Study on AA10 expression in *E. coli*

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Abstract

The GlcNAc-binding protein A (GbpA) has been known as a virulent factor of *Vibrio vulnificus* pathogen. Domain 1 of GbpA adhesion takes responsibility of binding both human intestine and the chitin surface. The domain 1 structure is similar to a polysaccharide monooxygenase (PMO) AA10-type (PMO), which catalyzed oxidation toward the recalcitrant chitin polymer. The role of the VvPMO10 module in catalytic functions has not been fulfilled characterized. To aim at the VvPMO10, this protein was cloned to the pET22b system and transformed into the *E. coli* BL21 (DE3) strain. The recombinant protein was expressed at 37 ^{0}C with induced IPTG. Total protein was checked by SDS-PAGE method and stained using Coomassie blue solution. The target band showed a band of 20 kDa as expectation. Thus, the heterologous protein was expressed successfully in *E. coli* BL21 (DE3) strain and becomes the materials for future study.

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1 Introduction

Vibrio vulnificus belongs to the Vibrionaceae family. They are negative Gram, rod-shaped and motile bacteria, and living in marine environments with a saprophytic lifestyle [1]. The *Vibrio* strains are usually found attached to zooplankton, an abundant of chitin, like algae cell walls and crustacean exoskeletons. The bacteria, capable of utilizing chitin as a sole carbon source, can survive under nutrient-poor environments [2]. Besides, the presence of chitin in aquatic environment effects *Vibrio*'s physiology, including chemotaxis, biofilm formation, and virulence factors. In human, consuming or being exposed to contaminated seafood can lead to serious diseases like primary septicemias and wound infections [3]. In order to infect the host, *V. vulnificus* strain possesses a variety of adhesive molecules to human epithelial cells, including GbpA, type IV pilus, flagellum and Omp protein. Among them, the GlcNAc-binding protein A (GbpA) was reported as a crucial factor as mediates adhesion to both intestinal human and the chitinous surface [2,4]. The GbpA general structure consists of four domain protein with domain 1 is essential for the initial stage of colonization. The most of conserved residues in GbpA domain 1 are similar to CBM21, a kind of chitin binding protein, take a responsivity in determining the binding specificity for certain types of chitin. Thus, this domain was predicted as the lytic polysaccharide monooxygenase AA10-type (VvPMO10). Although a few researches have been conducted to characterize GbpA, the role of VvPMO10 module in catalytic functions still hitherto limited [5-7].

PMO plays a role in the catalytic oxidation of recalcitrant carbohydrates. These enzymes cleave the glycosidic bonds of insoluble polysaccharide chains. The C1- and C4-oxidized products were an aldonic acid and keto 4-ketoaldose, respectively. The PMO reaction generated new chain ends of the crystalline structure for boosting the hydrolytic enzyme activity. In the CAZy database, PMO was classified into seven

auxiliary activity (AA) families (AA9-AA11, AA13-AA16) by similarities in sequence [8,9]. Each group has a different origin and/or substrate specificity. In the AA10 family, the substrate has been shown both chitin and cellulose, specific to C1- chitin or C1/C4 cellulose [10]. A large amount of protein is required for activity assays and spectroscopic techniques.

Recombinant protein expression is a powerful tool to achieve high yield protein. Herein, the VvPMO10 was fused to pET22b plasmid for the periplasmic secreted strategies. The vector was cloned in the *E. coli* BL21 (DE3) system. The heterologous protein will be the materials for the further study of GbpA domain 1 evaluation.

2 Materials and methods

2.1 Strains and plasmid

Escherichia coli DH5α strain [F^- φ80*lacZ*ΔM15 Δ (*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup* E44 λ^- *thi*-1 *gyr*A96 *rel*A1] (NEB) was used as the host for plasmid cloning. The *Escherichia coli* BL21 *(DE3)* [F^- *ompT gal dcm lon hsd*S_B($r_B^-m_B^-$) λ(DE3) strain was used to express the target protein. This strain was obtained from NEB and plasmid pET22b was ordered from Biobasic. The vector pET22b has the size of 5.5 kb containing T7 promoter for protein expression through isopropyl thiogalactosidase (IPTG) inducing.

