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Hydrolyzing-Extracting and Determining structure of the pigment R-phycoerythrinfrom *Mastocarpus stellatus*

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Abstract: This study reports the hydrolysis and extraction of R-Phycoerythrin (R-PE) from red algae Mastocarpus stellatus. R-PE is a phycobiliprotein which is used for natural colorant in the food and cosmetic industries. For freeze-dried algae, acetate buffer 50mM, pH 6 presented better results for R-PE extraction following hydrolysis by enzyme xylanase. The R-PE yield was 1.94 mg. g^{-1}dw and the Purity Index (PI) was 0.25. ThenR-PE purificationwas performed by DEAE sepharose Fast Flow chromatography with a three-step increase in ionic strength. The crude extract was separated into three fractions: AE-150, AE-200 and AE-1000.By SDS-PAGE the AE-200 fraction was determined (αβ)6γγ' for R-phycoerythrin from Mastocarpus stellatus.

Keywords: Mastocarpus stellatus, Purification, SDS-PAGE, R-phycoerythrin, xylanase.

I. INTRODUCTION

Mastocarpus stellatus (Stackhouse)Guiry (*M.stellatus)* is a member of the Rhodophyta with kappa/iota hybrid carrageenan[1]. *M.stellatus* is also a resource of protein and R-phycoerythrin [2].The carbohydrates content of *M.stellatus*were around 50% [3]. These compounds caninterfere with protein and R-phycoerythrinextraction [2].R-phycoerythrin (R-PE) is a nature pigment and one of four phycobiliproteins from the red seaweed. Phycobiliproteins have four groups: phycoerythrins (λmax=490-570nm), phycocyanins (λmax= 610-625nm), phycoerythrocyanins (λmax= 560-600nm) and allophycocyanins (λmax= 650-660nm). Phycoerythrins (PEs) have three main classes, including: B-phycoerythrin (B-PE; λmax = 545-563 nm), R-phycoerythrin (R-PE; λmax = 498-565 nm) and C-phycoerythrin (C-PE; λmax = 565 nm) [4][5]. R-phycoerythrin is an oligomeric water-soluble chromoprotein which has the subunits including 6α, 6β, 1γ, 1 γ' subunits (260kDa) [6]. For this structure, the α subunit has two phycoerythrobilins (PEBs), the β subunit obtain 2 or 3 PEBs and one phycourobilin (PUB), the gamma subunits include 3 PEBs and 2 PUBs (γ) or 1 or 2 PEBs and 1 PUB (γ') [4]. R-phycoerythrinwas used and applied as natural colorant for food and cosmetics. Moreover, this pigment is also used in biology such as fluorescent energy transfer, flow cytometry, fluorescent inmunoassays,… It has some biological activities, including antioxidant, antidiabetic, antitumoral,… [2][4][6]. This pigment cost depend on the purity index (PI), from 64 euros to 400 euros per mg (Sigma Aldrich 2018). R-PE was extracted by macerating the algae in water, phosphate buffer, acetate buffer,…Beside, R-PE was also extracted by hydrolysis enzyme [2][4].In this paper, the effects of xylanase degrading the polysaccharides on the extraction of protein and R-phycoerythrin from *Mastocarpus stellatus* were performed.

II. MATERIAL AND METHODS

2.1 Material

Mastocarpus stellatus was collected in the intertidal zone of Batz-sur-Mer on the Atlantic coast, France. Epiphytes were removed and washed and cleaned in seawater, tap water and distilled water. The red seaweed were then freeze-dried and homogenized in liquid nitrogen. We have the algae powder for R-phycoerythrin extraction.

The polysaccharidase, enzyme xylanase X2629 from *Trichodermalongibrachiatum* purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

2.2 R-PE extraction and purification

Hydrolysis –Extraction

Hydrolysis experiments were performed using a 500 mL glass reactor under controlled conditions (temperature and stirring speed) and in darkness. Around 2 g of algae powder was homogenized with 200 mL

acetate buffer 50 mM, pH 6. Xylanase (16.5 mg. g^{-1}dw) was added to the mixture and stirred continuously at 150 rpm during the 6 h hydrolysis period, at 35°C (Dumay et al. 2013).Triplicate digestions were performed [2][4].

