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Effects of molecular weight and hydrolysis conditions on anticancer activity of fucoidans from sporophyll of *Undaria pinnatifida*

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ABSTRACT

Hydrolyzed fucoidans, from sporophyll of *Undaria pinnatifida*, were used to determine the effects of molecular weight (M_w) and hydrolysis conditions on cancer cell growth. Native fucoidans showed anticancer activity of 37.6%. When hydrolyzed in boiling water with HCl for 5 min, fucoidans (M_w = 490 kDa) significantly increased anticancer activity to 75.9%. However, fucoidans hydrolyzed in a microwave oven showed little improvement of anticancer activity and even exhibited the inhibition activity below 30% when treated more than 90 s. This suggests that anticancer activity of fucoidans could be significantly enhanced by lowering their M_w only when they are depolymerized by mild condition.

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1. Introduction

Fucoidans are water-soluble and sulfated-fucans of complicated chemical structures, commonly found in brown seaweeds. Their structures and compositions vary with the species of brown seaweeds, but they mainly consist of fucose and sulfate with small amounts of galactose, xylose, mannose and uronic acids [1–8]. Fucoidans have attracted steady attentions in the last few years because of their various biological activities such as anticoagulant, antiviral and anticancer activities. It was reported that fucoidans had anticoagulant activity *in vivo* and *in vitro* [9] and were found to be potent activators of both anti-thrombin III and heparin cofactor II [10–12]. Ponce et al. [13] reported that galacto-fucans obtained by the fractionation of fucoidans from *Adenocystis utricularis* with a cationic detergent, cetrimide, showed a high inhibitory activity against herpes simplex virus 1 and 2 with no cytotoxicity. A number of studies have been also reported on the anticancer activity of fucoidan polymers [14–16]. It was suggested by Takahashi [17] that the anticancer activity of fucoidans was mainly attributed to the enhancement of host defense mechanism to neoplasia. On the other hand, Koyanagi et al. [16] suggested that fucoidan polymers could also inhibit the growth of tumor cells by suppressing angiogenesis, which is the formation of new micro-blood vessel, of tumor cells.

The biological activities of fucoidans have been reported to be closely related to their sulfate content and molecular weight. Oversulfated fucoidans led to the higher stimulation of the glutamic-plasminogen (Glu-Plg) activation in comparison with native fucoidans [18]. It was also found that oversulfated fucoidans possessed higher anti-angiogenic activity than native fucoidans, and thus more effectively inhibited the growth of tumor cells [16]. On the other hand, partially desulfated fucoidans with sulfate contents less than 20% showed drastic decreases in both anticoagulant and anticancer activities [19]. It was reported by Nishino and Nagumo [20] that the molecular weight of fucoidan polymers from *Ecklonia kurome* was related to their anticoagulant activity. The authors found that fucoidan polymers with molecular weights ranging from about 10 to 300 kDa showed the most potent anticoagulant activities. The mechanism why this range of molecular weight had greater anticoagulant activities appeared to be very complex and has not been clarified. To the best of our knowledge, however, the effects of the molecular weight of fucoidan polymers on the anticancer activity have not been reported yet. In the study of other polysaccharides, Lin et al. [21] observed a relationship between the

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molecular weight and the anticancer activity of sulfated α-glucans from *Poria cocos* mycelia. The authors found that the sulfated α-glucans having a moderate range of molecular weight from 20 to 400 kDa with relatively high chain stiffness and good water solubility could enhance the anticancer activity, compared to native and lower molecular weight polysaccharides. However, the relationship between the molecular weight and the anticancer activity was not clearly understood.

In the current study, fucoidan polymers were extracted from the sporophyll of *U. pinnatifida* and subsequently hydrolyzed by heating in boiling water or in a microwave in the presence of 0.01N HCl for various times. The objective of this study was to determine the anticancer activity of partially hydrolyzed fucoidan polymers and to investigate the effects of molecular weights and different hydrolysis conditions on the inhibition of cancer cell growth.

2. Materials and methods

2.1. Materials

The dried sporophyll of brown seaweed (*Undaria pinnatifida*) originated from the coast of Youngdukgun, Kyoungbuk province, South Korea was purchased, milled using a blender, sieved (<0.5 mm), and then stored at −20 °C before analyses. All chemicals and reagents used were of analytical grade.

2.2. Isolation of fucoidan

The milled sample (20 g) was treated with 85% ethanol (EtOH, 200 mL) with constant mechanical stirring for 12 h at room temperature to remove pigments, proteins and low molecular weight compounds, washed with acetone, centrifuged at 1800 × *g* for 10 min, and then dried overnight at room temperature. The dried biomass (5 g) was extracted twice with distilled water (100 mL) at 65 °C with stirring for 1 h. After centrifuging the extracts (18,500 × *g*, 10 min), the supernatant was collected. The supernatant was mixed with 1% $CaCl_2$ and the solution was kept at 4 °C overnight to precipitate alginic acid. After centrifugation at 18,500 × *g* for 10 min, EtOH (99%) was added into the supernatant to obtain the final EtOH concentration of 30% and then the solution was left at 4 °C for 4 h. After centrifugation at 18,500 × *g* for 10 min, additional EtOH was added to the collected supernatant to obtain the final EtOH concentration of 70%, and the solution was placed at 4 °C overnight. The native fucoidan was obtained by the filtration of the solution with a nylon membrane (0.45 μm pore size, Whatman International Ltd., Maidstone, England), followed by washing with EtOH (99%) and acetone, and then dried at room temperature overnight. The yield (8.8%) of fucoidan was calculated based on the dried biomass obtained after the treatment of the milled sample with 85% EtOH.

