African Journal of Biotechnology Vol. 10(39), pp. 7640-7649, 27 July, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2223
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

The subunits analysis of R-phycoerythrin from marine red algae by isoelectric focusing

Xuejun Fu[1], Li Sun[1]*, Guanpin Yang[2], Wang Lu[1], Mingri Zhao[1] and Xueqin Gong[1]

[1]College of Chemistry and Biology, Yantai University, (Shandong Yantai 264005, Fax 86-35-6902063, P. R. China).
[2]College of Marine Life Sciences, Ocean University of China, (Shandong Qingdao 266003, Fax 86-32-82031636, P. R. China).

Accepted 25 May, 2011

Subunit components of R-phycoerythrins (R-PEs) prepared from five marine macro red algae were analyzed by sodium dodecyl sulfate -polyarylamide gel electrophoresis (SDS-PAGE) and by isoelectric focusing (IEF) in pH gradients range of 3.0 to 9.5, 2.5 to 5.0 and 4.0 to 6.5. Riboflavin was used to catalyze polymerization of IEF gel in acidic pH gradients, and ethanolamine and HEPES were selected as cathode buffers for IEF. The pIs of the R-PE subunits existed between pH 4.9 and 5.7. A larger number of bands could be identified from IEF relative to SDS-PAGE, demonstrating that some subunits of the R-PEs which showed a certain apparent molecular weight have different pIs. This revealed that local net charge differences exist among the subunits of the R-PEs which have even the same molecular weight as well as those with various molecular weights, therefore charge-charge interaction among the subunits of the R-PEs may play an adequate role in R-PE assembly, which is consistent with the fact that the R-PEs are insensitive to surface-active reagents.

Key words: Phycobiliprotein, phycoerythrin, red alga, isoelectric focusing, polyacrylamide gel electrophoresis, spectral properties.

INTRODUCTION

Phycobiliproteins are predominant light-harvesting pigment-protein complexes widely existing in cyano-bacteria, red algae, some cryptophytes and dino-flagellates. In cyanobacteria and rhodophyceae, they are present as aggregation of macromolecular particles called phycobilisome (PBS). The PBS anchor on the outer surface of thylakoids and lie near Chlorophyll a (Chl a) reaction centers of Photosystem Ⅱ (Sun et al., 2003). Phyco- biliproteins from cyanobacteria and rhodophyceae are generally divided into three types based on their absorption properties: phycoerythrin (PE, λmax: 480 to 580 nm), phycocyanin (PC, λmax: 600 to 640 nm) and allophycocyanin (APC, λmax: 620 to 655 nm) (Sun et al., 2004; Bermejo, 2003; Glazer, 1984). Based on their origins and absorption properties, phycoerythrin is also classified into four classes: R-PE (λmax: 567, 545 and 495 nm), B-PE (λmax: 540 to 560 nm, shoulder: 495 nm), b-PE (λmax: 567 and 563 nm) and C-PE (λmax: 563, 543 and 492 nm) (Marsac, 2003). PEs has intensive fluorescence emission at about 578 nm. The PEs purified from red macro algae are commonly R-PE and composed of three subunits α, β and γ, which have apparent molecular weight about 18, 20, and 30 to 33 kDa respectively (Sun et al., 2003, 2004; Adir, 2005). Aggregation structure of R-PEs often appears as a hexamer, $(\alpha\beta)_3\gamma(\alpha\beta)_3$ or $(\alpha\beta)_6\gamma$, in the PBS of fresh red algae and was suspended in diluting buffer (Sun et al., 2004). Five red algae, *Polysiphonia urceolata* Grev, *Heterosiphonia japonica* Yendo, *Callithamnion corymbosum* Lynge, *Gloisiphonia capillaris* Carm , *Ceramium japonicum* Okamura are selected in this study. All of them possess "three-peak I" R-phycoerythrins (R-PEs) that show three absorbance maxima of 498 nm, 538 to 541 nm and 566 to 568 nm (Zhang et al., 2002). PEs from red algae has been

*Corresponding author: E-mail: sunliytu@yahoo.com.cn. Fax 86-35-6902063, Tel 86-35-6902063.

Abbreviations: R-PE, R-phycoerythrin; **IEF,** isoelectricfocusing; **PC,** phycocyanin; **APC,** allophycocyanin; **PBS,** phycobilisome; **Chl a,** chlorophyll a; **TEMED,** tetramethyl ethylene diamine;**SDS-PAGE,** sodiumdodecyl sulfate-polyarylamide gel electrophoresis; **AP,** ammonium per sulfate; **MB,** methyl leneblue; **DPIC,** dipheyliodonium chloride; **STS,** sodium toluene sulfinate;**pI,** isoelectric point; **PBS,** phycobilisome.

widely used as natural pigment in food and cosmetics, as fluorescent probes in immunodiagnostics and as analytical reagents in electrophoresis (Liu et al., 2005).

Polypeptide analysis of phycobiliproteins is commonly performed by SDS-PAGE that based on different apparent molecular weight of polypeptides (Lyubimova, 1993b). There are limited knowledge on isoelectric point (pI) properties of individual polypeptides in phycobiliproteins from red macro algae. Only a few studies of pI on polypeptides analysis using IEF were reported (Glazer, and Craig, 1975; Everroad et al., 2006; Six et al., 2005; Ohki, 1985). From the polypeptide analysis by SDS-PAGE, the composition of multiple-subunit proteins can be determined by proportions of the polypeptides, as well as individual apparent molecular weight of the polypeptides, and polypeptide organization of the complex proteins is deduced according to the polypeptide proportions (Guo, 1999), such as $(\alpha\beta)_3\gamma(\alpha\beta)_3$ for a PE hexamer and $(\alpha\beta)_3$ for a PC trimmer ($(\alpha\beta)$ is designated monomer for phycobiliproteins (Sun et al., 2003, 2004). By IEF, pIs of multiple-subunit proteins and their polypeptides can be detected in native and denaturing situation respectively. The pIs variation of the polypeptides of the multiple-subunit proteins reveals net charge difference of the polypeptides at a certain pH. The net charge difference is closely related to charge-charge interaction between polypeptides in the complex proteins assemble. From this point of view, the polypeptide analysis of phycobiliproteins by IEF may give some information about possible interaction between polypeptides which contributes to the assembly of multiple-subunit proteins.

