Efficient Purification and Characterization of Phycoerythrin from Caspian Sea Red Macroalgae (Osmundea caspica)

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Research Article

Keywords:

Posted Date: September 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2025108/v1

Abstract

Phycoerythrin (red pigment) (PE) is a naturally occurring colorant with excellent fluorescent and antioxidant properties that has a wide range of applications. More studies are needed, however, to improve the purification yield and chemical properties of PE. This study aimed to compare the use of different purification methods, which are activated charcoal (AC), ammonium sulfate precipitation (ASP), or anion-exchange chromatography (IE) alone and a combination of AC, ASP, and IE, for PE extraction from the Caspian Sea red macroalgae (*Osmundea caspica*). Response surface methodology (RSM) was employed for preliminary purification, with the independent variables being activated charcoal content (0.1–1% w/v) and stirring time (2–10 min). With an AC content of 0.4% and a stirring time of 2 min, PE purity and concentration were optimized. The predicted values from the equations agreed well with the experimental values, demonstrating the model's robustness. A three-step increase in the ionic strength of IE was also evaluated at three different treatments (PECE, PECE + AC + IE, and ASP + IE). The highest PE purity and lowest Fluorescence intensity (FI) and Antioxidant activity (AA) were obtained in the fraction of AE-200 (second fraction) with an index of 2.8 and a recovery yield of 48% in ASP + IE, whereas an index purity of 2 and the highest recovery yield of 67% were obtained using PECE + AC + IE.

Introduction

A small number of macrophytobenthos plants have been identified in the Caspian Sea, including 13 species of brown algae and 25 species of red algae (Karpinsky et al. 2005). According to reports, *Osmundea caspica* is the only red macroalgae species found on rocky shores of the southern Caspian Sea, and its distribution is temperature dependent (Mehdipour et al. 2015). A recent taxonomic reclassification has moved this species from the genus *Lurencia* to *Osmundea* (Rousseau et al. 2017). As the least studied species of this genus, *O. caspica* is characterized by lacking ‘corps en cerise’, secondary pit connections between cortical cells and a solid discoid holdfast, terete axes, which are identical to those found in other species of the genus (Rousseau et al. 2017). The biomedical properties of *O. caspica*'s alcoholic extract, such as antioxidant activity (AA), anticancer, and antibacterial properties, have been evaluated, and it seems that this species has great potential to be used in the cosmetics, food and pharmaceutical industries (Moshfegh et al. 2019; Tahmasebi et al. 2021). The analysis of the chemical profile of this red macroalgae showed that it contains high levels of proteins and carbohydrates and low levels of lipids (Mehdipour et al. 2014). The most important bioactive compounds in macroalgae are generally divided as follows: pigments, polysaccharides, and phenolic compounds.

In general, three major pigments in macroalgae have been identified: chlorophylls, carotenoids, and phycobilins. The water-soluble supramolecular chromoproteins known as phycobiliproteins are a major constituent of phycobilins. The phycobiliproteins, including phycocyanin (PC), phycoerythrin (PE), and allophycocyanin (APC), play an important role in the electron/energy transfer chain in photosynthesis. Phycoerythrin is a red protein pigment complex (Rastogi, Sonani, et al., 2015). PE's strong light absorption and intense fluorescence emission in the visible spectrum in hexamer ((αβ) - γ-(αβ) 3) configuration are caused by covalently attached linear tetrapyrrole chromophores known as phycobilins. The chromophores are generally linked to the apoproteins of α, β, and γ subunits at conserved positions by two cysteinyl thioether linkages through the bilateral pyrrole ring to form a more stable hexamer (Sun et al. 2009). PE is a protein with a molecular weight of 240 kDa. It is made up of two major subunits (α, β) with molecular weights of 20 and 21 kDa, respectively, and a minor subunit with a molecular weight of 30 (γ) kDa (Galland-Irmouli et al. 2000).

PE presents a double absorption peak at 498 nm and 565 nm and a shoulder at 540 nm (Hilditch et al. 1991), with a maximum fluorescence emission at 575 nm due to fluorescence resonance energy transfer (FRET) from phycourobilin to phycoerythrobilin chromophores (Liu et al. 1999). PE has attracted commercial attention and is used in medicine (as a bioactive), biomedical studies (as fluorescent marker), medical diagnostic (as immune-fluorescent probe), and cosmetics and food processing (as natural colorant). The antioxidant, anti-tumor, neuroprotective, anti-inflammatory, hepatoprotective, and anti-diabetic properties of the PE pigment (Sekar and Chandramohan 2008; Fitzgerald et al. 2011; Senthilkumar et al. 2013a) make R-PE a highly valuable low-volume compound (Mittal et al. 2019). Economically speaking, the price of one mg of PE varies from 180 to 250 USD depending on its purity ratio (A565/A280) (Deniaud et al. 2003). For example, purity ratios of 0.7, 3.9, and over 4.0 in R-PC (another phycobiliprotein) are regarded as the food grade, reactive grade, and analytical grade, respectively (Rito-Palomares et al. 2001). Therefore, the process of PE purification should be tailored to its expected applications. Different non-chromatographic and chromatographic methods have been developed to purify PE from both microalgae and macroalgae (Bermejo et al. 2013). However, simplicity, yield, cost, scale-up issues, selectivity, molecular structure of PE, and complicated process create differences between these purification methods.

