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Abstract. *Kappaphycus alvarezii* is type of red seaweed that widely cultivated in Indonesia. *K. alvarezii* contains phycobiliproteins mainly phycoerythrin which is commonly used as natural dyes in food and cosmetics, fluorescent, and analytical reagents. Phycoerythrin is generally extracted from red seaweed using buffer phosphate with freeze thawing process. The purpose of this study was to determine the effect of extraction time using ultrasonic waves on the characteristics of phycoerythrin. The maximum absorbance was formed at a wavelength of 499, 534, and 564 nm. Ultrasonic extraction treatment for 30 min and precipitation 60% ammonium sulfate were the best treatments, which had a pigment concentration of 1.9102 mg/mL, purity index 0.8311, and protein concentration of 0.5206 mg/mL. The antioxidant activity possessed by the phycoerythrin pigment is categorized as strong to very strong.

Keywords: *Kappaphycus alvarezii*; phycoerythrin; ultrasonication,

1. Introduction

Indonesia is known as on one of the largest producer of seaweed. Data FAO 2018 showed that, in 2015 Indonesia had contribution 34.6% in the global seaweed production and it was no 2 after China [1]. However on the carrageenophyte seaweed, which can produce carrageenan, Indonesia was the biggest producer since 2008. The total production of carrageenophyte seaweed was 10.2 million tones of wet seaweed.

Carrageenophyte seaweed is grouped as red seaweed which the pigment content is dominated by phycoerythrin. Phycoerythrin is one of phycobiliproteins which has high economic value. Phycoerythrin pigment has a high potential to be developed as natural dyes replacing carcinogenic synthetic dyes. Phycoerythrin were reported possess variety of biological activities, such as antiviral, antioxidant, anti-inflammatory, antidiabetic, antitumor, antihypertensive, immunosuppressive, and neuroprotective [2-5]. Unfortunately, the pigment in the carrageenan processing is almost not recovered and it is released as wastewater.

Phycoerythrin has sensitive with high temperature. For this reason, phycoerythrin is generally obtained from red seaweed using freeze thawing. This process will need long time and consumes high

energy. The innovation of extraction techniques is now being developed with the concept of "green extraction concept" such as ultrasonic extraction [6]. The extraction has various benefits, including time reduction, increasing of the mass transfer process and extraction yield, improving extract quality, and reducing energy consumption used in the extraction process [7-9].

Ultrasonic extraction was reported on phycocolloid extraction such on Laminaran [10], carrageenan [11] and agar [12]. This method could increase yield of phycocolloid at shorter time than conventional method. To the best of our knowledge no study has yet been reported on the phycoerythrin extraction using ultrasonication on fresh *Kappaphycus alvarezii* seaweed. In this study, phycoerythrin was extracted.

2. Method

2.1. Material and Tools

K. alvarezii seaweed was obtained from the waters of Lontar Village, Serang Regency, Banten. The chemical materials used in this study were phosphate buffer pH 7 0.1 M, distillated water, 1,1-diphenyl- 1-picrylhydrazyl (DPPH) Sigma, Sigma bovine serum albumin (BSA) standard protein, coomassie brilliant blue (Sigma), 95% ethanol, 85% phosphoric acid, Tris-HCl 1.5 M (pH 8.8), 30% acrylamide (Sigma), 10% sodium dodecyl sulfate (SDS), N, N, N, N'-tetramethyl-ethylenediamine (TEMED), ultrapure water, Tris-HCl pH 6.8 0.5 M, marker protein 10-250 kDa (Bio-Red), glacial acetic acid, 25% glutaraldehyde, 5% sodium thiosulfate, sodium acetate, 2.5% AgNO3, 37% formaldehyde, sodium carbonate, EDTA.Na2.2H2O, 87% glycerol.

The equipment used in this study consisted of a batch of ultrasonic cleaner at 40 kHz with 200 W power (Desen DSA100-SK4.0L, China), magnetic stirrer (Scilogex MS-H280-Pro, USA), centrifuge (Shimadzu CR21G Himac, Japan), UV-Vis spectrophotometer (Shimadzu UV-1700 PharmaSpec, Japan), High Performance Liquid Chromatography (HPLC) Shimadzu CMB 20A, and SDS-Page equipment (To AE-6500) , Japan).

2.2. Research procedure

This study consisted of four steps, namely *K. alvarezii* preparation, extraction of phycoerythrin, purification phycoerythrin, and characterizations of phycoerythrin.

2.2.1. Seaweed Preparation

Seaweed preparation was carried out by separating the material from various impurities. The seaweed was stored at -18 °C until the extraction process will be carried out. Seaweed that will be extracted should be thawed. Fresh/wet seaweed was blended to obtain smaller particle size.

