CLONING AND EXPRESSION OF PHYCOCYANIN ENCODING GENE FROM CYANOBACTERIA

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

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วันวิสาข์ สุภาพ : การโคลนและแสดงออกของยีนไฟโคไซยานินจากไซยาโนแบคทีเรีย (CLONING AND EXPRESSION OF PHYCOCYANIN ENCODING GENE FROM CYANOBACTERIA) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.มารินา เกตุทัต-คาร์นส์, 73 หน้า.

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาคุณลักษณะของยีนไฟโคไซยานินของสาหร่ายสีเขียวแกมน้ำเงินในระดับโมเลกุล โดยการโคลนยีนไฟโคไซยานินของสาหร่าย *Anabaena siamensis* TISTR8012 ซึ่งเริ่มจากการสร้างนิวคลีโอไทด์สายสั้นจากการเปรียบเทียบลำดับนิวคลีโอไทด์จากสาหร่ายสีเขียวแกมน้ำเงิน 7 สายพันธุ์ ได้แก่ *A. variabilis* ATCC 29413, *Arthrospira platensis, Spirulina maxima*, *A. kisseleviana*, *A. lemmermannii*, *A. flos-aquae* และ *A. planktonica* จากนั้น ทำการโคลนแอลฟา และบีตายีนโดยการเพิ่มปริมาณดีเอ็นเอด้วยปฏิกิริยา PCR แล้วโคลนเข้าสู่โคลนนิ่งเวคเตอร์ จากนั้น ถ่ายโอนเข้าสู่เว็คเตอร์เพื่อแสดงออกของโปรตีน เมื่อได้โปรตีนแอลฟา และบีตาแล้วได้ทำการทำโปรตีนให้บริสุทธิ์โดยการใช้ โคบอลต์ แอฟฟินิตี โครมาโตรกราฟี แล้วนำโปรตีนที่บริสุทธิ์แล้วนั้น มาทดสอบคุณสมบัติการต้านอนุมูลอิสระโดยวิธี ABTS scavenging ผลการทดลองที่ได้นำเสนอโดยค่า IC_{50} โดยวิตามินอีสังเคราะห์ โปรตีนแอลฟา และโปรตีนบีตา มีค่า IC_{50} 0.4, 13.8 และ18.6 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

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WANWISA SUPHAP : CLONING AND EXPRESSION OF PHYCOCYANIN ENCODING GENE FROM CYANOBACTERIA. THESIS ADVISOR : ASST. PROF. MARIENA KETUDAT-CAIRNS, Ph.D., 73 PP.

Anabaena siamensis TISTR8012/PHYCOCYANIN/ANTIOXIDANT PROPERTY

This research had objectives to clone and study phycoyanin properties. The genomic DNA of *Anabaena siamensis* TISTR8012 was used as a template for apo-α^{PC} and apo-β^{PC} amplification. The primers for apo-α^{PC} and apo-β^{PC} amplification were designed from the alignment of apo-α^{PC} and apo-β^{PC} of *A. variabilis* ATCC 29413, *Arthrospira platensis*, *Spirulina maxima*, *A. kisseleviana*, *A. lemmermannii*, *A. flos-aquae* and *A. planktonica*. The apo-α^{PC} and apo-β^{PC} were cloned into cloning vector by PCR reaction, subsequently sequenced and expressed using expression vector. The recombinantly expressed apo-α^{PC} and apo-β^{PC} were purified using cobalt affinity chromatography. The recombinant proteins were analyzed for antioxidant scavenging property using ABTS scavenging assay. The result showed that trolox, rTrx_apo-α^{PC} and rTrx_apo-β^{PC} proteins with IC_{50} value of 0.4, 13.8 and 18.6 mg/ml, respectively.

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LIST OF ABBREVIATIONS

ABTS	=	2, 2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid
Apo_α^{PC}	=	alpha protein
Apo_β^{PC}	=	beta protein
C-PC	=	cyanobacterial phycocyanin
cpcA	=	alpha gene
cpcB	=	beta gene
DPPH	=	2, 2-diphenyl-1-picrylhydrazyl
PBPs	=	phycobiliproteins
Trx	=	thioredoxin protein

CHAPTER I

INTRODUCTION

Anabaena siamensis is a member of cyanobacteria isolated from rice paddy field in Thailand. Recently this strain has been used in the Thai agricultural market as an algal bio-fertilizer for rice fields. It shows high capacity to fix atmospheric N_2 and subsequently increase the rice growth rate. The Cyanobacterial C-phycocyanin (C-PC) is a non-toxic biliprotein consisted of α and ß subunits that act as photoharvesting pigment in various cyanobacteria (Phunprunch *et al*., 2006).

The C-PC from many cyanobacteria has been reported to work as antioxidant scavenger. For example the recombinant apo-α^{PC} from *Anabaena* PCC 7120 can inhibit cell proliferation and induced apoptosis by promoting cytoskeleton depolymerization and activating the caspase activities that are associated with the extrinsic cell death pathway (Wang *et al*., 2006). The C-PC holo subunit (holo-α^{PC}) has been successfully expressed as a fluorescent holo- α^{PC} in *E.coli* BL21. The heme oxygenase 1 and 3Z-phycocyanobilin: ferredoxinoxidoreductase enzymes required for conversion of heme to phycocyanobilin (PCB), and *cpcA* gene encoding apo-α^{PC} along with *cpcE* and *cpcF* both of which necessary and sufficient for the correct addition of PCB to *cpcA* to occur blue colour. So *cpcA, cpcE* and *cpcF* were cloned into the same plasmid for produced blue colour protein (Tooley *et al*., 2001). Native C-PC has variety of properties, however it is difficult to purify. In this research the sequences of *cpcA* and *cpcB* genes of C-PC from *A. siamensis* TISTR8012 were constructed to

produce recombinant holo-α^{PC} to further used for antioxidant and colorance, which should be easier to purified than the native C-PC.

However, no study on sequence of phycocyanin α and ß subunits of *A. siamensis* TISTR8012 has been done. In this research, two pairs of primers of *cpcA* and *cpcB* genes were designed from *Anabaena* PCC7120, used as reference sequence. *Anabaena* 7120 biliprotein phycocyanin operon (X05239) and other 7 cyanobacteria such as *A. variabilis* ATCC 29413, *Arthrospira platensis*, *Spirulina maxima*, *A. kisseleviana*, *A. lemmermannii*, *A. flos-aquae* and *A. planktonica* were also used to compare and find the conserved sequence. The primers were used to amplify the *cpcA* and *cpcB* genes from *A. siamensis* TISTR8012 cloned into cloning vector, sequenced and submitted to NCBI database as accession number EU815327 and EU815328, respectively. Recombinant proteins were produced and the antioxidant properties of rTrx_apo-α^{PC} and rTrx_apo-β^{PC} were tested; the result showed that recombinant phycocyanin can exhibit antioxidant when tested with ABTS radical scavenging assay.

CHAPTER II

LITERATURE REVIEW

2.1 Cyanobacteria or blue-green algae

Microalgae play an important role in biological ecosystems because of their photosynthetic activity. They are the major producers of biomass and organic compounds on earth. Many species of microalgae are known. They can be separated into five major divisions: *Chlorophyta* (green algae), *Chrysophyta* (golden-brown, yellow algae and diatoms), *Pyrrhophyta* (dinoflagellates), *Euglenophyta* and *Cyanophyta* (blue-green algae) (Rippka *et al*., 1979).

The *Cyanophyta* are a diverse group of prokaryotic organisms that evolved in the Pre-Cambrian period, approximately 3.5 billion years ago. They are thought to be responsible for the oxygenation of the earth's atmosphere, 1 billion years after their appearance in the fossil record. The ability to survive in warm temperatures, high light, and low carbon dioxide concentrations has allowed the cyanobacteria to radiate into a broad range of habitats, including hot springs, frigid lakes and soils. Although few species of cyanobacteria are found in the open ocean, a large range of species can be found at the surface of estuarine mud and in shallow, eutrophic freshwater lakes and rivers. Formerly referred to as blue–green algae, cyanobacteria possess a sturdied cell envelope consisting of three layers, similar to other gram-negative bacteria: the (i) outer membrane; (ii) the peptidoglycan layer, which is thicker than traditionally associated with other gram-negative bacteria; and (iii) the cytoplasmic membrane (Christa *et al*., 2005).

Rippka *et al*., (1979) divided cyanobacteria by its morphology into five sections.

Section 1: Unicellular cyanobacteria that reproduced by binary fission or by budding such as *Synechococcus* Type I, II, III; *Glloeothce*; *Gloeocaps*; *Gloeobacter*; *Chanaesiphon* and *Synechocystis* Type I, II.

Section 2: Unicellular cyanobacteria that reproduced by multiple fission such as *Dermocarsa*; *Xenococcus*; *Dermocarpella*; *Chroococcidiopsis*; *Pleurocapsa* group Type I, II and *Myxosarcina*.

Section 3: Filamentous non-heterocystous cyanobacteria that divide in only one plane such as *Spirulina*; *Arthrospira*; *Oscillatoria*; *Pseudonabaena* and LPP group A, B and *Plectonema baryanum* type.

Section 4: Filamentous heterocystous cyanobacteria that divide in only one plane such as *Anabaena*; *Nodularia*; *Cylindrospermum*; *Nostoc*; *Scytonema*; *Calothrix* and *Tolypothrix tenuis* type.

Section 5: Filamentous heterocystous cyanobacteria that divide in more than one plane such as *Chlorogloeopsis fritschii* and *Fischerella*.

In this thesis, only the filamentous heterocystous cyanobacteria that divide in only one plane will be discuss in more detail.

Anabaena sp. is a filamentous and heterocyst-forming cyanobacteria. Heterocysts are metabolically highly active cells that have the capacity to fix nitrogen, an oxygen-sensitive process, in an oxygen-containing environment. Under conditions of nitrogen deficiency, heterocyst cells differentiate from vegetative cells at semi-regular intervals along the filaments, generating a pattern; *Anabaena* sp. has long been

used to study the genetics and physiology of cellular differentiation, pattern formation, and nitrogen fixation (Kaneko *et al*., 2001). *A. siamensis* was isolated from a rice paddy field in Thailand. Nowadays this strain is used in the Thai agricultural market as an algal bio-fertilizer for rice fields (Phunpruch *et al*., 2006).

2.2 Photosynthesis of cyanobacteria

Photosynthesis electron transfer reactions that take place in thylakoid membranes is the most abundant energy-storing and life supporting process on earth. Cyanobacteria are a valuable ubiquitous component of aquatic system that contributes significantly to the total photosynthetic products (Huseynova *et al*., 2007, Madhyastha and Vatsala, 2007).

Harvesting light energy from the sun is the first step of photosynthesis. The harvested light energy then is funneled to a trap or photosynthetic reaction center where the primary chemical reactions of photosynthesis occur. There is significant diversity in the types of light-harvesting proteins and the pigments they bind in various photosynthetic organisms. There are two major light-harvesting systems in cyanobacteria. Most classical cyanobacteria use external water-soluble bilin-bound protein complexes, phycobiliproteins (PBP), as their major light-harvesting systems are closely associated with the reaction centers of photosystem I (PSI) and photosystem II (PSII) and are similar to the proximal-antennae in other oxygen-producing plants; the new class of cyanobacteria, prochlorophytes (includind *Prochlorococcus*, *Prochloron*, *Prochlorothrix*) use integral membrane Chl-bound protein complexs, prochlorophyte chlorophyll *a/b* binding protein-Pcb, as their major antenna system, Chlorophyll a (Chl a) is the predominant pigment in nearly all

oxygenic photosynthetic organisms from cyanobacteria to land plants. (Gomez-Lojero *et al*., 1997 and Yang *et al*., 2009).

Phycobilisomes (PBS) serve as the distal antennae for PSII and, to a lesser extent, for PSI in cyanobacteria and red algae. PBS are supramolecular complexes of phycobiliproteins (PBPs) and linkers with smaller amounts of proteins like ferredoxin-$NADP^+$ oxido-reductase (Gomez-Lojero *et al*., 1997). PBPs are generally attached on the stromatic surface of the thylakoid membranes of both prokaryotic (cyanobacteria) and eukaryotic (red algae) organisms, these complexes represent the most abundant soluble proteins and act as major light-harvesting antennae for photosynthesis. PBPs are a major constituent of red algae and cyanobacteria, representing 40 – 60% of the total protein content of cell (Pilot and Fox, 1984). Cyanobacterial PBPs are classified into three main groups: phycocyanin (C-PC, λ_{max} = 620 nm), phycoerythrin (C-PE, λ_{max} = 560 nm) and allophycocyanin (C-APC, λ_{max} = 650 nm) depending on inherent color and absorbance properties. Here prefix 'C' indicates its cyanobacterial origin (Edwards *et al*., 1997 and Soni *et al*., 2008).

2.3 Component of Phycobiliproteins (PBPs)

The photosynthetic apparatus of cyanobacteria is localized on intracellular membranes termed thylakoids. Proteins, lipids, carotenoids and chlorophyll a are major components of thylakoids (Yu *et al*., 1981). PBPs are light-harvesting pigments normally associated with PSII (Edwards *et al*., 1997) or protein-chromophore conjugates found in thylakoids of cyanobacteria (Pilot and Fox, 1984). PBPs are the brilliantly colored, water soluble proteins, bearing covalently attached open chain tetrapyrroles attached to the apoproteins, are the major photosynthetic accessory

pigment in cyanobacteria (Patel *et al*., 2006). PBPs aggregate to form complexes called phycobilisomes, which have an allophycocyanin core with rods of phycocyanin and sometimes phycoerythrin or phycoerythrocyanin (Edwards *et al*., 1997). The assembly of phycobilisomes is mediated by PBPs and linker polypeptides. These linkers also alter the spectral properties of PBPs so as to ensure an efficient transfer of absorbed light energy to the membrane. PBS are found attached to the outer surface of the photosynthetic lamellae in thylakoid membrane, perhaps in association with photosystem II light harvesting complex; LHC (Lorimier *et al*., 1984, Patel *et al*., 2006). PBPs are a small group of highly conserved chromoproteins, a macromolecular protein complex whose main function is to serve as a LHC for the photosynthetic apparatus of cyanobacteria. In addition, associated with the PBPs in these complexes are small amounts of linker polypeptides, most of which do not bear chromophores (Liu *et al*., 2005, Mishra *et al*., 2008). The bilin groups are attached to the polypeptides through thioether linkages to specific cysteinyl residues. The basic structural unit of the proteins contains one β chain and one α chain (Pilot and Fox, 1984).