Ammonium sulfate precipitation (ASP) (Soni et al. 2008; Manirafasha et al. 2017), aqueous two-phase extraction using polyethylene glycol (Liu et al. 2012), and Rivanol-sulfate (Minkova et al. 2007) are categorized under the conventional non-chromatographic methods and technique. ASP is the most widely used and conventional technique for the preliminary stage of phycobiliprotein purification (Ventura et al. 2012). Depending on the species, different ASP saturation percentages (25 to 85%) have been reported as a preliminary purification step (Munier et al. 2015). This technique yielded PE purity index values of 4.53, 3.5, and 2.7 for *Dasysiphonia japonica* (formerly *Heterosiphonia japonica*) (Sun et al. 2009), *Portieria hornemannii* (Senthilkumar et al. 2013b), and *Polysiphonia stricta* (formerly *Polysiphonia urceolata*) (Liu et al. 2005), respectively. The second purification step can be carried out using chromatographic methods or ultrafiltration. The most widely used method for purifying proteins is ion-exchange chromatography. Linear increasing or step gradient of salt concentration (Kaixian et al. 1993; Senthilkumar et al. 2013a) and pH gradient (Liu et al. 2005) are done for elution. Extensive studies have been carried out through ASP followed by diethylaminoethyl (DEAE) column chromatography. PE purification from *Ceramium isogonum* and *Polysiphonia stricta* using these methods yielded a purity index of 2.1 and 5.6, respectively. Similarly, Denis et al. (2009) managed to retain PE by 100% in *Grateloupia turuturu* using single-stage ultrafiltration with a polysulfone membrane and a molecular weight cut-off of 30 kDa, whereas other proteins and carbohydrates were retained by 32.9 and 64.6%, respectively. It is hence commercially interesting and important to develop a rapid and efficient PE purification technique that produces biologically active PE in high yield under mild conditions. An affinity precipitation technique (APT) based on chitosan (CS) and AC is a potential alternative to existing methods that can address the above-mentioned objectives and limitations (Patil et al. 2006; Gupta and Sainis 2010; Liao et al. 2011; Lee et al. 2016). This method offers some advantages over conventional methods, such as time savings, simplicity, safety (non-toxic biosorbents), and the ability to physically and efficiently adsorb impurities (Dastkhoon et al. 2015; Kaixian et al. 1993; Kanatt et al. 2013). In addition to being environmentally friendly, it can be operated in a non-continuous/continuous mode. There are a few reports on the use of APT for purifying and concentrating PC and PE from a variety of sources (Kaixian et al. 1993; Pan-utai and Iamtham 2019; Fekrat et al. 2019). To the best of our knowledge, the literature contains little to no information on many aspects of using the APT method to purify PE from macroalgae. The optimal AC content and stirring time are one of these less addressed aspects that influence the PE purity, yield, color, and activity.

In this study, *O. caspica*, which is the only marine red macroalgae of the Caspian Sea, was selected as a source of PE. This study aims to optimize PE purification based on AC content using RSM to simultaneously elevate the purity and concentration of the product. This study also compares the purity, concentration, antioxidant activity (AA), and fluorescence intensity (FI) of the purified PE under optimal conditions with those of the most conventional and non-conventional methods, i.e., ASP (fractionation) and ion-exchange chromatography. Finally, the effects of temperature, alcohol, and light on the stability of the obtained PE will be investigated.

Materials & Methods

Specimens of the red seaweed *Osmundea caspica* were collected from Chamkhaleh beach, Langroud, Iran at 36°51´30" N, 53°23´26" E during the winter of 2021. The macroalgae samples were taken to the lab and rinsed with tap water and distilled water to remove epiphytes, sand particles, and other unwanted foreign materials. The samples were then stored at -20 °C until the extraction process began. The extracted seaweed should be thawed.

Extraction procedure:

The extraction conditions were exerted using defrosted thalli that had been ground in liquid nitrogen (average size < 1 mm). Following the procedure of Pereira et al. (2020), the samples were suspended in sodium phosphate buffer (50 mM; pH 7.1) and biomass: buffer ratio (20X) using freezing-thawing methods. Then, the suspension was centrifuged at 15,000 rpm for 25 min at 4 °C to obtain the phycoerythrin-rich supernatant, called phycoerythrin crude extract (PECE). A UV–V spectrophotometer was used to measure absorption spectra from 200 to 900 nm (Biochrom Inc., United Kingdom). The equations proposed by Sampath-Wiley and Neefus (2007) were also employed to calculate the PE purity ratio, concentration, and yield (Eqs 1, 2, and 3, respectively).

$$\text{Purity ratio} = A565/A280 \quad (1)$$

$$\text{R-PE concentration (mg ml}^{-1}) = 0.1247\,[(A\ 565nm - A730) - 0.4583(A618-A730)] \quad (2)$$

Yield (%) = (3)

(The concentration of pure PE (mg mL^{-1})× collected volume (mL))/(The concentration of PECE (mg mL^{-1})× collected volume (mL))×100

The maximum absorbance (λAmax) values of PE and total proteins (contaminant proteins) were 565 and 280 nm, respectively.

AC-based Purification of PE

The resulting PECE was purified by adding five different concentrations of AC powder (100–400 mesh) (i.e., 0.1, 0.32, 0.55, 0.72, and 1% w/v). At each run, AC was added to 25 mL of PECE and the mixture was vigorously stirred (1500 rpm). The samples were then collected at five different time intervals, 2, 4, 6, 8, and 10 minutes, by centrifugation at 15,000 rpm for 20 min. After the pure bright PE was separated from the pellets, the PE purity and concentration were measured, and the optimal conditions were determined. Finally, PE was purified under optimal conditions for further studies and the purity and concentration were measured, and the product was then dried by an Eppendorf Concentrator Plus (Eppendorf, Germany) and stored until further analysis.

Ammonium sulfate precipitation (ASP)

With some modifications, the ASP method described by Moraes and Kalil. (2009) was used to purify PE. Briefly, PECE was saturated up to 30%, mixed with finely powdered $(NH_4)_2SO_4$ at room temperature (30 g in 100 mL of PE) and the mixture was left for 12 h and then centrifuged at 4000 rpm for 5 min. The pellets were re-suspended in 25 ml of 50 mM sodium acetate. Finally, the precipitate was dissolved in 25 mL of 50 mM sodium phosphate buffer (pH=7.1) to obtain ammonium sulfate extract and dialyzed overnight against the same buffer. The extract was then dried by a concentrator.

