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One-step purification of R-phycoerythrin from the red macroalga *Palmaria palmata* using preparative polyacrylamide gel electrophoresis

A.V. Galland-Irmouli[a,b], L. Pons[a], M. Luçon[b], C. Villaume[c], N.T. Mrabet[a], J.L. Guéant[a,*], J. Fleurence[b,1]

[a]*Laboratoire de Pathologie Cellulaire et Moléculaire en Nutrition, EP CNRS 0616, Faculté de Médecine, BP 184, 54505 Vandoeuvre lès Nancy, France*

[b]*Laboratoire de Qualité et Physico-Chimie, IFREMER, Rue de l'Ile d'Yeu, BP 1105, 44311 Nantes Cedex 03, France*

[c]*Equipe de Recherches Aliment et Comportement, INSERM U308, 38 Rue Lionnois, 54000 Nancy, France*

Abstract

Phycoerythrin is a major light-harvesting pigment of red algae and cyanobacteria widely used as a fluorescent probe. In this study, phycoerythrin of the red macroalga *Palmaria palmata* was extracted by grinding the algal sample in liquid nitrogen, homogenisation in phosphate buffer and centrifugation. Phycoerythrin was then purified from this crude extract using preparative polyacrylamide gel electrophoresis (PAGE) with a continuous elution system and detected by its pink colour and fluorescence. The pigment presented a typical spectrum of R-phycoerythrin, with three absorbance maxima at 499, 545 and 565 nm, and displayed a fluorescence maximum at 578 nm. The absorbance ratio A_{565}/A_{280}, a criterion for purity, was 3.2. A single protein of relative molecular mass 240 000 was detected on native-PAGE with silver staining. Sodium dodecyl sulphate–PAGE demonstrated the presence of two major subunits with M_r 20 000 and 21 000, respectively, and a very minor subunit of M_r 30 000. These observations are consistent with the $(\alpha\beta)_6\gamma$ subunit composition characteristic of R-phycoerythrin. Phycoerythrin of *Palmaria palmata* was determined to be present in larger amounts in autumn and showed a good stability up to 60°C and between pH 3.5 and 9.5. In conclusion, phycoerythrin of *Palmaria palmata* was purified in a single-step using preparative PAGE. Obtaining pure R-phycoerythrin of *Palmaria palmata* will allow one to evaluate its fluorescence properties for future applications in biochemical techniques.

Keywords: *Palmaria palmata*; Phycoerythrin

1. Introduction

Phycobiliproteins are major light-harvesting pigments found in the chloroplasts of red algae and cyanobacteria [1,2]. They are divided into three classes based on their absorption properties: phycoerythrins (λ_{max}=565 nm), phycocyanins (λ_{max}=620 nm) and allophycocyanins (λ_{max}=650 nm) [3,4]. These proteins are assembled into an organised cellular structure, the phycobilisome. This complex consist of a core and a set of rod substructures which radiate from the core. Phycoerythrin is located at the tip of the rod, phycocyanin in the middle and allophycocyanin forms the core of the phycobilisome [2–4]. Phycobiliproteins absorb light from 450 nm to

*Corresponding author. Fax: +33-3-8359-2798.

[1]Author for reprint requests.

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650 nm, where chlorophyll a absorbs poorly, and transfer the energy to Photosystem II [2,4,5]. Energy is transferred successively from phycoerythrin to phycocyanin, then to allophycocyanin, and finally to chlorophyll a, with an overall quantum efficiency close to 100% [2–4,6,7]. Because of its spectral properties, phycoerythrin is widely used in biochemical techniques and clinical diagnoses. Phycoerythrins can be divided into three main classes, depending on their absorption spectrum: B-phycoerythrin (peaks at 545, 565 nm with a 499 shoulder), R-phycoerythrin (peaks at 499, 565 nm and a 545 shoulder) and C-phycoerythrin (peak at 565 nm) [4,8,9]. B- or R-phycoerythrins are the most abundant phycobiliproteins in marine red algae.

