Protein R-phycoerythrin from marine red alga *Amphiroa anceps*: extraction, purification and characterization

Sarkar M. A. Kawsar[1], Yuki Fujii[2], Ryo Matsumoto[2], Hidetaro Yasumitsu[2] & Yasuhiro Ozeki[2]

[1] Laboratory of Carbohydrate and Protein Chemistry, Department of Chemistry, Faculty of Science, University of Chittagong, Chittagong-4331, Bangladesh, e-mail: akawsarabe@yahoo.com (corresponding author)

[2] Laboratory of Glycobiology and Marine Biochemistry, Department of Genome System Sciences, Graduate School of Nanobiosciences, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan

Received: October 13, 2011 ▷ Accepted: November 28, 2011

Abstract. The paper reports an efficient procedure for the extraction and purification of R-phycoerythrin (R-PE) from marine red algae *Amphiroa anceps*. *A. anceps* was extracted in 50 mM phosphate buffered saline and precipitated by $(NH_4)_2SO_4$. The R-PE was isolated by ion-exchange chromatography loaded with Q-Sepharose Fast Flow, which was developed by linear ionic strength gradients. Then it was purified by gel filtration Sepharose CL-6B column chromatography. SDS-PAGE showed the presence of two major subunits with 18 kDa and 20 kDa, respectively, and a minor subunit of 30 kDa. The observations are consistent with the $(\alpha\beta)_6\gamma$ subunit composition characteristic of R-PE. The absorption spectrum of R-PE was characterized by three absorbance maxima at 563, 538 and 495 nm, respectively, and the fluorescence emission spectrum at room temperature was 580 nm. Furthermore, R-PE showed good stability between pH 3.5 and 9.5. However, under oxidative conditions R-PE lost its shining. The results indicate that using ion-exchange chromatography with Q-Sepharose and gel filtration Sepharose CL-6B chromatography, R-PE can be purified on large scale from the red alga *A. anceps*.

Key words: *Amphiroa anceps*, extraction, purification, R-phycoerythrin, SDS-PAGE

Introduction

Phycobiliproteins are a family of light-harvesting pigment protein complexes widely found in the chloroplasts of red algae and cyanobacteria (MacColl 1998). They are divided into three classes according to their absorption properties: phycoerythrin (PE; λ_{max}=565 nm), phycocyanins (PC; λ_{max}=620 nm) and allophycocyanins (AP; λ_{max}=650 nm) (Sidler 1994). Phycobiliproteins absorb light at 450 nm to 650 nm, when chlorophyll-a absorbs poorly, and transfer the energy to Photosystem II (Redlinger & Gantt 1982). Energy is transferred successively from PE to PC, then to AP, and finally to chlorophyll-a, with an overall quantum efficiency close to 90 % (Glazer 1989). Because of its spectral properties, PE is widely used in biochemical techniques and clinical diagnoses. Phycoerythrins (PEs) can be divided into three main classes, depending on their absorption spectrum: B-phycoerythrin (B-PE), R-phycoerythrin (R-PE) and C-phycoerythrin (C-PE) (Marsac 2003).

R-phycoerythrins (R-PEs) are the most abundant phycobiliproteins in the marine red algae. They

are fluorescent, with high quantum efficiency, a large Stokes shift and excitation and emission bands at visible wavelengths. Their special absorption spectrum in native state is a three-peak spectrum with absorption maxima at 565, 539 and 498 nm, respectively. Concerning immunodiagnostics in which 488 nm is often used as an exciting wavelength, R-PEs are better than B-PEs because the 498 nm maximum is more available (Telford & al. 2001). They are highly water-soluble and stable proteins that can be easily linked to antibodies and other proteins by conventional protein cross-linking techniques, without altering its spectral characteristics. Thus, R-PEs are currently widely used in the production of food and cosmetics, and play an important role in many biochemical techniques due to their fluorescence properties (Albertsson 2003). R-PE is an oligomeric protein of 240 kDa, with 6 α (~20 kDa), 6 β (~20 kDa) and 1 γ (~30 kDa) subunits, i.e. as $(\alpha\beta)_6\gamma$. R-PE is commonly used as a fluorescent label in immunology, cell biology (Kronik 1986) and flow cytometry (Wilson & al. 1991). It is also applied as a natural food dye (D'Agnolo & al. 1994) and as a marker in gel electrophoresis and isoelectrofocusing (Araoz & al. 1998).

