Thesis for Degree of Master of fisheries science

Expression and Analysis of Recombinant Gas Vesicle in *Halobacterium*

by

Chowon Kim

Department of Fisheries Biology

The Graduate School

Pukyong National University

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재조합 gas vesicle 단백질의 발현과 특성 분석

Advisor: Jong-Myoung Kim

by

Chowon Kim

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A dissertation

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Chowon Kim

Approved as to style and content by:

(Chairman) Chang-Hoon Kim

(Member) Sung-Min Kang

(Member) Jong-Myoung Kim

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Abstract

Halobacterium is an extremophilic microorganism that lives in high concentration of salt. *Halobacterium* could survive only under high salinity but lyses in freshwater and seawater, due to the difference in osmotic pressure. Therefore, *Halobacterium* is an organism of interest for expressing recombinant proteins without concerns about ecosystem disturbance by GMO. Gas vesicles are hollow proteins structures that enable the Halobacterial cells float to the surface of the water. In order to study the structure and function of gas vesicle, gene encoding gas vesicle subunits were modified to contain cysteine and hexa-Histidine tags. Gene encoding gas vesicle subunit C was modified to fuse with the lipase to test its potential for applying gas vesicle on the removal of lipid from watery environment. Recombinant gas vesicle genes were expressed in *Escherichia coli* and *Halobacterium*. Recombinant proteins of GvpC and GvpC fused lipase are purified in *E. coli* and their

lipase activities were confirmed by various assay. Effects of the mutations on functional gas vesicle formation and their accessibility to cysteine-specific reagents were analyzed.

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Ⅰ. Introduction

Halobacteria are salt-loving organism that grows optimally under the conditions of extremely high salinity, 5-10 times that of seawater. At lower salt concentrations, cell shape becomes distorted and commencing lysis at about 1M NaCl. Haloarchaea are halophilic members of the archaeal domain and called a halophilic archaea. Most Halobacteria are colored red or orange due to the presence of carotenoids, but some strains are colorless, and those with gas vesicles form opaque colonies with white or pink color.

Gas vesicles are hollow proteins structures surrounding a gas filled space. A small number of species of extremely halophilic Archaea of the family *Halobacteriaceae* (8 out of the 137 species having names with standing in the nomenclature as of August 2012) are able to produce gas vesicles. The Gas vesicle is a lipid-free membrane which forms a barrier to liquids but is permeable to many dissolved gases such as nitrogen, oxygen, carbon dioxide and methane. The function of gas vesicles is to enable the Halobacteria cells float to the surface of the water in concentrate brines in which the solubility of molecular oxygen is low. This increases not only for its exposure to the more aerobic phase but also for the availability of light for purple membrane mediated photophosphorylation.

Mature gas vesicles are spindle or cylinder shaped organelles that are 0.045~0.2 μm wide and 0.1~2 μm long. During gas vesicle formation, small

bicone structures appear and grow to become the spindle or cylinder shaped gas vesicles. Gvp proteins are part of the gas vesicle structure and involve 8–14 different Gvp proteins in total, all of which are encoded in *gvp* gene clusters.

GvpA protein has a molecular weight of 7~8kDa. GvpA has a very high content of hydrophobic amino acid, and is highly conserved in all prokaryotes and archaea that produce gas vesicles. GvpA protein forms 4.6nm wide ‘ribs’ that run nearly perpendicular to the long axis of the vesicle. The GvpC protein has a molecular weight of 31~42kDa. GvpC is located on the vesicle outer surfaces and provides strength, stability and shape to the vesicle. Previous findings suggested that GvpC is present on the external surface of the vesicle and functions as “Molecular glue” to enhance the membrane stability and strengthens the structure of the vesicles.

The gas vesicle is like a viral shell without harmful properties and genetic materials. Moreover, gas vesicle has been reported to have characteristics similar to the structure of the nanoparticles closely resembling the epidermis of the virus but without genetic materials. Despite of its ample possibility of their applications, major constraints conducting the research with gas vesicle has been attributed due to poor understanding regarding its structure as well as associated technical difficulties for analysis. Thus, the practical application of gas vesicle has been limited in genetic field till now.

One of the goals in the present study is to investigate the impact of the gas vesicle by structure engineering the cysteine mutants. The emphasis was placed to focus on GvpA, subunit which is a major protein that forms the gas vesicle. This study aimed to establish useful vector system for the expression and recombination gas vesicle genetic manipulation. This study also included the constitution and expression analysis of protein, GvpN and GvpC as well as attachment with the His6-tag for prepare at detection and purification. The overall observation from the present experiment, confirmed the lipase activity of the fusion protein of GvpC fused lipase and purified by *E. coli*. The second part of the study includes functional expression and analysis of gas vesicle mutants from *Halobacterium*.

Ⅱ. Materials and Methods

1. Materials

Topcloner^{TM} TA core kit and Topspeed^{TM} DNA Ligation kit were purchased from Enzynomics (Korea). Plasmid purification and gel extraction kits were purchased from Bio-solution (Korea). Various restriction endonucleases were purchased from New England Biolabs (Beverly, MA) and Enzynomics (Korea). Plasmids pET-19b and pGEX4T vectors were obtained from Novagen (Darmstadt, Germany). pFM104D vector was obtained from Dr. DasSarma Laboratory, University of Maryland, Baltimore, USA. Kit used for the purification of Histidine-tagged protein was purchased from TAKARA (Japan). Monoclonal antibodies raised against 6-His Epitope (Mouse) was purchased from Thermo Scientific (Rockford, USA) together with 6-His Antibody Rabbit polyclonal purchased from Bethyl (USA). Immunopure Goat Anti-Mouse IgG was purchased from Thermo Scientific (Rockford, USA). Rabbit-IgG-heavy and light chain Antibody was purchased from Bethyl (USA). Nitrocellulose Membranes, 0.45μm and Clarity^{TM} Western ECL substrate were purchased from Bio-Rad (USA).

2. Methods

2-1. Expression GVP lipase hybrid gene in *E. coli*

(1) Preparation of competent cells

E. coli DH5α were used for general cloning and together with *E. coli* BL21 for recombinant protein expression. Competent *E. coli* cell was prepared by using Inoue method (Inoue, et. al., 1990). For this, 1 mL of *E. coli* seed culture fully grown in Luria-Bertani (LB) medium was inoculated into 200 mL of LB for further growth at 37℃ with moderate shaking (160 rpm). When OD_{600} of *E. coli* culture reached 0.6, the culture was chilled on ice for 10 min followed by centrifugation at 2,500 x g for 10 min at 4℃. Cells were resuspended in 20 mL of ice-cold transformation buffer (TB: 10 mM Hepes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7) followed by centrifugation at 2,500 X g for 10 min. Cell pellets were resuspended in 20 mL of ice-cold TB, and incubated on ice for 10 min and then harvested by centrifugation at 7,000 rpm for 10 min at 4℃. Cells were resuspended in 10 mL of ice-cold TB together with 7% DMSO (1.5 mL), and aliquot into small amounts. Competent cells were frozen in liquid nitrogen and then stored at -75℃ until use.

(2) Transformation into *E. coli*

E. coli strains DH5α and BL21 were used for the cloning and expression of gvp, respectively. *E. coli* competent cells (100 μl) were mixed with and incubated on ice for 30 min. Upon heat-shock treatment at 42℃ for 1 min, sample was chilled on ice for 3 min. With an addition of 600 μl of LB medium, the mixture was incubated at 37℃ for 1hr. Then plated on the LB agar medium containing Ampicillin (100 μg/mL).

