INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
11–384/AWB/2012/14–1–121–125
http://www.fspublishers.org

Full Length Article

Purification and Characterization of a Thermostable Phycoerythrin from Hot Spring Cyanobacterium *Leptolyngbya* sp. KC45

CHAYAKORN PUMAS, YUWADEE PEERAPORNPISAL†, PANMUK VACHARAPIYASOPHON†, PIMPORN LEELAPORNPISID‡, WALAILUCK BOONCHUM†, MASAHARU ISHII¶ AND CHARTCHAI KHANONGNUCH[1]
Division of Biotechnology, School of Agro-Industry, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai 50100, Thailand
†Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand
‡Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai 50200, Thailand
¶Department of Biotechnology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-8657, Japan
[1]Corresponding author's e-mail: aiickhnn@chiangmai.ac.th

ABSTRACT

This study aimed to understand characteristics of thermostable phycoerythrin from hot spring cyanobacteria *Leptolyngbya* sp. KC45. Phycoerythrin was purified with the purification index at 17.38 of A565/A280 ratio and demonstrated as two protein bands of 21 and 18 kDa under SDS-PAGE analysis. The native protein was assumed to be hexamer with a molecular mass of approximately 235 kDa based on the results from gel filtration. N-terminal amino acid sequences shared the highest percent of identities with *Fremyella diplosiphon* Fd33 at 100% and 90% for α- and β-subunit, respectively. Phycoerythrin and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity remained at approximately 80% of the original level after being heated at 60°C for 30 min, indicating that it can be considered as the promising thermostable phycoerythrin.

Key Words: Antioxidant activity; *Cyanophyceae*; N-terminal analysis sequence; Phycobiliprotein
Abbreviations: PBP, phycobiliprotein; APC, allophycocyanin; PC, phycocyanin; PE, phycoerythrin; DPPH, 2,2-diphenyl-1-picrylhydrazyl

INTRODUCTION

The phycobiliproteins (PBPs) are a group of light harvesting proteins, which are covalently bound with bilin from the tetrapyrrole prosthetic groups. These pigments are commonly found in rhodophyta and cyanophyta. PBPs and can be classified into three major classes based on the distinct maximum spectra absorption: phycoerythrins (PE: λ_{max} ~565 nm), phycocyanins (PC: λ_{max} ~620 nm) and allophycocyanin (APC: λ_{max} ~650 nm) (Yamanaka *et al.*, 1978). PBPs assemble to be supramolecular complexes called phycobilisomes and perform in the light harvesting and energy transfer processes in PSII. The pigments serve in collecting solar energy in the range beyond chlorophyll *a* absorption (MacColl, 1978). PBPs are generally formed of two chromophore-linked subunits, α and β, and found in trimer $(\alpha\beta)_3$ (about 120 kDa) or hexamer $(\alpha\beta)_6$ (about 240 kDa), where (αβ) (about 40 kDa) is defined as the PBPs monomer. PE normally occurs in the form of hexamers in the aqueous solution as found in phycobilisomes, while PC and APC mainly exist in trimers (Sun *et al.*, 2009). PE can be used as a natural colorant in food, pharmaceuticals, cosmetics, textiles and as printing dyes (Sekar & Chandramohan, 2008). Due to its fluorescent properties, PE has been widely used as a fluorescent labeling agent for biological molecules and also used in immunolabeling experiments and fluorescent microscopy (Spolaore *et al.*, 2006). In addition, PE has been reported to possess both antioxidant activities (Yuan *et al.*, 2005) and pharmacological activities (Soni *et al.*, 2008). Nowadays, commercial PE is obtained from mesophile rhodophyta *Porphyridium* (phycoerythrin) and available from various companies including AnaSpec Inc., Sigma-Aldrich Corporation and Invitrogen Corporation. However, the PBPs from mesophile organisms are sensitive to heat and display a lower stability level at high temperatures (Zhao & Brand, 1989; Sekar & Chandramohan, 2008).