It is necessary to adopt a method for obtaining effectively high purity of R-PEs for its application in life and research. However, the traditional methods applied in the purification of PEs, involving chromatography on hydroxylapatite, expanded-bed adsorption (Rossano & al. 2003), or preparative polyacrylamide gel electrophoresis (Galland-Irmouli & al. 2000), are lengthy and complex procedures. It is desirable to develop a rapid and efficient process for separation and purification of R-PE from good red algae with a high degree of purity. A method based on chromatography on hydroxylapatite connected to Sephadex 75 gel filtration obtained pure R-PE in two steps from *Corallina elongata* (Rossano & al. 2003), with a purity index A_{566}/A_{280} higher than 5.0, and containing α/β (20 kDa) and γ (30 kDa) subunits according to SDS-PAGE. Among ttraditional separation methods, ion-exchange chromatography showed some advantages in the overall process.

Amphiroa anceps is a marine red alga of the macroalga type and grows luxuriantly in the local sea from January to April, when the temperature of sea water ranges from 5° to 15 °C. To the best of our knowledge, no R-PE has been purified so far from *A. anceps*. Thus, this paper describes a two-steps chromatography method for efficient extraction and purification of R-PE from *Amphiroa anceps*, involving fraction precipitation with ammonium sulfate, ion-exchange chromatography with a gradient of NaCl and gel filtration Sepharose CL-6B chromatography. Phycoerythrin stability as a function of pH is also investigated for future applications in biochemical techniques.

Material and methods

Plant material

Red sea macroalgae of *Amphiroa anceps* (Lam.) Decne. were collected at a depth of 3 to 5 m in the inter-tidal zone of along Zushi coast, Kanagawa Prefecture, Japan. The species was rinsed with seawater and the contaminating algae and alien substances were removed.

Chemicals

Standard protein markers for gel permeation chromatography were purchased from Sigma, USA. A standard protein marker mixture (Daiichi-III) for SDS-PAGE was purchased from Daiichi Pure Chem. Co. Ltd., Japan. Sepharose CL-6B and Q Sepharose were obtained from GE Health Sciences and Sigma, USA. A bicinchoninic acid (BCA) kit was purchased from Pierce Co. Ltd., USA. Ammonium sulfate, sodium chloride and all other chemicals were from Wako Pure Chem. Ind. Ltd., Japan. All buffers and reagents used in the purification were prepared in Milli Q water (MILLIPORE, USA).

Extraction of R-PE from *Amphiroa anceps*

The phycobiliproteins were extracted from the fresh alga *A. anceps* (35 g wet weight) in 500 ml of 50 mM phosphate buffered saline (PBS), pH 7.4, containing 150 mM NaCl at 4 °C. To prevent the biliproteins from degrading during the experiments, 4 mM NaN_3, 2 mM 2-mercaptoethanol and 2 mM EDTA were added to the 50 mM PBS. The fresh alga was cut into small pieces and crushed in a mortar at first; then it was homogenized in a commercial blender (Waring, USA). The extract was roughly filtrated via a filter bag to remove debris and the filtrate was centrifuged at 14,720 g for 30 min at 4 °C with a Suprema 21 centrifuge equipped with a NA-18 HS rotor (TOMY Co. Ltd., Japan). Biliproteins in the alga residue were further extracted in the same way. After that the combined biliprotein solution was again centrifuged at 27,500 g for 20 min at 4 °C. The

red supernatant, called R-PE extract, was collected and stored at 4 °C in the dark.

The supernatant was further fractioned with $(NH_4)_2SO_4$ at 80 % (w/v) saturation at 4 °C. Solid ammonium sulfate (56.1 g per 100 ml of the extract) was added slowly by gentle stirring, and the solution was left to rest for 2 h and then centrifuged at 27,500 g for 20 min at 4 °C. Subsequently, solid $(NH_4)_2SO_4$ was again added by stirring to the red supernatant. A precipitate was obtained by the above-mentioned centrifuging process. Then the precipitate was dissolved in 50 mM PBS containing 150 mM NaCl (pH 7.4) and was dialyzed overnight against the same buffer. The dialyzed, red-colored solution was passed through a 0.22 μm Millipore membrane filter.

Purification of R-PE by ion-exchange chromatography

The dialyzed samples of R-PE were subjected to ion exchange chromatography in a loaded with Q Sepharose Fast Flow column (26 mm×100 mm), which was pre-equilibrated with 50 mM PBS (pH 7.4) containing 150 mM NaCl. After the samples passed through it, the column was extensively washed with equilibrium buffer, until the 280 nm absorbance of the following solution was stable. Ion-exchange chromatography was carried out at an elution rate of 1 ml/min, by using linear gradient elution from 0.0 mM, 30 mM, 60 mM, 100 mM, 200 mM, 300 mM, 400 mM, and 600 mM NaCl. The eluted red-colored fractions were collected. The elution was monitored by absorption at 280 nm. The obtained R-PE fractions were examined by an absorption spectrum from 200 to 700 nm and were combined and concentrated by TOMY concentrator (TU-055, Japan).