(3) Purification of recombinant plasmid DNA

A single colony was picked for the plate of LB medium containing Ampicillin (100 μg/mL) and incubated at 37℃ overnight. Plasmids purification was carried out by using plasmid purification mini kit according to the protocol provided by manufacturer. Briefly, 3 mL of cells were collected by centrifugation at 14,000 rpm for 1 min. Cells were resuspended with 250 μl resuspension solution containing RNaseA (100 μg/mL) by vortexing. Upon the addition of 250 μl of lysis solution followed by several times inversion and incubation for 5 min until cell lysate became clear, the lysate was mixed with 350 μl of Neutralization Solution by inversion. After, centrifugation at 14,000 rpm for 10 min, supernatant containing recombinant plasmid was transferred into the spin

column. The column was washed using 750 μl of Washing A solution followed by centrifugation at 14,000 rpm for 1 min. After removal of flow-through, the column was centrifuged for 2 min, and then transferred to a new microcentrifuge tube. Plasmid DNA was eluted 50 μl of distilled water or elution buffer.

(4) Expression of gvp in *E. coli*

For the expression of gvp genes. *E. coil* BL21 cell was used. The transformants were cultivated in an LB medium containing ampicillin at 37℃, 200 rpm overnight. Cells were grown until the cell density at 600nm reached around 0.6. Isopropylthiogalactoside (IPTG) was added to induce the expression of gvp in *E. coli* BL21 transformants cell. The growth temperature and time were optimized for the each sample. [GvpC: 37℃ 12hours, GvpC + Lipase: 25℃ 24hours, Lipase: 15℃ 24hours]. For analysis, cells were harvested by centrifugation at 14,000 rpm for 2 min, resuspended in 200 μl of 0.1M Tris (pH 8.0), and then mixed with 150 μl of 2.5 X SDS loading dye with β-mecaptoethanol. Samples were heated in boiling water for 5 min, centrifuged at 14,000 rpm for 3 min, and subjected to SDS-PAGE.

(5) Protein gel electrophoresis

Recombinant gvp proteins were analyzed by using 10% and 8% SDS-PAGE. SDS-PAGEs were stained by Coomassie Blue (CB). For CB staining, gel was soaked into staining solution [Methanol 50%, Acetic acid 10%, and 0.25 g of Coomassie Brilliant Blue R-250 in 100mL solution] and then destained in solution containing [Methanol 50% and Acetic acid 10%].

(6) Lipase protein Purification

Recombinant lipase protein fused with His6 tag was purified by using Chelating Exellose spin kit (TAKARA, Japan). Upon induction of cells by IPTG, cells were harvested by Centrifugation at 6,000 rpm 15 min. Cells were resuspended in Equilibration buffer [50mM Tris-HCl (pH 8.0), 0.5M NaCl, 10mM Imidazole]. Together with PMSF (1mM) and Triton X-100 (0.5%). The mixture was stirred for 30 min on ice with addition of Lysozyme (1mg/mL) followed by. Repeated (5-10 times) freezing and thawing in liquid nitrogen until become clear. When the lysate was still viscous, DNase I (1mg/mL) was added to 5 μg/mL and stirred on ice 15 min. Upon removal of cell debris by centrifuge at 6,000 rpm for 30 min, the supernatant was added with β-Mecaptoethanol (30mM) and then. Applied to metal-chelating chromatography using Chelating Exellose spin kit (TaKaRa). The IDA exellose 500 μl was

transferred on to a column followed by centrifugation at 2,000 rpm for 3 min. Column was washed with 500 μl 1 X Equilibration buffer [10mM Tris-HCl (pH 8.0), 0.1M NaCl] followed by centrifugation at 2,000 rpm for 3 min. Then supernatant (treatment cell) was mixed with resin and incubated for 2 min at RT. Upon centrifugation at 2,000 rpm for 3 min, eluate (F-T) was transferred to new 1.5mL tube. Column was washed three times with 400 μl Wash buffer [50mM Tris-HCl (pH 8.0), 0.5M NaCl, 50mM Imidazole]. Tagged proteins were eluted with 200 μl Elution buffer [50mM Tris-HCl (pH 8.0), 0.5M NaCl, 100mM Imidazole, 250mM Imidazole]. All fractions were collected and stored at -20℃ until further analysis.

2-2. Lipase activity assay

(1) Protein quantitation

Detergent compatible formulation based BCA protein assay was used for the colorimetric detection and quantitation. Protein quantification using a microplate was carried out according to the protocol provided by company by BCA protein assay kit (Pierce). Calibration curve was employed to measure the quantification of BSA. All samples were diluted 6-fold and were measured replicated three times. The results were analyzed to calculate the average protein content of each sample.

(2) Rhodamine B plate assay

LB agar plates containing olive oil and rhodamine B (Sigma) were used for analyzing lipase activity in the cell. Rhodamine B (1mg/mL) was dissolved in distilled water and sterilized by filtration. LB agar medium containing 1% Arabic gum was autoclaved and cooled to approximately 60℃. Plates were prepared with 1% (W/V) olive oil and 0.001% (W/V) rhodamine B upon emulsification with stirring. Cells were spotted on Rhodamine B plated and incubated at 37℃ and lipase, activity was monitored by irradiating plates with UV light at 350nm.

(3) Color metric method assay

Color metric method was used to measure changes in color according to pH. Lipase hydrolyzed the fat to glycerol and fatty acids which results in lowering pH. The pH indicator color was blue at higher than pH 8.0, green in pH 6.0 and yellow at lower than pH 6.0. Seawater 5mL was added with pH indicator 100 μl, olive oil 300 μl and enzyme. The control was included with 50mM Tris-HCl (pH 8.0) instead of the enzyme. Enzyme activity was measured based upon the changes color over time at RT.

(4) Spectrophotometric assay

Lipase activity was measured by using a spectrophotometric assay with 4-Nitro phenyl decanoate (Sigma) as substrate. 4-Nitro phenyl decanoate was dissolved in acetonitrile at a concentration of 10mM. Subsequently, absolute ethanol and 50mM Tris-HCl (pH 8.0) were added to make a final composition of 1: 40: 950 (V/V) acetonitrile ethanol buffer. The reaction mixture comprised of 20 volume diluted sample and 200 μl substrate solutions. The hydrolysis reaction was carried out at 37℃ for 5 min. The amount of released deaconate was determined by an absorption increase at 405nm measured using microplate.

2-3. Analysis of recombinant gvp in *Halobacterium*.

(1) *Halobacterium* transformation

Halobacterium sp. SD109 which do not have gene encoding functional Gvps was used for transformation. Cells were cultured at 42℃ for 3~4 days and then inoculated 1/50 volume into a fresh CM+ media. Upon growth at 42℃ for 20hrs followed by. Incubation on ice for 10 min, cell was centrifuged at 7000 rpm at 4℃ for 10 min. Cells were resuspended in 1/10 volume of medium Spheroplasting solution [2.0M NaCl, 27.0mM KCl, 50.0mM Tris-HCl (pH 8.75), 15% sucrose] on ice. Upon addition of 0.5M EDTA to 1/200 total volume, the mixture was

incubated at RT for 10 min. Upon confirming circular shape of transformed cells examined by microscopy, 200 ㎕ of spheroplasting were mixed with 1 ㎍ of recombinant DNA at RT for 5 min. Equal volume of PEG-600 solution [40% spheroplasting solution and 60% PEG-600] was added to the mixture was and incubated at RT for 20 min. One mL of spheroplast dilution medium [4.3M NaCl, 27.0mM KCl, 50mM Tris-HCl (pH 7.4), 80.0mM $MgSO_4$, 10mM Na-citrate, 1.4mM $CaCl_2$, 15% sucrose, 1% peptone] was added to the mixture on mixed by inversion. Upon centrifugation at 6,000 rpm for 4 min, cell pellet was resuspended in 1mL of spheroplast dilution medium by pipetting into test tube (15mL), and then incubated at 37℃ for 24hrs. 1 ㎖ of CM+ medium [250 g NaCl, 20 g MgSO4, 3 g (Tris)Sodium citrate, 2 g KCl, 10 g Oxoid Neutralized Bacteriological peptone in 1L (pH 7.2) with trace metal] was added and incubated for 24hrs. Cells were plated out on CM+ plate with mevinolin (50 ㎛M) and incubated at 42℃ for 1 week. In a container to avoid evaporation in during the incubation.

