รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาความสัมพันธ์เชิงโครงสร้างและหน้าที่ของเอ็นไซม์เอซิลทรานสเฟอเรสจากเชื้อก่อโรคไอกรนโดยอาศัยกระบวนการชีวสังเคราะห์โปรตีนที่ไม่ใช้เซลล์

โดย ผู้ช่วยศาสตราจารย์ ดร.สรวง รุ่งประกายพรรณ และคณะ

กุมภาพันธ์ พ.ศ.2553

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ผู้วิจัย

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สังกัด

คณะเภสัชศาสตร์ มหาวิทยาลัยศิลปากร

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

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Abstract

Project Code : MRG4880107

Project Title : Structure-function relationship study of acyltransferase (CyaC) from Bordetella pertussis using cell-free protein synthesis

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CyaC is an acyltransferase needed for activation of the adenylate cyclase toxin CyaA in *Bordetella pertussis*. Despite the rapidly increasing knowledge about the *B. pertussis*, the exact molecular mechanisms of the acylation and structural-function relationship of the CyaC are largely unknown to date. Our research group is interested in elucidating the structure and function of the CyaC. In this research, we investigated the possibility of expression the CyaC in vitro using a cell-free protein synthesis system called in vitro coupled transcription/ translation. The CyaC protein encoding gene was obtained by PCR amplification of the B. pertussis genome, and cloned into a plasmid vector suitable for in vitro coupled transcription/ translation. The constructed plasmid was tested as the DNA template for cell-free protein synthesis and the results showed that CyaC was expressed as soluble protein when there was no DTT present in the in vitro coupled transcription/ translation system. One limitation of applying the cell-free protein synthesis in research is how to detect expressed protein. One of the most sensitive methods is using radioisotope-labeled amino acid. However, it requires special facilities and well trained personel. To overcome this limitation, we appended a histidine tag to CyaC protein by recombinant DNA technology. However, the histidine tag system did not provide good result. Therefore, we attempted to produce antibody fragments that bind the CyaC using phage display technology. Pre-made antibody-phage display libraries, Tomlinson I and J, were panned and anti-CyaC scFvs with high affinity were obtained. Those anti-CyaC-scFvs are not only useful for CyaC detection, but also for therapeutic use if needed.

Keywords : Cell-free protein synthesis, in vitro coupled transcription/ translation, CyaC, phage display, single-chain variable fragment

บทคัดย่อ

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ชื่อโครงการ : การศึกษาความสัมพันธ์เชิงโครงสร้างและหน้าที่ของเอ็นไซม์เอซิลทรานสเฟอเรสจากเชื้อก่อโรคไอกรนโดยอาศัยกระบวนการชีวสังเคราะห์โปรตีนที่ไม่ใช้เซลล์

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CyaC เป็นเอนไซม์สำคัญที่ทำหน้าที่เติมหมู่เอซิลให้สารพิษ CyaA ในเชื้อก่อโรคไอกรน แม้ความรู้และวิทยาการเกี่ยวกับเชื้อไอกรนจะก้าวหน้าไปมาก แต่โครงสร้างและกลไกการทำงานของเอนไซม์ CyaC นั้นยังไม่เป็นที่ทราบแน่ชัด กลุ่มวิจัยของเราจึงมีความสนใจศึกษาเกี่ยวกับโครงสร้างและหน้าที่ของเอนไซม์ CyaC ในงานวิจัยชิ้นนี้ทางกลุ่มวิจัยได้ศึกษาและทดสอบความเป็นไปได้ที่จะประยุกต์ใช้ระบบการสังเคราะห์โปรตีนในหลอดทดลองโดยไม่ใช้เซลล์ชนิดควบการถอดรหัสและแปลรหัสเพื่อการสังเคราะห์เอนไซม์ CyaC โดยเริ่มด้วยการโคลนยีนของ CyaC จากเชื้อไอกรนที่แยกได้ในประเทศไทยเข้าสู่พลาสมิดที่เป็นเวกเตอร์ที่เหมาะสมสำหรับการสังเคราะห์โปรตีนในหลอดทดลอง โดยไม่ใช้เซลล์ชนิดควบการถอดรหัสและแปลรหัส เมื่อนำพลาสมิดที่ได้มาทำการทดสอบพบว่าเราสามารถสังเคราะห์เอนไซม์ CyaC ได้ด้วยระบบการสังเคราะห์โปรตีนในหลอดทดลองโดยไม่ใช้เซลล์ชนิดควบการถอดรหัสและแปลรหัส อย่างไรก็ตาม เราพบว่าปริมาณของ DTT ในปฏิกิริยาจะมีผลต่อการละลายของเอนไซม์ CyaC ที่ถูกสังเคราะห์ขึ้น ข้อจำกัดหนึ่งของการศึกษาการสังเคราะห์โปรตีนในหลอดทดลองโดยไม่ใช้เซลล์คือ ทำอย่างไรจึงจะตรวจสอบโปรตีนที่ถูกสังเคราะห์ขึ้นได้ วิธีที่มีความไวสูงสุดคือการติดฉลากกรดอะมิโนด้วยสารกัมมันตรังสี อย่างไรก็ตามการใช้สารกัมมันตภาพรังสีต้องอาศัยห้องและเครื่องมือจำเพาะและบุคคลากรที่ได้รับการอบรมพิเศษเพื่อป้องกันอันตราย เพื่อแก้ปัญหานี้เราได้ทดสอบการติด histidine-tag เข้ากับเอนไซม์ CyaC เพื่อใช้สำหรับการติดตามการสังเคราะห์เอนไซม์ CyaC อย่างไรก็ตาม เมื่อทดสอบแล้วพบว่าวิธีนี้ไม่ได้ผลที่ดี เราจึงได้ทำการพัฒนาแอนติบอดีที่จำเพาะต่อเอนไซม์ CyaC ด้วยเทคนิคฟาจดิสเพลย์ โดยทำการคัดเลือกแอนติบอดีจากไลบรารี Tomlinson I และ J ได้แอนติบอดีที่มีสัมภัคภาพสูงต่อเอนไซม์ CyaC แอนติบอดีที่ได้จะเป็นประโยชน์สำหรับการศึกษาวิจัยเอนไซม์ CyaC และสามารถนำไปพัฒนาเพื่อการรักษาโรคไอกรนอีกด้วย

คำสำคัญ: Cell-free protein synthesis, in vitro coupled transcription/ translation, CyaC, phage display, single-chain variable fragment

Structure-function relationship study of acyltransferase (CyaC) from *Bordetella pertussis* using cell-free protein synthesis

Introduction

Bordetella pertussis, the whooping cough agent, secretes a 1706-residue-long adenylate cyclase toxin-hemolysin (CyaA, ACT, or AC-Hly), a repeat in toxin (RTX) family protein, which can invade a variety of eukaryotic cells (Landant and Ullmann, 1999; Hanski, 1989). Catalytic adenylate cyclase domain (AC) of CyaA is delivered into cells and is activated by intracellular calmodulin to catalyze unregulated conversion of ATP to cAMP (Wolff et al., 1980; Confer and Eaton, 1982; Hanski and Farfel, 1985; Moss et al., 1984). This induces apoptosis of lung macrophages and impairs microbicidal functions of immune effector cells (Khelef et al., 1993; Gueirard et al., 1998). In addition, CyaA has weak hemolytic activity accounted by its capacity to form small cation-selective membrane channels (Khelef et al., 1993; Gueirard et al., 1998; Bellalou et al., 1990; Rogel et al., 1991; Ehrmann et al., 1991; Szabo et al., 1994; Benz et al., 1994).

The CyaA polypeptide, encoded by *cyaA*, is synthesized as an inactive protoxin (proCyaA). The physiologic activity of CyaA to form hemolytic cation-selective channels and to penetrate target cell membranes and deliver the AC domain (cell-invasive activity) depends on a covalent post-translational fatty-acyl modification (Barry et al. 1991; Sebo, et al., 1991; Hackett, 1994) (Figure 1). This is catalyzed by a specific acyltransferase, CyaC, which can acylate the ε-amino groups of two internal lysine residues of CyaA, Lys-983 and Lys-860,

located within conserved RTX acylation sites (Barry et al. 1991; Sebo, et al., 1991; Hackett, 1994). Acylation process also can occur in vitro by incubating cell-extract containing proCyaA with cell-extract containing CyaC in presence of acylated acyl-carrier protein (Westrop et al., 1996). The mechanism of this novel type of protein acylation was recently analyzed in substantial detail for the prototype RTX toxin-activating and acylACP-dependent protein acyltransferase HlyC, which acylates the homologous lysines 564 and 690 of the *Escherichia coli* α-hemolysin HlyA (Issartel et al., 1991; Ludwig et al., 1996; Stanley et al., 1994 and 1998). Several residues, including Ser-20 and His-23, were identified as being potentially involved in acyl transfer catalysis by HlyC (Trent et al., 1999a, b, c). However, for the CyaC itself, there are very few reports studying on its structure and mechanism of action (Hackett et al., 1995; Basar et al., 1999 and 2001). In addition, it is still unclear how the acylation associating with functionality of the CyaA.