Gel filtration Sepharose CL-6B chromatography using FPLC

Gel filtration chromatography with Sepharose CL-6B (1.0 × 65 cm) connected with a FPLC system was employed for further R-PE purification after the ion-exchange column chromatography. The gel column was equilibrated with 50 mM PBS (pH 7.4) at 1 ml/min. R-PE samples amounting to 10 % of the total column volume were loaded into the column. The loaded column was eluted at 1 ml/min with the same phosphate buffer as the one used in the column equilibrium. The fractions eluted by the column were monitored by absorption at 280 nm. The bright-red fractions rich in R-PE were collected as purified R-PE and used for characterization.

Absorption and fluorescence emission spectrum identification of R-PE

The absorption spectrum of the eluting PE fractions was determined by using UV-2000 monitor (ATTO Co. Ltd., Japan). The scan wave length was 200–700 nm. The purity was evaluated according to the absorbance ratios A_{565}/A_{280}. The fluorescence emission spectrum was determined by using FP-2020 Plus spectrofluorometer (JASCO Co., Japan) with an excitation wavelength of 498 nm. All spectra were recorded at room temperature.

Electrophoretic identification of R-PE

The polypeptide components of purified R-PE were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Purified R-PE was mixed with an equal amount of sample buffer (20 mM Tris-HCl, pH 6.8; 0.2 % SDS, and 20 % glycerol) and then heated at 70 °C for 15 min. Aliquots of 30 μl were applied to the well of a mini-slab gel (gel size: 80 mm×100 mm with 1 mm thickness; 12 % and 5 % polyacrylamide was used in separation and upper gels, respectively). The molecular mass of the polypeptide was determined by SDS-PAGE, according to an earlier report (Laemmli 1970). After SDS-PAGE, the red bands of R-PE were examined by enhancement of the bright-yellow fluorescence emitted by R-PE under ultraviolet light at 365 nm.

Protein determination

The total protein concentration of extracts was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, USA), according to the manufacturer's instructions (Smith & al. 1985; Wiechelman & al. 1988). Bovine serum albumin (BSA) was used as a standard in measuring absorbance at 562 nm with ND-1000 spectrophotometer (Nano Drop Tech. Inc., USA). The concentration of R-PE (mg/ml) in all extracts was estimated by absorbance at 565 nm.

Effect of pH and oxidative-reductive reagent

PE stability as function of pH and oxidative-reductive reagents were monitored spectrophotometrically (UV-2000 monitor; ATTO Co. Ltd., Japan). PE stability was investigated within the range of pH 3 to 11. Purified R-PE was dissolved in 20 mM sodium acetate-acetic acid (pH 3–5), 20 mM sodium phosphate-HCl (pH 6–7), 20 mM Tris-HCl (pH 8–8.5), and 20 mM glycine-NaOH (pH 9–10) for 2 h at room tempera-

ture, and the differentiation of absorption was measured by UV spectroscopy. PE stability to oxidative-reductive reagents was also investigated by using $CuSO_4$ solution and β-mercaptoethanol, respectively. Adsorption spectra of R-PE were determined by adding different concentration of $CuSO_4$ solution and β-mercaptoethanol for 1–2 h at room temperature.

Results

Isolation of R-PE from *A. anceps*

The complete R-PE isolation and purification method is shown in Fig. 1. Thirty-five grams of lyophilized algae were re-suspended in three volumes of buffer containing 50 mM phosphate buffer PBS/150 mM NaCl/pH7.4, blended in a cold Waring blender and centrifuged. The red-colored supernatant was fractioned with 80% $(NH_4)_2SO_4$ and the resulting slurry was mixed with a stirrer. The slurry was then centrifuged and the supernatant obtained after centrifugation and the absorption spectrum of the supernatant showed maximum corresponding proteins. The supernatant was subjected to ion-exchange and gel filtration Sepharose CL-6B column chromatography for purification. The supernatant revealed R-PE content of 1.65 g (data not shown).

Purification of R-PE from *A. anceps*

Purification by ion-exchange chromatography

The Q-Sepharose captured R-PE was eluted with 50 mM phosphate buffer (pH7.4) containing NaCl with a gradient from 0 to 600 mM successively. The eluates were collected and then the fractions were measured for the following spectroscopic features by using a UV-2000 spectrophotometer: peak 1 (Fig. 2) fawn-