(2) Gas vesicle isolation

Transformants were cultivated at 37℃ on CM+ agar [250 g NaCl, 20 g MgSO4, 3 g (Tris)Sodium citrate, 2 g KCl, 10 g Oxoid Neutralized Bacteriological peptone in 1L (pH 7.2) with agar 20 g] plates containing trace metals [440 ㎛g $ZnSO_4.7H_2O$, 330 ㎛g $MnSO_4.H_2O$, 10 ㎛g

$CuSO_4.5H_2O$, 1.75 mg $FeSO_4$ and 23 mg $FeCl_2.4H_2O$] and the selective antibiotic, mevinolin (CM+Mev). Colonies were selected from each transformation and grown in liquid media under the same selective conditions. Gas vesicle mutant strains were grown in liquid cultures in 5mL CM+Mev medium, in incubator at 42℃ for 4~7 days.

(3) Gas vesicle purification

Transformants cultured in liquid media were spread onto 15 ㎝ diameter CM+Mev plates. Incubation at 42℃ for 10 days. Cells were lysed by water containing 1.0mM $MgSO_4$ and DNase Ⅰ (2 ㎍/mL). Upon incubation for 3hrs at 37℃, cell lysates were adjusted to 10% (w/v) NaCl, overlayed with 5% NaCl and centrifuged at 60 X g overnight. Floating gas vesicles were carefully collected and resuspended in 5% NaCl. The isolated gas vesicles were washed by centrifugally accelerated flotation for three times.

(4) Protein gel electrophoresis & Western blotting

Proteins were analyzed by using 10% SDS-PAGE. Cells were harvested by centrifugation at 14,000 rpm 2 min and resuspended in 50 ㎕ basal salt and 150 ㎕ sterile distilled water. The mixtures were added with an equal volume of 2 X SDS loading dye with β-Mecaptoethanol.

Upon heating in boiling water for 7 min followed by centrifugation at 14,000 rpm for 5 min, supernatant was loaded on to SDS-PAGE. Gels were stained with Coomassie Blue R250 or electroblotted for Western blot analysis. For western blots, proteins were transferred from the SDS-PAGE gel Nitrocellulose membrane (Bio-Rad) using Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) at 18volts for 1hr. Subsequently, membranes were blocked with 5% non-fat dry milk in TBS-T [150mM Tris-HCl, 150mM NaCl, 0.1% (v/v) Tween-20] 20mL for 1hr. After three times washing with TBS-T 20 ㎖, membrane was incubated with Anti-His_6 (IGtherapy, Mouse) diluted 1:5000 for 1hr. Then membrane washed three times with TBS-T 20mL for 3 min. Antibody was detected by incubation with either horseradish peroxidase conjugated Goat Anti-Mouse IgG (thermo) or Goat anti-Rabbit (Bethyl). Each conjugate was diluted 1:20000 in TBS-T and incubation continued for 30 min. Membrane was washed three times with TBS-T 20mL and antibodies on the membrane were detected by chemiluminescent solutions and upon exposure onto X-ray film.

2-4. GvpA structure formation

GvpA monomers have a high tendency to aggregate and dissolve only in 80% formic acid. Dialysis to remove the formic acid caused amorphous precipitates of GvpA rather than a refolded protein structure of even a reassembly of the gas vesicles (Belenky et al., 2004).

(1) GvpA mutants

GvpA was the major gas vesicle formation protein and composed of 7- amino acids (Fig.1). Analyses of gvpA structure have been hampered by its difficulty at resolution the component in gel electrophoresis. Since no there are no cysteine among 70 amino acids, amino acid residues at N-terminal, central and C-terminal regions of GvpA were replaced by the cysteine. These include amino acid at 3th, 31th, 33th and 76th. Expression GVPA in *E. coli* GvpA was induced by IPTG using the p19b and GEX4-T vectors which contain glutathione S-transferase (GST) fusion vector and N-terminal His-tag. Induced cell was identified by 10% SDS-PAGE. For functional analysis, GvpA mutant vector was transformed to Halobacterium (SD109).

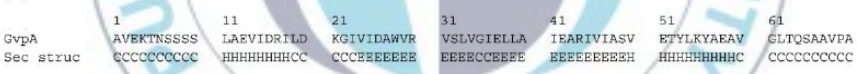


Fig.1 GvpA sequence

(2) PDS assay

Structural analysis of gvp was carried out with gas vesicle GvpA mutants using PDS assay. PDS (2,2'-Dithio-dipyridine) was used for quantification of SH groups. Stock solutions were made as 1mM 2-PDS in 0.2M NaOAc (pH 4.0) and 1M NaOAc (pH 4.0) as shown (Table.1). Gas vesicle was mixed with for 15 min and then spectra were measured at 343nm.

Sample	Blank	NNH(con)	Q3C	G33C	A76C
Gvp	0 ㎕	12.8 ㎕	13 ㎕	20 ㎕	17.7 ㎕
1mM 2-PDS	100 ㎕	100 ㎕	100 ㎕	100 ㎕	100 ㎕
1M NaOAc	180 ㎕	180 ㎕	180 ㎕	180 ㎕	180 ㎕
3'DW	720 ㎕	707.2 ㎕	707 ㎕	700 ㎕	702.3 ㎕
Total	1000 ㎕				

Table.1 Reaction scheme used for PDS assay.

Fig2. PDS assay

(3) Gas vesicle purification with His-tag system

Chelating Exellose spin kit (TAKARA, Japan) resin was used to purify recombinant GVP fused with His6 tags. In order to find conditions for binding to the resin, samples were mixed with 5 X Equilibration buffer [250mM Tris-HCl (pH 8.0), 2.5M NaCl] depending on the amount of protein in the gas vesicles described in Table (Table.2). For IDA exellose column using centrifugation at 2,000 rpm for 3 min, column was adjusted to 1 X Equilibration buffer [50mM Tris-HCl (pH 8.0), 0.5M NaCl]. Upon three times washing with the column of Wash buffer [50mM Tris-HCl (pH 8.0), 0.5M NaCl, 10mM Imidazole] 500 μl (GvpC), 125 μl (gas vesicle) and protein eluted of Elution buffer[50mM Tris-HCl(pH8.0), 0.5M NaCl, 250mM Imidazole] 200 μl (GvpC), 50 μl (gas vesicle). All fractions were collected and stored at 4℃ for further identification of the recombinant proteins.

