การศึกษาชีวสารสนเทศและการแสดงออกของ Pyridoxal kinase ในแบคทีเรียชอบร้อน *Geobacillus* sp. H6a ที่ผลิตวิตามินบีหก

Bioinformatics and Expression Analysis of Pyridoxal Kinase in Vitamin B-6-producing Thermophilic Bacterium, *Geobacillus* sp. H6a

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บทคัดย่อ

เอนไซม์ไพริดอกซอล ไคเนส (pdxK) เร่งปฏิกิริยาการเติมหมู่ฟอสเฟตของวิตามินบีหกในรูปอิสระจาก ATP และ ไอออนโลหะไดวาเลนต์ เครื่องมือชีวสารสนเทศหลายชนิด ใช้ในการศึกษาลักษณะทางกายภาพและทางเคมี รวมถึงโครงสร้างทางชีวภาพและหน้าที่ของไพริดอกซอล ไคเนสจากเชื้อ *Geobacillus* sp. H6a (Gh) ยีน *GhpdxK* มีความยาว 810 คู่เบส ถอดรหัสได้เป็นโปรตีน 269 กรดอะมิโน คำนวณน้ำหนักโมเลกุลเท่ากับ 29.06 และมีจุดไอโซอิเลกทริกที่ 5.56 โดเมนที่ทำหน้าที่ของโปรตีน GhpdxK มีความเหมือนกับโดเมนไพริดอกซอล ไคเนส ซึ่งจัดอยู่ในกลุ่มโดเมนใหญ่ ไรโบไคเนส/ pfkB-like การทำนายโครงสร้างสามมิติแสดงให้เห็นว่าโปรตีน GhpdxK เป็นโฮโมไดเมอร์ และมีโครงสร้างเหมือนกับโปรตีน pdxK จากเชื้อ *Bacillus subtilis* การวิเคราะห์การจัดเรียงลำดับกรดอะมิโนและแผนภาพต้นไม้เชิงวิวัฒนาการ แสดงความเหมือนของโปรตีน GhpdxK อยู่ในกลุ่มเดียวกับโปรตีน pdxK จากเชื้อในกลุ่มสปีชีส์ Geobacillus รูปแบบการแสดงออกของยีน *GhpdxK* ในสภาวะการเพาะเลี้ยง และภายใต้ภาวะเครียดออกซิเดชั่นที่เหนี่ยวนำด้วย H_2O_2 ตรวจสอบด้วย RT-PCR ในการเจริญของเชื้อ *Geobacillus* sp. H6a ยีน *GhpdxK* แสดงระดับการแสดงออกสูงที่สุดในระยะเจริญเติบโตเต็มที่ ระดับการแสดงออกของยีน *GhpdxK* ลดต่ำลงอย่างมีนัยสำคัญทางสถิติ ภายใต้ภาวะเครียดออกซิเดชั่นที่เหนี่ยวนำด้วย H_2O_2 แต่ปริมาณวิตามินบีหกเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ เมื่อเปรียบเทียบกับกลุ่มควบคุม งานวิจัยนี้เป็นการรายงานครั้งแรกของการศึกษาเอนไซม์ ไพริดอกซอล ไคเนส ในเชื้อชอบร้อนที่ผลิตวิตามินบีหก

ABSTRACT

Pyridoxal kinase (pdxK) catalyzes the phosphorylation of vitamin B-6 with ATP and divalent metal ions. The physical and chemical characteristics, biological structure and the function of pyridoxal kinase of *Geobacillus* sp. H6a (Gh) were studied using multiple bioinformatics tools. The full length of *GhpdxK* was 810 bp encoding a protein of 269 amino acids with a calculated molecular weight of 29.06 kDa and isoelectric point of 5.56. The function domain of GhpdxK showed a high confidence level with pyridoxal kinase domain, a member of ribokinase/pfkB-like

superfamily domain. The predicted three-dimensional structure of GhpdxK was a homodimer and very similar to pdxK of *Bacillus subtilis*. The analysis of multiple sequence alignment and phylogenetic tree supported that GhpdxK shared high similarity with pdxK from Geobacillus spp. The *GhpdxK* expression pattern in culture condition and H_2O_2-induced oxidative stress were examined using RT-PCR. In the growth of *Geobacillus* sp. H6a, *GhpdxK* expression showed the highest expression level during the exponential phase. Under oxidative stress by hydrogen peroxide, *GhpdxK* expression significantly decreased but total extracellular vitamin B-6 was significantly increased compared to the control. This is the first time to study pyridoxal kinase in vitamin B-6 producing thermophilic bacterium.

