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Methods of phycobiliprotein extraction from *Gracilaria crassa* and its applications in food colourants

M.P. Sudhakar [a], A. Jagatheesan [b], K. Perumal [a,*], K. Arunkumar [b]

[a] Shri AMM Murugappa Chettiar Research Centre, Chennai, Tamilnadu, India
[b] Marine Algae Research Division, Post Graduate and Research Department of Botany, Alagappa Government Arts and Science College (Alagappa University), Karaikudi, Tamil Nadu, India

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ABSTRACT

Phycobiliproteins from red seaweeds are pigment–protein complexes constituting three different pigments such as phycocyanin, allophycocyanin and phycoerythrin. The present investigation is focused on phycobiliproteins in red seaweed *Gracilaria crassa* collected from Pudumadam coast, Rameswaram. Three different solvents such as phosphate buffer, distilled water and seawater were used for extraction of pigments and quantity and purity were compared. Among the three different solvents, distilled water showed better results for phycoerythrin extraction; seawater proved to be a good solvent as well as preservative for all phycobiliproteins. The purity index (PI) was 3.79 for R-phycoerythrin (R-PE), purified by DEAE (Diethylaminoethyl) cellulose 52. The stability of phycobiliproteins was assessed using different preservatives. NaCl at 5% proved to be suitable for long-term preservation of phycoerythrin.

1. Introduction

Phycobiliproteins are light harvesting pigment–protein complexes [1] predominantly present in red seaweeds such as *Hypnea* sp., *Acanthophora* sp., *Porteria* sp., and *Sarconema* sp., and different species of *Gracilaria* such as *G. corticata* [2], *G. crassa* [3], *G. edulis* [4] and *G. verrucosa* [5]. Based on their absorption properties, phycobiliproteins are generally divided into three classes: phycoerythrins (PEs), which absorb light at 495 and 540–570 nm; phycocyanins (PCs), which absorb light at 610–620 nm; and allophycocyanins (APCs) with an absorption range of 650–655 nm [6]. Phycobiliproteins are aggregated into two subunits (α and β) and the third subunit (γ), a linker peptide which is found in R-phycoerythrin (R-PE). The structure of R-phycoerythrin (R-PE) can be described as $(\alpha\beta)_6\gamma$, while phycocyanin and allophycocyanin have the structure $(\alpha\beta)_3$ and they are bound to specific cysteines by thioether bonds [7]. Apart from seaweeds, red microalgae such as *Porphyridium*, *Rhodella*, and *Rhodosorus*, are also cultivated in large quantity for phycobiliprotein extraction [8,9].

There are numerous protocols available for extraction and quantification of phycobiliproteins, but all these methods differ considerably [10–12]. Literature reveals that phosphate buffer is the widely used solvent for phycobiliprotein extraction. Apart from phosphate buffer, other solvents such as distilled water [5,13,14] and seawater also can be used for the extraction. R-phycoerythrin can also be extracted using enzymes, such as xylanase, that breakdown the cell wall of seaweeds [15]. However the quantity and purity of the pigments are likely to vary among these solvents. Since pigments are highly sensitive to oxygen, temperature, pH, and light, and as the stability of the pigments differs in various solvents, it is important to store the pigments using appropriate preservatives [16–18].

Phycobiliproteins can be used as food colourants [19,20] and in many other food applications [21–23]. Puddings are available in different permitted colours but they are made of chemicals. Natural colour such as phycobiliproteins can be used as an alternate colour for puddings. The annual market of phycocyanin is around 5–10 million US dollars [24].

The present study deals with extraction and quantification of phycobiliproteins from red seaweed, *Gracilaria crassa*, using different solvents, and estimation of their purity and stability in suitable preservatives for food applications. The fluorescent property of R-

Abbreviations: PC, phycocyanin; APC, allophycocyanin; R-PE, R-phycoerythrin; BHT, butyl hydroxyl toluene; DEAE, diethylaminoethyl; FW, fresh weight; TCSPC, time-correlated single photon counting spectrometer.

* Corresponding author at: Shri AMM Murugappa Chettiar Research Centre, Taramani, Chennai 600 113, India.
E-mail address: perumalk@mcrc.murugappa.org (K. Perumal).

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phycoerythrin can be used as fluorescent markers in medical applications.

2. Materials and methods

2.1. Extraction of phycobiliproteins

Fresh thalli of red seaweed, *G. crassa*, (Fig. 1) collected from Pudumadam coast, Rameswaram, Tamilnadu, India, were cleaned thoroughly with distilled water to remove unwanted debris, epiphytes, etc., and stored at 4 °C. Phycobiliproteins were extracted using 0.1 M phosphate buffer (pH 6.8), distilled water (pH 7.0) and seawater (pH 8.13) separately under room temperature in the ratio of 1:13 w/v. Fresh thalli 20 g in 0.1 M phosphate buffer, 15 g in distilled water and 1 g in seawater were ground in mortar and pestle repeatedly to extract crude pigment and centrifuged at 10,000 rpm for 20 min and later filtered through a 0.2 μm syringe filter (Millipore, polyethersulfone membrane) in order to remove insoluble particles present in the crude extract [25]. The filtrate was stored at 4 °C until further studies. Fig. 2 shows a schematic representation of the overall experiment.