2.2 Design and collection of *VvPMO10* from *Vibrio vulnificus* ATCC 27562

Genomic of *V. vulnificus* ATCC 27562 was isolated using soil DNA Isolation Kit (Norgen biotek). The *VvPMO10* was amplified from *V. vulnificus* ATCC 27562 genome by PCR amplification. To target gene fusion vector, restriction enzymes were used. *Nco*I and *Xho*I were added to 5' and 3' terminals of the target gene by PCR reaction. Two of primer (Forward primer (fw): 5'- *Nco*I- TTATGTTTCTGCAGTGGA-3'; Reverse primer (Rv): 5'-CAATGTCAT TGATGTCAAATTC- *Xho*I-3') were constructed using the putative GbpA gene encoding from *V. vulnificus* ATCC 27562 as a reference. Additionally, plasmid pET22b was used as a template for PCR amplified with the primer pair adding restriction site to the ends of the linearized vector. Next, the plasmid PCR product was treated with 1 µL DpnI (NEB) at 37 ^{0}C for 1 hour in order to destroy plasmid template. All of fragment was purified by Monarch® PCR & DNA Cleanup Kit (NEB).

2.3 Vector construction

The gene fragment and linearized plasmid were treated with *Nco*I and *Xho*I enzymes before performing a ligase reaction. The mixture ligation used the molar ratio of 1:3 vector to the fusion gene and the process carried out by T4 ligase. The final construct was transferred into *E. coli* DH5α competent cells following the heat shock protocol. Briefly, the strain *E. coli* DH5α was cultured in liquid Luria Bretani (LB) medium until OD_{600} of 0.5 - 0.7. Then, the bacterial cells were harvested by centrifugation at 4,000 rpm, 4 ^{0}C for 10 min. The cell pellet was washed two times with 100 mM $CaCl_2$ before being resuspended to the mixture containing 100 mM $CaCl_2$ and 10 % glycerol. Then, adding 10 µL the ligated product to 50 µL the competent cell, mixing and incubated on ice for 5 minutes. Next, heat shock the cells for 60 sec at 42 ^{0}C and place the tubes immediately on ice for 5 min. Following chemical transformation, 1 mL LB medium was added to the tubes. The transformants are allowed to regenerate through being shaken for 1h at 37 ^{0}C and 150 rpm. The bacterial cells were collected with centrifugation at 8,000 rpm for 30 s and subsequently spread on LB-Amp-agar plates.

DH5α recombinants was tested for the presence of the target gene by colony PCR method using the gene-specific primer. The product was electrophoresis on the agarose gel. The clones containing the desired plasmid were selected. The plasmid recombinant was extracted according to the introduction of Monarch® Plasmid Miniprep Kit (NEB). These plasmids are continuously confirmed by PCR reaction before analyzing by sequence.

2.4 Protein expression

The vector recombinant (pET22b-VvPMO10) was introduced into *E. coli* BL21 (DE3) competent cells by the chemical transformation. The colonies were selected on LB containing ampicillin (100 µg/mL) and checked by PCR with the gene-specific primer. The single BL21 clone carrying pET-VvPMO10 was incubated overnight on LB liquid containing 100 µg/mL ampicillin at 37 ^{0}C, 150 rpm. In the next step, 50 mL LB-Amp was inoculated (1/10) from an overnight culture and got cultured again at 37 ^{0}C, 150 rpm for (3 - 4) hours. Protein expression was induced by adding of 0.5 mM IPTG (final concentration) to the cultured medium after the optical density at 600 nm reached the appropriate value (0.5 - 0.6). The mixture continuously held at 37 ^{0}C, 150 rpm and obtained after

4 hours of growth. The centrifugating was applied to remove pellet cells (6,000 rpm, 10 minutes). The cells were harvested by centrifugation (6,000 rpm, 10 minutes, 4 ^{0}C) and resuspended in NPI10 solution (50 mM pH 8.0 NaH_2PO_4, 0.5 M NaCl, 10 mM imidazole) following the sonicated process. Total protein extracted from cells was centrifuged at 13,000 rpm for separating the soluble protein and the pellets. In the next step, transfer the supernatant to the new tube and resuspend the pellets in the same NPI10 volume. The total, soluble, and pellet proteins were analyzed by SDS-PAGE.

3 Result

3.1 Collection *VvPMO10* from *Vibrio vulnificus* ATCC 27562

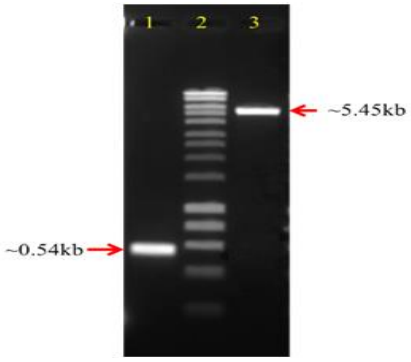

Fig. 1 PCR reaction to obtain the target gene and linear plasmid pET22b. 1-VvPMO10 gene; 2-Marker hyperLadder 1kb; 3-Linear plasmid

In this study, we cloned the *VvPMO10* to the plasmid pET22b. Since N-terminal residue is one of the conserved PMO motifs, it is crucial to obtain recombinant LPMOs with histidine as an N-terminus. Thus, the *Xho*I and *Nco*I are suitable restriction enzymes added to both the end of gene *VvPMO10* and plasmid pET22b by PCR amplification. The PCR product of the gene and plasmid expected fragments of 0.54 kb and 5.4 kb, respectively. The length of expected fragments is similar to bands showed on agarose gel (Fig. 1). These indicated bands were treated with *Xho*I and *Nco*I before purified for the ligation reaction.

