Optimization of decolorization process in agar production from *Gracilaria lemaneiformis* and evaluation of antioxidant activities of the extract rich in natural pigments

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Abstract

Gracilaria lemaneiformis is mainly used as a raw material source for agar industry, and its extract is rich in natural pigments with antioxidant activities. In this study, a solvent reflux extraction method for decolorization of *G. lemaneiformis* has been developed in agar production. The extraction conditions were optimized as follows: solvent-to-material ratio, 50:1; ethanol concentration, 70%; number of extractions, 3; extraction time, 0.5 h, under which the total antioxidant yield of the extract reached 2.89 ± 0.88 mg/g dried seaweeds. Their IC_{50} values of DPPH radical scavenging activity, hydroxyl radical scavenging activity and superoxide anion scavenging activity were 21.91 ± 1.8 mg/L, 40.59 ± 1.5 mg/L and 160.87 ± 2.8 mg/L, respectively. Further isolation and spectroscopic analysis of natural pigments suggested the strong antioxidant capacities were attributed to chlorophyll derivatives. The results indicate that the decolorization process was able to improve the agar quality, and the extract containing lots of natural pigments had antioxidant activities which may be used in functional food and cosmetics.

Keywords *Gracilaria lemaneiformis* · Agar · Decolorization · Pigments · Antioxidant

Introduction

A marine red macroalgae, *Gracilaria lemaneiformis*, is increasingly cultivated in the coast from Liaodong peninsula, northern China, to Leizhou Peninsula, southern China, because of its high commercial value (Jin et al. 2017). This seaweed is a good food source which contains lots of beneficial proteins, mineral and trace elements to humans (Han et al. 2013). Moreover, it is also an important raw material to produce high-quality agar, which is widely used as

gelling agent in processed foods, cosmetics and pharmaceutical products (Wu et al. 2015). Since the raw seaweed contains lots of photosynthetic pigments, decolorization is need in the agar production process. For decolorization the sodium hypochlorite and other chemicals are used as bleaching agents during the extraction process in China. Unfortunately, this process produces poisonous chloride gas which is harmful to human health and the environment (Warburton 2005). Besides, as natural antioxidants bioresource, the pigments and other antioxidant compounds in the seaweed were wasted and not economically used. For the purpose of manufacturing high-quality agar, several methods including surfactant-induced coagulation (Meena et al. 2014) and bio-ionic liquids for selective precipitation are applied in the production process (Sharma et al. 2015). An eco-friendly photobleaching extraction process was developed for workers' health and environment (Li et al. 2008), and a more economical recovery by the biorefinery which is complete utilization of raw material to obtain the products (Baghel et al. 2016). It seems a good idea that to firstly get rid of the pigments from raw material seaweeds and then to recover them as natural antioxidants.

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Natural pigments and other non- or slight-polar compounds in many other kinds of seaweeds are natural antioxidants with beneficial effects such as prolonging the shelf life of food and cosmetics by inhibiting oxidation (Zubia et al. 2014; Paliwal et al. 2015). In recent years, biologically active compounds in algae have been numerous isolated and identified, the antioxidant compounds in brown and green algae are massively reported (Cho et al. 2010; Balboa et al. 2013). The study related to red algae is mainly focused on polysaccharides and their bioactivities (Souza et al. 2012), while the report about natural antioxidant compounds in this red algae is few. Natural pigments are slightly polar compounds in chemical structure, which are easily removed by using the method of organic solvent extraction (Rapsomanikis et al. 2016). In this study, a solvent reflux method has been developed for the extraction of the pigments and other antioxidant compounds from the red algae *G. lemaneiformis*, and the optimum extraction condition is obtained by an L9(3)4 orthogonal experimental array. The antioxidant compounds in the crude extract are analyzed and identified by spectroscopic methods, and the antioxidant activities are provided by DPPH radical scavenging assay, hydroxyl radical scavenging assay, and superoxide anion scavenging assay. The extract is likely to be used in functional food and cosmetics. The extraction process of pigments can not only improve the agar quality, but also can reduce the effluents, which makes them a resource of natural antioxidants rather than a waste of agar industry.

Materials and methods

Chemicals and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma, St, Louis, MO, USA). 1,10-Phenanthroline monohydrate was purchased from Aladdin Reagent Database Inc. (Shanghai, China). Hydroxyl radical detection kit and superoxide anion detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Methanol, acetonitrile (Fisher Scientific, USA) was of LC grade. Deionized water was supplied by a Milli-Q water purification system from Millipore (USA). All other reagents used in the experiment were of analytical grade.