2.2. Quantification of phycobiliproteins

Crude and filtered (0.2 μm) phycobiliproteins from different solvent extracts such as 260 mL (buffer), 200 mL (distilled water) and 15 mL (seawater) were analysed by a UV–visible spectrophotometer and are quantified using the following equations of Bennett & Bogorad [26].

$$\text{PC}\ \left(\text{mg mL}^{-1}\right) = [A_{615} - 0.474\ (A_{652})]/5.34$$

$$\text{APC}\ \left(\text{mg mL}^{-1}\right) = [A_{652} - 0.208\ (A_{615})]/5.09$$

$$\text{PE}\ \left(\text{mg mL}^{-1}\right) = [A_{562} - 2.41\ (\text{PC}) - 0.849\ (\text{APC})]/9.62$$

2.3. Quality/purity of pigments

The crude pigment was extracted using 260 mL of 0.1 M phosphate buffer (pH 6.8) followed by ammonium sulphate precipitation (65% saturation) and dialysed (molecular weight cut-off: 12–14 kDa, HiMedia, India) overnight at 4 °C against 1 L of 0.1 M potassium phosphate buffer (pH 6.8). After dialysis, PE was separated and purified through column chromatography by using DEAE (Diethylaminoethyl) cellulose 52 (HiMedia, India). Phycoerythrin was eluted using 50 mL of NaCl gradient from 50 mM NaCl to 300 mM NaCl in 0.1 M potassium phosphate buffer at pH 6.8. The purity of the R-phycoerythrin pigment was analysed by UV–visible spectrophotometer (Varion 300) scan in the range between 450 and 650 nm (see Supplementary material Figs. 1 and 2). The purity index (PI) for each class of phycobiliprotein was calculated by using the formula as given by Senthilkumar et al. [27].

Fig. 1. Fresh seaweed *Gracilaria crassa*.

$$\text{PC} = A_{615}/A_{280}$$

$$\text{APC} = A_{652}/A_{280}$$

$$\text{PE} = A_{565}/A_{280}$$

where, A_{615} = maximum absorbance of PC, A_{652} = maximum absorbance of APC, A_{565} = maximum absorbance of PE and A_{280} = the absorbance of total proteins.

2.4. Phycobiliprotein stability in preservatives

Preservatives such as glucose, sucrose, citric acid, ascorbic acid, sodium chloride, sodium azide and butyl hydroxyl toluene were used to check the stability of the pigments under various temperatures. The percentage losses were calculated for:

1. Crude phycobiliproteins (0.028 mg/mL) extracted in phosphate buffer (pH 6.8), filtered through a 0.2 μm syringe filter and stored at 0 ± 5 °C in different preservatives such as glucose (20%), sucrose (20%), sodium chloride (20%), ascorbic acid (0.4%), citric acid (0.4%), sodium azide (0.05%) and BHT (0.4%) under dark condition for 65 days.
2. Crude phycobiliproteins (0.038 mg/mL) extracted in distilled water (pH 7.0) and stored at 0 ± 5 °C in different concentrations of sodium chloride (2%, 5%, 10%, 15%, 20%, and 25%) under dark for 50 days.
3. Crude phycobiliproteins (0.024 mg/mL) extracted in seawater (pH 8.13) and stored at two different temperatures 30 ± 5 °C and 0 ± 5 °C in dark for 30 days.
4. Crude phycobiliproteins (0.024 mg/mL) extracted in seawater, filtered through 0.2 μm syringe filter and stored at two different temperatures 30 ± 5 °C and 0 ± 5 °C in dark for 30 days.

The above-mentioned studies were conducted mainly to find the phycoerythrin stability in different concentrations of preservatives. The control pigment is used for comparison without any of the above-mentioned preservatives.

2.5. Application of R-phycoerythrin in puddings

Dialysed crude pigment was used for application studies. The purity index (PI) of the pigment was found to be 0.91. Since phycobiliproteins is used for food colouring, dialysed crude phycobiliproteins were subjected to filtration (0.2 μm PES syringe filter, Merck) and then used as food colourant. White colour puddings, commercially available, were used for colour testing. About 70 g of white coloured pudding (Manufactured by Jellico Food Co., Ltd, Taiwan) was mixed with 3 mL of dialysed pigment thoroughly under sterile condition, sealed and stored at 0 ± 5 °C for 50 days. The colour of the pudding was visually monitored on a daily basis.

2.6. Spectral properties of R-phycoerythrin

Absorption and emission wavelength of R-phycoerythrin were analysed using UV–visible spectrophotometer and spectrofluorometer, respectively. Fluorescence property (time resolved fluorescence spectrum) of R-PE was measured using a time-correlated single photon counting spectrometer (TCSPC). The light used in TCSPC was nano-LEDs and excitation, emission wavelength was set as 490 nm and 578 nm, respectively (see Supplementary material Fig. 3) [28].