Phycoerythrin, as other biliproteins, is highly water-soluble [2,3]. Phycoerythrin is classically purified by a combination of several techniques such as ammonium sulphate precipitation, ion-exchange chromatography, gel filtration and chromatography on hydroxylapatite [9–11]. Purification procedures are often long and complex. For example, R-phycoerythrin of the red macroalga *Corallina officinalis* was purified by extraction in phosphate buffer, ammonium sulphate precipitation (35–45%), Sepharose 4B-Cl-200 chromatography, DEAE–cellulose chromatography and finally Sephacryl S-200 chromatography [9]. This purification procedure yielded a total of 12 mg of pure phycoerythrin from 150 g of wet algae and the purified phycoerythrin was used to prepare a derived phycofluor probe for detecting sugar-binding sites on cell membranes.

In this paper, we describe a single-step purification procedure, convenient and fast, and concise characterisation of phycoerythrin from the red macroalga *Palmaria palmata*. Phycoerythrin stability as a function of pH and temperature is also investigated for future applications in biochemical techniques.

2. Experimental

2.1. Material

The *Palmaria palmata* specimens were collected at Belle Ile (French Brittany coast). Epiphytes were removed and samples were successively rinsed with seawater and distilled water. The algae were then freeze–dried.

2.2. Purification

All procedures were carried out at 4°C or below.

2.2.1. Preparation of crude algal extract

Freeze–dried algae (10 g dry mass) were ground in liquid nitrogen. The resulting algal powder was then suspended in 40 ml of 0.02 *M* phosphate buffer (pH 7.0) and homogenised with an UltraTurax for 3×1 min at 24 000 rpm. The homogenate was clarified by centrifugation at 20 000 *g* for 20 min. The resulting supernatant was filtered through a glass microfibre filter (Whatman, Maidstone, UK) and subjected to preparative electrophoresis.

2.2.2. Preparative electrophoresis

Preparative electrophoresis was performed using a Prep Cell (Bio-Rad, Hercules, CA, USA), with a stacking gel of 4% and a separating gel of 7% acrylamide. The sample (8 mg crude algal extract, 1.5 ml) contained 20 m*M* Tris–HCl (pH 6.8), 4% (v/v) glycerol, 0.009% (w/v) bromophenol blue. The column (7×2.8 cm I.D.) was eluted with 25 m*M* Tris–HCl buffer (pH 8.3), 0.192 *M* glycine at a constant flow-rate of 0.4 ml/min. The separation was done under 20 mA for 20 h. Fractions were collected in 2-ml aliquots. Phycoerythrin elution was detected by its pink colour and fluorescence. Fractions corresponding to phycoerythrin (retention time of 720–760 min) were pooled and concentrated to 3 ml in an Amicon ultrafiltration apparatus using an M_r 5000 cut-off filter (Amicon, Beverly, MA, USA).

2.3. Absorbance and fluorescence determination

Absorbances at 280, 499, 545 and 565 nm and absorption spectra were determined using a double-beam UV–Vis spectrophotometer (Uvikon 923, Kontron Instruments, Zurich, Switzerland). Fluorescence emission spectra were recorded using a spectrofluorometer, with an excitation wavelength set at 565 nm.

2.4. Protein determination

The protein content of algal powder was determined using the Kjeldhal method ($N\times6.25$) [12]. The total protein content of extracts was determined with the bicinchoninic acid (BCA) protein reagent assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions [13]. Bovine serum albumin (BSA) was used as a standard. The content of R-phycoerythrin (mg/ml) in all extracts was estimated from absorbance at 565 nm ($\epsilon=2\times10^6\ M^{-1}\ cm^{-1}$).