คำสำคัญ: ไพริดอกซอล ไคเนส จีโอบาซิลลัส ชีวสารสนเทศ การแสดงออกของยีน
Keywords: Pyridoxal kinase, *Geobacillus*, bioinformatic, gene expression

INTRODUCTION

Vitamin B-6 is a water-soluble vitamin consisting of six forms: pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and their phosphate ester forms: pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate (PMP). PLP is the important form that serve as a cofactor for many enzymes in metabolism of protein, lipid, and carbohydrate.

Mammals do not have a biosynthesis pathway for vitamin B-6 and consequently require it from food. In contrast, microorganisms and plants can produce vitamin B-6 from intermediates of carbohydrate and protein metabolisms by *de novo* biosynthesis pathway. Most organisms can convert other forms of vitamin B-6 to PLP by salvage pathway. The salvage pathway involves PL kinase, phosphatase and oxidase. In the presence of divalent cation (Zn^{2+} or Mg^{2+}) and ATP, PL kinase catalyzes the phosphorylation of PN, PL and PM to form PNP, PLP and PMP, respectively. PNP oxidase catalyzes the oxidation of PNP and PMP to form PLP. The phosphatase can dephosphorylate PNP and PLP (Rosenberg et al., 2017).

PL kinase has been purified and characterized from many organisms (Rosenberg et al., 2017; Huang et al., 2011; Cao et al., 2006; Lum et al., 2002; Safo et al., 2006; Kim et al., 2016). The overall structure of *Bacillus subtilis* PL kinase shows that it forms a dimer with identical subunits with 29 kDa comprising 271 amino acid residues. The monomer of *B. subtilis* PL kinase consisted of a central eight-stranded β-sheet with seven parallel strands and one antiparallel strand surrounded by eight α-helices. This characteristic is a highly conserved core tertiary structure in the ribokinase superfamily (Newman et al., 2006).

In previous studies, *Geobacillus* sp. H6a (Gh) was isolated from a hot spring in Thailand, producing extracellular vitamin B-6 in PMP and PM forms (Anutrakunchai et al., 2010a). The low concentration (0.4 – 4 mM) of hydrogen peroxide (H_2O_2) could stimulate extracellular vitamin B-6 production in *Geobacillus* sp. H6a (Anutrakunchai et al., 2010b). The expression analysis of *Ghpdx1* and *Ghpdx2* genes which encode PLP synthase, the key enzyme in *de novo* biosynthesis pathway, was also studied. The effect of oxidative stress induced by H_2O_2 on *Ghpdx1* expression showed no significant difference between control and treatments, while the *Ghpdx2* expression significantly increased over the range of 0.8 – 4.0 mM H_2O_2 treatment (Anutrakunchai et al., 2010b).

There are little known about expression of PL kinase gene in H_2O_2-induced oxidative stress to vitamin B-6 production, especially in thermophilic bacterium. In this study, the full length of PL kinase gene (*pdxK*) from

Geobacillus sp. H6a was sequenced and examined for its expression pattern in culture conditions and H_2O_2-induced oxidative stress. The protein structure of *Geobacillus* sp. H6a PL kinase was also determined by bioinformatics analysis.

RESEARCH METHODOLOGY

Full-length *GhpdxK* Isolation

Glycerol stock of *Geobacillus* sp. H6a kept at -70°C was cultured overnight in 2 ml LB broth medium. Total genomic DNA was extracted using High Pure PCR Template Preparation Kit (Roche, Germany) according to the manufacturer's protocol. The quantity and quality of the extracted DNA was analyzed by agarose gel electrophoresis. DNA solutions were stored at -20 °C.

