FUNCTION AND PROTEIN-PROTEIN INTERACTION OF CHITIN CATABOLIC SENSOR/ KINASE FROM *VIBRIO HARVEYI*

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biochemistry

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การศึกษาหน้าที่และความสัมพันธ์ของไคตินแคตาบอลิกเซนเซอร์/ไคเนสจากเชื้อแบคทีเรีย *Vibrio harveyi*

นางสาวประกายฝัน อุบนบาล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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FUNCTION AND PROTEIN-PROTEIN INTERACTION OF CHITIN CATABOLIC SENSOR/ KINASE FROM *VIBRIO HARVEYI*

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master Degree.

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การศึกษานี้เป็นการศึกษาหน้าที่และความสัมพันธ์ของเพอริพลาสมิคเซนเซอร์โปรตีน ซึ่งเป็นโปรตีนที่อยู่ในส่วนเพอริพลาสซึมของโปรตีนไคตินแคตาบอลิกเซนเซอร์/ไคเนสที่มีชื่อว่า *Vh*ChiS_SM และโปรตีนที่มีความจำเพาะต่อน้ำตาลไคโตโอลิโกแซคคาไรด์ที่มีชื่อว่า *Vh*CBP จากเชื้อแบคทีเรีย *Vibrio harveyi* จากการศึกษาเบื้องต้นพบว่าการทำงานของโปรตีน *Vh*ChiS_SM จะถูกควบคุมโดยโปรตีน *Vh*CBP ซึ่งจะกระตุ้นให้เกิดการทำงานของโปรตีน ChiS และทำให้เกิดการแสดงออกของยีนส์ที่มีความเกี่ยวข้องกับกระบวนการย่อยสลายไคตินภายในเชื้อแบคทีเรีย *V. harveyi* ซึ่งในระบบของ *Escherichia coli* สายพันธ์ BL21(DE3) โดยโปรตีนจะถูกทำให้บริสุทธิ์ด้วยการแยกตามความจำเพาะต่อนิกเกิลเรซิน การแยกตามความแตกต่างของประจุ และสุดท้ายเป็นการแยกตามขนาดของโปรตีน จากนั้นนำโปรตีนที่ได้ไปผลิตโพลีโคลนอลแอนติบอดี โดยนำโปรตีนที่บริสุทธิ์ฉีดเข้าไปในกระต่ายเพศเมียพันธุ์ White- New Zealand จำนวน 2 ตัว เพื่อให้สร้างโปรตีน *Vh*ChiS_SM และ *Vh*CBP โพลีโคลนอลแอนติบอดี้ จากการทดลองพบว่าโพลีโคลนอลแอนติบอดีชนิด *Vh*ChiS_SM และ *Vh*CBP มีความจำเพาะต่อโปรตีน *Vh*ChiS_SM และ *Vh*CBP ตามลำดับ แต่ไม่พบความจำเพาะต่อโปรตีนชนิดอื่นจากเชื้อแบคทีเรีย *Vibrio* นอกจากนี้ยังพบว่าแอนติบอดีชนิด *Vh*CBP มีความจำเพาะต่อโปรตีน CBP ที่มาจากเชื้อ *Vibrio harveyi* สายพันธุ์ 650 หลังจากการกระตุ้นด้วยไคติน จากการตรวจหาความสัมพันธ์ระหว่างโปรตีน *Vh*CBP และ *Vh*ChiS_SM ด้วยเทคนิค pull-down พบว่า *Vh*ChiS_SM ถูกชะออกมาพร้อมกับ CBP ที่มาจาก *V. harveyi* 650 อธิบายได้ว่าทั้งสองโปรตีนมีความสัมพันธ์กัน

สาขาวิชาเคมี
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ลายมือชื่อนักศึกษา ประกายฝัน
ลายมือชื่ออาจารย์ที่ปรึกษา
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PRAKAYFUN UBONBAL : FUNCTION AND PROTEIN- PROTEIN INTERACTION OF CHITIN CATABOLIC SENSOR/KINASE FROM *VIBRIO HARVEYI*. THESIS ADVISOR : ASST. PROF. PANIDA KHUNKAEWLA, Ph.D. 76 PP.

CHITIN/ PERIPLASMIC SENSOR DOMAIN CHITIN/ CATABOLIC SENSOR/ KINASE

The periplasmic sensor domain of two-component chitin catabolic sensor/kinase from *Vibrio harveyi* (namely *Vh*ChiS_SM) is located in the periplasmic region and it-controls the gene expression involved in the chitin utilization system. The recombinant *Vh*ChiS_SM was highly expressed in the *Escherichia coli* strain BL21(DE3) express on the system and purified by Ni-NTA, anion exchange and gel filtration chromatography. The purified *Vh*ChiS_SM was proven to be a monomer of 36.5 kDa. A synthetic gene encoding *Vh*CBP was used to produce a monomer of 61 kDa molecular mass. The purified proteins were used to successfully immunize two New Zealand white female rabbits to obtain anti-*Vh*ChiS_SM and anti-*Vh*CBP polyclonal antibodies, and the *Vh*ChiS_SM and *Vh*CBP polyclonal antibody were found to react strongly with *Vh*ChiS_SM and *Vh*CBP, respectively. they did not cross-react with other *Vibrio* proteins. Furthermore, an anti-*Vh*CBP antibody was also found to react strongly with native CBP in *V. harveyi* type strain 650 after induction. Pull-down assay was used to observe the interaction between *Vh*ChiS_SM and *Vh*CBP, which shows that *Vh*ChiS_SM co-elute with the native *Vh*CBP, indicating that they are endogenous binding partners.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CBP	Chito-oligosaccharide binding protein
cDNA	Complementary DNA
°C	Degree Celsius
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic
ESI	Electronspray ionisation
GlcNAc	*N*-acetyl-glucosamine
$(GlcNAc)_2$	Chitobiose
$(GlcNAc)_3$	Chitotriose
$(GlcNAc)_4$	Chitotetraose
$(GlcNAc)_5$	Chitopentaose
$(GlcNAc)_6$	Chitohexaose
HK	Histidine kinase
HRP	Horseradish peroxidase
HPt	Phosphorylatable histidine
IPTG	Isopropyl-β-D-thiogaltopyranoside
kDa	Kilo Dalton
LB	Luria-Bertani

LIST OF ABBREVIATIONS (Continued)

LC	Liquid chromatograph
Mw	Molecular weight
µg/ml	Microgram per milliliter
mg/ml	Milligram per milliliter
(µ, m) l	(micro, milli) liter
(n, µ, m) l	(nano, micro, milli) liter
(µ, m) M	(micro, milli) Molar
Ni-NTA resin	Nickel-Nitrilotriacetic Acid resin
OD	optical density
PB	Phosphate buffer
PBS	Phosphate buffered saline
PDB	Protein data bank
Pfam	Protein families
rpm	Revolutions per minute
RR	Respond Regulator
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPBs	Periplasmic Solute Binding Proteins
*Vh*CBP	*Vibrio harveyi* chito-oligosaccharide binding proteins
*Vh*ChiS	*Vibrio harveyi* chitin catabolic sensor/kinase
*Vh*ChiS_SM	*Vibrio harveyi* chitin catabolic sensor/kinase sensor module
WT	Wild-type

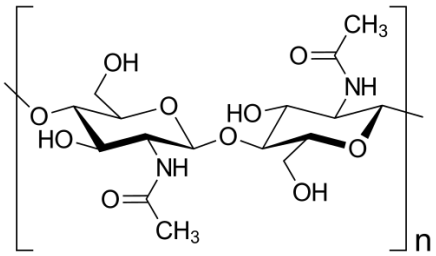
CHAPTER I

INTRODUCTION

1.1 Chitin and applications

Chitin $(C_8H_{13}O_5N)_n$ is a long-chain polymer of *N*-acetylglucosamine linked by β-(1→4)-glycosidic bonds (Figure 1.1A) (Younes and Rinaudo, 2015). Chitin molecules can be designed as α-, β- and γ-chitin (Figure 1.1B). In α-chitin, the chain is arranged in an antiparallel configuration. Chains are arranged in parallel is β-chitin. The γ-chitin is a mixture of both α- and β-chitin. The most abundant of chitin form on the earth is α-chitin (Brigham, 2017). Chitin is found as a component of natural composites throughout the biosphere, such as the cell wall component for many filamentous fungi, the exoskeleton in crustaceans and the cuticle of insects (Laribi-Habchi et al., 2015). Chitin in exoskeletons of invertebrates is found as a combination with proteins and, often, calcium salts.

A



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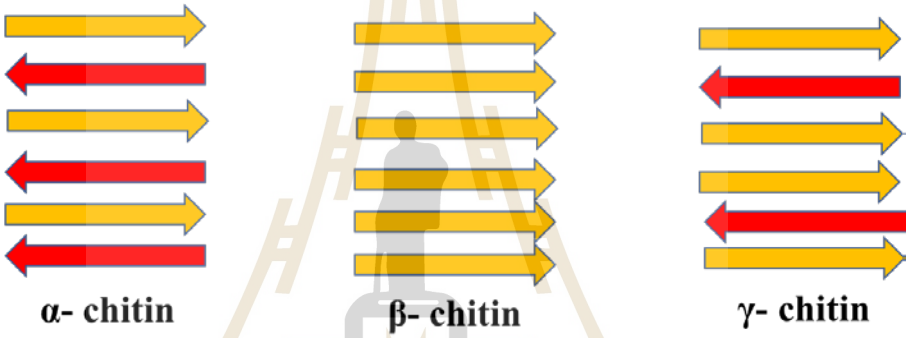

Figure 1.1 The structure of chitin. A) The chemical structure of chitin, poly (*β*-(1-4)-N-acetyl-D-glucosamine). (http://en.wikipedia.org/wiki/Chitin) B) The general organization of *α*-, *β*-, and *γ*-chitin structures. Each colored arrow represents a single chitin chain.

Chitin is one of the most abundant biopolymers in nature. Simultaneously, Chitin wastes produced from the aquatic food industry cause a serious environmental problem. On the other hand, chitin and the oligomers have been attracting much attention for biomedical, agricultural, pharmacological and biotechnological application due to the physiological functionality of the oligomers of chitin and the deacetylated compound, chitosan (Kaya et al., 2013). The most common process of

depolymerization of chitin can be hydrolysis by acids or enzymes, such as lysozyme, some glucanases, and chitinases.

1.2 Vibrionaceae

Vibrionaceae or Vibrios, are gram negative, usually rod-shaped, mesophilic and chemoorganotrophic gamma-proteobacteria having a facultative fermentative metabolism. Vibrios are found in aquatic environments, estuaries, marine water, and aquaculture setting (Thompson, Iida and Swings, 2004). Vibrios appear particularly on marine organisms, e.g., corals, fish, mollusks, seagrass, sponges, shrimps, and zooplanktons. *Vibrio harveyi* is a serious pathogen of both marine fish and invertebrates (e.g., shrimp, sea cucumber). For shrimps, the pathogen is associated with luminous vibriosis.

1.3 Chitin utilization system of Vibrios

In the marine ecosystem, chitin is the major source of carbon and nitrogen for marine vibrios. The degradation pathway comprises many steps (Nemat O. Keyhani and Roseman, 1999), which involves:1) chitin binding, the chemotaxis system specific for chitin oligosaccharide that allows the cell to bind to chitin in the extracellular environment (Yu, Lee and Basslers, 1991). 2) Chitin degradation by chitinase, chitin fragment is transported through the outer membrane by diffusion through chitoporin or porin depending on the size (Wipa Suginta et al., 2013). 3) Then, in the periplasm, *β*-N-acetylglucosaminidase (N. O. Keyhani et al., 1996) and chitodextrinase degrade the chitin fragment to producing GlcNAc and $(GlcNAc)_2$, (N. O. Keyhani et al., 1996). The binding of $(GlcNAc)_2$ to chitooligosaccharides binding protein (CBP) that is usually attached to chitin catabolic sensor/kinase (ChiS) at the outer part of the inner membrane, CBP bind to $(GlcNAc)_2$ activates the ChiS sensor, regulating transcription

of the gene under control of the $(GlcNAc)_2$ catabolic operon (Meibom et al., 2004) (X. Li and Roseman, 2004). 4) active transport of GlcNAc and $GlcNAc_2$ to cytoplasm, GlcNAc is transported to the cytoplasm by the phosphoenolpyruvate transferase system (PTS) (Bouma and Roseman, 1996), while $(GlcNAc)_2$ is transported through the inner membrane by the $(GlcNAc)_2$ ABC permease (N. O. Keyhani et al., 1996). 5) In the cytoplasm, both products are phosphorylated and finally converted to fructose-6-P, acetate and NH_3 (Suginta et al., 2013). which can readily be metabolized as a carbon and nitrogen source for the cells (Figure1.2).

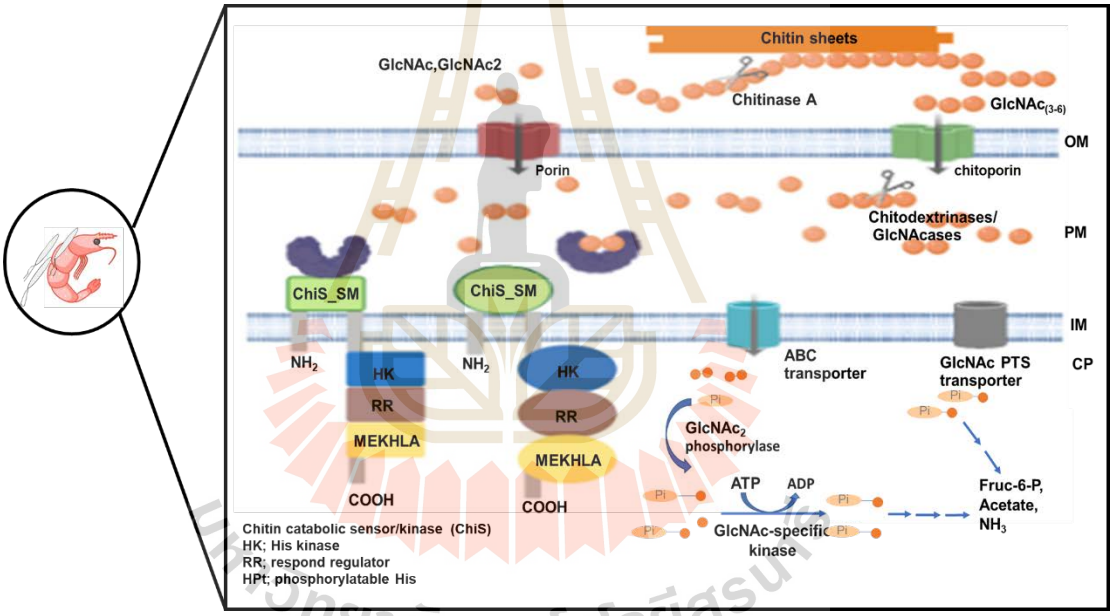

Figure 1.2 The chitin utilization system of Vibrio species (Modified from Suginta et al., 2013).

1.4 Periplasmic-solute binding proteins (SBPs)

Periplasmic-solute binding proteins (SBPs) are localized in the periplasmic space of Gram-negative bacteria (Davidson et al., 2008). The SBPs play an important role in assisting the transport of small solute from the outer membrane to the inner

membrane are involved in the active transport of nutrient into the cytoplasm (Suginta et al., 2013) and signal transduction (G.Scheepers et al., 2016). In recently, Suginta and co-workers have reported the chitooligosaccharide-binding protein from marine *V. harveyi* namely *Vh*CBP, the isothermal titration calorimetry (ITC) analysis was used to demonstrate the binding affinity of $(GlcNAc)_n$ for chitin-binding protein. The reported values of the binding free energy changes ($\Delta G°$) for *Vh*CBP binding to $(GlcNAc)_2$, $(GlcNAc)_3$ and $(GlcNAc)_4$ (-38 to -40 kJ/mol), also dissociation constants (K_d) of *Vh*CBP binding to $(GlcNAc)_{2-4}$ (31-66 nM). Which the $\Delta G°$ values of *Vh*CBP binding to $(GlcNAc)_{2-4}$ were much larger than chitinase in GH18 (Norberg et al., 2010) and GH19 families (Ohnuma et al., 2011). The higher affinity of *Vh*CBP can be described the interaction mechanism. That specifically recognizes chitooligosaccharides binding proteins (CBPs) of namely *Vh*CBP (Suginta et al., 2018).