The α polypeptide is covalently linked to one bilin derivative; the β polypeptide is covalently linked to two bilin derivatives. In vivo, PBPs exist in higher aggregation states with the $(\alpha\beta)_6$ form generally believed to be the functionally important aggregate called hemidiscoidal shape (Pilot and Fox, 1984). The role of PBPs is to trap light energy in the 500 to 650nm wavelength range and to transfer it to chlorophyll a of PSII. Measurements of energy transfer in both intact algal cells and isolated PBS have shown that light energy is normally first absorbed by to chlorophyll phycoerythrin, then transferred to phycocyanin, then to allophycocyanin, and finally

a. It has further been established that the transfer of energy within a PBPs is from the β chain to the α chain (Romay *et al*., 2003).

Cyanobacterial Phycocyanin (C-PC) isolated from cyanobacteria is an oligomeric biliprotein with the linear tetrapyrrole chromophore known as bilins. It covalently attached to the apoprotein by a thioether linkage (Mishra *et al*., 2008). It is a major component of the PBPs family. C-PC is gaining increasing importance for its promising antioxidant property (Patel *et al*., 2006). The native C-PC is stable at pH 4.5-8.5 and temperature between -4 to 45°C. When the secondary, tertiary and quaternary structures of the protein are denatured, the visible absorption band as well as the fluorescence drops in intensity (Romay *et al*., 2003).

2.4 *Anabaena siamensis*'s Research

A. siamensis, is as filamentous heterocystous cyanobacteria found in rice paddy in Thailand. Very few researchers works has been done on it, so in this review will showed various ability such as N_2–fixing cyanobacteria which play vital roles in the buildup and maintenance of soil fertility in natural and also in agricultural ecosystems (Antarikanonda, 1984). Thomas, (1990) used *A. siamensis* as nitrogen biofertilizers in rice field in countries where rice is the major staple diet. Nagase, 1997 used *A. siamensis* as cadmium removal from hard water. Phunpruch, (2006) used *A. siamensis* as dihydrogen a new source of clean energy. Firstly, In 1984 Antarikanonda, studied *A. siamensis* Antarikanonda, it produces of extracellular and intracellular free amino acid. The result showed difference of amino acid exudates between intracellular and extracellular free amino acid pool in cyanobacteria (Antarikanonda, 1984).

cyanobacteria which release ammonium continuously, Thomas *et al*., (1990) studied ammonium–excreting mutant; SS1 (SS1 released ammonium due to the high activity of nitrogenase) ammonium excretion by an L-Methionine-DL-Sulfoximine-resistant (MSX) mutant. The researcher used *A. siamensis* which has fast growth rate and high nitrogen-fixing capacity. This strain also adapts well to temperature fluctuations (25 to 42°C) prevailing in rice fields and to the salinity ranges (1 to 2%) existing in most of the tropical wetland soils. In this respect SS1 exhibited C-PC levels (14.0 μg ml^{-1}) significantly higher than in the wild type strains (9.6 μg ml^{-1}). During the whole growth cycle *A. siamensis* proved promising and is already marketed as an algal biofertilizer for rice fields, its efficiency in increasing the growth and yield of rice plants is apparently due to its high N_2-fixing capacity (Thomas *et al*., 1990).

An industrial reactor for mass cultivation of microalgae was at present nearly without exception all designed as open raceways. A major weakness of the open raceway were difficulties in harvesting the algal biomass, become rather easily contaminated, relatively large running costs involved in maintaining a large volume of water with a low concentration of cells, water temperature cannot be readily controlled. Comparing the productivity in terms of dry biomass per volume *of A. siamensis* in different culturing method the advantage of the tubular system over open raceways becomes ever so evident because the open raceways had more density effects related to mutual shading exert new limitations on growth in addition to the limitation imposed by light (Richmond *et al*., 1993).

The contamination of surface and ground water by heavy metals as a result of

an alternative, in recent years the use of microorganisms for metal removal has received increasing attention because of its high efficiency and low cost requirements. The selective cadmium removal from hard water using NaOH-treated cells choose be one in that *A. siamensis* TISTR8012 the result showed that NaOH-treated cells were thus found to have a high selectivity for Cd adsorption in hard water. Pretreatment with NaOH increased the level of Cd adsorption *A. siamensis* (57% Cd removal) (Nagase *et al*., 1997).

In N_2-fixing cyanobacteria, there are three enzymes involved in the hydrogen metabolism. Nitrogenase, uptake hydrogenase and reversible hydrogenase. Uptake hydrogenase genes that perform alter H_2, by product from N_2 fixation by nitrogenase for future prospects of biohydrogen use as source of clean energy. From the reason above Phunpruch *et al*., (2006) interested on uptake hydrogenase initial cloned and sequenced on structural genes encoding both the small (HupS) and the large (HupL) subunits of the uptake hydrogenase in *A. siamensis* the result showed that the presence of whole *hupSL* was identified *hupSL* from *A. variabilis* ATCC 29413 (Y13216) and *Anabaena* sp. PCC 7120 (U08013). The upstream gene, *hupS*, consisted of 963 bp encoding 320 amino acids whereas the downstream gene, *hupL*, comprised 1,596 bp encoding 531 amino acids. *A. siamensis hupS* showed higher than 74% nucleotide identity and 90% amino acid similarity. The *hupL* were higher than 73% nucleotide identity and 88% amino acid similarity of *A. variabilis* ATCC 29413 and *Anabaena* sp. PCC 7120 (Phunprunch *et al*., 2006).

From the review above *A. siamensis* had very few researcher studied on it, so in this thesis was focus on *A. siamensis* TISTR8012 for studied on basic knowledge

2.5 Natural C-Phycocyanin purification

C-PC is the major PBPs in cyanobacteria. C-PC comprises a protein and chromophore and the protein moiety consists of *cpcA* and *cpcB* genes encoded alpha and beta proteins of molecular weights in the range of 18 and 20 kDa, respectively. C-PC is mainly used as fluorescent markers in biomedical research, nutrient ingredient, and natural dye for food and cosmetics and also as a potential therapeutic agent such as cyclooxygenase-2 inhibition, antioxidant and anti-inflammatory effect (Reis *et al*., 1998, Minkova *et al*., 2003, Madhyastha *et al*., 2006 and Patil *et al*., 2006). A lot of methods used for the purification of C-PC involve a number of steps such as precipitation, centrifugation, dialysis, ion-exchange chromatography, gel filtration chromatography, etc. (Patil *et al*., 2006). In 1998, Reis and their group studied the method for purification of PBPs from *Nostoc* sp. The researchers used ultrafiltration 30kDa cut off, ammonium sulfate $(NH_4)_2SO_4$ precipitation, gel filtration chromatography and ion-exchange chromatography. The result showed that 66% protein losses was due to damage by thermal and the hollow fiber membrane and permeate losses from ultrafiltration method (Reis *et al*., 1998). Minkova *et al*., 2003 studied purification of C-PC by 40% ammonium sulfate $(NH_4)_2SO_4$ precipitation, gel filtration and 70% $(NH_4)_2SO_4$ precipitation. The result showed that last step of purification yield of C-PC was 45.7% from crude extract and determine molecular masses 19.5 and 21.5 kDa, corresponding to the *cpcA* and *cpcB* genes products, respectively (Minkova *et al*., 2003). In 2006, Madhyastha and their group studied method for purification of C-PC from *Spirulina fusiformis* by hydroxypatite column

chromatography and gel filtration chromatography the result showed molecular masses of the *cpcA* and *cpcB* genes products of 16 and 17 kDa, respectively. The homology of the alpha and beta proteins when compare with other cyanobacteria PBPs, *S. fusiformis* shared the highest degree of homology with *S. platensis* (93% for *cpcA* and 95% for *cpcB*) followed by *Mastigocladus nidulans* (57% for *cpcA* and 64% for *cpcB*) (Madhyastha *et al*., 2006). Patil and their group, 2006 studied purification of C-PC by aqueous two phase extraction (ATPE) combination with ion-exchange chromatography the result shown that C-PC from ATPE purification yield of C-PC was 73% and high purity of protein, not only enabled an increase in product purity without the need of multiple steps but also reduced the volume of the contaminant protein (Patil *et al*., 2006). Soni and their group studied one single step hydrophobic interaction chromatography (HIC) for purification of C-PC from *Phormidium fragile* the result show that HIC had advantage of binding the protein to the resin in the presence of high concentration of $(NH_4)_2SO_4$ salt. Since hydrophobic interactions are influenced by the presence of salt concentration, decreasing $(NH_4)_2SO_4$ concentration caused elution of protein and molecular masses of the *cpcA* and *cpcB* genes product were 19 and 20 kDa, respectively. (Soni *et al*., 2008). In 2009, Moraes and Kalil evaluated the use of $(NH_4)_2SO_4$ precipitation, gel filtration chromatography and ion-exchange chromatography (IEC) to purify C-PC in a variety of sequences. The final design included the C-PC extraction step, precipitation, gel filtration and ion-exchange chromatography the result shown that precipitation step used in first method for remove contaminant protein from C-PC protein gave high recovery rate, very simple and inexpensive technique. After that the used of gel filtration chromatography to remove salt from C-PC for high absorb ability between C-PC and resin. The last

step major increment purity occurred in the IEC step. Gel filtration was not efficient for purification (Moraes and Kalil, 2009).

A lot advantage of C-PC studied by many researchers but the scale-up of these methods is difficult and expensive so some of the researchers try to studied in the field of molecular biology to clone and express C-PC for more convenience and inexpensive to scale up protein desire.

2.6 Cloning of C-Phycocyanin

Lorimier *et al*., (1984) used *Agmenellum quadruplicatum* PR-6 and plasmid pUC8 to cloned the *cpcA* and *cpcB* genes. They synthesized 3'-T-A-C-C-A-T-C-T-C-A-T-G-G-A-5' (oligonuclotide probe encoded following sense-strand: mRNA-complementary) that could encode a particular pentapeptide in *cpcA* gene from *A. quadruplicatum*, and used as probe on southern blots of *Ag. quadruplicatum* DNA. The probe-hybridized to a 3.1-kilobase pair *Hind*III fragment which was cloned. The nucleotide sequence of a 1.7-kilobase pair segment of this clone was determined. The deduced amino acid sequences of the gene products show considerable homology with the *cpcA* and *cpcB* genes of C-PC subunits from other species compare with the other three complete sets of C-PC sequences: those of *Synechococcus* 6301, *Mastigocladus laminosus*, and *Cyanidium caldarium*, the average homologies between all pair-wise combinations are 71.5 ± 4.2% for *cpcB* subunits and 72.5±5.4% for *cpcA* genes (Lorimier *et al*., 1984).

Pilot and Fox, (1984) used *Ag. quadruplicatum* and plasmid pUC9 to cloned and sequenced of the genes encoding the *cpcA* and *cpcB* genes of C-PC. The result showed that the *cpcB* gene is upstream from the *cpcA* gene, with a 108-bp

gene coding region was 486 bp. Just upstream from the identified initiation site is a sequence identical to the consensus prokaryotic RNA polymerase binding site, T-A-T-A-A-T. The *cpcB* gene contains 172 amino acids and has a calculated molecular weight of 18.3 kDa; the *cpcA* gene subunit contains 162 amino acids and has a calculated molecular weight of 17.6 kDa (Pilot and Fox 1984).

Belknap and Haselkorn, (1987) used *Anabaena* PCC 7120 and plasmid pAn410 and pAn420 to cloned and studied the light regulation expression of the phycocyanin operon of the cyanobacterium *Anabaena*. The result showed that the single-copy C-PC genes are part of a larger operon which consists of five subunits of C-PC, namely; *cpcA*, *cpcB*, *cpcD*, *cpcE* and *cpcF* genes. In this experiment, they defined the complete sequence of C-PC of *Anabaena* PCC 7120. The light intensity used to alter the transcription of the five genes in *Anabaena* PCC 7120 operon was also shown. They also demonstrated that starvation or fixed nitrogen do not alter the transcription of these five genes in *Anabaena* PCC 7120 operon (Belknap and Haselkorn, 1987).

The major covalent modification of the biliproteins is the formation of thioether linkage between bilin chromophores and cysteine residues of C-PC apoprotein subunits. C-PC apoprotein contains three bilin sites; the Cys-82 and Cys-153 are in C-PC/β subunit of the apoprotein and the Cys-85 in C-PC/α subunit of the apoprotein. Toole *et al*., (1998) used *Synechocystis* sp. Strain 6803 and plasmid pSTV02K to study the bilin deletions and subunit stability in cyanobacterial light-harvesting proteins. The result of the experiment of mutation by site directed bilin mutagenesis to change three bilin sites from cysteine to alanine show that the three

contributes to the efficiency of folding and assembly of monomer to form hexamer of PC-holoprotein (Toole *et al*., 1998).

Tooley *et al*., (2001) used *Synechocystis* sp. Strain 6803 and plasmids pBS414V and pAT101 to studied the biosynthesis of a fluorescent C-PC holo-α, subunit the result showed that they tried to clone C-PC apoprotein bind with chromophore to form holoprotein that gave blue fluorescent. The researcher cloned C-PC/α subunit from *cpcA* gene (sll1578), C-PC/α Phycocyanobilin Lyase from *cpcE* gene (slr1878) *cpcF* gene (sl1051), Heme Oxygenase 1 (HO1) from *hox1* gene (sll1184) and 3Z-Phycocyanobilin: Ferredoxin Oxidoreductase from *pcyA* gene (slr0116). So plasmid pBS414V containing *cpcA-cpcE-cpcF* cassette and plasmid pAT101 containing Phycocyanobilin (PBP) biosynthesis cassette, *hox1* gene and *pcyA* gene (Tooley *et al*., 2001). The mechanism of biosynthetic pathway for the production of phycocyanobilin showed in Figure 2.1.