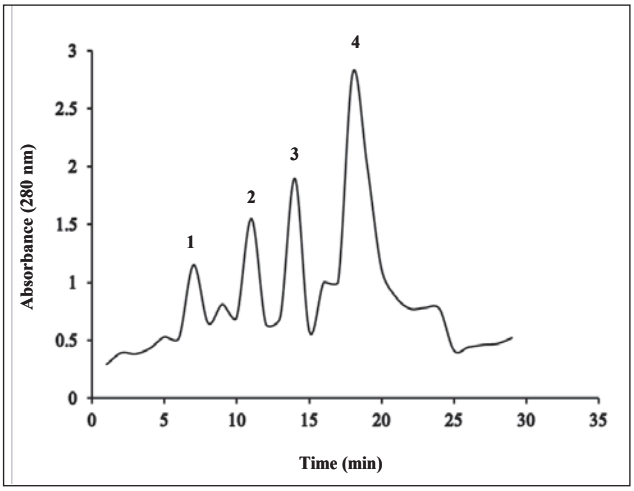

Fig. 2. Ion exchange chromatography of R-PE from *A. anceps*. The elution profile was monitored at 280 nm. Peak 4 was of R-PE containing solution.

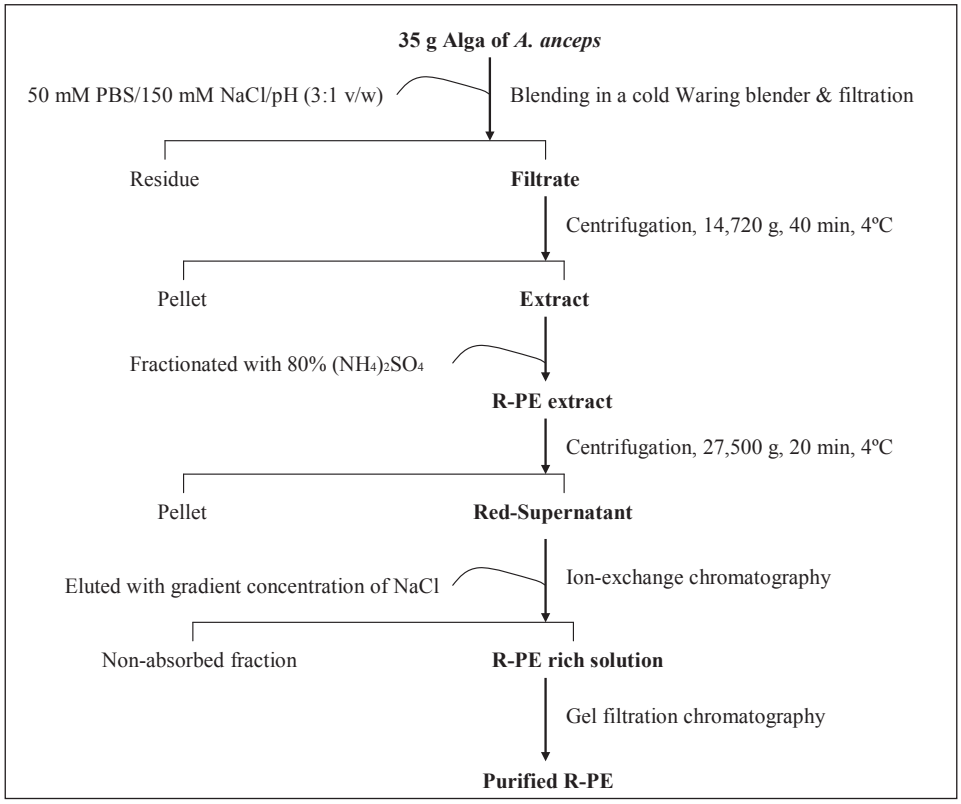

Fig. 1. Flow sheet for R-PE extraction, isolation and purification from *A. anceps*.

colored solution containing some impurities and pigments; peak 2 colorless solution, probably some unbound proteins with maximum of 280 nm; peak 3 light-blue solution, with maxima at 280, 350 and 545 nm, containing some of the impurities and R-PE; peak 4 red-colored solution, with maxima at 280, 374, 498, 539, and 565 nm, containing R-PE. The R-PE-rich fraction was subjected to gel filtration CL-6B column chromatography for further purification.

Purification by gel filtration Sepharose CL-6B chromatography

The gel filtration Sepharose CL-6B chromatography for R-PE purification is shown in Fig. 3. When the Sepharose CL-6B column was eluted with 50 mM phosphate buffer (pH 7.4), two fractions were obtained: one rich in R-PE and the other containing some small molecular substances with strong absorbance at 280 nm. The R-PE-containing fraction was collected and re-chromatographed in the same column. After elution, the column was further eluted with 50 mM PBS (pH 7.4) as a single band of R-PE. The purified R-PE was taken for characterization.