In the chitin degradation by marine *Vibrios*, CBPs is bind to a small molecule of chitooligosaccharides such as $GlcNAc_2$ in the periplasmic space, and then translocated them to the specific ABC transporter localized in the inner membrane (N. O. Keyhani et al., 1996).

The structure of CBP from *V. cholerae* (*Vc*CBP) was first registered in the PDB databases (PDB code, 1ZU0) and the overall structure of *V.harveyi* (PDB code, 5YQW) in complex with $(GlcNAc)_2$ has since been coded. The amino acid sequence is highly homologous about 83% with *Vc*CBP (Suginta et al., 2018). Figure 1.3 shows the amino acid sequence and overall structure two domains of *Vh*CBP, Figure 1.3A protein sequence alignment of *Vh*CBP. The individual domain is designated as the upper domain and the lower domain. the two domains are connected by two flexible linkers as a hinge that forms the sugar-binding cleft, where $(GlcNAc)_2$ was bound. The

$GlcNAc_2$ is bound, triggering a conformation change of *Vh*CBP. The residues are involved in $(GlcNAc)_2$ binding written in red. The overall structure of *Vh*CBP in complex with $(GlcNAc)_2$ was shown in figure 1.3B, secondary structure separates into two domains, the upper domain (amino acid residues 1-241 and 488-530) is composed of sixteen antiparallel *β*-sheet surrounded by nine α-helices shown in cyan and magenta, respectively. For the lower domain (amino acid 242-481) a three stands antiparallel *β*-sheet and two-stand parallel *β*-sheet are surrounded by twelve α-helices, which are shown in orange and red color. The bound $(GlcNAc)_2$ is represented by a stick model in green. A metal ion located in the central part of the lower domain was observed as Ni^{2+} is present in a dark blue color.

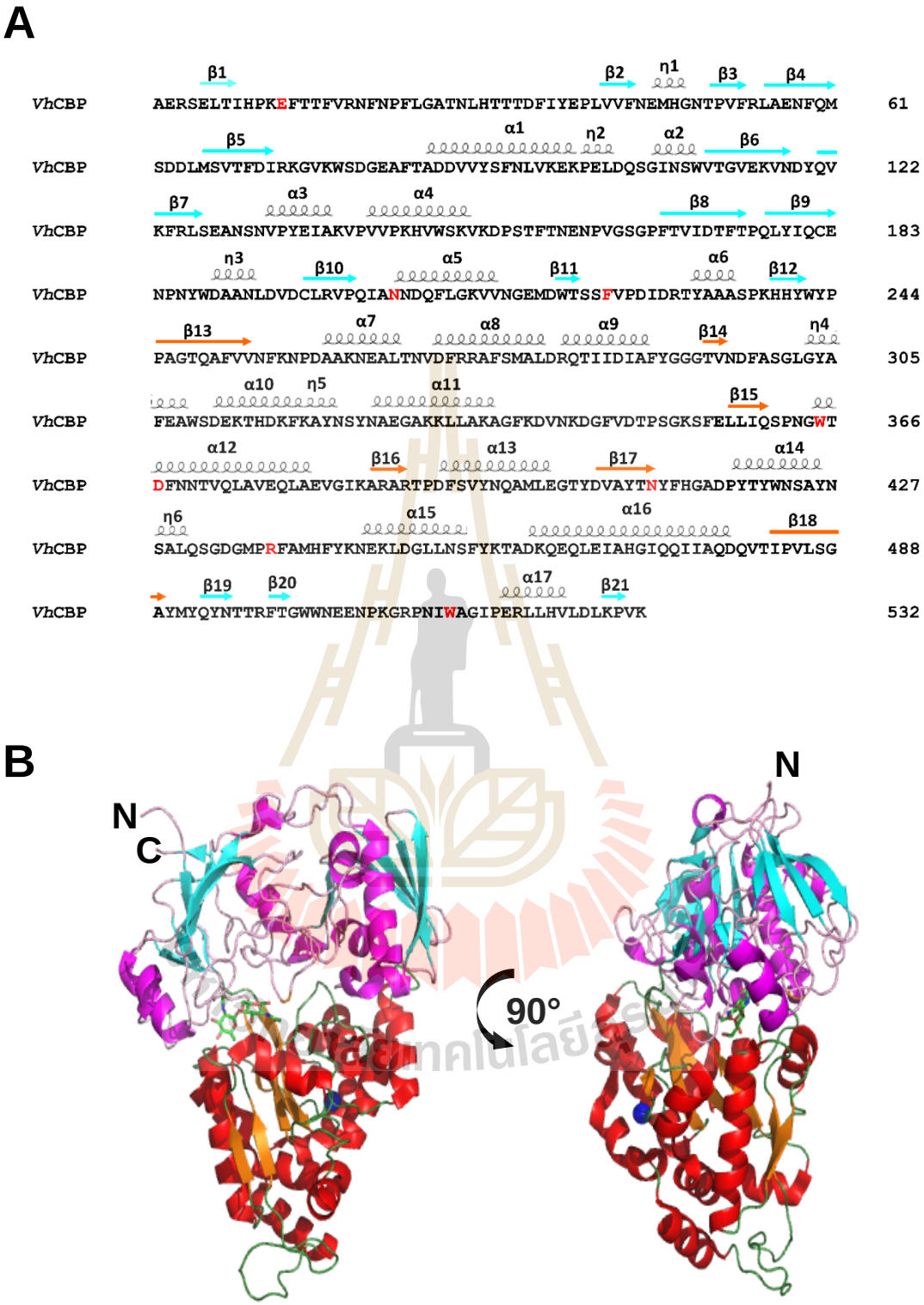

Figure 1.3 Amino acid sequence and overall structure of *Vh*CBP in complex with $(GlcNAc)_2$. A) protein sequence alignment of *Vh*CBP. The residues are involved in

$(GlcNAc)_2$ binding written in red. The secondary structure is show in the same color in panel B. B) The overall structure of *Vh*CBP in complex with $(GlcNAc)_2$.

1.5 Two-component system of bacteria

Two-component systems (TCS) are the major signal transduction system that is the predominant means by which bacteria adapt to change in the surroundings. The TCS are composed of sensor histidine kinase (HK) and a response regulator (RR) (Krell et al., 2010). The HK is regulated by environment, and the HK domain becomes auto-phosphorylated at a histidine residue and then transfers the phosphoryl group to the RR domain, as shown in Figure 1.4. Phosphorylation induces a conformation change in RR that results in the activation of an associated domain that the effect the response (Zschiedrich et al., 2016). The TCS controls the transcription of the genes involved in stress response by the cytosolic RR, which is activated by the auto-phosphorylated from HK (Kwon et al., 2012).

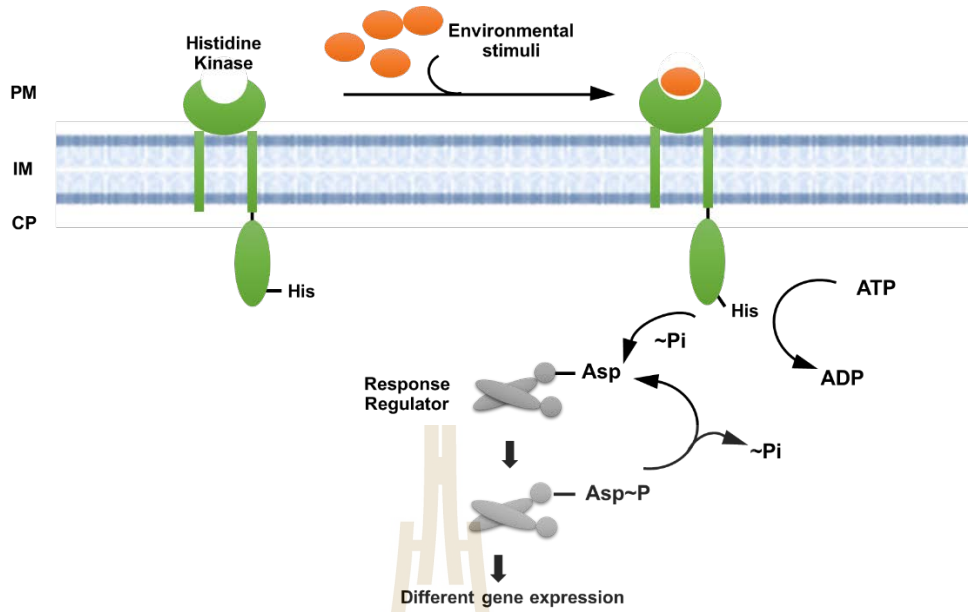

Figure 1.4 Domain organization of a two-component system and signal transduction (modified from M.Zúñiga et al., 2017) .

In *Escherichia coli*, the Cpx two component system is the global modulator of cell envelope stress in Gram-negative bacteria, and consists of the kinase CpxA, the regulator CpxP and the periplasmic accessory protein CpxP (Zhou et al., 2011). The Karolin group reported the dynamic interaction between the CpxA sensor kinase and the periplasmic protein CpxP in unstressed cells (shuts off) in *E. coli* using two different method, membrane-strep-tagged protein interaction experiment (m-SPINE) (Müller et al., 2011) and bacteria two hybrid assay (BACTH) (Kit n.d.). They found the interaction in dependency of three specific stimuli, such as salt concentration, the positive charge of CpxP and misfolded pilus subunit PapE displaces CpxP from CpxA (Tschauner et al., 2014). The periplasmic domain of *Vibrio parahaemolyticus* CpxA (*Vp*CpxA-peri) was solved by crystal structure and the interaction characterized by NMR, which the interaction was not detected (Kwon et al., 2012).

1.6 Chitin catabolic sensor/kinase from *V. harveyi* (*Vh*ChiS)

Chitin catabolic sensor/kinase, ChiS plays an essential role in transferring information from the environment to the genome of prokaryotes and some eukaryotes. ChiS consists of a N-terminal short peptide chain in the cytoplasm, a transmembrane domain, periplasmic domain, and a long polypeptide chain extending into the cytoplasm (Roseman et al., 2003). The latter comprise three subdomains; Histidine Kinase (HK), response regulator (RR) and HK phosphotransfer domain (HPt) as shown in Figure 1.5A. The phosphoryl group is transferred from ATP to HK, and RR, to a His in HPt, and finally to Asp in a separate cytoplasmic cognate response regulator that interacts with the genome.

Li and Roseman (2004) reported the ChiS sensor in the chitin utilization system (Figure 1.5A) in *Vibrio cholera* and *Vibrio furnissi*. They proposed the binding of chitooligosaccharides binding protein (CBP) to the periplasmic sensor domain (ChiS sensor module) and change in the conformation of ChiS to an inactive form (Figure 1.5B). This CBP-ChiS interaction is assumed to be inhibited by $(GlcNAc)_2$ through binding to CBP followed by a conformational change in ChiS to an active form, resulting in the expression of chitinolytic gene cassettes (Figure 1.5C). $(GlcNAc)_2$ is the major product of chitin degradation by chitinase. Since the accumulation of $(GlcNAc)_2$ means the presence of chitin in the environment. Thus, recognition of $(GlcNAc)_2$ as a positive inducer was a reasonable explanation for the mechanism of this system.

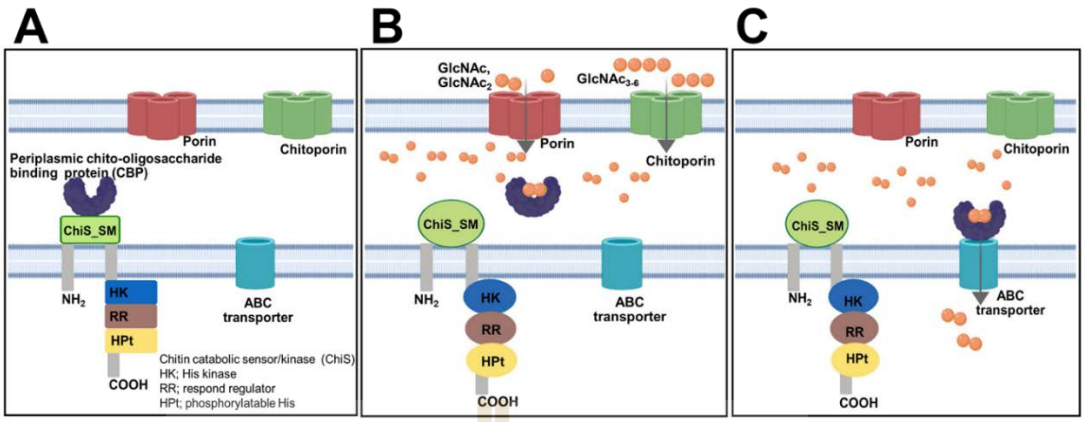

Figure 1.5 Model for regulation of activity of the chitin catabolic sensor/kinase (ChiS) in marine vibrios (Modified from Li and Roseman, 2004). A) In Off mode, chitin utilization system before chitooligoseccharide uptake through the pore. Three compartments, separated by two membrane contain the extracellular space, the periplasmic space and cytoplasmic. The outer membrane contains the porins and chitooligosaccharide specific porin or chitoporin are shown in red and dark green, respectively. The periplasmic contain the chitooligosaccharide-binding protein (CBP) in purple. ChiS consists of a N-terminal short peptide chain in the cytoplasm, a transmembrane domain in gray, the periplasmic domain in green, and three subdomains extending into the cytoplasm; Histidine Kinase (HK) in dark blue, response regulator (RR) in brown and HK phosphotransfer domain (HPt) in yellow. In inner membrane contain ABC transporter shown in blue. B) In On mode, $GlcNAc_2$ binds to periplasmic chitooligosaccharide binding protein (CBP), dissociating from ChiS, which activates ChiS to control transcription of the gene. C) The $GlcNAc_2$ is transferred through the inner membrane by an ABC transporter to the cytoplasm for phosphorylation and finally converted to fructose-6-P, acetate and NH_3.

1.7 Research objectives

This study initially focuses on functional characterization to determine the interaction of *Vh*CBP and *Vh*ChiS_SM. we established an expression protocol for these in proteins in an *E. coli* heterologous expression system. This study aims to clarify the mechanism of regulation of *Vh*ChiS through binding to *Vh*CBP.

The objectives of this study are:

1. To express and purify the ChiS sensor model from *V. harveyi* (*Vh*ChiS_SM).
2. To produce of anti-*Vh*CBP and anti-*Vh*ChiS_SM polyclonal antibody.
3. To study the endogenous expression and purification of native CBP from *V. harveyi* type strain 650
4. To study the binding interaction of *Vh*ChiS_SM and *Vh*CBP by a pull-down assay.

CHAPTER II

MATERIAL AND METHODS

2.1 Materials

2.1.1 Bacteria strains and vector

The pET23a(+) vector was used as the expression vector. *E. coli* DH5α strain was used as a routine host for cloning, subcloning and preparation of recombinant plasmid. Four strains of *E. coli* BL21 (DE3), C43(DE3), Omp8 Rosetta and BL21(DE3) Origami were used as the expression hosts. *Vibrio harveyi* type strain 650 was used for endogenous protein expression.

Table 2.1 Recombinant plasmids and bacteria strains used in this study.

