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Efficient Expression and Purification of Vacuolating Cytotoxin A (VacA) from an s1m2-type Thai Clinical Isolate

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Abstract**:** *Vacuolating cytotoxin A, VacA, is one of the major virulence factors produced by H. pylori and its gene polymorphism might play a major role in strain virulence of H. pylori itself. VacA is produced as a 140 kDa protoxin which undergoes multiple cleavage reactions to give an 88 kDa mature toxin. The 88 kDa protoxin is further cleaved by unknown proteases into 55 kDa C terminal domain (p55) and 33 kDa N terminal domain (p33). The crystal structure of p55 was determined in 2007 by Gangwer et al, while p33 structure is yet to be solved. Several studies focusing on p33 reported that most of the expressed proteins are in insoluble form. Earlier work in our laboratory has demonstrated that expression of the pore-forming p33 domain in recombinant E. coli results in marked effects on cell growth which is stalled upon induction of protein biosynthesis. The scope of this project is to outline the strategy for optimum expression of recombinant s1m2 strain and to lay a ground work for the study of p33 domain, which crystal structure is yet to be solved. Here, we report the enhanced protein expression of Thai isolate VacA (s1m2 strain) by placing a DHFR fusion protein at the N- terminus of protein and efficient purification strategy for functional studies.*

Keywords: *Helicobacter pylori, Vacuolating cytotoxin A, Dihydrofolate reductase*

1. Introduction

Helicobacter pylori is a toxin producing Gram-negative, spiral shaped, microaerophilic bacteria that can infect the human stomach (1). It was first identified in 1982 by Australian scientists Barry Marshall and Robin Warren, who proved that certain bacterium can colonizes the human stomach (2). Approximately half of the world's populations are infected by *H. pylori*, therefore marking it the most widespread infection in the world (3, 4). Its prevalence is highly variable in accordance with geography, ethnicity, age, and socioeconomic factors. Globally, different strains of *H. pylori* associated with differences in virulence, and the complex interplay with host genetic background and environmental factors leads to subsequent variations in the disease progression. About ~85% of the affected population can develop only asymptomatic acute gastritis, ~10-15% can develop severe chronic gastric disorders like gastric atrophy and peptic ulcer while only ~1-2% can develop gastric adenocarcinoma and primary gastric cell lymphoma (5-7). The different in clinical outcomes may be related to bacterial virulence factors, environmental factors and host genetic backgrounds (8). The geographic variation in the seroprevalence rates of *H. pylori* infection makes it an important etiological factor for the occurrence of gastric adenocarcinoma (9, 10). The incidence rate of gastric adenocarcinoma also reflects the seroprevalence rate of *H. pylori* infection. The route of transmission for *H. pylori* is unclear, possibly oral-oral route or oral-

faecal route (11). Socio economic status, population density and living conditions can also be important factors for *H. pylori* infection, as prevalence rates in developing countries are higher than developed countries (8).

H. pylori produce several virulence factors, such as urease, CagA, VacA and secretory enzymes (mucinase, lipase, and catalase) (12). The virulence factor productions vary depending on the individual strain of *H. pylori*, and might also influence the disease progression (13). For example, CagA (+) strains are more associated with gastric adenocarcinoma patients whereas CagA (-) ve strains show little or no association with gastric cancer (14). The vacuolating cytotoxin A, VacA is one of the major virulence factors produced by *H. pylori* (15). VacA can be found in every strain of isolated *H. pylori* bacteria and displays high levels of gene polymorphisms (16-18). Mature VacA toxin contains two domains, p33 and p55, named according to their molecular weights in kDa (19). The precise identification of mechanism for VacA depends on the structure of the VacA itself. While crystal structure for p55 domain was solved in 2007, which contains mainly of right handed beta helix and small C terminal globular domain (20), the structure for p33 is still under investigation. The presence of VacA in every *H. pylori* isolates makes it a possible candidate for vaccine production. And several researchers are working on the molecular characterization of VacA to understand the mechanism and host pathogen interaction. Although much works have been focusing on s1m1 strains, regarding their virulence and mechanism of action, little has been known about the s1m2 strain which is mainly found in East Asia region (21, 22). Our lab have been working on the VacA, both s1m1 strain (60190) (s1/m1 allelic type) (GenBank accession number-U05676) and Thai isolate (s1m2 strain) (GenBank accession number-KC529337). Previous students have successfully cloned and characterized the s1m1 and s1m2 strains of VacA, but the expression of recombinant s1m2 strain is quite low for further studies. So, the scope of this thesis is to outline the strategy for optimum expression of recombinant s1m2 strain and lay a ground work for the study of p33 domain, which crystal structure is yet to be solved.