One of the key steps in studying molecular characteristics and mechanism of actions of a protein is high-throughput expression and purification of the target protein. The CyaC is usually expressed in vivo either by *B. pertussis* or recombinant *E. coli* (Sebo et al., 1991; Westrop et al., 1996) which depends on cell growth and is labor intensive. In addition, to study structural and functional importance of certain amino acids by mutagenesis, the in vivo expression requires a lot effort on cloning. On the other hand, high-throughput expression and purification of the target protein can hasten the studies.

First developed in 1960s, expression of proteins in vitro, without intact cell involved (cell-free protein synthesis), has become a very powerful technology for genomics and

proteomics (DeVries and Zubay, 1967; Endo and Sawasaki, 2003; Nathans et al., 1962; Nirenberg and Matthaei, 1961; Sawasaki et al., 2002). There are three well-established cell-free protein synthesis systems classified by cell-extract used; *E. coli* S30 extract, wheat germ extract (Roberts and Paterson, 1973), and rabbit reticulocyte lysate (Pelham and Jackson, 1976). Typical cell-free systems comprise two consecutive processes, in vitro transcription to produce mRNA and in vitro translation (Figure 2). However, mRNA can also be made directly in the translation reaction in a so-called coupled transcription/translation system (Chen and Zubay, 1983; Nevin and Pratt, 1991). In the coupled system, plasmid DNA or even PCR product can be used directly as the template for expression (Martemyanov et al., 1997). The process of in vitro expression (transcription/translation) could be finished within as short as one hour, which is much faster than the classical in vivo over expression.

The scalability of the cell-free systems allows large-scale production of the selected protein. Cell-free protein synthesis on a larger scale can provide unique possibilities for structural analyses of proteins. The effective incorporation of stable-isotope (13C, 15N)-labeled amino acids into proteins synthesized in cell-free systems (Kigawa et al., 1999; Klammt et al., 2004; Guignard et al., 2003; Morita et al., 2003) allows structure determination of the proteins in solution by high-resolution NMR spectroscopy. Similarly, the incorporation of a cytotoxic unnatural amino acid, selenomethionine, into a protein by cell-free synthesis (Kigawa et al., 2002 and Wada et al., 2003) provides a powerful approach for the phasing of crystal protein structures and the atomic structure determination of cell-free synthesized proteins using X-ray crystallography.

In addition to structural studies, the possibility of incorporating unnatural and chemically modified amino acids into proteins synthesized in cell-free systems (Kanda et al., 2000; Kiga et al., 2002; Budisa, 2003) raises new possibilities in functional studies, protein engineering and pharmaceutical research. Also the absence of cell control permits the preparative scale synthesis of cytotoxic proteins and polypeptides, including various cell regulators and other factors crucial for cell life.

According to beneficial characteristics of cell-free protein synthesis mentioned above, an established cell-free system for in vitro expression of CyaC combining with PCR mutagenesis technologies provide a rapid production (expression/purification) and amino acid labeling of the protein and their mutants, which are highly valuable for both structural and functional characterization of the protein. This project initially aims to establish a cell-free protein synthesis system for the CyaC and employ it for rapid structural and functional characterization of the protein. First CyaC encoding gene (*cyaC*) from a *B. pertussis* clinical strain isolated in Thailand was amplified by PCR technology and its sequence was determined. The amplified gene was thereafter cloned into a T7 system expression vector. The in vitro expression systems using *E. coli* S30 extract were tested for expression of the recombinant plasmid.

In order to detect protein expressed in vitro, usually radioisotope labeled amino acid is incorporated in the reaction. However, this method required special facility to do the experiment. Therefore, after we first test the possibility of in vitro expression of the CyaC, we adjusted our research plan to produce monoclonal antibody fragment against the CyaC which

will be further used for detection of the protein. The antibody fragment also will be valuable for further study on functions of the CyaC. The anti-CyaC antibody fragments were selected using phage display technology.

Invented by Gorge P. Smith group in 1985, phage display is a molecular technology that enables molecules of foreign protein or peptide to be displayed at surface of phage particles (Smith, 1985). A phage display protein library is a pool of phage particles displaying distinguish protein on their surface, i.e. a phage particle carrying only a protein of it surface, and also carrying the gene of the displayed protein inside the particle. In application of antibody selection, monoclonal antibody fragments are displayed of the surface of phage particles. The displayed antibody fragment can interact and bind with its specific antigen if present (Figure 8). Thus the selection process is carried out by coating a target antigen on solid support, and let the phage displayed antibody fragments bind to the antigen (Figure 9). Unbound phages are washed out, and the bound phages which are displaying antibody fragments specific to target antigen are collected. The collected phages are then amplified. The amplified phages are put through selection process again for a few rounds. Finally phages with high affinity antibody fragments are obtained. We can construct an antibody phage display library in house using basic recombinant DNA techniques, or use pre-made libraries which are available commercially and freely on research request (Clackson, 1991)

There are two formats of antibody fragment that are well used in phage display technology; Fab and single chain variable fragment (scFv) (Figure 10). The Fab is larger and the heavy chain and light chain are linked by disulfide bonds. For the scFv, the heavy chain

and light chain are covalently linked through a short peptide linker. The origin of antibody gene is important to its application. If we want to use the antibody with a human being, the antibody gene from human is preferable because of low immunogenicity. To use as diagnostic kit or ex vivo research experiments, there is no limitation on the source of the antibody gene. In this research, we used a pre-made antibody fragment library name Thomlinson I+J distributed by the MRC laboratory of molecular biology (Cambridge, UK) (Nissim, 1994). It is a semi-synthetic scFv library. The scFv framework is from human origin. Therefore, scFv selected from this library can be used for both detection and future therapeutics if needed.

2. Material and Methods

2.1 Cloning of CyaC encoding gene from genomic DNA

A pET17b based recombinant plasmid pCyaC was prepared Dr.Busaba at Institute of Molecular Biology and Genetics, Mahidol University. Briefly, a *B. pertussis* clinical strain isolated in Thailand was cultured and its genomic DNA was extracted. Using the genomic DNA as template, *cyaC* was amplified by PCR using a pair of primers containing BamH I site for cloning. The PCR product was checked by 1.2% agarose gel electrophoresis. The cyaC fragment was purified. Both cyaC fragment and pET17b vector were digested by BamH I, and then purified. Ligation and transformation were performed yielding pCyaC (Figure 3).

2.2 Subcloning of CyaC encoding gene to T7 vector with histidine tag

Using the pCyaC as template, *cyaC* was amplified by PCR using a pair of primers CyC-F-Bam: GATAA*GGATCC*GATGCTTCCGTCCGCCCAAGCGCC and CyC-R-Kho: GCAGAT*CTCGAG*TTATCAGGCGGTGCCCCGGCCTC which anneal to each end of the *cyaC*. The primers contain restriction sites *Bam*H I and X*ho* I, respectively. The 50 µl PCR reaction contains 5 µl of 10x Pfu buffer, 5 µl of 2.5 mM dNTPs, 0.5 µl of 100 mM primers, 50 unit of Pfu DNA polymerase, 100 ng of genomic DNA, and sterile water adjusting the volume to 50 µl. The cycle condition is as following: Preheat at 95°C for 10 mins; 25 cycles of 95°C 3 mins, 43°C 1 min, 72°C 1 min; and final extension at 72°C for 7 min before cooling down to 4°C. The PCR product was checked by 1.2%

agarose gel electrophoresis. The cyaC fragment was purified using HiYield™ Gel/PCR DNA Fragments Extraction Kit (Real Biotech Corp., Taiwan) according to manufacturer's protocol. PCR amplified cyaC and the expression vector pRSET-A were double digested by the restriction enzyme B*am*HI and X*ho*I (in a standard reaction using supplied buffer, New England Biolab, USA) at 37°C for 2 hours. The digested fragments (insert and vector) were purified using HiYield™ Gel/PCR DNA Fragments Extraction Kit (Real Biotech Corp., Taiwan) according to manufacturer's protocol.