Purification by anion-exchange chromatography

The PE purification was done based on the method proposed by Munier et al. (2015). PE was further purified from three different resulting mixtures (PECE, PECE+AC, and PECE+ASP) by anion exchange chromatography using a DEAE-Sepharose Fast Flow column (100×25 mm) on a fast protein liquid chromatography (FPLC, AKTA purifier 10, GE, USA). Following equilibration with the same buffer, a three-step increasing ionic strength buffer consisting of 25 mM phosphate, 1 M NaCl (pH=7.1) (0-150 mM, 150-200 mM, and 200-1000 mM) was used for PE elution at a flow rate of 1.0 ml/min. At 150 mM, 200 mM, and 1000 mM NaCl, three fractions containing PE were combined and named AE-150, AE-200, and AE-1000, respectively. Each chromatogram containing the red color was collected separately and monitored using UV-Visible spectrophotometry. The resulting mixtures were desalted overnight by the dialysis method with the same buffer, which lacked NaCl, and then freeze-dried for further studies. The absorbance and fluorescence spectra of each fraction were also monitored.

Water-soluble proteins

The method proposed by Bradford (1976) was employed to compare AC, ASP, chromatography, and PECE methods in terms of the water-soluble proteins of purified PE. The total protein content was determined using bovine serum albumin as a standard ranging from 0 to 10 mg L^{-1} by measuring the absorbance at 595 nm. To prepare the Bradford reagent, 0.1 g of Coomassie Brilliant Blue G-250 was mixed with 50 mL of 95% ethanol and 20 mL of 85% phosphoric acid. Then, the mixture was diluted with distilled water until it reached a volume of 1 L. Finally, a 100 L sample was mixed with 5 mL of Bradford reagent.

AA Analysis

AA was measured using DPPH free radical scavenging assay, as described previously by Burits and Bucar (2000) to compare the AA of the PE purified through different extraction methods (the AC, ASP, chromatography, and PECE). The reaction mixture consisted of 0.1 mL of PE at different concentrations and 3.9 mL of the DPPH reagent (0.002 w/v) dissolved in MeOH. Finally, the solution was kept in the dark at room temperature. The sample's absorbance was measured at 517 nm, and the AA was calculated using the formula below: inhibition (%) = [(A517 of control–A517 of the sample)/A515 of control] × 100.

Where Acontrol denotes the absorbance of the blank solution and Asample represents the absorbance of the sample.

The fluorescence intensity (FI)

The fluorescence emission spectra of PE in different extraction methods (AC, ASP, chromatography, and PECE) in sodium phosphate buffer (PBS) were monitored using a fluorescence spectrometer (Agilent Cary Eclipse) with an excitation wavelength of 498 nm. The FI of different PE concentrations (10, 50, 100 mg mL^{-1} PBS) was measured.

Experimental design and statistical analysis

Based on our preliminary studies and screening experiments, the purification parameter ranges were determined, and a two-factor five-level Central Composite Design (CCD) was used to explore and optimize the purity and concentration of the extracted PE. Table 1 shows the data related to the two independent variables: AC content (0.1–1% w/v) and stirring time (2–10 min). It is noteworthy that purity optimization without considering product concentration can be misleading. As a result, RSM's multi-objective optimization was used to simultaneously elevate both responses to optimal values. To estimate the experimental errors, RSM was performed using the Design-Expert software (trial version 10.0.6.0) with six replicates at the center point. The statistical differences between the experiments were examined by using the one-way analysis of variance (ANOVA) in SPSS -21.0. Anion-exchange chromatography, AA and FI experiments were performed in three replicates, the data are expressed as the mean ± SD and were compared using the Duncan test at a significance level of 0.05 and Graphpad prism 9 used for drawing the graphs.

Results

CCD statistical analysis and model fitting

The effects of AC content (X1) and time (X2) on PE purity, concentration, and water-soluble proteins were determined by RSM to achieve the optimal purity and concentration of PE. A total of 13 experimental responses were evaluated as shown in Table 2. The results indicated that PE purity, concentration, and water-soluble proteins ranged between 0.64 and 0.8, 0.035 and 0.067 mg mL^{-1}, and 0.229 and 0.456 mg mL^{-1}, respectively. According to the developed model, linear regression was used to determine the optimal conditions for each response simultaneously, with overall desirability of 79%. When the independent variable was 0.43% (charcoal) at an exposure time of 2 min, the optimum for PE purity, concentration, and water-soluble proteins, the values obtained were around 0.66, 0.068 mg mL^{-1}, and 0.393 mg mL^{-1}, respectively. The accuracy of the model was further tested by conducting another experiment under the conditions suggested by the model, and the results revealed that the PE purity, concentration, and water-soluble proteins were 0.67±0.09, 0.053±0.05 mg mL^{-1}, and 0.35±0.1 mg mL^{-1}, respectively, under the above-mentioned conditions. The one-way ANOVA results on purity, concentration, and water-soluble proteins demonstrated the high F-value of purity (28.18), concentration (39.52), and water-soluble proteins (108.17) as well as the related low P-values (< 0.0001) (Table S1). For all responses, the observed values agreed with the predicted values, indicating the model's robustness and the relevance of prediction equations. This finding suggested a link between the charcoal level and the purification of water-soluble proteins, in general, and R-PE extraction, in particular. Equations 4, 5, and 6 provide mathematical models in terms of coded variables for purity, concentration, and water-soluble proteins, respectively. It should be noted that insignificant terms were omitted in order to achieve a better fit.

4) Purity= 0.6751 +0.0777 X1 - 0.0115 X2

5) Concentration = 0.0554 - 0.0099 X1-0.0038 X2

6) Water-soluble proteins = 3.41 - 0.5825 X1 - 0.1092 X2

Where the coded independent factors are X1 = Charcoal S% and X2 = time.

