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Preparative isolation of sargachromanol E from *Sargassum siliquastrum* by centrifugal partition chromatography and its anti-inflammatory activity

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ABSTRACT

Centrifugal partition chromatography (CPC) can be used to isolate various bioactive compounds from natural materials by one-step. We confirmed antioxidative compounds existed in chloroform ($CHCl_3$) fraction of *Sargassum siliquastrum* using online-HPLC. Fractions (A, B, C, D and E) were separated from the CHCl3 fraction by preparative CPC (n-hexane:ethyl acetate:methanol:water, 5:5:7:3, v/v). In this study, we proved that the isolated compounds exhibit anti-inflammatory activities using lipopolysaccharide (LPS) stimulated RAW 264.7 macrophages. The fraction A which exhibited the strongest inhibitory effect on nitric oxide (NO) production level, was confirmed as sargachromanol E by LC–MS–ESI, ^{1}H NMR and ^{13}C NMR data. The sargachromanol E significantly reduced the inflammatory response in LPS induced macrophages, decreasing LPS-induced transcription factor of pro-inflammatory cyclooxygenase-2, NO synthase, phosphate P38, phosphate ERK1/2, LPS-stimulated tumor-necrosis factor alpha, interleukin-1 beta and prostaglandin E2 release. In conclusion, it was suggested that sargachromanol E inhibited inflammation in LPS induced RAW 264.7 cells via MAPK pathway.

1. Introduction

Inflammation represents a highly coordinated set of events that allow the tissues to respond against an injury or infection. It involves the participation of various cell-types expressing and reacting to the diverse mediators along a very precise sequence of events (Babu et al., 2009). Usually, inflammation is initiated through the production of specific cytokines or chemokines characterized by the recruitment of leukocytes to the damage sites. However, the sustained or excessive inflammation can lead to various diseases including rheumatoid arthritis, psoriasis and inflammatory bowel disease (Simon and Green, 2005). Macrophages play a key role in the inflammatory and immune reactions by releasing a variety of inflammatory mediators such as nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-1 β (IL-1β), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Yoon et al., 2003; Ramana et al., 2006; Nunez Miguel et al., 2007). Lipopolysaccharide (LPS), a component of the gram negative cell wall, was used to stimulate peritoneal macrophages and induce the production of anti-inflammatory cytokines in the infection response (Pan et al., 2009).

Brown seaweed is a marine organism with various biological activities including the antioxidative and anti-inflammatory effects (Yang et al., 2011; Kang et al., 2012). *Sargassum* sp. is a brown algae found throughout the tropical and subtropical regions and are reported to produce the metabolites of structural classes such as plastoquinones (Segawa and Shirahama, 1987; Mori et al., 2005; Ishitsuka et al., 1979), chromanols (Kato et al., 1975), chromenes (Kikuchi et al., 1975; Jang et al., 2005), phlorotannins (Kubo et al., 1992), phlorethols (Banaimoon, 1992), steroids (Tang et al., 2002a), and glycerides (Tang et al., 2002b). Especially, many researchers have reported that the polar and nonpolar extracts from *Sargassum wightii* in the winter exhibit significant anti-inflammatory properties. The dichloromethane, ethanol and boiling water extracts of *Sargassum fulvellum* and *Sargassum thunbergii* inhibited the inflammatory symptoms in the mouse ear edema. However, the definite anti-inflammatory compounds of *Sargassum* sp. have not been confirmed. *Sargassum siliquastrum* used in this study has only been reported for its chromenes, farnesylacetones

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and fucoxanthine exhibiting the anticancer and vasodilatation effect as well as the protective effect for the UV-B induced cell damage (Heo et al., 2011; Park et al., 2008; Heo et al., 2009).