2.3 DNA sequencing and analysis

Cycle Sequencing reactions were performed using primers pair T7 promoter/ T7 reverse or In-F/ In-R (their sequences listed in table 1) and the BigDye™ Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, USA) according to the manufacturer's protocol. The cycle sequencing products were analyzed on the ABI PRISM 310 Automated DNA Sequencer and electropherograms were analyzed with Sequencing Analysis Software v5.1 (Applied Biosystems, USA). Sequences were edited and analyzed using Bioedit version 7.0.8

2.4 Cell-free protein synthesis of the CyaC

In this research, the cell-free protein synthesis system is a coupled transcription/translation based on Shultz's method with some modifications. The reactions were carried out in 20 ml reactions with contain which contains components

as following: DNA template, 54.6 mM Tris-acetate buffer, 1.22 mM ATP, 0.85 mM of each of GTP, CTP, and UTP, 3.2mM of each of 20 amino acids, ^{14}C-luecine, *E. coli* tRNA, 7.7μg/ml T7 RNA polymerase, 40 mM creatine phosphate, 0.15 μg/ml creatine kinase, 34.6 μg/ml folinic acid, 4% w/v polyethylene glycol 6000, magnesium acetate, potassium acetate, 36 mM ammonium acetate, rifampicin, and *E. coli* S30 extract, all at amount as previously reported. The cell-free protein synthesis reactions were incubated at 37°C for 60 minutes and subsequently put on ice to stop the reaction. Control reactions were performed under identical conditions without the DNA template. 0.016 mM 14C-labeled leucine was added to the reactions for later detection by gel analysis.

2.5 SDS-PAGE and autoradiography

^{14}C-Labeled proteins were analyzed by electrophoresis in a 10% polyacrylamide gel. 5 μl of reaction mixture from cell-free protein synthesis was precipitated with ice-cold acetone. Pellet was the dissolved in 5 μl of distilled water, mixed with and equal volume of 2x loading buffer, and boiled for 3 mins. All the sample-buffer mix was loaded onto SDS-polyacrylamide 12.5% gel and electrophoresized alongside a marker. The gel was dried and exposed to Fuji imaging plate for 20 hours, and was analyzed by a FUJIX BAStation (FUJI photo film Co.Ltd., Tokyo).

2.6 In vivo expression of the CyaC

CyaC encoding gene from the isolated strain was amplified using PCR as mention above. The gene was then cloned into an expression vector pET17b yielding a recombinant plasmid named pCyaC. Cloning was confirmed using restriction digestion and DNA sequencing. The recombinant plasmid was transformed into E.coli BL21(DE3)pLys for expression. The transformed E.coli was grown at 37°C overnight. Twenty ml of the culture was then transfer into fresh 1L LB medium with ampicillin, and cultured until OD 0.9. The expression of CyaC was induced by 0.1mM IPTG, and incubated at 30°C for 6 hours. Expression of the CyaC was checked using 12% SDS-PAGE.

2.7 Purification of the recombinant CyaC

Cells were harvested by centrifugation at 4000 rpm 5 mins. The collected cells were broken using French press, and centrifuged to collect soluble and insoluble fraction. The inclusion body was wash several times using phosphate buffer pH 7.4, and later solubilized by citrate buffer pH 10.5. The solubilized CyaC was purified using gel filtration, and concentrated using a spin column.

2.8 Anti-CyaC scFv selection using phage display

Antibody phage display library Thomlinson I+J was distributed from the MRC (Cambridge, London). The library was propagated and panned according to supplied protocol with some modification (see appendix). Briefly, 5 μg/ml of the purified CyaC

was coated onto immunotube (Nunc, Denmark) and incubated at 4°C overnight. The immunotube was washed twice using phosphate buffer saline (PBS; 5.84 g NaCl, 4.72 g Na_2HPO_4 and 2.64 g $NaH_2PO_4.2H_20$, pH 7.2, in 1 litre). The immunotube was blocked using blocking buffer (PBS with 3% skimmed milk) at 37°C for 2 hours, then washed twice using washing buffer (PBS with 1% skimmed milk and 0.1% tween20). Freshly prepared phage (10^{12}-10^{13}) in MPBS (2% skimmed milk in PBS) was added to the coated immunotube, incubated at room temperature for 2 hours on an under-and-over turntable. Unbound phage was discarded and the tube was washed using washing buffer 10 times. Shake out the PBS and elute phage by adding of 500 μl trypsin-PBS (50 μl of 10 mg/ml trypsin stock solution in 450 μl PBS) and rotate the tube for 20 mins. Take 250 μl of elute phage and put into 1.75 ml of precultured E.coli TG1 (K12 Δ*(lac-proAB) supE thi hsdD5/*F' *traD36 proA+B lacIq lacZ*Δ*M15*) OD 600 of 0.4, incubate at 37°C for 30 min without shaking to let the phage infection. Go on to prepare the phage for the next round of selection. Select the phage for 5 rounds. After 5 rounds of selection infect the eluted phage to E.coli TG1, and spread the infected E.coli TG1 on TYE agar (15g Bacto-Agar, 8g NaCl, 10g Tryptone, 5g Yeast Extract in 1 litre) with selective antibiotics to isolate single colonies. Pick 384 single colonies for further analysis.

2.9 Screening phage particles by ELISA

Produce phage from single colonies selected from the library in 96 cell-well plates according to supplied protocol in the appendix. ELISA plate was coated with 5 μg/ml of the purified CyaC at 4°C overnight. The coated plate was washed twice using phosphate buffer saline (PBS). The plate was blocked using blocking buffer (PBS with 3% skimmed milk) at 37°C for 2 hours, then washed twice using washing buffer (PBS with 1% skimmed milk and 0.1% tween20). Prepared phage from individual colonies in MPBS (2% skimmed milk in PBS) was added to the coated ELISA plate, incubated at room temperature for 2 hours. Unbound phage was discarded and the plate was washed using washing buffer 10 times. Anti-M13 conjugated with HRP was added to ELISA plate, and incubated at room temperature for 2 hrs. Discard the anti-M13-HRP solution from the plate and wash 10 times using washing buffer. ABTS (colorimetric HRP substrate) solution was added to the plate and the green color developed as signal of presence of anti-CyaC scFv.

2.10 Production of soluble antibody fragment

E. coli HB2151 (K12 *ara* Δ*(lac-proAB) thi/*F' *proA+B lacIq lacZ*Δ*M15*) was grown until OD 600 reaching 0.4. Let selected phage infect the E.coli for 30 min in 2xTY (16g Tryptone, 10g Yeast Extract and 5g NaCl in 1 litre). Plate the infected E. coli onto TYE medium with 1% glucose and 100 μg/ml ampicillin, and incubate at 37°C overnight. Pick individual colonies and inoculate into 2 ml 2xTY with 1% glucose and 100 μg/ml ampicillin, and incubate shaking at 37°C overnight. Subculture the overnight grown into

50 ml 2xTY with 0.1% glucose and 100 μg/ml ampicillin, and incubate shaking at 37°C until OD600 reaching 0.9 (around 3 hours). Add IPTG to the final concentration of 1 mM to induce expression, and incubate the culture at 30°C overnight.

Results and Discussion

Cell-free protein synthesis of CyaC

The synthesis of proteins in vitro (in cell-free extracts) is an important tool for molecular studies. It has been applied to a variety of research, including the rapid identification of gene products, localization of mutations, protein folding studies, and incorporation of modified or unnatural amino acids for functional studies. The use of in vitro expression systems can have advantages over in vivo gene expression when the over-expressed product is toxic to the host cell, when the product is insoluble or forms inclusion bodies, or when the protein undergoes rapid proteolytic degradation by intracellular proteases. One remarkable advantage is speed when we need just small amount of protein for analysis. In principle, a cell-free extract for in vitro translation are derived from cells engaged in a high rate of protein synthesis. In standard translation reactions, purified RNA is used as a template for translation. On the other hand, "linked" and "coupled" systems use DNA as a template. RNA is transcribed from the DNA and subsequently translated without any purification. Such systems typically combine a prokaryotic phage RNA polymerase and promoter (T7, T3, or SP6) with eukaryotic or prokaryotic extracts to synthesize proteins from exogenous DNA templates. DNA templates for transcription/translation reactions could be cloned into plasmid vectors or generated by PCR. The "linked" system is a two-step reaction based on transcription with a bacteriophage polymerase followed by translation, while the “coupled” system the transcription and translation occur simultaneously in a reaction tube as it occur in the prokaryotic cell. In this study, we employed the E. coli

coupled transcription/ translation system with T7 promoter because our target protein CyaC is also from bacterial origin.