Some hot spring cyanobacteria were studied for PBP content, as well as their stability. The result revealed that the red-violet cyanobacterium *Leptolyngbya* sp. KC45 showed

To cite this paper: Pumas, C., Y. Peerapornpisal, P. Vacharapiyasophon, P. Leelapornpisid, W. Boonchum, M. Ishii and C. Khanongnuch, 2012. Purification and characterization of a thermostable phycoerythrin from hot spring cyanobacterium *Leptolyngbya* sp. KC45. *Int. J. Agric. Biol.*, 14: 121–125

the highest content of PE moreover, PE from the crude extract of this strain also showed the attractive thermal stability as it could retain up to 80% stability after being incubated at 60°C for 30 min. The results suggested that *Leptolyngbya* sp. KC45 may serve as a prospective source for thermostable PE (Pumas *et al.*, 2011). To obtain the certain properties and biochemical characteristics, the purification of PE from *Leptolynbya* sp. KC45 is necessary.

In this paper, purification of *Leptolynbya* sp. KC45 phycoerythrin and its biochemical characteristics including N-terminal amino acid sequence, thermal stability and also 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity were described. This is the first report described the purification and properties of purified PE from cyanobacteria in the genus *Leptolynbya*.

MATERIALS AND METHODS

Microorganisms, culture conditions and media: A hot spring cyanobacterium, *Leptolyngbya* sp. KC45, was obtained from the Applied Algal Research Laboratory, Chiang Mai University, Thailand. This strain was maintained in Castenhölz's medium D (Castenhölz, 1981) at 40°C under the constant lighting of a fluorescent lamp (8.1 μmol photons·m^{-2}·s^{-1}).

Protein purification: All buffers and reagents used in purification were prepared in deionized water with 0.01% (w/v) sodium azide added to prevent photobleaching (Bermejo Román *et al.*, 2002) *Leptolyngbya* sp. KC45 was cultivated in 1,000 mL of Castenhölz's medium D with 140 rpm rotary agitation at 40°C under the constant lighting of a fluorescent lamp (8.1 μmol photons·m^{-2}·s^{-1}) for 1 month. Algal cells were harvested from this culture. The cells (0.5 g dry biomass) were rinsed with 1 mM phosphate buffer (pH 7.0), 0.2 M NaCl and resuspended in the same buffer. The resuspended cells were disrupted using the sonicator: Sonics (Vibra-cell™) under about 250W at 0°C. Fifteen second ultrasonication followed a 30-sec interval. Cell debris was removed by centrifugation at 6,000×g at 4°C for 30 min. The supernatant containing phycobiliproteins were precipitated by 85% saturation of ammonium sulfate at 4°C and precipitants were collected by centrifugation at 6,000×g at 4°C for 30 min. The precipitated biliproteins were resuspended in 1 mM phosphate buffer (pH 7.0), 0.2 M NaCl. Ammonium sulfate was eliminated by centrifugation through Microsep® centrifugal concentrator filter size 3 kDa at 6,000×g at 4°C for 30 min. The desalted PE was applied on a hydroxylapatite (HA) column pre-equilibrated with 1 mM phosphate buffer (pH 7.0) supplemented with 0.2 M NaCl. The unbound fraction was washed with the equilibrated buffer and the bound fractions were eluted with 100 mM phosphate buffer (pH 7.0) supplemented with 0.2 M NaCl. The colored fractions were pooled and concentrated by centrifugation through Microsep® centrifugal concentrator filter size 3 kDa at 6,000×g at 4°C for 30 min. The concentrated fraction was applied on a column of Q-sepharose pre-equilibrated with 50 mM acetate buffer (pH 5.5), and then eluted using linear gradient elution from 0 – 1 M NaCl in 50 mM acetate buffer (pH 5.5). The PE fractions were pooled and concentrated using the same method described previously and then applied on a Sephacryl™ S-200 HR pre-equilibrated with 0.1 M phosphate buffer (pH 7.0), 0.2 M. The pink fractions were collected and analyzed as purified PE.

Phycoerythrin measurement: The absorption spectra of the PE samples obtained from the purification steps were measured by Microplate reader Spectra MR (Dynex technologies) with a 1-cm light path. PE content was calculated using absorbance at 565, 615 and 650 nm with the simultaneous equations of Bennet and Bogorad (1973), and with the extinction coefficients from Bryant *et al*. (1979). The purity of the PE was estimated using the following two indexes: A565/A280 and A615/A565.

