glutamic acid. Moreover, D-PhgAT has been used in analytical and pharmaceutical applications, such as for the determination of L-glutamate and monosodium glutamate (MSG) in various foods by spectrophotometric enzymatic cycling method with L-glutamate dehydrogenase (Khampha et al., 2004) and the quantitative determination of amoxicillin by UV-spectrophotometric method with penicillin acylase (Rojanarata et al., 2010).

Due to the benefits mentioned above, D-PhgAT has been studied and genetically engineered for improving enzymatic performance. Many studies have reported that truncation of some amino acid residues at the carboxyl-terminus (C-terminus) of target enzymes can enhance their thermal stability (Vihinen et al., 1994; Ohdan et al., 1999; Liao et al., 2002), solubility (Krueger et al., 2006) and specific activity (Lee et al., 1997; Sanoja et al., 2000). The lecithin-cholesterol acyltransferase was truncated at the C-terminus of 5 amino acid residues (Lee et al., 1997) and the specific activity of the truncated mutant was increased 8-fold.

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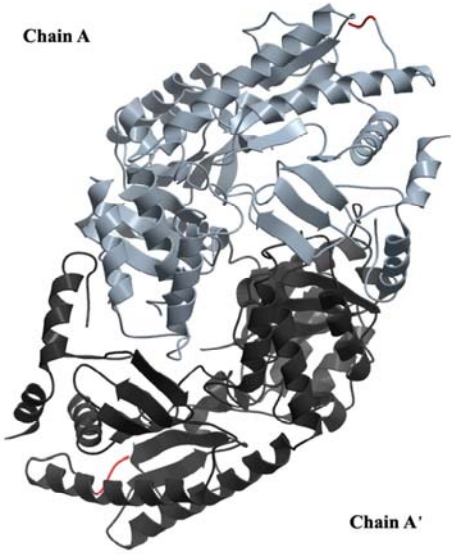

Figure 1. Three-dimensional structure of D-PhgAT, consisting of two identical subunits (Chains A and A′). Red region represents the 10 amino acid residues at each C-terminus. The diagram was generated from the crystal structure data of D-PhgAT (PDB ID: 2CYB) using ICM-Browser 3.7 software.

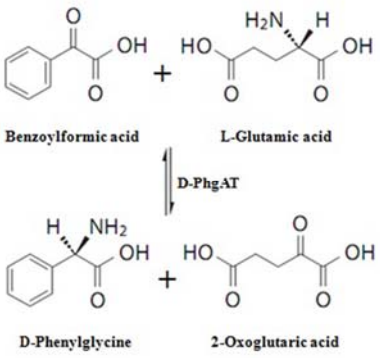

Figure 2. Stereo-inverting transamination reaction of D-PhgAT. The diagram was modified from Chantarasiri et al. (2012)

To address the function of the C-terminus on the catalytic performance of D-PhgAT, the C-terminal truncated enzyme was constructed using structure-guided engineering and monitored for the resulting effect.

2. Materials and Methods

2.1 In silico analyses

The target amino acid region for C-terminal truncation was predicted by bioinformatics tools. A comparison of amino acid sequences between D-PhgAT (GenBank Accession number: AAQ82900.1) and other related proteins in the National Center for Biotechnology Information (NCBI) database was completed employing a BLAST program (Altschul et al., 1990). The 3D Similarity program (Prlić et al., 2010; Ye and Godzik, 2003) was applied on the three-dimensional structure of D-PhgAT (PDB ID: 2CY8) to compare the identity with other enzymes in the RCSB Protein Data Bank (PDB). The intimated enzymes from databases were chosen for amino acid sequence alignment. The sequences were aligned focusing on the C-terminal region by ClustalW 2.1 program (Larkin et al., 2007).

2.2 Construction of C-terminal truncated D-PhgAT

Plasmid pEPL, carrying the *dpgA* gene (GenBank Accession number: AY319935.1) encoding the full-length D-PhgAT from *Pseudomonas stutzeri* ST-201, was kindly provided by Dr. Poramaet Laowanapiban. The *dpgA* gene was genetically modified by QuikChange® Lightning Mutagenesis Kit (Agilent Technologies, USA) according to the prediction provided by bioinformatics analyses. To amplify the modified *dpgA* gene, a forward primer (5'-GACGAAAACCT GTTGTCTTGGTGAAAACTAGCCTGAAACTT G-3') and a reverse primer (5'-CAAGTT TCAGGCTAGTTTTCACCAAGACAACAGGTT TTCGTC-3') were designed using QuikChange® Primer Design Program. DNA sequencing of the modified *dpgA* gene was completed at Macrogen Inc., Korea. The modified *dpgA* gene encoding the C-terminal truncated D-PhgAT was placed under the controlled expression system of the pET-17b plasmid (Novagen, Germany) and expressed in expression host *Escherichia coli* BL 21 (DE3).