To prepare DNA template for in vitro expression of CyaC, a B. pertussis clinical strain isolated in Thailand was cultured and its genome was extracted. Using the genome as template, *cyaC* was amplified by PCR using a pair of primers annealing to each end of the *cyaC*. The forward and reverse primers contain B*amH* I restriction sites for cloning. Success of cloning was confirmed using restriction digestion and DNA sequencing. The recombinant plasmid pCyaC contains a promoter, a ribosome-binding site (SD), a target gene, and a terminator sequence consecutively, which is capable of being *in vitro* transcribed and translated (Fig 1).

In the coupled transcription/translation, there are pCyaC (DNA template for expression), Tris-acetate buffer, NTPs, 20 amino acids, E. coli tRNA, T7 RNA polymerase, creatine phosphate, creatine kinase, folinic acid, polyethylene glycol 6000, magnesium acetate, potassium acetate, ammonium acetate, rifampicin, and E. coli S30 extract. Usually, the S30 extract is prepared under the presence of DTT, especially commercially available in vitro expression kit, which helps increasing expressed protein yield. However, in many cases the presence of DTT make expressed protein insoluble. Therefore, in addition to possibility of expression of the CyaC in vitro, we also investigated effect of DTT presence in the reaction.

Two in vitro coupled transcription/ translation of CyaC using E.coli S30 extract with and without DTT each were carried out. After 1 hour incubation at 37°C, the reaction mixtures

were spin at 8000 rpm to separate soluble and insoluble fractions. Supernatant and precipitate from both reactions were checked presence of expressed CyaC by SDS-PAGE and autoradigraphy. For the reaction with DTT, there were large amount of expressed CyaC (22 kDa), but it was in the insoluble fraction (Figure 5). On the other hand, the expressed CyaC was in soluble fraction when expressed in the reaction without DTT, even though the amount of expressed CyaC is much lower. Other two smaller bands present on the gel were possibly degraded portion of the expressed CyaC. From this result we can concluded that the presence of DTT in the reaction affects solubility and amount of expressed CyaC. Although the reaction with DTT gave high amount of expressed protein, it was insoluble and presumably not functional. Although refolding of insoluble protein is possible, it is time consuming and not possible to every protein. Therefore, optimization of amount of DTT in the reaction to produce the highest amount of soluble protein should be the first priority. Unfortunately, commercial in vitro expression kits using E. coli S30 extract usually contain certain amount of DTT which cannot be adjusted by user. A commercial in vitro expression kit using E. coli S30 extract was tested for expression of the CyaC, and the result was not successful (data not shown). The best way is to prepare the S30 extract in house. However, it needs a well trained researcher to prepare the high efficiency extract.

Subcloning of the cyaC from pCyaC to pRSET A

To track proteins expressed in small-size cell-free reaction which normally give low protein yields, the most sensitive method is radioisotope labeling. However, handling radioisotope needs special training and special facilities for protection of the handler and

environment. Therefore, we tried to develop an alternative system for monitoring the *in vitro* expression of CyaC protein. The straightforward strategy is to use an antibody against the CyaC protein for detection (Western blot or ELISA). However, anti-CyaC antibody is not commercially available. Another option is to append a peptide tag to the recombinant CyaC protein and use an antibody against the tag. We decided to adopt the latter strategy appending a histidine tag to the N-terminal of the CyaC protein by subcloning the *cyaC* into pRSET A which possess c-terminal histidine tag (Figure 6). The *cyaC* was amplified by PCR using *Bam* HI site containing forward primer and *Xho*I site containing backward primer. The amplicon was cloned into the pRSET A via Bam HI/ Xho I sites to construct the recombinant plasmid pRSET-*cyaC*. The construct was checked by restriction analysis and DNA sequencing.

Prior to using the new construct pRSET-cyaC as DNA template for cell-free protein synthesis, in vivo expression was carried out and the detection via the histidine tag appened to the recombinant CyaC protein was performed to verify the strategy. The pRSET-cyaC was transformed into E. coli BL21(DE3) pLys, T7 system expression host. The transformed host was cultured at 37°C until OD 600 was around 0.9, and then IPTG was add to the final concentration of 0.1 mM to induce the protein expression. The cultured was incubated at 30°C with shaking overnight. CyaC protein expression was analyzed by SDS-PAGE with coomassie blue staining. The SDS-PAGE with coomassie blue staining showed that the recombinant protein (tagged-CyaC protein) was expressed by the BL21(DE3)pLysS as shown in figure 7. However, consequence analysis of protein

expression by Western blot using anti-His/ anti-mouse IgG-HRP/ DAB detection system gave negative result. Although the Western blot was repeated with new detection system, His-Probe-HRP®/ DAB, the result has not changed. It is likely that the folding of the new construct (His tag-CyaC) hinder the c-terminal histidine tag from exposing the outside.

Selection of anti-CyaC scFv by phage display

In parallel with developing a detection system based on a peptide tag, antibodies against the CyaC protein in the single-chain variable fragment format (figure 10) were also being developed using phage-display technology (figure 8, 9). Recombinant CyaC, to be used as the antigen for antibody selection, was expressed in vivo under IPTG induction. The CyaC was expressed in large amount as inclusion bodies as shown in figure 11. Thereafter, the inclusion bodies were solubilized in citrate buffer and purified by gel filtration (Figure 12).

Phage-scFv libraries Thomlinson I+J from the Medical Research Center (MRC), UK, (the complete detail of the library is attached in the appendix) were panned to select scFv antibody fragments that bind to immobilized CyaC protein. After four rounds of bio-panning, 384 clones of scFv showing binding to the CyaC protein were picked for further analysis by phage ELISA. Five clones with highest ELISA signal, I2A7, J1B8, J1C4, J1F2, and J2A12, were chosen for binding confirmation (Figure 13) and the result showed that they really bound to the recombinant CyaC with high affinity (Figure 14). The clone J1B8 which showed highest affinity to the recombinant CyaC was chosen to test its ability to be

expressed as soluble protein in the E.coli HB2151, and the result is shown in Figure 15 that it was expressed successfully.

Phage display is a powerful technique for selection of proteins base on affinity. Using the phage display technology, we have succeeded in selection of anti-CyaC scFv from the library Thomlinson I+J. These anti-CyaC scFv will be valuable for study on the structure, function, structure-function relationship, including future therapeutics aspect of the CyaC.

Table 1 List of primers used in this experiment

Primer	Sequence
T7 promoter	TAATACGACTCACTATAGGG
T7 reverse	TAGTTATTGCTCAGCGGTGG
In-F	CCAA TACGCAAACCGCCTCTCC
In-R	TACAGGGCGCGTCCCATTCG
CyC-F-Bam	GATAA*GGATCC*GATGCTTCCGTCCGCCCAAGCGCC
CyC-R-Xho	GCAGAT*CTCGAG*TTATCAGGCGGTGCCCCGGCCTC