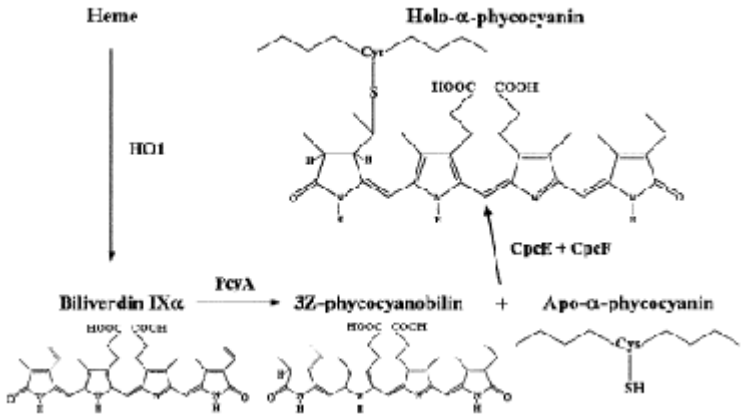


Figure 2.1 Minimal biosynthetic pathway for the production of phycocyanobilin from heme and its addition to the C-phycocyanin apo-α subunit (Tooley *et al*., 2001).

Wang *et al*., (2006) used *Anabaen*a PCC7120 and plasmid pGEX-2T to study the recombinant *cpcB* gene of C-PC in inhibition of cell proliferation and apoptosis induction. The result showed that the recombinant C-PC inhibited cell proliferation and induced apoptosis. They believe that the C-PC/β inhibits cell proliferation and promotes apoptosis by promoting cytoskeleton depolymerization and activating the caspase activities that are associated with the extrinsic cell death pathway. Growth of all cancer cells were largely inhibited by treatment with C-PC/β. The C-PC/β showed the greatest inhibitory ratio of 60.4 % whereas the C-PC/β had minor inhibitory effects on the growth of non-cancer cells which show inhibitory ratio of 29.7 % and 25.2 %. The results suggested that the C-PC/β inhibits cell growth (Wang *et al*., 2006).

Guan *et al*., (2007) used *Synechocystis* sp. Strain 6803 and plasmids pCDF to studied the biosynthesis of a fluorescent C-PC holo-α subunit of C-PC using one expression vector the result showed that they they tried to clone C-PC apoprotein bind with chromophore to form holoprotein that gave blue fluorescent. The researcher used this vector pCDF-*cpcA*-*cpcE*-*cpcF*-*hoxI*-*pcyA* for expressed the holo-α subunit of C-PC, this vector constructed from pET28a+-*cpcA*-*cpcE*-*cpcF* and pCDF-*hoxI*-*pcyA* cut genes from pET28a+ and ligated to pCDF-*hoxI*-*pcyA*. The recombinant holo-α subunit had calculated molecular weight of 21.1 kDa (Guan *et al*., 2007).

In 2009, Guan and their group used vector pCDF-*cpcA*-*cpcE*-*cpcF*-*hoxI*-*pcyA* for expressed the holo-α subunit of C-PC and the researcher used recombinant holo-α subunit tested antioxidant properties, the result showed that recombinant holo-α subunit can act against hydroxyl radicals with IC_{50} values of 277.5 µg/ml and IC_{50} values of 20.8 µg/ml against peroxyl radicals. The recombinant holo-α subunit had

The recombinant protein tends to accumulate in the form of insoluble aggregation and the most common way to improve the solubility of recombinant protein in *E. coli* is to reduce the induction temperature. In addition, Liu *et al*., (2009) studied and compare the improvement of solubility and stability of two vectors for expressed the holo-α subunit of C-PC. The researchers used pHPC and pHMPC that recombinant were fused hexahistidine and maltose-binding protein tag, the result showed that maltose-binding protein tag can increase soluble and stability than hexahistidine binding protein tag (Liu *et al*., 2009).

2.7 Property of C-Phycocyanin

Antioxidant property:

The oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, believed to be a primary factor in various diseases as well as in-aging which start chain reactions that damage cells (Patel et al., 2006). Antioxidants terminate these chain reactions by removing radical intermediates, and inhibit other oxidation reactions by being oxidized themselves via enzymetic and non-enzymatic antioxidant mechanism. (Erel, 2004, Benedetti *et al*., 2004). Enzymetic antioxidant mechanism, key metabolic steps are the superoxide dismutases (SOD), enzymes that catalyze the dismutation of superoxide radical to hydrogen peroxide (H_2O_2) and oxygen, and the conversion of H_2O_2 into water and oxygen is produced by catalase (CAT). Glutathione (GSH) plays a key role as an essential cellular antioxidant in the defence of brain cells against oxidative damage induced by reactive oxygen species (ROS) (Paloma *et al*., 2008).

damage of macromolecules within the cell. It is this damage to lipid, proteins and DNA that can rise to pathological consequences (Paloma *et al*., 2008 and Madhyastha *et al*., 2009).

Antioxidants are molecules that slow down or prevent the oxidation of other molecules (Benedetti *et al*., 2004), to prevent the formation and oppose the actions of reactive oxygen and nitrogen species which are generated in vivo and cause damage to DNA, lipids, proteins and other biomolecule (Paloma *et al*., 2008). Natural antioxidants, particularly in fruits and vegetables have increasing interest among consumers and scientific communities because frequent consumption of natural antioxidants is associated with a lower risk of cardiovascular disease and cancer (Thaipong *et al*., 2006).

Oxidative stress is an important factor in the genesis of much pathology from cancer to cardiovascular and degenerative diseases. In order to protect the body against the consequences of oxidative stress, an efficacious approach consists in improving the antioxidant nutrition. In this regard, scientific studies have shown that the synergistic action of a wide spectrum of antioxidants is better than the activity of a single antioxidant and that antioxidants from natural sources (primarily foods) have a higher bioavailability and therefore higher protective efficacy than synthetic antioxidants (Wang *et al*., 2006).

Benedetti *et al*., (2004) studied the antioxidant of C-PC from *Aphanizomenon flos-aquae* (AFA) the result showed that the efficacy of a novel natural extract from

AFA enriched with C-PC in protecting human erythrocytes and plasma samples against the oxidative damage induced by 2,2V-Azobis (2-amidinopropane) dihydrochloride (AAPH). In Red Blood Cell (RBC) treated with 50 mM AAPH, free radicals attack erythrocyte membrane components such as proteins and lipids cause changes in the structure and function of membranes. A time-dependent RBC hemolysis result was observed during cell incubate with AAPH. In RBC suspensions pre-incubated with the natural AFA extract, clearly indicated that the extract significantly reduced in a time- and dosage-dependent manner and extent of lipid peroxidation and hemolysis of RBC treated with AAPH, thus protecting the cell against the oxidative damage. C-PC extract from cyanobacteria has recently been reported to have many pharmacological characteristics (Benedetti *et al*., 2004).

Wang *et al*., (2006) studied the antioxidant property of recombinant C-PC by looking at the cell proliferation inhibition and the promotion of apoptosis. They demonstrated that the recombinant C-PC / β from *Anabaena* PCC 7120 can inhibit cell proliferation and induced apoptosis by promoting cytoskeleton depolymerization and activating the caspase activities that are associated with the extrinsic cell death pathway. The result shown that growth of all cancer cells were largely inhibited by treatment with C-PC/β. The C-PC/β show the greatest inhibitory ratio of 60.4% on the other hand, the C-PC/β had minor inhibitory effects on the growth of non-cancer cells which show the inhibitory ratio of 29.7%. The results suggested that the C-PC/β inhibits cell growth. C-PC induces apoptosis reduce tumor cell. The C-PC/β also inhibited cell proliferation (Wang *et al*., 2006).

Roy *et al*., (2007) demonstrated that C-PC can scavenge ROS and reduced drug resistance in cancer cell. So Roy and their group studied C-PC property of *S. platensis*. The result showed that C-PC scavenged ROS and stops expression of

various drugs primarily by the over expression of MDR1, it creates pores in this cell membrane which causes drug to be removed from drug treated cells, causes drug resistance to occur in those cells showed in below equation (Roy *et al*., 2007).

$$\text{ATP} + H_2O + \text{xenobiotic (In)} \xrightarrow{\text{MDR1}} \text{ADP} + \text{phoshate} + \text{xenobiotic (Out)}$$

Xenobiotic: substances foreign to the body, includind drugs and some food additives.

Soni *et al*., (2008) demonstrated the antioxidant propertied of C-PC in *Phormidium fragile* by Ferric Reducing Ability of Plasma assay (FRAP assay). They demonstrated the correlation of the antioxidant capacity. They used various well established non-enzymatic antioxidant; ferrous sulfate, ascorbic acid, galic acid, uric acid, α-tocopherol and C-PC. The result showed that antioxidant activity of C-PC was either equal or higher than all of these antioxidant. The fold antioxidant capacity of C-PC to ferrous sulfate, ascorbic acid, galic acid, uric acid, α-tocopherol was 4.25, 1.78, 0.94, 3.98 and 2.65, respectively (Soni *et al*., 2008).

The researchers also discuss about the linear tetrapyrrol prosthetic group commonly called “bilins” of C-PC that gives the ability to scavenge ROS and the molecule can act as an antioxidant (Soni *et al*., 2008 and Paloma *et al*., 2008).

Madhyastha *et al*., (2009) demonstrated the comparative antioxidant properties of C-PC from *S. fussiformis* by various antioxidant assay such as 2,2-azino-bis(3)ethylbenzthaiazoline-6-sulfonic acid (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH). The observed antioxidant activities were compared with that of ascorbic

acid, a standard antioxidant. ABTS and DPPH have been used extensively as free radicals to assess the scavenging activities of compounds. DPPH assay is based on the reduction of stable DPPH radical to yellow colored diphenyl picryl hydrazine in the presence of a hydrogen donor. However, C-PC failed to scavenge the ABTS radical significantly. The perceivably difference between ABTS and DPPH scavenging assay may be due to the differences in scavenging reactions occurring in aqueous phase for ABTS and in organic phase for DPPH assay. The researchers also discuss about the modification of the polypeptide with higher cysteine for increase antioxidant property (Madhyastha *et al*., 2009).

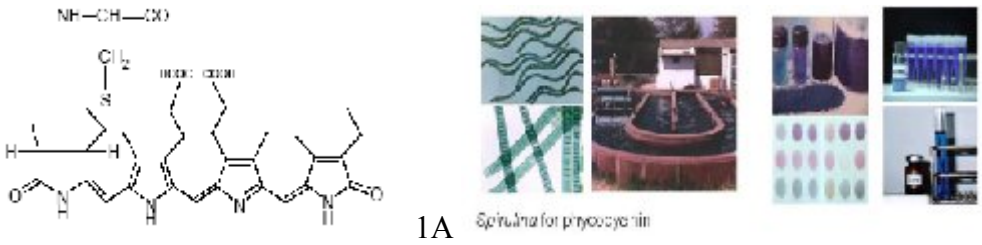
Anti-inflammatory property:

The other researchers studied about anti-inflammatory of C-PC in *S. platensis* by inhibited cyclooxgenase-2. Cyclooxygenase (COX) is bifunctional enzymes that catalyzing in the biosynthesis of prostaglandins (PGs), the COX biosynthesis respect with tumor tissues that contain high levels of PGs. The COX had two form, cyclooxgenase-1 (COX-1) and cyclooxgenase-2 (COX-2). The COX-2 is a key enzyme involved in the biosynthesis of PGs which plays an important role in inflammation, pain and variety of other disorders. The result demonstrated that the holoprotein and the apoprotein of C-PC which contain only the proteins play critical role in anti-inflammatation whereas the chromophore cannot inhibit COX-2 biosynthesis. From this result suggested that the apoprotein plays a role in the inhibition of COX-2 (Reddy *et al*., 2000).

Colorant property:

Microalgal use by indigenous populations has occurred for centuries. Indeed, edible blue green algae including *Nosto*c, *Arthrospira* (*Spirulina*) and *Aphanizomenon*

species have been used for food for thousands of years (Jensen et al.,2001). Batista *et al*., (2006) demonstrated the use of carotenoids as a main source of natural colorings (yellow to red). Carotenoids have been used by the food industry for foods and beverages color (e.g. orange juice). Carotenoids are also precursors of many important chemicals responsible for the flavor of foods and the fragrance of flowers. Another source of natural colourings is PBPs, which are water soluble fluorescent proteins derived from cyanobacteria. The blue phycobiliprotein (absorption maximum at 610–650 nm) from *S. maxima* (*Arthrospira*) widely used as a fluorescent marker in clinical diagnosis and also as coloring in the pharmaceutical and cosmetics industry. Also one type of PBPs; C-PC used as food coloring is not yet permitted within Europe and USA but it is authorized in Japan where it has been used in products like chewing gums, candies and dairy product (e.g. yoghurts). The antioxidant, anti-inflammatory properties have been attributed to C-PC (Batista *et al*., 2006). Another researcher studied the blue color from *Porphyridium aerugineum*. The C-PC extract of *P. aerugineum* can be extracted by centrifuge separation of the biomass, cell breakage and extraction. Use of salt solution as an extraction medium increases stability of the color during extraction. The blue color has the maximum absorbance at a wavelength of 620 nm. The shade of the blue color produced from *P. aerugineum* does not change with pH, stable under light but sensitive to heat. Within a pH range from 4.0 to 5.0, the blue color produced from *P. aerugineum* is stable at 60 °C for 40 min (Arad and Yaron, 1992). The C-PC structure had comprised of apoprotein and chromophores to form holoprotein. When the chromophores are composed of four tetrapyrrol, conjugated to an apoprotein via thioether bonds to form holoprotein that showed blue color protein showed in Figure 2.2 (Toole *et al*., 1998; Tooley *et al*., 2001; Guan *et al*., 2007; Guan *et al*., 2009).



1A 1B

Figure 2.2 Structure of C-PC (1A) and C-PC products from *Spirulina* sp. (1B) (Arad and Yaron, 1992).

This thesis aim to clone, express and purify the *cpcA* and *cpcB* genes from *A. siamensis* TISTR8012. This is the beginning of a bigger project to engineer and produce heat tolerant C-PC. However, after cloning, expression and purification of the C-PC, the properties of C-PC were tested for antioxidant properties. C-PC has several properties e.g. anti-inflammatation and color formation. The antioxidant property is the property of the apoprotein of C-PC. However, the color formation property requires the formation of thioether linkage between bilin chromophores (open chain tetrapyrrol) and cysteine residues of apoprotein bind to the *cpcA* and *cpcB* genes. In the second phase the *cpcA* and *cpcB* genes will be co-express to produce color chromophores. Later on screening for heat tolerant chromophores to use in high temperature food industries is the final goal. Therefore, this Master thesis proposes to only clone, expression and purify the single subunit of C-PC.