Plasmid and bacteria	Description	Source
Recombinant plasmid		
pET23a(+)/*Vh*ChiS_SM	Wild type	GenScript Co.
pET23a(+)/*Vh*CBP	Wild type	GenScript Co.
Bacterial strains		
E. coli strain DH5α	Cloning host	In our lab
E. coli strain BL21(DE3)	Expression host	In our lab
Origami		
E. coli strain BL21 (DE3)	Expression host	In our lab

Table 2.1 Recombinant plasmids and bacteria strains used in this study (Continued).

Plasmid and bacteria	Description	Source
E. coli strain C43 (DE3)	Expression host	In our lab
E. coli strain BL21(DE3) Omp8 rosetta	Expression host	In our lab
V. harveyi type strain 650	Wild type	In our lab

2.1.2 Chemicals and reagents

Chemicals and reagents used for protein expression, purification, antibody production and immunological analysis were of analytical grade unless otherwise stated. Isopropyl - β -D- thiogaltopyranoside (IPTG), Ni-NTA agarose resin (GenScript), bacto tryptone, bacto yeast extract, agar, potassium chloride, potassium phosphate, sodium chloride, sodium tetraborate, sodium acetate, sodium hydroxyl disodium ethylene diamine tetraacetate (EDTA), glycerol, glycine, imidazole, tris-base, sodium dodecyl sulphate (SDS), acrylamide, *N,N´*- methylene bisacrylamide, ammonium persulfate, Tetramethylethylenediamine (TEMED), coomassie brilliant blue R-250, 2-mercaptoethanol, Complete and Incomplete Freund's adjuvant are products of Sigma– Aldrich (St. Louis, MO, USA). DNaseI was purchased from Pacific Science Co., LTD. Ampicillin and kanamycin are the products of USB Corporation (Cleveland, OH, USA). *p*-coumaric acid and 3-aminophthalhydrazide are product from Fluka® Analytical. Skimmed milk is a product from Hardy diagnostics (West McCoy Lane, Santa Maria, CA).

2.1.3 Instrumentation

Instrument that are required throughout the study include a Sonopusl Ultrasonic homogenizer with a 6–mm and 13 mm diameter probe (Sonics, Connecticut, USA), a DNA gel apparatus (Myrun^{nc}, Ontario, Canada), a protein gel apparatus plus with a compatible power supply (BIO-RAD, Califonia, USA), a Genway UV–VIS spectrophotometer (Thermo, Dreieich, Germany), a Gel Document system (BIO-RAD, Milan, Italy), AKTA prime a purification system (GE Healthcare, Uppsala, Sweden), AKTA start protein purification system (GE Healthcare, Uppsala, Sweden), a semi-dry gel blotting system (Bio-Rad, USA).

2.2 Methodology

2.2.1 Gene identification and construction of *Vh*ChiS_SM recombinant plasmid

The nucleotide sequence encoding of catabolic sensor/kinase in the sensor module part from *V. harveyi* (*Vh*ChiS_SM, accession: WP_101904505) was obtained from GenBank. The gene fragment includes the location AL538-06259 on the *V. harveyi* genome was searched from KEGG Database (https://www.genome.jp/kegg-bin). The signal peptide was predicted from SignalP 3.0 server. The transmembrane region was predicted by SOSUI: Submit a protein sequence (http://harrier.nagahama-i-bio.ac.jp/sosui/). The pET23a(+) plasmid containing a synthetic gene encode *Vh*ChiS_SM was obtained from GenScript Oc,. And recombinant was expressed as a hexahistidine-tagged at C-terminal so that it could be purified by affinity chromatography. Bacteria *E. coli* DH5α strain was the host strain for routine subcloning and plasmid preparation.

2.2.2 Optimization of recombinant *Vh*ChiS_SM expression

To optimize the expression, we were determined with three factors, the firstly, try to optimize the *E. coli* host cell expression, four strains of *E. coli*, BL21(DE3), C43(DE3), Omp8 Rosetta, and Origami (DE3) we have was used. And try to optimize the temperature at 18 °C and 25 °C in the same way. 1 µL of recombinant plasmid pET23a(+)/ *Vh*ChiS_SM was added into 100 µL of the competent cell and spread onto LB (Luria-Bertani) agar plate containing appropriate antibiotic resistance to each *E. coli*. single colonies of transformed bacterial cell were grown in 4 mL of LB media containing the appropriate antibiotic resistance at 37 °C for 16 hr. The starting culture was transferred to 50 mL of LB media containing antibiotic resistance, and pre-incubate at 37 °C and shake 200 rpm until the OD_{600} reached 0.8. Then, add 1.0 mM isopropyl thio–*β*–D–galactoside (IPTG) to induce the cultivation, and continuous incubate at 25 °C for 18 hr. Afterward, the cultivate was centrifuged at 4,500 for 20 min to collect cell pellet. Pellet was resuspended with 10 mL of lysis buffer (20 mM Tris-HCl, pH 8.5, 150 mM NaCl, 10 % glycerol, 0.1 % Triton X-100, 1 mM phenyl methylsulfonyl (PMSF) and 10 µg/mL DNase) and sonicate for 10 min, and centrifuge the broken cell using 12,000 rpm, at 4 °C for 45 min. protein expression was check by SDS-PAGE analysis.

To optimize inducer concentration, in this experiment, IPTG was used for induce expression, the concentration of IPTG; 0.01, 0.05, 0.5, and 1.0 mM we have used and we were performed in the same way with the host cell and temperature condition.

2.2.3 Expression and purification of recombinant *Vh*ChiS_SM

Recombinant plasmid pET23a(+)/ *Vh*ChiS_SM was transformed into 100 µl of competent cells *E. coli* BL21 (DE3) strain and spread onto the LB ager plate containing 100 µl/ml ampicillin. single colonies of the transformed bacterial cells harboring pET23a(+)/*Vh*ChiS_SM was grown in 50 ml of LB medium contain 100 µg/ml ampicillin and shake it at 37 °C for 16 hr for starting culture. Afterward, the starting culture was transferred to a large volume of LB medium containing 100 µg/ml ampicillin, and shake at 37 °C, 200 rpm until OD_{600} reaches 0.8. Then, IPTG was added into the cell culture to 0.5 mM final concentration for *Vh*ChiS_SM induction. Incubation was continued at 18 °C by shaking at 200 rpm for an additional 24 hr. After that, the cells were harvested by centrifugation at 4,500 rpm for 20 min at 4 °C. for protein extraction, the cell pellet was re-suspended with lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 % glycerol, 0.1 % Triton X-100, 1 mM PMSF and 10 µg/mL DNase). Lyse the cell with sonication on ice. Then, remove the cell debris with a centrifuge at 12,000 rpm, 4 °C for 45 min and collect the supernatant. After that, the crud supernatant containing protein *Vh*ChiS_SM was purified by using Ni-NTA agarose affinity chromatography (GenScript). The Ni-NTA affinity column (1.0 x 10 cm.) was equilibrated with 50 ml of the equilibration buffer (20 mM Tris-HCl buffer, pH 7.4 and 150 mM NaCl). Then, incubate the lysate with equilibrated Ni-NTA resin at 4 °C for 1 hr. collect the flow-through fraction, wash the column with 50 ml of equilibration buffer, followed by 50 ml of equilibration buffer containing 10 mM imidazole and collect the solution. After that, apply 2 mL of the equilibration buffer containing 100 mM imidazole and collect into a test tube. Change the test tube and repeat this process until the protein is completely eluted. Fractions, which contain

*Vh*ChiS_SM was pooled and dialyzed thoroughly against 20 mM Tris-HCl, pH 8.5 for removal of imidazole and salt. After that, the *Vh*ChiS_SM was purified by using anion exchange chromatography (HiTrap Q HP, 5ml). Equilibrate the column with 20 mM Tris-HCl, pH 8.5, 100 mM NaCl, apply sample with the flow rate of 3.0 ml/min. then wash column with 10 CV of equilibration buffer. Elute protein by linear gradient of NaCl concentration from 100 mM - 600 mM within 100 mL of the buffer at the flow rate of 3.0 mL/min and fractionate the eluent into 3.3 ml fractions. Collect the fraction containing *Vh*ChiS_SM and concentrate with Amicon Ultra-15 at 3,000 xg, 4 °C Fractions, which contain *Vh*ChiS_SM was purified by gel – filtration chromatography (Hiprep Sephacryl S-300 26/60 High resolution). Equilibrate the column with 20 mM Tris-HCl, pH 8.0, 150 mM NaCl. Then, apply the sample with a flow rate of 2.1 ml/min. Isocratically elute the protein at the same flow rate and fractionate the eluent into 6 mL fractions. the protein purity of eluted was confirmed by SDS-PAGE, the purified *Vh*ChiS_SM was pooled snap freeze. Then keep the sample at -80 °C for use in the experiment. Protein concentration was determined by reading absorbance at 280 nm. Using the extinction coefficients.

2.2.4 Expression and purification of recombinant *Vh*CBP

The expression and purification of recombinant *Vh*CBP was carried out following Suginta et al. (Suginta et al., 2018), the plasmid pET23a(+)/*Vh*CBP was added into 100 µl of *E. coli* Origami(DE3), incubate on ice for 30 min, then incubate at 42 °C for 45 seconds and move the tube to incubate on ice for 2 min. and add 900 µl of LB media (no antibiotic) and shake at 37 °C for 45 min, 150 rpm. After that, centrifuge at room temperature at 4,500 rpm for 5 min. spread 200 µl of culture into LB plate contain 100 µg/ml of ampicillin, and incubate at 37 °C for overnight. Pick colonies

into 30 ml of LB media containing 100 µg/ml ampicillin to make the pre-culture. Then cultivate the cell at 37 °C, 180 rpm for 16 hr. Inoculate 1 % of pre-culture to 1 L of LB media containing 100 µg/mL ampicillin and shake it at 37 °C until OD_{600} reached 0.8. induce the expression by added 0.05 mM IPTG and continued shaking at 18 °C for 24 h. collect the cell culture into centrifuge bottles by centrifuge at 4,500 rpm, 4 °C for 20 min. and keep cell pellet at -20 °C. The cell pellet was re-suspened in 50 mL (1/20 of the culture) of lysis buffer (20 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl, 10 % glycerol, 0.1 % Triton X-100, 1 mM PMSF and 10 µg/mL DNase). Lyse the cell with sonication on ice for 30 min. Remove the cell debris with a centrifuge at 12,000 rpm, 4 °C, 45 min and collect the crude supernatant. For protein purification, Re-suspend the cell pellet into lysis buffer (20 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl, 10 % glycerol, 0.1 % Triton X-100, 1 mM PMSF and 10 µg/mL DNase). Then, crude supernatant was applied to Ni-NTA chromatography, which the column was equilibrated with 20 mM sodium phosphate, pH 7.4, 150 mM NaCl (equilibration buffer). Incubate the lysate with the equilibrated Ni-NTA resin at 4 °C for 1 h. Collect the flow-through fraction. Wash the column with 50 mL of the equilibration buffer containing 10 mM imidazole and collect the solution. Apply 2 mL of the equilibration buffer containing 100 mM imidazole and collect into a test tube. Change the test tube and repeat this process until the protein is completely eluted. Combine elution fraction and dialyze with 20 mM sodium phosphate, pH 7.4, 50 mM NaCl. After that, protein *Vh*CBP was purified by anion exchange chromatography (HiTrap Q HP, 5ml). The column was equilibrated with 20 mM sodium phosphate, pH 7.4, 50 mM NaCl, apply sample with a flow rate of 3 mL/min. then wash the column with 5 CV of dialysis buffer. Proteins were eluted by a linear gradient of NaCl concentration from 50 mM -

500 mM within 100 mL of the buffer at the flow rate of 3.0 mL/min and the eluent was fractionated into 3. 3 mL fractions. The fraction containing *Vh*CBP was collected**.** Finally, *Vh*CBP was purified by gel-filtration chromatography, concentrate protein with Amicon Ultra- 15 at 3,000 xg, 4 °C. Apply *Vh*CBP into the gel -filtration column (HiPrep Sephacryl S-300, 26/60 HR). Which the column was equilibrated with 20 mM sodium phosphate, pH 7.4, 150 mM NaCl in the flow rate of 2.1 mL/min. Protein was isocratically eluted at the same flow rate and fractionate the eluent into 6 mL fractions**.** The purity of eluted protein was confirmed by SDS-PAGE, the purified *Vh*CBP was pooled and snap freeze by liquid nitrogen and keep at -80 °C to use for the experiment. Protein concentration was determined by reading absorbance at 280 nm. Using the extinction coefficients.

2.2.5 Expression and purification of native *Vh*CBP from *Vibrio harveyi* stain 650

The expression of native *Vh*CBP was carried out following Suginta et al. (Suginta et al., 2000). Stick Vibrios stain 650 onto Vibrio Complex Medium (VCM) plat and incubate at 30 °C for overnight. After that pick a single colony into 50 ml Marine Medium (MM) (Bousfield and Graham, 1975), pH 7.6 and shake at 180 rpm, 30 °C for overnight to prepare the pre-culture cell. For cell cultivation, add 1 % of pre-culture into 500 ml MM (in 2 L flask) containing 1 % crystalline chitin (w/v) to induce protein expression. then, incubate the cell at 30 °C, 180 rpm for 24 hr. The cell pellet was collected by centrifuge at 4,500 rpm at 4 °C for 1 hr. Add 50 ml of lysis buffer (20 mM sodium phosphate buffer, pH 7.4, 10 % glycerol, 0.1 % TritonX-100, 1 mM PMSF, 10 µg/ml DNase I) and sonicate 20 min on ice to break the cell. Then centrifuge at

12,000 rpm at 4 °C for 1 hr. to collect crude protein. The protein expression was confirmed by SDS-PAGE and follow by western blotting analysis.

The crude supernatant of native *Vh*CBP was measured the volume. after that, purified by salting out with ammonium sulfate carried out following Krisna C. (Duong-Ly and Gabelli, 2014). add 0-30 % (w/v) of $(NH_4)_2SO_4$ into cell lysate, stir at 4 °C for 30 min. Collect the precipitated proteins by centrifugation at 10,000 rpm for 30 min and remove supernatant and keep at 4 °C. Then add 30-70 % (w/v) of $(NH_4)_2SO_4$ into pellet, stir at 4 °C for 30 min. And harvest the precipitated protein by centrifugation at 12,000 rpm for 30 min, remove supernatant and keep at 4 °C. Run SDS-PAGE and immunoblotting analysis. Afterward dissolve the pellet with suitable volume of 20 mM sodium phosphate buffer, pH 7.4 and dialysis the sample with 2 liter of 20 mM sodium phosphate buffer 3 times. Before applying the sample into column, the sample was centrifuged at 12,000 rpm for 30 min and filtrated. After that, the sample was purified by anion exchange chromatography (HiTrap Q FF, 5 ml). The column was equilibrated with 20 mM sodium phosphate buffer, pH 7.4 (5 CV), sample was applied with the flow rate at 3.0 ml/min. then wash the column with 5 CV of dialysis buffer. Protein was eluted by a linear gradient of NaCl concentration from 0 – 1 M within 100 mL of the buffer at the flow rate of 3.0 mL/min and the eluent were fractionated into 3.3 mL fractions. The fraction containing native *Vh*CBP were collected and check the purity by SDS-PAGE follow by western blotting analysis.

2.2.6 Production of anti-*Vh*ChiS and anti-*Vh*CBP polyclonal antibodies

Production of anti-*Vh*ChiS and anti-*Vh*CBP polyclonal antibodies was carried out using the in-gel method (Amero, James and Elgin, 1994). Partially purified *Vh*ChiS_SM (~2 μg per well) was separated by 12 % SDS-PAGE. Following

electrophoresis, the protein bands were stained with Coomassie Brilliant Blue R-250. After thorough de-staining with distilled water, the protein band (Mr = 36.5 kDa) was excised from the gel. The excised band (~80 µg) was homogenized with 200 µl of 1x PBS (pH 7.2), and emulsified with 500 µl Freund's complete/incomplete adjuvant. The emulsified mixture was injected subcutaneously into a female white rabbit to produce anti-*Vh*ChiS antisera and another female white rabbit to produce anti-*Vh*CBP. Multiple injections were performed and bleeds will be collected as described below:

Week 0: Collection of pre-immune serum (10 ml).

