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Efficient approach to purification of octaphlorethol A from brown seaweed, *Ishige foliacea* by centrifugal partition chromatography

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ABSTRACT

The brown seaweed, *Ishige foliacea* is distributed along the coast of Jeju Island. The main compound that this seaweed produces, octaphlorethol A (OPA), has been recently reported to exhibit strong antidiabetic and whitening activities. However, obtaining pure OPA requires repetitive complex processes and its antioxidant effect *in vivo* has not been demonstrated. Therefore, we devised a method to efficiently detect and isolate OPA from *I. foliacea* using 2.2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^+$) on-line HPLC and centrifu-gal partition chromatography (CPC), respectively. And we evaluated its antioxidative properties using zebrafish as an *in vivo* model system. OPA among compounds from *I. foliacea* ethyl acetate (EtOAc) fraction had the lowest absorbance in ABTS$^+$ on-line HPLC. A total of 11.2 mg OPA was rapidly isolated from the EtOAc fraction (500 mg) of *I. foliacea* extract by using CPC with a two-phase solvent system. In particular, OPA strongly inhibited reactive oxygen species and lipid peroxidation in 2.2′-azobis (2-amidinopropane) dihydro-chloride-induced zebrafish embryos.

1. Introduction

Marine algae, which are abundant in the coast areas over the world and very popular as food in Korea and Japan, can be a useful source of therapeutic compounds. *Ishige foliacea*, a brown seaweed belonging to the family *Ishigeaceae*, grows at the interface of the eulittoral and sublittoral zones in Korea and Japan, and has been known to have various biological activities, including antioxidative, antifouling, algicidal, and anti-inflammatory properties [1,2]. In particular, octaphlorethol A (OPA) mainly isolated from *I. foliacea* has been reported to possess inhibitory activity against α-glucosidase *in vitro* [3].

Phlorotannins such as OPA are organic phloroglucinol (1,3,5-trihydroxybenzene) polymers (Fig. 1) unique to the brown algae. These phlorotannins exhibit a variety of activities, which have been shown to be antidiabetic [4], *anti*-oxidative [5,6], anti-cancerous [7], anti-inflammatory [8,9,10,11], tyrosinase inhibitory [12] and anti-HIV activities [13]. However, complex and lengthy processes were previously required to detect and isolate phlorotannins, which had various biological effects, from brown seaweeds, including *Ecklonia cava*, *I. foliacea*, and *I. okamurae* [3,14,15].

The 2.2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS$^+$) on-line HPLC and preparative centrifugal partition chromatography (CPC) system have allowed the efficient detection and isolation of antioxidative compounds, including phlorotannins [16,17,18]. In this study, therefore, we focused on purifying OPA with antioxidant activity in *I. foliacea* detected by ABTS$^+$ on-line HPLC system using preparative CPC, and evaluated its antioxidative effect *in vivo* using zebrafish embryos as a model.

2. Materials and methods

2.1. Sample material

I. foliacea, was collected on the coast of Jeju Island, south Korea in April 2014, and then was ground and sifted using a 50-mesh standard testing sieve after being freeze-dried using the freeze dryer SFDSMO6 (Samwon Freezing Engineering Co., Gyeonggi, Korea), at which point the sample was stored at 4 °C until further use.

All solvents used for preparation of crude samples and CPC separation were of analytical grade (Daejung Chemicals &Metals Co., Seoul,

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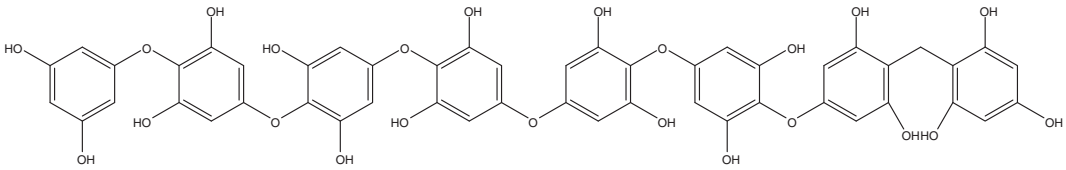

Octaphlorethol A

Fig. 1. Chemical structures of OPA from *I. foliacea*.

Korea). HPLC grade solvents were purchased from Burdick & Jackson (Muskegon, MI, USA).

2.2. Apparatus

High Performance CPC 240 (System Instruments co., Ltd., Japan) was consisted with total cell volume of 240 mL, and the system was equipped with a FLO 214 dual pump (FLO, Tokyo, Japan), an S-3702 UV/Vis detector (Soma Optics, Ltd., Tokyo, Japan), and an Advantec fraction collector, model CHF 122SC (Toyo Seisakusho Kaisha Ltd., Tokyo, Japan).

The HPLC system in this experiment consisted of two mono Waters 515 HPLC pumps, a Waters 2998 photodiode array detector, a Waters 2707 autosampler, and the Waters pump control module II (Waters Corporation).