2.8 Research Objectives

The objectives of this thesis include

1. To clone cyanobacterial phycocyanin (C-PC), *cpcA* and *cpcB* genes from *A. siamemsis* TISTR8012.
2. To express, purify and test antioxidant property of recombinant proteins (rTrx_apo-α^{PC} and rTrx_apo-β^{PC} proteins) from *A. siamemsis* TISTR8012.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial strains:

- *Anabaena siamensis* TISTR8012 (purchase from Thailand Institute of Scientific and Technological Research (TISTR)) was cultured in BG11 medium.

- *Escherichia coli* (*E. coli*)

1. DH5alpha (DH5α) used for cloning purpose.

2. BL21 produces lower levels of proteases and thus better for expression of recombinant proteins. BL21(DE3)pLysS; host strain was used for protein expression in the *E. coli* system. BL21 is the strain of choice for high-level gene expression and production of recombinant proteins in bacterial systems. Strains designated as "DE3" carry a copy of the T7 RNA polymerase under control of the IPTG inducible; are ideal for controlled expression of T7 promoter driven constructs. Tighter control is provided by strains carrying the pLysS plasmids encoding T7 lysozyme, a natural inhibitor of T7 RNA polymerase, which reduces background levels of polymerase activity in uninduced cells.

3.1.2 Chemicals

All chemical used were molecular grade or analytical grade. 10 mM

dNTP mix (Invitrogen), 10X PCR buffer (200 mM Tris-HCl (pH8.4), 500 mM KCl, 15 mM $MgCl_2$) (Promega), 25 mM $MgCl_2$ (Invitrogen), *Taq* DNA polymerase (Home-made), TAE buffer (Appendix 2.5.1), Agarose low EEO (Research organics), 1 kb Ladder marker DNA (BioLabs), 100 bp Ladder marker DNA (Fermentas), 6X Gel Loading Dye (Biolabs), Stainning solution; 0.5 µg/ml ethidium bromide in distilled water, 5X stock SDS-gel loading buffer ((2.5 M Tris Base, 10% Sodium Dodecyl Sulphate (SDS), 0.5% Bromophenol blue and 50% glycerol)), 1.5 M Tris-Cl, pH8.8 (Promega), 0.5 M Tris-Cl, pH6.8 (Promega), UltraPure™ Acrylamide (Invitrogen), UltraPure™ N, N'-Methylenebisacrylamide (Invitrogen), Glycine Ultrapure, MB Grade (USB corporation), Coomassie brillant blue R-250 (Carlo Erba), Ammonium persulfate, MB Grade (Promega), 2-Mercaptoethanol (Sigma), SDS (Carlo Erba), TEMED (Bio-rad), Unstained Protein Molecular Weight Marker (Fermentas), Isopropyl-beta-D-thiogalactopyranoside (IPTG) (Fluka), IMAC Sepharose™ 6 Fast Flow (GE Healthcare), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Fluka)

3.1.3 Media (Appendix A)

- LB-LS medium
- BG11 medium

3.1.4 Vectors

- pENTR/D-TOPO (Invitrogen) and pET32a(+)/DEST (a gift from J.R. Ketudat-Cairns)

Entry clone

- The pENTR Directional TOPO® Cloning Kit was used to directionally clone a blunt-end PCR product, which includes the four base pair sequence (CACC) necessary for directional cloning on the 5' end of the forward primer. This vector allows the enter into Gateway cloning system available from Invitrogen.

Destination clone

- pET32a(+)/DEST (a gift from J.R. Ketudat Cairns) is plasmid constructed from pET32a (Novagen) by inserting Gateway cloning reading frame A into *Eco*R V (206) site. It contains the *att*R1 and *att*R2 site to allow recombination with Gateway entry clones by LR cloning technique, so it serves as a destination vector of expression of the DNA transferred from the entry clone (Invitrogen).

3.1.5 Primers; Gene specific primers were designed from *Anabaena* billiprotein phycocyanin operon accession number X05239

Table 3.1 The oligonucletides primers used for DNA amplification and sequencing.

Primer name	Sequence	Primer Size (bp)	Tm (°C)
1_Sai_phyco_beta_F	CAC CAT GAC ATT AGA CGT ATT TAC	24	48
2_Sai_phyco_beta_R	TTA ACC AAC AGC AGC AGC GC	20	62
3_Sai_phyco_alpha_F	CAC CAT GGT TAA AAC CCC CAT TAC	24	57
4_Sai_phyco_alpha_R	CAT GCT GAG AGG GTT GAT AGC G	22	56

3.2 General methods

3.2.1 Genomic DNA extraction

A. siamensis TIRTR8012 filaments from BG 11 medium (100 ml) were collected by centrifuge at 10,000 x g for 1 min. The collected pellets were washed with 500 µl of TEN buffer (50 mM Tris pH8.0, 0.1 M EDTA pH8.0, 0.5 M NaCl) two times. Then 320 µl extraction buffers (200 µl of 20% sucrose in TE buffer + 100 µl of 10% SDS + 20 µl of 2 mg/ml lysozyme) were added and then incubated at 37°C for 30 min. The samples of lysed cells were adjusted to 100 mM NaCl and were gently extracted with phenol-chloroform-isoamylalcohol (25:24:1). Aqueous DNA solutions were transferred to new containers by pipetting. The DNA was precipitated with 0.1 volumes of 3 M sodium acetate and 2.5 volumes of absolute ethanol and then kept overnight at 4°C. The samples were centrifuged 10,000 x g for 10 minutes and the pellet collected. The precipitated DNA was rinsed with 70% ethanol, and dissolved in 25 – 50 µl of TE buffer pH8.0.

3.2.2 Amplification of *cpcA* and *cpcB* genes of C-phycocyanin

The Polymerase Chain Reaction (PCR) was used to amplify *cpcA* and *cpcB* genes of phycocyanin gene by designing two pair of primers (Table 3.1) from the *Anabaena* billiprotein phycocyanin operon. The annealing temperature for amplified *cpcA* and *cpcB* genes were 50°C for 30 sec (Table 3.2). In the first round of PCR, primers 1_Sai_phyco_beta_F and 4_Sai_phyco_beta_R were used to amplify first amplicon. In the second round of PCR (semi-nested PCR), the first amplicon were used as template to amplified *cpcA* and *cpcB* genes by 3_Sai_phyco_beta_F and 4_Sai_phyco_beta_R; 1_Sai_phyco_beta_F and 2_Sai_phyco_beta_R primers,

respectively. *Taq* DNA polymerase was used in the PCR reaction to generate PCR products. The bands of PCR products were excised from agarose gels and purified by DNA extraction kit (Invitrogen).

Table 3.2 The PCR conditions used to amplify *cpcA* and *cpcB* genes for pENTR/D-TOPO.

Condition	Temperature (°C)	Time	Number of cycle
Initial denaturation	94	3 min	1
Denaturation	94	30 sec	35
Annealing	50	30 sec	
Extension	72	30 sec	
Final extension	72	7 min	1
Hold	4	∞	1

The expected size when amplified with 3_Sai_phyco_alpha_F and 4_Sai_phyco_alpha_R, 1_Sai_phyco_beta_F and 2_Sai_phyco_beta_R should be 480 bp and 500 bp for *cpcA* and *cpcB* genes, respectively. The PCR products were cloned into pENTR/D-TOPO cloning vector and transformed into *E. coli* (strain DH5α) by the electroporation technique and plated on LB agar contain 50 μg/ml of kanamycin.

3.2.3 Expression and purification of recombinant phycocyanin in *E. coli*

Expression of recombinant phycocyanin in *E. coli*

The enzyme LR clonase from Gateway® technology was used to exchange *cpcA* and *cpcB* genes from the pENTR/D-TOPO cloning vector to pET32a(+)/DEST. Transformation of pET32a(+) containing *cpcA* and *cpcB* genes into *E.coli* strain DH5α and transformed again into BL21(DE3)pLysS were done by electroporation technique. The clones were checked by colony PCR, after that the

samples were sequenced.

Two clones each of *cpcA* and *cpcB* genes in pET32a expression vector were selected to use in protein expression and one clone of pET32a was used for expression control, the selected cloned were grown at 37°C overnight in LB-LS broth containing amplicilin 100 µg/ml. The cultures were inoculated into LB-LS broth containing the same antibiotic and the cultures were grown in a shaking incubator at 37°C. After the optical density at wavelength 600 nm of the cultures reached to 0.5-0.6, IPTG was added to a final concentration of 0.4 mM and the cultures were shaken at 200 rpm 20°C for 4 h. Induced cultures were transferred to 50 ml centrifuges, the cell were harvested by centrifugation at 4,000 x g 4°C for 30 min and the cell pellet was kept at -70°C for at least 30 min or longer until used for protein extraction.

To extract the protein from induced recombinant cell, the bacterial pellet was thawed and suspended in 5 ml per gram cell freshly prepared extraction buffer (50 mM Tris-Cl pH8.0, 200 µg/ml lysozyme, 1 mM phenylmethylsulfonylfluoride (PMSF) and incubated at room temperature for 30 min. The cell suspensions were disrupted by sonication on ice until it seen transparent. The soluble protein fraction was recovered by precipitation of the insoluble fraction by centrifugation at 10,000 x g 4°C for 10 min. The supernatant is the soluble fraction.

Purification of recombinant phycocyanin

Purification of soluble recombinant phycocyanin alpha and beta protein were done with immobilized-metal affinity chromatography (IMAC) superflow resin (QIAGEN) at 4°C. Ten millilites of soluble protein from recombinant pET32a system was added to a 1 ml Co-NTA superflow column equilibrated with 10 ml (10 volumes) of equilibration buffer (20 mM Tris-Cl, pH8.0 + 150 mM NaCl). The column was

washed with 5 ml (5 volumes) of washing buffer (20 mM Tris-Cl, pH8.0 + 150 mM NaCl + 5 and 10 mM imidazole). The recombinant protein was eluted with 5 ml (5 volumes) of elution buffer (20 mM Tris-Cl, pH8.0 + 150 mM NaCl + 150 and 500 mM imidazole). The 5 volumes of eluted recombinant proteins were concentrated and exchanged with 50 mM Tris-Cl, pH8.0 in 10 kDa cutoff centrifugal ultracentrifugation membrane (Amicon).

3.2.4 Test antioxidant propertied of recombinant phycocyanin

1. Determination of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Any substance that can donate a hydrogen atom (antioxidant) to the solution of $DPPH^{\cdot}$ can reduced the stable free radical and change the color of solution from violet to pale yellow. Nonreacted radical form DPPH absorbs in the visible range, and spectroscopic method is based on the measurements of color intensity at 517 nm (Milardovic *et al*., 2006).

DPPH solution of 0.1 mM in distilled water was prepared and added to 1.0 ml of recombinant C-PC of 3 different concentrations (50, 100 and 150 μg/ml) in 50 mM Tris-Cl, pH8.0. The absorbance of 517 nm was measured after 30 min incubation. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Ascorbic acid (50, 100 and 150 μg/ml) were used as positive control. And estimated IC_{50} value was used to compare (DPPH) radical scavenging of positive control and other sample.

DPPH radical scavenging activity (%) = [($Abs_{control}$ − Abs_{sample})]/($Abs_{control}$)] × 100

where:

$Abs_{control}$ is the absorbance of DPPH radical + 50 mM Tris-Cl, pH8.0 and

Abs_{sample} is the absorbance of DPPH radical + sample

2. Determination of 2, 2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging assay

The most widely used colorimetric method for free radical scavenger is ABTS based methods, in which a colorless molecule, reduced ABTS, is oxidized to a characteristic blue-green ABTS. When the colored ABTS is mixed with any substance that can be oxidized, it is reduced to its original colorless ABTS form again; in contrast the reacted substance is oxidized (Erel., 2004)

ABTS reagent (7 mM ABTS, 2.5 mM potassium persulfate ($K_2(SO_4)_2$ and 10 mM sodium phosphate buffer, pH7.4) were kept at room temperature in dark condition for 12-16 h for radical generation. Before use, ABTS reagent was diluted with 10 mM sodium phosphate buffer, pH7.4 until the absorbance of 734 nm signal to 0.7±0.02 at were obtaining.

The reaction mixture contained 1980 µl of ABTS reagent and 20 µl of sample at different concentration. The mixture were gently mixed and incubated at room temperature in dark condition for exact 5 min, the absorbance were measured at 734 nm. Trolox was used as positive control and IC_{50} value was used to compare (ABTS) radical scavenging of positive control and other sample.

ABTS radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample})]/(Abs_{control})] \times 100$

where:

$Abs_{control}$ is the absorbance of ABTS radical +50 mM Tris-Cl, pH8.0 and

Abs_{sample} is the absorbance of ABTS radical + sample

CHAPTER VI

RESULTS AND DISCUSSIONS

4.1 Genomic DNA extraction

The extracted genomic DNA (gDNA) of *A. siamensis* TISTR8012 by phenol: chloroform: isoamylalcohol (25: 24: 1) were shown to be of high quality of more than 10.0 kb with high molecular weight on 1% agarose gel (figure 4.1).

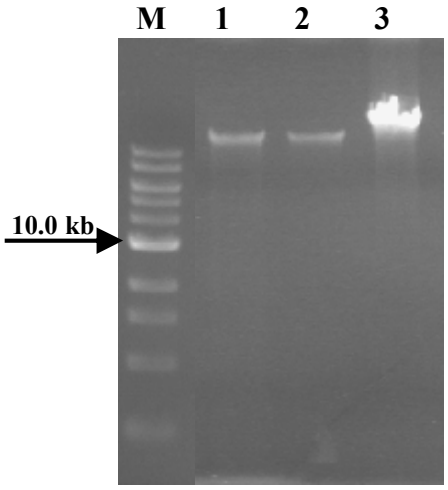


Figure 4.1 Agarose gel electrophoresis of extracted genomic DNA. M: 1 kb marker (Biolabs); Lane 1-3: triplicate of genomic DNA extraction.