Week 1: First boosting with the *Vh*ChiS and *Vh*CBP (500 µg) antigen mixed with the complete Freund's adjuvant (Sigma-Aldrich, USA).

Week 2: Collection of blood serum 10 ml for first bleeding.

Week 3: Second boosting with the antigen (100 µg) was mixed with the incomplete Freund's adjuvant (Sigma-Aldrich, USA).

Week 4: Collection of blood serum 10 ml for second bleeding.

Week 5: Third boosting with the antigen (100 µg) was mixed with the incomplete Freund's adjuvant.

Week 6: Collection of blood serum 10 ml for third bleeding.

Week 7: Fourth boosting with the antigen (100 µg) was mixed with the incomplete Freund's adjuvant.

Week 8: Collection of blood serum 20 ml for fourth bleeding.

Week 9: Collection of blood serum 40 ml for fifth bleeding

Week 10: Collection of blood serum 40 ml for sixth bleeding.

Week 11: Collection of blood serum 15 ml for seventh bleeding.

The bleeds were collected from an ear vein of the immunized rabbit. The serum was obtained after centrifugation at 2,500 rpm, at 4 °C for 20 min. After centrifugation, the serum was collected and used for immunoblotting analysis. *Vh*ChiS and *Vh*CBP were analyzed by Western blotting and detected with the enhanced chemiluminescence reagent.

2.2.7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The protocol for SDS-PAGE analysis was carried out following Laemmli (LAEMMLI, 1970). Take protein samples and mix with protein sample buffer (150 mM Tris-HCl, pH 6.8, 6 % SDS, 30 % glycerol, 5 % beta-mercaptoethanol, 0.03 % bromophenol blue). Then, the protein sample was boiled at 95 °C for 5 min and spin down the sample by centrifugation a 12,000 rpm for 1 min. 10 µl of protein sample and 3 µl of standard protein markers were loaded onto 12 % SDS-PAGE gel with a discontinuous tris-glycine buffer system in Mini-Protean Tetra Cell (BioRAD), and then run the sample by apply electric power at a constant current 120 volt for 70 min from cathodic (-) end to anodic (+). After electrophoresis, the gel was stained with coomassie blue R250 for 30 min and then the gel was destained with destaining gel solution I (40 % Methanol, 7 % Acetic acid) and follow by destaining gel solution II (5 % Methanol, 7 % Acetic acid) until the black ground was clear. The size of protein band was compared with the unstained protein molecular weight marker (Thermo Scientific™ 26610) compose of beta-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase Bsp98I (25.0 kDa), beta-lactoglobulin (18.4 kDa), lysozyme (14.4 kDa).

2.2.8 Immunoblotting analysis

Immunoblotting was performed following the standard ECL protocol (Mruk & Cheng, 2011). First, the purified proteins *Vh*ChiS and *Vh*CBP samples (1 μg) were mixed with a one-fourth volume of the 4x SDS-gel loading buffer and heated to 100 °C for 10 min. The protein sample was loaded onto 12 % SDS-PAGE. After electrophoresis, the nitrocellulose membrane was soaked in a blotting buffer (25 mM Tris, 192 mM glycine, and 20 % methanol). The protein band was transferred onto a nitrocellulose membrane (Bio-Rad, USA) using a semi-dry gel blotting system (Bio-Rad, USA). The transferred membrane was incubated in a blocking solution containing 1x phosphate-buffered saline (1xPBS), pH 7.2, 5 % Skimmed milk for 1 hr. at room temperature. Remove the blocking solution and rinse the blocked membrane once with 1xPBS. Then, add diluted anti-*Vh*CBP or anti-*Vh*ChiS_SM polyclonal antibody (primary antibody) in 2 % Skimmed milk in 1xPBS, incubate for 1 hr. at room temperature with shaking. Then, remove the primary antibody solution and wash the transferred membrane with 0.1 % Tween20 in 1x PBS (0.1 % PBST) by shaking at room temperature for 5 times (5 min for each time). After that, add Horseradish peroxidase conjugated-goat-anti-rabbits (goat antirabbit IgG (HRP)) (secondary antibody) in 2 % Skimmed milk in 1xPBS and incubate at room temperature for 1 hr. by shaking. Afterward, the membrane was washed with 0.1 % PBST for 3 times and 1xPBS for 2 times at room temperature (5 min for each time). Remove the solution from the membrane and put the membrane on dry transparency plastic and the chemiluminescent substrate reagent (mixing equal parts of the Peroxide Solution and the Luminol Enhancer Solution). Incubate at room temperature for 2 min and detect the protein band by developing with X-ray film at various times.

2.2.9 Antibody titer test

After protein was transferred, remove the membrane form transfer apparatus and cut the membrane to strips membrane. The strips membrane was incubated in a blocking solution containing 1x PBS, pH 7.2, 5 % Skimmed milk for 1 hr. at room temperature. Rinse the blocked membrane once with 1xPBS. Incubate each membrane strips in polyclonal antisera that was diluted with 2 % Skimmed milk in 1xPBS with the two-fold serial dilution of 1:2,500, 1:5,000, 1: 10,000, 1:20,000, 1:40,000 and 1:18,000 to test the production of anti-*Vh*ChiS and anti-*Vh*CBP polyclonal antibodies (primary antibody). After incubation with the antiserum, the membrane was wash five times with 1x PBS containing 0.1 % Tween20 (0.1 % PBST), followed by incubation in 1: 10,000 dilution of the secondary antibody (goat antirabbit IgG (HRP)) in 2 % Skimmed milk in 1xPBS for 1 hr. at room temperature. The membranes strips were washed three times with 0.1 % PBST, then another two times with 1x PBS with 5 min of incubation per wash. Detection using chemiluminescence was carried out by incubating the membrane with a small volume of chemiluminescence substrate for 2 minutes at room temperature. The membrane was wrap with dry transparency plastic, then exposed to an X-ray film in the dark room.

2.2.10 Cross-reactivity antibody

After protein was transferred onto the membrane. Then, the membrane was remove from transfer apparatus and incubate the transferred membrane in 5 % skimmed milk in 1xPBS on the rocker shaker for 1 hr. to block the non-specific sites. Rinse the blocked membrane once with 1xPBS. Incubate each membrane in primary antibody solution 1:20,000 of anti-*Vh*CBP and 1:5,000 of anti-*Vh*ChiS_SM in 2 % Skimmed milk in 1xPBS and shake at room temperature for 1 hr. Wash the membrane

in 0.1 %Tween 20 in 1x PBS (0.1 % PBST) by shaking at room temperature for 5 times (5 mins for each washing) . Incubate the membrane in Horseradish peroxidase conjugated-goat-anti-rabbit (secondary antibody) with a dilution of 1: 10,000 in 2 % skimmed milk-PBS and shake at room temperature for 1 hr. Then, wash the membrane with 0.1 % PBST for 3 times at room temperature (5 mins for each washing) and twice with PBS at the same condition. Remove the solution from the strips and put the strip on dry transparency plastic. Apply a small volume of chemiluminescent substrate reagent to cover all surfaces of the membrane. Incubate at room temperature for 2 minutes and remove with tissue paper. then, detect the protein band by developing with X-ray film at various times.

2.2.11 Protein-protein interaction by pull-down assay

The pull-down assay uses to determines a physical interaction between two or more proteins (Berggård, Linse and James, 2007). For protein-protein interaction of *Vh*CBP and *Vh*ChiS_SM. Which utilize a His-tag protein of recombinant *Vh*ChiS_SM was immobilized to Ni-NTA affinity resin as the bait protein. purified of native *Vh*CBP and cell lysate from *V. harveyi* is prey protein that can be captured and pulled down when target protein flow through. The Ni-NTA resin was equilibrated with 5 column volume of equilibration buffer (20 mM sodium phosphate buffer, pH 7.4 50 mM NaCl and 1 % glycerol) and then 2 mg of purified recombinant *Vh*ChiS_SM was added into the Ni-NTA resin and the purified of native *Vh*CBP and cell lysate from *V. harveyi* were pulled in the same resin. After that incubate at 4 °C for 1 hr., and then flow through fraction was collected and the resin was washed with equilibration buffer until completely wash by checking A_{280} (should be lower than 0.1). the proteins partner were eluted with 250 µl of equilibration buffer containing 250 mM imidazole within 2

ml of elution buffer. The protein was separated by 12 % SDS-PAGE followed by immunoblotting analysis.

CHAPTER III

RESULTS

3.1 Gene identification and sequence analysis of *Vh*ChiS_SM

The amino acid sequence of full length *Vh*ChiS (WP_101904505) comprised of 3,384 bps, which was translated to polypeptide of 1,128 amino acid, The amino acid sequence was analyzed by protein families database (Pfam) (Figure 3.1), the result showed that the full-length *Vh*ChiS comprise of HAMP domain (amino acid residues 374-417), Histidine Kinase A (phospho-acceptor) domain (amino acid residues 459-525), HATPase (amino acid residues 573-683) and Response regulator receiver domain (726-836 amino acid residues) are localized in the cytoplasm. The SOSUI result was displayed full length *Vh*ChiS including two transmembrane regions, first contains amino acid 16-38 amino acid residues and the second contains 352-374 amino acid residues.

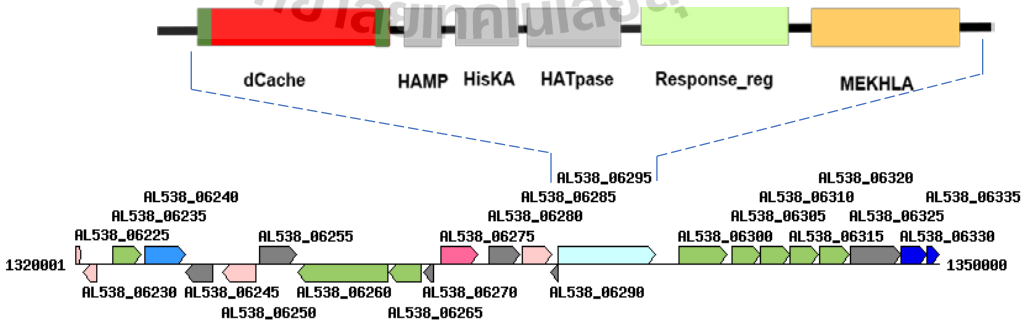

Figure 3.1 Schematic diagram of chitin catabolic sensor/kinase from the *Vibrio harveyi* gene cluster (https://www.genome.jp/keggbin/show_genomemap).

The 3D structure of *Vh*ChiS_SM was predicted by SWISS-MODEL and aligned with the sequences of the structural homologs shown in Table 3.1. We use the PDB code for protein structure 4WY9 is the chemoreceptor from *Campylobacter jejuni* as the template and it has 12.45 % sequence identity. Which the structure comprising ten-stranded antiparallel *β*-sheet flanked by seven-helices containing the longest α-helix, which is composed of 42 amino acid residues (residues 31-73) (Mayra A. Machuca et al., 2017).

Table 3.1 Structure alignment of the *Vh*ChiS_SM with the structure in the Protein Data Bank (PDB).

PDB ID	Seq identity (%)	Protein name	Organism	Reference
4wy9	12.45	Chemoreceptor	*Campylobacter jejuni*	(M.A. Machuca et al., 2017)
3lid	12.15	Histidine kinase	*Vibrio paraphaemolyticus*	(Zhang & Hendrickson, 2010)
5t65	15.32	Chemoreceptor	*Pseudomonas aeruginosa*	(Gavira et al., to be published)
5ltx	15.32	Chemoreceptor	*Pseudomonas aeruginosa*	(Nwachukwu et al., 2017)
4xmq	11.76	Chemoreceptor (Tlp3/Ccml)	*Campylobacter jejuni*	(Liu et al., 2015)

Table 3.1 Structure alignment of the *Vh*ChiS_SM with structure in the Protein Data Bank (Continued).

PDB ID	Seq Identity %	Protein name	Organism	Reference
5wbf	14.47	Chemoreceptor	*Helicobacter pylori*	(M.A. Machuca et al., 2017)
5avf	10.78	Chemoreceptor (Mlp37)	*Vibrio cholerae serotype 01*	(Nishiyama et al., 2016)
3c8c	11.45	Chemoreceptor	*Vibrio cholerae serotype 01*	(Patskovsky et al., to be published)
6f9g	14.59	Chemoreceptor (McpU)	*Pseudomonas putida*	(Gavira et al., 2018)
5ltv	13.62	Chemoreceptor	*Pseudomonas aeruginosa*	(Gavira, Gimenez-Rico, and Krell, to be published)

The nucleotide sequence of identified *Vh*ChiS_SM gene comprised of 1,023 bps. The gene was translated to a polypeptide of 346 amino acid, including the 25 amino acid signal sequence. The theoretical mass of *Vh*ChiS_SM with 39,088.85 Da, was predicted the *p*I about 6.28. The amino acid sequence of *Vh*ChiS_SM was aligned with other periplasmic sensor domain of chitin catabolic sensor/kinase in the family *Vibrionaceae*, such as *V. cholerae* (accession number WP_000488279) as show in Figure 3.2. The sequence identity of *V. harveyi* with *V. cholerae* is 77.43 %. Submission

of the amino acid sequence of *Vh*ChiS_SM through the Swiss-Model database generated a structural model of *Vh*ChiS_SM using that of *Campylobacto jejuni* chemoreceptor as a template (PDB 4WY9) (M.A. Machuca et al., 2016). The comparison with all periplasmic sensor domains with known 3D-structures, *Vh*ChiS_SM is closest to the sensing domain with sequence identity of 12.45 %. Figure 3.3, shows the secondary structure features predicted for in *Vh*ChiS_SM, In the structure comprises 12 *β*-stranded antiparallels flanked by 9 helices containing the longest α-helix, which its composed of 46 amino acid residues (residues 7-42).

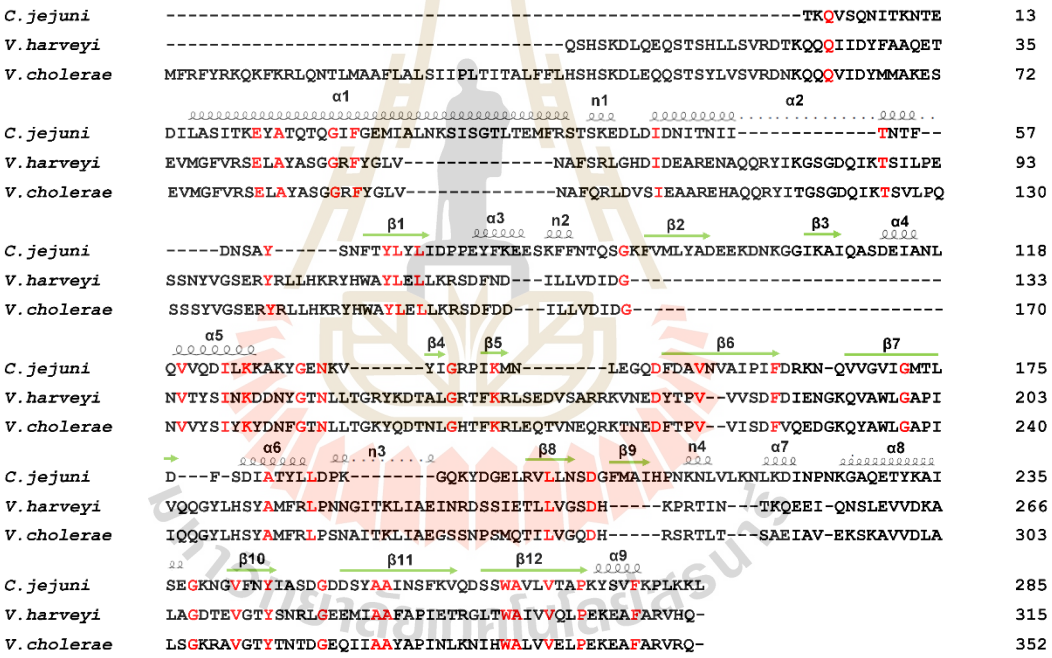

Figure 3.2 Amino acid sequence and secondary structure alignment of *Vh*ChiS_SM. The amino acid sequence was aligned using “CLUSTALW”. The secondary structure of *Vh*ChiS_SM was constructed by ESPript v.3.0 according to the structure of *Campylobacto jejuni* (PDB code 4wy9). Amino acid residues conserved within three sequences highlighted in red. *β*-stand are present as a green arrow.