Gel electrophoresis: The purity and polypeptide components of the PE prepared by the gel filtration chromatography on Sephacryl™ S-200 HR were evaluated by SDS–PAGE (Bermejo *et al.*, 2001). The gel was composed of a 16.5% (w/v) polyacrylamide separating gel containing 0.1% (w/v) SDS with a 4% polyacrylamide stacking gel. Before loading, each sample was denatured by incubation with 50 mM Tris, pH 6.8 containing 4% (w/v) SDS, 12% (v/v) glycerol, 2% (v/v) β-mercaptoethanol, 0.025% (w/v) bromophenol blue at 95°C for about 2 min. Gels were run at ambient temperatures with electrode buffer containing 192 mM Tris-Glycine (pH 8.3) with 0.1% (w/v) SDS. Protein bands were detected by staining with a Biorad Silver Stain kit (Bio-Rad Laboratories, CA, USA) following the instruction manual. PageRuler™ prestained protein ladder (Fermentas, MD, USA) was used as a guide to determine molecular mass. Native-PAGE was analyzed for native protein color with the method of SDS–PAGE, without initial denaturation and SDS addition.

Native protein size estimation: Gel filtration was used to estimate the molecular mass of the purified PE. The samples were loaded on a Superose™ 6 HR column (Amersham Pharmacia Biotech, NJ, USA) pre-equilibrated with 0.1 M phosphate buffer (pH 7.0), 0.2 M and then the column was eluted by the same buffer at a rate of 0.5 mL·min^{-1}. In the experiment, gel filtration reference proteins (Bio-Rad Laboratories, CA, USA) containing thyroglobulin (670 kDa), bovein gamma globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa) and vitamin B-12 (1.35 kDa) were employed as standard markers.

N-terminal amino acid sequences analysis: Two subunits of protein, α- and β-subunits, were separated by SDS-PAGE and transblotted to polyvinylidine fluoride (PVDF) membrane using a semi-dry electroblotter apparatus (Bio-Rad Laboratories, CA, USA). N-terminal amino acid sequences of both α- and β-subunits were analyzed by Procise™ protein sequencer (PE Biosystems, UK). The amino acid sequences obtained were compared with α- and

β-subunits of PE from other species of Cyanophyta and Rhodophyta using SWISSPROT and Genbank databases.

Effects of temperature on PE stability and 2,2-diphenyl-1-picrylhydrazy (DPPH) radical scavenging activity: The purified PE powder was dissolved in deionized water to get the final concentration of 1 $mg \cdot mL^{-1}$, then, 1 mL sample was heated for 30 min at six different temperatures that were 25, 40, 50, 60, 70 and 80°C, respectively. Then, the remaining quantity of PE and the DPPH radical scavenging activity of each temperature were determined and the remaining PE quantity (%) was calculated and compared with the original values before being heated. After being heated at 25°C, the remaining solution was used as the control to observe protein denature by non-heat factors.

DPPH radical scavenging activity: The scavenging activity on DPPH radical scavenging activity was measured according to the method of Hou *et al*. (2001) with some modifications. Either 0.8 mL of the preheated purified PE or distilled water were added to 0.067 mL of 1 M Tris-HCl buffer (pH 7.9), and then mixed with 0.8 mL of 130 μM DPPH in methanol for 20 min under light protection. The absorbance at 517 nm was determined. The inhibition (%) was calculated by the following formula:

$$\% \text{ Inhibition} = [(A_{517 \text{ control}} - A_{517 \text{ test sample}})/A_{517 \text{ control}})] \times 100$$

RESULTS AND DISCUSSION

PE purification, molecular mass determination and N-terminal sequences analysis: The PE was purified from cell free extracts obtained from harvested cells as a soluble protein via sonication, ammonium sulfate precipitation, hydroxyapatite column, Q-Sepharose column and Sephacryl™ S-200 HR (Table I). The PE was purified and the purity of the PE was estimated using the following two indexes: A565/A280 and A615/A565. The first ratio of A565/A280 is an indicative index of the PE purity compared to other contaminating proteins. This index should be higher than 5.3 (Rossano *et al.*, 2003). The purified PE showed a purity index as high as 17.38, which is about 3-times higher than the recommendation for commercial PE products (AnaSpec Inc., Sigma-Aldrich Corporation & Invitrogen Corporation). Another index of A615/A565 showed that the purity of PE compares to PC, which is the closest contaminated protein. Our PE showed a PC/PE index as 0.006 that revealed very low PC contamination. The absorption spectra of purified PE (Fig. 1) showed the characteristic single peak of C-PE at 565 nm (Alberte *et al.*, 1984).