As shown in Table S1, the effect of all the independent variables on PE purity, PE concentration, and water-soluble proteins was significantly linear, as well as the interaction between charcoal and time (P< 0.05), except for the linear interaction between time and PE purity (P>0.05). More specifically, AC (linear term) exhibited the greatest effect on PE purity, PE concentration, and water-soluble proteins (Table S1). The results showed that the PE purity increased significantly from the lower level of AC at 0.1% to the higher level of AC at 1% and that the PE purity decreased as the time, at 0.325% AC, increased from 2 to 10 min. Maximum AC level and shortest incubation time resulted in higher purity. After 10 min of incubation with AC at 0.1-1%, the purity ratio ranged from 0.4 to 0.8 (Figure 1 A). The same trends were observed with PE concentration and water-soluble proteins, as they increased with decreasing incubation time for all AC concentrations (Figures 1 B and C). The combination of about 1% AC and medium levels of incubation time (around 6 min) resulted in the highest PE purity (Fig. 1 A) but reduced the PE concentration (Fig. 1B). According to the findings,

AC had a more severe effect on all responses than the incubation time did, as higher AC levels resulted in maximum PE purity but minimum PE concentration and water-soluble proteins. A negative correlation was found between incubation time and all responses, implying that longer incubation times affected the purification process by absorbing PE rather than the impurities, as previously explained. The PE purified by AC under ideal conditions was used for further analyses.

Purification using anion-exchange chromatography

The purification process was also carried out by the anion-exchange chromatography on DEAE Sepharose Fast Flow. To allow separation and purification of the R-PE, a three-step increasing gradient in buffer ionic strength (0-150 mM, 150-200 mM, and 200-1000 mM) was performed. Three different resulting mixtures (PECE, PECE+AC, and PECE+ASP) were analyzed on DEAE Sepharose Fast Flow to compare purification methods, as described by Munier et al. (2015). The anion-exchange elution pattern by NaCl gradient enabled the separation of the above-mentioned resulting mixture into three fractions. Table 3 presents the results of separate analyses of each fraction monitored for three different treatments. Purified PE from each fraction was collected at 150 mM, 200 mM, and 1000 mM NaCl concentrations and labeled as AE-150, AE-200, and AE-1000, respectively. Figure 2 depicts the qualification absorbance and fluorescence of each fraction in the three treatments (PECE, PECE+AC, and PECE+ASP). The resulting mixtures of one of these peaks were then dialyzed for further analysis.

Only the fraction AE-200 (second fraction) showed a higher PE purity and concentration in all treatments after ion-exchange chromatography, which is close to the R-PE purification standard of 3.2 (Chuner *et al*., 2012). Purity was 1.46, 2.04, and 2.8, with PE concentrations of 0.23, 0.32, and 0.18 mg/g of FW obtained in PECE, PECE+AC, and PECE+ASP, respectively. To monitor the efficacy of the purification, three ratios were evaluated. The purity of the R-PE was calculated using the ratio A565/A280, with the ratios A620/A565 and A650/A565 indicating the presence of allophycocyanin and phycocyanin contaminants, respectively. These ratios must be lower than 0.001 (Nguyen et al. 2020). The remaining ratios of A620/A565 and A650/A565 were variable depending on fraction and treatment, with the former accounting between 0.002 and 0.4 and the latter between 0.01 and 0.3, demonstrating the low levels of phycocyanin and allophycocyanin contaminants (Table 3). The absorbance and fluorescence spectra of the three fractions obtained after the purification step in all treatments are shown in Figure 2. The UV/Visible spectrum of the first and second fractions in all treatments (Fig. 2) showed a high absorbance at 565 nm and no maximum at 280 nm, indicating the presence of water-soluble proteins, except for Treatment 1, which had a higher absorbance at 280 nm than at 565 nm, indicating the large concentration of contaminating proteins.

The highest value in PE recovery was observed in Treatment 2 (PECE+AC), which contributed 67% at AE-200. This fraction represented native PE. Furthermore, despite having the highest purity in Treatment 3 (PECE+ASP) at AE-200, PECE+AC had the highest yield of PE concentration and the lowest A620/A565 and A650/A565 ratios. At AE-200, the maximum fluorescence was also detected at 575 nm in all three treatments.

AA- and fluorescence intensity-based PE evaluation:

The AA and FI features of the PE obtained from the above-mentioned treatments were evaluated *in vitro* at five concentrations (2, 4, 6, 8, and 10 mg mL^{-1}) of PECE and PECE+ASP as well as the PE purified under optimal conditions in two following treatments (PECE+AC+chromatography). The results are shown in Table 4. Increased PE concentration in all treatments increased DPPH radical scavenging activity and FI. PE purified by PECE, PECE+AC+IE and PECE+ASP methods inhibited DPPH radicals in the ranges of 65.03-78.36, 58.26-75.83, and 43.99-58.5%, respectively. Among the three purification methods, the PECE+AC+ Chromatography method demonstrated the highest FI activity, whereas the purified PE by PECE+ASP demonstrated the lowest FI. Unlike FI, there was a statistically significant difference between the three purified PE specimens in AA (PECE method > PECE+AC+ Chromatography > PECE+ASP method) (Table 4).

Figure 3 shows the strong linear regression relationships observed between the PE (PECE+AC+ Chromatography) concentration and AA as well as between AA and FI (R2 = 0.97 and 0.98, respectively). The R2 for the correlation between PE concentration and FI was 0.98, and similar trends were observed for other treatments.

Discussion

This study aimed to find the optimal conditions for the PE purification process, including AC content, and to achieve maximal PE purity and concentration through multi-objective optimization. The model's analysis of variance revealed low P values (0.0001) and high F-values for the responses, confirming that the developed model was statistically significant. The purification process increased PI values significantly (p 0.05) in all experiments. Furthermore, the non-significant lack of fit ($p > 0.05$) and high values of R2, adj-R2, and pre-R2 suggested that second-order regression could establish a good correlation between the responses and the independent variables (Table S1). The model's validation revealed that the experimental and theoretical values were highly accurate and applicable, indicating the model's suitability and efficacy. In general, all levels of AC terms outperformed the time terms. The low P-value and high F-value of linear functions in all variable responses strongly confirmed that changes in those variables could directly lead to a decrease or increase in the investigated response, i.e. PE purity, PE concentration, and water-soluble proteins, resulting in deteriorated purity and concentration.