Biological material

Gracilaria lemaneiformis was collected from the Zhanjiang Naozhou coastline of Guangdong Province (N 21°12', E 110°4'), southern China in June 2016. Sample was washed thoroughly with tap water to remove salt and impurities, then dried in an air oven at 60 °C until a constant weight. The dried sample was cut and stored in a refrigerator until

use. The species was identified by School of Marine Science, Guangdong Ocean University, China.

Decolorization process and conditions optimization

The natural pigment compounds were extracted as previously described (Kim et al. 2011) with slight modifications. Briefly, the dried *G. lemaneiformis* was put into a flask with some volume ethanol, varied according to the solvent-to-material ratios, extracted for setting time and then filtered. The filtrate was concentrated in a rotary vacuum at 40 °C to obtain the crude extract, and stored at 4 °C. Since various factors affected the total antioxidant content in the extract from *G. lemaneiformis*, optimizing the operating conditions to obtain the highest total antioxidant content was the crucial step. According to our previous single-factor test (Gan et al. 2016), in each experiment the dried sample (6 g) and the temperature (60 °C) were constant in a condensing reflux flask, and other impact factors are shown in Table 1. The extraction was collected after filtration, then the filtrate was used for the determination of the total antioxidant content.

Isolation and analysis of natural pigments

The crude extract was dissolved in deionized water and membrane filtered (0.45 µm), and then the filtrate was sequentially partitioned with petroleum ether and ethyl acetate. Natural pigments were extracted into ethyl acetate fraction (EAF). The EFA was further purified using silica gel column chromatography by step-gradient elution with CHCl₃/MeOH solution successive ratios were 100:0, 90:10, 80:20, 70:30 and 60:40 (v/v), and each elution was 100 mL to yield five subfractions. The solvent in each subfraction was evaporated by a rotary vacuum at 40 °C, and the pigments were eluted into the subfraction (70:30). And then the eluate was further purified using a RP-C₁₈ column of 250 × 4.6 mm i.d., with 5 µm particle size (Thermo, USA). The mobile phase consisted of a mixture of methanol (A) and 1% glacial acetic acid aqueous (B) solution (90:10, V/V) for 20 min. Flow rate was fixed at 1.0 mL/min, column temperature was maintained at 30 °C, injection volume was 20 µL, and elution profile was monitored at 400 nm. The pigment compound was analyzed with

Table 1 Factors and levels for orthogonal test

Factor	Level		
	1	2	3
A. Ratio of solvent-to-raw material	10:1	30:1	50:1
B. Ethanol content (%)	70	80	90
C. Number of extractions	1	2	3
D. Extraction time (h)	0.5	1.0	1.5

UV–Vis light spectrophotometer (UV-3101PC, Shimadzu, Japan), HPLC–MS (LC/MS6430B, Agilent, USA) and NMR (AVANCE II 400M, Bruker, USA).

Determination of total antioxidants content

The total antioxidants content (TAC) of crude extract was determined with Folin–Ciocalteu reagent, according to the reported method (Duan et al. 2006) with slight modifications. A 1.0-mL aliquot of sample, 1.5 mL deionized water and 0.5 mL of 0.1 mol/L Folin–Ciocalteu reagent were mixed thoroughly for 1 min, and then 1.0 mL of 20% sodium carbonate solution was added to the mixture. The controls contained all the reaction reagents except the sample. After 30-min incubation at 37 °C, the absorbance was measured at 750 nm and compared to a gallic acid calibration curve. TAC was expressed as gallic acid equivalent:

$$\text{TAC (mg/g)} = CV/M.$$

In this formula, *C* means the antioxidant content of the extract, mg/mL; *V* means the volume of the extract, mL; *M* means the weight of dried seaweed, g.

Antioxidant activities

DPPH radical scavenging activity

DPPH radical scavenging activity assay was adopted to investigate the antioxidant activity of the crude extract and the EAF. The assay was performed according to a modified method (Cho et al. 2010). An aliquot of each sample (100 µL) was mixed with 100 µL of 0.1 mM DPPH solution (prepared with ethanol), and then incubated for 30 min. The absorbance of each sample was recorded at 515 nm using a microplate reader (EL-800, BioTek Instruments, USA). The percentage of scavenged DPPH was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)} = [(A_c - A_s)/A_c] \times 100,$$

where *A_c* was the absorbance value of the control (the mixture of 100 µL of the DPPH solution and 100 µL of ethanol) and *A_s* was the absorbance value of the sample. Gallic acid was used as the reference.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity (HOR) of the crude extract and the EAF was determined according to the method with slight modification (Cho et al. 2010). Briefly, 1 mL of 6 mM ferrous sulfate solution, 1 mL of 6 mM hydrogen peroxide, 0.5 mL of 2 mM salicylic acid and 1 mL of sample solutions at various concentrations were mixed well and incubated at 37 °C for 30 min. The absorbance of the

mixture was then measured at 510 nm. The hydroxyl radical scavenging activity was calculated as follows:

$$\text{HOR scavenging activity (\%)} = [(A_1 - A_2)/(A_1 - A_0)] \times 100,$$

where *A₀* is the absorbance of the reagent blank absorbance, *A₁* is the positive control absorbance and *A₂* is the absorbance of the sample. Gallic acid was used as the reference.