PCR was performed in a GeneAmp® PCR system 9700 (Applied Biosystem, USA) using 2.5 U Taq DNA polymerase (Real-Biotech, Taiwan), 200 µM dNTPs (Eppendorf, Germany), 0.2 µM of each primer (5PdxKNde: 5′-AACATATGATGCCAAAAGCG TTAACGATCG CC-3′, 3PdxKBam: 5′-AAAAGGATCC TTATTGTTCGCCGTA GCGG-3′) and 960 ng *Geobacillus* sp. H6a genomic DNA. The reactions were subjected to 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, a touchdown step from 62 to 58 °C for 1 min, 72 °C for 2 min, and finally 72 °C for 10 min. The amplified fragment was purified after gel electrophoresis and then sequenced using 5PdxKNde, 3PdxKBam, and 2 internal primers (R1-PdxK: 5'- TGATCG TCTCAAGCTGGGC-3' and F2-PdxK: 5'- GGTTG ACGTCCTTTACGACG-3') by Biomolecular Analysis Service Unit, Department of Biochemistry, Faculty of Medicine, Khon Kaen University.

Bioinformatic analyses of putative protein

Physicochemical properties were analyzed by ProtParam (http://web.expasy.org/ protparam/). The signal peptide was predicted by SignalP-4.1 (http://www.cbs.dtu.dk/services/ SignalP). The trans-membrane structure was predicted by TMpred. Prediction of the secondary structure and disulfide bond was performed by PredictProtein server (https:// www.predictprotein.org/) and PSIPRED software (http:// bioinf.cs.ucl.ac.uk/psipred/). CDD (http:// www.ncbi.nim. nih.gov/cdd) was used to predict the function domain. Phyre 2 and Swiss-model (http://swissmodel.expasy. org/) to predict the three-dimensional structure of the putative protein. The predicted model of GhpdxK was validated by RAMPAGE (http://mordred.bioc. cam.ac. uk/) server.

Homology analysis and phylogentic tree construction

The full nucleotide sequence of the *GhpdxK* was assembled using BioEdit 7.2.6 *(*Alzohairy, 2011*).* The deduced amino acid sequence of *GhpdxK* was aligned and compared with other proteins using BLASTX program with default parameters. The multiple sequence alignment was subjected to phylogenetic analysis with the CLUSTAL W in the Molecular Evolutionary Genetic Analysis software (MEGA version 7.0.14) and a phylogenetic tree was constructed using the neighbor-joining method with 1,000 bootstrap replications to assess nodal support in the tree.

Determination of vitamin B-6.

Samples and vitamin B-6 standard (PN) were measured using the Agar Diffusion Assay technique (Sangsai et al., 2016). Briefly, the yeast *Saccharomyces carlsbergensis* TISTR 5345 (ATCC 9080) was mixed with a Pyridoxine assay medium (PAM, Difco) at a ratio of 1:100 and incubated at 30 °C for 6 h. After acid hydrolysis according to AOAC (AOAC official method 961.15), the samples were applied into each well punched on the yeast-PAM agar plate. The plates were then incubated at 30 °C for 12 h. The diameter of the growth zone around the well was measured and compared with that of standard vitamin B-6 (1 – 5 ng).

Growth conditions for analysis of *GhpdxK* expression

Geobacillus sp. H6a was grown in fresh 50 ml SM under continuous shaking condition at 60 ºC for 24 h. Optical density (600nm) and the total amount of vitamin B-6 were measured every 2 h. Expression levels of *GhpdxK* and the housekeeping gene *dnaA* were detected by RT-PCR after incubation in culture growth medium at 8, 16 and 24 h, respectively.

The effect of oxidative stress conditions was analyzed by growing *Geobacillus* sp. H6a in fresh 5 ml SM under continuous shaking conditions at 60 ºC. Our previous study showed 1.2 mM H_2O_2 triggered the maximum amount of vitamin B-6 production among the various concentration (0.4 – 4 mM) of H_2O_2 (Anutrakunchai et al., 2010b). When the OD_{600} reached a value of 0.7, the 1.2 mM of H_2O_2 was added and continuously incubated for a further 3 h. The pellet cells were collected and washed twice with phosphate buffer saline (PBS) pH 7.4 for RNA extraction. Since the amount of vitamin B-6 could not be determined by Agar Diffusion Assay at 3 h, the supernatant was collected by centrifugation at 3,000 g for measuring amount of vitamin B-6 after 24 h.