Because of its highly specific surface area, AC is commonly used for decolorization and odor removal. Furthermore, it is simple to use and does not affect the extract's bioactivity (Liao et al. 2011). R-PE concentration in the crude extract was approximately 0.8 mg g^{-1} fresh wt. Based on seaweed species and extraction method, previous studies found that the concentration of PE ranged from 1.23 mg g^{-1} fresh wt. in *Portieria hornemannii* to 0.05 mg g^{-1} fresh wt. in *Kappaphycus alvarezii* (Senthilkumar et al. 2013b; Dewi et al. 2020). A few studies have investigated the effect of AC on PE extracted from seaweed. The use of AC resulted in a threefold increase in PE purity from 0.25 to 0.67 under optimized conditions. It should be noted that the increased PE purity achieved in this study was the same as the results on phycocyanin for *Arthrospira platensis* (Cyanobacteria) with an optimum level of AC (80 g L^{-1}) (Pan-utai and Iamtham 2019), whereas purification with a mixture of activated coal (AC) and chitosan (CS) was as efficient as crude extraction at their pre-purification stages of phycocyanin (by 7 times in *Arthrospira platensis* and 2 times in *Asterocapsa nidulans* – formerly *Anacystis nidulans* BD1) (Cyanobacteria) (Gupta and Sainis 2010; Fekrat et al. 2018). Phycocyanins have been previously purified from various cyanobacteria using a variety of absorbents such as AC and CS, yielding different optimum levels of AC as well as different responses in purity and concentration. The results of this study, however, are different from those obtained for phycocyanin purification, which required lower AC and time (Gupta and Sainis 2010; Pan-utai and Iamtham 2019). There is no information on the effects of AC on PE purification in seaweed. These contradictory results could be attributed to the type of algae (microalgae or seaweed), species of algae, extraction method, nature and level of contamination (low molecular protein and carbohydrate), and type of phycobiliproteins (Kaixian et al. 1993; Gupta and Sainis 2010; Ghosh and Mishra 2020). The purification of the crude extract is a complex model in which many factors come into play. To take advantage of the absorbing effect of AC, high levels of AC were prepared, which allowed for greater purification than lower levels, reducing PE concentration and water-soluble proteins. This phenomenon is most likely due to the simultaneous adsorption of PE and low molecular weight protein or contaminating proteins (Kaixian et al. 1993; Fekrat et al. 2018). It seems that the extra concentrations of AC absorb PC (along with other impurities present) and thus eliminate it from the solution. As a result, the optimal values of AC and time for concurrently optimum purity and concentration were 0.43% and 2 min, respectively.

Phycobiliproteins are the most highly sought-after natural chromophores-containing proteins, with numerous applications in the food, diagnostic, therapeutic, biotechnology, and cosmetic industries. As a result, it is critical to optimize their isolation and purification procedure (Gupta and Sainis, 2010). Further purification of PE was performed in this study using anion-exchange chromatography (IE) on PECE as well as AC (at optimal conditions) and ASP methods. Tables 3 show the purification yield and purity of the PE obtained after IE treatment and adsorption on PECE, AC, and ASP, respectively, and are labeled IE + PECE, IE + AC, and IE + ASP. The purity of the fraction AE-200 from IE + PECE, IE + AC, and IE + ASP improved further to 1.48, 2.04, and 2.8, respectively, but concentrations of 0.23, 0.28, and 0.33 decreased with the loss of PE. Furthermore, AC + IE had the lowest absorbance ratios A_{620}/A_{565} and A_{650}/A_{565} and the highest recovery, demonstrating negligible contamination with phycocyanin and allophycocyanin.

PE purification from red algae using single or combined methods is derived from many conventional methods such as ammonium sulfate precipitation, ion-exchange chromatography, gel filtration, hydrophobic interaction chromatography, hydroxyapatite chromatography, electrophoresis, and ultrafiltration. According to previous reports, purity and concentration ranged from 1.07 to 6.7, and concentration ranged from 0.038 to 8.9 mg g^{-1} of dry weight (Rossano et al. 2003; Denis et al. 2009). On the one hand, the use of a single and combined method of purification in this study resulted in the multiplied PE purity by 3.5 and 4 times at the end of purification with ASP + IE and AC + IE, respectively, whereas the highest purity was observed in one-step purification with IE + PECE by 6 times. In addition, AC + IE produced the highest PE purification yield and concentration when compared with other treatments. *Gracilaria gracilis* (Nguyen et al. 2020) and *Grateloupia turuturu* (Munier et al. 2015) were purified by 7 and 11 times, respectively,

using a single method of anion-exchange chromatography, and produced a purity index of 3.2 and 2.89. There is little information on the effects of combining the AC and IE methods on PE purity and concentration levels in red seaweed, and more research is needed in this area. Our optimum protocol (AC + IE) managed to reduce the time and cost of purification when compared to the ASP method, around 1 h vs. 48 h, respectively, due to implementing more complex and costly procedures than in previous studies and separation time in the purification, which play a critical role in maintaining PE stability. These contradictory results could be due to an earlier extraction method and/or algae species, the nature and level of contamination in the solution, or the purification method chosen. Absorption spectroscopy, fluorescence spectroscopy, SDS-PAGE, and ion-exchange chromatography were used to confirm PE purity.