2. Methods

2.1. Construction And Protein Expression Of Vaca-DHFR

The fusion protein DHFR-VacA-His was synthesized by TOP Gene® CANADA. The gene was subcloned into pTrcHis2A vector (Invitrogen) using *E. coli* TOP 10 bacterial strain and the transformants were screened by restriction digestion and sequencing. The protein expression was carried out by 0.1mM IPTG in a LB media containing Ampicillin (final concentration at 100µg/ml) at 37°C incubator shaker for 6 hours at 250 rpm. The cell pellet was collected by centrifugation at 8000 rpm for 10 minutes at 4°C. The pellet was resuspended in a buffer A (20mM HEPES, 500mM NaCl, pH-7.4) with lysozyme at a final concentration of 300µg/ml. The soluble and insoluble fractions were separated by centrifugation at 8000 rpm for 10 minutes at 4°C. The fractions were then subjected to SDS-PAGE and Western blot analysis. The VacA-DHFR fusion protein approximately 116 kDa was overexpressed mainly as inclusion bodies.

2.2. Western Blot analysis

Protein samples were separated by SDS-PAGE and soaked in the transfer buffer (25mM Tris, 150mM Glycine, 10% ethanol) for 5 minutes. The nitrocellulose membrane and filter papers were also soaked in the transfer buffer. The protein was transferred onto nitrocellulose membrane by semi dry transfer with constant current for 1-2 hours. The membrane was then blocked by 5% BSA in PBS buffer (140mM NaCl,10.1mM Na2HPO4, 1.8mM KH2PO4, 2.7mM KCl, pH-7.4) at room temp for 1 hour followed by addition of primary antibody at dilution of 1:3000 and incubated for 1 more hour. The membrane was washed with 0.1% tween 20 in PBS solution for 5 min *3 times. The Membrane was then incubated in PBS solution with 5% BSA including 1:5000 ratio of secondary antibody for 1 hour. Secondary antibody signal was developed with chemiluminescent substrate solution (Thermo Scientific, USA). The developed membrane was subjected to autoradiography film in dark room for analysis.

2.3. Purification Of Vaca By High Performance Liquid Chromatography

The inclusion bodies were partially solubilized in 20mM HEPES, 500mM NaCl, pH-7.4 by ultrasonication. The solubilized proteins were purified by Ni-NTA chromatography using HPLC (Spectrosystem, p2000, SN 4000, Thermo Scientific).The partially solubilized inclusion bodies were subjected to purification by HPLC

using Ni-NTA affinity chromatography. The Ni-NTA columns (GE) were equilibrated with buffer A (20mM HEPES, 500mM NaCl, pH-7.4) for 20 column volumes followed by sample injection at 1ml/min. The flowthrough was collected and column was washed with buffer B (20mM HEPES, 500mM NaCl, 50mM Imidazole, pH-7.4) to remove contaminants. The sample was eluted by buffer C (20mM HEPES, 500mM NaCl, 250mM Imidazole, pH-7.4) and the eluted fractions (1ml/min) were subjected to SDS-PAGE gel electrophoresis (Bio-Rad). The eluted fractions were then pooled and subjected to dialysis with buffer A to remove imidazole from the sample. The dialysis was carried out at 4°C for 2 hours and the procedure was repeated. The protein sample was then subjected to enzymatic assay by DHFR activity assay kit (Sigma).

2.4. DHFR Activity Assay

The purified fractions were pooled and the DHFR enzymatic activity was determined by a spectrophotometric assay of the VacA-DHFR fusion protein using a DHFR assay kit (SIGMA). The assay is based on the conversion of dihydrofolate to 5, 6, 7, 8-tetrahydrofolate (THF) utilizing NADPH as cofactor. Activity of DHFR was calculated following the following formula:

$$\text{Units/mg P} = \frac{(\Delta OD/min(sample)) - (\Delta OD/min(blank)) \times d}{12.3 \times V \times mg\ P/ml}$$

ΔOD/min blank = ΔOD/min/min (blank) for the blank, from the spectrophotometer readings;

ΔOD/min (sample) = ΔOD/min-for the reaction, from the spectrophotometer readings;

12.3 = extinction coefficient (ε, mM-1 cm-1) for the DHFR reaction at 340 nm;

V = Enzyme volume in ml (the volume of enzyme used in the assay);

d = dilution factor of the enzyme sample;

mg P/ml = enzyme concentration of the original sample before dilution;

Units/mg P = specific activity in mmol/min/mg protein.