2.3 Overexpression and purification of D-PhgAT

The full-length D-PhgAT was prepared as described previously (Kongsaeree et al., 2003). For expression of the C-terminal truncated D-PhgAT, *E. coli* BL 21 (DE3) cells containing the modified *dpgA* gene were cultured in LB-Miller broth (Difco, USA) supplemented with 50 µg/ml of ampicillin at 37°C with 200 rpm shaking until an OD_{600} of 0.6 was achieved. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the final concentration of 0.4 mM to induce C-terminal

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truncated D-PhgAT expression. Induced *E. coli* cells were further incubated for 16 hours at 20°C with 100 rpm shaking. The induced cells were harvested by centrifugation. The pellet of induced cells was re-suspended in 9 volumes of 20 mM TEMP buffer (pH 7.6) (Chantarasiri et al., 2012) and disrupted by ultra-sonication. Crude enzyme solution was clarified by centrifugation and purified by ammonium sulfate precipitation at 25-45% saturation, followed by hydrophobic interaction chromatography on a Phenyl agarose (CL-4B) column (Amersham Pharmacia Biotech, Sweden) and a DEAE sepharose anion-exchange column (GE Healthcare, Sweden). All chromatography was carried out on a BioLogic Controller Fast Protein Liquid Chromatography system (BIO-RAD, USA). The active fractions were combined and concentrated using Amicon Ultra-15 (30 kDa) centrifugal filter devices (Millipore, Ireland). The purity of enzymes was determined by SDS-PAGE and the concentration was determined by Bradford assay (Bradford et al., 1976) using bovine serum albumin (BSA) as the standard. The purified enzymes were kept at 4°C in Protein LoBind tubes until use (Eppendorf, Germany).

2.4 D-PhgAT activity assay

The D-PhgAT activity assay was determined using a kinetic spectrophotometric method in the direction of 4-hydroxy-BZF formation as described previously (Chantarasiri et al., 2012). The 1,000-µl volume of reaction mixture contained 20 µl of appropriate diluted D-PhgAT, 10 mM D-HPG, 10 mM 2-oxoglutaric acid, 50 mM CAPSO buffer (pH 9.5), 5 µM PLP and 5 µM EDTA. The rate of 4-hydroxy-BZF formation was measured as a function of time by monitoring the increase in absorbance at 340 nm for a 180-s time period using a Heλios spectrophotometer (Spectronic Unicam, UK). The measurements were performed in triplicate. One unit (U) of D-PhgAT activity was defined as the amount of the enzyme required to release 1 µmol of 4-hydroxy-BZF per 1 min under specific reaction conditions, with 1 unit of specific activity (U/mg) defined as the activity of D-PhgAT per milligram of total enzyme.

3. Results and Discussions

3.1 Target region for D-PhgAT truncation at C-terminal end

Bioinformatics analyses revealed that D-PhgAT had an amino acid sequence closely related to glutamate-1-semialdehyde 2, 1-aminomutase (EC 5.4.3.8; GenBank Accession number: WP_010046875.1) with 43% identity. The three-dimensional structure of D-PhgAT was nearly identical to glutamate-1-semialdehyde 2, 1-aminomutase (PDB ID: 2ZSL) with 21% identity and β-phenylalanine aminotransferase (PDB ID: 4AO9) with 19% identity, respectively. The amino acid sequence alignment of three intimated enzymes was done by Clustal W program. D-PhgAT was longer than glutamate-1-semialdehyde 2, 1-aminomutase at the C-terminal end by 15 amino acid residues and β-phenylalanine aminotransferase by 10 amino acid residues. To avoid any unfavorable effect on the three-dimensional structure, activity and stability of D-PhgAT from abundant amino acid truncation (Vihinen et al., 1994), only 10 amino acid residues from position Q444 to S453 (Q444-P445-T446-N447-L448-S449-G450-N451-Q452-S453) at each C-terminus of D-PhgAT subunit were preferred for truncation. The predicted region for truncation at the C-terminus of D-PhgAT is shown as red in Figure 1.