IEF is an electrophoretic method for separation and analysis of amphoteric molecules based on their pIs, which were set up in the 1960s. Since then, IEF has become the most sensitive technique of electrophoresis and was widely used in practice at present (Guo, 1999). In a steady, continuous and linear pH gradient, IEF separates and analyzes proteins by their differences in pI. In this study, subunits of R-PEs purified from several marine red macro algae by ion exchange chromatography or native polyacrylamide gel electrophoresis were analyzed by using polyacrylamide gel IEF in pH ranges from 3.0 to 9.5, 2.5 to 5.0 and 4.0 to 6.5. In acidic pH gradients, the gel polymerization was catalyzed with only riboflavin under UV-light for both native and denaturing polyacrylamide gel IEF. This overcomes the problem that the polyacrylamide gel is hard to polymerize in acidic pH when it is catalyzed by tetramethyl ethylene diamine (TEMED) and ammonium persulfate (AP) (Jordan and Raymond, 1969). The polymerization catalyzed by riboflavin also resolves the problem that some proteins are precipitated in the gel because the proteins and the catalyzer formed insoluble complexes when $AgNO_3$-AP is used as catalyzer in acidic pH ranges (Guo, 1983). The IEF analysis showed that pIs of the subunits of the five prepared R-PEs were between pH 4.9 and 5.7. A larger number of colored subunit bands could be identified from IEF relative to SDS-PAGE. This reveals that among the subunits of R-PEs, charge-charge interaction which originates from local positive or negative charge variant distribution of the subunits of a phycoerythrin may play roles when R-PEs assemble hexameric aggregates.

MATERIALS AND METHODS

Sephadex G-150 and ampholine are from Phamacia co. (Sweden); DEAE Sepharose Fast Flow and HEPES are from Beijing Xinjingke co. (China); the other reagents are from Shanghai GuoYao co. ltd (China).

Fresh algae of *P.urceolata* Grev, *H. japonica* Yendo, *C. corymbosum* Lynge, *G. capillaris* Carm, *C. japonicum* Okamura were collected from the intertidal zone of local sea of Yantai (Northern Yellow Sea). Five phycoerythrins from *P. urceolata*, *H. japonica*, *C. corymbosum*, *G. capillaris* , *C. japonicum* were prepared by biological macromolecular lab of Yantai University. The method is as follows (Sun et al., 2009).

The phycobiliproteins were released out from fresh alga by treatment with ultrasonication (JY92-II ultrasonic cell disruptor, China) in 0.05 M phosphate buffer (pH 7.0) for 10 min at about 350 W at 0°C, and were salted out with $(NH_4)_2SO_4$ at saturation of 60% at 4°C and collected by the centrifugation at 25,000 g for 15 min at 4°C. The precipitated biliproteins were resuspended in 0.05 M phosphate buffer (pH 7.0) and stored at 4°C in dark. The gel filtrations with Sephadex G-150 (3.7× 65 cm) were employed for R-PE isolation from the phycobiliprotein extracts. The phycobiliprotein samples were eluted by 0.05 M phosphate buffer (pH 7.0) at rate of 30 ml/h and monitored by the absorption at 280 nm. The bright red fractions rich in the R-PE were collected and used as the samples for the R-PE purification by using the ion exchange chromatography on DEAE Sepharose Fast Flow column (2.6×6 cm). The ion exchange chromatography was carried out by using 500 ml of 0 to 0.4M NaCl linear gradient elution (pH7.0, 0.025 M phosphate buffer). The samples were eluted at rate of 30 ml/h and monitored at 280nm.The obtained R-PE fractions were examined with the absorption spectrum from 250 to 750 nm. The purity of the R-PE was evaluated by the native PAGE and the IEF under native conditions.

Spectrum measurement

The absorption spectra of the five purified R-PE samples suspended in 0.05 M phosphate buffer (pH 7.0) were determined from 250 to 750 nm by using TU-1900 UV-VIS Spectrophotometer (Beijing Puxitong Instrument Co. Ltd, China) at room temperature. The fluorescence spectra were determined using fluorescence spectrometer Cary Eclipse (Hitachi Co. Ltd, Japan) in phosphate buffers (pH 7.0) at room temperature. The fluorescent emission spectra of R-PEs were determined with an excitation wavelength of 498 nm and the scan wavelength was 520 to 800 nm; the fluorescent excitation spectrums of R-PEs were determined at wavelength of maxim emission peak and recorded from 350 nm-wavelength of maxim emission.

Polyacrylamide gel electrophoresis

Native PAGE was employed to evaluate the purity of the five R-PEs. The gel was composed of 3% (w/v) stacking gel in Tris-phosphonic acid buffer (pH 5.5) and 6% (w/v) resolving gel in Tris–HCl buffer (pH 7.5). Tris-diethylbarbituric acid buffer (pH 7.0) was used as electrode buffer. After the native PAGE, the red band of the R-PE in

native state was examined by enhancement of the bright yellow fluorescence emitted by the R-PE under ultraviolet light at 365 nm; Moreover, the gel was stained with 0.1% (w/v) Coomassie Blue G-250 in 40% (v/v) methanol and 10% (v/v) acetic acid to examine colorless protein bands together with the red R-PE. SDS-PAGE was employed for analysis of subunit components of the five R-PEs and the results were compared with those obtained by IEF. The SDS-PAGE was performed with 13% (w/v) separating gel in pH 9.3 Tris-HCl buffer containing 0.1% (w/v) SDS and 4% (w/v) stacking gel in pH 6.8 Tris-HCl buffer containing 0.1% (w/v) SDS. Tris- glycine buffer of 0.05 M (pH 8.3) containing 0.1% (w/v) SDS was used as electrode buffer.

The fluorescent bands from the chromophore-carrying subunits were observed under ultraviolet light at 365 nm. Moreover, the other polypeptides without the chromophores, together with the subunits, were further examined by staining the slab gel with 0.1% (w/v) Coomassie Blue G-250 in 40% (v/v) methanol and 10% (v/v) acetic acid.

Isoelectric focusing electrophoresis

pIs of the five prepared R-PEs in natural aggregation were detected by native IEF. The IEF was performed on 6% (w/v) polyacrylamide gel containing 2% (v/v) ampholine in pH ranges from 4.0 to 6.5 and from 2.5 to 5.0. The gel was polymerized for about 20 to 30 min by the catalysis of 0.67 μg/ml riboflavin under cool-white light. Samples with low ion strength were added onto the basic end of the gel. Cathode buffer was 0.4 M HEPES and anode buffer was 1.0 M phosphoric acid. The IEF was performed at a constant voltage of 100 to 300 V for about 3 h.

Subunit components of the five prepared R-PEs were analyzed by denaturing polyacrylamide gel IEF in pH ranges of 3.0 to 9.5, 4.0 to 6.5 and 2.5 to 5.0. The polyacrylamide gel of 7% (w/v) with 2% (v/v) ampholine in 8 M urea was polymerized by catalysis with riboflavin of 0.67 μg/ml under UV-light. 10 μl of every sample was loaded on the basic end of the gel. Cathode buffer was 0.5 M ethanolamine or 0.4 M HEPES and anode buffer was 1.0 M phosphoric acid. The IEF was performed at voltage of 500 to 580 V until current was down to zero. After the IEF, the polypeptides were examined by the natural color of the subunits, by the emission fluorescence of the subunits under ultraviolet light at 365 nm and by the blue bands of the polypeptides after the gels were stained with 0.2% (w/v) Coomassie Blue G-250 in 40% (v/v) methanol and 10% (v/v) acetic acid.