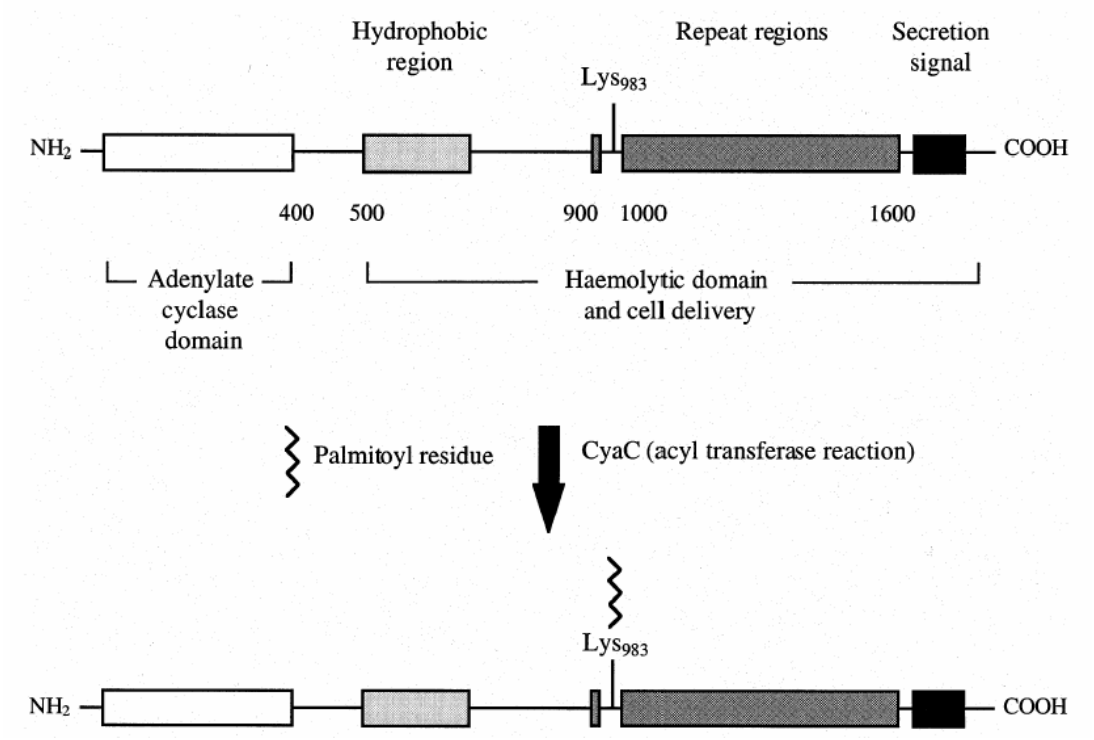

Figure 1. Schematic diagram shows simple structure of CyaA and acyl transferase reaction catalyzed by CyaC (A.M. Smith et al. /FEMS Microbiology Reviews 25 (2001) 309-333)

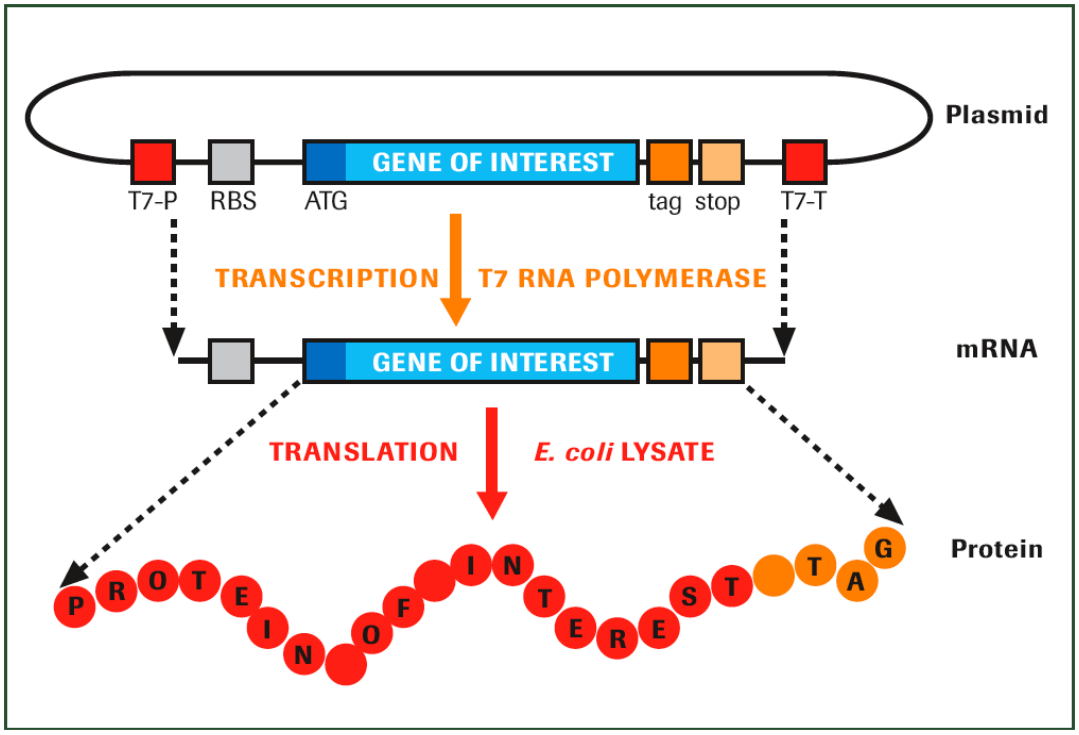

Figure 2. Schematic diagram showing structure of DNA template suitable for in vitro coupled transcription/ translation. Transcription is initiated by T7 RNA polymerase to produce mRNA, followed by translation to produce the target protein. In the coupled transcription/ translation reaction, the transcription and translation occur simultaneously in the E.coli lysate.

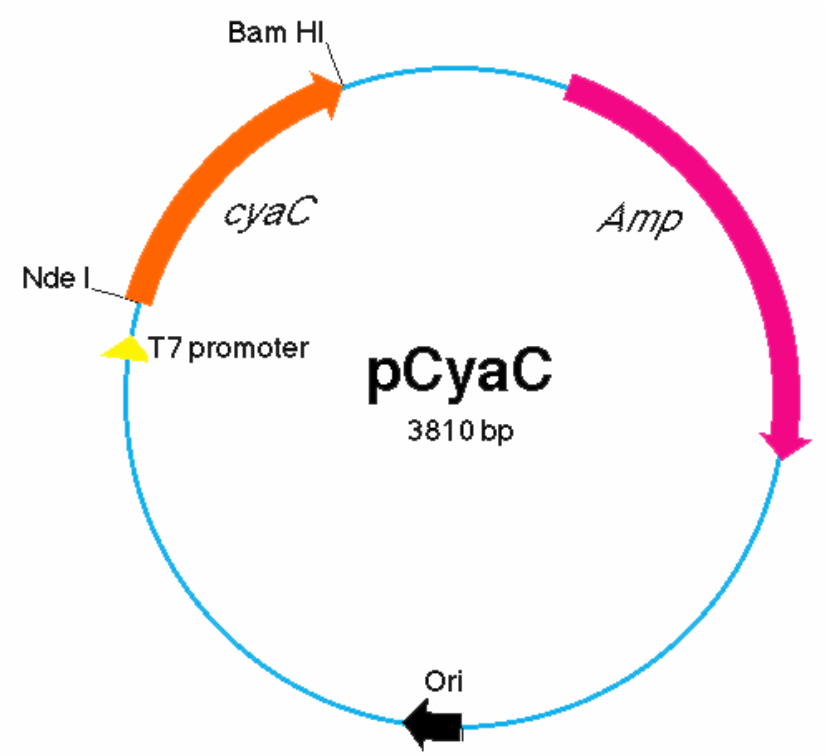

Figure 3. The map of the plasmid pCyaC which was constructed by cloning of *cyaC* encoding gene into pET-17b via the *Nde* I and *Bam*H I site.

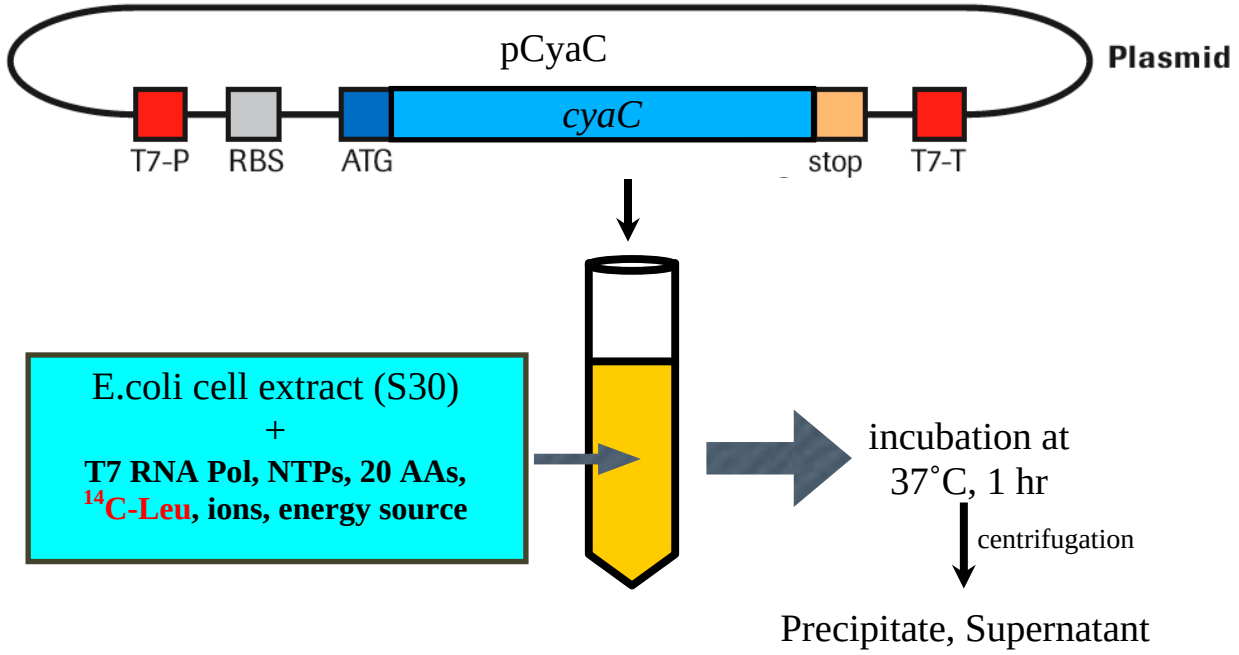

Figure 4. In vitro coupled transcription/translation of Cyac reaction. DNA template (pCyaC) is put into the reaction mixture containing all components necessary for simultaneous transcription and translation. The ^{14}C-Leu is incorporated into the polypeptide chain during protein synthesis, enabling radioisotope detection.