3.2 Effect of C-terminal truncation on overexpression and enzyme purity

E. coli BL21 (DE3) cells containing the modified *dpgA* gene could be induced and expressed the C-terminal truncated D-PhgAT. It was evident that the designed engineering had no unfavorable effect on the expression or folding processes of D-PhgAT in expression host. The purity of D-PhgAT, with a molecular weight of 47.5 kDa, is shown in Figure 3.

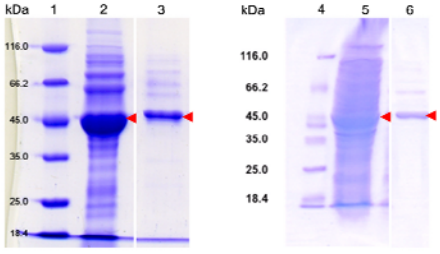

Figure 3. SDS-PAGE analysis of D-PhgAT after the purification processes. Lanes 1 and 4 were the

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protein markers with molecular weight (Fermentas, USA). Lanes 2 and 3 were a crude enzyme solution and purified full-length enzymes, respectively. Lanes 5 and 6 were a crude C-terminal truncated enzyme solution and purified C-terminal truncated enzymes, respectively.

3.3 Effect of C-terminal truncation on enzyme activity

The catalytic performance of full-length and C-terminal truncated D-PhgAT was monitored in the direction of 4-hydroxy-BZF formation. The specific activity was 63.89 ± 2.39 U/mg for full-length D-PhgAT and 87.84 ± 0.04 U/mg for the C-terminal truncated enzyme. The truncation of 10 amino acid residues at C-terminus had a positive effect on the catalytic performance of D-PhgAT by 37.49%. Other previous studies have discussed truncation at the C-terminus of the target enzymes as having increased slightly or causing no negative effect on catalytic performance (Evans et al., 1990; Ohdan et al., 1999; Sanoja et al., 2000; Lin et al., 2009). For D-PhgAT, the 10 amino acid residues at the C-terminal end (the position Q444 to S453) were far apart from the active site and catalytic domain. However, how is it possible that they improved the catalytic performance? The explanation is supported by a previous study (Chantarasiri et al., 2012), which showed that the Q444 residue in the C-terminal region of D-PhgAT was the highly solvent exposed residues and involved in crystal contact residues. The crystal contact is an intermolecular interface that is critical to holding the protein molecule together when forming a protein crystal and protein aggregation (Chantarasiri et al., 2012). The Q444 residue is able to form the hydrogen bonds that interact with amino acid residues of different D-PhgAT subunits, such as K10 and D55 (Figure 4) (Chantarasiri et al., 2012). This interaction results in protein aggregation and protein precipitation that shows no catalytic performance after the protein purification processes. The truncation of 10 amino acid residues, which includes the Q444 residue at the C-terminal region of D-PhgAT, could abolish protein-protein interactions and prevent protein aggregation after protein purification processes, resulting in the increased catalytic performance of D-PhgAT in the solution.

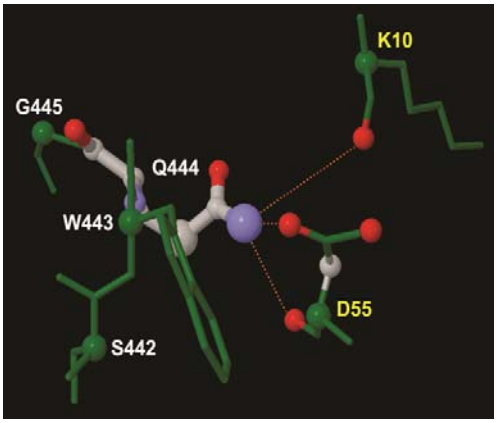

Figure 4. In silico predicted crystal contacts between Q444 and K10/D55 from another D-PhgAT molecule (red dotted line denotes hydrogen bonds). The diagram was modified from Chantarasiri et al (2012).

4. Conclusions

We have shown an approach for increasing the catalytic performance of D-PhgAT by truncation of 10 amino acid residues at the C-terminal region guided by bioinformatics prediction for amino acid sequence and three-dimensional structure. With the development of nucleotide and protein databases, knowledge from this study could be applied to develop and improve the catalytic performance of other enzymes.

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