R-PE samples for denaturing polyacrylamide gel IEF of the subunit analysis were prepared by the following procedures:

(1) R-PEs were denatured and precipitated in 20% (w/v) trichloroacetic acid, and then collected by centrifugation at 20000 g for 15 min.
(2) Wash the collected pellet with 0.004 M EDTA-Na_2 and 10% (v/v) glycerol, and the denatured R-PEs were collected again by centrifugation at 20000 g for 15 min.
(3) Finally, the subunits of R-PEs in denaturing situation were fully suspended by the sample lysate which was composed of 2% (v/v) Ampholine, 0.5% (v/v) NP-40 (or Triton X–100), 0.12 M mercaptoethanol, 8 M urea and 0.003% (w/v) bromphenol blue, and the suspension was centrifuged at 20000 g for 15 min so that insoluble substances were removed. The supernatant was the subunit samples prepared for the denaturing IEF.

RESULTS

Spectral properties of the five prepared phycoerythrins

Spectral properties of R-PEs prepared from *P. urceolata, H. japonica, C. corymbosum , G. capillaris , C. japonicum* are shown in Figure 1 (a) to 1(e). Absorption spectra of the five purified R-PEs were measured from 750 to 250 nm at room temperature. In visible light region, three strong absorption maximums occurred at 498nm, 538 to 541nm and 566 to 568 nm, respectively. The fluorescence emission and excitation spectra were also given in Figure1 (a) to 1(e) with dash dot lines and dash lines respectively. The fluorescence emission maximums occurred at 577 nm for the R-PE from *P. urceolata*, at 573 nm for that from *H. japonica*, at 576 nm for that from *C. corymbosum*, at 575 nm for that from *G. capillaries*, and at 576 nm for that from *C. japonicum* with an exciting wave-length at 498 nm. The fluorescence excitation spectra were examined at 580 nm; the R-PEs showed three peaks at about 498, 538 to 541, 566 to 568 nm. These spectroscopic characteristics indicated that the five prepared R-PE were all three peaks I type R-PEs.

Polyacrylamide gel electrophoresis

The purity of R-PEs from the five red algae was examined by the native gel electrophoresis. As shown in Figure 2A (Lane a to e), the R-PEs from the five algae except *C. japonicum* not only showed a single red band with bright yellow fluorescent band under ultraviolet light at 365 nm(not shown), but also showed a single blue band after the gel was stained with Coomassie Blue G-250. The result demonstrated that the R-PEs prepared from those four algae except *C. japonicum* were in high purity for polypeptide analysis by SDS-PAGE and IEF. The R-PE from *C. japonicum* showed more than one red bands, so it is needed for further purification. After filtrated with 30 K ultrafiltration tube, the R-PE from *C. japonicum* was purified by a discal native PAGE with 6.5% (m/v) slab gel in pH 7.5 Tris-Hcl buffer and 4% (m/v) stacking gel in pH 5.5 Tris-H_3PO_4 buffer. Tris-Barbitone (pH 7.0) was used as electrode buffer. The result is shown in Figure 2B. There showed a single red band with bright yellow fluorescent, R-PE of *C. japonicum*. The red band was collected and analyzed by using denature SDS-PAGE.

Polypeptide analysis of the five purified R-PEs by SDS-PAGE is shown in Figure 3. The results demonstrated that the fluorescent bands from colored polypeptides (chromophore-containing subunits) observed after $ZnSO_4$ staining (Figure 3A) were exactly consistent with the blue bands reflected by Coomassie Blue G-250 staining (Figure 3B). This proved that the purified R-PE was composed of three or four chromophore-containing subunits, $\alpha\beta\gamma/\gamma'$ and other colorless polypeptides (Figure 3A and 3B).

The R-PE from *C. corymbosum* (Lane a) had five bands, which were subunit α (17.6 kDa), β (20 kDa), γ (31 kDa) and two dissociated subunit complex (38 and 43.5 kDa). The R-PE from *P. urceolata* (Lane b) had three

bands, corresponding subunit α (16.8 kDa), β (20.1 kDa)