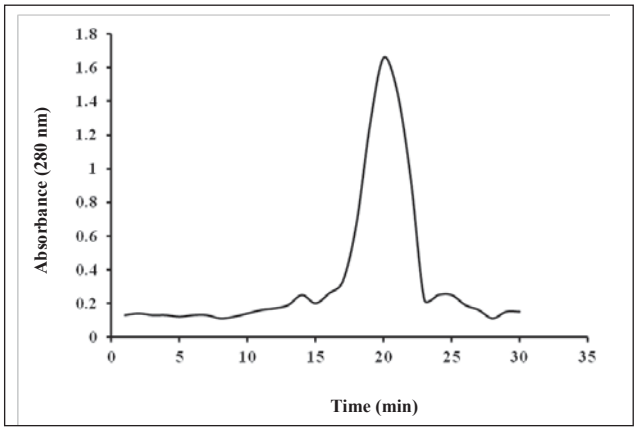

Fig. 3. Gel filtration column chromatography of R-PE. Gel filtration was achieved with 50 mM phosphate buffer (pH 7.4) containing 150 mM NaCl at a flow rate of 1ml/min, and monitored by absorption at 280 nm.

Identification of purified R-PE

Spectroscopic identification

The absorption spectrum of the eluting R-PE solution was obtained by using UV-2000 spectrophotometer. The scan wavelength was from 200 to 700 nm. The R-PE obtained was typically a "three peak" PE with absorption maxima at 565, 539, and 498 nm, as shown in Fig. 4. It showed an absorption spectrum typical of R-PE and perfectly related to earlier works (Yu & al. 1981; Apt & al. 1993). The fluorescence emission spectrum is also shown in Fig. 4 by the broken line and the fluorescence emission maximum was at 580 nm, with an exciting wavelength of 498 nm. This result was consistent with the earlier publications on the fluorescence spectrum of PE (Bermejo & al. 2002). Thus, the obtained PE was confirmed to as typical R-PE from the spectra information above.

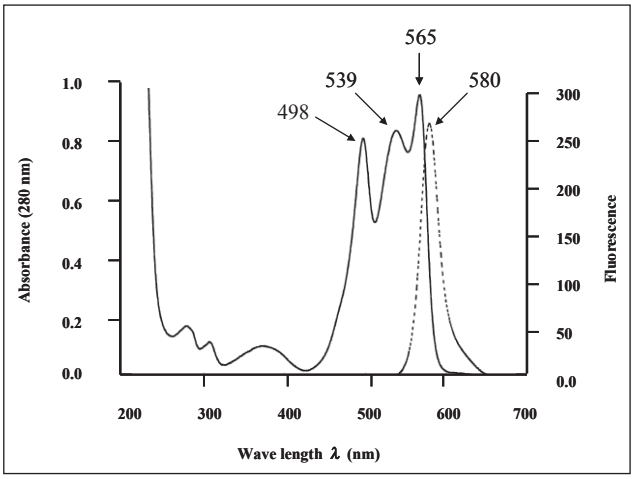

Fig. 4. Absorption spectrum and fluorescence emission spectrum of R-PE from *A. anceps*. The absorption maxima were at 498, 539 and 565 nm, respectively in the absorption spectrum (–). The exciting wavelength was 498 nm in the fluorescence spectrum (--) and the fluorescence emission maximum was at 580 nm.

Electrophoretic identification

The purity of R-PE was also confirmed by electrophoresis. Crude extraction (C) and a R-PE containing fraction (P) were subjected to SDS-PAGE with 12% acrylamide (Fig. 5) and showed three bands corresponding to its three subunits: α, β and γ with the molecular weights determined as 18, 20 and 30 kDa, respectively. Therefore, pure R-PE with three subunits (α, β, γ) was obtained.

R-PE recovery

R-PE recovery is expressed as percentage of the total amount of R-PE obtained per amount of R-PE before each treatment step. The PE content in the different solutions was estimated from measurements of absorbance at 565 nm. Apparently, it is difficult to obtain a high recovery yield, if there are too many steps within the entire process. In the present work, a high recovery yield was obtained by efficient chromatographic methodology.