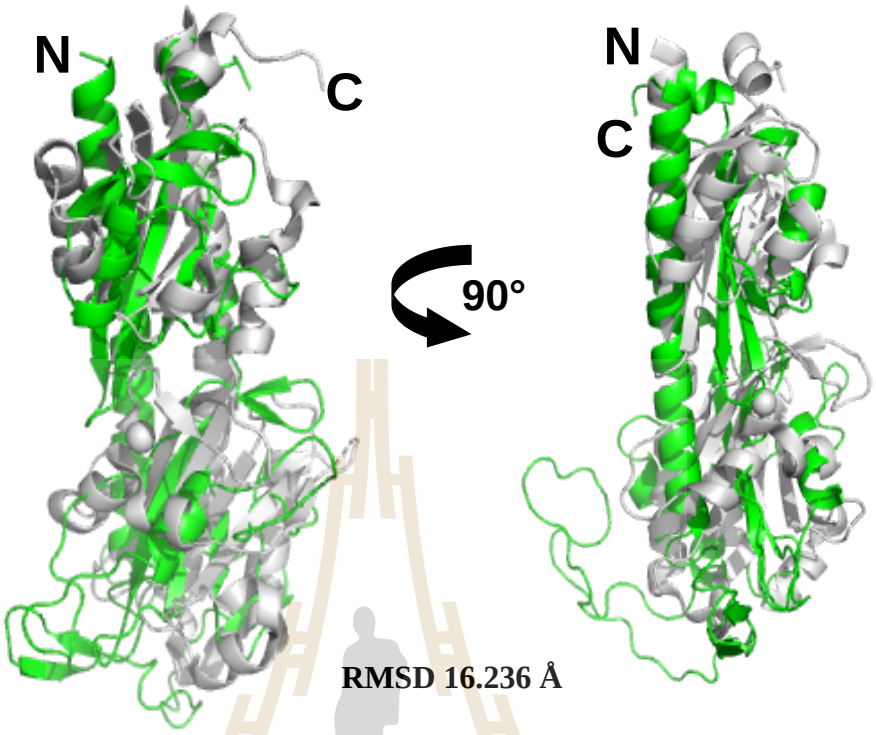

Figure 3.3 Superimposition of the structure model of *Vh*ChiS_SM show in green color with the *C. jejuni* Tlp1-PTPSD show in gray color (PDB code 4WY9), the sequence identity of 12.45 %.

3.2 Optimization of recombinant *Vh*ChiS_SM expression

The pET23a(+) expression plasmid encode *Vh*ChiS_SM gene, the recombinant was expressed with the 25 amino acid signal peptide at N-terminus to aid the protein targeting to the bacteria envelope. After the proteolytic removal of the signal peptide, the *Vh*ChiS_SM containing 321 amino acid residues and Mw of 36,508.74 Da. The optimization of *E. coli* host cell expression included the *E. coli* strains: C43(DE3), BL21(DE3), BL21(DE) Omp8 Rosetta, and Origami (DE3), show in Figure 3.4A. The cells were induction by 1 M IPTG at 25 °C for 18 hr, the SDS-PAGE analysis displayed the protein band of recombinant protein was highly expressed in *E. coli* BL21(DE3)

strain. After that the host cell *E. coli* expression was selected to optimize the concentration of IPTG as inducer (Figure 3.4B). The final step optimization of protein expression, we various concentration of IPTG, including 0.05 mM, 0.1 mM, 0.5 mM and 1.0 mM IPTG at 18 °C for 24 hr. We found that the protein of *Vh*ChiS_SM as high level of expression with 0.5 mM IPTG at 18 °C for 24 hr, shown the protein in SDS-PAGE.

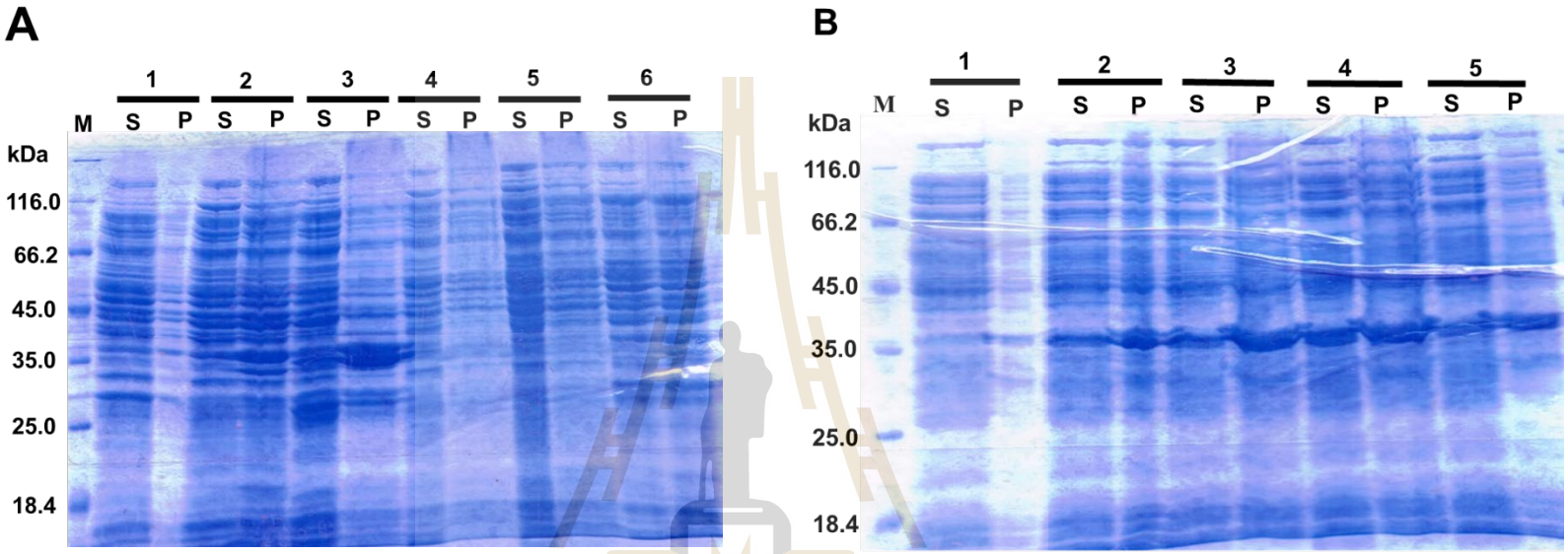

Figure 3.4 SDS-PAGE analysis of optimization expression of *V. harveyi* ChiS_SM. A) SDS-PAGE of optimization of host cell expression in LB medium (Amp^+) induce with 1 M IPTG at 25 °C for 18 hr. lane M; Molecular-weight size marker, lane 1; *E. coli* C43(DE3), lane 2; *E. coli* BL21 (DE3) (large colony), lane 3; *E. coli* BL21 (DE3) (small colony), lane 4; *E. coli* BL21(DE3) Omp8 Rosetta (large colony), lane 5; *E. coli* BL21 (DE3) (small colony), lane 6; *E. coli* Origami (DE3). B) SDS-PAGE of optimization of IPTG concentration express in *E. coli BL21* (DE3) LB medium(Amp^+) at 18 °C for 24 hr. lane M; Molecular-weight size marker, lane,1 non-induction; lane 2, 0.01 mM; lane 3, 0.05 mM; lane 4,0.5 mM; lane 6, 1.0 mM of IPTG concentration, S= supernatant, P= pellet.

3.3 Expression and purification of recombinant *Vh*CBP

The pET23a(+) plasmid containing encoded gene *Vh*CBP fused with His-tag at the C-terminus was transformed into *E. coli* Origami(DE3) to produce *Vh*CBP protein, then purified by Ni^{2+}-affinity chromatography followed by anion exchange chromatography (HiTrap Q) and gel filtration chromatography (26/60 Sephacryl S200). The SDS-PAGE displayed the single band of purified *Vh*CBP about 61 kDa of molecular mass (61,223.41 Da) (Figure 3.5). The chromatographic profile of the final step of purification was shown in Figure 3.5C, the proteins were eluted with 20 mM sodium phosphate, pH 7.4, 150 mM NaCl**,** proteins fractions were analyzed by SDS-PAGE (Figure 3.5C; see in an insert). After purified by gel filtration chromatography, the yield of purified proteins was approximate 10-15 mg/ml per liter of bacterial culture.

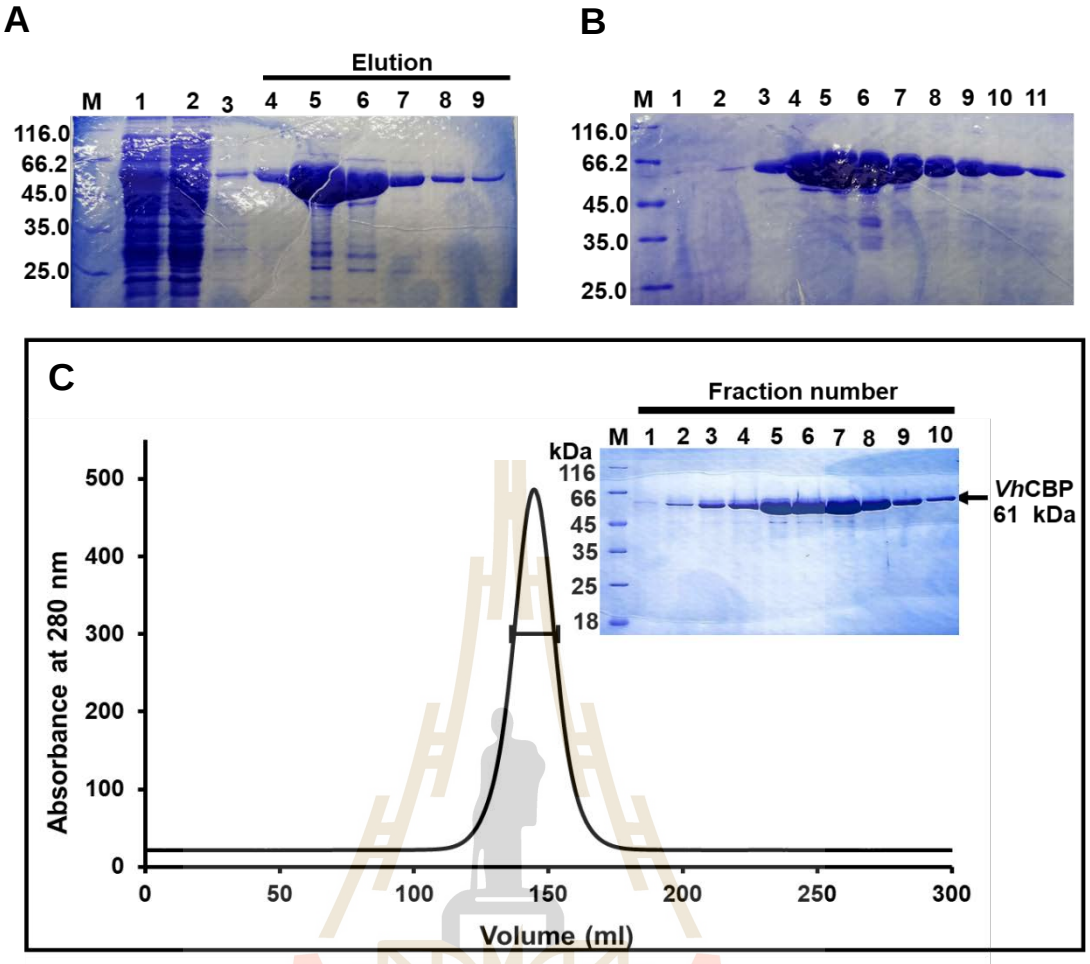

Figure 3.5 Purification of recombinant *Vh*CBP. A) Ni-NTA affinity chromatography. B) Anion exchange chromatography (HiTrap Q HP column). C) Gel filtration chromatography, the proteins were eluted in 20 mM sodium phosphate, pH 7.4, 150 mM NaCl, Protein fraction was analyzed by SDS-PAGE (insert) and stained with Coomassie Brilliant Blue R-250. Lane M= Molecular-weight size marker; Lanes 1-10= the elution fractions obtained from a 26/60 Sephacryl S200 gel filtration column.

3.4 Expression and purification of recombinant *Vh*ChiS_SM

The pET23a(+) plasmid containing encode gene *Vh*ChiS_SM was transformed into *E.coli* BL21(DE3) to produce *Vh*ChiS_SM protein. The six histidine residues tagged at *C*-terminus allowed the protein to be purified by Ni- affinity chromatography, *Vh*ChiS_SM was further purified by anion exchange chromatography using a HiTrap Q resin column followed by gel filtration chromatography (26/60 Sephacryl S200). The profile of final step of purification was show in the Figure 3.6, the proteins fractions were eluted with 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, which in the SDS-PAGE show the single band of purified *Vh*ChiS_SM about 36.5 kDa of molecular mass (36,508.74 Da) (Figure 3.6; see in an insert). After purified by gel filtration chromatography, the yield of purified proteins was estimate to be approx. 5-8 mg/ml per liter of bacterial culture

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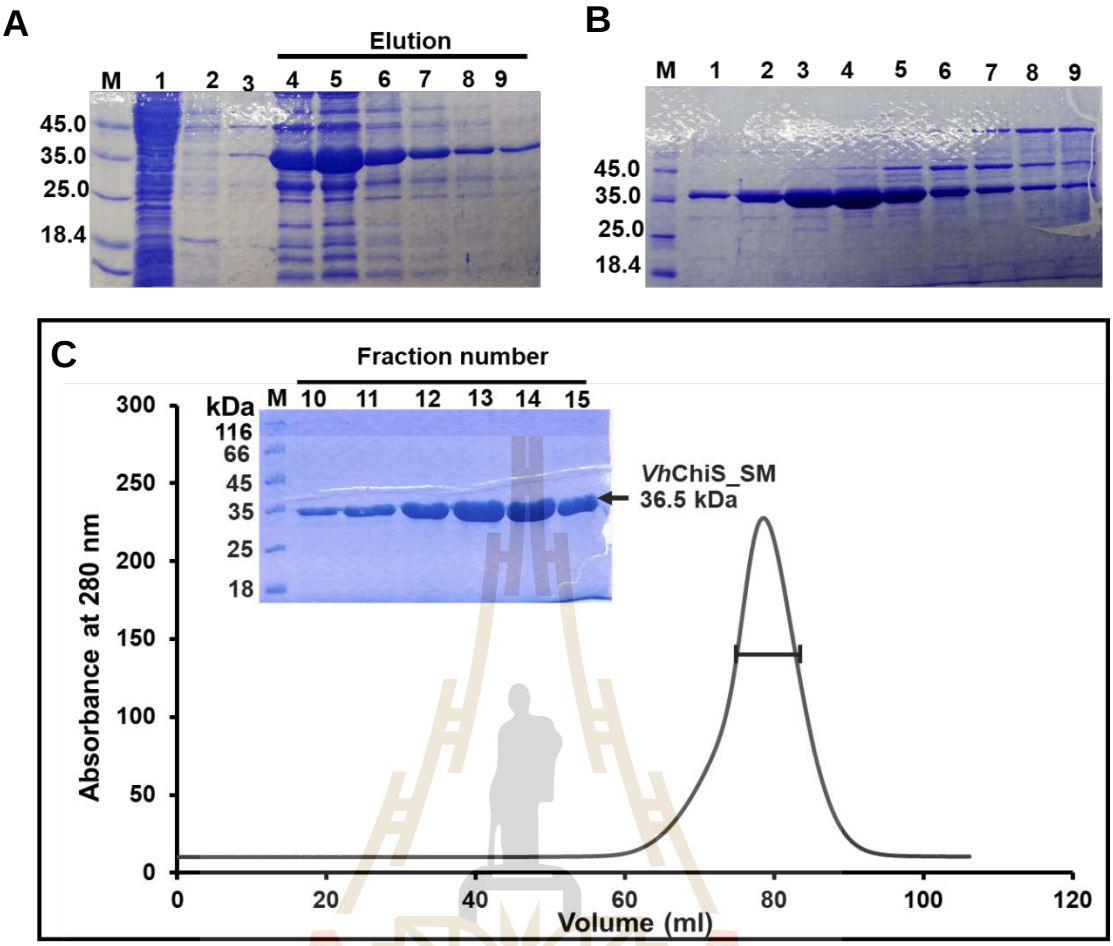

Figure 3.6 Purification of recombinant *Vh*ChiS_SM. A) Ni-NTA affinity chromatography. B) Anion exchange chromatography (HiTrap Q HP column). C) Gel filtration chromatography protein was eluted in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl. Fractions, containing eluted proteins were electrophoresed and stained with Coomassie Brilliant Blue R-250 (insert). Lane M= Molecular-weight size marker; Lanes 10-15= the elution fractions obtained from a 26/60 Sephacryl S200 gel filtration column.