RNA extraction and RT-PCR

Semi-quantitative two-step RT-PCR was carried out to investigate the expression profiles of *GhpdxK* to the growth phase and oxidative treatment. Total RNA was extracted using the RNeasy® Mini Kit (Qiagen, Germany) according to manufacturer instructions. The integrity of total RNAs was verified by electrophoresis on a 1% agarose gel.

The mRNA was reverse transcribed using reverse transcriptase as supplied in the LongRange 2Step RT-PCR Kit (Qiagen, Germany). Fragments of *GhpdxK* were amplified from cDNAs using primers as with the PCR reaction. The housekeeping gene, *dna*A, which encoded the chromosomal replication initiation protein, was used as an internal control. The PCR primers were dnaA-FW (5′-TTAATGCACGCGATCGGCC-3′) and dnaA-RV (5′-TGCCTTTGCAGCTGCGTATC-3′). Amplifications were carried out as described in handbook of this Kit. The reactions were subjected to 94 ºC for 3 min, followed by 30 cycles of 94 ºC for 30 sec, 55 ºC for 30 sec, and finally 68 ºC for 90 sec. RT-PCR products were analyzed by 2% agarose gel stained with ethidium bromide. The bands were quantified using Gel Doc 2000 (Bio-Rad, USA). SPSS version 19 for Windows was used for statistical analysis.

RESULTS

DNA sequence of *GhpdxK*

The PCR product of *pdxK* from *Geobacillus* sp. H6a was 810 base pairs, including the position of start (ATG) and stop codons (TAA). The DNA sequence of *GhpdxK* was deposited in GenBank under accession number FJ497251.

Characterization of putative GhpdxK

The ProtParam tool was used for physiochemical analysis of the GhpdxK. The *GhpdxK* encoded 269 amino acids with a predicted molecular weight of 29.06 kDa and theoretical isoelectric point of 5.56. The most abundant amino acids were Ala, Thr and Gly with proportions of 11.9%, 8.9%, 8.6%, respectively. The theoretical extinction coefficients (at 280 nm measured in water) were 19,035 M^{-1} cm^{-1} (assuming all pairs of Cys residues form cystines) and 18,910 M^{-1} cm^{-1} (assuming all Cys residues are reduced). The calculated instability index was 25.90. The predicted grand average of hydropathicity (GRAVY) of GhpdxK was -0.016. GhpdxK had no signal peptide and was not a transmembrane protein. PSIPRED server shows an image of the secondary structure of GhpdxK (Figure 1) which consisted of 9 helices and 7 strands. Disulfide bonds did not exist in the protein sequence.

The function domains of GhpdxK were also analyzed by CDD. GhpdxK had a high homology with ribokinase/6-phosphofructokinase (pfkB)-like super-family domain and had a high confidence level with pyridoxal kinase (PRK12412) domain model (Figure 2). The position of the substrate binding site (residue 9, 21, 42, 80 and 214) and ATP binding site (residue 105, 142, 176, 211 and 213) were identified through CDD. The dimer interface also showed on the position of 3 – 67.