4.2 Amplification of *cpcA* and *cpcB* genes of phycocyanin

The primer for *cpcA* and *cpcB* genes of phycocyanin were designed from reference sequence; *Anabaena* 7120 biliprotein phycocyanin operon (X05239) (figure 4.2) and alignment with *cpcA* and *cpcB* genes of *A. variabilis* ATCC 29413,

Arthospira platensis, Spirulina platensis A. kisseleviana, A. lemmermannii, *A. flos-aquae* and *A. planktonica* for align conserved cysteine that found in many cyanobacteria. Total gDNA was used as a template for the first PCR reaction. First amplicon was synthesized by 1_Sai_phyco_beta_F and 4_Sai_phyco_alpha_R. Semi-nested PCR was done by using the first PCR amplicon as a template with primer 3_Sai_phyco_alpha_F and 4_Sai_phyco_alpha_R to amplify the *cpcA* gene and primer 1_Sai_phyco_beta_F and 2_Sai_phyco_beta_R to amplify the *cpcB* gene. The expected size when amplified with 3_Sai_phyco_alpha_F, 4_Sai_phyco_alpha_R and 1_Sai_phyco_beta_F, 2_Sai_phyco_beta_R should be 480 bp and 500 bp for *cpcA* and *cpcB* genes, respectively. The results of the above are shown in figure 4.3. Pilot and Fox, 1984 also amplified *cpcA* and *cpcB* genes from *Agmenellum quadruplicatum* PR-6 resulted from their group showed that *cpcA* and *cpcB* genes consist of 486 and 516 bp, respectively and indicated that the *cpcB* gene was upstream from the *cpcA* gene (Pilot and Fox., 1984). Lorimier *et al*., (1984) studied C-PC and indicated that *cpcA* and *cpcB* genes were in the same orientation, with *cpcB* gene located upstream of the *cpcA* gene (Lorimier *et al*., 1984; Lu and Zhang, 2005). From the PCR results, the major bands of expected size were found after that DNA size around 480 bp and 500 bp were excised and eluted from agarose gel using DNA extraction kit (Invitrogen). The DNA was then transferred into pENTR/D-TOPO vector and transformed to *E. coli* DH5α. The transformants were screened on LB-kanamycin (50 μg/ml) plate.

Several cloned of both *cpcA* and *cpcB* genes in cloning vector were checked for DNA insertion by colony PCR (figure 4.3). After that two clones of *cpcA* and four Clones of cpcB genes were sequenced by DNA Macrogen Company in Korea. The

A. lemmermannii (AY886906) and 60% identity to *A. sphaerica* UTEX (DQ439645). And results from DNA sequences indicated that the clones of *cpcB* gene similar to 100% identity to *A. variabilis* ATCC 29431 (CP000117), 100% identity to *Anabaena* 7120 biliprotein (X05239) and 98% identity to *Fremyella diplosiphon* (DQ848352) (figure 4.4).

Phylogram of the phycocyanin *cpcB* gene indicated that the *cpcB* gene of *A. siamensis* TISTR8012 is closely related to the *cpcB* gene of *A. variabilis* ATCC19413 (figure 4.6). However the phycocyanin *cpcA* gene of *A. siamensis* TISTR8012 sequence is more similar to *Anabaena* sp. PCC7120 than *Arthospira platensis* and *A. lemmermannii* (figure 4.5). The *cpcA* gene may be the same ancestor with *A. lemmermannii* more than *A. variabilis* ATCC 29431 that showed in phylogram of *cpcA* gene in figure 4.5.

The sequence of *cpcA* (figure 4.7) and *cpcB* (figure 4.8) genes were submitted to NCBI database and got the accession number EU815327 and EU815328 for *cpcA* and *cpcB* genes were received, respectively. The sequence of *cpcA* and *cpcB* genes accession number EU815327 and EU815328 are shown in figure 4.7 and figure 4.8; the underline sequences represent primer sequences and the solid triangle indicates the conserve cystiene (Cys) 85 of the bilin binding site for apo-α^{PC} (αCys85) and the conserve Cys 83 and 154 of the bilin binding site for apo-β^{PC} (βCys83 and βCys155).

The deduce amino acid sequence of the open reading frame corresponded to a protein of 163 and 172 amino acids of *cpcA* and *cpcB* genes product, respectively. Conserved cysteine of alpha and beta proteins consist of αCys85, βCys83 and βCys155

molecular weight of alpha and beta proteins of phycocyanin are approximately about 19 and 20 kDa with an isoelectric point of 6.08 and 5.74 (Compute pI/Mw program available: http://www.expasy.org/egibin/pi.tool).

The amino acid sequence of alpha of phycocyanin protein show 87%, 85%, 85% and 84% identity with *Nostoc* sp. PCC 7120, *Cylindrospermopsis raciborskii* CS-505, *Raphidiopsis brookii* D9 and *Nostoc azollae* 0708. The amino acid sequence of beta of phycocyanin protein show 92%, 89%, 88% and 87% identity with *Nostoc* sp. PCC 7120, *Cylindrospermopsis raciborskii* CS-505, *Raphidiopsis brookii* D9 and *Nostoc azollae* 0708. The amino acid sequence of alpha and beta proteins of phycocyanin aligned with various phycocyanin are shown in figure 4.9 and 4.10.

The other researchers demonstrated conserve cysteine in native phycocyanin that contain the conserve αCys85, βCys82 and βCys153 found in *Synechocystis* sp. 6701 (Toole *et al*., 1998), αCys84, βCys84 and βCys155 found in *Cyanidium caldarium* (Stec *et al*., 1999), αCys84, βCys84 and βCys155 found in *Spirulina platensis* (Padyana *et al*., 2001) and αCys84, βCys84 and βCys155 found in *Mastigocladus luminosus* (Adir *et al*., 2006), the result from conserve Cys binding site indicated that in alpha protein contain only one site (on Cys83 or 84 or 85) whereas beta protein contain two site (on Cys82 or 83 or 84 and Cys153 or 154 or 155). The positions of Cys depend on species of cyanobacteria.

(A)

ATGGTTAAAACCCCCATTACCGAAGCAATTGCAGCTGCTGACACCCAAGGTCGTTTCTTAGGCAACACCGAA
CTCCAATCAGCTCGTGGTCGTTATGAGCGCGCTGCTGCTAGTTTAGAAGCTGCTCGTGGTTTGACTTCCAAC
GCTCAACGCTTGATCGATGGTGCAACCCAAGCTGTTTACCAAAAATTCCCTTACACAACCCAAACCCCCGGT
CCTCAGTTCGCTGCTGACAGCCGTGGTAAATCCAAGTGCGCTCGTGACGTTGGTCACTACCTACGCATCATC
ACCTATAGCTTAGTTGCTGGTGGTACTGGCCCCTTGGATGAATACCTAATTGCTGGTTTGGCTGAAATCAAC
AGCACCTTTGACCTATCTCCTAGCTGGTATGTTGAAGCTCTAAAGCACATCAAAGCTAATCATGGTTTAAGT
GGTCAAGCTGCTAACGAAGCTAACACCTACATCGACTACGCTATCAACGCTCTCAGCTAG

(B)

ATGACATTAGACGTATTTACCAAGGTGGTTTCTCAAGCTGACTCCAGAGGCGAGTTCCTGAGCAACGAACAA
CTAGACGCATTGGCAAATGTTGTTAAAGAAGGCAACAAACGCTTGGATGTTGTTAACCGCATCACCAGCAAC
GCTTCTGCGATCGTTACCAACGCTGCTCGTGCGTTGTTTGAAGAACAACCCCAGTTGATTGCTCCTGGTGGT
AACGCTTACACCAACCGTCGCATGGCTGCTTGTTTACGCGACATGGAAATCATCTTGCGTTACGTCACCTAC
GCTATCCTCGCAGGCGATGCTAGCGTTCTAGACGATCGCTGCTTGAACGGCTTGCGCGAAACATACCAAGCT
TTGGGAACTCCTGGTTCTTCCGTAGCTGTTGGCGTTCAAAAAATGAAAGATGCTGCTGTTGGCATCGCTAAC
GACCCCAACGGAATCACCAAAGGTGATTGCAGCCAATTGATCTCTGAAGTTGCTTGCTACTTTGATCGCGCT
GCTGCTGTTGGTTAA

Figure 4.2 Reference sequence for primer designe; *Anabaena* 7120 biliprotein phycocyanin operon (X05239), alpha subunit; *cpcA* gene (A) and beta subunit; *cpcB* gene (B).

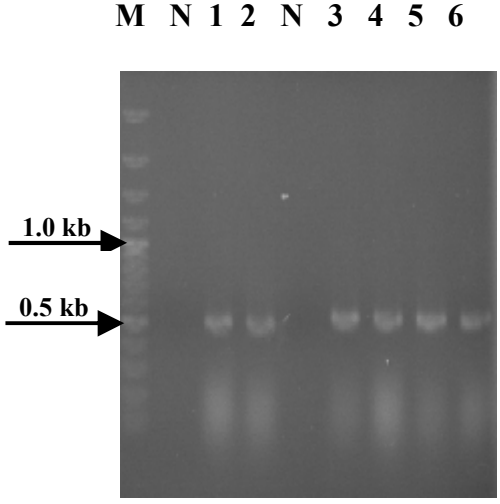


Figure 4.3 Agarose gel electrophoresis of PCR product of *cpcA* and *cpcB* genes. M: 100 bp marker (Fermentas); N: negative control; lane 1-2: PCR product 480 bp of *cpAB* gene and lane 3-6: PCR product 500 bp of *cpcB* gene.

Accession	Description	Query coverage
	***cpcA* gene**	
CP000117	*Anabaena variabillis* ATCC 2941	99%
AF178757	*Anabaena* PCC7120 phycobilisome rod structure	99%
X05239	*Anabaena* 7120 biliprotein phycocyanin operon	99%
BA000019	*Nostoc* sp. PCC 7120 DNA, complete genome	99%
DQ439645	*Anabaena sphaerica* UTEX'B 1616'phycocyanin	60%
AY078437	*Cylindrospermopsis raciborskii* strain Florida phycocyanin beta	56%
AY886906	*Anabaena lemmermanni* 'BA Ana 0014' phycocyanin	61%
	***cpcB* gene**	
CP000117	*Anabaena variabillis* ATCC 2941	100%
X05239	*Anabaena* 7120 biliprotein phycocyanin operon	100%
AF178757	*Anabaena* PCC7120 phycobilisome rod structure	100%
BA000019	*Nostoc* sp. PCC 7120 DNA, complete genome	100%
AF068771	*Synechocystis* PCC9413 beta-phycocyanin (*cpcB*)	97%
DQ848352	*Fremyella diplosiphon* Fd33 phycobilisome linker-core	98%
X07012	*Tolypothrix* sp. PCC7602 inducible *cpcB*2 and *cpcA*1	77%

Figure 4.4 Result of NCBI blast alignment the DNA sequencing of *A. siamensis* TISTR8012 *cpcA* and *cpcB* genes in pENTR-D/TOPO when compared with other cyanobacteria.

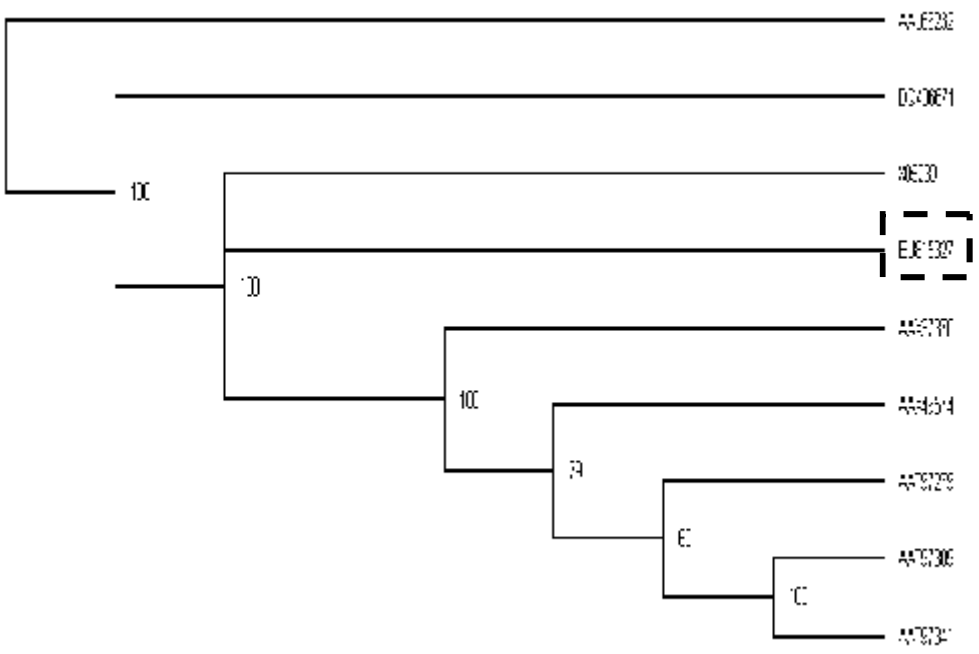


Figure 4.5 Phylogram of apo_α^{PC}, the accession number from AAL66232; *Spirulina maxima*, DQ406671; *Arthospira platensis*, X05239; *Anabaena* sp. PCC7120, EU815327; *A. siamensis* TISTR8012, AAX37370; *A. lemmermannii*, AAX49574; *A. variabilis* ATCC 29413, AAT97279; *A. planktonica*, AAT97309; *A. kisseleviana* and AAT97341; *A. flos-aquae*. The numbers in branches are the percentage of these trees with a particular branch.