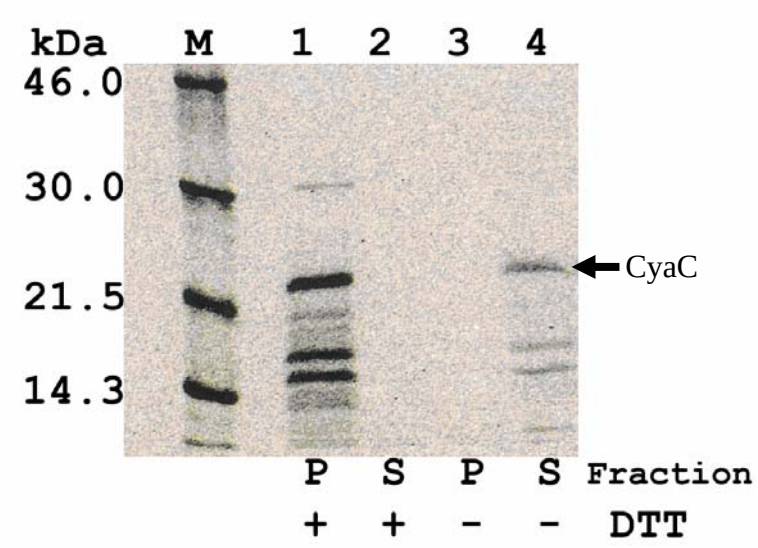

Figure 5. Autoradiography of CyaC protein expressed by an in vitro coupled transcription/ translation system with DTT (lane 1 and 2) and without DTT (lane 3 and 4) on SDS-PAGE. Cell-free reactions were centrifuged to separate the pellet (P) and supernatant (S) fractions prior to electrophoresis.

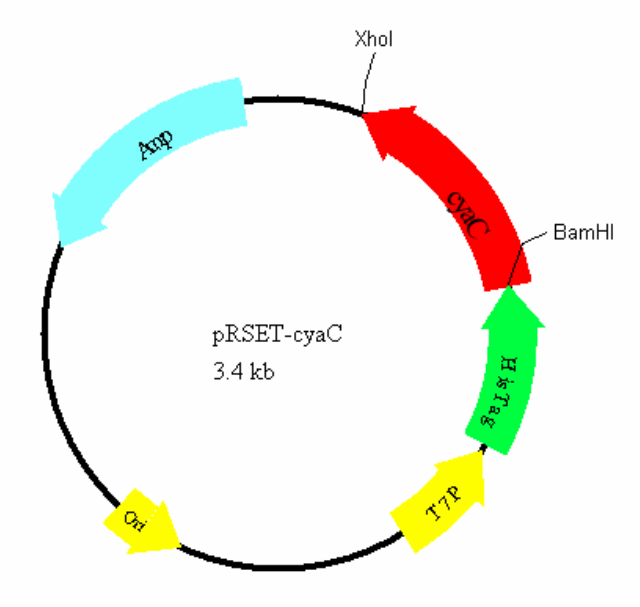

Figure 6. The map of the plasmid pRSET-*cyaC* constructed by subcloning of the *cyaC* from pCyaC into the pRSET A via the BamHI/XhoI sites.

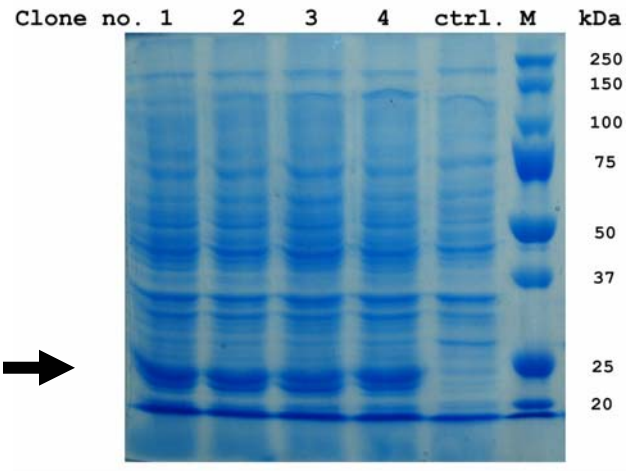

Figure 7. SDS-PAGE with coomassie blue staining shows expression of recombinant protein CyaC in 4 clones of BL21(DE3)pLysS with pRSET-*cyaC* (lane 1-4, arrow indicated). The control is proteins expressed in BL21(DE3)pLysS with pRSET A (vector without *cyaC*). Marker was loaded in lane M.

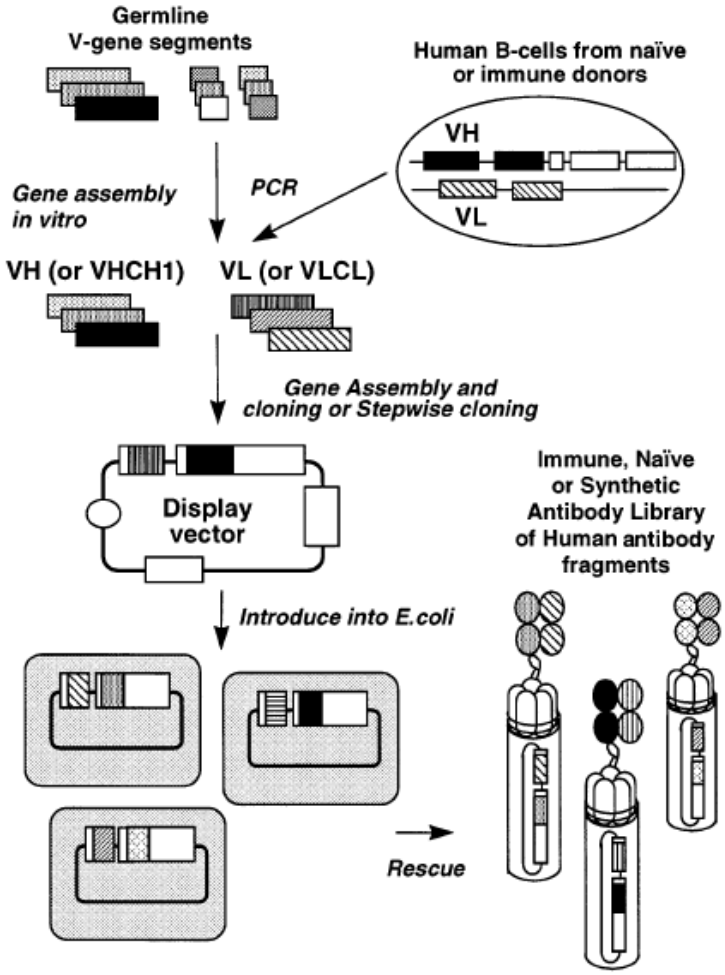

Figure 8. Schematic representation of antibody-phage display library by recombinant DNA techniques. The antibody fragment encoding genes (Vh/Vl or VhCh/VlCl) are cloned by PCR and assemble into a phage display vector. The recombinant phagemid then is transferred into an E.coli host to produce phages displaying antibody fragment. (Hoogenboom et. al 1998)