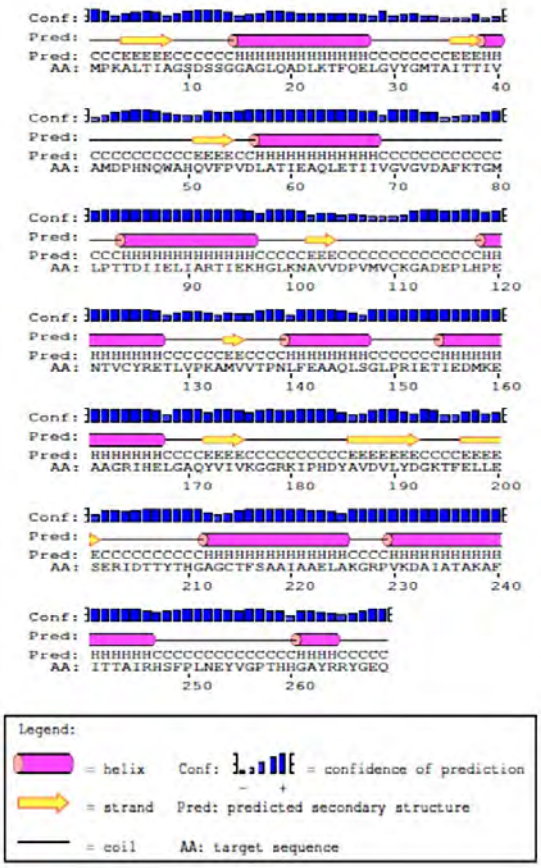

Figure 1. Prediction of the secondary structure of GhpdxK by PSIPRED server.

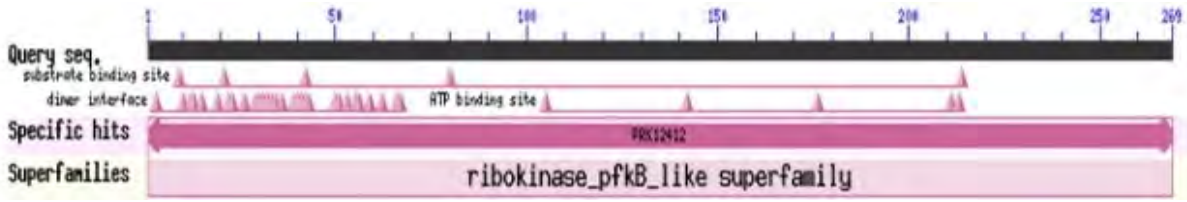

Figure 2. CDD predicted domain of GhpdxK.

Structure prediction of GhpdxK

Figure 3 showed the predicted three-dimensional structure of GhpdxK. It has a homodimer and is structurally very similar to PL kinase of *B. subtilis* with 69.7% and 97% using Swiss-model and Phyre2 methods, respectively. Structural validation of these predicted models was carried out using RAMPAGE server. The Ramachandran plot obtained from RAMPAGE for each of the generated pdb structures of GhpdxK (Figure 4). The resulting homology models revealed that 94.7 – 95.5% of residues were found in favored regions, 3.7% - 5.2% of residues were located

in allowed regions and 0.2% - 0.7% of residues were found in outlier regions.

Multiple sequence alignment and phylogenetic tree analysis

Comparison of the deduced amino acid sequences of GhpdxK with available amino acid sequences in a public database (GenBank, NCBI) showed that GhpdxK was remarkably similar to both *Geobacillus* and *Bacillus* species.

The results of the phylogenetic tree confirmed that GhpdxK was in *Geobacillus* sp and closely related to G. *strearothermophilus* strain 10 (Figure 5). The amino acid sequences of GhpdxK with pdxK of others *Geobacillus* sp.and *B. subtilis* sp.168 were aligned as shown in Figure 6. The amino acid sequences of pdxK were more conserved residues in this group.

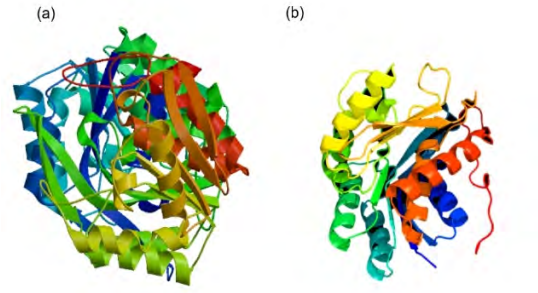

Figure 3. The predicted the three-dimensional structure of GhpdxK using (a) Swiss-model (dimer) and (b) Phyre2 (monomer). Each secondary structure would be shown in a different color.