3.1. Electrophoresis

3.1.1. Native polyacrylamide gel electrophoresis (native-PAGE)

Native-PAGE was performed using a mini-protean II electrophoresis unit (Bio-Rad) with a stacking gel of 4% and a separating gel of 5.5, 6.5 or 7.5% acrylamide in 25 m*M* Tris–HCl (pH 8.3), 0.192 *M* glycine. Each sample contained 20 m*M* Tris–HCl (pH 6.8), 4% glycerol, 0.009% bromophenol blue. The separation was carried out at 30 mA for 1 h. The following polypeptides were used as native molecular mass markers: thyroglobulin (M_r 669 000), ferritin (M_r 440 000), catalase (M_r 232 000), lactate dehydrogenase (M_r 140 000), BSA (M_r 67 000) (HMW electrophoresis calibration kit, Pharmacia, Uppsala, Sweden). After separation, protein bands from the algal protein extract were detected by silver staining and protein bands corresponding to phycoerythrin were identified both by their pink colour and fluorescence.

3.1.2. Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis

SDS–PAGE was performed using a mini-protean II electrophoresis unit (Bio-Rad) with a stacking gel of 4% and a separating gel of 12% acrylamide in 25 m*M* Tris–HCl (pH 8.3), 0.18 *M* glycine, 0.1 SDS. Each sample contained 10 m*M* Tris–HCl (pH 8.0), 1 m*M* EDTA, 1% SDS, 40 m*M* dithiothreitol (DTT), 10% glycerol, 0.015% bromophenol blue and was heated at 100°C for 3 min before loading. The separation was carried out under 30 mA for 1 h. The following polypeptides were used as molecular mass markers: phosphorylase b (M_r 94 000), albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 100) and lactalbumin (M_r 14 400) (LMW electrophoresis calibration kit, Pharmacia). After separation, protein bands corresponding to phycoerythrin were identified by their pink colour and fluorescence, and the protein bands of the algal protein extract were detected by silver staining.

3.1.3. Silver staining

Proteins were fixed in 40% methanol, 10% acetic acid for 1 h. The gel was then washed with 30% ethanol for 3×20 min. Proteins were reduced with 0.02% sodium thiosulphate for 1 min, washed with ultrapure water for 3×20 s, stained with 0.2% silver nitrate, 0.02% formaldehyde for 20 min. The gel was washed and developed with 3% sodium carbonate, 0.05% formaldehyde, 0.0005% sodium thiosulphate for 3–5 min. Colouration was stopped with 0.5% glycine for 5 min and the gel was washed with ultrapure water for 2×30 min.

3.2. Phycoerythrin stability to pH variations and heat

Phycoerythrin stability as a function of pH and temperature was monitored spectrophotometrically (Uvikon 923, Kontron Instruments). Phycoerythrin stability was investigated using a large pH range. Absorption spectra of phycoerythrin were determined after a gradual fall or increase in pH (pH 7, 6, 5, 4, 3, 2, 1 and pH 7, 8, 9, 10, 11, 12). Phycoerythrin stability to heat was also investigated. Absorption spectra of phycoerythrin were determined after an increase in temperature from 20 to 110°C, by 10°C increments over 10 min.

4. Results and discussion

We report here a single-step procedure for the purification of phycoerythrin from a crude extract of the red macroalga *Palmaria palmata*.

The phycoerythrin content of crude extracts from *Palmaria palmata* collected at different months was estimated by absorbance at 565 nm. *Palmaria palmata* was confirmed to be a good source of phycoerythrin. Seasonal variation of the phycoerythrin

content was observed with a clear maxima in autumn and a lowest amount in the summer, as described previously for this and other red algae [9,14]. For example, phycoerythrin content of crude extract from February, May, July and October was evaluated at 6.7%, 7.6%, 5.9% and 12.2% of total protein, respectively. Light intensity is known to be an important factor in the control of pigmentary content [14,15]. An inversely proportional relationship occurs between sunlight intensity and total biliprotein quantity. An environment deficiency in micronutrients may have a dramatic effect on cellular pigmentation. For example, nitrogen deficiency leads to a biliprotein synthesis inhibition [14].