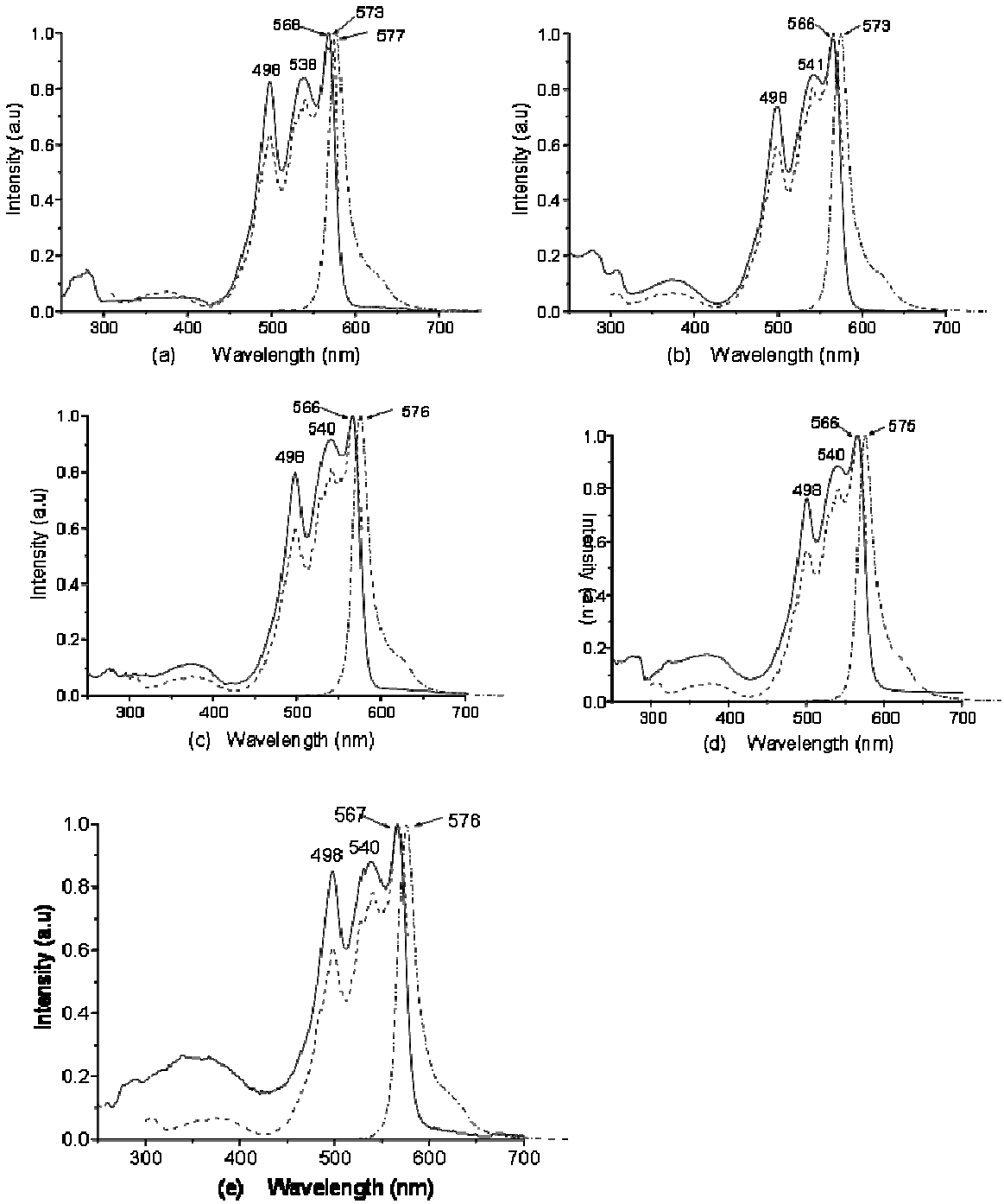

Figure1. Absorption (solid line), fluorescence emission (dash dot line) and fluorescence excitation (dash line) spectra of R-PEs in 50 mM phosphate buffer (pH 7.0). The R-PEs were prepared from: a) *Polysiphonia urceolata*, b) *Heterosiphonia japonica*, c) *Callithamnion corymbosum*, d) *Gloisiphonia capillaris*, e) *Ceramium japonicum*. The fluorescence emission spectra were recorded on excitation at 495 nm and the fluorescence excitation spectra were measured at 580 nm.

and γ (33.3 kDa). The R-PE from *H. japonica* (Lane c) had four bands, corresponding subunit α (17.6 kDa), β (20 kDa), γ (30.2 kDa),γ' (31.6 kDa). The R-PE from *G. capillaris* (Figure 3A, Lane d) had six colored bands, which were subunit α/β (16.8 to 19.8 kDa), γ (31 kDa),γ' (33 kDa)and three dissociated subunit complexes, c (41.2

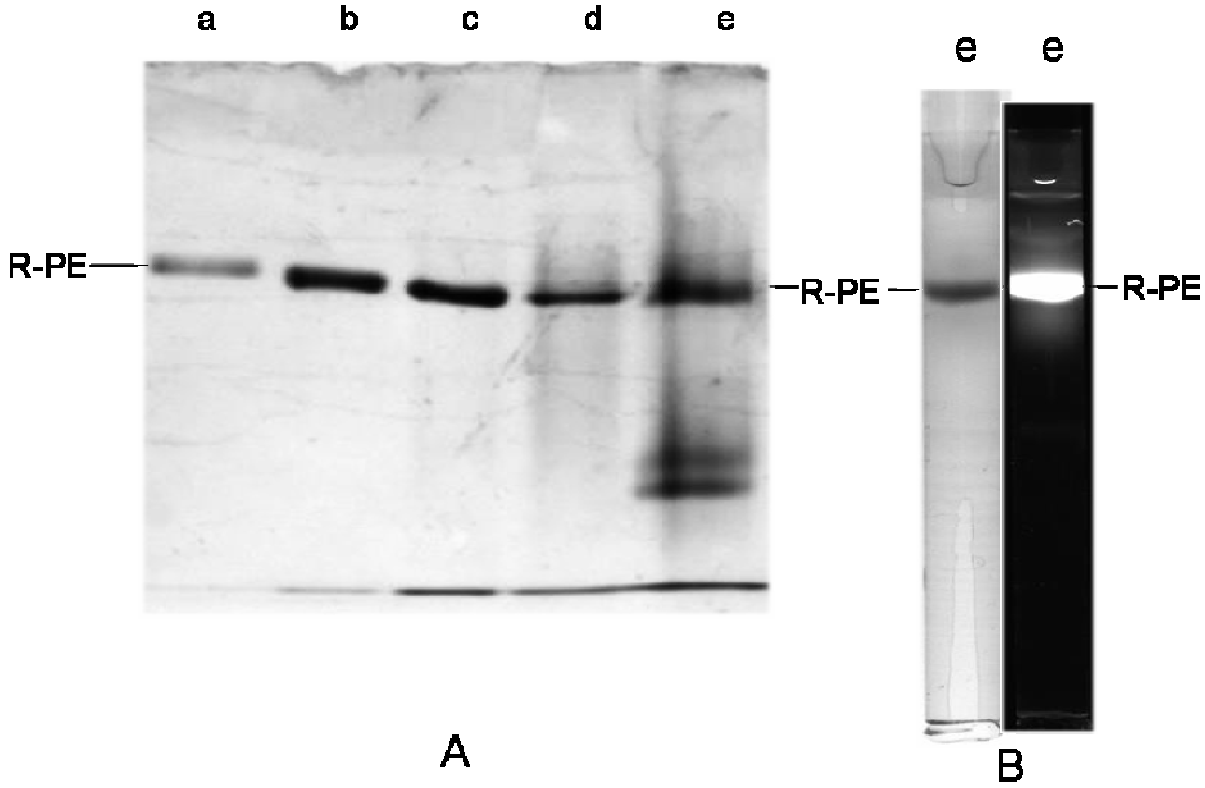

Figure 2. A was image of Native-PAGE of R-PEs from *Polysiphonia urceolata* (lane a), *Heterosiphonia japonic* (lane b), *Callithamnion corymbosum* (lane c), *Gloisiphonia capillaris* (lane d), *Ceramium japonicum* (lane e). After the electrophoresis, the gels were stained by Coomassie brilliant blue; B was result of discal Native-PAGE of R-PE from *Ceramium japonicum*. Left gel had single red band under cool-white light and right gel had single yellow fluorescence band under UV-wavelength at 365 nm.

kDa), c' and c" (43 to 66 kDa). After Coomassie blue staining, there have two blue colored bands L (27 kDa) and L' (38 kDa) (Figure 3B, Lane d) which were colorless under cool-white light and with no fluorescence under UV wavelength (Figure 3A, Lane d). They are assumed linker peptides associated with the R-PE. The R-PE from *C. japonicum* (Lane e) had five bands, which were subunit α (17.6 kDa), β (20.1 kDa), γ (31.3 kDa) and dissociated subunit complexes (38, 43 kDa).