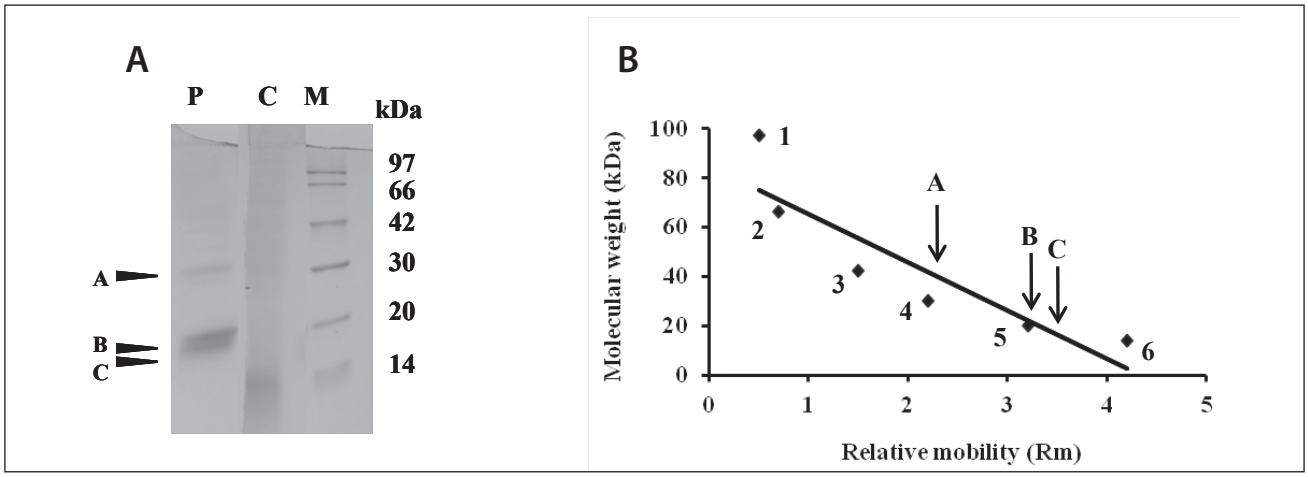

Fig. 5. SDS-PAGE of R-PE from *A. anceps*. (A) C: crude extract of *A. anceps* in the phosphate buffer (pH 7.4); P: purified R-PE. Electrophoresis was run in 12 % polyacrylamide as a separating gel. M: molecular weight markers (from top to bottom): 1: Phosphorylase-b (M_r 97 kDa); 2: Bovine serum albumin (M_r 66 kDa); 3: Ovalbumin (M_r 42 kDa); 4: Carbonic anhydrase (M_r 30 kDa); 5: Trypsin inhibitor (M_r 20 kDa), and 6: Lysozyme (M_r 14 kDa). Molecular weights of the three subunits α, β, γ corresponding to the three bands with 18, 20 and 30 kDa, respectively. (B) calibration curve obtained by R_f values.

Optimal pH range and oxidative-reductive state of R-PE activity

R-PE was stable between pH 3.5 and 9.5. In acidic medium, R-PE lost the red color or its activity shown in Fig. 6. When R-PE was treated with an oxidizing agent, the red color, i.e., shining was also lost. On the other hand, the red color remained when R-PE was treated with b-marcaptoethanol. The absorption spectra of the protein characteristics peaks were lost.

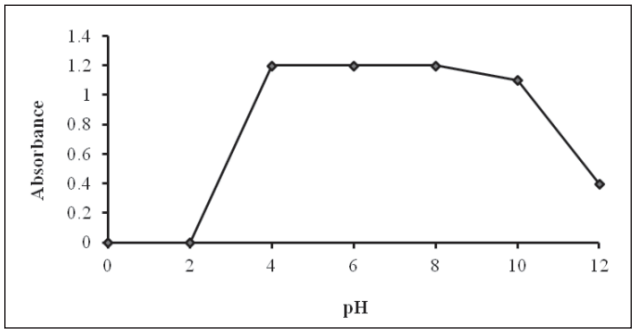

Fig. 6. pH stability of R-PE from *A. anceps*. The effects of various pH on protein activity.

Discussion

The aims of this study were to find both a good natural source of R-PE and a simple procedure for its purification (Fig. 1). For that purposes, R-PE was purified from red alga *A. anceps* by ion-exchange (Fig. 2) and gel filtration Sepharose CL-6B column chromatography (Fig. 3). R-PE has high fluorescent properties and is thus widely used in food and cosmetics industry, in immunodiagnostics and as analytical reagent. Different methodologies have been proposed for purifying phycobiliproteins from algae, namely hydroxylapatite chromatography, expanded-bed chromatography, preparative polyacrylamide gel electrophoresis, etc., but few of them proved useful for scaling up and the resolution ratio was relatively low. For instance, hydroxylapatite chromatography is a better way to purify phycobiliproteins as reported in many papers (Rossano & al. 2003), but separation ability depends on the quality of particles of hydroxylapatite. Regeneration capacity of hydroxylapatite was not very good and other complexes were easily absorbed at the top of the column, which even made the elute asymmetry influence the separation effects. Therefore, hydroxylapatite chromatography is often performed repeatedly, or combined with other separation techniques. Galland-Irmouli & al. (2000) reported that pure R-PE with an absorbance ratio of 3.2 was obtained from *Palmaria palmata* by using one-step preparative polyacrylamide gel electrophoresis. It provided a good way for separation of phycobiliproteins but the yield of R-PE obtained was small and the purification value of this method was low. Ion-exchange chromatography and gel filtration chromatography were developed for separation and purification of PEs and ion-strength gradient elution was adopted. This kind of chromatography has shown many advantages, such as simple and rapid purification. We have studied purification of R-PE from *Amphiroa anceps* with Q-Sepharose Fast Flow ion-exchange chromatography and