	Purified GvpC	Gas vesicle NNH
Sample	50 ㎕	70 ㎕
5 X Equilibration buffer	200 ㎕	50 ㎕
3'DW	750 ㎕	130 ㎕
Total volume	1mL	250 ㎕

Table.2 Compositions used for His-tag purification of GVP

Ⅲ. Results and Discussion

1. Expression of GVP

For expression of recombinant GVP from *E. coil* BL21 cell transformed with recombinant plasmids, transformants were cultivated in an LB medium containing ampicillin at 37℃. Cells were grown further in a fresh LB media up to an OD_{600} of 0.6. Upon addition of IPTG, cells were further incubated at 37℃, 200 rpm and 3hrs. In order to prevent insoluble aggregation of an exogenous proteins formed at 37℃, proteins expressions were also carried at a lower temperatures at 37℃, 25℃, 18℃ and 15℃. Upon analysis of optimum time for the induction, more proteins were found in soluble form at 15℃. Recombinant proteins were analyzed by SDS-PAGE. Upon induction with IPTG, extracts prepared from cells transformed with recombinant DNA containing GvpC gene and Lipase gene fused with His-tag were compared with samples prepared from cells transformed with vector only.

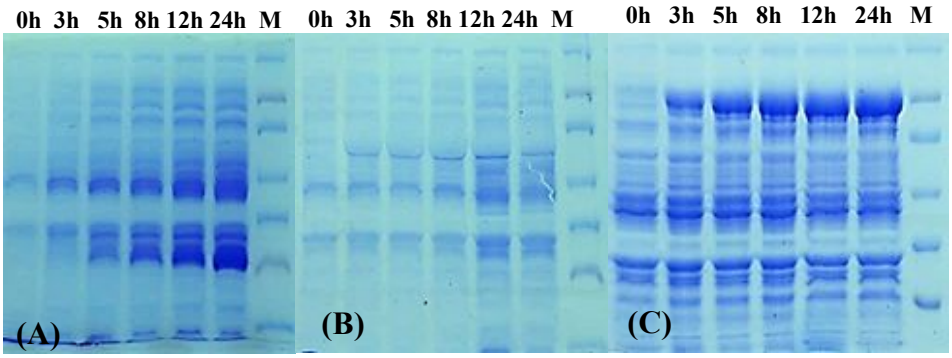


Fig3. Expression of GVP upon IPTG induction

SDS-PAGE analysis of proteins prepared form *E. coli* transformed with (A)recombinant pET19 containing Lipase gene 15℃, (B)recombinant pET19 containing GvpC gene at 25℃ and (C)recombinant pET19 containing GvpC gene fused with Lipase gene at 15℃.

2. Purification of recombinant protein

Since recombinant GvpC gene, Lipase gene and GvpC fused Lipase gene were designed to contain His-tag, purification of recombinant proteins were carried out by Ni-IDA chromatography. Amount of protein eluted from the column was the highest in Elution fraction Ⅰ. Level of expression was slightly less in GvpC fused His-tag sample. These results indicated that recombinant GvpC Lipase gene fused with His-tags can be expressed in *E. coli* and purified by using affinity chromatography.

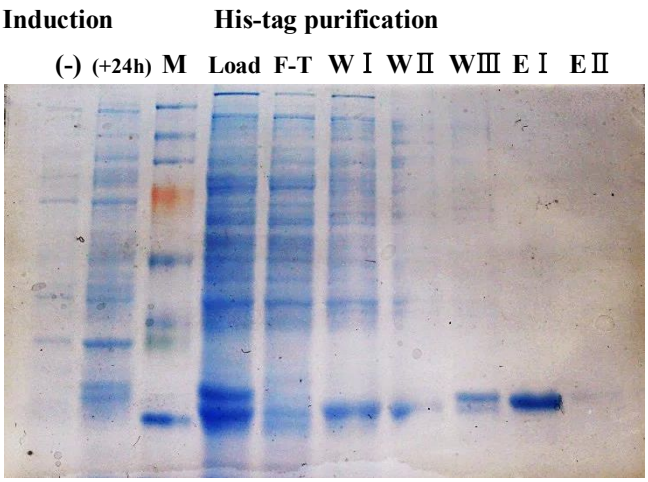


Fig4. SDS-PAGE analysis of purified Lipase fused with His6-tag

Sample included were total proteins extracted from cells transformed by recombinant containing Lipase genes without (-) or with IPTG induction for 24hrs at 15℃ (+24h). Lanes include the fractions for loading (load) and the fractions obtained from flow through (F-T), wash (W) and Elution (E) of the column

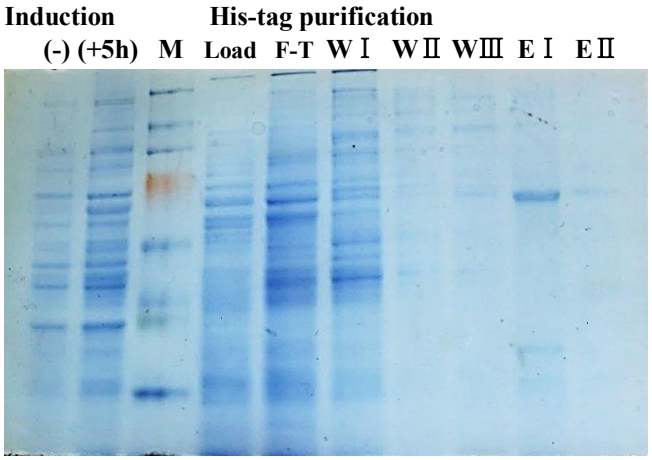


Fig5. SDS-PAGE analysis of purified GvpC gene fused with His6-tag

Sample included total proteins extracted from cells transformed with recombinant containing GvpC genes without (-) or with IPTG induction for 5hrs at 25℃ (+5h). Lanes include the fractions for loading (load) and the fractions obtained from flow through (F-T), wash (W) and Elution (E) of the column.

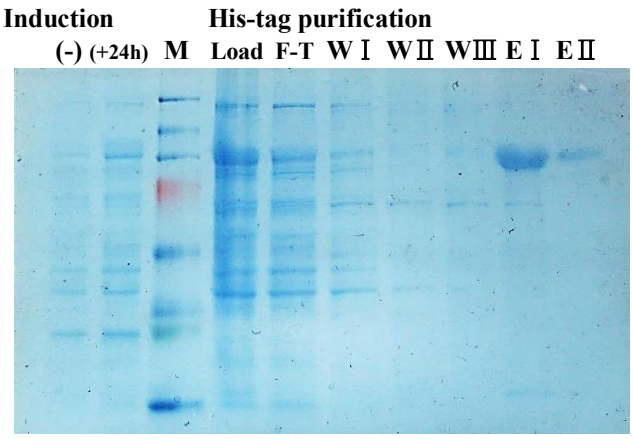


Fig6. SDS-PAGE analysis of purified GvpC and Lipase fusion construct

Sample included total proteins extracted from cells transformed by recombinant containing GvpC genes without (-) or with IPTG induction for 5hrs at 15℃ (+24h). Lanes include the fractions for loading (load) and the fractions obtained from flow through (F-T), wash (W) and Elution (E) of the column.

3. Protein quantitation

Protein quantitation was carried out by using the BCA assay method. Calibration curve was obtained using by BSA (Bovine serum albumin) with dilution. Based on the absorbance of the fractions eluted from the column, amount of purification protein was similar to that of SDS-PAGE result. GvpC gene fused lipase sample was the most abundant among the samples while the amount of GvpC protein alone was the least.