After hydrolysis, the samples were centrifuged at 25,000 x g for 20 min, 4°C to separate into pellets and supernatants. Then theywere determined the water- soluble protein and R-phycoerythrin yields (Fig. 1).

R-phycoerythrin determination

R-PE yield and purity index were determined spectrophotometrically using the method of Beer and Eshel Eq (Beer and Eshel 1985). The absorption spectra of R-PEpresent three peaks: 498 nm, 545 nm and565 nm. R-PE yield was expressed as mg g^{-1}dw (dry algae)[7].

[R-PE] (mg.mL-1) = $[(A_{565}-A_{592}) - (A_{455}-A_{592}) \times 0.20] \times 0.12$ (Eq.1)

Purity Index or PI = A_{565}/A_{280} (Eq. 2)(Galland-Irmouli et al. 2000).

Water-soluble proteins determination

Water-soluble proteins were determined by the method of 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^{-1}) enabled the protein content to be determined[8].

Purification by anion-exchange chromatography and separation of fractions

The HPLC system consisted of a piston pump (Schimadzu) interfaced with a diode array detector (DAD SPD-M20A, Shimadzu). Chromatograms were monitored at 280 nm and 565 nm. Data were acquired using the Labsolutions/LC solution software. The mobile phase was constituted of two buffers: buffer A consisted of 20 mM sodium phosphate pH 7.1 and buffer B consisted of 20 mM sodium phosphate, 1 M NaCl pH 7.1. The CE was applied on a DEAE-Sepharose Fast Flow column (26 x 100 mm), which was pre-equilibrated with buffer A. The elution of R-PE was carried out using a three-step increase in the buffer ionic strength (0-150 mM, 150-200 mM and 200, 1000 mM). The fractions containing R-PE were collected at 150 mM, 200 mM and 1000 mM NaCl and were named AE-150, AE-200 and AE-1000, respectively [6] (Fig. 1).

Gel electrophoresis SDS-PAGES

After purified by DEAE chromatography, three fractions AE-150, AE-200 and AE-1000were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).The gel was composed of a 12% (w/v) polyacrylamide separating gel (Mini-Protean II, Bio-Rad) in 1.5 M Tris HCl buffer (pH 8.8) containing 0.1% SDS (w/v) and a stacking gel of 4% (w/v) in 0.5 M Tris-HCl buffer (pH 6.8) with 0.1% SDS (w/v). Before loading, the R-PE samples were denatured by incubation in buffer containing 6.25 mM Tris buffer (pH 6.8), 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 25% (v/v) glycerol and 0.01% (w/v) bromophenol blue, and then the samples were incubated at 100°C for 10 min. The incubated suspension was centrifuged at 500 g for 10 sec to remove the insoluble substances. After SDS-PAGE, protein bands were detected by staining with Coomassie Blue G250, 2.5% (w/v) brilliant blue G250 in methanol/acetic acid/water (45/10/45; v/v/v). Precision Plus Protein™ All Blue Standards (Bio-Rad) were used as a guide to determine molecular mass[6].