2.2.2. Phycoerythrin extraction using ultrasonication

Phycoerythrin from seaweed was extracted with 0.1 M phosphate buffer solvent at pH. The solvent was added to the sample with a ratio of 1:10 (b/v) [13]. Seaweed was blended to obtain a paste form. The paste was extracted at 4 °C with a frequency of 40 kHz ultrasonic with the treatment time 0, 15, 30, and 60 min. The paste extracted was filtered with 150 mesh of nylon cloth. The filtrate was centrifuged at 10,000 g at 4 °C for 30 min. Supernatant was obtained as a crude extract of the phycoerythrin.

2.2.3. Precipitation of Phycoerythrin

The crude extract of the phycoerythrin was precipitated with ammonium sulfate with saturation 60%. The crude extract of the phycoerythrin and ammonium sulfate was incubated at 4 °C for 1 hour. The result of precipitation was centrifuged at 4 °C and 10,000 g for 30 min [14]. The precipitate was dialyzed with a 14 kDa membrane bag using distilled water at 4 °C. During the dialysis process, distilled water was replaced every 4 hours for 24 hours.

2.2.4. Characterizations of Phycoerythrin extracts

Characterizations were carried out on the crude and precipitated of the phycoerythrin pigments. The characterizations parameters included concentration of phycoerythrin, protein content, antioxidant activity was analyzed.

2.2.4.1. Determination of concentration and purity index of phycoerythrin

Analysis of the concentration and purity index of phycoerythrin pigments was carried out using a UV-Vis Shimadzu UV-1700 spectrophotometer at wavelength 200 to 700 nm. The phycoerythrin purity index can be determined by a ratio of A565/A280 [15]. The concentration of phycoerythrin can be calculated using the following equation.

Phycoerythrin (µg / mL) = 155.8 A498.5 - 40.0 A614 - 10.5 A651

2.2.4.2. Protein content

Protein concentrations in the phycoerythrin pigments were measured using Bradford reagents. The reagent was prepared by mixing 0.20 g of Comassie Briliant Blue G-250 dissolved with 10 mL of 95% ethanol and 20 mL of 85% phosphoric acid. The mixture was then diluted with distilled water to 1 L. A sample of 100 µL was added with 5 mL of Bradford reagent. The blue complex formed is incubated for 5 min at room temperature, then its absorbance is measured at a wavelength of 595 nm. Calculation of protein concentration was carried out with standard protein Bovine Serum Albumin (BSA) which was given the same treatment [16].

2.2.4.3. Analysis of antioxidant activity

DPPH 0.1 mM reagent was prepared by dissolving 0.0006 g of DPPH powder with ethanol up to a volume of 15 mL. Preparation of sample stock was carried out at concentrations of 20, 40, 60, 80, and 100 ppm dissolved in ethanol. Each sample stock of 4.5 mL was added with 0.5 mL of a 0.1 mM DPPH reagent. The mixture was vortexed for 30 seconds, then incubated for 1 hour in dark conditions. Absorbance measurements were carried out at a wavelength of 517 nm. The blank was made by mixing 4.5 mL ethanol with 0.5 mL DPPH reagent, then the same process was carried out with the sample [17]. Antioxidant activity can be calculated by the following formula.

Inhibition (%) = (Absorbance of the sample blanks) / (Absorbance of the blank) × 100%

3. Results and discussion

3.1. Characteristics crude extract of phycoerythrin

The phycoerythrin pigments obtanined from K alvareezii had a pink color (Figure 1B). The intensity of pigment color increased as time extraction increased. The highest intensity was obtained from the the pigment extracted using ultrasonication for 60 min (Figure 1A). The increase of intensity color may increase of concentration of phycoerythrin as ultrasonication time was increased.

We measured the crude extract pigments using spectrophotometer UV vis at wavelength of 200 to 700 nm. Some strong peaks were observed at 499, 534 and 564 nm. The peak at 499 nm show the subunit structure of phycourobilin and 534 and 564 nm indicated phycoerythrobilins [18]. In addition, the weak peak also observed at 609 nm (Figure 1B). Galland-Irmouli et al. [19] reported that the maximum peak absorbance of phycoerythrin pigments was at 499 nm, 545 nm, and 564 nm. A similar study by Rossano et al. [20] stated that the maximum peak absorbance of phycoerythrin pigment was at 495 nm and 566 nm. Dumay et al. [21] stated that phycoerythrin is in the wavelength range of 540-570 nm.