Polyacrylamide gel IEF

The R-PEs purified from *P. urceolata* and *H. japonica* by the ion exchange chromatography was also evaluated by the isoelectric focusing (IEF) in pH range from 4.0 to 6.5 and from 2.5 to 5.0 under native conditions, respectively. The results of native polyacrylamide gel IEF for the R-PEs from *P. urceolata* and *H. japonica* are shown in Figure 4. The R-PE of *P. urceolata* showed two bands on IEF both in pH 2.5 to 5.0 (Figure 4A) and 4.0 to 6.5 (Figure 4B) gradients. The two bands were very close to each other and focused on about pH 4.1 in pH 2.5 to 5.0 gradients and pH 4.7 in pH 4.0 to 6.5 gradients, respectively. The different pH values of R-PE on the aforementioned two different pH-gradients (Figure 4) attributed to the different pH gradients foamed in the gel. The result demonstrated that the R-PE of *P. urceolata* has two kinds of complexes. The two R-PE complexes with the same ratio of charge to mass have different pIs but very near each other. The R-PE of *H. japonica* showed a little broad band focused on about pH 4.2 in pH 2.5 to 5.0 gradients (Figure 4A) and pH 4.8 in pH 4.0 to 6.5 gradients (Figure 4B). It showed that the pIs of two kinds complexes of the R-PE from *H. japonica* which were deduced based on its two γ subunits are too near to be separated each other (Apt,1993; Wang et al., 2008).

Denaturing polyacrylamide gel IEF was performed in pH 3.0 to 9.5 (not shown), pH 2.5 to 5.0 (not shown) and pH 4.0 to 6.5 (Figure 5). In pH 3.0 to 9.5, subunit bands of the purified R-PEs from the five marine macro-algae were too close to clearly resolved each other. Subunits pIs of the five purified R-PEs are at about pH 5.0. R-PE subunits of *H. japonica* formed clear and separated bands in pH 2.5 to 5.0. But R-PE subunits of *P. urceolata* focused only two clear bands in this pH gradient, and even some samples could not run into the gel. In contrast, all of the R-PE samples could focus into clear and separated bands in pH

4.0 to 6.5 with both 0.5 M ethanolamine (Figure 5) and 0.4 M HEPES (not shown) used as cathode buffers. The

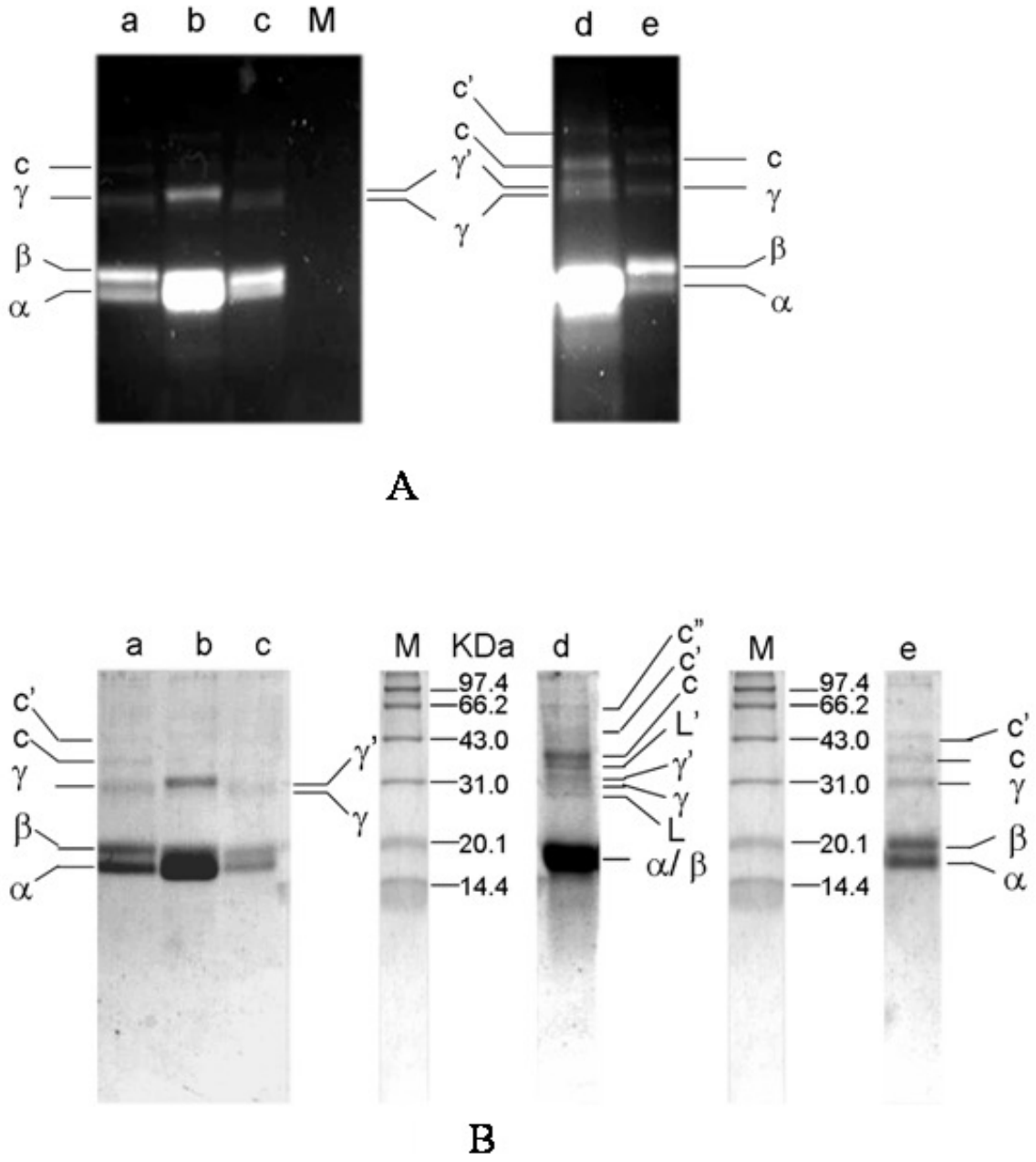

Figure 3. Subunit analysis of R-PEs by SDS-PAGE; lane M: marker proteins; Lanes a to e: the R-phycoerythrins from *Callithamnion corymbosum* (Lane a), *Polysiphonia urceolata* (Lane b), *Heterosiphonia japonica* (Lane c), *Gloisiphonia capillaris* (Lane d) and *Ceramium japonicum* (lane e). Samples were incubated at 100°C for 5 min. After the electrophoresis, the gels were stained by $ZnSO_4$-imidazole (A), and then stained by Coomassie brilliant blue (B). L/L′ represents colorless link peptides and c dissociated subunit complexes.

subunit pIs of the five prepared R-PEs were between 4.9 and 5.7.The R-PEs all exhibited two thick and some thin colored bands polypeptide bands. The two thick bands are assumed α/β subunits. The thin bands may be γ subunits and a certain subunit complex which is incompletely dissociated. These results are identical to those from the SDS-PAGE. Result of IEF of *G. capillaris* (Figure 5d) has several bands which were colorless under cool-white light, no fluorescence under UV wavelength at 365 nm and blue color after stained with Coomassie Brilliant Blue G-250. These bands are assumed linker peptides.