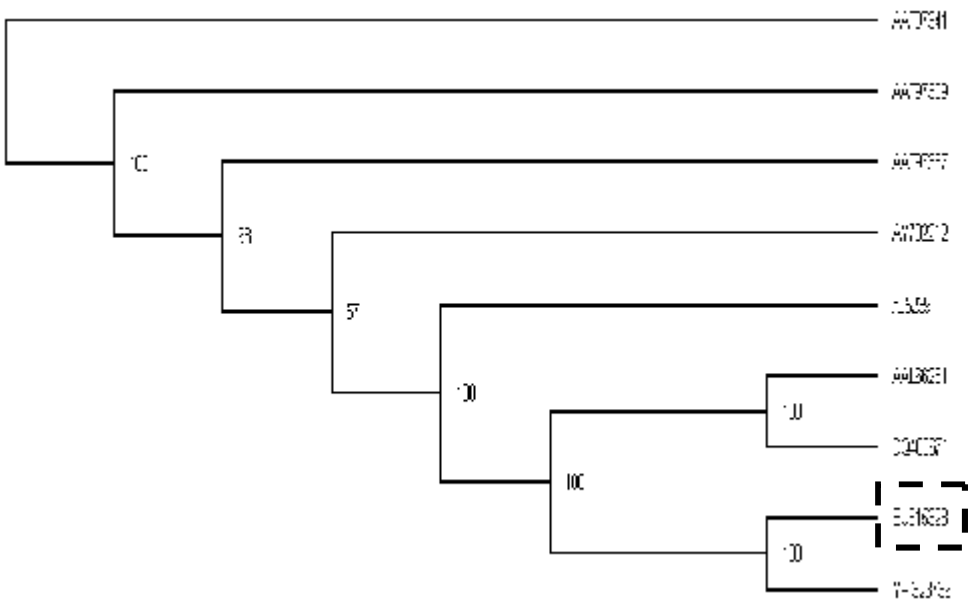


Figure 4.6 Phylogram of apo_β^{PC}, the accession number from AAT97341; *A. flos-aquae*, AAT97309; *A. kisseleviana*, AAT97337; *A. lemmermannii*, AY702212; *A. planktonica*, X05239; *Anabaena* sp. PCC7120, AAL66231; *Spirulina maxima*, DQ406671; *Arthrospira platensis*, EU815328; *A. siamensis* TISTR8012 and YP323436; *A. variabilis* ATCC 29413. The numbers in branches are the percentage of these trees with a particular branch.

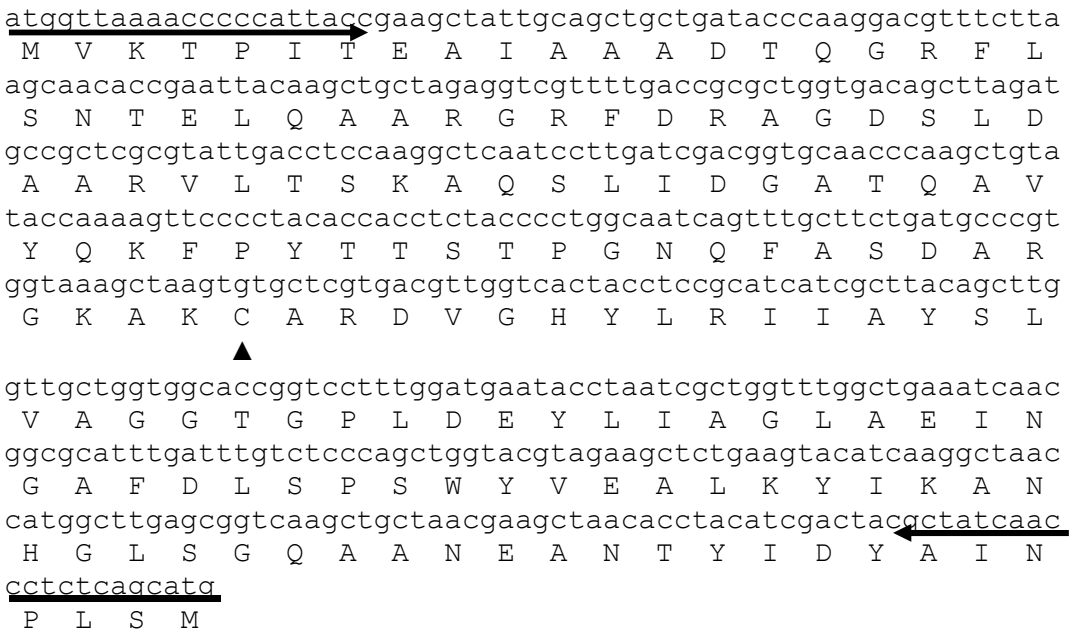


Figure 4.7 The nucleotide sequence of *cpcA* gene and deduce amino acid sequence. Nucleotide residues and amino acid residues are arrange from 5' end. The solid triangle indicates the conserve Cys 85 comprising the bilin binding position and underline sequence represent the sequence of primer.

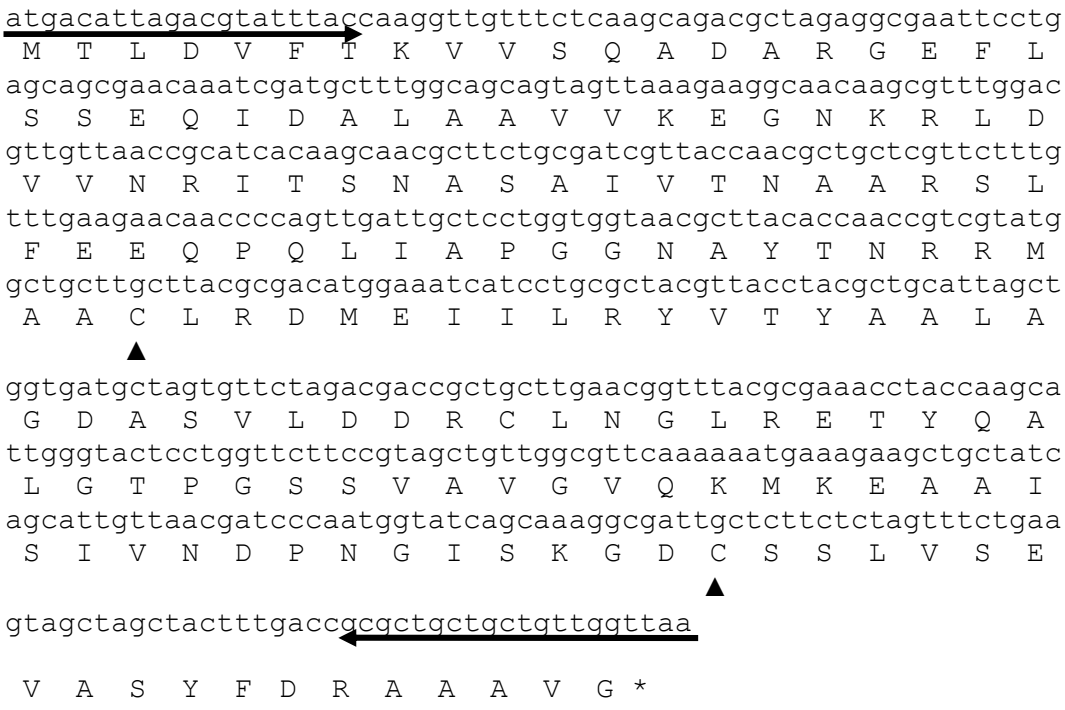


Figure 4.8 The nucleotide sequence of *cpcB* gene and deduce amino acid sequence. Nucleotide residues and amino acid residues are arrange from 5' end. The solid triangles indicate the conserve Cys 83 and Cys 154 comprising the bilin binding position and underline sequence represent the sequence of primer.

```
CLUSTAL 2.0.12 multiple sequence alignment

ZP_06308539      -MKTPITEAIAAADTQGRFLSNTELQAVNGRFVRAAASMEAARGLTANAQKLIDGATNAV 59
ZP_06304364      -MKTPITEAIAAADTQGRFLSNTELQAVNGRFVRAAASMEAARGLTANAQKLIDGATNAV 59
ZP_03764119      -MKTPITEAIASADTQGRFLSNTELQAVNGRFARAVASMEAARGLTANAQKLVDGATNAV 59
EU815327         MVKTPITEAIAAADTQGRFLSNTELQAARGRFDRAGDSLDAARVLTSKAQSLIDGATQAV 60
NC_003272        MVKTPITEAIAAADTQGRFLGNTELQSARGRYERAAASLEAARGLTSNAQRLIDGATQAV 60
                  :*********:********.*****:..**: **  *::*** **::** *:****:**

ZP_06308539      YQKFPYTTSTPGAQYAADSRGKSKCARDVGHYLRIVTYSLVAGGTGPLDEFLIAGLAEIN 119
ZP_06304364      YQKFPYTTSTPGAQYAADARGKSKCARDVGHYLRIVTYSLVAGGTGPLDEFLIAGLAEIN 119
ZP_03764119      YQKFPYTTNTPGNQFAADVRGKSKCARDVGHYLRIITYSLVAGGTGPLDEFLIAGLAEIN 119
EU815327         YQKFPYTTSTPGNQFASDARGKAKCARDVGHYLRIIAYSLVAGGTGPLDEYLIAGLAEIN 120
NC_003272        YQKFPYTTQTPGPQFAADSRGKSKCARDVGHYLRIITYSLVAGGTGPLDEYLIAGLAEIN 120
                 ********.*** *:*:* ***:************::**************:*********
                                          ▲
ZP_06308539      GAFNLSPSWYVEALKYIKANHGLSGQAANEANTYIDYAINALS 162
ZP_06304364      GAFNLSPSWYVEALKYIKANHGLGGQAANEANTYIDYAINALS 162
ZP_03764119      SAFDLSPSWYVEALKYIKGNHGLSGQAANEANTYIDYTINALS 162
EU815327         GAFDLSPSWYVEALKYIKANHGLSGQAANEANTYIDYAINPLS 163
NC_003272        STFDLSPSWYVEALKHIKANHGLSGQAANEANTYIDYAINALS 163
                 .:*:************:**.****.**************:**.**
```

Figure 4.9 Multiple sequence alignment of apo_α^{PC}, the accession number from EU815327: *Anabaena siamensis* TISTR8012; NC_003272: *Nostoc* sp. PCC 7120; ZP_0630853: *Cylindrospermopsis raciborskii* CS-505; ZP_06304364: *Raphidiopsis brookii* D9 and ZP_03764119: *Nostoc azollae* 0708. Symbols represent; "*", identical; ".", conserved substitutions and ":", semi-conserved substitution.

Figure 4.10 Multiple sequence alignment of apo_β^{PC}, the accession number from EU815328: *Anabaena siamensis* TISTR8012; NC_003272: *Nostoc* sp. PCC 7120; ZP_06308538: *Cylindrospermopsis raciborskii* CS-505; ZP_06304363: *Raphidiopsis brookii* D9 and ZP_03764118: *Nostoc azollae* 0708. Symbols represent; "*", identical; ".", conserved substitutions and ":", semi-conserved substitution.

4.3 Expression of recombinant phycocyanin in *E. coli*

The *cpcA* and *cpcB* genes in pENTR/D-TOPO were transferred into pET32a using LR reaction. They were transformed into *E. coli* DH5α and selected on LB amplicilin resistant plate. Then, the recombinant plasmids in DH5α were extracted using QIAprep miniprep (Invitrogen; company constructed) and transformed again into *E. coli* BL21(DE3)pLysS. After that colonies containing recombinant plasmid were screened on amplicilin resistant plate.

Most recombinant proteins over-expressed in *E. coli* tend to accumulate in the

form of insoluble aggregation and the most common way to improve the solubility of recombinant proteins in *E. coli* is to reduce the induction temperature. Both native and recombinant apo-α^{PC} are prone to self-aggregate. Aggregation-prone may be the native characteristics of apo-α^{PC} as it forms in a trimeric aggregate *in vivo* (Liu et al., 2009). The induction temperature of this research varied from 30°C to 20°C and the result showed that insoluble fractions of recombinant pET32a; pET32a_apo-α^{PC} and pET32a_apo-ß^{PC} (Trx, rTrx_ apo-α^{PC} and rTrx_ apo-β^{PC}) proteins were clearly reduced in 20°C. The induction time, in this research were varied from 0, 2, 4, 6, 8 h. and overnight, the result showed that after 4 h. the recombinant protein (Trx, rTrx_ apo-α^{PC} and rTrx_ apo-β^{PC}) were expressed. And the isopropyl-beta-D-thiogalactopyranoside (IPTG) concentration, in this research were varied from 0.1, 0.2, 0.3, 0.4, and 1.0 mM, the result showed 0.4 mM of IPTG can expressed at a high level recombinant protein (Trx, rTrx_ apo-α^{PC} and rTrx_ apo-β^{PC}). Other researchers used difference conditions such as 0.1 mM IPTG for 4.5 h. (Wang *et al*., 2006); 0.1 mM IPTG for 6 h. at 28°C (Guan *et al*., 2007); 1.0 mM IPTG for 7.4 h. at 28°C (Guan *et al*., 2009); and 0.2 mM IPTG for 12 h. at 25°C (Liu *et al*., 2009). Wang *et al*., 2006 studied the recombinant β subunit of C-PC of *Anabaena* PCC7120; the expression separately in bacterial *E. coli* reveals that the α-subunit of C-PC is easily self-aggregated during expression and purification at physiological conditions, indicated that interaction between the α-subunit may contribute to the force for C-PC trimeric aggregation. On the other hand, the C-PC/β can be expressed and purified as soluble recombinant protein in *E. coli* (Wang *et al*., 2006). However, this thesis indicated that rTrx_apo-β^{PC} protein from *A. siamensis* TISTR8012 was more self-aggregated during expression and purification (figure 4.11B lane 8) when compared with recombinant

apo-α^{PC} protein (figure 4.11A lane 8).

The result of protein expression showed in figure 4.11 (A and B) lane 3, 4 were soluble and insoluble fractions of induced pET32a showed thioredoxin (Trx) protein band size of around 20 kDa when compared with lane 1 and 2 which is soluble and insoluble fractions of uninduced pET32a. At the same time lane 7 (A and B) and lane 8 (A and B) were soluble and insoluble fractions of induced *cpcA* and *cpcB* genes with the rTrx_apo-α^{PC} and rTrx_apo-β^{PC} proteins.

The appropriate condition for this protein expression was 0.4 mM IPTG 20°C for 4 h. Since the calculated molecular masses of Trx, rTrx_apo-α^{PC} and rTrx_apo-β^{PC} were 20 kDa, 39 kDa and 40 kDa, respectively; Guan *et al*., 2007 showed purified recombinant protein of apo-α^{PC} corresponding to the calculated molecular massed of 21.1 kDa in *Synechocystis* sp. PCC6803 (Guan *et al*., 2007) and 21 kDa in *Spirulina* (Guan *et al*., 2009). The 39 kDa and 40 kDa proteins revealed by SDS-PAGE was expected to be the rTrx_ apo-α^{PC} and rTrx_ apo-β^{PC} fusion protein (figure 4.11A and B lane 7).