3.5 Antibody production and immunoblotting analysis

After the proteins were isolated and purified by using a 26/60 Sephacryl S200 gel filtration column. The protein band corresponding to the *V. harveyi* CBP and ChiS_SM monomer was excised from a gel and used to raise a rabbit polyclonal antiserum as described in the methodology section. Initially, to measure and detect the amount and the presence of antibodies against immunizing antigen in the female rabbit was estimated by an antibody titer test using western blotting analysis. This experiment was performed using the serial dilution of anti-*Vh*CBP antiserum from 1:2,500 to 1:80,000. Figure 3.7A show a Coomassie blue stained gel of purified *Vh*CBP, corresponding to the immunoblot with anti-*Vh*CBP antiserum in Figure 3.7B, revealed that the anti-*Vh*CBP antiserum could recognized with *Vh*CBP in the minimum dilution of 1:80,000 anti-*Vh*CBP antiserum. and anti-*Vh*ChiS_SM could recognize with *Vh*ChiS_SM in 1:40,000 of minimum antibody dilution in Figure 3.7C. Furthermore, do not have protein was recognized by rabbit pre-immune serum, suggesting that the rabbit can produce only anti-*Vh*CBP and anti-*Vh*ChiS_SM polyclonal antibody after immunization.

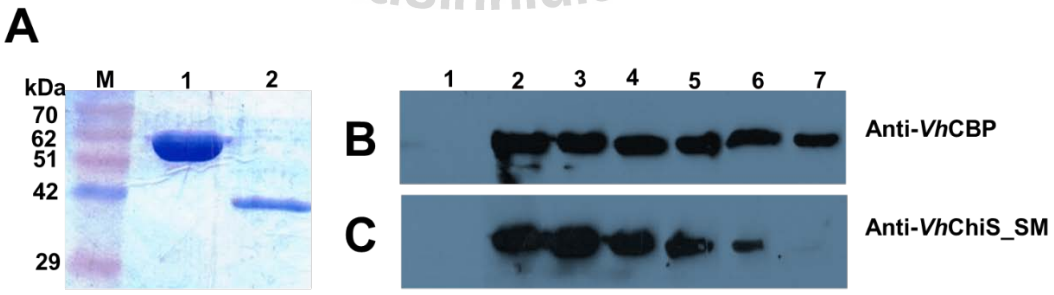

Figure 3.7 Immunoblotting analysis for titer test of anti-*Vh*CBP and anti-*Vh*ChiS_SM. A) Coomassie blue-stain SDS-PAGE of purified recombinant *Vh*CBP and *Vh*ChiS_SM. lane M; standard marker, 2; purification recombinant *Vh*CBP. 3; purified recombinant

*Vh*ChiS_SM. B) The corresponding immunoblot detect with anti-*Vh*CBP antibody. C) The corresponding immunoblot detect with anti-*Vh*ChiS_SM antibody. The reactivity of diluted sera (serial dilution from 1: 2,500 to 1: 80,000) form immunized rabbit was detected. lane 1; pre-immune, lane 2; 1: 2,500, lane 3; 1: 5,000, lane 4; 1: 10,000, lane 5; 1: 20,000, lane 6; 1: 40,000, lane 7; 1: 80,000.

Figure 3.8 shows a Coomassie blue-stained gel of purified *Vh*CBP and *Vh*ChiS_SM as a single band about 61 and 36.5 kDa of molecular mass, respectively. Figure 3.6B show an immunoblot of the corresponding protein band of *Vh*CBP, detected with anti-*Vh*CBP polyclonal antiserum and corresponding of the immunoblot with anti-*Vh*ChiS_SM polyclonal antiserum could be recognized with only *Vh*ChiS_SM (Figure 3.6C). The result suggested that no cross-reactivity of the anti-*Vh*CBP polyclonal antiserum with *Vh*ChiS_SM and anti-*Vh*ChiS_SM with *Vh*CBP. The anti-*Vh*CBP polyclonal antiserum recognized only *Vh*CBP and anti-*Vh*ChiS_SM interacted with only *Vh*ChiS_SM.

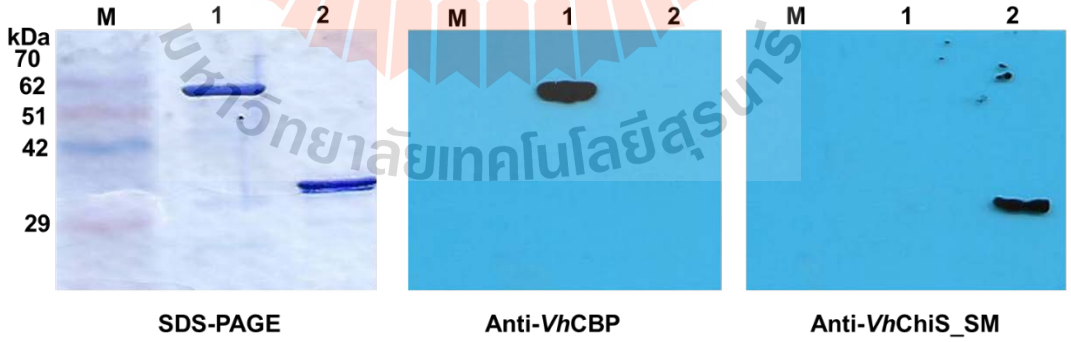

Figure 3.8 Immunological cross-reactivity with anti-*Vh*CBP and anti-*Vh*ChiS_SM. sample of purified recombinant *Vh*CBP and *Vh*ChiS_SM, using 1: 20,000 in the dilution of anti-*Vh*CBP and serum using 1: 5,000 in the dilution of anti-*Vh*ChiS_SM

serum. lane 1; standard marker, lane 2; 1: purified recombinant *Vh*CBP, lane 2; purified recombinant *Vh*ChiS_SM.

3.6 Endogenous expression and purification of native *Vh*CBP from *V. harveyi* strain 650

To determine the expression of native *Vh*CBP in *V.haveyi* strain 650, the expression was estimated after the bacterial growth in the presence of chitin. Coomassie blue stained gel of the bacterial cell growth with and without chitin shown in Figure 3.9A, while Figure 3.9B shown the corresponding immunoblot with an anti-*Vh*CBP antibody. The antibody recognized with protein band in the position of purified recombinant *Vh*CBP after the cell was induced with 0.2 % crystalline chitin and none protein band was recognized by the anti-*Vh*CBP antibody in the absence of chitin.

The purification of native *Vh*CBP was performed by protein precipitation with ammonium sulfate followed by anion exchange chromatography using HiTrap Q resin column. Figure 3.9C shown the chromatographic profile of purification, the proteins fractions were eluted with the gradient concentration of NaCl 0 to 1 M and peak fraction were analyzed by 12 % SDS-PAGE followed by western blot (Figure 3.9C; see in an insert), it is seen that correspond to the molecular mass of about 61 kDa and anti-*Vh*CBP antibody recognized protein band in the position of purified recombinant *Vh*CBP.

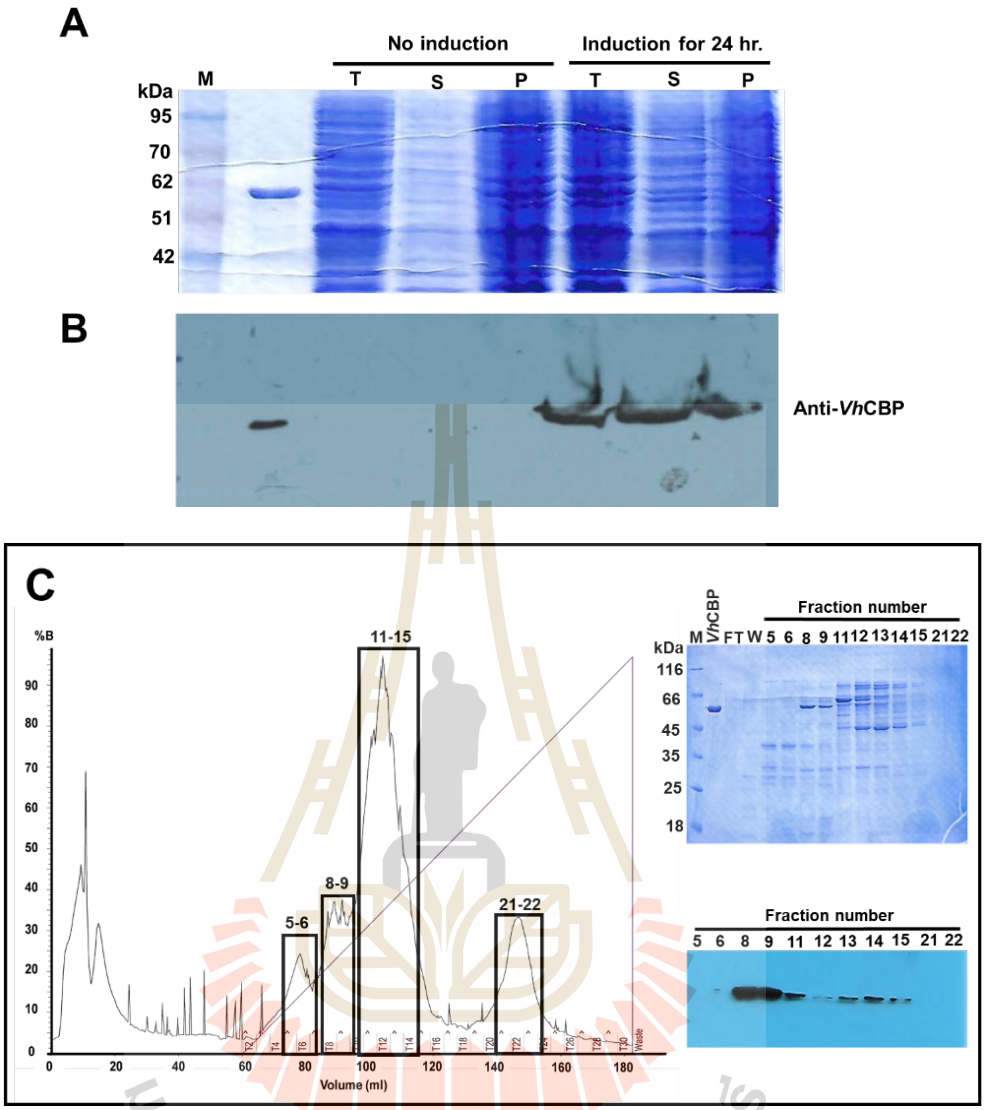

Figure 3.9 Immunoblotting analysis of endogenous expression and purification of CBP in *V. harveyi* 650 in the absence and presence of 0.2 % (w/v) crystalline chitin. A) Coomassie blue-stained SDS-PAGE. B) immunoblot of the same sample as in panel A. using an anti-*Vh*CBP serum. M; standard maker, T; a total of cell lysate, S; supernatant, P; precipitate. C) Chromatographic profile of native *Vh*CBP purification with HiTrap Q HP column (5 ml) connecting to ÄKTA start. The column was eluted with 20 mM sodium phosphate, pH 7.4 containing gradient concentration of 0- 1M

NaCl. Coomassie blue-stained SDS-PAGE analysis, Elution fraction was analyzed by immunoblotting analysis using anti-*Vh*CBP serum (insert).

3.7 Protein-protein interaction study by pull-down assay

To study protein-protein interaction between *Vh*CBP and *Vh*ChiS_SM using pull-downs assay, which pull-downs assay is a commonly use affinity purification method for determining protein-protein interaction. The pull-down involves coupling the protein of interest to beads to serve as affinity resins. Cell lysates that contain target protein that associate with the bound protein was incubated with the beads and protein interactions were detected after washing and elution fractions were analyzed by SDS-PAGE followed by immunoblotting analysis. After the protein *Vh*ChiS_SM was purified by 26/60 Sephacryl S200 gel filtration column. The protein was used to determine the protein-protein interaction between recombinant *Vh*ChiS_SM and CBP from *V. harveyi* 650. Figure 3.8 shown protein interaction by pull-downs assay. In these studies, The his-tagged fusion of purified recombinant *Vh*ChiS_SM was immobilized in the Ni-NTA affinity resin and their protein partner (CBP) was pulled downs from *V. harveyi* 650. The binding was eluted with 20 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 % glycerol containing 250 mM imidazole and analysis by SDS-PAGE followed by immunoblotting. Western blot was probed with a polyclonal anti-*Vh*CBP antiserum and using enhanced chemiluminescence after incubated with the anti-HPR-conjugated-goat-anti-rabbit antibody.

Figure 3.10A-C displays the pull-down assay, which His-tagged fusion of recombinant *Vh*ChiS_SM was immobilized in the Ni-NTA resin and cells lysate of *V. harveyi* 650 were pulled down. After the binding was wash and elute, the binding was detected by SDS-PAGE analysis as shown in Figure 3.10A, to investigate that the

binding was flowed out in the same fractions, the interaction was detected by immunoblotting analysis using anti-*Vh*CBP and anti-*Vh*ChiS_SM polyclonal antibody. As expected, the antibody can recognize the CBP from cell lysate of *V. harveyi* 650 and recombinant *Vh*ChiS_SM after eluted with 20 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 % glycerol containing 250 mM imidazole (Figure 3.10B-C, lane 8-10), but a few amounts of endogenous CBP from cell lysate was detected in the flow-through and wash fraction (Figure 3.10B, lane 5 and 6) and recombinant *Vh*ChiS_SM was eluted completely (Figure 3.10C, lane 11-14). However, an interaction between endogenous CBP from *V. harveyi* 650 and *Vh*ChiS_SM shows the binding but signal is not strong binding.