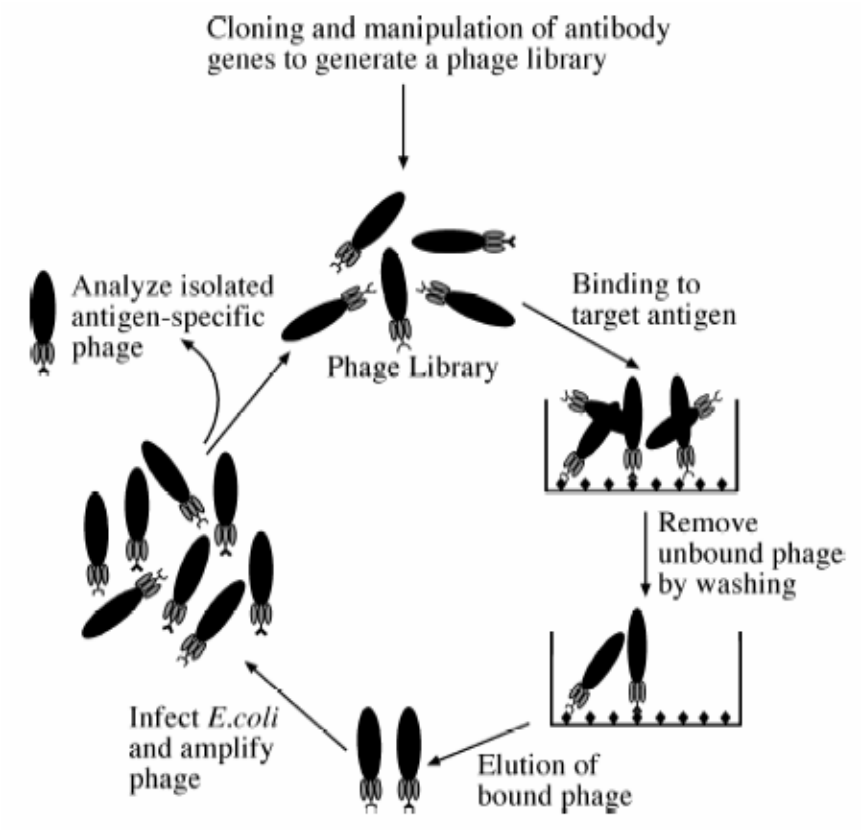

Figure 9. Schematic representation of affinity-selection by phage display. An antibody library is generated by recombinant DNA techniques. The resulting phage library is subjected to several cycles of affinity selection including capture of phage with antigen, washing to remove unbound phage, elution to release antigen-bound phage, and amplification in *E. coli*. When adequate enrichment has been obtained, individual antigen-specific clones are isolated and characterized.

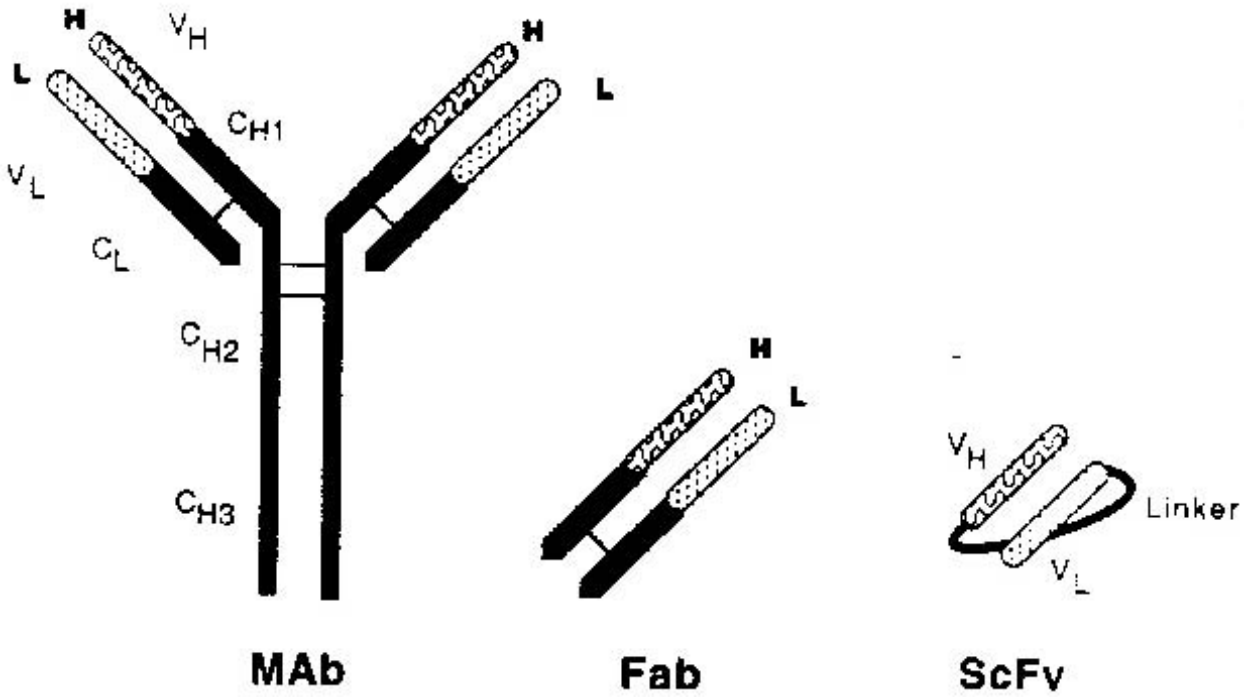

Figure 10. Schematic representation of antibody molecule. Fab and scFv are common format in antibody phage display. (http://dels.nas.edu/ilar_n/ilarjournal/37_3/37_3Recombinant.shtml)

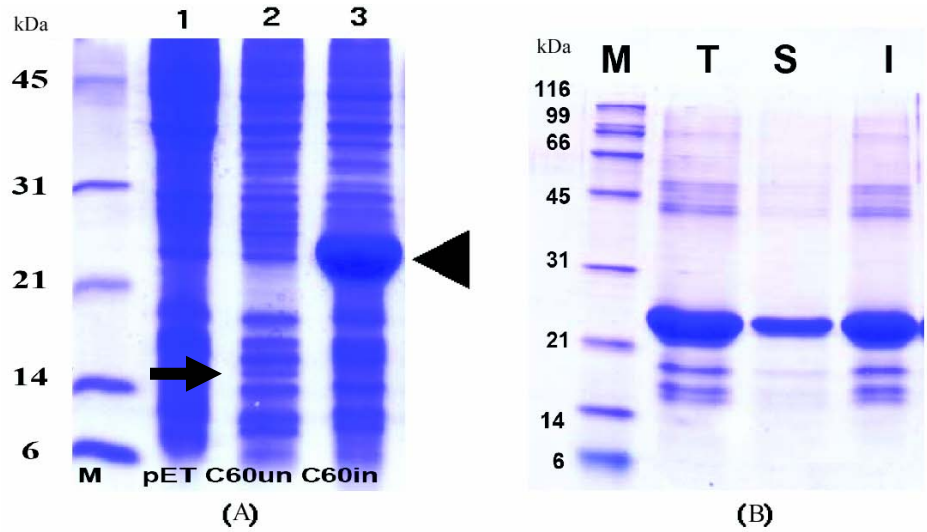

Figure 11. A) Cell lysates were analyzed by SDS-PAGE in 12% gel. lane 1; pET-17b in *E.coli* BL21(DE3) pLys, lane 2; pCyaC in *E.coli* BL21(DE3) pLys, uninduced, lane 3; pCyaC in *E.coli* BL21(DE3) pLys, induced with IPTG. Marker was loaded in lane M. **B**) Solubilization of CyaC inclusion bodies in carbonate buffer pH 10.5; T: total CyaC in carbonate buffer, S: soluble fraction, and I: insoluble fraction