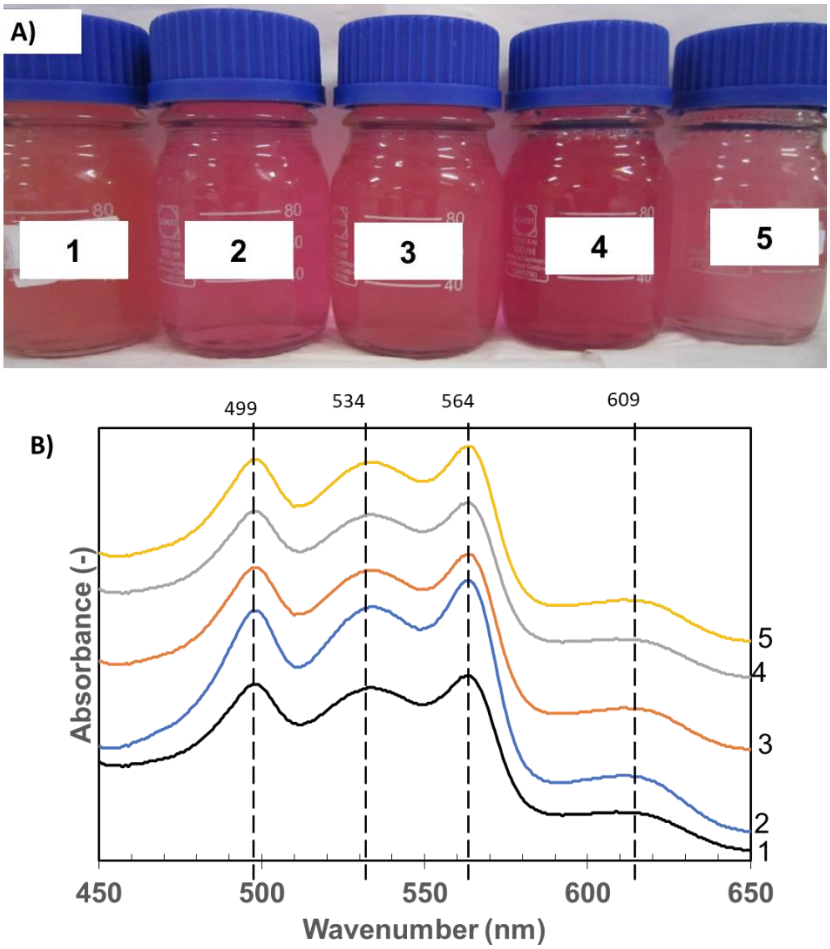

Figure 1. Crude phycoerythrin pigments at different extraction times. A) Visual appearances and B) Spectra of phycoerythrin. (1-4) extraction using ultrasonication for 0, 15, 30 and 60 minutes, respectively and (5) Phycoerythrin extraction for 60 min without ultrasonication.

3.2. Concentration and purity index for pigment phycoerythrin

The phycoerythrin concentrations increased during ultrasonication. The crude extracts of phycoerythrin (PE) increased from 0.054 mg/mL to 1.121 mg/mL. Interestingly, in the shorter time (15 min extraction), the extraction using ultrasonication could produce higher concentration of PE compared with the extraction, which was carried out without ultrasonication for min extraction (Figure 2A). Falleh et al. [22] reported that ultrasonic waves could break cell membranes therefore they could reduce extraction time and increased yield. Furthermore, the concentrations of PE increased significantly after the crude extracts were precipitated using ammonium sulfate.

Crude extracts of phycoerythrin had purity index in the range 0.1946-0.2255. This finding was similar as reported by Sudhakar et al. [13]. However after the crude extracts were precipitated using ammonium sulfate, the purity index of the extract increased 3-6 times than their crude extracts with the maximum values of purity 1.24 (Figure 2B). The increasing of purity index of PE presumably was caused by ammonium sulfate could remove cellular protein and other components in a material as a result increasing of protein concentration. Pan et al. [23] reported that the use of phycoerythrin in the food field must have a minimum purity index of 0.7 and for drugs with minimum purity index 2.0, for example for antitumor phycoerythrin with purity index 2.45 was used.