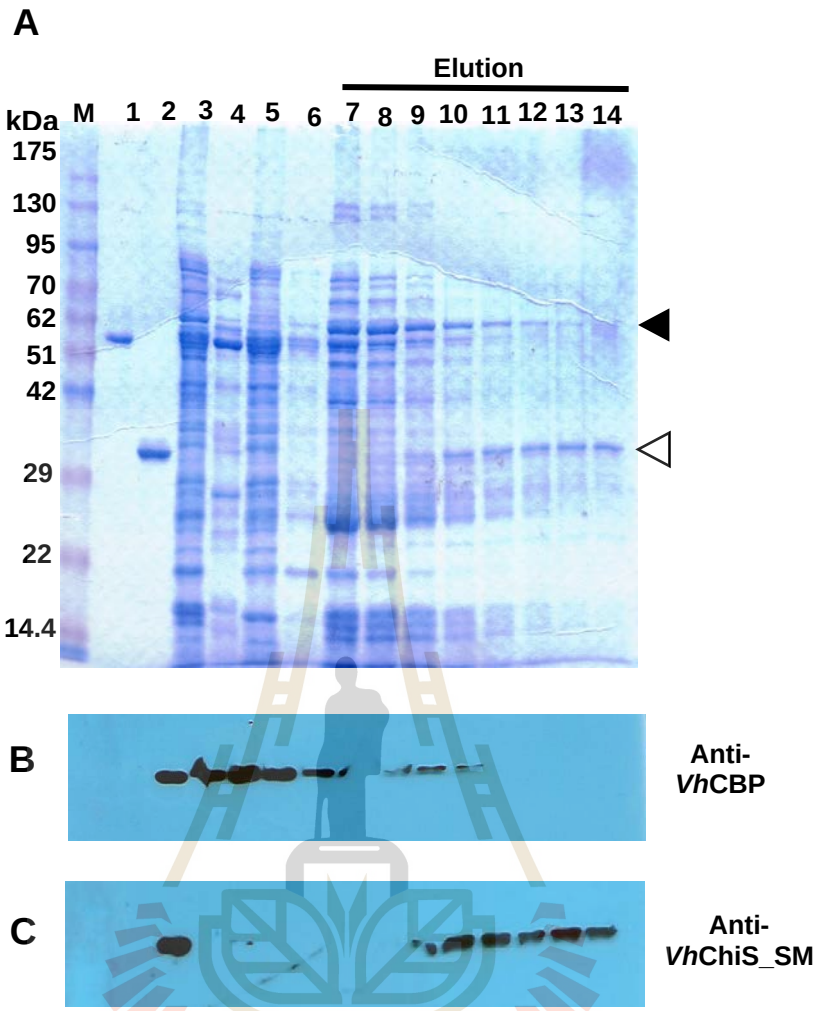

Figure 3. 10 Protein-protein interaction by pull-down assay, Utilized the Ni-NTA resin was immobilized by 2 mg recombinant *Vh*ChiS_SM is a bait protein and crude lysate of *V. harveyi* 650 is prey protein. A) Coomassie blue stained SDS-PAGE, lane M; standard marker, lane 1; purified recombinant *Vh*CBP, lane 2; purified recombinant *Vh*ChiS_SM, lane 3; crude lysate of *V. harveyi* 650, lane 4; native *Vh*CBP, lane 5; flow through fraction, lane 6; wash fraction, lane 7-14; elution fraction, Black triangle show *Vh*CBP and white triangle show *Vh*ChiS_SM. B) immunoblot of the same sample as in panel A. using anti-*Vh*CBP. C) immunoblot of the same sample as in panel A. using anti-*Vh*ChiS_SM.

We also observed the interaction by the pull-down assay, the purified of endogenous CBP from *V. harveyi* 650 was pulled. After the binding was wash and elute, the binding was detected by SDS-PAGE analysis as shown in Figure 3.11A, to investigate that the binding was flowed out in the same fractions, the interaction was detected by immunoblotting analysis using anti-*Vh*CBP and anti-*Vh*ChiS_SM polyclonal antibody. As expected, the antibody can be recognized the CBP from cell lysate of *V. harveyi* 650 and recombinant *Vh*ChiS_SM after eluted with 20 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 % glycerol containing 250 mM imidazole (Figure 3.11B-C, lane 5-12), but a few amounts of purified endogenous *Vh*CBP was detected in the flow-through and wash fraction (Figure 3.11B, lane 3 and 4) and recombinant *Vh*ChiS_SM was eluted completely (Figure 3.11C, lane 9-12). However, an interaction between endogenous CBP from *V. harveyi* 650 and *Vh*ChiS_SM shows the binding but the signal not strong.

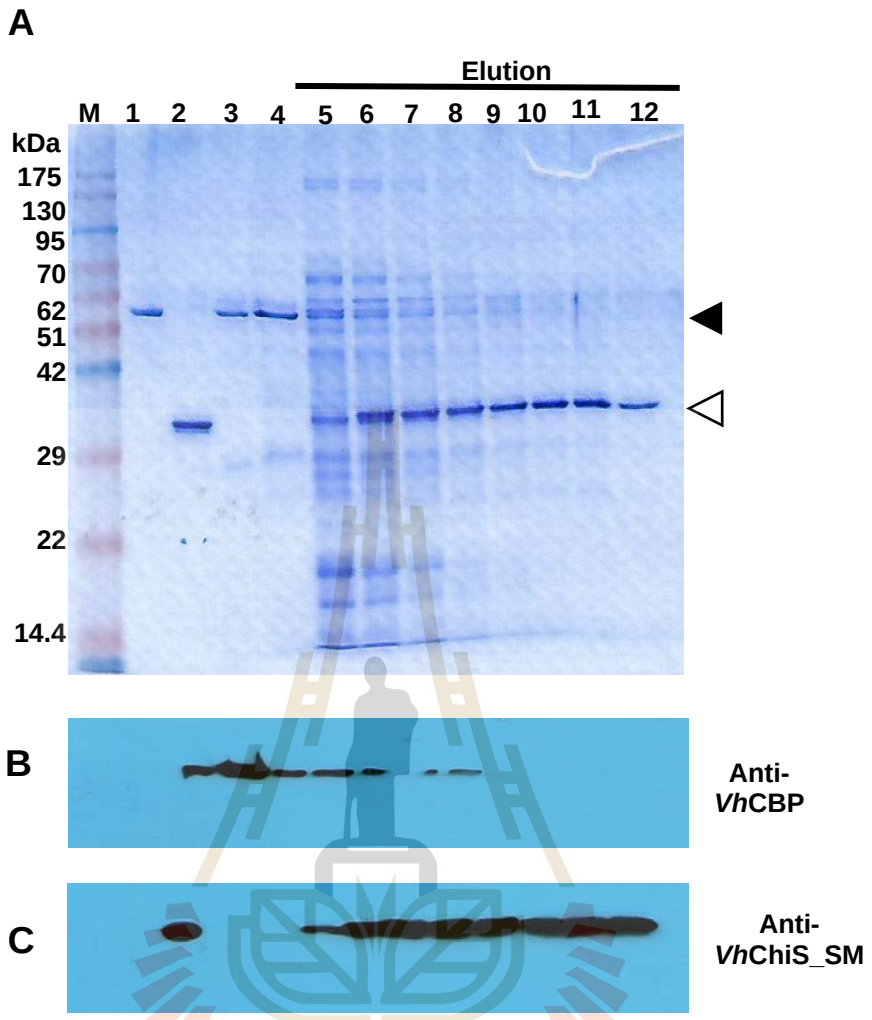

Figure 3.11 Protein-protein interaction by pull-down assay 2 mg recombinant *VhChiS*_SM immobilized in the Ni-NTA is a bait protein and native *Vh*CBP of *V. harveyi* 650 is prey protein. A) Coomassie blue stained SDS-PAGE, lane M; standard marker, lane 1; purified recombinant *Vh*CBP, lane 2; purified recombinant *Vh*ChiS_SM, lane 3; flow through fraction, lane 4; wash fraction, lane 5-12; elution fraction., Black triangle shown *Vh*CBP and white triangle show *Vh*ChiS_SM. B) immunoblot of the same sample as in panel A using anti-*Vh*CBP. C) immunoblot of the same sample as in panel A. using anti-*Vh*ChiS_SM.

CHAPTER IV

DISCUSSION

4.1 Gene identification and sequence analysis of *Vh*ChiS

The periplasmic sensor domain of two-component catabolic sensor/kinase is the protein in the periplasmic region and it controls the gene expression involved in the nutrient utilization system. For example, *Vf*ChiS and *Vc*ChiS are the two-component hybrid sensor/kinase in *Vibrio furnissii* and *Vibrio cholerae* (Xibing Li et al., 2007). Recently, *Vh*ChiS was isolate from the marine bacterium *Vibrio harveyi*. The *Vh*ChiS was separated and expressed by cloning some part of the gene, namely *Vh*ChiS_SM (chitin catabolic sensor module form *V. harveyi*) was tagged with His_6 at the C-terminus of pET23a(+) expression vector. The sequence with other periplasmic sensor domains suggested that *Vh*ChiS_SM most similar to *V. cholerae* is 77.43 % (Pao and Saier, 1995) but both proteins do not have the 3D-structure. The recently, the crystal structure of chemoreceptor in *Campylobacto jejuni* (PDB 4WY9) (Mayra A. Machuca et al., 2016) as similar to *Vh*ChiS with sequence identity only 12.45 % which has 12 antiparallel β-strand flanked by 9 α-helices containing the longest α-helix, which is composed of 46 amino acid residues.

4.2 Expression and purification of *Vh*ChiS_SM and *Vh*CBP

The recombinant *Vh*ChiS_SM encodes 315 amino acid residues (Pao and Saier, 1995), expressing a M_w 36.5 kDa protein. The *VhChiS*_SM was tagged with a C-terminal 6x-His of pET23a(+) expression vector. In this study, the recombinant *Vh*ChiS_SM was showed the highest level of the protein expression when the recombinant plasmid was transformed into *E. coli* BL21(DE3). Proteins *Vh*ChiS_SM were purified by affinity chromatography followed by anion exchange chromatography and size exclusion chromatography, the major band of M_r ~36.5 kDa obtained from SDS-PAGE analysis. A complete purification of *Vh*ChiS_SM yielded approx. 5-8 mg/ml per liter of bacterial culture. For the expression and purification of *Vh*CBP, recombinant plasmid *Vh*CBP has been expressed and purified with transient overexpression system, which *Vh*CBP was expressed in *E. coli* Origami (DE3). In this study, we attempted to obtain the sufficient amount an of *Vh*CBP production with varied concentration of IPTG, temperature and time for protein induction. The result showed highest level of *Vh*CBP expression when the cells were induced with 0.05 mM IPTG at 18 °C for 24 hr. The recombinant *Vh*CBP was tagged with six histidine residues at C-terminus of pET23a(+), so that the protein could be purified by Ni-affinity chromatography. Afterward, anion exchange chromatography and gel filtration chromatography was used in the final step for *Vh*CBP purification. The protein band of *Vh*CBP about 61 kDa was detected by SDS-PAGE gel. A purified *Vh*CBP approx. 10-15 mg/ml per liter of bacterial culture.

4.3 Polyclonal antibody production and endogenous expression of vibrio proteins

The immunoblotting was used for determined antibody titer test, the *Vh*CBP and *Vh*ChiS_SM antiserums reacted strongly with *Vh*CBP and *Vh*ChiS_SM target at the dilution upto 1:80,000 and 1:40,000, respectively. In addition, no signal was detected from rabbit pre-immune serum, event at the highest concentration tasted. Assessment of cross-reactivity with other vibrio proteins used a 1:20,000 dilution of *Vh*CBP and 1:5,000 dilution of anti-*Vh*ChiS_SM. The western blots revealed no cross-reactivity of polyclonal antiserum with *Vh*CBP and *Vh*ChiS_SM, confirming that the antiserum specific for target proteins, *Vh*CBP and *Vh*ChiS_SM. The endogenous expression of vibrio proteins from *V. harveyi* 650 such as chitinase or *Vh*ChiA (Suginta W. et al., 2000) and chitoporin (namely *Vh*ChiP) (Suginta W. et al., 2013) was seen expressed when the cells were grown and induction with chitin-containing medium at 30 °C, suggesting that the *CBP* gene is regulated by *chiS* regulon (Meibom et al., 2004). The western blotting analysis gave evidence of the endogenous CBP expression, which anti-*Vh*CBP polyclonal antibody raised against the endogenous CBP when using 1:20,000 dilution of anti-*Vh*CBP.

4.4 Interaction between *Vh*ChiS_SM and *Vh*CBP detection by pull down assay

In the model for regulation of activation the chitin catabolic sensor ChiS in *Vibrio*, the chitin utilization system of *Vibrios* is controlled by a two-component system histidine kinase (namely ChiS) (Meibom et al., 2004). ChiS must be activated for regulate the chitinolytic genes (X. Li and Roseman, 2004). In an unstressed cell or

minus phenotype, the periplasmic binding protein associates either directly and indirectly with the periplasmic sensor domain (Isaac et al., 2005). The pull-down assay demonstrated physical interaction between *Vh*ChiS_SM and *Vh*CBP in the unstressed cell. Our result estimated that the interaction specific determination of protein expression (Gonsior et al., 1999).

CHAPTER V

CONCLUSION

The periplasmic sensor domain of the two-component chitin catabolic sensor/kinase (ChiS_SM) plays an important role to control the expression of the genes that are involved in the chitin utilization system. It has been suggested that ChiS_SM might bind to chitooligosaccharide-binding protein (CBP), the major chitin-specific solute-binding protein, which transports chitin into the cytoplasmic region through the ABC transporter. In the present study, we expressed and purified the CBP and periplasmic ChiS_SM from *V. harveyi*, the recombinant *Vh*ChiS_SM gene was successfully cloned into the pET23a(+) vector. The *Vh*ChiS_SM was suitable to be expressed in the *E. coli* BL21 (DE3) host strain and further purified to homogeneity by 1) Ni-NTA affinity, 2) anion exchanger, and 3) gel filtration chromatography. When the proteins were purified, *Vh*CBP proved to be a monomer of 61.0 kDa and *Vh*ChiS_SM a monomer of 36.5 kDa. Each purified proteins as used to successfully immunize a New Zealand white female rabbit to obtain anti-*Vh*CBP and anti-*Vh*CBP polyclonal antibodies. Anti-*Vh*CBP and anti-*Vh*ChiS_SM polyclonal antibodies were found to react strongly with CBP and ChiS_SM, respectively, but did not cross react with other *V. harveyi* proteins. Pull-down assays showed that ChiS-SM co-eluted with the endogenous *Vh*CBP, confirming that they are endogenous binding partners.

Further work is underway to find how the genes involved in the chitin degradation pathway are controlled by the interactions between ChiS_SM and CBP in

the *Vibrio* system. The interaction will be confirmed by the appropriate biochemical techniques, such as Isothermal Titration Calorimetry (ITC) and Bio-Layer Interferometry (BLI).

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APPENDICES

APPENDIX A

COMPETENT CELL PREPARATION AND PLASMID TRANSFORMATION

1. Preparation of calcium chloride competent cells

The *E. coli* DH5α, BL21(DE3), Origami (DE3), C43 (DE3) and omp8 Rosetta strain are bacterial strain used for the competent cell preparations. The single colony was picked up from LB agar plate and grown in 5 mL of LB broth and incubated at 37 °C for overnight (18 hr) at 200 rpm. Then 1 mL the overnight cell cultured was subjected into 100 mL of LB broth (ratio 1:100) and grown at 37 °C until OD_{600} reached about 0.4-0.6. The cell cultured was transferred into a pre-chilled polypropylene tube, chilled on ice for 10 min, and the cell pellets were collected by centrifugation at 4,500 rpm at 4 °C for 10 min. The cell pellets were gently resuspended in 10 mL of pre-chilled $CaCl_2$ solution (100 mM CaCl2 and 15 % glycerol) on ice, then centrifuged at 4,500 rpm at 4 °C for 10 min. then, the cell pellets were gently resuspended again in 10 mL of pre-chilled $CaCl_2$ solution. The cell pellets were collected as describe above, then resuspended in 4 mL of pre-chilled $CaCl_2$ and kept on ice for 10 min. aliquot 100 μL of suspension competent cells into 1.5 mL eppendroft tube. The competent cells were frozen using snap-freeze technique under liquid nitrogen and store at −80 °C.