gel filtration by Sepharose CL-6B chromatography. The obtained R-PE has proved pure, after using an absorption spectrum, fluorescence spectrum (Fig.4), and SDS-PAGE (Fig.5). Out of 35 g of lyophilized alga, we have recovered 25 mg of pure R-PE by the above-described method (data not shown). This methodology provides better results than the others. Galland-Irmouli & al. (2000) for instance, have indicated 18 % recovery, when the biomass is frozen with liquid nitrogen and extracted with phosphate solution, whereas Sarada & al. (1999) have indicated that 50 % of the proteins were lost, when the biomass was dried. Only Tchernov & al. (1999) have reported a high yield of 60 % with frozen biomass, phosphate buffer and rivanol in precipitation of colorless proteins. Sodium dodecyl sulfate-PAGE with 12 % acrylamide showed three bands: two predominant pink and fluorescent bands of equal intensity, namely M_r estimated at 18 and 20 kDa and a much smaller slightly fluorescent band of M_r at about 30 kDa (Fig. 5). Therefore, this observation is consistent with a (αβ)6γ subunit composition, characteristic of R-PE. In fact, R- and B-PEs have three types of subunits: α and β (about 20 kDa, acidic isoelectric point, p*I*) and γ (about 30 kDa, basic p*I*), and both have a (αβ)6γ subunit composition, whereas the oligomeric composition of C-PE is (αβ)6 (Glazer 1989). The crystal structure of R-PE from *Polysiphonia urceolata* was determined (Jiang & al. 1999). The γ subunit is assumed to be a hydrophobic linker protein located in the central channel of the PE αβ torus (Glazer 1989). PE is also particularly appropriate for use as a protein marker in such electrophoretic techniques as SDS-PAGE, or isoelectric focusing and size-gel exclusion chromatography and probably could be used as alimentary or cosmetic colorant (Araoz & al. 1998).

PE stability to pH variations (Fig. 6) was monitored spectrometrically. R-PE of *A. anceps* displayed a good stability at pH variations, and unimportant modifications of color and fluorescence were observed between pH 3.5 and 9.5. Variation of pH changed the absorption spectrum of this protein, indicating change in the aggregation state or denaturation of the protein. The color was lost at pH lower than 1–3. The spectrum properties of phycobiliproteins are pH-dependent. Thus, R-PE (pH 3.5–9.5) can be used as a colorant in food products and also as a fluorescent tag or probe. In fact, PEs are widely used in biochemical techniques, due to their exceptionally high molar absorption coefficient near 2.4×10^6 m^{-1} cm, and quantum yield near 0.8, which contribute to high sensitivity (Oi & al. 1982, Kronik 1986). Therefore, PE emits in the orange-red band (fluorescence emission maximum of 580 nm), where background fluorescence is exceptionally low (Oi & al. 1982). Thus, PE conjugate, as an antibody-phycoerythrin complex, protein A-phycoerythrin and avidin-phycoerythrin conjugates can be used as probes in fluorescence flow cytometry, fluorescence microscopy and fluorescence immunoassay (Oi & al. 1982, Kronik 1983). PE can also be used as a probe in the proximity or interaction assays between two molecules by fluorescence resonance energy transfer (Ha & al. 1996), where one of the molecules is conjugated to PE and the other to allophycocyanin. Purification of R-PE from the red alga *Amphiroa anceps* has been achieved with ion-exchange and gel filtration chromatography. Thus purified R-PE showed good stability between pH 3.5 and 9.5. The fluorescence, spectral and stability properties of R-PE from *A. anceps* present a good opportunity for investigation of its future applications in biochemical techniques.

Conclusions

The aims of this study were to find both a local natural source of R-PE and a new simple procedure for its purification. This method can be effectively and economically used in large-scale isolation and purification of R-PE. Our results indicate that *A. anceps* could be used as a good alternative source of R-PE.

Acknowledgements. This work was partially supported by a Grant-In-Aid for scientific research of the Japan Society for the Promotion of Science (JSPS). The authors are grateful to Dr. Robert A. Kanaly (USA & YCU) for his critical notes on the manuscript.

References

Albertsson, P.A. 2003. The contribution of photosynthetic pigments to the development of biochemical separation methods: 1900–1980. – Photosynth. Res., **76**(1-3): 217-225.