Purification of *Palmaria palmata* phycoerythrin was carried out on the *Palmaria palmata* specimen collected in October 1998, which presented a high level of phycoerythrin. The absorption spectrum of the crude extract between 300 and 700 nm showed six peaks. Of these, three peaks corresponded to the maxima of phycoerythrin (565 nm, 499 nm and a 545-nm shoulder). Two other peaks were found at 620 nm (A_{max} of phycocyanin) and 650 nm (A_{max} of allophycocyanin); the latter peaks were significantly less important compared to the phycoerythrin peaks. Finally, the most important peak was observed at 320 nm, but its nature has not yet been identified. The absorbance ratio A_{565}/A_{280}, an index for phycoerythrin purity, [9] was 0.5. The total protein content of the crude extract was estimated at 8.2 mg/ml, corresponding to 18.5 mg/g of algal powder. The protein content of the algal powder being equal to about 100 mg/g, protein extraction yield was calculated to be 18.5%. From the absorbance at 565 nm, the phycoerythrin content of the crude extract was calculated to be 1 mg/ml, corresponding to 12.2% of the total protein content.

Purification of phycoerythrin was carried out by preparative electrophoresis with a separating gel of 7% acrylamide as this percentage gave a good resolution on acrylamide mini-gel. The pink colour and fluorescence of phycoerythrin allowed to easily locate it during elution. The purified phycoerythrin of *Palmaria palmata* was eluted with a retention time of 720–760 min. It presented an absorption spectrum typical for R-phycoerythrin with two peaks at 499 and 565 nm and a shoulder at 545 nm (Fig. 1) [3,4]. The absorbance ratio A_{565}/A_{280} was 3.2,

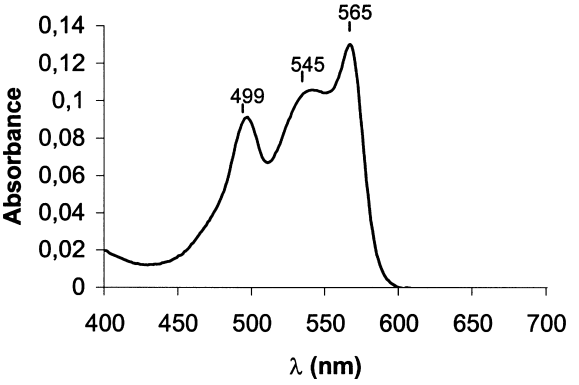

Fig. 1. Absorption spectrum of R-phycoerythrin from *Palmaria palmata*. The protein content was approximately 0.02 mg/ml.

consistent with purity. Absorbance ratios A_{620}/A_{280} and A_{670}/A_{280} were <0.001 which demonstrated the absence of phycocyanin and allophycocyanin contaminants. The purified phycoerythrin presented an intense orange fluorescence with emission maxima at 578 nm (Fig. 1). Intense red colour and fluorescence properties are a consequence of the interactions between multiple covalently bound chromophores and apoprotein.

Phycoerythrin purity was confirmed by electrophoresis. A single protein was found in silver stained native-PAGE (Fig. 2). It presented a pink colour and was fluorescent. The relative molecular mass determined from comparison of relative migration (Rm) on native-PAGE at various acrylamide gel concentrations (5.5, 6.5 and 7.5%) was 240 000, consistent with the literature [2,4]. Moreover, silver-stained SDS–PAGE with 12% acrylamide showed three bands: two predominant pink and fluorescent bands of equal intensity and M_r estimated at 20 000 and 21 000 and a much smaller slightly fluorescent band of M_r at about 30 000 (Fig. 3). This observation is therefore consistent with an $(\alpha\beta)6\gamma$ subunit composition, characteristic of R-phycoerythrin. In fact, R- and B-phycoerythrins have three types of subunit: α and β (about 20 000, acidic isoelectric point, pI) and γ (about 30 000, basic pI) and both have a $(\alpha\beta)_6\gamma$ subunit composition, whereas the oligomeric composition of C-phycoerythrin is $(\alpha\beta)_6$ [2,4]. The crystal structure of R-phycoerythrin from *Polysiphonia urceolata* has been determined [16,17]. The γ subunit is assumed to be a hydrophobic linker