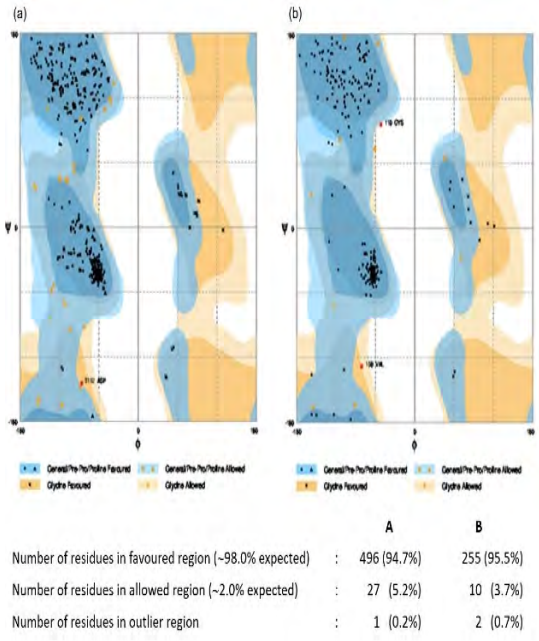

Figure 4. The Ramachandran plot of GhpdxK using RAMPAGE according to the results of (a) Swiss-model and (b) Phyre2.

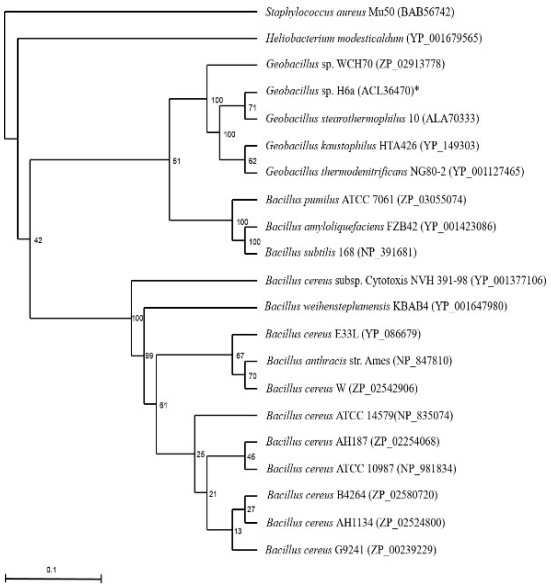

Figure 5. Phylogenetic tree of GhpdxK. The GhpdxK is asterisked. The numbers indicate bootstrap values. The scale bar indicates an evolutionary distance of 0.1 amino acid substitutions per position in the sequence. The sequence of Staphylococcus aureus Mu50 was used as the outgroup.

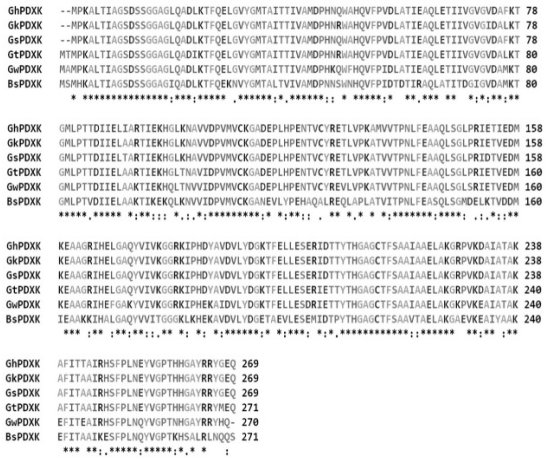

Figure 6. Multiple alignment of amino acid sequence of GhpdxK with other pdxK sequences. The conserved amino acids are shown: (*) are conserved residues, (:) are strongly similar residues, (.) are weakly similar residues. Gh: Geobacillus sp. H6a (ACL36470), Gs: G. stearothermophilus strain 10 (ALA70333), Gk: G. kaustophilus HTA426 (YP_149303), Gt: G. thermodenitrificans NG80-2 (YP_001127465), Gw: Geobacillus sp. WCH70 (ZP_02913778). B. subtilis sp.168 (NP_391681).