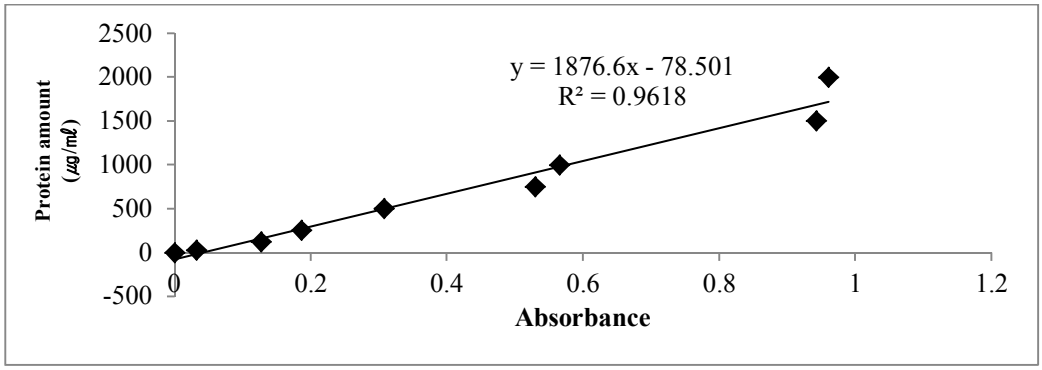


Fig7. BCA standard curve

	Absorbance	Protein amount (㎍/mL)
LipH E Ⅰ	0.1186	2883.78
LiPH E Ⅱ	0.048	230.52
GvpC E Ⅰ	0.074667	1232.36
GvpC E Ⅱ	0.054333	469.22
GV+L E Ⅰ	0.127333	3209.06
GV+L E Ⅱ	0.066	307.1

Table3. Quantitation of protein based on BCA assay

Sample was measured with 20-fold dilution and subtracted blank (measured 3'DW). Absorbance value was measured at 562 nm.

LipH: Lipase gene, GvpC: GvpC gene, GV+L: GvpC gene fused lipase, E Ⅰ: Elution Ⅰ fraction, E Ⅱ: Elution Ⅱ fraction

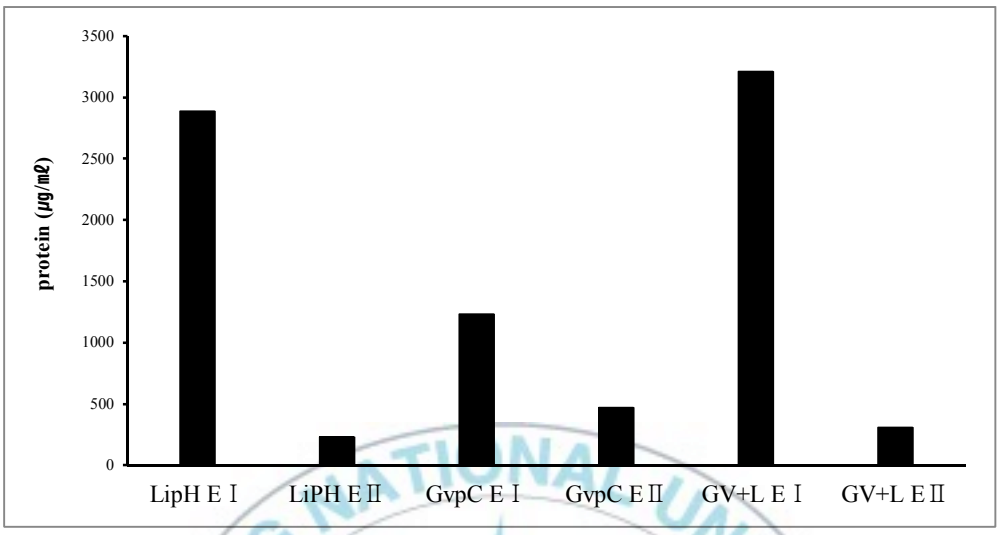


Fig8. Quantitation of purified proteins.

Purified proteins were diluted to 20-fold and subject to the quantitation. Absorbance were measured at 562nm and subtracted from the blank. E Ⅰ and E Ⅱ refer to the fractions eluted from the column, respectively.

4. Rhodamine B plate assay

For plate assay, transformants were grown in LB containing ampicillin at 37℃ overnight and then streaked onto Rhodamine plate. Upon further growth at 37℃ plates were illuminated under the UV light to confirm lipase activity. Lipase gene and GvpC fusion protein expressed from *E. coli* showed lipase activity. Negative control, GvpC protein showed no lipase activity.

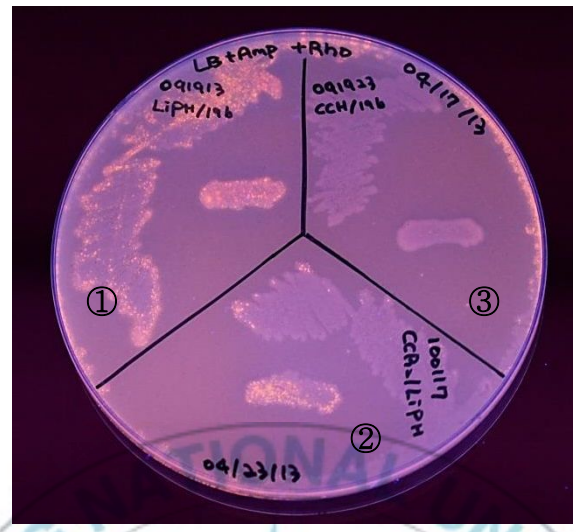


Fig9. Rhodamine B plate assay for lipase activity analysis

E. coli cells tested were transformed with plasmids containing ① Lipase gene streaking, ② GvpC gene fused with Lipase, and ③ GvpC gene.

5. Color metric assay

Lipase activity was also confirmed by using a pH indicator. This was based on oil decomposition into glycerol and fatty acids by lipase lowering the pH in solution. Time course experiment results showed increase in lipase activity in lipase gene sample and GvpC fused lipase sample. This was shown changes to yellow for period over time. On the other hand, GvpC sample and control sample showed no changes from blue color, the first indicator color.

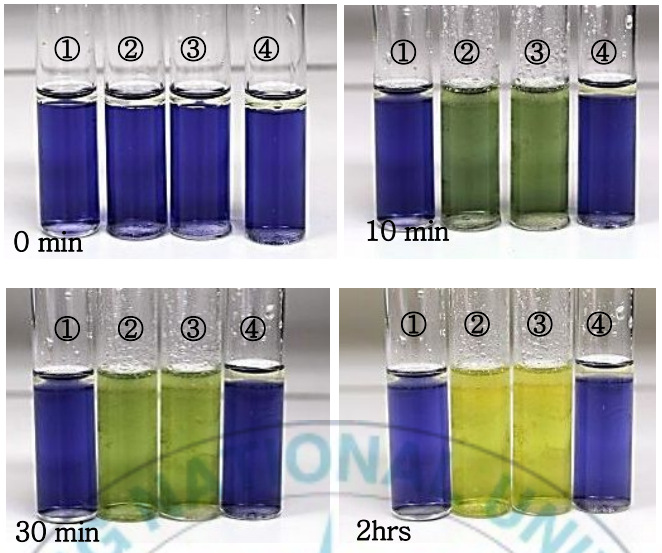


Fig10. Lipase activity analysis of the purified protein

①Control sample: pH indicator + Seawater + Olive oil + 3'DW

②Lipase sample: pH indicator + Seawater + Olive oil + LipaseE Ⅰ

③GvpC + Lipase sample: pH indicator + Seawater + Olive oil + GvpC + LipaseE Ⅰ

④GvpC sample: pH indicator + Seawater + Olive oil + GvpC E Ⅰ

6. Spectrophotometric lipase activity analysis

Lipase activity was also analyzed by the amount of 4-nitrophenol in microplate assay using 20-fold diluted protein. While GvpC + Lipase and Lipase sample produced a similar amount of 4-nitrophenol, GvpC sample did not show any change as similar to the control sample (3'DW instead of protein). Lipase sample showed the highest activity followed by GvpC + Lipase fusion protein. Similar level of lipase activity was detected in GvpC + Lipase fusion protein based on molar ratio.