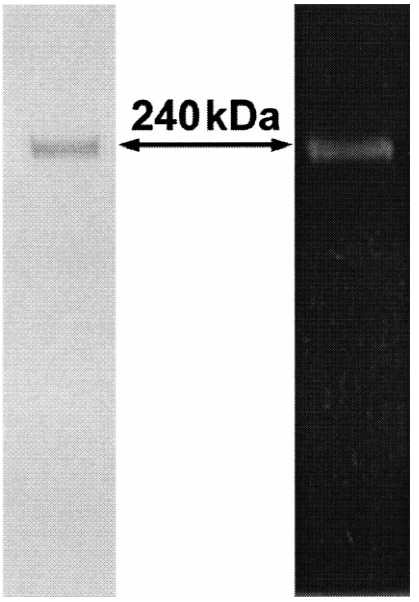

Fig. 2. Native-PAGE of R-phycoerythrin from *Palmaria palmata*. Electrophoresis was run in a 7.5% acrylamide gel (30 mA per gel). Protein band was detected by silver staining. Fluorescence was observed under UV.

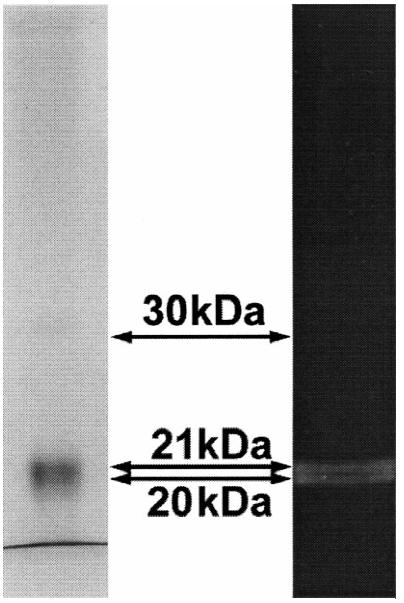

Fig. 3. SDS–PAGE of R-phycoerythrin from *Palmaria palmata*. Electrophoresis was run in a 12% acrylamide gel (30 mA per gel). Protein bands were detected by silver staining. Two main protein bands were observed, with respective M_r of 20 000 and 21 000. A very minor protein band was detected with M_r of 30 000. Fluorescence was observed under UV.

protein located in the central channel of the phycoerythrin αβ torus [2,4,16].

So preparative electrophoresis gave a good resolution to purify phycoerythrin in a single step. This purification procedure yielded a total of 200 μg of pure phycoerythrin from 1 ml of crude extract, corresponding to 20% of its amount in the crude extract, and to a yield of 450 μg phycoerythrin per g of algal powder. Preparative electrophoresis was already used for the purification of an M_r 41 000 cod-allergenic protein [18]. Clearly, however, a large scale purification is difficult to envisage using this technique. The obtained phycoerythrin can easily be used on a laboratory scale for biochemical studies. By comparison, purification of R-phycoerythrin of *Corallina officinalis* was done by a combination of three chromatography steps, but allowed a large scale purification and obtained phycoerythrin showed a higher A_{565}/A_{280} ratio (4.7 versus 3.2) [9].

Phycoerythrin stability to heat and pH variations was monitored spectrometrically (Fig. 4). R-Phycoerythrin of *Palmaria palmata* displayed a good stability to pH and temperature variations as no important modification of colour and fluorescence was observed up to 60°C and between pH 3.5 and 9.5. Phycoerythrin spectrum was not modified in this range, showing the three absorption maxima at 499, 545 and 565. Absorbance was slightly decreased when temperature was increased and when pH differed from neutrality. Beyond this range, however, spectral modifications occurred probably as a result of denaturation and peaks at 565, 545 and 499 nm and fluorescence progressively disappeared. The 499 nm peak showed the great stability to high temperature. Beyond 90°C, the phycoerythrin solution was almost colourless. R-phycoerythrin stability up to 60°C is consistent with thermal stability of R-phycoerythrin from *Gracilaria longa* [19].