The final purified PE showed two bands in SDS-PAGE (Fig. 2A) with molecular masses of approximately 21 and 18 kDa, respectively. From native-PAGE without staining (Fig. 2B), PE was visualized as two bands of the brilliant pink color. The native PE mass analysis was determined by gel filtration, existing as a hexameric $(\alpha\beta)_6$ subunit and trimer $(\alpha\beta)_3$ with a molecular mass of approximately 235 and 125 kDa, respectively (Fig. 3). The protein migration on native electrophoresis could depend on the pH, ionic strength, and shape and size of the protein. However, the lower molecular weight complex tends to result in a greater mobile on the PAGE. Therefore, it is reasonable to consider the dense band in native-PAGE as a band of PE trimer, while the other can be considered a band of PE hexamer.

Comparison of the N-terminal amino acid sequences of α-subunit (18 kDa band) and β-subunit (21 kDa band) using SWISSPROT database revealed a high percentage of

Table I: Purification of *Leptolyngbya* sp. KC45 phycoerythrin

Purification steps	Total protein (mg)	Total PE (mg)	Yield PE (%)	A565/ A280	A565/ A615
Cell free extract	313.13	40.72	100.00	1.52	0.431
Ammonium sulfate precipitation	215.22	28.15	69.13	2.16	0.433
Hydroxyapatite	65.31	15.21	37.35	6.75	0.234
Q-Sepharose	16.38	3.93	9.65	15.48	0.055
Sephacryl ™ S-200 HR	1.25	0.56	1.36	17.38	0.006

Fig. 1: The absorption spectra of the purified PE, eluent fraction from Sephacryl ™ S-200 HR column

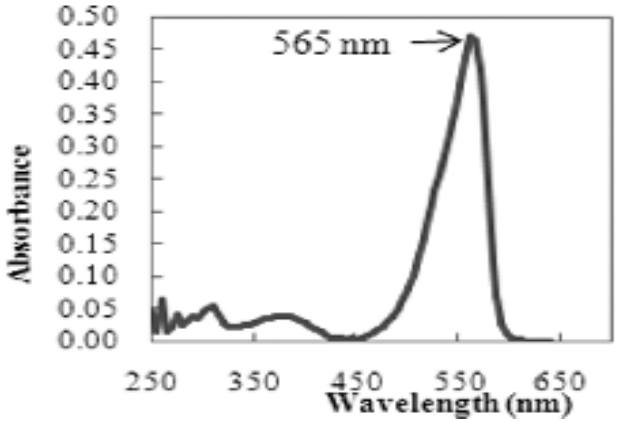


Fig. 2: Detection of the purified PE from *Leptolyngbya* sp. KC45 from each purification step

(A) SDS-PAGE stained with Biorad Silver Stain kit, (B) Native-PAGE without staining. Lane M, Page Ruler™ prestained protein ladder (170, 130, 100, 70, 55, 40, 35 25, 15 and 10 kDa); lane 1, crude extract; lane 2, salt precipitation; lane 3, Hydroxyapatite column; lane 4, Q-sepharose column; lane 5, Sephacryl ™ S-200 HR column

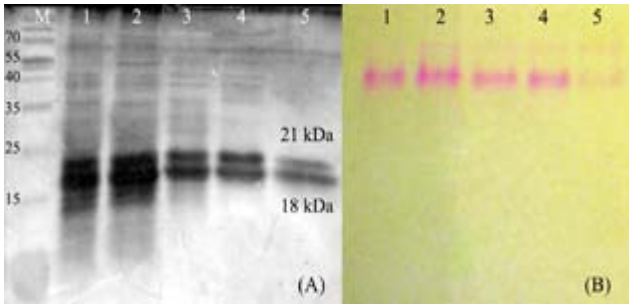


Fig. 3: Determination of molecular mass of PE from *Leptolyngbya* sp. KC45 by gel filtration chromatography using a Superose 6 HR 10/30 column