Superoxide anion radical scavenging activity

Superoxide anion radical scavenging activity was determined by the described method with some modifications (Duan et al. 2007). All solutions were prepared in 0.2 M phosphate buffer (pH 7.4). The crude extract and the EAF with different content were, respectively, mixed with 3 mL of reaction buffer solution (pH 7.4) containing 1.3 mM riboflavin, 0.02 M methionine and 5.1 µM NBT. The reaction solution was illuminated by exposure to fluorescent lamps for 20 min and then the absorbance was measured at 560 nm using a spectrophotometer. The reaction mixture without any sample was used as control. The superoxide anion radical scavenging activity (%) was calculated as follows:

$$\text{SOA scavenging activity (\%)} = [1 - (A_s/A_c)] \times 100,$$

where *A_c* was the absorbance value of the control and *A_s* was the absorbance value of the sample. Gallic acid was used as the reference.

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan's test (SPSS 12.0, SPSS Inc., USA). Data were expressed as mean ± standard deviation (SD). Experiments were performed in triplicate. The *p* < 0.05 and *p* < 0.01 were considered statistically significant.

Results and discussion

Optimization of extraction conditions

In the present study, all the selected factors were examined using the orthogonal L9(3)4 tests, the results of orthogonal test and extreme difference analysis are listed in Tables 2 and 3.

As seen from Tables 2 and 3, the influences on the total antioxidant content were decreased in the order *A* > *C* > *B* > *D* according to the *R* values. The ratio of solvent-to-raw material was the most important determinant. The optimized extraction conditions were A3B1C3D1, in which the ratio of solvent-to-raw material, ethanol content,

Table 2 Analysis of the orthogonal test

No.	A	B	C	D	TAC (mg/g raw material)
1	1	1	1	1	1.07
2	1	2	2	2	1.05
3	1	3	3	3	1.35
4	2	1	2	3	1.54
5	2	2	3	1	2.28
6	2	3	1	2	0.70
7	3	1	3	2	3.37
8	3	2	1	3	1.74
9	3	3	2	1	2.11
K1	1.157	1.993	1.170	1.820	
K2	1.507	1.690	1.567	1.707	
K3	2.407	1.387	2.333	1.543	
R	1.250	0.606	1.163	0.277	

Table 3 Variance analysis results

Variation sources	SS	V	MS	F	p
A	2.495	2	1.248	21.509	*
B	0.552	2	0.276	4.759	
C	2.098	2	1.049	18.086	
D	0.116	2	0.058	1.00	
Error	0.12	2			

* $F_{0.05}(2, 2) = 19.0$, ** $F_{0.01}(2, 2) = 99.0$, *** $F_{0.005}(2, 2) = 199.0$

number of extractions, and duration of extraction were 50:1, 70%, 3, and 0.5 h, respectively.

With the optimized conditions, the extraction process was carried out in triplicates. The dried *G. lemaneiformis* (20 g) was extracted three times with 70% ethanol (1 L) at 60 °C for 0.5 h by a condensing reflow device. The extracted solution was concentrated in a rotary evaporator and a vacuum drier

at 40 °C to obtain the crude extract (58.8 mg). The total anti-oxidant content was 2.89 ± 0.88 mg/g. After ethanol extraction, the dark seaweeds became light gray in color because the pigments were mostly dissolved in the extract (Fig. 1).

Isolation and analysis of natural pigments

During decoloring process, the natural pigments of *G. lemaneiformis* were extracted into ethanol, and then they were further isolated with ethyl acetate. The EAF was dark green oily substance containing mostly hydrophobic pigments which are chlorophyll derivatives (Zhou et al. 2016). The pigments were further purified with silica gel column chromatography and a green-colored compound was obtained. Since only a single peak was seen in HPLC chromatogram (Fig. 2), it may be that the pigment compound was of high purity.