We used the centrifugal partition chromatography (CPC) as a fast and effective method to purify the active compounds such as chromanols and fucoxanthin from *S. siliquastrum*. It demanded the repetitive chromatography processes on the Sephadex LH-20 column chromatography and reversed-phase HPLC (Zang et al., 2005; Heo et al., 2009). Traditional isolation methods such as the liquid solid chromatography have various problems when separating the secondary metabolites from the plants. These problems such as time-consuming, tedious repeated chromatography and adsorption in stationary phase do not affect the preparative CPC technology of the liquid–liquid chromatographic techniques. The preparative CPC system is a non-solid support preparative liquid–liquid separation process chromatographic technique which is based on the difference in the distribution of components over two immiscible liquid phases. It is use for the large isolation and able to purify large quantities of compounds with purity over 90% in the one step process (Michel et al., 1997; Delannay et al., 2006; Bourdat-Deschamps et al., 2004). In addition, the solutes are separated according to their partition coefficient (K), expressed as the ratio of their concentrations in the stationary phase to their concentration in the mobile phase (Berthod and Roussel, 1988). The CPC system has been widely used for the separation of bioactive compounds from the land plants (Marston et al., 1988, Bourdat–Deschamps et al., 2004, Kim et al., 2005). However, in the case of the seaweeds, only a few algae species such as *Ascophyllum nodosum* have been subjected to CPC (Chevolota et al., 1998, 2000).

In the present study, the CPC system was applied for the large isolation of the active compounds from *S. siliquastrum*. The isolated compounds were confirmed by the online HPLC-ABTS$^+$ system for rapidly analyzing the free radical scavenging activities (Koleva et al., 2000, 2001). We evaluated for anti-inflammatory effect on the LPS-induced RAW 264.7 cells.

2. Materials and methods

2.1. Materials

S. siliquastrum was collected on the coast of Jeju island, South Korea in June 2009, was ground and shifted through a 50 mesh standard testing sieve after dried by freeze dryer, and then the dried *S. siliquastrum* was stored in refrigerator until use. All solvents used for preparation of crude sample and centrifugal partition chromatography (CPC) separation were of analytical grade (Daejung Chemicals & Metals Co., Seoul, Korea). High performance liquid chromatography (HPLC) grade solvents were purchased from Burdick & Jackson (MI, USA). Lipopolysaccharide (LPS) was purchased from sigma chemical Co., Ltd. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) penicillin–streptomycin and trypsine-EDTA were obtained from Gibco/BRL (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). M-MuLV reverse transcriptase were purchased from Promega (Madison, WI, USA). The enzyme-linked immunosorbent assay (ELISA) kit for interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and Prostaglandin E_2 (PGE_2) were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Protein assay kit and ECL detection reagent were bought from Bio-Rad (Richmond, CA, USA) and Amercham Biosciences (Piscataway, NJ, USA), respectively. Other all reagents and solvents were purchased from Sigma (St. Louis, MO, USA).

2.2. Apparatus

LLB-M high performance CPC (Sanki Engineering, Kyoto, Japan) was used as preparative CPC. The total cell volume is 240 mL. A four-way switching valve incorporated in the CPC apparatus allows operating in either the descending or the ascending mode. This CPC system was equipped with a Hitachi 6000 pump (Hitachi, Japan), an L-4000 UV detector (Hitachi), and a Gilson FC 203B fraction collector (Gilson, France). The samples were manually injected through a Rheodyne valve (Rheodyne, CA, USA) with a 2 mL sample loop.

^{1}H NMR spectra were measured with a JEOL JNM-LA 300 spectrometer and ^{13}C NMR spectra with a Bruker AVANCE 400 spectrometer. Mass spectra (FAB-MS and EIMS) were recorded on a JEOL JMS 700 spectrometer. The HPLC system in this experiment consisted of a binary FLEXAR UHPLC pump, a FLEXAR PDA detector, a FLEXAR PDA auto sampler (PERKIN ELMER, USA).

2.3. Preparation of crude extracts from S. siliquastrum

Dried *S. siliquastrum* (600 g) was extracted three times for 3 h with 80% MeOH under sonication at room temperature. The extract, concentrated in a rotary vacuum evaporator, partitioned with $CHCl_3$, and then the concentrated $CHCl_3$ fraction (62 g) was stored in a refrigerator for CPC separation.