DISCUSSION

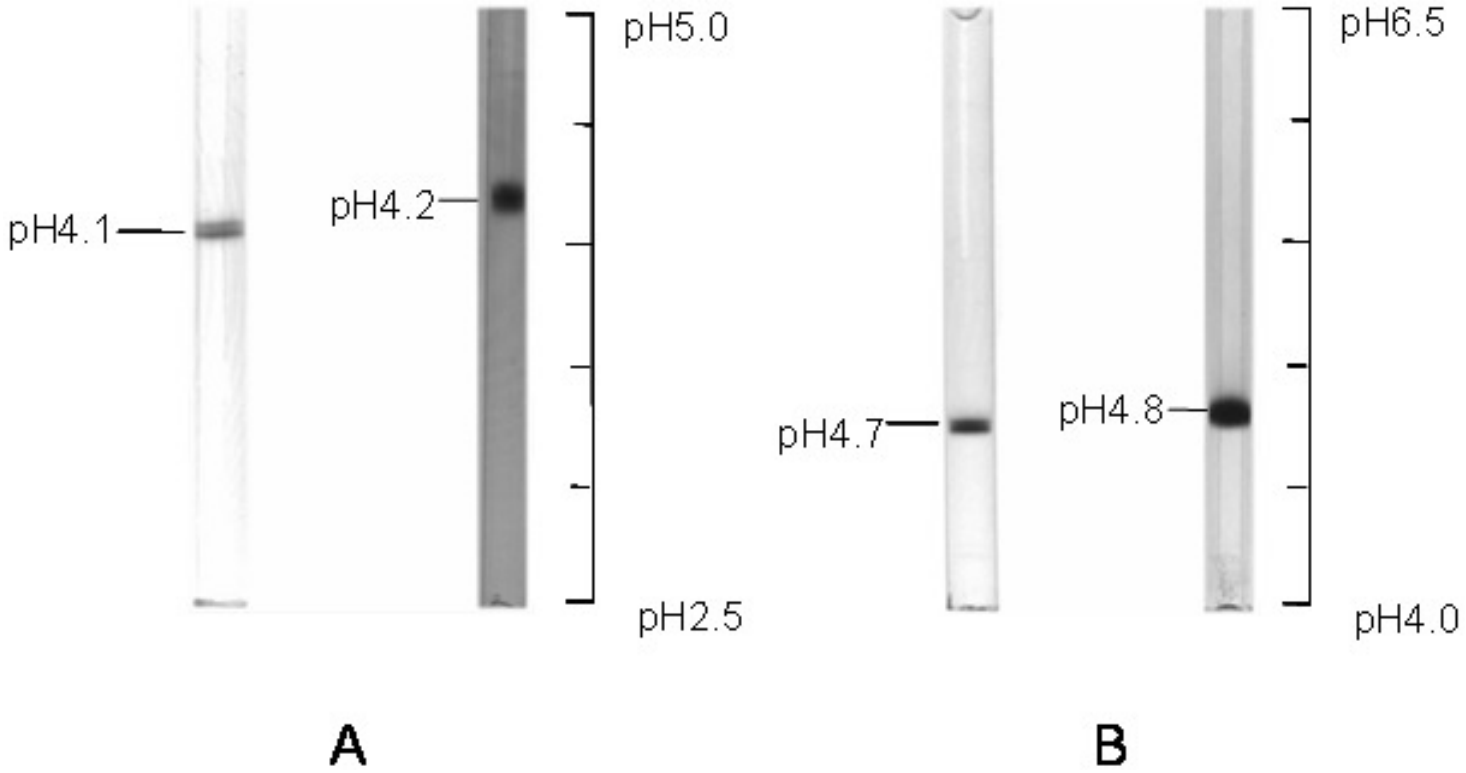

Figure 4. Native polyacrylamide gel IEF of R-PEs in pH gradient 2.5-5.0 (A) and 4.0 to 6.5 (B). The R-PEs were from *Polysiphonia urceolata* (left) and *Heterosiphonia japonica* (right). Samples were loaded at the basic end of the gel. 0.5 M ethanolamine was used as cathode buffer and 1.0 M phosphoric acid was used as anode buffer.

R-PEs and differences of R-PE subunits in molecule weight. On the basis of these data, subunit organizations of R-PE complexes can be deduced, but almost no information on subunit interaction for aggregation formation can be obtained. IEF analysis demonstrated subunit differences of R-PEs in pI. The denaturing IEF result showed that a larger number of subunit bands could be identified based on pI difference than those determined by SDS-PAGE, and the subunit pIs were in pH range between 4.9 and 5.7. This revealed that some certain subunits with the same apparent molecular weight have various pIs and revealed that among the subunits of R-PEs, charge-charge interaction which originates from local positive or negative charge variant distribution of R-PE subunits may play adequate roles when R-PEs assemble hexameric aggregations. Band by IEF corresponds to subunitα, β or γ cannot be confirmed. To confirm the corresponding relationship of bands based on p*I* difference and subunits of R-PE, two-dimensional electrophoresis or other experiment should be done.

The pI variation represents net charge differences of the polypeptides at a certain pH. The net charge differences are closely related to charge-charge interaction between polypeptides in multi-subunit proteins assembly. Therefore, among the subunits of R-PEs, charge-charge interaction which originates from net charge variation of the subunits of an R-phycoerythrin participates in assembly of R-PE hexameric aggregations. In other words, electrostatic interaction between subunits is assumed to play a role to a certain degree in R-PE hexamer formation as hydrophobic interaction. It is also proved by the fact that R-PEs are insensitive to non-ionic surface-active reagent and exist stable in 0.4% (v/v) NP-40 or Trion-X 100. In contrast, the R-PC from *P. urceolata* is sensitive to surface-active reagent and can be denatured in 0.4% (v/v) Trion-X 100. Moreover, the R-PC showed two subunit bands by denaturing IEF, which were consistent with those by SDS-PAGE (Figure 6). Two subunits β have the same pI but different apparent molecular weight. From this point of view, the polypeptide analysis of phycobiliproteins by IEF gives information on the contribution of charge-charge interaction between polypeptides to assembly of the multiple-subunit proteins.