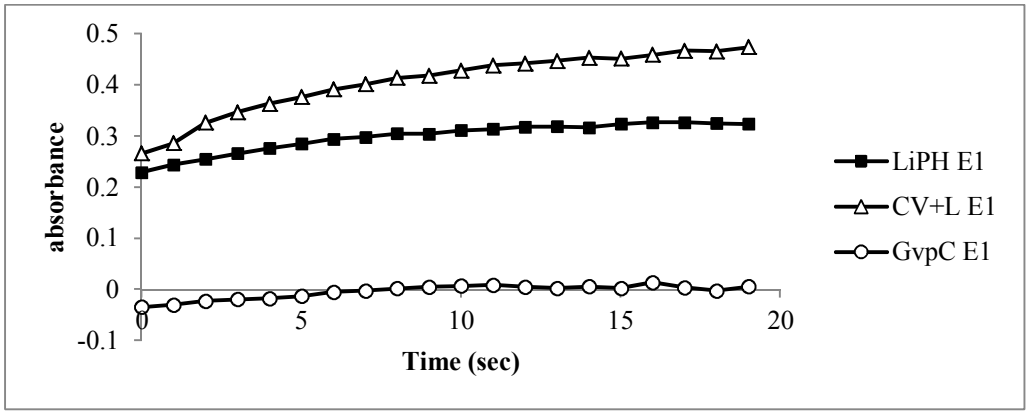


Fig11. Lipase activity analysis by using plate assay

The amount of 4-nitrophenol has the highest GvpC + Lipase sample. The GvpC did not show any lipase activity.

$$VA = \frac{\Delta A410Vs}{VIS\ L\varepsilon 410}$$

Fig12. Lipase activity formula

A_{410} was the absorbance at 410nm. The Vs was total volume, VIS was the volume of the enzyme (the volume of the protein). The L was length of the cuvette, ε_{410} was extinction coefficient 18.8.

	Average	Gradient	Activity
LiPH E1	0.005	0.0046	8.922259
LiPH E2	0.00585	0.0065	0.902651
CCR2 E1	0.01065	0.0097	5.571527
CCR2 E2	0.01375	0.0136	2.239232
CCH E1	0.00215	0.0019	6.895718
CCH E2	0.00285	0.0025	0.376696

Table4. Lipase activity

Measure the amount of 4-nitrophenol by the absorbance per 1 min.

7. GvpA expression

For expression of recombinant proteins, *E. coil* BL21 cell was used. The transformants were cultivated in an LB medium containing ampicillin at 37℃. When the OD_{600} (0.6), IPTG was added the culture. While the induction of gvpA proteins was detected in clones derived from GEX4-T vector, genes cloned into pET19b vector showed little expression.

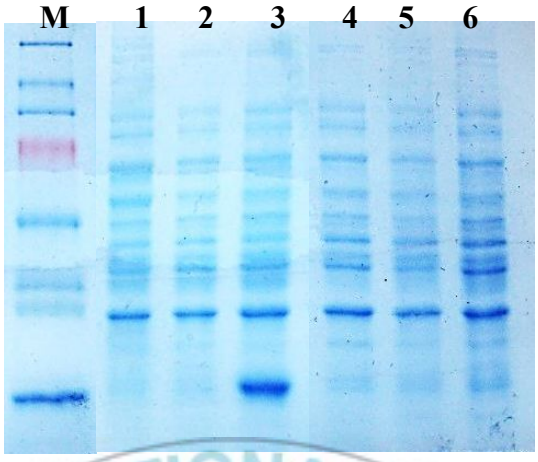


Fig13. SDS-PAGE analysis of total cell lysate

Tasted were cells transformed with vector only (lane 1 and lane 4), with plasmids containing recombinant GvpA (lane 2, lane3, lane 5 and lane 6). Transformants were treated with (lane 3, lane 6) or without IPTG (lane 1, lane 2, lane 4 and lane 5).

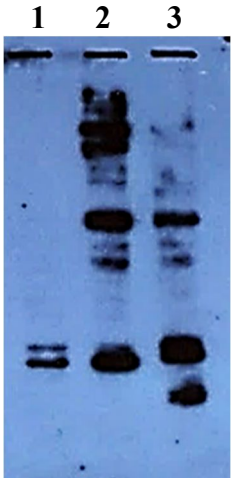
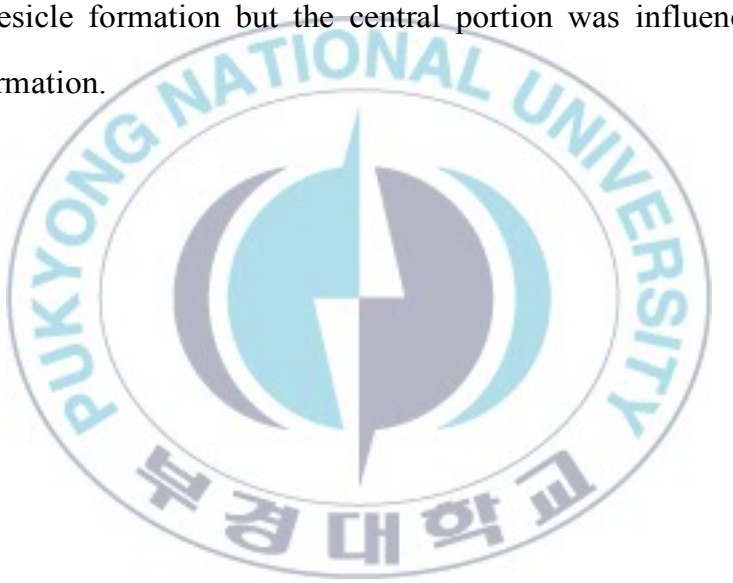


Fig14. Western blot analysis of total cell lysate

Tastes were cells transformed with vector only (lane 1), with plasmids containing recombinant GvpA (lane 2, lane3). Transformants treated with (lane 3) or without IPTG (lane 1, lane 2).

8. GvpA mutation

In order to obtain structural information by using cysteine accessibility analysis, gvpA gene was modified to contain cysteine replacement at position Q3, L31, G33 and A76 of gvpA. Upon cloning into pFM104d followed by transformation into *Halobacterium* SD109, cells were first tested for functional GVP formation. While gvpA, G3C and A76C showed GVP formation, L31C showed -little GVP formation. The results indicate that N-terminal and C-terminal regions of gvpA did not significantly affect the gas vesicle formation but the central portion was influenced by gas vesicle formation.