Finally, the resulting mixture of three treatments was compared in terms of AA and FI at five different concentrations. The AA and FI were used to establish the efficacy values of different treatments and compare responses under five different concentrations (Table 4). The results for the AA and FI that were tested in this study revealed that PECE had the highest level of AA, but AC + IE had the highest level of FI among the others. However, the PECE had the highest level of AA. Due to the higher amounts of PE molecules in the AC-purified PE sample as discussed earlier, the PE purified by the AC + IE method had a higher AA than the PE purified by the ASP method. PE AA has been demonstrated in a variety of red seaweed species with varying levels of AA. The study findings are consistent with those reported for *Portieria hornemannii* (Senthilkumar et al. 2013 a), which exhibited moderate to low antioxidant activities, whereas the antioxidant activity of PE extracted from *Kappaphycus alvarezii* (Dewi et al. 2020) was categorized as strong to very strong. In general, the contradictory results of these studies can be attributed to the presence of other components involved in AA (such as phycocyanin and allophycocyanin) and unwanted or different impurities (such as free polyphenols and polysaccharides), pH and ionic strength in solution, PE aggregation, and partial denaturation of its structure precipitated with ammonium sulfate caused by the purification method (Mishra et al. 2008: Zhou et al. 2012; Amid et al. 2012; Fekrat et al. 2018)

PE is a fluorescent compound that exhibits fluorescence properties when extracted into an aqueous buffer (Eriksen 2008). Our previous study results showed that when excited at 495 nm, the maximum fluorescence emission wavelength of IE + PECE, IE + AC, and IE + ASP is around 575 nm, which was consistent with previous reports (Wang et al. 2015). The low FI of purified PE, particularly PE purified by the ASP method, could be due to unfavorable changes in PE's spatial structure during purification processes, but it was higher in PE purified by AC + IE. The study findings on phycocyanin were consistent with the results of Patil et al. (2006) and Fekrat et al. (2018) who found that the FI reduced when the native form of phycocyanin was manipulated. Finally, it can be concluded that the AA and FI of PC have a linear regression relationship. The high coefficient of determination suggests that PE's AA and FI can be used interchangeably.

The optimized procedures for *Osmundea caspica* PE purification can be conclusively summarized based on the experimental results: (1) phycobiliprotein pre-purification with AC at 0.4 percent in 2 min phosphate buffer (pH 7.0); (2) R-PE salting-out with $(NH_4)_2SO_4$ at 30% saturation; and (3) PE purification from the AC treatment using DEAE Sepharose Fast Flow chromatography developed with a step ionic strength gradient of 200 mM NaCl per ml. The single and combined chromatographic modes were used in this study. Pre-purification with different concentrations and times suggested that such a system with IE was the best approach for high PE yield and purity with fewer contaminants. Finally, these optimized AC + IE methods improve the efficiency, convenience, and ease of scaling-up of PE purification from *O. caspica* in practical applications, while also providing other benefits such as being a time-efficient and eco-friendly alternative to conventional methods.

Declarations

Data availability statements

All data generated or analyzed during this study are included in this published article and its supplementary information file.

Conflict of interest

There is no conflicts of interest to declare in this work.

Author Contribution Statement

All of the experiments, data analysis, and manuscript preparation were conducted by Hamid Eshaghzadeh. In addition, Sobhan Akhavan assisted in macroalgae collection and laboratory activities, and Mohammad Amin Hejazi advised in microalgae cultivation

and strain selection Maryam Shahbazi and Leonel Pereira were responsible for the conceptualization and supervision of the experiments and the critical revision of the manuscript.

Acknowledgments

The author wishes to thank the center of Dr. Keyvan Marine Science and Technology Research Center (Gilan province, Iran) managers for their support and provision of the facilities and Mr. Sadeghipour for their kind help. Leonel Pereira thanks to MARE, Marine and Environmental Sciences centre, and to the Associate Laboratory ARNET.

Funding

This research activities were carried out under the support of Iran National Science Foundation, INSF, Iranian Deputy of Science and Technology.

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Tables

Table 1 Experimental design levels of independent variables (activated charcoal content, and stirring time) used in the central composite design

Factor	Unit	Parameter sign	Variable levels				
			− α	-1	0	+1	+α
AC content	w/v%	X1	0.1	0.325	0.55	0.775	1
Time	min	X2	2	4	6	8	10

Table 2 Purification conditions and responses for purity index, R-PE contents and water-soluble protein obtained for the central composite design

	Responses		Independent variables		
(mg ml^{-1}) Soluble protein	Concentration (mg ml^{-1})	Purity	X_2 (min)	X_1(%)	Run
2.29	0.0355396	0.804348	6	1	1
3.44	0.0563854	0.72561	6	0.55	2
3.95	0.0677124	0.64	4	0.325	3
4.56	0.0747788	0.481928	6	0.1	4
3.46	0.0578818	0.709302	6	0.55	5
2.67	0.0465132	0.711111	8	0.775	6
3.12	0.0543902	0.71875	6	0.55	7
3.15	0.0424812	0.664122	10	0.55	8
2.96	0.0412966	0.781818	4	0.775	9
3.75	0.063722	0.660194	2	0.55	10
4.13	0.059877	0.565022	8	0.325	11
3.43	0.0609578	0.640394	6	0.55	12
3.37	0.0584222	0.673913	6	0.55	13

Table 3 Purification step of the PECE, PECE+AC and PECE+ASP by semi-preparative anion exchange chromatography on DEAE Sepharose. R-PE was eluted at 150, 200, and 1000 mM NaCl using a three-step increase in ionic strength

	Anion Exchange Chromatography				Anion Exchange Chromatography				Anion Exchange Chromatography			
Fraction name	**PECE**	AE-100	AE-150	AE-1000	**PECE+AC**	AE-100	AE-150	AE-1000	**PECE+ASP**	AE-100	AE-150	AE-1000
The volume of sample (mL)	15	23	17	7	15	23	17	7	15	23	17	7
Purity index	0.35±0.00	1.38±0.04	1.46±0.08	0.15±0.01	0.49±0.005	1.64±0.20	2.04±0.13	0.35±0.00	0.90±0.00	2.51±0.09	2.79±0.06*	0.42±0.01
Ratio A620/A565	0.18±0.01	0.015±0.02	0.10±0.01	0.10±0.18	0.17±0.01	0.002±0.0	0.003±0.00	0.03±0.00	0.20±0.00	0.09±0.00	0.20±0.02	0.42±0.03
Ratio A650/A565	0.06±0.00	0.01±0.00	0.02±0.01	0.17±0.01	0.06±0.00	0.010±0.00	0.01±0.01	0.04±0.01	0.09±0.00	0.05±0.01	0.07±0.01	0.34±0.02
[R-PE] (mg mL^{-1})	0.1±0.00	0.04±0.00	0.06±0.00*	0.01±0.00	0.07±0.00	0.01±0.00	0.05±0.00	0.005±0.00	0.08±0.00	0.02±0.00	0.03±0.00	0.01±0.00
R-PE extraction amount (mg g^{-1} fw)	0.77±0.01	0.11±0.00	0.24±0.01	0.01±0.00	0.65±0.00	0.06±0.02	0.32±0.01*	0.006±0.00	0.73±0.00	0.1±0.02	0.18±0.00	0.06±0.00
R-PE purification yield(%)	100	15	30	1	100	9	67	1	100	15	24	8
Water-soluble protein (mg.g^{-1} fw)	0.49±0.04 (100%)	0.16±0.01 (31.1%)	0.21±0.02 (41.90%)	0.13±0.03 (27.12%)	0.31±0.04 (100%)	0.11±0.02 (33.75%)	0.12±0.05 (37.26%)	0.08±0.01 (25.47%)	0.29±0.02 (100%)	0.07±0.00 (23.61%)	0.09±0.01 (32.6%)	0.06±0.01 (19.44%)