2. Plasmid transformation (Heat shock method)

The frozen competent cells were gently thawed on ice and then added 50-100 ng recombinant plasmid DNAs of *Vh*ChiS_SM and *Vh*CBP into 100 µL of the competent cells and kept on ice. The mixture were immediately placed at 42 °C for 45 second and then rapidly placed on ice again for 3 min. adding 900 µL of pre-warmed LB broth at 37 °C into the transformed cells and incubated at 37 °C for 45 min. centrifugation at 4,500 rpm for 5 min, the 900 µL of supernatant were removed. The 100 µL of cells were spread on a LB agar plate containing the appropriate antibiotic and then incubated at 37 °C overnight.

APPENDIX B

SOLUTION AND REAGENT PREPARATION

1. Solution for bacteria culture

1.1. Luria-Bertani (LB) media

Dissolve 10 g of Bacto-tryptone, 5 of Yeast extract, and 10 g of NaCl in 950 ml distilled water. Stir And dH_2O to 1 L (Autoclave)

1.2. LB plates

Mix 500 mL of LB media with 7 g of Agar (Autoclave). Cool to ~55-65 °C prior to pouring. The addition of antibiotics should be made before pouring and at a temperature not higher than 55 °C.

1.3. Luria-Bertani (LB) media containing 100 µg/ml ampicillin

Dissolve 10 g of Bacto-tryptone, 5 of Yeast extract, and 5 g of NaCl. And dH_2O to 1 L (Autoclave)

1.4. LB plates (0.5 L) containing 100 µg/ml ampicillin

Mix 500 mL of LB media with 7 g of Agar (Autoclave). Cool to ~55-65 °C prior to pouring. The addition of antibiotics should be made before pouring and at a temperature not higher than 55 °C.

1.5. Luria-Bertani (LB) media containing 100 µg/ml ampicillin and 25 µg/ml kanamycin

Dissolve 10 g of Bacto-tryptone, 5 of Yeast extract, and 5 g of NaCl. And dH_2O to 1 L (Autoclave)

1.6. LB plates (0.5 L) containing 100 µg/ml ampicillin and 25 µg/ml kanamycin

Mix 500 mL of LB media with 7 g of Agar (Autoclave). Cool to ~55-65 °C prior to pouring. The addition of antibiotics should be made before pouring and at a temperature not higher than 55 °C.

1.7 Vibrio Complex Medium (VCM), pH 8.5 (1 L)

Dissolve 5 g bacteriological peptone, 5 g bacto-yeast extract and 30 g NaCl in 500 ml of deionised H_2O. Stir until solutes have dissolved. Tris-HCl was added in to the medium to a final concentration of 50 mM. Adjust the pH to 8.5 with 5 N NaOH. Adjust the volume of the solution to 1 litre with deionised H_2O. Sterilise by autoclaving for 20 min at 15l b/sq. in. on liquid cycle.

1.8 Marine Medium 2216E (MM) (1 L)

Dissolve, 5 g Bacteriological peptone, 5 g bacto-yeast extract and 0.10 g $FePO_4$ in 500 ml of 500 ml of filtered, aged sea water. Stir until solutes have dissolved. Adjust the pH to 7.5-7.6 with 1 N NaOH. Adjust the volume of the solution to 1 liter with filtered, aged sea water. Sterilise by autoclaving for 20 min at 15l b/sq. in. on liquid cycle.

1.9. VCM, pH 8.5 plates (0.5 L)

Mix 500 mL of VCM media with 7 g of Agar (Autoclave). Cool to ~55-65 °C prior to pouring.

1.10. MM plates (0.5 L)

Mix 500 mL of MM media with 7 g of Agar (Autoclave). Cool to ~55-65 °C prior to pouring.

1.11 Antibiotic stock solution

1.11.1 Ampicillin stock solution (100 mg/ml)

Dissolve 1 g ampicillin in sterile distilled water to a volume of 10 ml. Sterilize by filter the solution through a 0.2 µM cut-off membrane disc, aliquot and store at -30°C.

1.11.2 Kanamycin stock solution (50 mg/ml)

Dissolve 1 g ampicillin in sterile distilled water to a volume of 10 ml. Sterilize by filter the solution through a 0.2 µM cut-off membrane disc, aliquot and store at -30 °C.

1.12 Isopropyl thio-*β*-D-galactoside (IPTG) stock solution (1 M)

Dissolve 2.38 g of IPTG in distilled water and make up to a final volume of 10 mL. The stock solution is filtered to sterilisation and aliquoted to small volume and stored at −20 °C.

2. Reagents for competent *E. coli* cell preparation

2.1 $CaCl_2$ solution (100 mM $CaCl_2$ contains 15 % (v/v) glycerol)

Preparation of 100 mL CaCl2 working solution, mixed the stock solution as follows:

- 10 mL of 1 M CaCl2 (14.7 g/100 mL, filtered to sterilisation)

- 15 mL of 100 % (v/v) sterilised glycerol (autoclaved at 121 °C, for 15 min)

Adding sterile distilled water to bring a volume to 100 ml. Store the solution at 4 °C.

3. Reagent for agarose gel electrophoresis

3.1 50x TAE buffer

Mix 242 g Tris-base, 57.1 mL glacial acetic acid, and 100 mL of 0.5 M EDTA (pH 8.0). Adjust the final volume to 1,000 mL with distilled water. Store the solution at room temperature.

3.2 6x DNA loading solution (10 mL)

Mix 0.025 g Bromophenol blue and/or 0.025 g xylene cyanol and 3 mL of 100 % (v/v) of glycerol. Adjust to the final volume of 10 mL with ditilled water and store at 4 °C.

4. Solutions for protein expression and purification

4.1 Lysis buffer for *Vh*CBP (20 mM phosphate buffer, pH 7.4, 150 mM NaCl, 10 % glycerol, 0.1 % TritonX-100, 1 mM PMSF, 10 µg/ml DNase I) (200 mL)

Mix solution of 20 ml of 100 % glycerol, 2 ml of 100 % TritonX-100, 2 ml of 100 mM PMSF and make up the volume to 200 mL with 20 mM phosphate buffer, pH 7.4 containing 150 mM NaCl and stored the solution at 4 °C.

4.2 Lysis buffer for *Vh*ChiS_SM (20 mM Tris base, pH 7.4, 150 mM NaCl, 10 % glycerol, 0.1 % TritonX-100, 1 mM PMSF, 10 ug/ml DNase I) (200 mL)

Mix solution of 20 ml of 100 % glycerol, 2 ml of 100 % TritonX-100, 2 ml of 100 mM PMSF and make up the volume to 200 mL with 20 mM Tris base, pH 7.4 containing 150 mM NaCl and stored the solution at 4 °C.

4.3 0.2 M Na_2HPO_4 (M_r = 358.14 g mol^{-1})

Dissolve 71.63 g of $Na2HPO_4$ in 500 mL of distilled water and make up the volume to 1,000 mL with distilled water.

4.4 0.2 M $Na_2H_2PO_4$ (M_r = 136 g mol^{-1}) Dissolve 27.2 g of NaH_2PO_4 in 500 mL of distilled water and make up the volume to 1000 mL with distilled water.

4.5 0.1 M phosphate buffer (PB), pH 7.4

Preparation of 100 mL of 0.1 M PB, pH 7.4 working solution, mixed the stock solution as follows:

- 40.5 mL of 0.2 M Na_2HPO_4
- 9.5 mL of 0.2 M NaH_2PO_4

Adjust the volume to 100 mL with distilled water and stored the solution at room temperature.

4.6 20 mM phosphate buffer, pH 7.4 (1 L)

Mix the solution of 200 ml of 0.1 M PB with 800 ml of distilled water and stored the solution at room temperature.

4.7 20 mM phosphate buffer, pH 7.4 containing 150 mM NaCl (Equilibration buffer, 1 L)

Dissolve 8.77 g of NaCl in 1 L of 20 mM phosphate buffer, pH 7.4.
store at 4 °C.

4.8 20 mM phosphate buffer, pH 7.4 containing 150 mM NaCl and 10 mM imidazole (Wash buffer, 1 L)

Dissolve 1.36 g of imidazole in 1 L of equilibration buffer. store at 4 °C.

4.9 20 mM phosphate buffer, pH 7.4 containing 150 mM NaCl and 100 mM imidazole (Elution buffer, 250 ml)

Dissolve 1.702 g of imidazole in 250 mL of equilibration buffer. store at 4 °C.

4.10 20 mM phosphate buffer, pH 7.4 containing 50 mM NaCl (Buffer A for IEC, 1 L)

Dissolve 2.922 g of NaCl in 1 L of 20 mM phosphate buffer, pH 7.4. store at 4 °C.

4.11 20 mM phosphate buffer, pH 7.4 containing 500 mM NaCl (Buffer B for IEC, 1 L)

Dissolve 29.22 g of NaCl in 1 L of 20 mM phosphate buffer, pH 7.4. store at 4 °C.

4.12 20 mM Tris base, pH 7.4 (1 L)

Dissolve 2.42 g of Tris base in 800 ml distilled water. Adjust pH to 8.0 with 6 M HCl and the volume to 1 L with distilled water. Store at 4 °C.

4.13 20 mM Tris base, pH 7.4 containing 150 mM NaCl (equilibration buffer, 1 L)

Dissolve 8.77 g of NaCl in 1 L of 20 mM Tris base buffer, pH 7.4. store at 4 °C.

4.14 20 mM Tris base, pH 7.4 containing 150 mM NaCl and 10 mM imidazole (Wash buffer, 1 L)

Dissolve 1.36 g of imidazole in 1 L of equilibration buffer. store at 4 °C.

4.15 20 mM Tris base, pH 7.4 containing 150 mM NaCl and 100 mM imidazole (Elution buffer, 250 ml)

Dissolve 1.702 g of imidazole in 250 mL of equilibration buffer. store at 4 °C.

4.16 20 mM Tris base, pH 8.5 containing 100 mM NaCl (Buffer A for IEC, 1 L)

Dissolve 5.84 g of NaCl in 1 L of 20 mM Tris base, pH 8.5. store at 4 °C.

4.17 20 mM Tris base, pH 8.5 containing 600 mM NaCl (Buffer B for IEC, 1 L)

Dissolve 35.06 g of NaCl in 1 L of 20 mM Tris base, pH 8.5. store at 4 °C.

4.18 DNase I (10 mg/mL)

Dissolve 0.001 g of DNase I in 20 mM PB (pH 7.4) to the final volume of 100 µL and kept at −20 °C before used.

4.19 Phenylmethylsulfonyl Fluoride (PMSF) (0.1M)

Dissolve 0.174 g of PMSF in isopropanol to the final volume of 10 mL. then aliquot and store at −20 °C before used.

4.20 SDS-gel loading buffer (3x stock) contains 0.15 M Tris-HCl (pH 6.8), 6 % SDS, 0.1 % bromophenol blue and 30 % glycerol

Dissolve 6 g of SDS, 0.1 g bromophenol blue, 30 mL of glycerol and add 0.15 M Tris-HCl (pH 6.8) to the final volume of 100 mL. Store the solution at -30 °C. Before used, add 20 µL of 2-mercapthoethanal to the final volume of 40 µL of the solution mixture.

4.21 1.5 M Tris-HCl (pH 8.8)

Dissolve 18.17 g of Tris-base in 80 mL distilled water. Adjust pH to 8.8 with 6 M HCl and bring the volume up to 100 mL with distilled water and stored at 4 °C.

4.22 1.0 M Tris-HCl (pH 6.8)

Dissolve 12.10 g of Tris-base in 80 mL distilled water. Adjust pH to 6.8 with 6 M HCl and bring the volume up to 100 mL with distilled water and stored at 4 °C.

4.23 30 % (w/v) Acrylamide solution

Dissolve 29 g of acrylamide and 1 g N, N'-methylene-bis-acrylamide in distilled water to a final volume of 100 mL. Mix the solutionby stirring for 1 hr until

the solution is homogeneous and filter through a whatman filter paper membrane No. 1. Store the solution in the dark bottle at 4 °C.

4.24 Tris-glycine electrode buffer (5x stock solution)

Dissolve 30.29 g of Tris-base, 144 g of glycine, 5 g of SDS in distilled water. Adjust pH to 8.3 with 6 M HCl and bring the final volume up to 1 liter with distilled water.

4.25 Staining solution with Coomassie Brilliant Blue for protein

Mix 1 g of Coomassie Brilliant Blue R-250, 400 mL methanol, 500 mL distilled water and 100 mL glacial acetic acid and filter through a whatman filter paper membrane No. 1 and Store the solution in the dark bottle at room temperature.

4.26 Destaining solution for Coomassie stain

Mix 400 mL methanol, 100 mL glacial acetic acid, and then add distilled water to the final volume of 1,000 mL

4.27 10 % (w/v) Ammonium persulfate

Dissolve 100 mg of ammonium persulfate in 1 mL of distilled water. Store the solution at −20 °C.

4.28 12 % (w/v) Separating SDS-PAGE gel (10 ml)

Mix the solution as follows:

- 1.5 M Tris-HCl (pH 8.8) 2.5 mL
- Distilled water 3.3 mL
- 10 % (w/v) SDS 0.1 mL
- 30 % (w/v) Acrylamide solution 4.0 mL
- 10 % (w/v) Ammonium persulfate 0.1 mL
- TEMED 0.004 mL

4.29 5 % (w/v) Stacking SDS-PAGE gel (5 ml)

Mix the solution as follows:

- 0.5 M Tris-HCl (pH 6.8) 0.63 mL
- Distilled water 3.4 mL
- 10 % (w/v) SDS 0.05 mL
- 30 % (w/v) Acrylamide solution 0.83 mL
- 10 % (w/v) Ammonium persulfate 0.05 mL
- TEMED 0.005 mL

APPENDIX C

AMINO ACID SEQUENCE

1. Amino acid sequence of *Vh*CBP

ATTGFASVATAAERSELTIHPKEFTTFVRNFNPFLGATNLHTTTDFIYEPLVVFNEM

HGNTPVFRLAENFQMSDDLMSVTFDIRKGVKWSDGEAFTADDVVYSFNLVKEKPELD

QSGINSWVTGVEKVNDYQVKFRLSEANSNVPYEIAKVPVVPKHVWSKVKDPSTFTNE

NPVGSGPFTVIDTFTPQLYIQCENPNYWDAANLDVDCLRVPQIANNDQFLGKVVNGE

MDWTSSFVPDIDRTYAAASPKHHYWYPPAGTQAFVVNFKNPDAAKNEALTNVDFRRA

FSMALDRQTIIDIAFYGGGTVNDFASGLGYAFEAWSDEKTHDKFKAYNSYNAEGAKK

LLAKAGFKDVNKDGFVDTPSGKSFELLIQSPNGWTDFNNTVQLAVEQLAEVGIKARA

RTPDFSVYNQAMLEGTYDVAYTNYFHGADPYTYWNSAYNSALQSGDGMPRFAMHFYK

NEKLDGLLNSFYKTADKQEQLEIAHGIQQIIAQDQVTIPVLSGAYMYQYNTTRFTGW

WNEENPKGRPNIWAGIPERLLHVLDLKPVK

2. Amino acid sequence of *Vh*ChiS_SM

QSHSKDLQEQSTSHLLSVRDTKQQQIIDYFAAQETEVMGFVRSELAYASGGRFYGLV
NAFSRLGHDIDEARENAQQRYIKGSGDQIKTSILPESSNYVGSERYRLLHKRYHWAY
LELLKRSDFNDILLVDIDGNVTYSINKDDNYGTNLLTGRYKDTALGRTFKRLSEDVS
ARRKVNEDYTPVVVSDFDIENGKQVAWLGAPIVQQGYLHSYAMFRLPNNGITKLIAE
INRDSSIETLLVGSDHKPRTINTKQEEIQNSLEVVDKALAGDTEVGTYSNRLGEEMI
AAFAPIETRGLTWAIVVQLPEKEAFARVHQ

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