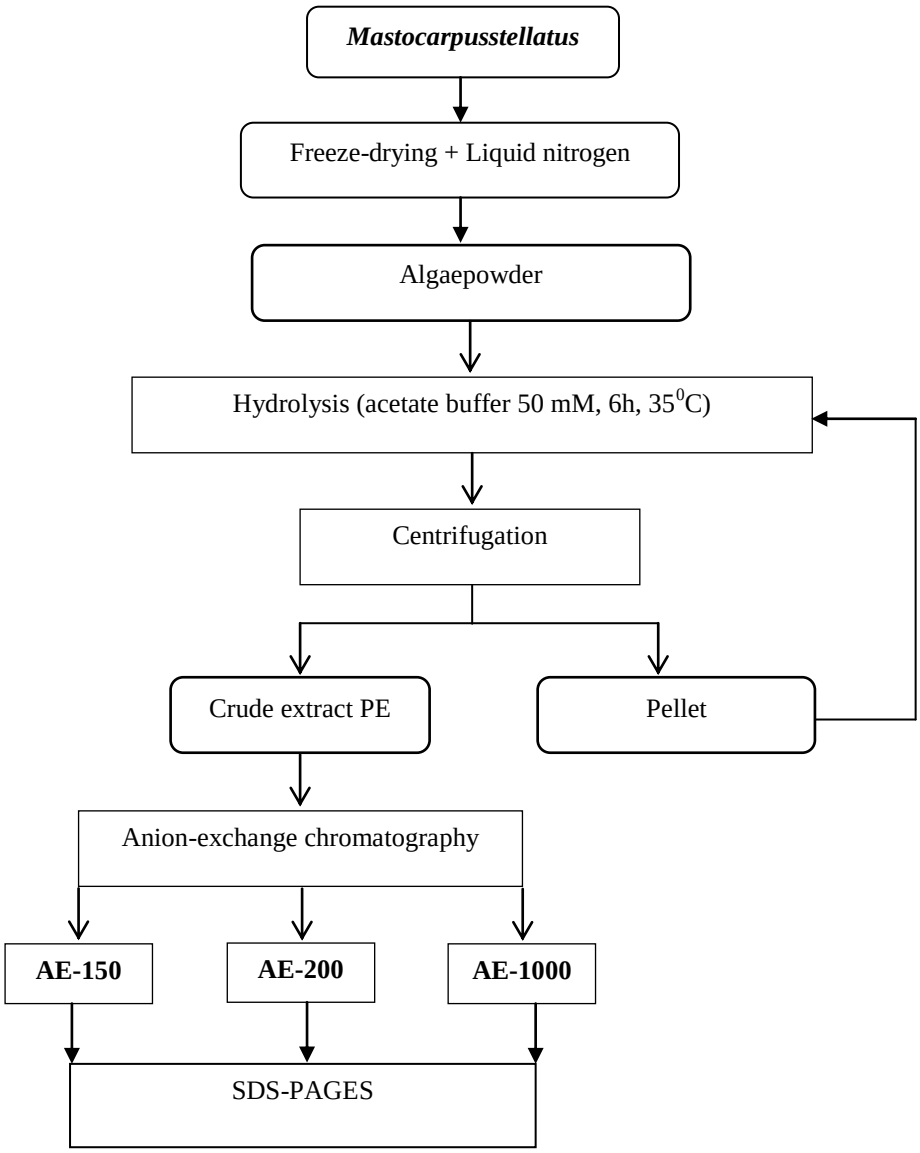

Fig. 1.*Mastocarpusstellatus*: protocol for the extraction, purification and gel electrophoresis of R-phycoerythrin.

III. RESULTS AND DISCUSSION

3.1 Extraction

The powder algae*M.stellatus* were hydrolysed by enzyme xylanase in four acetate buffers (pH 4, pH 5, pH 6, pH 7) (Fig. 2).Quantities of water-soluble proteins extracted ranged from 3.1 ± 0.02 mg. g-^{1}dw (xylanase, acetate buffer pH 4) to 4.44 ± 0.03 mg. g^{-1}dw (xylanase, acetate buffer pH 6). In addition, the results obtained were very similar to those for water-soluble proteins; R-PE extraction significantly ranged from 1.06 ± 0.02 mg. g-^{1}dw (xylanase , acetate buffer pH 4) to 1.94 ± 0.08 mg. g^{-1}dw (xylanase, acetate buffer pH 6). However, there was no significant difference in Purity index of R-PE results was obtained between the four hydrolysis procedures and the values were from 0.17 to 0.25.