ND: Data are expressed as the mean ± SD (n = 3). Significantly different results with p < 5% are indicated by (*) for each fraction in all sample within Anion Exchange Chromatography

Table 4 the comparison of the purity, concentration, AA (antioxidant activity), and FI (fluorescence intensity) of PECE, PECE+AC+IE and PECE+ASP

Data are expressed as the mean of triplicate experimental values (n = 3) ± standard deviation (SD). The same letters in the same column indicate that no significant difference between the different PE samples at a 95% confidence level (P < 0.05)

Figures

Extract value (mg)	A280	A565	A618	A730	purity	PE (mg ml^{-1})	Yield (%)	AA (%)	FI (a.u.)
PECE	0.604	0.221	0.048	0.007	0.36±0.0003a	0.097±0.0002[a]	100		
2								65.03±0.7[a]	940.12±15.58
4								67.4±2.35[a]	1641.17±30.08
6								70.3±0.94[a]	2368.33±42.08
8								75.7±1.8[a]	3040.436±54.55
10								78.3±0.92[a]	4216.611±76.37
PECE+AC+IE	0.056	0.115	0.026	0.002	2.04±0.132[c]	0.051±.001[c]	52		
2								58.26±0.37[a]	1447.98±3.6
4								61.4±0.47[a]	2750.61±129.11
6								65.8±0.8[a]	4207.85±17.70
8								71±1.73[a]	5396.18±84.41
10								75.83±1.6[a]	6097.28±105.76
PECE+ASP	0.179	0.202	0.062	0.027	1.134±0.09[b]	0.079±0.003[b]	81		
2								43.99±2.7[b]	873.76±108.81
4								46.7±1.11[b]	1671.61±95.87
6								50.1±2.34[b]	2131.24±63.94
8								54.4±1.02[b]	2413.96±56.33
10								58.5±3.61[b]	3519.60±50.77

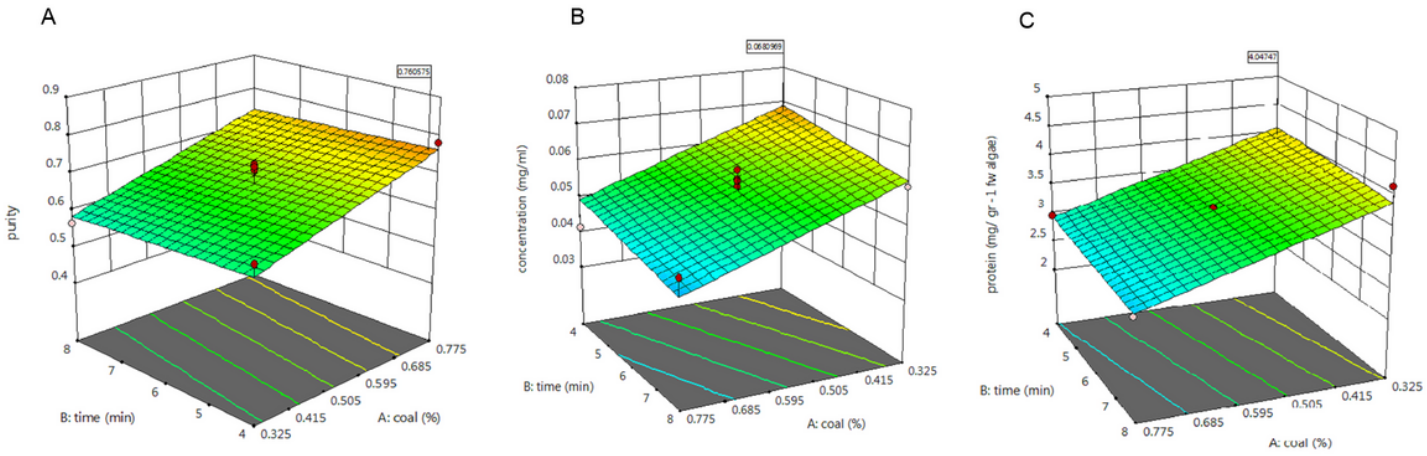

Figure 1

Effect of 100–400-mesh AC (activated charcoal) (0.1–1% w/v) and stirring time from 2 to 10 min as independent variables on purity (A), concentration (B), and water-soluble protein (C) of phycoerythrin from *O. caspica*