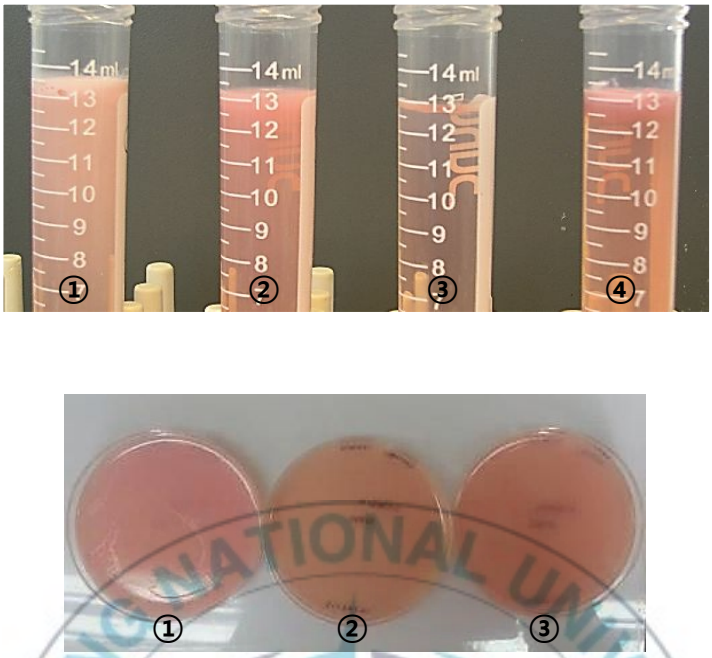


Fig15. Gas vesicle formation in gvpA mutants

①GvpA 3th amino acid substituted with cysteine

②GvpA 33th amino acid substituted with cysteine

③GvpA 76th amino acid substituted with cysteine

④GvpA 31th amino acid substituted with cysteine

While gas vesicle formation was detected in mutants A76C and Q3C, little gas vesicle formation was detected in another mutant G33C.

9. PDS assay

Experiment has been carried out to obtain indirect structural information of gas vesicle using GvpA. PDS was used to react with the exposed cysteine. Therefore, it will be possible to know indirectly that portion of GvpA through was exposed. The experiments were performed using a Q3C, G33C and A76C that GvpA mutant was formed gas vesicle. Through the protein quantification, experiments were used the same amount of protein. PDS assay results, G33C and Q3C samples were similar value. However, A76C sample had a similar value to that NNH sample of GvpA without cysteine mutant. Amino acid of GvpA, 33th and 3th positions were appeared to be exposed to the outside.

	NNH(Con)	Q3C	G33C	A76C
Absorbance	0.209	0.20575	0.1335	0.15125
Protein amount (μg/mL)	193.2304	186.4171	34.9522	72.1633

Table5. Protein amount of GvpA mutants

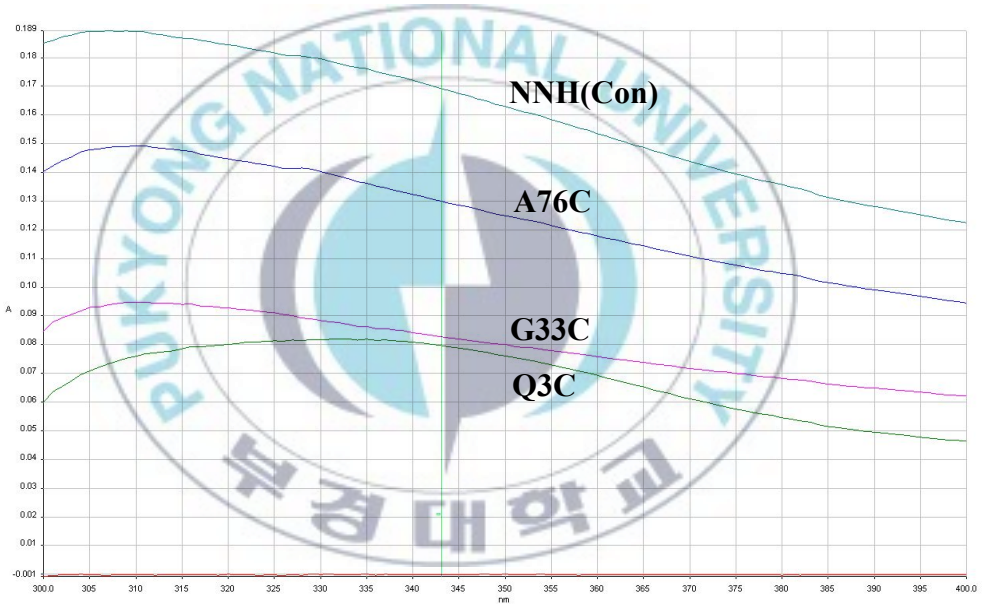


Fig16. Spectroscopic measurement of PDS reactivity

NNH	0.16916A
Q3C	0.079656A
G33C	0.082716A
A76C	0.12994A

Table6. PDS analysis using absorbance at 343nm

$$[\text{Protein}] = \{(A_{343,\text{sample}} - A_{343,\text{blind}}) \times 125\} / \{7600 * x * n_{SH}\} \; {}^{mol}/_{L}$$

n_{SH} : number of SH groups in the protein / molecule

Fig17. PDS assay formula

NNH	0
G3C	0.0014721
G33C	0.0014248
A76C	0.0006458

Table7. PDS assay

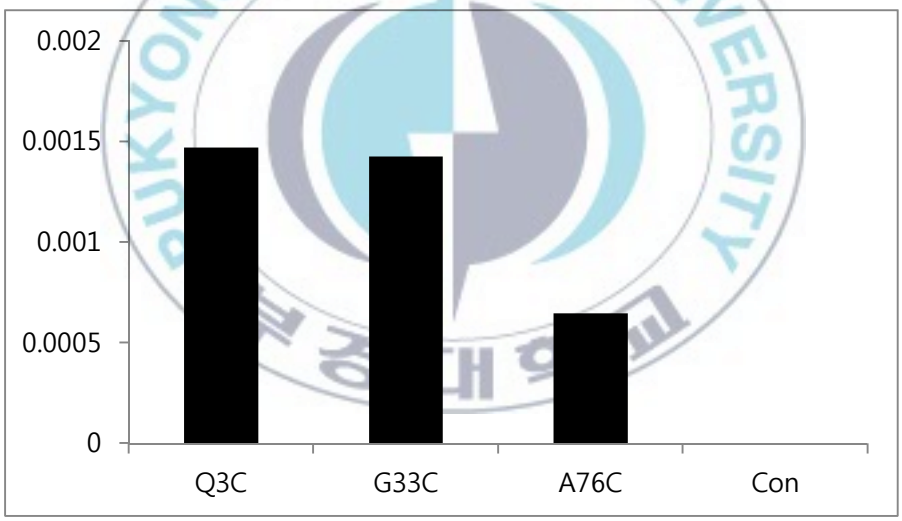


Fig18. PDS assay

10. Gas vesicle purification with His-tag system

In the present study, proteins tagged with His6 residues were purified. In order to test whether we could purify gas vesicle by using His-tag purification column, gas vesicle were mixed with resin. GvpC sample showed binding to the resin and could be purified by using His-tag. In order to test whether- NNH mutant containing His-tag attached to GvpN could be purified, experiments were carried out with gas vesicle purification followed by western blot. The results indicated that gvpN was found to be at the same position as detection of gas vesicle and cell extract. Furthermore, His-tag was not observed to be bound to the resin when gas vesicles were mixed with buffer without using other processing. Although bound to resin in the case of a sonication. Gas vesicle sample treated with sonication showed that viscosity disappeared and turned into opaque form with transparent appearance.