The best results for R-PE and water-soluble proteins contents were obtained with the acetate buffer 50mM, pH 6 ($p<0.05$) (1.94 ± 0.08 mg. g^{-1} dw and 4.44 ± 0.03 mg. g^{-1} dw, respectively). From these results, acetate buffer 50mM, pH 6 was preferable for hydrolysis and R-PE extraction. Then, we performed hydrolyzing three times for the samples pH 6.

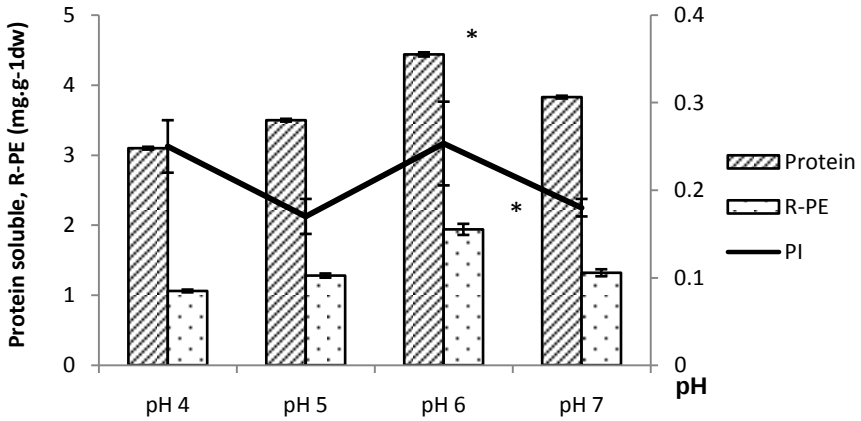

Fig. 2R-PE,water-soluble proteins yields and purity index PI of *Mastocarpusstellatus* after hydrolysis in acetate buffer 50 mM, pH 4, pH 5, pH 6, pH 7. Values are expressed as the mean ± SD (n=3).
NB: Anova one way significantly different results with p<5% are indicated by *.

After centrifuged and obtained the crude extract, the residue will be extracted repeatedly. After three times hydrolysis and extraction, the yield of R-phycoerythrin of CE were calculated (Fig.3).The R-PEcontent after first hydrolysis with enzyme xylanase, acetate buffer50mM pH 6 obtained the highest value, 1.94 ± 0.08 mg. g^{-1} dw. In addition, the R-phycoerythrin yields (Fig.3) show that there is a significant difference between the yields of the first step (PE), and the yield of steps 2 and 3 (SE, TE) in all threeextraction ($p<0.05$). The extraction yields of the R-PE after the second hydrolysis are low. Besides, for the samples in acetate buffer 50mM pH 6 and enzyme xylanase, during the hydrolysis and extraction, it was observed that the supernatants of the extractions SE and TE have a light pink, non-pink color respectively, displaying a denaturation of this pigment, as revealed by the data illustrated in Fig.3.

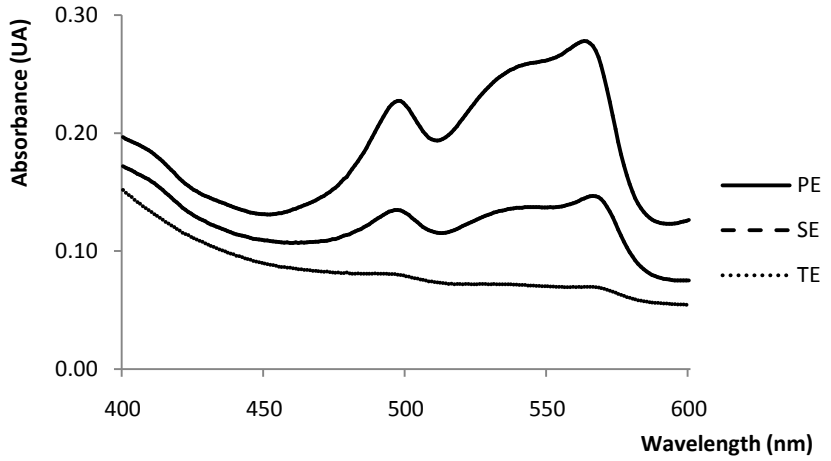

Fig. 3.The numbers of hydrolysis: 3 times (acetate buffer 50 mM, pH 6)
PE: First extraction; SE: Second extraction; TE: Third extraction.