This biliprotein can, hence, be used as fluorescent probe. In fact, phycoerythrins are widely used in biochemical techniques due to their exceptionally high molar absorption coefficient near $2.4\times10^6\ M^{-1}\ cm^{-1}$ and quantum yield near 0.8, which allow a high sensitivity [20–22]. Therefore, phycoerythrin emits in the orange-red (fluorescence emission maxima=580 nm), where background fluorescence is

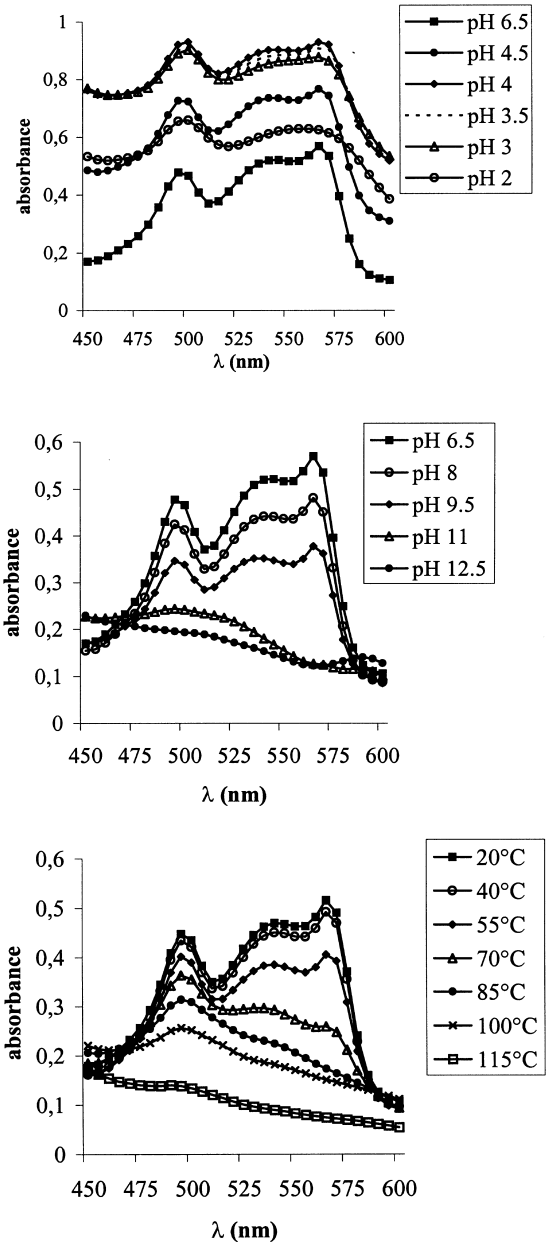

Fig. 4. Absorption spectrum of R-phycoerythrin of *Palmaria palmata* at various pH and temperatures. The protein content was approximately 0.07 mg/ml.

exceptionally low [21]. So, phycoerythrin conjugates, as antibody–phycoerythrin complex, protein A–phycoerythrin and avidin–phycoerythrin conjugates, can be used as probe in fluorescence flow cytometry, fluorescence microscopy and fluorescence immunoassay [9,21–24]. Phycoerythrin can also be used as probe in proximity or interaction assays between two molecules by fluorescence resonance energy transfer (FRET) [25], where one of the molecules is conjugated to phycoerythrin and the other to allophycocyanin. Phycoerythrin is also particularly appropriate for use as a protein marker in electrophoretic techniques (SDS–PAGE and isoelectric focusing) and size-gel exclusion chromatography and may probably be used as alimentary or cosmetic colourant [26,19].

In conclusion, the purification of R-phycoerythrin from the red macroalga *Palmaria palmata* has been achieved in a single-step preparative electrophoresis. This purified R-phycoerythrin showed a good stability up to 60°C and between pH 3.5 and 9.5. The fluorescence, spectral and stability properties of R-phycoerythrin from *Palmaria palmata* suggests good opportunity to investigate future applications in biochemical techniques.

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