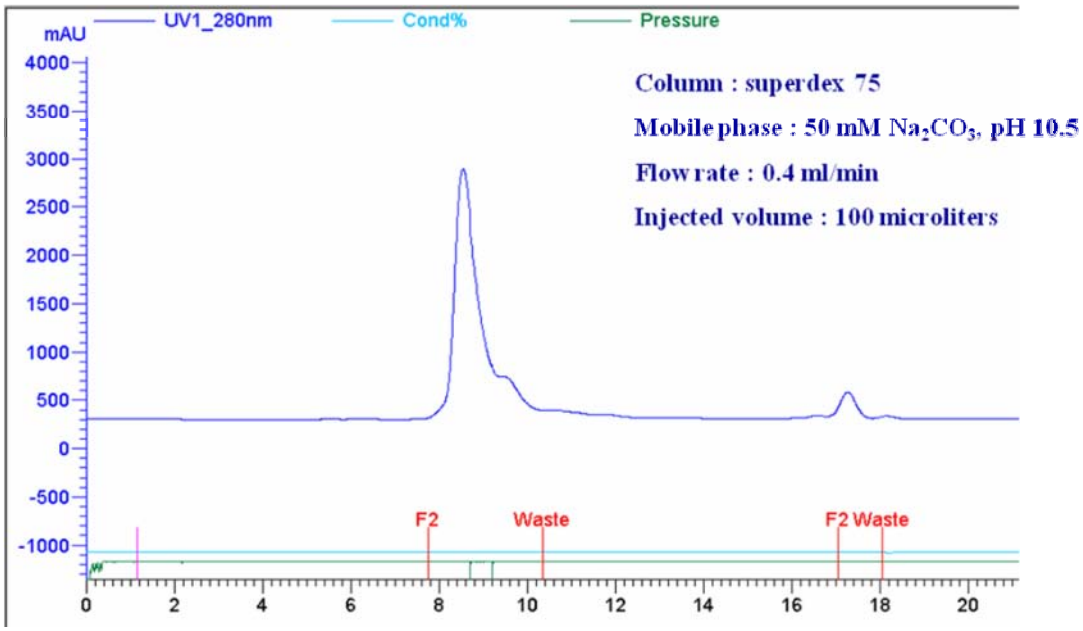

Figure 12. Gel filtration chromatogram of solubilized CyaC on Akta explorer. The sharp UV peak shows the fraction of the CyaC.

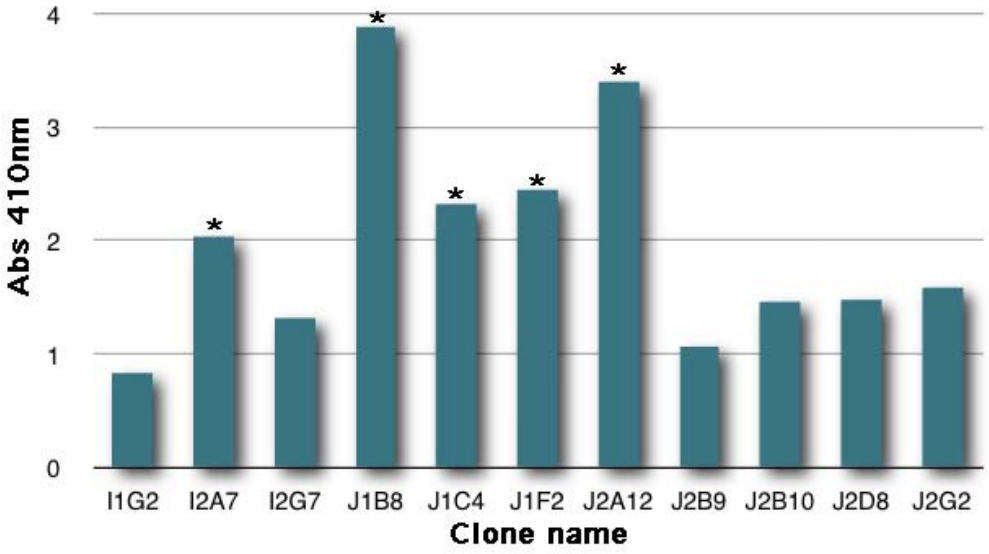

Figure 13. ELISA screening of positive clones from the antibody phage library. Starred clones were selected for further analysis

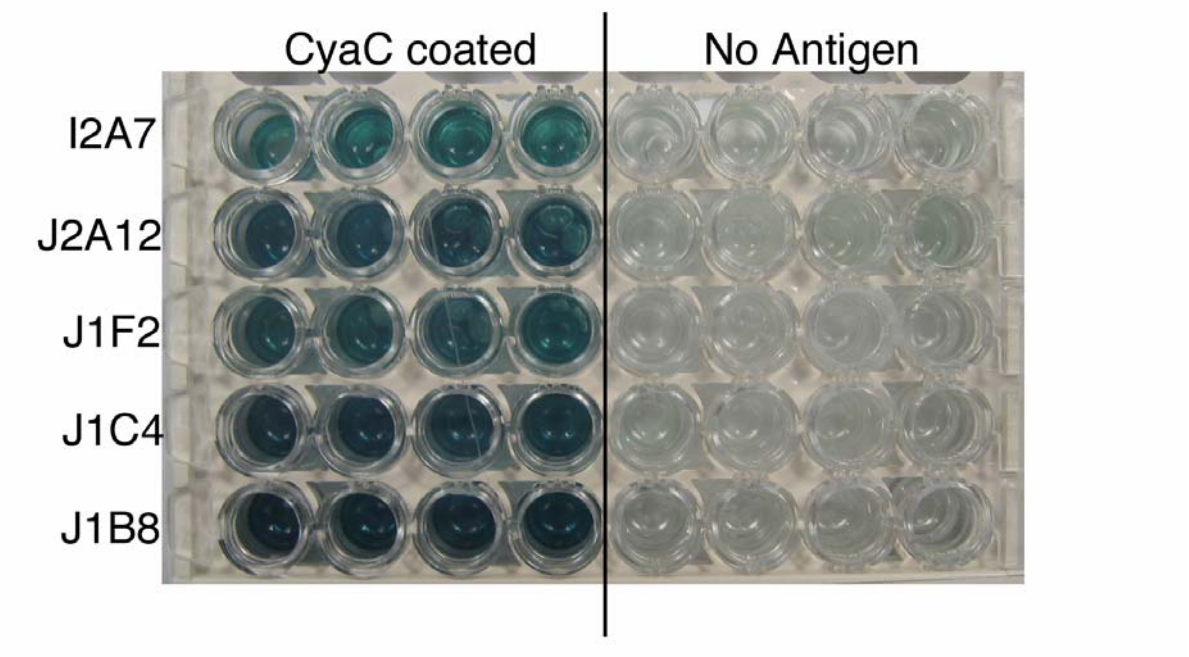

Figure 14. Confirmation of binding of scFv selected from the library by ELISA using ABTS as colorimetric substrate. Wells on the left half were coated with antigen CyaC, while wells on the right half were uncoated (negative control).

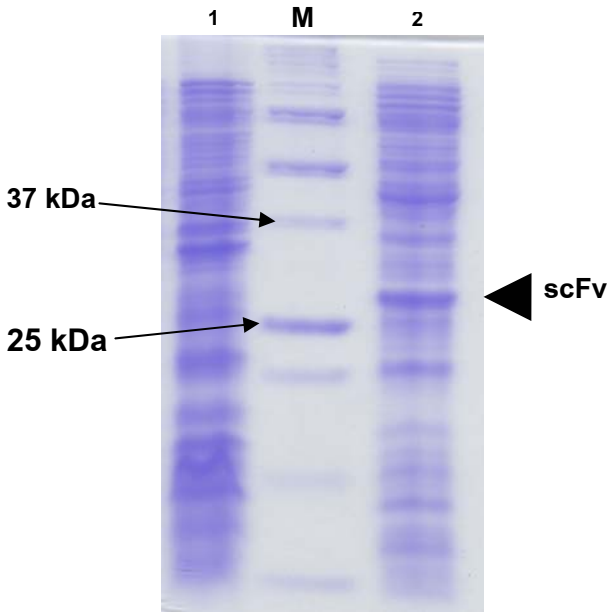

Figure 15. Expression of soluble anti-CyaC scFv. Cell lysates were analyzed by SDS-PAGE in 12% gel. lane 1; lysate from *E.coli* HB2151 (negative control), lane 2; anti-CyaC scFv phagemid in *E.coli* HB2151.Marker was loaded in lane M. Arrow indicates the presence of scFV.

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Output

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ
    - In vitro coupled transcription/ translation of CyaC (an acyltransferase) from *Bordetella pertussis* (manuscript in preparation)
2. การนำผลงานวิจัยไปใช้ประโยชน์
    - สร้างนักวิจัยใหม่ที่มีความรู้เรื่อง cell-free protein synthesis โดยมีนักศึกษาฝึกปฏิบัติงานวิจัยในหัวข้อดังกล่าวเป็นเวลา 1 ปีการศึกษา
    - สร้างนักวิจัยใหม่ที่มีความรู้เรื่อง phage display โดยมีนักวิจัยจากศูนย์วิจัยและพัฒนาการสัตวแพทย์ภาคเหนือ (ตอนล่าง) จังหวัดพิษณุโลก จำนวน 2 ราย
3. อื่นๆ
    - รีคอมบิแนนท์พลาสมิดสำหรับการสังเคราะห์ CyaC โดยวิธี cell-free protein synthesis.
    - Anti-CyaC scFv