In this study, riboflavin was experimentally proved to catalyze the gel polymerization in acidic pH under cool-white light with high simplicity and efficiency. This overcomes the problem that the polyacrylamide gel is hard to polymerize in acidic pH when it is catalyzed by tetramethyl ethylene diamine (TEMED) and ammonium persulfate (AP) (Jordan and Raymond, 1969; Guo, 1999). This also resolves the problem that some proteins formed insoluble complexes when $AgNO_3$-AP is used as catalyzer

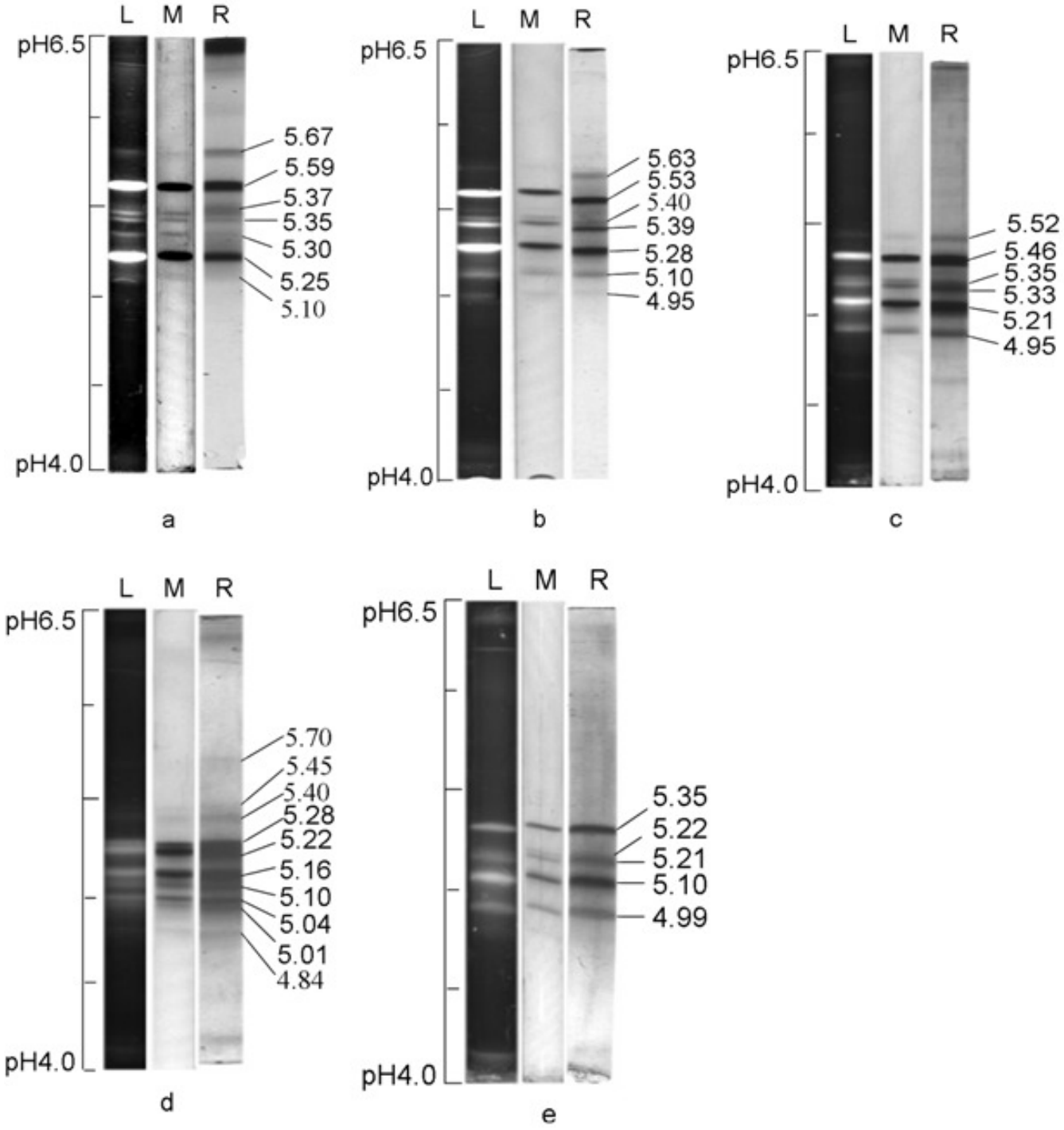

Figure 5. Denaturing polyacrylamide gel IEF of R-PEs in pH gradient 4.0 to 6.5. The R-PEs were from (a) *Polysiphonia urceolata*, (b) *Heterosiphonia japonica*, (c) *Callithamnion corymbosum*, (d) *Gloisiphonia capillaris*, (e) *Ceramium japonicum*. The samples in 8 M urea were loaded at the basic end of the gels. 0.5 M ethanolamine was used as cathode buffer and 1.0 M phosphoric acid was used as anode buffer. The gels were imaged under UV wavelength (L) at 365 nm, under cool-white light (M) and strained by Coomassie brilliant blue G-250 (R).

and their subunits are sensitive to extreme pH, and the denaturizing of phycobiliprotein was observed when 0.5 M NaOH was used as cathode buffers for the IEF. When weaker base, 0.5 M ethanolamine or 0.4 M HEPES, was

selected as cathode buffers for the IEF, the result showed clear and separated bands. The method of IEF in acidic