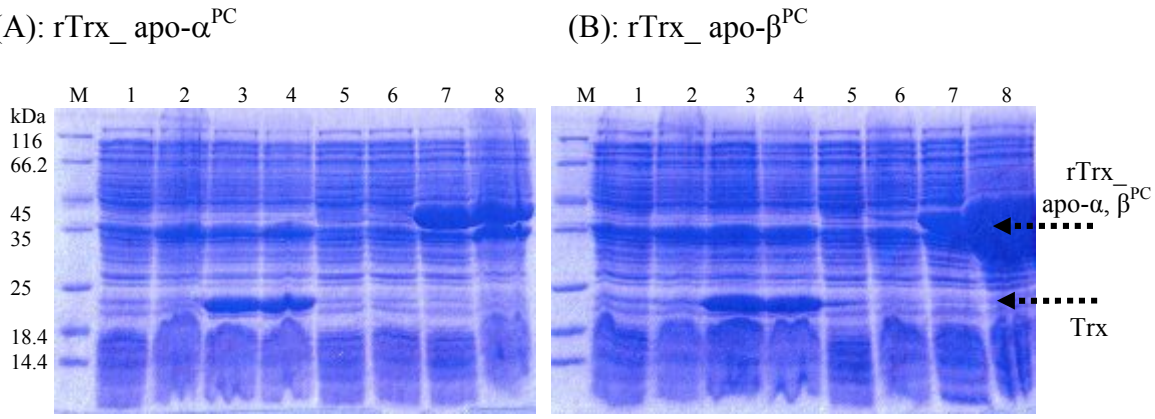


Figure 4.11 SDS-PAGE analysis of rTrx_ apo-α^{PC} and rTrx_ apo-β^{PC} expression in *E. coli* BL21(DE3)pLysS strain. Lane M: Protein standard (Fermentas); Lane 1-2: the soluble and insoluble fraction of un-induced cell containing pET32a, respectively; Lane 3-4: the soluble and insoluble fraction of induced cell containing pET32a, respectively; Lane 5-6: the soluble and insoluble fraction of un-induced cell containing rTrx_apo-α^{PC} (lane 5-6 (A)) and rTrx_apo-β^{PC} (lane 5-6 (B)), respectively; Lane 7-8: the soluble and insoluble fraction of induced cell containing rTrx_apo-α^{PC} (lane 7-8 (A)) and rTrx_apo-β^{PC} (lane 7-8 (B)), respectively. Arrow demonstrated the rTrx_apo-α^{PC} and rTrx_apo-β^{PC} in the soluble fraction, dash arrow (◄·····) demonstated Trx (fusion partner) in the soluble fraction.

4.4 Purification of recombinant phycocyanin from *E. coli* by immobilized-metal affinity chromatography (IMAC) superflow

Purification recombinant protein were done with Cobalt-NTA (Co-NTA) superflow resin at 4°C cobalt affinity column were used to purify the Trx, rTrx_apo-α^{PC} and rTrx_apo-β^{PC} proteins. The rTrx_apo-α^{PC} protein consists of 6XHis-tag at both N and C terminal parts (figure 4.7) of the protein whereas the rTrx_apo-β^{PC} protein consists of 6XHis-tag at only the N terminal parts of the protein (figure 4.8). This might cause some different in the elution fraction of the desire protein showed in lane 14 (figure 4.12A lane 14) of rTxn_apo-α^{PC} protein compare with lane 14 of rTrx_apo-ß^{PC} protein (figure 4.12B lane 14). rTxn_apo-α^{PC} protein can bound tightly with cobalt bead more than rTxn_apo-β^{PC} protein. Figure 4.12 indicated that low concentration of imidazole (5, 10 and 150 mM) in lane 4 – 6 and 11 – 13 cannot elute the recombinant protein from cobalt resin. Imidazole of 150 mM was used to elute the Trx in lane 7 whereas rTrx_apo-α^{PC} and rTrx_apo-β^{PC} proteins must to use higher imidazole concentration, 500 mM imidazole (figure 4.12A and B lane 14). Figure4.12A and B lane 14 indicated that rTrx_apo-α^{PC} and rTrx_apo-β^{PC} proteins can be purified to some extent.

After purification step the 5 volumes of eluted fraction recombinant proteins were concentrated and exchanged with 50 mM Tris-Cl, pH8.0 in 10 kDa cutoff centrifugal ultracentrifugation membrane. The result indicated that recombinant protein had more concentrated. They were then dissolved in 50 mM Tris-Cl, pH8.0 (figure 4.13 lane 6 A and B).

(A) Trx_apo-α^{PC} (B) Trx_apo-β^{PC}

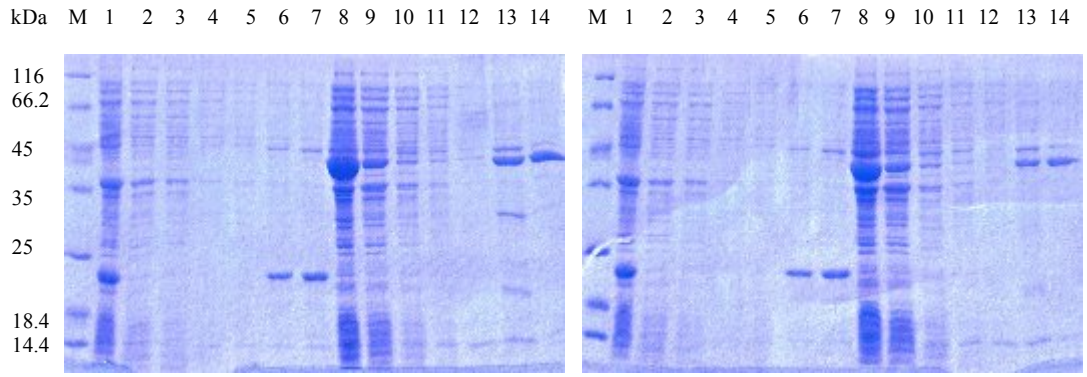


Figure 4.12 The SDS-PAGE analysis of rTrx_apo-α^{PC} and rTrx_apo-β^{PC} in BL21(DE3)pLysS strain. Fractions from purification using cobalt bead. Lane M: Protein standard (Fermentas); Lane 1: Soluble fraction pET32a; Lane 2: Flow-through pET32a; Lane 3: Wash fraction 0 (0 mM imidazole); Lane 4: Wash fraction 1 (5 mM imidazole); Lane: 5 Wash fraction 2 (10 mM imidazole); Lane 6: Elution fraction (150 mM imidazole) and Lane 7: Elution unbound from cobalt bead (500 mM imidazole); Lane 8 Soluble fraction rTrx_apo-α^{PC} and rTrx_apo-β^{PC} (lane 8A and 8B); Lane 9: Flow-through rTrx_apo-α^{PC} and rTrx_apo-β^{PC} (lane 9A and lane 9B); Lane 10: Wash fraction 0 (0 mM imidazole) of rTrx_apo-α^{PC} and rTrx_apo-β^{PC} (lane 10A and lane 10B); Lane 11: Wash fraction 1 (5 mM imidazole) of rTrx_apo-α^{PC} and rTrx_apo-β^{PC} (lane 11A and lane 11B); Lane 12: Wash fraction 2 (10 mM imidazole) of rTrx_apo-α^{PC} and rTrx_apo-β^{PC} (lane 12A and lane 12B); Lane 13: Wash fraction 3 (150 mM imidazole) of rTrx_apo-α^{PC} and rTrx_apo-β^{PC} (lane 13A and lane 13B); Lane 14: Elution fraction (500 mM imidazole) of rTrx_apo-α^{PC} and rTrx_apo-β^{PC} (lane 14A and lane 14B).

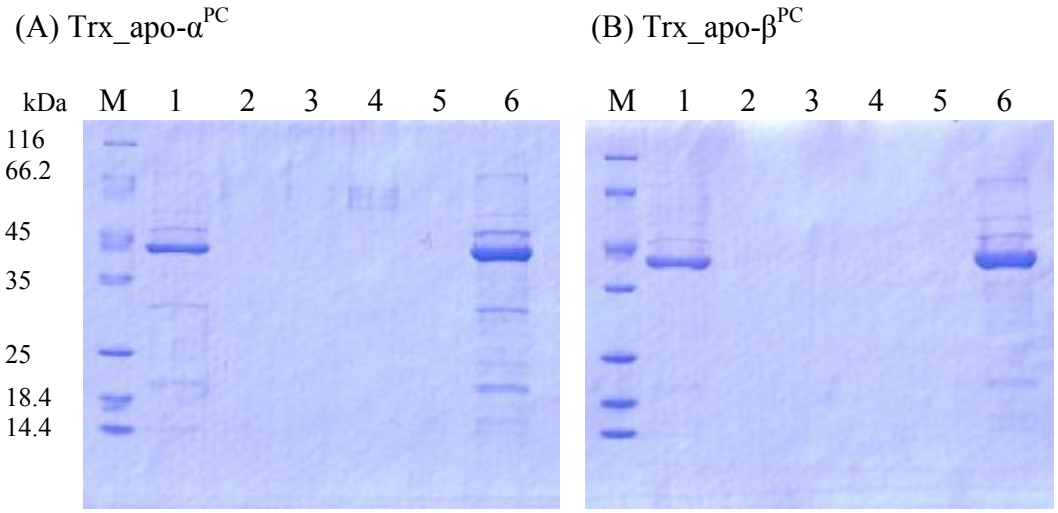


Figure 4.13 The SDS-PAGE analysis of rTrx_apo-α^{PC} and rTrx_apo-β^{PC} concentrated and exchanged with 50 mM Tris-Cl, pH8.0 in 10 kDa cutoff. Lane 1: Elution fraction (500 mM imidazole) ofrTrx_apo-α^{P} and rTrx_apo-β^{PC}; Lane 2: Filtrated fraction at 15 min; Lane 3: Filtrated fraction at 30 min; Lane 4: Filtrated fraction at 45 min; Lane 4: Filtrated fraction at 60 min; Lane 6: Concentrated fraction of recombinant protein (rTrx_apo-α^{PC} and rTrx_apo-β^{PC}) in 50 mM Tris-Cl pH 8.0.

This report represents the purification of recombinant C-PC proteins, although they were not fluorescent. Recently co-expression of several transgenes has allowed the biosynthesis of fluorescent holo-α-subunit of cyanobacterial phycocyanin in *E. coli* (Tooley *et al*., 2001, Guan *et al*., 2007 and Guan *et al*., 2009). On the basis of the present work, it is feasible that in the future the biosynthesis of holo-subunits of cyanobacteria C-PC in heterologous hosts, which will fluoresce with a unique spectroscopic property (λ_{max} = 620 nm), may be accomplished.

4.5 Test antioxidant propertied of recombinant phycocyanin

4.5.1 Determination of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The result from DPPH scavenging assay showed that ascorbic acid showed DPPH scavenging activity with estimated IC_{50} value 0.14 mg/ml (figure 4.14). Whereas Trx , rTrx_apo-α^{PC} and rTrx_apo-β^{PC} proteins did not showed DPPH scavenging (figure 4.14). This may be due to the solubility of recombinant proteins in the assay buffer. They all aggregated and form cloudy solution blocking all the light absorbant (figure 4.15B) after 30 minutes of DPPH assay.

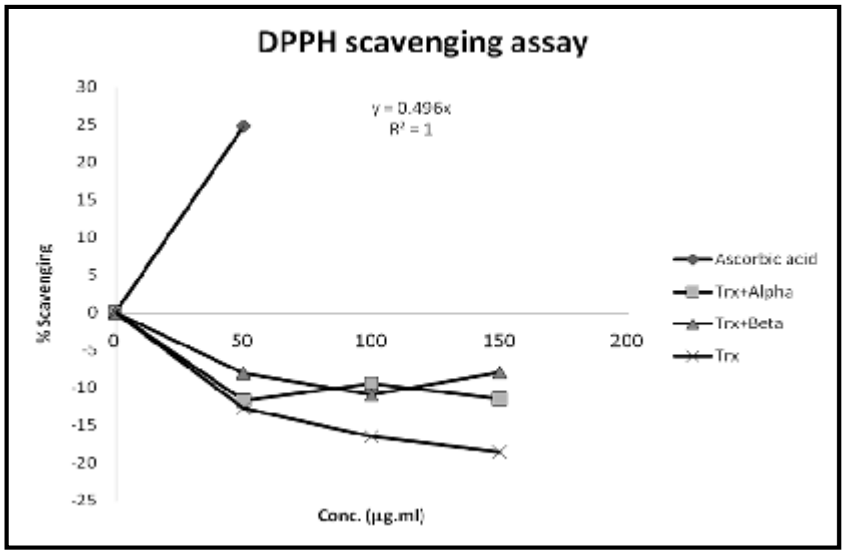


Figure 4.14 DPPH scavenging assay. The target oxidative substance varies in difference concentrations (50, 100 and 150 µg/ml) of ascorbic acid, Trx, rTrx_apo-α^{PC} and rTrx_apo-β^{PC} proteins were tested. The target oxidative substances values represent the IC_{50} of scavenging activity.


(A)

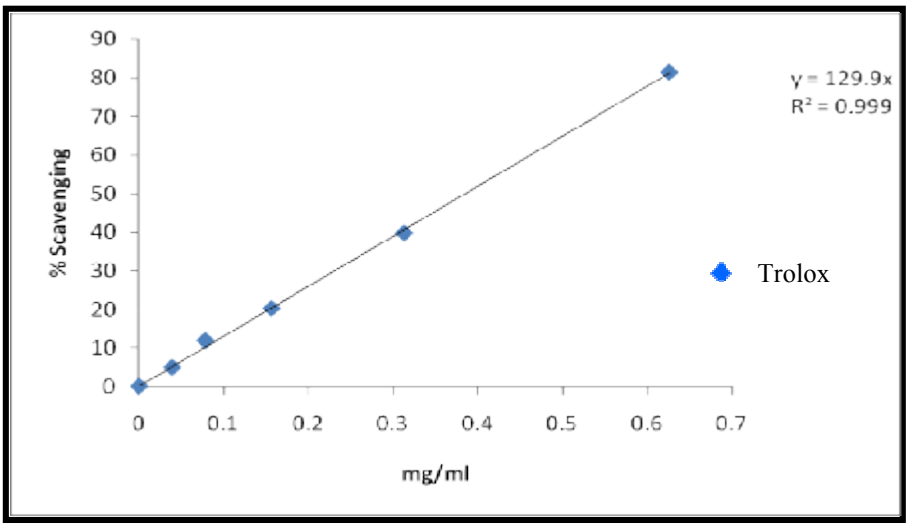
(B)

Figure 4.15 DPPH reactions after 30 min of positive control and recombinant protein. DPPH reaction of ascorbic acid (positive control) (A) and DPPH reaction of recombinant protein (B).

