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# Optimization of hydrolysis conditions of *Palmaria palmata* to enhance R-phycoerythrin extraction

Justine Dumay *, Nathalie Clément, Michèle Morançais, Joël Fleurence

*LUNAM Université, Université de Nantes, MMS, Nantes, 2 rue de la Houssinière, BP 92208, 44322 Nantes Cedex 3, France*

## HIGHLIGHTS

- Enzymatic engineering was investigated to improve R-phycoerythrin extraction.
- Enzyme ratio, temperature and hydrolysis time have been considered for the hydrolysis optimization.
- Enzymatic treatment led to improve significantly the R-phycoerythrin extraction yield and purity.
- Roughly cut wet algae provides the most interesting results in terms of extract quality and economic cost.
- Optimization of hydrolysis conditions led to less time and enzyme-consuming process.

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## ABSTRACT

In this study, response surface methodology was applied to optimize R-phycoerythrin extraction from the red seaweed *Palmaria palmata*, using enzymatic digestion. Several algal treatments prior to digestion were first investigated. The extraction yield and the purity index of R-phycoerythrin, and the recovery of proteins and reducing sugars in the water-soluble fraction were then studied in relation to the hydrolysis time, the temperature and the enzyme/seaweed ratio.

Enzymatic digestion appears to be an effective treatment for R-phycoerythrin extraction. Moreover, using the seaweed roughly cut in its wet form gives the most interesting results in terms of extract quality and economic cost. The R-phycoerythrin extraction yield is 62 times greater than without enzyme treatment and 16 times greater than without optimization. Enzymatic optimization enhanced the purity index up to 16 times.



## 1. Introduction

Two hundred and twenty one species of seaweed are used worldwide for human consumption or as phycocolloids (Lindsey Zemke-White and Ohno, 1999). Seaweeds are traditionally consumed in Asian countries, especially China, Japan and Korea (Denis et al., 2010). Brown and red algae are the phyla mainly used for human consumption and represent 64 and 125 different species, respectively (Lindsey Zemke-White and Ohno, 1999). Red seaweeds are well known for containing large amounts of protein; up to 47% of dry weight for *Porphyra yezoensis* while *Palmaria palmata* protein content can reach 35% (Fleurence et al., 2012). Unfortunately, seaweed proteins are not as bioavailable as expected due to their polysaccharide content (xylans, agar, carrageenan or alginates), which limits the digestibility of protein fractions (Horie et al., 1995), and also due to the strong covalent bonding between mix-linked xylans and glycoproteins complexes, as reported for *P. palmata* (Deniaud et al., 2003a).

R-phycoerythrin (R-PE) is one of the soluble proteins specific to red algae and belongs to the phycobiliproteins. These are major light-harvesting pigments found in the chloroplasts of red algae and cyanobacteria (Galland-Irmouli et al., 2000). According to their light absorption properties, phycobiliproteins can be classified into four groups: phycoerythrins ($\lambda_{max}$ = 490–570 nm), phycocyanins ($\lambda_{max}$ = 610–625 nm), phycoerythrocyanins ($\lambda_{max}$ = 560–600 nm) and allophycocyanins ($\lambda_{max}$ = 650–660 nm) (Sun et al., 2009). Phycoerythrin, like other phycobiliproteins, is highly water-soluble and its proteinic nature has led to many applications and much interest (Sekar and Chandramohan, 2008). In addition to its colorant properties, phycoerythrin possesses a yellow fluorescence. As a food colorant, R-PE is widely used in Asian countries and is now used for entertainment, recreation and amusement (toys, paint, cosmetics and soft drinks). R-PE and phycobiliproteins can also be exploited as a moiety in fluorescent energy transfer, fluorescent labels, tags, tracers and markers, which are useful in flow cytometry, fluorescent immunoassays, immunophenotyping and other

* Corresponding author. Tel.: +33 (0)251 12 56 70; fax: +33 (0)251 12 56 68.
*E-mail address:* justine.dumay@univ-nantes.fr (J. Dumay).

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such fluorescent studies (Glazer, 1994; Isailovic et al., 2006; Sekar and Chandramohan, 2008). Lastly, R-PE also possesses some biological activities, such as antitumoral ones in which R-PE subunits have been indicated as an attractive option for improving the selectivity of photodynamic therapy in mouse tumor cells and human liver carcinoma cells (Sekar and Chandramohan, 2008). Other effects reported include antioxidant (Fitzgerald et al., 2011; Pangestuti and Kim, 2011), antidiabetic, antitumoral (Fitzgerald et al., 2011), immunosuppressive and antihypertensive effects (Cian et al., 2012). In addition, red seaweeds are reputed for their bioactive molecules with a wide range of activity (cytotoxic, antiviral, anthelmintic, anti-inflammatory, free radical scavenger, neurophysiological, insecticidal, antimicrobial) (El Gamal, 2010; Plaza et al., 2008). *P. palmata* also possesses antioxidant or antiproliferative activities as reported in several studies (Wang et al., 2010; Yuan et al., 2005).