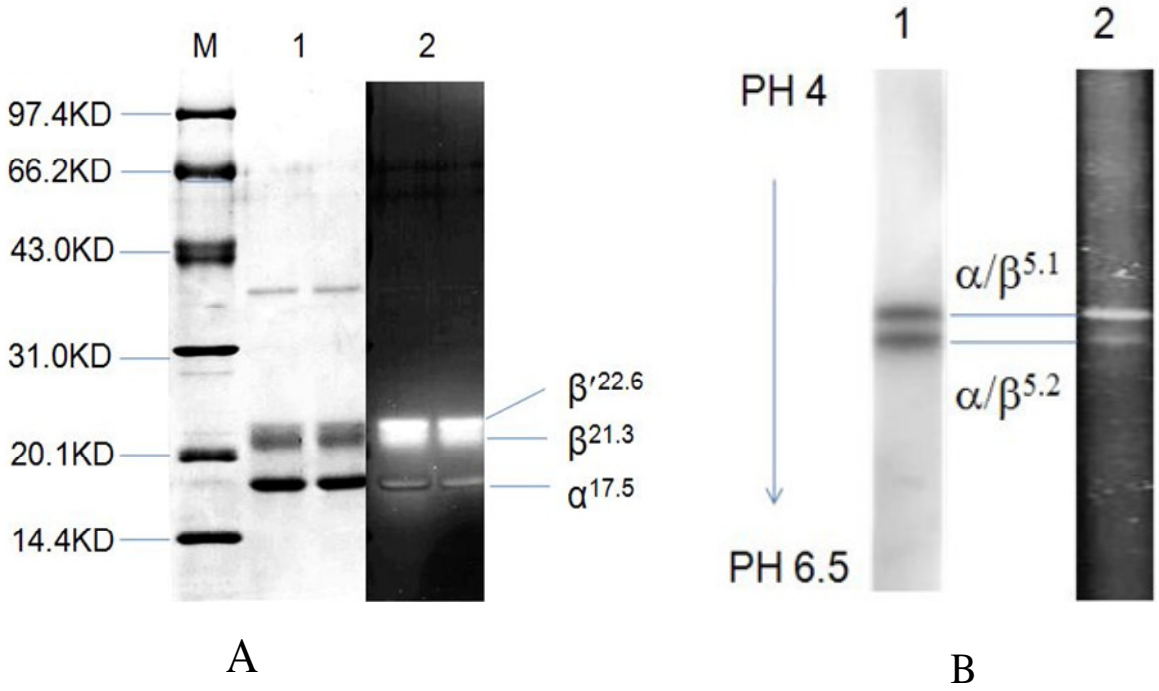

Figure 6. A is subunit analysis of R-PC from *Polysiphonia urceolata* by SDS-PAGE. M: Mark; 1: the gel was stained by Coomassie blue; 2: the gel was stained by $ZnSO_4$-imidazole. B is denaturing polyacrylamide gel IEF of R-PC from *Polysiphonia urceolata* in pH gradient 4.0 to 6.5. The R-PC in 8 M urea was loaded at the basic end of the gel. 0.4 M HEPES was used as cathode buffer and 1.0 M phosphoric acid was used as anode buffer. The gel was strained by Coomassie brilliant blue G-250 (1), The gel was imaged under UV wavelength at 365 nm (2)

pH range in this study is also a simple method for studyon other proteins.

ACKNOWLEDGEMENTS

We wish to gratefully acknowledge the support of National Natural Science Fund of china (30571720) and Prize Fund for Excellent Middle Aged and Young Scientists of Shandong Province, PR China (2006BS02016) for this work.

REFERENCES

Apt KE, Hoffman NE, Grossman AR (1993). The γ Subunit of R-phycoerythrin and Its Possible Mode of Transport into the Plastid of Red Algae. J. Bio. Chem., 22: 16208-16215.

Adir N (2005). Elucidation of the molecular structures of components of the phycobilisome: reconstructing a giant. Photosynthesis Res., 85: 15-32.

Bermejo R, Acien FG, Ibanez MJ Fernandez JM, Molina E, Alvarez JM (2003). Preparative purification of B-phycoerythrin from the microalga *Porphyridium cruentum* by expanded-bed absorption chromatography. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 790: 317-325.

Everroad C, Six C, Partensky F, Thomas JC, Holtzendorff J, Wood (2006). Biochemical Bases of Type IV Chromatic Adaptation in Marine *Synechococcus* spp. J. Bacteriol., 188: 3345-3356.

Glazer AN, Craig SH (1975). Characterization of R-PC: chromophore content of R-phycocyanin and C-phycoerythrin. J. Bio. Chem., 250: 5487-5495.

Glazer AZ (1984). Phycobilisome: a macromolecular complex optimized for light energy transfer. Biochem. Biophy. Acta., 768: 29-51.

Guo YJ (1999). Experimental Technique in Protein Electrophoresis. Science Press. Beijing: 161-208.

Guo YJ (1983). Method and principle of thin-layer isoelectric focusing. Progress Biochem. Biophys., 51: 50-55.

Jordan EM, Raymond S (1969). Gel electrophoresis: a new catalyst for acid systems. Anal. Biochem., 27: 205.

Liu LN, Chen XL, Zhang XY, Zhang YZ, Zhou BC (2005). One-step chromatography method for efficient separation and purification of R-phycoerythrin from *Polysiphonia urceolata*. J. Biotechnol., 116: 91-100.

Lyubimova T, Caglio S, Gelfi C, Righetti PG, Rabilloud T (1993a). Photopolymerization of polyacrylamide gels with methylene blue. Electrophoresis, 14: 40.

Lyubimova T, Righetti PG (1993b). On the kinetics of photopolymerization: a theoretical study. Electrophoresis, 14: 191-201.

Marsac NT (2003). Phycobiliproteins and phycobilisomes: the early observations, J. Photosynth. Res., 76: 197-205.

Ohki K, Gantt E, Claudia A, Lipschultz CA, Marjorie CE (1985). Constant Phycobilisome Size in Chromatically Adapted Cells of the *Cyanobacterium* and Variation in *Nostoc sp*. J. Plant Phys. Biol., 79: 943-948.

Rabilloud T, Vincon M, Garin J (1995). Micropreparative one- and two-dimensional electrophoresis: improvement with new photopolymerization systems. Electrophoresis, 16: 1414-22.

Rabilloud T (1996a). Solubilization of proteins for electrophoretic analyses. Electrophoresis, 17: 813-829.

Rabilloud T, Girardot V, Lawrence J (1996b). One- and two-dimensional histone separations in acidic gels: usefulness of methylene

blue-driven photopolymerization. Electrophoresis, 17: 67-73.
Righetti PG, Bossi A, Giglio M, Vailaiti A, Lyubimova T, Briskman (1994).

Is gravity on our way? The case of polyacrylamide gel polymerization. Electrophoresis, 15: 1005-10013.
Six C, Thomas JC, Thion L, Lenoine Y (2005). Two Novel Phycoerythrin Associated Linker Proteins in the Marine *Cyanobacterium Synechococcus sp*. Strain WH8102. J. Bacteriol., 187: 1685-1694.
Sun L, Wang SM, Chen LX Gong XQ (2003). Promising Fluorescent Probes
from Phycobiliproteins. IEEE J. Select. Quantum Electron, 9: 177-188.
Sun L, Wang SM, Chen LX, Gong XQ (2004). A rod-linker-contained R-phycoerythrin complex from the intact phycobilisome of the marine red alga *Polysiphonia urceolata*. J. Photochem. Photobio. B: Biol., 76: 1-11.
Sun L, Wang SM, Gong XQ, Zhao MR, Fu XJ, Wang L (2009). Isolation, purification and characteristics of R-phycoerythrin from a marine macroalga *Heterosiphonia japonica*. Protein Exp. Purif., 64: 146-154.
Wang L, Sun L, Fu XJ, Zhao MR (2008). Phycobiliprtein components of phycobilisomes from marine red alga *Polysiphonoa urceolata* Grev. J. Yantai Unversity (Natural Science and Engineering Edition). 21: 94-99.
Zhang YZ, Chen LX, Wang LS, Zhou BC, He JA, Shi DX, Pang SJ (2002). *In vitro* assembly of R-phycoerythrin from marine red alga *Polysiphonia urceolata*. J. Biochem. Biophy., 34: 99-103.