For R-phycoerythrin from the red algae, the benefits of using enzymes were demonstrated by several references[9][10]. They concluded that enzymatic hydrolysis could improve and enhance for extraction process[9][11][12].According the study of Nguyen et al. 2017[2], the R-PE extraction from *M.stellatus* found to improve after hydrolyzingby xylanase. In this study,thepH factor has a stronger influence on R-PE extraction.pH can affect the enzymatic reaction as well as the stability of R-phycoerythrin. pH 6 was preferable for enzyme activity and R-PE extraction because low pH (pH 4 and pH 5) may denature for this pigment [13]. Besides, high pH (pH 7) could be unfavorable for xylanase activity [2].

3.2 Separation the fraction and SDS-PAGE

The crude extract of R-phycoerythrin (sample PE) after hydrolyzed by xylanase in acetate buffer 50mM, pH 6 and then centrifuged was purified by anion-exchange chromatography on DEAE-Sepharose Fast Flow column. The NaCl elution step allowed the sample PE to be separated into three fractions: AE-150, AE-200 and AE-1000.The absorbance spectra of each fractionpresent in the Fig. 4.

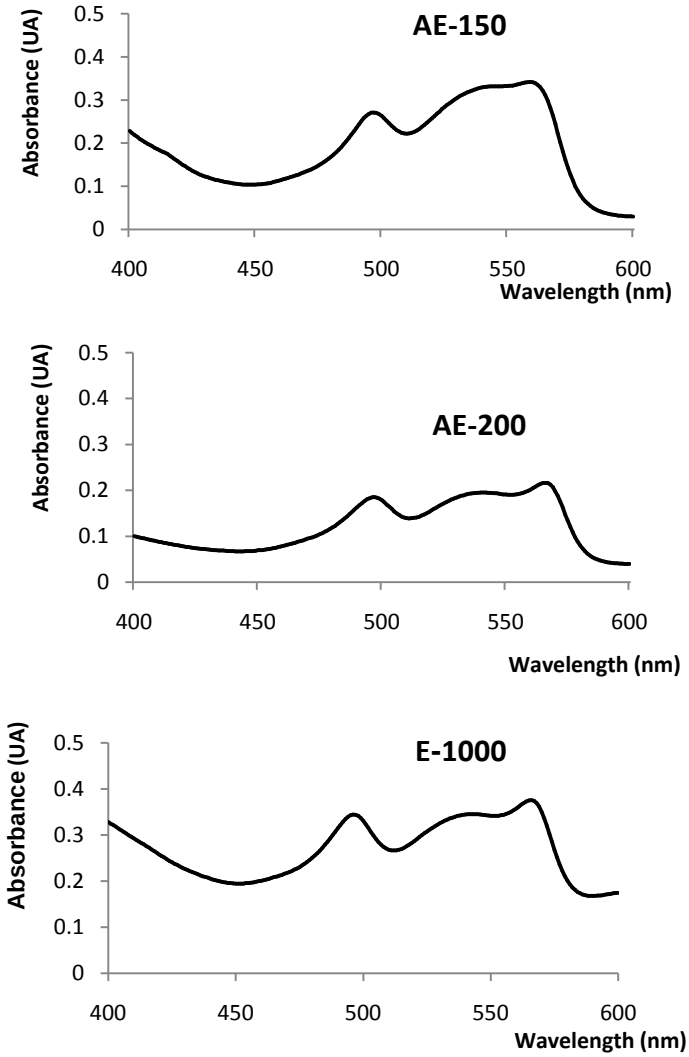

Fig 4. Absorbance spectra of fractions: AE-150, AE-200, AE-1000, obtained after the purification step.
NB: AE-150, AE-200 and AE-1000:the fractions containing R-PE were collected at 150, 200, 1000 mMNaCl.