The column was calibrated with thyroglobulin (670 kDa), bovein gamma globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa) and vitamin B-12 (1.35 kDa) as refence proteins (Biorad) and the molecular mass of the native PE was calculated by comparing with the migration length of reference proteins

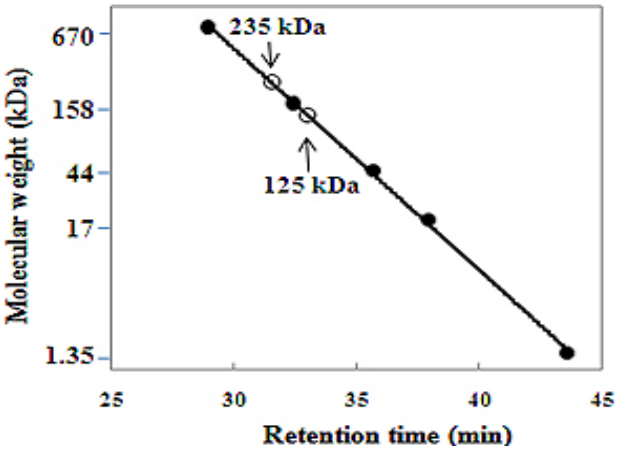
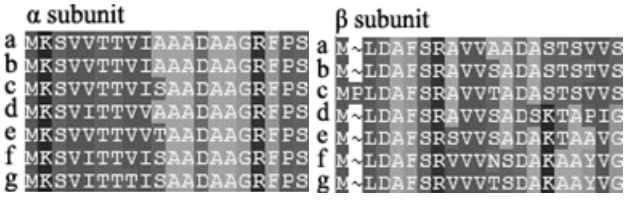


Fig. 4: Alignment of N-terminal amino acid sequences of subunits

(a) *Leptolyngbya* sp. KC45 with (b) *Fremyella diplosiphon*, (c) *Pseudanabaena* sp. PCC7409, (d) *Synechocystis* sp. PCC6701, (e) *Synechococcus* sp. WH7803, (f) *Porphyridium sordidum* and (g) *Griffithsia monilis*. Identical amino acid residues are highlighted. The sequence alignment was performed with the aid of SWISSPROT database for proteins. Accession numbers of *Fremyella diplosiphon* (α-subunit P05098, β-subunit P05097), *Pseudanabaena* sp. PCC7409 (α-subunit P29296, β subunit P29297), *Synechocystis* sp. PCC6701 (α-subunit P20778, β-subunit P20779), *Synechococcus* sp. WH7803(α-subunit Q08088, β-subunit Q08087), *Porphyridium sordidum* (α-subunit P29947, β-subunit P29948) and *Griffithsia monilis* (α-subunit O36005, β-subunit O36004)

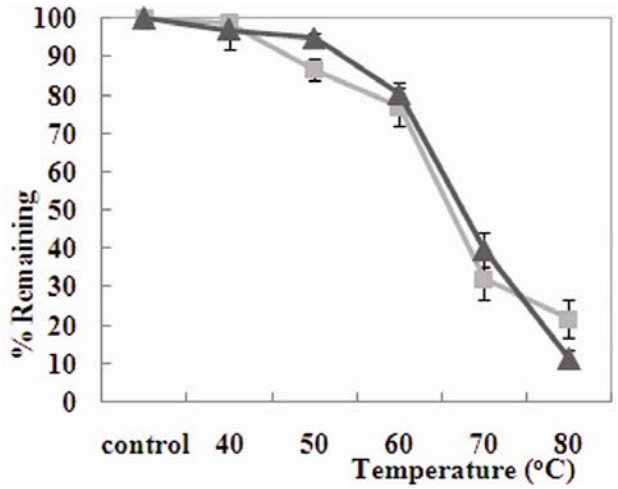


identities with other species of Cyanophyta and Rhodophyta (Fig. 4). PE of *Leptolyngbya* sp. KC45 shared the highest homology with *Fremyella diplosiphon* (100% for α- & 90% for β-subunit) followed by *Pseudanabaena* sp. PCC7409 (95% for α- & 90% for β-subunit), *Synechocystis* sp. PCC6701 (90% for α- & 81% for β-subunit), *Porphyridium sordidum* (90% for α- & 78% for β-subunit), *Griffithsia monilis* (85% for α- & 78% for β-subunit) and *Synechococcus* sp. WH7803 (90% for α- & 73% for β-subunit), respectively (Bairoch & Apweiler, 1996).