3.2 Vector construction

VvPMO10 gene was joined to the incomplete plasmid by T4 DNA ligase. The mixed reaction was transformed and screened in *E. coli* DH5α cells. Only the transformants received the plasmid carrying the gene coding of Ampicillin-resistance protein can survival in the selective LB-Amp medium. The recombinants were examined by colony PCR with the specific-gene primer pair. All the tested colonies had a band ranging from 0.4 kb to 0.6 kb (Fig.2, lane 4,5). The product size was similar to the DNA reference fragments (Fig.2, lane 3). Subsequently, the recombined plasmid isolated from the positive colonies was confirmed by PCR reaction, which results in a band of approximately 0.54 kb (Fig.3A, lane 4). Simultaneously, this plasmid was digested with *Nco*I and *Xho*I, producing expected bands at 5.5 kb and 0.54 kb (Fig.3B lane 2) respectively, matching the DNA plasmid and insert gene, Finally, DNA sequencing results had identified up to 100 % with the GbpA domain 1 sequence of *V. vulnificus* ATCC 27562 on the NCBI database. Therefore, the expressed vector (pET22b-VvPMO10) was constructed successfully.

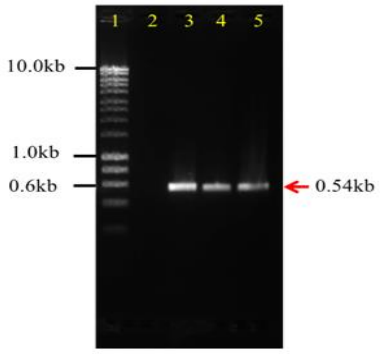

Fig. 2 Examination of the DH5α recombinants by PCR colony with the specific-gene primer pair. 1-Marker hyperLadder 1 kb; 2-Negative control; 3-Positive control; 4,5- The tested colony

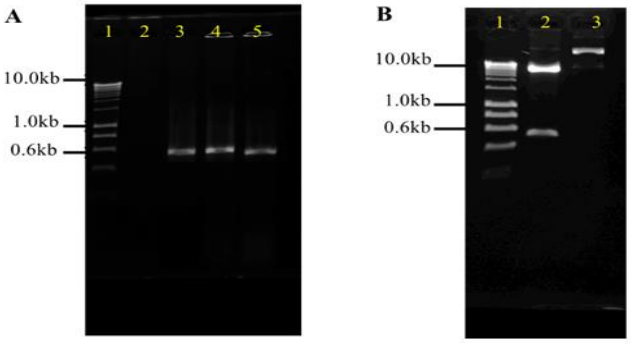

Fig. 3 Result of the confirmation recombined plasmid
A. PCR reation with vector template. 1-Marker hyperLadder 1 kb; 2-Negative control; 3-Positive control; 4,5- The purify plasmid from the positive colony
B. Digestion of vector with *Nco*I and *Xho*I enzymes. 1-Marker hyperLadder 1 kb; 2- The treated plasmid; 3- The purify plasmid before treating

3.3 Expression of VvPMO10 protein in *E. coli* BL21 (DE3)

The *E. coli* is well known as the host organism. These strains are preferred due to their rapid growth in simple conditions and using the inexpensive medium. In addition, the full comprehension of genome, well-characterized genetics, a large of cloning vectors made it simple to apply to protein production. In this study, *E. coli* BL21 (DE3) was chosen to express the target protein. These DE3 strains possess λDE3 lysogen containing T7 RNA polymerase gene regulated by the promoter of LacUV5. So, DE3 strains have not depended on *E. coli* RNA polymerase and using IPTG inducer for protein expression. Moreover, the BL21 strain was deficient *lon* and *ompT* proteases to prevent damage to the protein product. The pET22b-VvPMO10 vector was introduced to BL21 competent cells by chemical method. The colonies growth on LB medium containing Ampicillin was confirmed by colony PCR method with the gene primer pair. The agarose analysis of PCR product appears as a band of 0.54 kb (Fig. 4, lane 4) similar to the band of positive control (Fig. 4, lane 3).