Currently, most procedures used for the extraction of R-PE are based on cell wall breakage. Grinding in liquid nitrogen is used to facilitate the destruction of the cell wall, which is the main obstacle to accessing and extracting the algal proteins. However, this approach is not totally efficient for cell wall degradation and is also costly on an industrial scale (Fleurence, 2003). The presence of various polysaccharides in large quantities in the cell wall, and their bonding with glycoproteins (Deniaud et al., 2003a), reduces the efficiency of classic extraction methods. However, new kinds of extraction techniques have recently appeared, such as enzymolysis and microwave-assisted extraction (Wijesinghe and Jeon, 2012). Preliminary studies have demonstrated the benefits of using enzymes to recover high added-value seaweed compounds (Wijesinghe and Jeon, 2012), R-PE (Joubert and Fleurence, 2008) or seaweed proteins (Fleurence et al., 2012; Lahaye and Vigouroux, 1992) but none of them has tried to optimize the enzymatic conditions. It is well known that enzymatic tissue disruption may be an efficient alternative for releasing many kinds of compound, usually confined and inaccessible. In fact, some papers have reported the optimization of enzymatic hydrolysis conditions, such as pH, temperature and enzyme concentration, for protein or peptide recovery using response surface methodology (RSM) (Diniz and Martin, 1996; Guerard et al., 2007). Moreover, although the optimization of enzymatic processes on algal material has not yet been reported, RSM has appeared helpful in algal bioprocess optimization (Patil et al., 2011; Ryu et al., 2012).

As explained by Marrion et al., water-soluble xylan, present in high proportions in *P. palmata*, could explain the weak digestibility of proteins (Marrion et al., 2003). The purpose of this study was to develop an efficient method for the recovery of R-PE from *P. palmata* using xylanase. After determining the best algal pretreatment, an experimental design procedure was used to improve the enzymatic conditions (time, temperature and enzyme/substrate ratio) in order to extract the highest quantity of R-PE.

## 2. Methods

### 2.1. Materials

Seaweed, *P. palmata*, was harvested in February 2011 in the mediolittoral zone at Le Croisic (Atlantic coast, France). Samples were successively rinsed with seawater, tap water and distilled water. A portion of the thalli was freeze-dried (Edwards Super Modulyo, Thermo Electro Corporation, Waltham, MA, USA). The algae were then stored at −20 °C until use. To perform hydrolysis, wet or dried samples were roughly cut (average size < 5 $cm^2$), ground using an ULTRA-TURRAX (1 $cm^2$ < average size < 2 $cm^2$) or ground in liquid nitrogen (average size < 1 $mm^2$).

The enzyme used for hydrolyses was purified xylanase E.C.3.2.1.8. from *Trichoderma longibrachiatum* purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). Xylanase was selected due to its digestion potential for *P. palmata*, as widely described in the literature (Joubert and Fleurence, 2008; Lahaye and Vigouroux, 1992), induced by the cell wall composition of this seaweed (Deniaud et al., 2003a). According to the manufacturer, the highest enzymatic activity is obtained at 40 °C and pH 5.

### 2.2. Hydrolysis

Hydrolysis experiments were carried out using a 500-mL glass reactor under controlled conditions (temperature and stirring speed) and in darkness to prevent R-PE degradation. The equivalent of 2 g of dry seaweed samples was homogenized with 200 mL acetate buffer 50 mM, pH 5 (Joubert and Fleurence, 2008; Lahaye and Vigouroux, 1992). Enzyme was added to the mixture and the system was continuously stirred at 700 rpm during hydrolysis.

For the algal pretreatment determination, a fixed temperature of 40 °C was applied and 16.5 g $kg^{-1}$ dw of xylanase was added to the system. Triplicate digestions were carried out and a control (same experiment without enzyme) was performed simultaneously.

After hydrolysis, the hydrolysate was centrifuged at 25,000*g* for 20 min at 4 °C to separate undigested residues and solubilized compounds (Denis et al., 2009b). After centrifugation, supernatants were filtered then recovered and used to determine the reducing sugar, soluble protein and R-phycoerythrin contents.

### 2.3. R-phycoerythrin determination

R-PE concentration and purity were determined spectrometrically using the Beer and Eshel equation (1) (Beer and Eshel, 1985) and the $A_{565nm}/A_{280nm}$ ratio (=R-PE Purity Index or PI), respectively (Galland-Irmouli et al., 2000; Liu et al., 2005). R-PE yield was expressed as mg $g^{-1}$ dw.

$$[\text{R-PE}] = [(A_{565} - A_{592}) - (A_{455} - A_{592}) \times 0.20] \times 0.12 \quad (1)$$

The R-PE absorption spectra displayed three peaks: two at 495 and 545 nm and one main peak at 565 nm; 455 and 592 nm constituted the lower wavelengths. The spectral profile is commonly used to indicate the non-degradation of R-PE.

### 2.4. Water-soluble proteins

Total water-soluble proteins in the soluble fraction were analyzed by the method adapted from Bradford (Bradford, 1976). Bradford reagent (Sigma) (200 μL) was added to 800 μL of sample solution. The absorbance measurement at 595 nm (read immediately after the reaction) and the use of BSA (Sigma) as a standard (from 0 to 50 mg/L) enabled the protein content to be determined.

### 2.5. Reducing sugars

The reducing sugar concentrations were assayed using the ferricyanide method adapted from Kidby and Davidson (Denis et al., 2009b). In each assay, glucose was added as a standard.

### 2.6. Experimental design and evaluation

Response surface methodology (RSM) was used to investigate the influence of the hydrolysis variables, such as temperature, hydrolysis time and enzyme-substrate (*E*/*S*) ratio (g $kg^{-1}$ of dry algae), on the digestion of *P. palmata* thalli and to optimize the influence of these three variables. In a $2^3$ central composite design (CDD), the starting temperature, hydrolysis time and *E*/*S* ratio were varied systematically at high and low levels (Table 1). Medium