Effects of temperature on PE stability and DPPH radical scavenging activity: The thermal stability of the PE from *Leptolyngbya* sp. KC45 was examined by measuring the

Fig. 5: Thermal denaturation of purified PE

(■) and thermal inactivation of DPPH radical scavenging activity from the purified PE (▲) from *Leptolyngbya* sp. KC45. Data represent the means of three experiments and error bars represent standard deviation



absorbance 565 nm at temperatures ranging from 40 to 80°C, compared with its DPPH scavenging activity (Fig. 5). It was obviously revealed that PE thermal stability was found to be correlated with its DPPH scavenging activity. Both PE and its antioxidative activity showed high thermal stability as approximately 80% of the original level remained after it was incubated at 60°C for 30 min. Moreover, the purified PE from *Leptolyngbya* sp. KC45 showed thermal stability at the same level of the PE in crude extract of *Leptolyngbya* sp. KC45 (Pumas *et al.*, 2011) and could be stable at temperatures higher than the isolation and cultivation temperatures, 45°C and 40°C, respectively. In addition, the thermal stability of the purified PE was slightly more stable than PBPs from mesophiles. In this context, Zhao and Brand (1989) reported that PBPs in various strains of cyanobacteria and red algae: *Anacystis nidulans*, *Fremyella diplosiphon*, *Phormidium fragile*, *Synechocystis* sp. PCC6714 and *Porphyridium cruentum* were bleached after being incubated at 60-65°C for 5-8 min. From the thermal stability of PE and its antioxidative activity, PE from *Leptolyngbya* sp. KC45 is of increased interest for its potential use as a thermostable dye or as thermostable fluorescence labels.

Because of high molar absorbance coefficients, high fluorescent quantum yield, large Stokes shift, high oligomer stability and high photostability, PE has been widely used in industries and clinical or research immunology laboratories. Recently, many biotechnological techniques have been developed using PE as a fluorescent labeling agent, for example, as labels for antibodies, receptors and other biological molecules in a fluorescence-activated cell sorter and as well, they can be used in immunolabelling experiments and fluorescence microscopy or diagnostics (Spolaore *et al.*, 2006). In addition, PE is also of interest in the use of light fluorescent products in food applications, especially beverages (Dufosse *et al.*, 2005). Many PE

applications are continually being developed and patented. It seems likely that the demand of PE will continue to increase. However, the success in PE production depends on the mass production ability and efficiency of downstream processing, especially in the purification from other PBPs (Sekar & Chandramohan, 2008). Therefore, molecular cloning and high expressions of the recombinant PE is required to obtain large quantities and an easily purify PE in order to respond to its recent commercial demand as described by Qin *et al.* (2004).

CONCLUSION

This is the first report for the purification of PE from *Leptolyngbya* sp. The purified PE showed its thermal stability to be at the same level as its antioxidative activity. In addition, the N-terminal analysis sequences obtained in this study are available to be used for PE gene sequencing and expression in the further studies.

Acknowledgement: We acknowledge the Office of Higher Education Commission, Thailand for the scholarship under the Strategic Scholarships for Frontier Research Network for the Joint Ph.D. Program. This work was partially supported by a grant from the National Research Council of Thailand (NRCT) under management of the Institute for Science and Technology Research and Development, Chiang Mai University. Lastly, we would like to thank the Asian Core Program (ACP) on "Capacity Building and Development of Microbial Potential and Fermentation Technology towards New Era" for its assistance in the scientist exchange programs and the collaborative research. Lastly, we also acknowledge Mr. Russell K. Hollis at English Department, Faculty of Humanities, Chiang Mai University for English corrections.

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(Received 22 June 2011; Accepted 17 October 2011)