HPLC–DAD–ESI/MS analyses were equipped with an autosampler, a column oven, binary pump, DAD detector, and degasser (Hewlett-Packard, Waldbronn, Germany) combined with a Finnigan MAT LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA).

^{1}H and ^{13}C-NMR spectra were measured using a JEOL JNM-LA 400 spectrometer (Jeol Ltd., Tokyo, Japan)

2.3. Preparation of crude sample from I. foliacea

The 80% methanol (MeOH) extract from dried *I. foliacea* (100 g) was prepared by extracting three times for 3 h using 80% methanol in sonication condition at room temperature. And then, the dried EtOAc fraction was stored in a refrigerator for CPC separation.

2.4. CPC separation procedure

The CPC experiments were performed by modifying methods of the previous studies [17,18]. The CPC condition was selected with a two-phase solvent system composed of n-hexane:EtOAc:MeOH:water (1:9:2:8, *v*/v), the centrifuge force of 1000 rpm, the flow rate of 2 mL/min and ascending mode. When the mobile phase emerged from the column, indicating that hydrodynamic equilibrium had been reached (back pressure of 2.5 MPa and stationary phase of 150 mL), the dried EtOAc fraction (500 mg) from the 80% MeOH extract of *I. foliacea* was dissolved in 6 mL of a 1:1 (top phase:bottom phase, *v*/v) mixture of the two CPC solvent system phases and was manually injected through the Rheodyne injection valve equipped to 2 mL loop. The effluent from the CPC was monitored in UV range of 290 nm and the eluted fractions were collected with 6 mL in test tubes of 8 mL.

2.5. HPLC and ABTS^{+} on-line HPLC analysis of EtOAc fraction from I. foliacea

The mobile phase of HPLC was acetonitrile-water in gradient mode as follows: acetonitrile with 0.1% formic acid-water with 0.1% formic acid (from 0 min to 50 min ranging from 5:95 *v*/v to 100:0 *v*/v, then up to 60 min maintaining 100:0 *v*/v) and operated in an Atlantis T3 3 μm, 3.0 × 150 mm column (Waters Corporation, USA) using a gradient acetonitrile–water solvent system. The flow rate and UV absorbance range was set up with 0.2 mL/min and 290 nm, respectively.

HPLC coupled with an ABTS^{+} assay was performed using the method descripted by Lee et al. (2013). Data were analyzed using Empower Software (Waters Corporation).

2.6. HPLC–DAD–ESI/MS analysis of OPA

The HPLC conditions were identical to those described in Section 2.5 and the ESI/MS conditions were accompanied by the protocol of Lee et al. 2014. The conditions were as follows: soucrce voltae, 4.5 kV; capillary temperature, 250 °C; capillary voltage, −36.5 V; inter-octapole lens voltage, 10 V; sheath gas, 80 psi; auxiliary gas, 20 psi.

2.7. DCF-DA and DPPP measured in AAPH-induced zebrafish

Both ROS and lipid peroxidation were evaluated for antioxidant effects in the zebrafish model *in vivo* by slightly modifying the protocol described by Ko et al. [19] and Kang et al. [20]. The quantification of fluorescence intensity of individual zebrafish embryos was identified using a Perkin-Elmer LS-5B spectrofluorometer. The fluorescence images of stained embryos were taken a photograph using an Olympus microscope digital camera (Olympus America Inc., USA).

2.8. Statistical analysis

All of the measurements were made in triplicate and all values are represented as means ± standard error. The results were subjected to an analysis of the variance (ANOVA) using the Tukey test to analyze the difference. Values of $p < 0.05$, 0.01 and 0.005 were considered statistically significant.

3. Results

3.1. Chromatogram of ABTS^{+} on-line HPLC

From the *I. foliacea* extracted three times by 80% MeOH while being sonicated for 3 h, the following four fractions were obtained: n-hexane (IFH, 273.2 mg), chloroform (IFC, 288.2 mg), EtOAc (IFE, 1428.6 mg), and water (IFW). For the further experiments, the EtOAc fraction that was expected the highest concentration of OPA was utilized [3]. The EtOAc fraction was analyzed after processing in the described ABTS^{+} on-line HPLC conditions, from which we obtained a chromatogram (Fig. 2). We found that OPA exhibited stronger antioxidant activities than other peaks against ABTS^{+}. Therefore we isolated the targeted OPA using preparative CPC and evaluated its antioxidant effect on 2.2′-azobis (2-amidinopropane) dihydrochloride (AAPH)-zebrafish embryos.

3.2. Isolation and purification of OPA by preparative CPC

The partition coefficient (K) in a suitable two-phase solvent system is the most important variable for successful separations of the target samples by preparative CPC. For the most efficient isolation of OPA, we determined the K-values in several two-phase solvent systems with different volume ratios of two immiscible solvents as volume ratios of two immiscible solvents such as n-hexane:EtOAc:MeOH:water (*v*/v).