4.5.2 Determination of 2, 2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging assay

The result from ABTS radical scavenging assay showed that trolox (used as positive control), rTrx_apo-α^{PC}, rTrx_apo-β^{PC} and Trx proteins with IC_{50} value of 0.4 mg/ml (figure 4.16A), 13.8, 18.6 and 23.4 mg/ml (figure 4.16B), respectively. Madhyastha *et al*., 2009 indicated that native C-PC from *Spirulina platensis* had 12% scavenging DPPH radical whereas it could not scavenged ABTS radical. However, native C-PC failed to scavenge the ABTS radical significantly. The perceived difference between ABTS and DPPH scavenging assay may be due to the differences in scavenging reactions occurring in aqueous phase for ABTS and in organic phase of DPPH assay. The researchers also discuss about the modification of the polypeptide with higher cysteine for increase antioxidant property (Madhyastha *et al*., 2009). A few researchers tried to studied antioxidant and anti-inflammatory properties of recombinant protein; Reddy *et al*., 2000 suggested apoprotein of C-PC play a role in the inhibition of COX-2 in anti-inflammatory; Wang et al., 2006 suggested apo-β^{PC} could inhibited cell proliferation and promoted apoptosis of cell.

(A) ABTS assay.



(B)

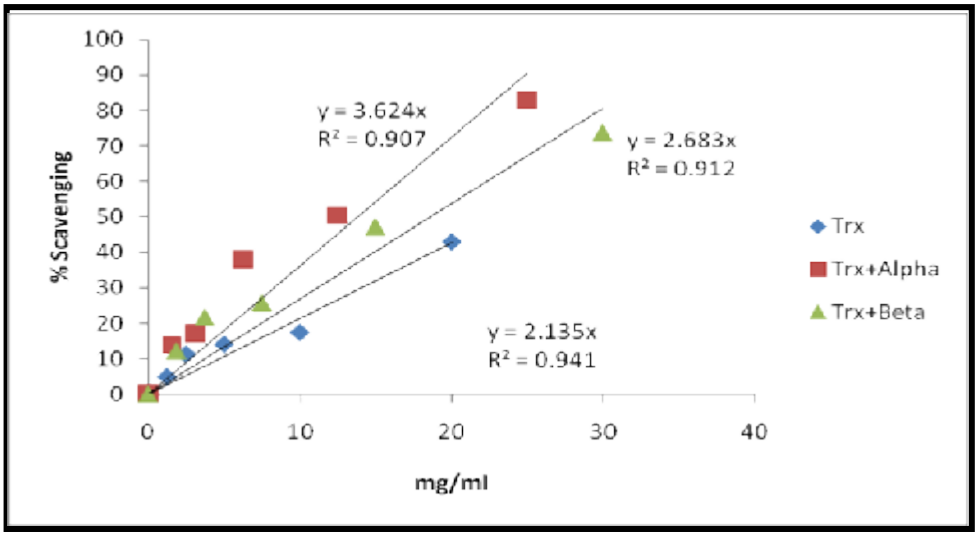


Figure 4.16 ABTS scavenging assay. Difference concentrations of Trolox (A), rTrx_apo-α^{PC}, rTrx_apo-β^{PC} and Trx proteins (B) were incubated along with ABTS reagent. The target oxidative substances values represent the IC_{50} of scavenging activity.

This method indicated that rTrx_apo-α^{PC} and rTrx_apo-β^{PC} proteins had little antioxidant property above background of Trx when tested with ABTS radical scavenging assay. The importance of antioxidant property assay based on simple, inexpensive, rapidly performed, and provide a high degree of precision (Thaipong *et al.,* 2006). There does not exist a standardized method for antioxidant capacity evaluation, rather, so the other researchers often employ several of antioxidant assay. Antioxidant methods differ in terms of the assay principles and experimental conditions; consequently, particular antioxidants have varying contributions to the total antioxidant potential in different methods (Samec *et al*., 2009). These results supported the possibility that phycobiliproteins; phycocyanin may have potential application in medicine and the recombinant C-PC might be potential used in the future to antioxidant for used in food industries.

CHAPTER V

CONCLUSIONS

1. The extracted genomic DNA (gDNA) of *A. siamensis* TISTR8012 by phenol: chloroform: isoamylalcohol (25: 24: 1) gave high quality DNA.

2. Two pairs of primers of *cpcA* and *cpcB* genes were designed from reference sequence; *Anabaena* 7120 biliprotein phycocyanin operon (X05239). These primers were able to amplify *cpcA* and *cpcB* genes from *A. siamensi*s TISTR8012.

The genes were clone, sequence and submitted to NCBI database as accession number EU815327 and EU815328, respectively. The results indicated that the clones of *cpcA* gene had 99% identity to that to *Anabaena variabilis* ATCC 29431 (CP000117), *Anabaena* PCC 7120 (AF178757) and *Anabaena* 7120 biliprotein (X05239). And the results of *cpcB* gene had 100% identity of *Anabaena variabilis* ATCC 29431 (CP000117), *Anabaena* 7120 biliprotein (X05239) and *Anabaena* PCC 7120 (AF178757).

3. The appropriate condition for recombinant protein expression in the pET32 system were 0.4 mM IPTG 20°C for 4 h.

4. The appropriate condition for recombinant protein purification via IMAC were 500 mM Imidazole for elution.

5. The antioxidant properties; DPPH scavenging assay showed that only ascorbic acid showed scavenging activity with estimated IC_{50} value of 193.80 µg/ml. The result from ABTS radical scavenging assay showed that trolox (positive control), rTrx_apo-α^{PC} and rTrx_apo-β^{PC} proteins with IC_{50} value of 0.4, 13.8 and 18.6 mg/ml. respectively.

6. This is first research to clone, sequencing and express *cpcA* and *cpcB* genes of phycocyanin from *Anabaena siamensis*.

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APPENDIX

APPENDIX

1. MEDIA

1.1 LB-LS medium (Luria-Bertani Low Salt medium) Per liter:

distilled water	950 ml
Tryptone	10 g
Yeast extract	5 g
NaCl	5 g

Stir until the solutes have been dissolved. Adjust the volume of the solution to 1 liter with deionized H_2O. Sterilized by autoclave for 15 min at 121°C. Store the solution at 4°C. Antibiotics were added into warm medium (50°C): amplicilin at 100 µg/ml and kanamycin 50 µg/ml; final concentrations after autoclaving. If the agar medium is desired 15 g/L was add into the medium before autoclaving.

1.2 BG11 medium Per liter:

Table 6.1 Composition per liter of BG11 medium as described below.

Component (ml)	Final conc. (g/L)	1000xstock (g/100 ml)	Final Stock	Stock no.
$NaNO_3$	1.5	-	1.5 g	-
K_2HPO_4	0.04	4.0	1.0 ml	Stock I
$MgSO_4.7H_2O$	0.075	7.5	1.0 ml	Stock II
$CaCl_2.2H_2O$	0.036	3.6	1.0 ml	Stock III
Citric acid	$6.56x10^{-3}$	0.656	1.0 ml	Stock IV
$Fe(NH_4)_3(C_6H_5O_7)_2$	$6.0x10^{-3}$	0.6	1.0 ml	Stock V
$Na_2EDTA.2H_2O$	$1.04x10^{-3}$	0.104	1.0 ml	Stock VI
Na_2CO_3	0.02	2.0	1.0 ml	Stock VII
A5 trace metal			1.0 ml	

Table 6.2 Composition per liter of A5 trace metal as described below.

Component	Final conc. (g/L)	Final Stock
H_3BO_3	-	2.36 g
$MnCl_2.4H_2O$	-	1.31 g
$ZnSO_4.7H_2O$	-	0.22 g
$CuSO_4.5H_2O$	79.0	1.0 ml
$Na_2MO_4.2H_2O$	-	0.391 g
$Co(NO_3)_2.6H_2O$	49.4	1.0 ml

Dissolved K_2HPO_4 into 100 ml dH_2O, Dissolved $Fe(NH_4)_3(C_6H_5O_7)_2$ into 100 ml distilled water and then add the remaining component into 800 ml distilled water

and sterilized by autoclave for 15 min at 121°C. After cooling down mix all components together before used. Store the solution at 4°C.

2. REAGENT

2.1 Electrophoresis and gel loading buffer

2.1.1 TAE buffer 50X

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5M EDTA (pH8.0)	100 ml

Store the solution at room temperature.

2.1.2 Gel loading buffer

Bromophenol blue	0.25% (w/v)
Xylene cyanol FF	0.25% (w/v)
Glycerol in distilled water	30 % (v/v)

Store the solution at room temperature.

2.2 SDS-PAGE gel electrophoresis

2.2.1 SDS-gel loading buffer 5X stock, 8 ml

Dissolve 0.30 g Tris Base, 1 g SDS, 0.05 g Bromophenol blue, 5 ml glycerol and adjust pH to 6.8 with HCL and the volume to 8 ml with distilled water. Before used add 20 µl of 2-mercapthoethanol to 80 µl of solution mixture. Store the solution at room temperature.

2.2.2 1.5 M Tris pH8.8 100 ml

Dissolve 18.17 g Tris Base in 80 ml distilled water. Adjust pH to 8.8

with HCl and adjust the volume to 100 ml with distilled water. Store the solution at 4°C.

2.2.3 0.5 M Tris pH6.8 100 ml

Dissolve 6.06 g Tris Base in 80 ml distilled water. Adjust pH to 6.8 with HCl and adjust the volume to 100 ml with distilled water. Store the solution at 4°C.

2.2.4 30% Acrylamide solution 100 ml

Dissolve 29 g acrylamide and 1 g N, N.-methyene-bis-acrylamide in distilled water and adjust the volume to 100 ml. Mix the solution by stirring in dark bottle for 1hour or till homogeneous and filter through Whatman membrane No. 1. Store the solution in dark bottle at 4°C.

2.2.5 Tris-Glycine electrode buffer 5x stock, 1 L

Dissolve 15.1 g Tris base, 94 g glycine and 5 g SDS in distilled water. Adjust pH to 8.3 with HCl and adjust the volume to 1 L with distilled water. Store the solution at room temperature.

2.2.6 Staining solution with coomassie brilliant blue for protein 1 L

Mix 1 g Coomassie brilliant blue R-250, 400 ml methanol, 500 dH_2O and 100 ml glacial acetic acid. Filter through Whatman No. 1.

2.2.7 Destaining solution for Coomassie Stain 1 L

Mix 400 ml methanol, 100 ml glacial acetic acid and add distilled water to a final volume of 1 L.

2.2.8 10% w/v Ammonium persulfate 1 ml

Dissolve 100 mg ammonium persulfate in 1 ml distilled water. Store the solution at -20°C.

2.2.9 15% separating gel SDS-PAGE 10 ml

Mix the solution as follow:

1.5 M Tris pH8.8	2.5 ml
Distilled water	2.3 ml
10% SDS	0.1 ml
30% acrylamide solution	5.0 ml
10% ammonium persulfate	0.1 ml
TEMED	8 µl

2.2.10 5% stacking gel SDS-PAGE 5 ml

Mix the solution as follow:

0.5 M Tris pH6.8	1.26 ml
Distilled water	2.77 ml
10% SDS	0.05 ml
30% acrylamide solution	0.83 ml
10% ammonium persulfate	50 µl
TEMED	5 µl

2.3 Measurement protein concentration by Bradford protein assay

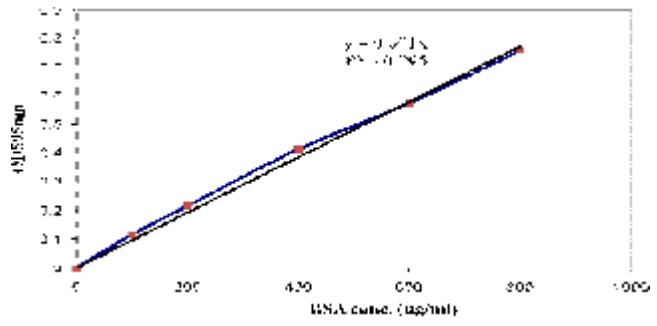

Figure 6.1 Standard curve of OD_{595} versus BSA concentration**.**

The reaction is initiated with adding 50 µl of sample into 2.5 ml of Bradford reagent (10 mg of comassie brilliant blue G-250 + 5 ml of 95% absolute

ethanol + 10 ml of phosphoric acid and 85 ml of distilled water). Then the reactions were gentle votexing for avoid foaming. The protein concentration is detected by spectrophotometer at 595 nm between 2 min – 1 h. The reaction mixture contains 50 mM Tris-Cl pH8.0 is used as the blank. The protein concentration is calculated as follow.

2.4 Antioxidant activity assay

2.4.1 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

Dissolve 5.9 mg DPPH in 1.0 ml absolute ethanol to make 10 mM stock solution of DPPH. Before used dissolved 10 µl of stock solution in 990 µl of absolute ethanol.

2.4.2 2, 2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS)

Dissolve 3.84 mg ABTS in 1.0 ml distilled water to make 7 mM stock solution of ABTS. The stock solution was kept at room temperature in dark condition for 12-16 h. Before use, 7 mM ABTS stock solution was diluted with 10 mM sodium phosphate buffer, pH 7.4 until the absorbance of 734 nm obtained at 0.7±0.02.

BIOGRAPHY

Wanwisa Suphap was born on March 26th, 1984 in Nakhon Ratchasima, Thailand. She graduated with a bachelor degree of science from school of Crop Production Technology, Institute of Agricultural Technology at Suranaree University of Technology, in 2007. After graduation, she received an opportunity to study master degree in the School of Biotechnology, Institute of Agricultural Technology at Suranaree University of Technology. The research in the topic of cloning and expression of phycocyanin from cyanobacteria was her thesis work. Wanwisa Suphap was support by SUT Outstanding graduate student scholarship (SUT OGSS).

In 2008, she went to Mahasarakham University to attended the 20th Annual Meeting and International Conference of the Thai Society for Biotechnology and presented her work in the title of cloning and expression of phycocyanin from cyanobacteria in the poster presentation section.

In 2009, she attended the 2nd SUT Graduate Conference at Suranaree University of Technology and presented her work in the title of cloning and expression of phycocyanin from cyanobacteria in the oral presentation section.