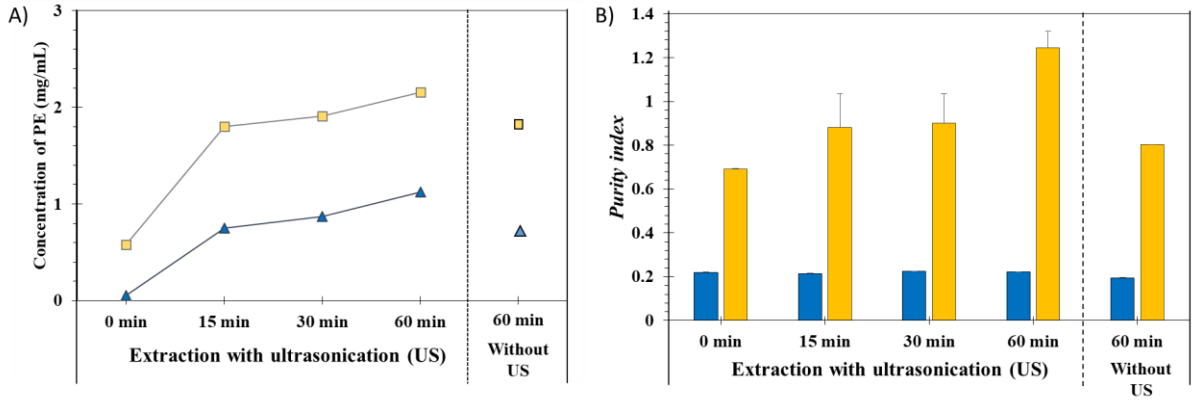

Figure 2. Concentration (A) and purity index (B) of phycoerythrin extracted with ultrasonication and without ultrasonication. Ballets or bars filled with blue color show crude extract, whereas filled with yellow color show extract after precipitated with ammonium sulfate. Mean ± SD; n=3.

3.2.1. Antioxidant activity of phycoerythrin

Crude extract and precipitated phycoerythrin had antioxidant activities in range 74.95-31.02 µg/mL (Figure 3). This finding agreed with Sangeetha *et al.* [24]. According to the range, the antioxidant activities of PE were grouped as strong to very strong antioxidant [25].

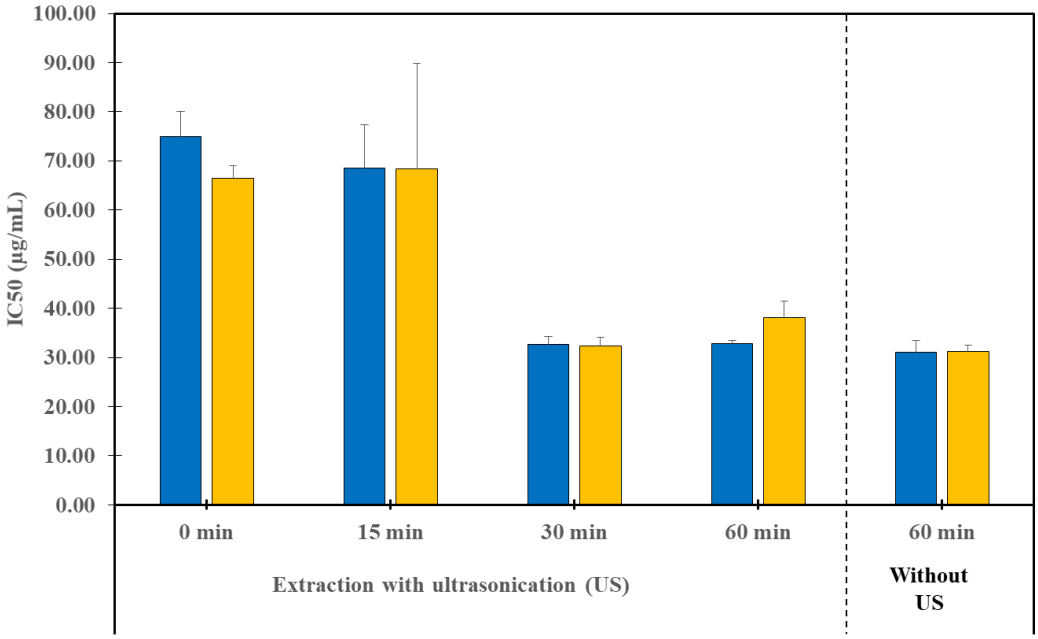

Figure 3. Antioxidant activities of extracted and precipitated of phycoerythrin. Bars filled with blue color show crude extract, whereas filled with yellow color show extract after precipitated with ammonium sulfate. Mean ± SD; n=3.

Time of extraction gave a significant effect on improving the value of IC 50 of phycoerythrin. The best antioxidant activity reached at 30 min extraction. Apparently, extending of extraction time till 60 min was note improving the IC 50 value. Furthermore, precipitation using ammonium sulfate could not improve the activities of crude extract PE. This is due to the fact that the extract of phycoerythrin, which is precipitated with ammonium sulfate, is still not pure, so it still contains other components, such as free polyphenols and polysaccharides [26].

4. Conclusions

Extraction using ultrasonication could increase the concentration and accelerate extracted phycoerythrin. Precipitation using ammonium sulfate significantly improves the purity index of phycoerythrin (3-6) folds of untreated. Phycoerythrin pigment had strong antioxidant activity. The best activity was obtained at 30 min extraction.

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