2.3. Preparation of native and partially hydrolyzed fucoidan solution

The native fucoidan solution was obtained by dissolving fucoidan polymers (20 mg) in 1 mL of 0.01N HCl without heat treatment, followed by neutralization with 1 mL of 0.01N NaOH. For obtaining the partially hydrolyzed fucoidan solutions, the native fucoidan (20 mg) was hydrolyzed with 1 mL of 0.01N HCl by heating in boiling water for 1, 5, 10 or 15 min or in a microwave oven (RE-552W, SamSung, Seoul, Korea) using a microwave bomb (# 4782, Parr Instrument Co., Moline, IL, USA) for 30, 60, 90 or 120 s, followed by neutralization with 1 mL of 0.01N NaOH.

2.4. Determination of weight average molecular weight of fucoidan

For measuring the weight average molecular weight (M_w) of the native and partially hydrolyzed fucoidans, the above fucoidan solutions were filtered through cellulose acetate membranes (3.0 μm pore size, Whatman International Ltd.), and then injected into the high performance size exclusion chromatography coupled to multiangle laser light scattering and refractive index detection (HPSEC–MALLS–RI) system. The HPSEC–MALLS–RI system consisted of a pump (model 321, Gilson, Middleton, WI, USA), an injector valve with a 100 μL sample loop (model 7725i, Rheodyne, Rohnert Park, CA, USA), a guard column (TSK PWxl, TosoBiosep, Mongomeryville, PA, USA), three SEC columns (TSK G5000 PW (7.5 mm × 600 mm), TSK G3000 PWxl and TSK G2500 PWxl (7.8 mm × 300 mm), TosoBiosep), a multiangle laser light scattering detector (HELEOS, Wyatt Technology Corp, Santa Barbara, CA, USA), and a refractive index detector (RI-150, Thermo Electron Corp., Yokohama city, Japan). The aqueous solution of 0.15 M $NaNO_3$ and 0.02% NaN_3 was used as a mobile phase at a flow rate of 0.4 mL/min. The normalization of MALLS detector and the determination of volume delay between MALLS and RI detectors were carried out with bovine serum albumin (BSA). The d*n*/d*c* value was set to 0.129 for fucoidan polymers [22]. The M_w of fucoidan polymers were calculated from the data collected from MALLS and RI detectors using ASTRA 5.3 software.

2.5. Anticancer activity assay

The anticancer activity of the native and partially hydrolyzed fucoidans was determined using sulforhodamine B (SRB) assay [23], which was based on the measurement of cellular protein content. The human lung cancer cell line, A549 (CCL-185, ATTC, Rockville, MD, USA), was used in this study. The cell line (100 μL) with concentration of 4–5 × 10^4 cells/mL was placed in a 96-well plate and cultured for 24 h at 37 °C in the presence of 5% CO_2. The above native and partially hydrolyzed fucoidan solution (100 μL) having various concentrations from 0.2 to 1.0 mg/mL was added to the cultured cell line in the plate and again cultured for 48 h. The supernatant was removed from the well and subsequently cold TCA (10%, 100 μL, 4 °C) was added into the well. The solution was left at 4 °C for 1 h. After removing TCA by washing with distilled water, the well was dried at room temperature. The SRB staining of the cell line was carried out with the addition of 100 μL of 0.4% SRB dissolved in 1% acetic acid into the dried well. The unstained SRB was removed by washing with 1% acetic acid and the well was dried again at room temperature. The dried well was filled with 100 μL of 10 mM Tris buffer and the absorbance of sample solution (As) was measured at 540 nm using a microplate reader (Molecular Devices, THERMOmax, Hayward, CA, USA). The percentage of inhibition of the cancer cell growth was calculated using the following equation: Growth inhibition (%) = $100(1 - (A_s/A_c))$, where A_s is the absorbance of sample solution and A_c is the absorbance of control (100 μL of H_2O was used instead of 100 μL sample solution).

3. Results and discussion

3.1. Effect of hydrolysis conditions on molecular weight of fucoidan

In the preliminary study, it was determined that the extracted fucoidan polymers used in this study mainly consisted of carbohydrates (54.9%) and sulfates (41.5%) with monosaccharide composition of 78.8% fucose and 21.2% galactose (data not shown) [24]. Fig. 1a shows the HPSEC chromatograms of native and partially

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