The green-colored compound was analyzed with combined spectroscopic methods. According to the UV–vis spectrum (Fig. 3a), the compound exhibited maximal absorption at 410 and 664 nm, the major spectral data on the compound were 410, 502, 534, 608, 664 nm. The data were in good agreement with those reported previously as hydroxyl-pheophorbide a, its main absorption data were 410, 496, 530, 614, 660 nm (Zhao et al. 2014). The ^1H -NMR spectral data (Fig. 3b) of the compound indicated the characteristic features, which showed the presence of three vinyl protons of the macrocycle, H- α , H- β , H- δ (δH 8.01, 7.68, 7.36), a monosubstituted double bond, H-2a, H-2b (δH 5.00, 4.62, 4.58), a methyl ester H-10b (δH 3.88), three olefinic methyl, H-1a, H-3a, H-5a (δH 1.82, 1.64, 1.46) and a methyl proton, H-8a (δH 0.98). The other signals observed in 0.50–1.50 ppm were the motions H-7, H-8 and methylenes, H-7a, H-7b protons. On the basis of the results of combined spectroscopic analyses, the compound exhibited closely a structure identical to the hydroxyl-pheophorbide a (Cheea et al. 2005; Zhao

Fig. 1 Photos of the raw material *Gracilaria lemaneiformis* **a** before extraction; **b** after extraction; **c** the crude extract with ethanol

Fig. 2 HPLC chromatogram (400 nm) of the pigment compound

et al. 2014). When the molecular mass of the compound was determined by MS (Fig. 3c), the spectrum exhibited the compound as the molecular ion, m/z 609.178 $[M + H]^+$, corresponding to the molecular formula of $C_{35}H_{36}N_4O_6$ in accordance with that reported as hydroxyl-pheophorbide a (Muller et al. 2011; Chen et al. 2017). Thus, the result implied that the green-colored compound may be hydroxyl-pheophorbide a which is one of chlorophyll derivatives. Through the literature retrieval, this compound was isolated from the *G. lemaneiformis* for the first time.

Antioxidant activities

DPPH radical scavenging activity

DPPH as a free radical has been used extensively to evaluate antioxidant substances, and is also a useful reagent for investigating the free radical scavenging activities of compounds. As shown in Fig. 4, the EAF showed a potent activity by inhibiting DPPH radical, and its IC_{50} was 21.91 ± 1.8 mg/L, while GA was 2.25 ± 1.0 mg/L only. The effective compounds reacting with the majority of reactive oxygen species (ROS) are of reductive chemical structures. The DPPH radical scavenging activity of the crude extract was comparable to that of other natural antioxidants; it is similar to *Callophyllis japonica* extract (51%) (Kang et al. 2005), but superior to ethanol extracts of some green seaweeds such as *Chaetomorpha moniligera* (29.1%) and *Ulva pertusa* (29.1%) (Cho et al. 2010). These considerable differences in DPPH radical scavenging activity of the extracts from various plants or seaweeds may be attributed to species-specific diversity.

Hydroxyl radical scavenging activity

Hydroxyl radical has strong oxidizing which can cause oxidative injury to biomacromolecules. Therefore, hydroxyl radical has been widely accepted as a tool for evaluating free radical scavenging activity (Wu et al. 2017). The HOR scavenging activity of the EAF was presented in Fig. 5. At the concentration of 200 mg/L, the HOR scavenging activity reached up to 78.93%. These results indicate that they have the ability to scavenge hydroxyl radical, and the IC_{50} was 40.59 ± 1.5 mg/L, while GA was 100.00 ± 2.0 mg/L only. The antioxidant activity could be ascribed to the fact that the EAF can donate electrons to the reactive groups, by reducing them to more stable and non-reactive species.

Superoxide anion scavenging activity

Superoxide anion is a weak oxidant and a precursor of reactive oxygen species (ROS) including singlet oxygen, hydroxyl radical, and hydrogen peroxide, it may combine with other reactive species, such as nitric oxide produced by macrophages, to give more reactive species (Ardestani and Yazdanparast 2007). In this study, the superoxide anion scavenging activity of the EAF from *G. lemaneiformis* were determined and the results are given in Fig. 6. The superoxide anion scavenging activity of the EAF was dose dependant, the IC_{50} of the superoxide scavenging activity of the EAF were 160.87 ± 2.8 mg/L, while GA was 250 ± 5.0 mg/L only. The results indicated the EAF was of antioxidant properties.

Fig. 3 Identification of the compounds isolated from the EAF: **a** UV-vis absorption spectrum; **b** ^1H -NMR spectrum and chemical structure of hydroxyl-pheophorbide a; **c** MS spectrum

Fig. 4 DPPH radical scavenging activity of the EAF

Fig. 5 Hydroxyl radical scavenging activity of the EAF

Fig. 6 Superoxide anion scavenging activity of the EAF

Conclusion

In this study, the ethanol extraction method is adopted to discard the pigment components in the raw material seaweeds. This decolorization process can bleach and improve the agar quality by reducing the amount of pollutants. Moreover, the crude extract and natural pigments fraction from the seaweed exhibit potent antioxidant activity, including high free radical scavenging capabilities, so it could be useful in functional food and cosmetic components. Thus, the crude extract from the seaweed is a natural antioxidant resource rather than an agar industrial waste.

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Compliance with ethical standards

Conflict of interest The authors have declared that no competing interests exist.

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