The UV/Visible spectrum showed that AE-200 fraction hasthree absorbancesphycoerythrin at 498 nm, 540 nm and 565 nm. However, AE-150 fraction showed no maximum at 565nm. The fractionAE-1000 displayed three peaks of R-PE but the absorbance at 280nm was too high.

After obtained these fractions (AE-150, AE-200 and AE-1000)analyzed by SDS-PAGES in 15% SDS-polyacrylamide gels (Fig.5).

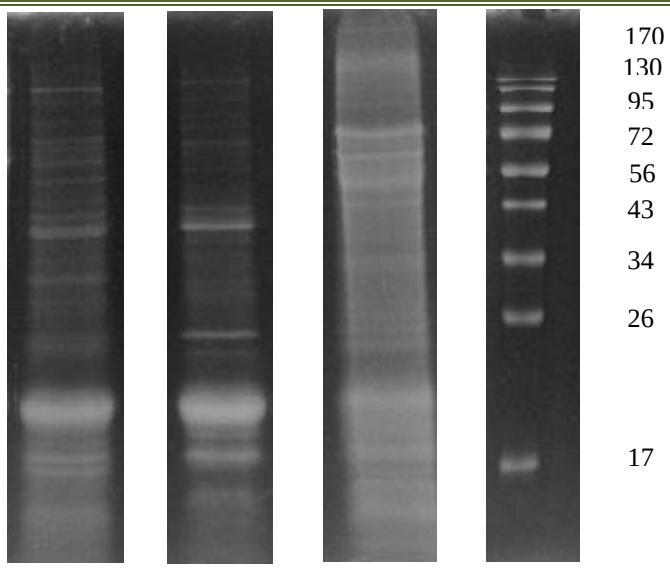

Fig. 5.SDS-PAGE analysis of three fractions obtained after purified and separated. Lane 1, 2 and 3: protein fractions AE150, AE-200, AE-1000, and Lane 4: Molecular weight markers (MW, kDa), respectively.

Three fractionspresented a broad band from 17 kDa to 26 kDa in sizebut the intensity was more clearly for the AE-200 fraction. This result may be corresponded to α (18kDa) and β subunits (21kDa) (Liu et al. 2005; Niu et al. 2006; Munier et al. 2015). This fraction has a low intensity band of 27kDa and 29 kDa corresponding to the γ' and γ subunit in the structure. According the study of Munier et al. (2015), the native R-PE of 260 kDa can be existed four dissimilar subunits (α, β, γ, γ'), corresponding to an oligomeric structure (αβ)6γγ' from *Grateloupia turuturu* [7].Beside, the studies of Rossano et al. (2003) [14], Galland-Irmouli et al. (2000) [15] and Wang (2002) [16] conluded that this pigment has the structure in a hexameric form (αβ)3 γ (αβ)from *Corallina elongate*[7],*Palmariapalmata*[15][16]. According the study of Wang et al. (2015) [17], R-PE is composed of 6α, 6β, 2γ and 1γ' subunits for *Polysiphonia urceolata*. In our study, the AE-200 fraction from *Mastocarpusstellatus* presented the peak of 260kDa and the structure (αβ)6γγ'.

IV. CONCLUSION

In this study, we can confirm that enzymatic hydrolysis couldincrease R-PE yield from *Mastocarpusstellatus*. However, the efficiency of the enzymatic procedure and pH factor differed according to the red algae. In this present work, we described an oligomeric structure (αβ)6γγ' of R-phycoerythrin from *Mastocarpus stellatus* by SDS-PAGES. Besides, next research will be needed to be stable this natural pigment for applications.

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