Expression of *GhpdxK* in *Geobacillus* sp. H6a in growth curve and oxidative stress condition

The growth curve and vitamin B-6 production of *Geobacillus* sp. H6a. was determined as shown in Figure 7. The increase in vitamin B-6 coincided with the growth of *Geobacillus* sp. H6a. Expression of *GhpdxK* and *dnaA* were analyzed during the growth curve, after incubation in culture growth medium at 8, 16 and 24 hours. The *GhpdxK* showed strong expression at the 16 hours and decreased at 24 hours of growth. To understand the role of GhpdxK in H_2O_2- treated conditions, the expression of *GhpdxK* was analyzed.

Figure 8 shows that the percent relative gene expression of *GhpdxK* significantly decreased when treated with H_2O_2, whereas the amount of extracellular vitamin B-6 (145.39 ± 21.23 µg/L) was significantly higher than those with control (121.66 ± 25.17µg/L).

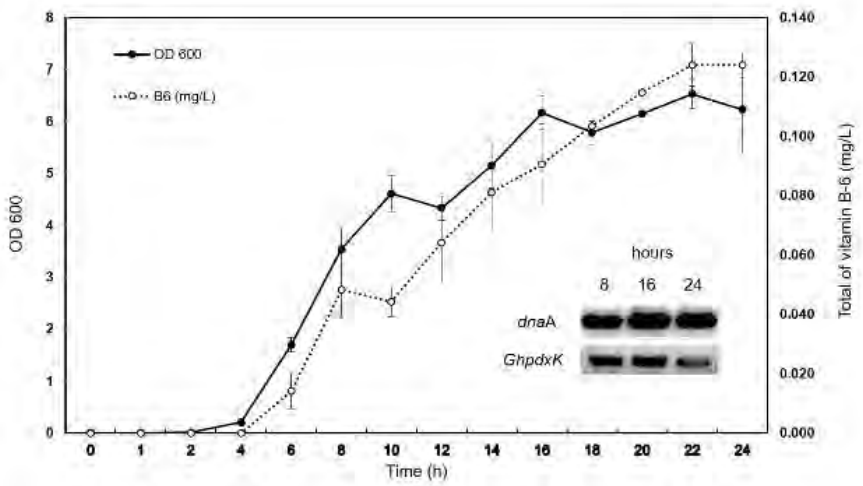

Figure 7. Amount of vitamin B-6 production during the growth of *Geobacillus* sp. H6a. The inset shows 2% agarose gel analysis of RT-PCR amplification of *GhpdxK* at 8, 16 and 24 h. *dnaA* was used as an internal control.

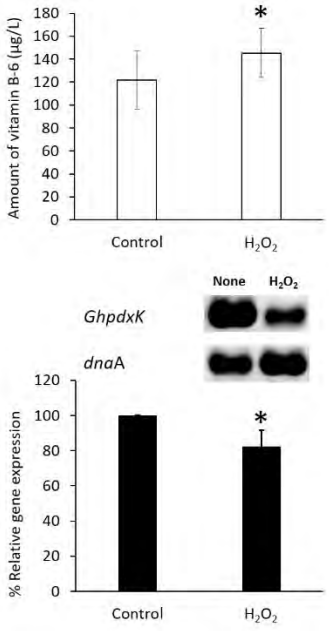

Figure 8. Amount of extracellular vitamin B-6 concentration and expression level of *GhpdxK*. *dnaA* was used as an internal control. * indicates significant differences from control and H_2O_2, respectively, at $p<0.05$.

DISCUSSION

PL kinase has been found in most organisms. It catalyzes the phosphorylation of vitamin B-6 (PN, PL and PM) with ATP and divalent metal ions. One form of its products, PLP, is an important cofactor for many reactions in the metabolism. PL kinase is of interest not only for its importance to our understanding of biochemistry but also for its medical significance; the antimalarial drugs, chloroquine and primaquine showed inhibition by binding to malaria pyridoxal kinase (Kimura et al., 2014).