3. Results and Discussion

The synthesized DHFR-VacA gene was subcloned into pTrcHis2A vector (Invitrogen) using *NcoI* and *PstI* restriction enzymes (Thermo scientific). The recombinant plasmid map can be seen in fig.1.

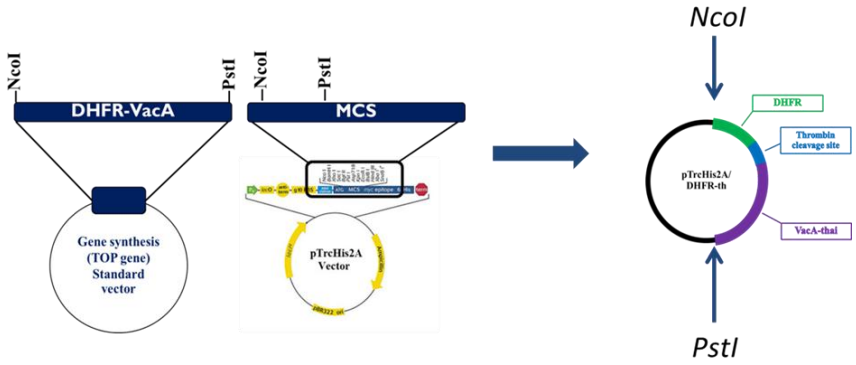

Fig.1: The recombinant protein DHFR- VacA was codon optimized and synthesized from TOP GENE® Canada, the restriction site *NcoI* was used at C-terminus and *PstI* at N-terminus. The gene was then subcloned into pTrcHis2A vector (Invitrogen).

The colonies were first screened by restriction digestion and then further confirmed by sequencing (Macrogen, Korea).The recombinant DHFR-VacA was expressed by induction with 0.1mM IPTG at OD 0.5-0.6 in LB media for 6 hr at 37°C. The expression profile of DHFR-VacA can be seen in fig.2.

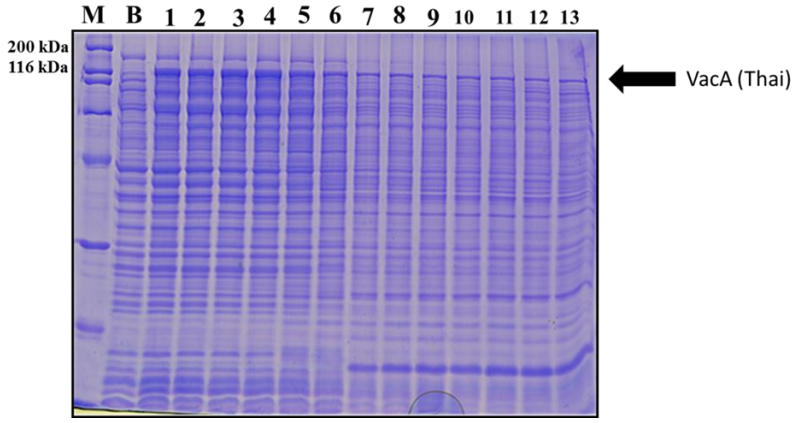

Fig.2: Expression profile of DHFR-VacA; protein expression was induced by 0.1mM IPTG at OD 0.5-0.6, M- markers; B-before induction; 1-13- hours after IPTG induction.

The expressed protein was separated into soluble and inclusion bodies by centrifugation. The identity of the target protein was confirmed by Western blotting using antiseras of His, DHFR and VacA. Results showed that the expressed target protein was mainly in insoluble fraction (inclusion bodies).Fig.3.