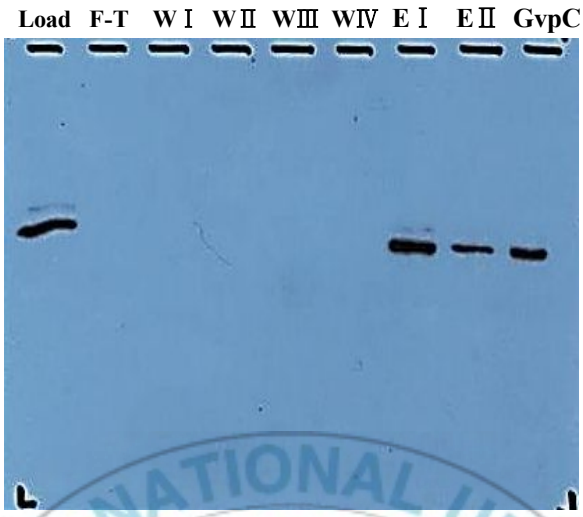


Fig19. **Western blot analysis of His-tag purified of GvpC**

Proteins were separated on 8% SDS-PAGE and the fractions obtained from the fractions for loading (load), flow through (F-T), washings (W), and elutions (E)

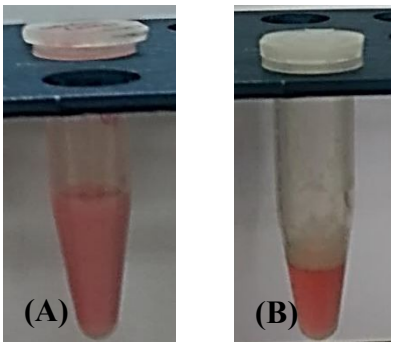


Fig20. Preparation of GVP with or without sonication

(A) Non treatment sonication sample, (B) sonication treatment sample

Sample was turned into a transparent pink color from opaque pink color.

Sonication was turned opaque form into transparent form in gas vesicle.

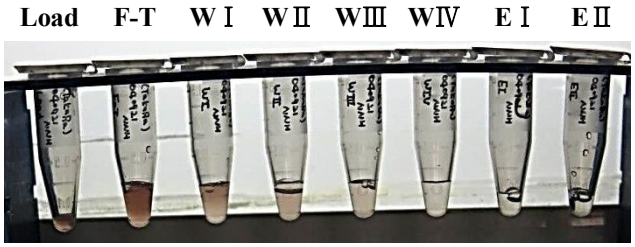


Fig21. His-tag purification of GvpN

The fractions for loading (load) and the fractions obtained from flow through (F-T), wash (W) and Elution (E) tube. Loading, flow through and wash fraction tubes were pale pink color. It turned into a transparent color closer to the Elution fraction tube. It was found that gas vesicle was not bound to the resin by the color of the tube.

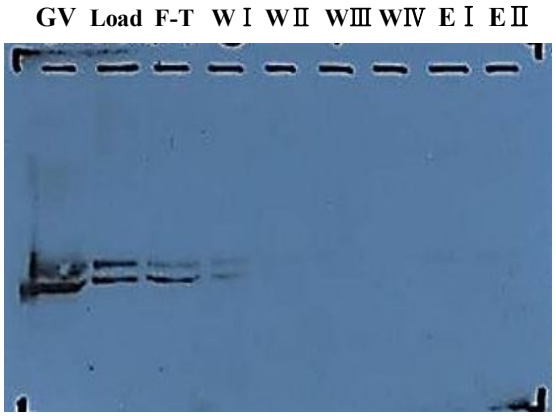


Fig22. Western blot analysis of His-tag purified of GvpN

Proteins were separated on 8% SDS-PAGE and the fractions obtained from the fractions for gas vesicle purification (GV), loading (load), flow through (F-T), washings (W), and elutions (E).

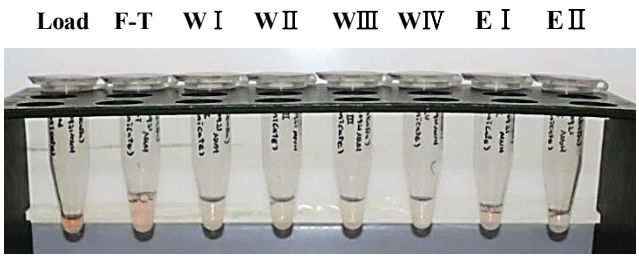


Fig23. His-tag purification of GvpN with sonication

The fractions for loading (load) and the fractions obtained from flow through (F-T), wash (W) and Elution (E) tube. Loading, flow through and Elution fraction tubes were pale pink color. But wash fraction tubes were transparent color. It could be analogized to see the color of the tube that the gas vesicle was elution.

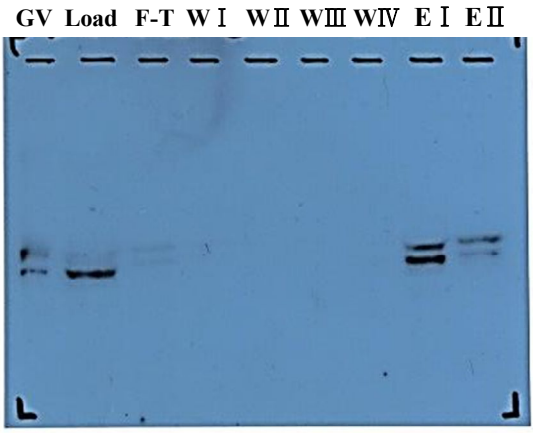


Fig24. Western blot analysis of His-tag purification of GvpN with sonication

Proteins were separated on 8% SDS-PAGE and the fractions obtained from the fractions for gas vesicle purification (GV), loading (load), flow through (F-T), washings (W), and elutions (E).

Ⅳ. Abstract (Korean)

재조합 gas vesicle 단백질의 발현과 특성 분석

김초원

부경대학교 대학원 수산 생물학과

Halobacterium 은 4M 이상의 포화염분 조건에서도 서식할 수 있는 극한미생물의 일종으로 단백질의 구조와 기능과 생명체 특성 연구의 대상의 하나이다. 해수나 담수의 염분 조건에서는 삼투압 차이로 인한 세포용해로 생존이 불가능하여, GMO 의 생태계 교란에 대한 우려를 극복하고 재조합 단백질 생산에 유용한 유용하게 이용될 수 있다. *Halobacterium* 의 단백질인 gas vesicle 은 세포에 부력을 줄 수 있는 단백질 (gvp)로, 유전자 cluster 중 GvpA 와 GvpC 가 gas vesicle 형성에 주요한 역할을 한다고 알려져 있다. 본 연구에서는 재조합 gas vesicle 유전자 조작과 발현에 유용한 벡터 시스템을 구축하고, gas vesicle 을 이루는 주요 단백질인 GvpA 의 구조 연구를 위하여 cysteine mutants 를 구성하고 gas vesicle 에 미치는 영향을 확인하였으며, His6 residue 가 부착된 gvpC 와 gvpN 단백질의 구성 및 발현 분석을 통하여 gas vesicle 연구를 진행하였다. Gas vesicle subunit A 단백질은 GST fusion

형태의 발현을 *E. coli* 에서 확인 하였으며, N-말단과 C-말단에 cysteine 이 도입된 gvpA 는 functional gas vesicle 을 구성함을 확인 하였다. His6-tag 이 융합된 gvpC 와 GvpC 에 Lipase 가 융합된 재조합 단백질을 *E. coli* 에서 분리 정제하였으며, 융합 단백질의 Lipase 활성을 확인하였다. His6-tag 이 융합된 gvpN 의 발현을 통하여 gvpN 과 gas vesicle 과의 상관 관계를 연구하였다.

Ⅴ. Acknowledgement

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마지막으로 제가 공부 할 수 있도록 정신적으로나 물질적으로나 많은 도움을 주신 부모님께 정말 감사합니다. 항상 부족한 딸을 믿어주고 용기를 주셔서 정말 감사하고 부모님의 기대를 져 버리지 않도록 언제나 최선을 다하는 딸이 되겠습니다.

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