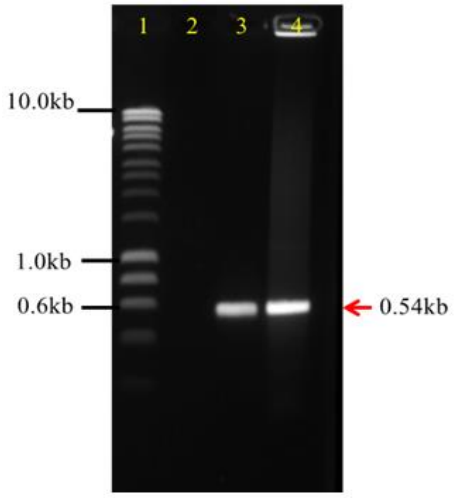

Fig. 4 Results of screening *E. coli* BL21 (DE3) recombinants by colony PCR.
1-Marker hyperLadder 1 kb; 2-Negative control;
3-Positive control; 4,5- The tested colony

The pET system is one of the most commonly used methods for the development of recombinant proteins. Under T7 promoter control, the target gene was transcribed to the mRNA and then translated to the amino acid sequence after induction by IPTG. The protein expression was checked by the SDS-PAGE method. The predicted molecular weight of VvPMO10 protein fusion 6xHis-tag was approximately 20.5kDa. Following the SDS-PAGE result, the band of 20 kDa appeared on the total protein from induced cells (Fig. 5, well 5) while the same band was not exerted in the negative control (BL21 (DE3)/pET-VvPMP10 without induced IPTG (Fig 5, well 2)). The significant proportion of target protein was concentrated in pellet form (Fig 5, well 7) when expressed at 37 ^{0}C. Thus, we can infer that the heterologous protein was expressed successfully in *E. coli* BL21 (DE3) strain.

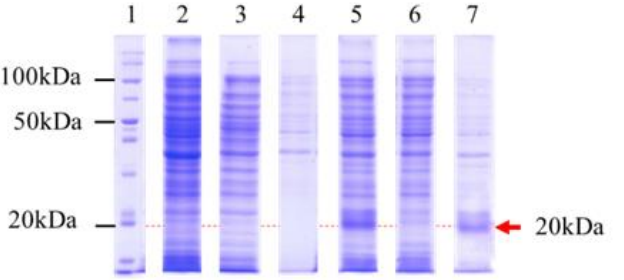

Fig. 5 SDS-PAGE analysis of the induced VvPMO10 protein.

Lane 1-Marker PageRuler™ Unstained Broad Range Protein
Lane 2- 3- 4- Total, solubled, pellet protein from the BL21 (DE3) recombinant without induced IPTG
Lane 5-6-7- Total, solubled, pellet protein from the BL21 (DE3) recombinant after 4 hours induction.

4 Conclusion

In this study, GbpA domain 1 (VvPMO10) from *V. vulnificus* was fused into pET22b system and expressed in the *E. coli* BL21 (DE3) strain. The molecular weight of expressed protein is approximately 20 kDa as the expectation. The recombinant protein was over-expressed mainly in the precipitation of the total protein sample at 37 ^{0}C. It is suggested that the protein expressed condition has to be optimized to achieve a soluble protein in higher yields. Further, biological activities will be carried out in the future.

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Conflict of Interest The authors declare that there is no conflict of interest.

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Nghiên cứu biểu hiện enzyme AA10 trong *E. coli*

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Tóm tắt Protein bám GlcNAc (GbpA) là một nhân tố độc lực của chủng gây bệnh *Vibrio vulnificus*. Domain 1 của GbpA bám dính ở cả bề mặt của ruột người và chitin. Cấu trúc của tiểu phần 1 tương đồng với một loại enzyme polysaccharide monooxygenase có thể thực hiện phản ứng oxy hóa đối với các chuỗi chitin khó phá vỡ. Vai trò của tiểu phần VvPMO10 trong hoạt động xúc tác hiện nay vẫn chưa được mô tả đầy đủ trong *in vitro*. Đối tượng của nghiên cứu này là VvPMO10, protein này được dòng hóa vào hệ thống biểu hiện pET22b và biến nạp vào trong tế bào *E. coli* BL21 (DE3). Protein tái tổ hợp được biểu hiện ở điều kiện 37℃ khi cảm ứng với IPTG. Protein tổng được kiểm tra bằng phương pháp SDS-PAGE và nhuộm phát hiện bằng dung dịch Coomassie blue. Vạch protein mục tiêu thu được có kích thước 20kDa tương tự kích thước lý thuyết, chứng tỏ protein tái tổ hợp đã được biểu hiện thành công trong chủng *E. coli* BL21 (DE3). Kết quả này đóng góp dữ liệu cho những nghiên cứu tiếp theo.

Từ khóa AA10, *E. coli*, biểu hiện, GbpA, Polysaccharide monooxygenase.