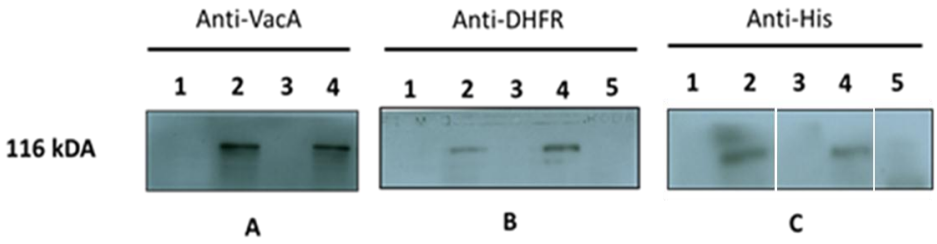

Fig. 3: Western Blot analysis of pTrc-VacA-DHFR protein using different antibodies

A. Anti-VacA; exposure time 5 seconds; lane 1, markers; lane 2, cell lysate; lane 3, soluble fraction; lane 4, inclusion bodies.

B. Anti-DHFR; exposure time 5 seconds; lane 1, markers; lane 2, cell lysate; lane 3, soluble fraction; lane 4, inclusion bodies; lane 5- pTrcHis2A vector (control).

C. Anti-His; exposure time 5 seconds; lane 1, markers; lane 2, cell lysate; lane 3, soluble fraction; lane 4, inclusion bodies; lane 5- pTrcHis2A vector (control).

The inclusion bodies were washed with 20mM HEPES, 500mM NaCl, pH-7.4, by ultasonication. The soluble fraction was achieved after centrifugation and subjected to purification by HPLC chromatography using Ni-NTA column. The elution profile and corresponding SDS PAGE gel can be seen in fig.4 and fig.5.

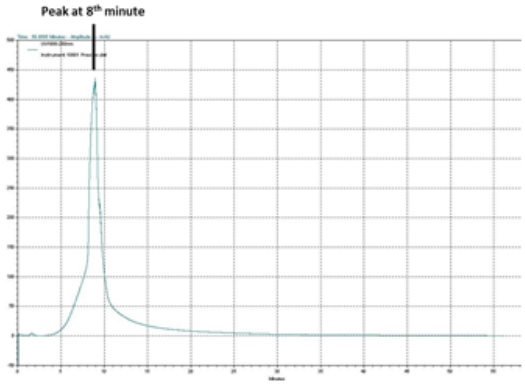

Fig. 4: Elution of DHFR-VacA, elution of target protein at 250 mM imidazole. The target protein was successfully eluted at 8th minute after elution with buffer C.

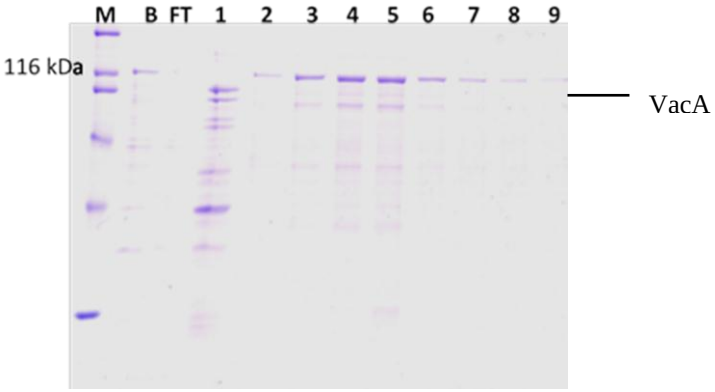

Fig. 5: Comassie blue-stained 10% SDS-PAGE gel of fractions collected after Ni-NTA affinity chromatography. M-molecular weight markers;

B-before injection onto the Ni-NTA column;

FT-flowthrough;

1-9- 250 mM imidazole elution fractions.

The DHFR activity of purified fractions was determined by DHFR activity assay kit (SIGMA) and the measured DHFR activity was calculated to 14.22 µmol/min/mg protein.

4. Conclusion

The fusion protein DHFR-VacA construct showed high expression levels of target protein with stable expression levels. The identity of target protein was confirmed by Western blotting and although most of the expressed proteins were in inclusion bodies, the subsequent purification strategy can give relatively pure protein profile of DHFR-VacA. The purified protein was proved to be enzymatically activity by DHFR activity assay and also have high enzymatic activity. Ongoing works includes assaying the VacA activity by functional assays such as fluorescence dye release from artificial liposomes. We hope this achievement might be of assistance for the expression and study of s1m2 strain of VacA while establishing a basal strategy for detailed molecular characterization of a p33 subunit.

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