2.4. CPC separation procedure

The CPC experiments were performed using a two-phase solvent system composed of *n*-hexane:ethyl acetate (EtOAc):methanol (MeOH):water (5:5:7:3, v/v). The two phases were separated after thoroughly equilibrating the mixture in a separating funnel at room temperature. The upper organic phase was used as the stationary phase, whereas the lower aqueous phase was employed as the mobile phase. The CPC column was initially filled with the organic stationary phase and then rotated at 1000 rpm while the mobile phase was pumped into the column in the descending mode at the flow rate used for the separation (2 mL/min). When the mobile phase emerged from the column, indicating that hydrostatic equilibrium had been reached (back pressure: 3.9 MPa), the concentrated ethyl acetate fraction (500 mg) from 80% MeOH extract of *S. siliquastrum*, which was dissolved in 6 mL of a 1:1 (v/v) mixture of the two CPC solvent system phases, was injected through the Rheodyne injection valve. The effluent from the CPC was monitored in the UV at 254 nm and fractions were collected with 6 ml in 10 ml tube by a Gilson FC 203 B fraction collector.

2.5. HPLC analysis

The HPLC system in this experiment consisted of a binary FLEXAR UHPLC pump, a FLEXAR PDA detector, a FLEXAR PDA auto sampler (PERKIN ELMER, USA). A 10 μl of 5 mg/ml sample solution was directly injected on Atlantis T3 3 μm 3.0 × 150 mm column (Waters, Ireland) using a gradient acetonitrile–water solvent system. The mobile phase was acetonitrile–water in gradient mode as follows: acetonitrile with 0.1% formic acid–water with 0.1% formic acid (0 min ~ 10 min: 10:90 v/v ~ 60:40 v/v, ~60 min: ~ 100:0 v/v). The flow rate was 0.2 mL/min with UV absorbance detection at 254 nm.

2.6. HPLC–DAD–ESI/MS analysis of purified compounds

HPLC–DAD–ESI/MS analyses were carried out using a Hewlett–Packard 1100 series HPLC system equipped with an autosampler, a column oven, a binary pump, a DAD detector, and a degasser (Hewlett–Packard, Waldbronn, Germany) coupled to a Finnigan MAT LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with a Finnigan electrospray source and capable of analyzing ions up to m/z 2000. Xcalibur software (Finnigan MAT) was used for the operation. The chromatographic conditions are identical to those described in Section 2.4 and the outlet of the flow cell was connected to a splitting valve, from which a flow of 0.2 mL/min was diverted to the electrospray ion source via a short length of fused silica tubing. Negative ion mass spectra of the column eluate were recorded in the range m/z 100–2000. The source voltage was set to 4.5 kV and the capillary temperature to 250 °C. The other conditions were as follows: capillary voltage, −36.5 V; inter-octapole lens voltage, 10 V; sheath gas, 80 psi (551.6 kPa); auxiliary gas, 20 psi (137.9 kPa).

2.7. On-line HPLC-ABTS$^+$ assay

HPLC coupled with ABTS assay was performed by using the method developed by Koleva et al. (2001) with some modifications. A stock solution containing 3.5 mM potassium persulphate and 2 mM ABTS was prepared and kept at room temperature in darkness for 12 h in order to stabilize the radical. The radical reagent was prepared by diluting the stock solution with pure water to an absorbance of 0.70 ± 0.02 at 680 nm. The extracts (10 μL) were injected into an Waters HPLC system. HPLC separation was carried out as described in the previous section. HPLC eluates from the column then arrived at a T-junction, where the 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) reagent was added. The ABTS reagent flow rate was 0.7 mL/min delivered by a Waters Reagent Pump (Waters Corporation, USA). After the eluates mixed with ABTS reagent in a reaction coil (15 m × 0.25 mm i.d. PEEK tubing), the negative peaks were measured by UV spectrometer at 680 nm. Water was used as the control by replacing ABTS$^+$ in terms of above procedure. Data were analysed using Empower Software.