Apt, K.E., Hoffman, N.E. & Grossman, A.R. 1993. The gamma subunit of R-phycoerythrin and its possible mode of transport into the plastid of red algae. – J. Biol. Chem., **268**(22): 16208-16215.

Araoz, R., Lebert, M. & Hader, D.P. 1998. Electrophoretic applications of phycobiliproteins. – Electrophoresis, **19**(2): 215-219.

Bermejo, R.R., Alvarez-pez, J.M., Acien Fernandez, F.G. & Molina, G.E. 2002. Recovery of pure B-phycoerythrin from the microalga *Porphyridium cruentum*. – J. Biotechnol., **93**(1): 73-85.

D'Agnolo, E., Rizzo, R., Paoletti, S. & Murano, E. 1994. R-Phycoerythrin from the red alga *Gracilaria longa*. – Phytochemistry, **35**: 693-696.

Galland-Irmouli, A.V., Pons, L., Lucon, M., Villaume, C., Mrabet, N.T., Gueant, J.L. & Fleurence, J. 2000. One-step purification of R-phycoerythrin from the red macroalga *Palmaria palmate* using preparative polyacrylamide gel electrophoresis. – J. Chromatogr. B: Biomed. Sci. Appl., **739**(1): 117-123.

Glazer, A.N. 1989. Light guides. Directional energy transfer in a photosynthetic antenna. – J. Biol. Chem., **264**(1): 1-4.

Ha, T., Enderle, T., Ogletree, D.F., Chemla, D.S., Selvin, P.R. & Weiss, S. 1996. Probing into the interaction between two single molecules: fluorescence resonance energy transfer between a single donor and a single acceptor. – Proc. Natl. Acad. Sci. USA., **93**(13): 6264-6268.

Kronik, M.N. 1986. The use of phycobiliproteins as fluorescent labels in immunoassay. – J. Immunol. Methods, **92**(1): 1-13.

Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. – Nature, **227**(5259): 680-685.

MacColl, R. 1998. Cyanobacterial phycobilisomes. – J. Struc. Biol., **124**(2-3): 311-334.

Marsac, N.T. 2003. Phycobiliproteins and phycobilisomes: the early observations. – Photosynth. Res., **76**(1-3): 193-205.

Oi, V.T., Glazer, A.N. & Stryer, L. 1982. Fluorescent phyconiliprotein conjugates for analyses of cells and molecules. – J. Cell Biol., **93**(3): 981-986.

Redlinger, T. & Gantt, E. 1982. A Mr 95000 polypeptide in *Porphyridium cruentum* phycobilisomes and thylakoids: possible function in linkage of phycobilisomes to thylakoids and in energy transfer. – Proc. Natl. Acad. Sci. USA., **79**(18): 5542-5546.

Rossano, R., Ungaro, N., D'Ambrosio, A., Liuzzi, G.M. & Riccio, P. 2003. Extracting and purifying R-phycoerythrin from the Mediterranean red algae *Corallina elongate* Ellis & Solander. – J. Biotechnol., **101**(3): 289-293.

Sarada, R., Pillai, M.G. & Ravisharkar, G.A. 1999. Phycocyanin from *Spirulina* spp: influence of processing of biomass on phycocyanin yield, analysis of efficacy of extraction methods and stability studies on phycocyanin. – Process Biochem., **34**: 795-801.

Sidler, W.A. 1994. Phycobilisome and phycobiliprotein structures – In: **Bryant, D.A.** (eds). The Molecular Biology of *Cyanobacteria*, Kluwer Acad. Publication, Netherlands, pp. 139-216.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. & Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. – Anal. Biochem., **150**(1): 76-85.

Tchernov, A.A., Minkova, K.M., Houbavenska, N.B. & Kovacheva, N.G. 1999. Purification of phycobiliproteins from *Nostoc* sp. by aminohexyl-Sepharose chromatography. – J. Biotechnol., **69**: 69-73.

Telford, W.G., Moss, M.W., Morseman, J.P. & Allnutt, F.C. 2001. Cyanobacterial stabilized phycobilisomes as fluorochromes for extracellular antigen detection by flow cytometry. – J. Immunol. Meth., **254**(1-2): 13-30.

Wilson, M.R., Crowley, S., Odgers, G.A. & Shaw, L. 1991. Immunofluorescent label in using covalently linked anti-Phycoerythrin antibodies and Phycoerythrin polymers. – Cytometry, **12**(4): 373-377.

Wiechelman, K.J., Braun, R.D. & Fitzpatrick, J.D. 1988. Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. – Anal. Biochem., **175**(1): 231-237.

Yu, M.H., Glazer, A.N., Spencer, K.G. & West, J.A. 1981. Phycoerythrins of the red alga *Callithamnion*. – Plant Physiol., **68**(2): 482-488.