In this study, we sequenced the full length of PL kinase gene from *Geobacillus* sp.H6a and characterized protein structure and function using bioinformatics analysis. GhpdxK did not have disulfide bond in the protein sequence, so that the theoretical extinction coefficient (at 280 nm measured in water) should be 18,910 $M^{-1}cm^{-1}$. The instability index of GhpdxK lower 40 was considered stable in vitro (Boutet et al., 2016). The GRAVY number of a protein is a measure of its hydrophobicity or hydrophilicity. The predicted GRAVY of GhpdxK was -0.016, which suggests this protein is more soluble in water. Using the PSIPRED tool, we obtained a secondary structure prediction of GhpdxK. It is structurally very similar to PL kinase of *B. subtilis.* It consisted of 8 β-strands and 8 α-helices which obtained from crystallization (Newman et al., 2006). The amino acid residues 36 – 40 which showed the second putative strand and the second putative α-helix by PSIPRED prediction were not determined because they showed a low confidence of prediction. PSIPRED tool builds models to predict the secondary structure class of a single residue position according to the information of its neighboring residues (Duan et al., 2008). As the result, their prediction performance around the ends of regular secondary structures are quite unsatisfactory, which remarkably limits the application of secondary structure prediction results (Duan et al., 2008).

The function domain of GhpdxK had a high confidence level with pyridoxal kinase domain, a member of ribokinase/pfkB-like superfamily domain. This superfamily domain accepts a wide variety of substrates, including carbohydrates and aromatic small molecules. All substrates were phosphorylated at a hydroxyl group (Marchler-Bauer et al., 2017). Similarly, structure prediction using Swiss-Model and Phyre2 methods showed that GhpdxK had a high similarity with the structure of PL kinase from *B. subtilis*. Both structure prediction models were validated using RAMPAGE server. The RAMPAGE server assessed the stereo-chemical quality of a protein structure and generated Ramachandran plot of the backbone structure (Goswami, 2017). The results suggested that both predicted models in this study were of acceptable quality, with over 90% in the most favored regions (Prajapat et al., 2014).

GhpdxK level of expression was high at 16 h that the cell growth reached the late exponential phase. The amount of extracellular vitamin B-6 was synchronized with the growth of *Geobacillus* sp. H6a, represented that vitamin B-6 is a primary metabolite. PL kinase catalyzed the reaction of PN, PL and PM to PNP, PLP and PMP, respectively. PLP is an important cofactor for many enzymes in various metabolisms, and is an integral part of the normal growth process. Our previous study showed the major form of vitamin B-6 excreted by *Geobacillus* sp. H6a is PMP (Anutrakunchai et al., 2010a). It postulated that over-expression of *GhpdxK* might involve in PLP and PMP production.

In our previous study, the 1.2 mM H_2O_2 could stimulate the maximum extracellular vitamin B-6 production in *Geobacillus* sp. H6a (Anutrakunchai et al., 2010b). The H_2O_2 generates detrimental hydroxyl radicals, used as inducer of oxidative stress. Vitamin B-6 is not only important for metabolisms, but it also serves as an antioxidant (Voziyan and Hudson, 2005; Merigliano et al., 2018). Our data showed the extracellular vitamin B-6 in H_2O_2-treated conditions was significantly higher than those with control. This result is in agreement with our previous study that vitamin B-6 *de novo* biosynthesis genes increased expression and produced more extracellular vitamin B-6 (Anutrakunchai et al., 2010b). It can be explained by defensive mechanism. Cell produced extracellular vitamin B-6 to protect cell

from hydroxyl radicals, while *GhpdxK* expression was low. *GhpdxK* might be not involved in defensive mechanism. The correlation between forms of extracellular vitamin B-6 and PL kinase activity of *Geobacillus* sp. H6a in H_2O_2-treated conditions should be future studied.

CONCLUSION

The full length of the PL kinase nucleotide sequence from *Geobacillus* sp.H6a was identified. The putative GhpdxK had PL kinase domain highly similar to the structure of PL kinase from *B. subtilis*. *GhpdxK* showed high expression in the exponential phase, indicating its essential role for cell growth. Oxidative stress by hydrogen peroxide could significantly decrease the expression of *GhpdxK*, indicating that its function may not be involved in the defense mechanism against oxidation. This work will lay the foundation for further functional studies of PL kinase from *Geobacillus* sp.H6a. This enzyme has potential as biocatalyst for use in industrial process.

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