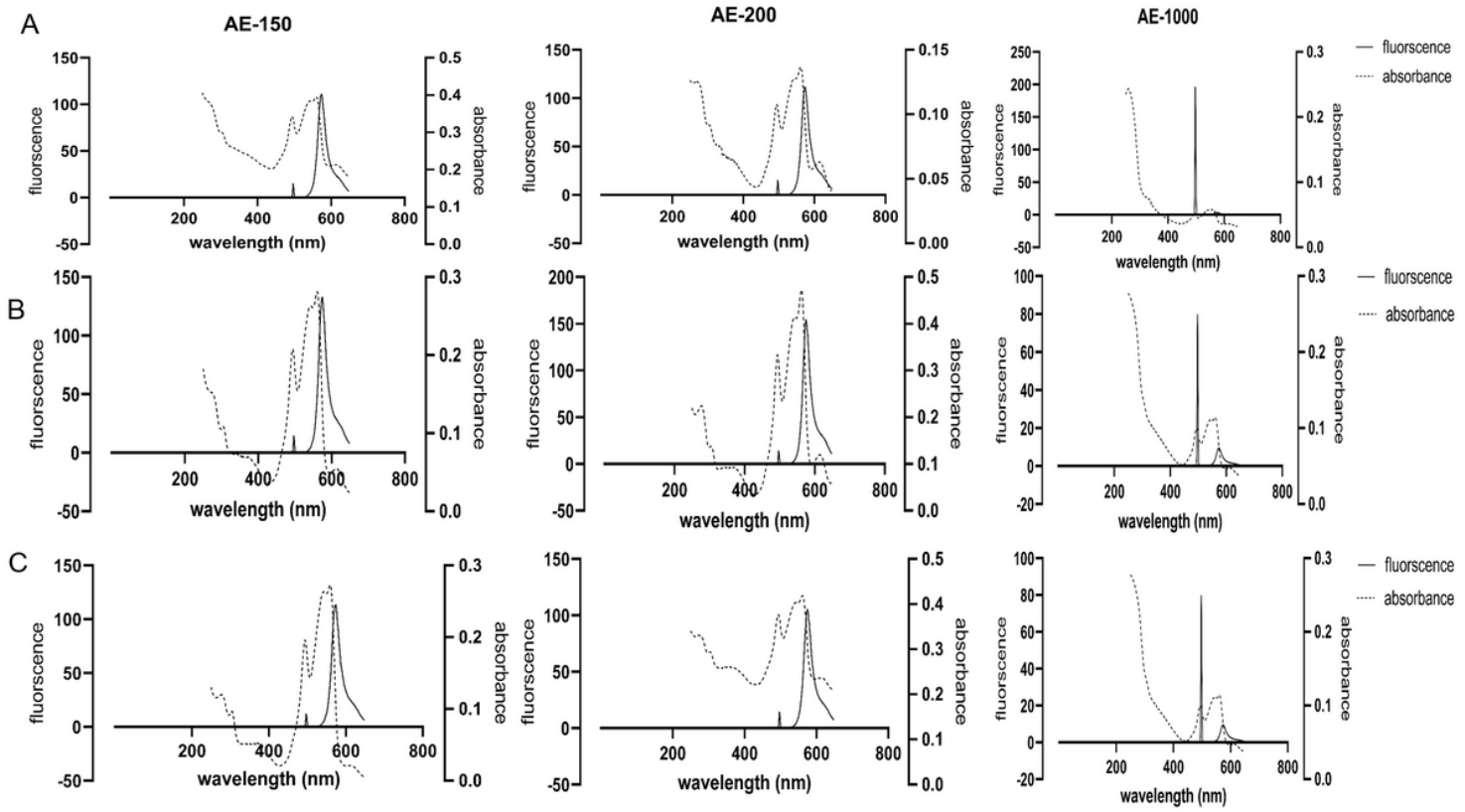

Figure 2

Evaluation of RP-E purity. Absorbance (dash line) and fluorescence (solid line) spectra of anion-exchange chromatograms at three different treatments of fractions AE-150, AE-200, and AE-1000, respectively, obtained after the purification step. Treatment 1 (PECE+IE) (A), Treatment 2 (PECE+AC+IE) (B) and Treatment 3 (PEC+ASP+IE) (C)

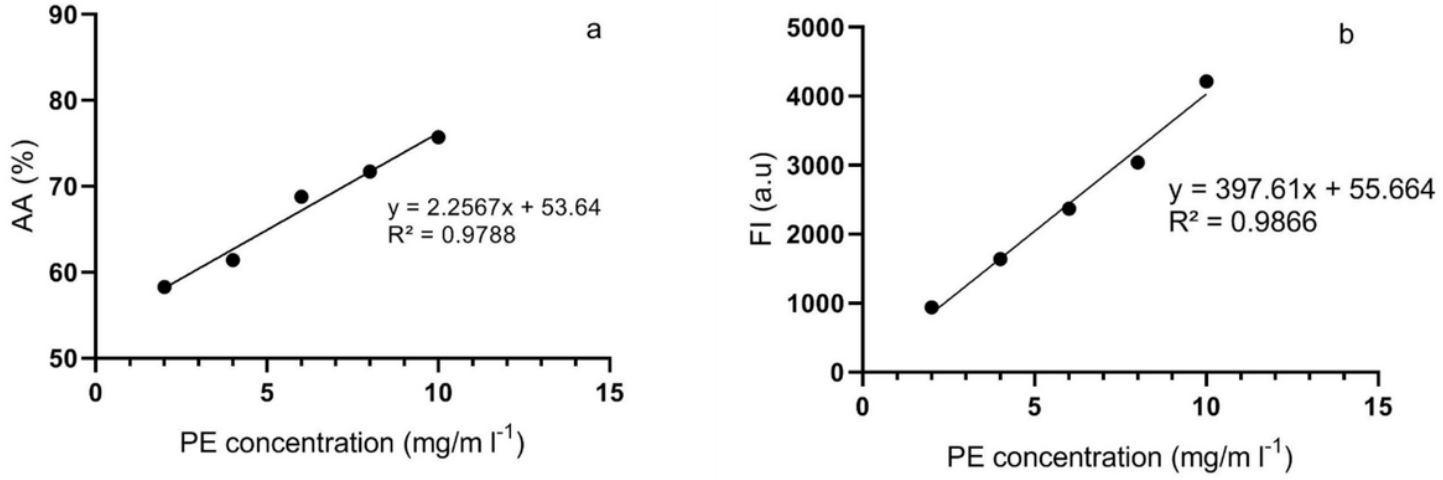

Figure 3

the relationships of PE obtained by PECE+AC+IE method in 2–10 mg mL−1 solutions. PE concentration and AA (antioxidant activity) (a), PE concentration and FI (fluorescence intensity) (b)

Supplementary Files

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