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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.

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

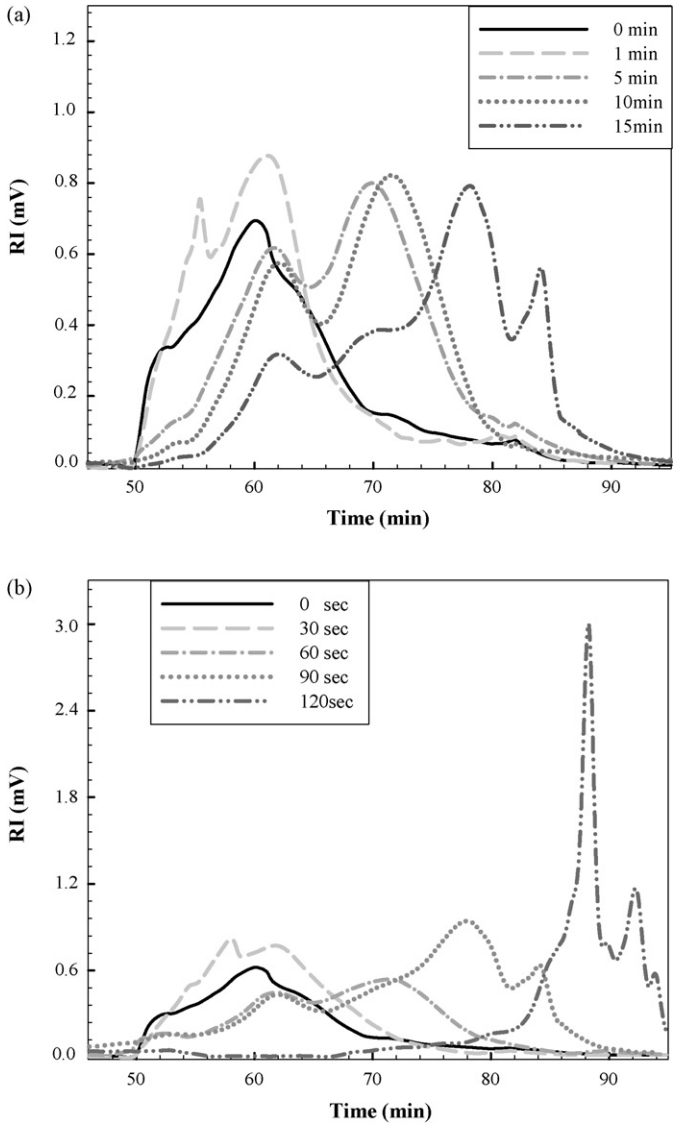

Fig. 1. The HPSEC chromatograms of partially hydrolyzed fucoidan polymers obtained by heating in boiling water (a) with 0.01N HCl at different times (0, 1, 5, 10, or 15 min), and by heating in a microwave (b) with 0.01N HCl at different times (0, 30, 60, 90, or 120 s).

hydrolyzed fucoidan polymers obtained by heating in boiling water with 0.01N HCl for various times from 0 to 15 min. The native fucoidan polymers with no heat treatment (0 min) were eluted from the SEC columns between elution times of 50 and 74 min. The acid and heat treatment in boiling water for 1 min did not significantly alter the elution time for major fucoidan fractions but slightly changed the shape of elution profile with the loss of a shoulder at 52 min and the appearance of a peak at 55 min in the HPSEC chromatogram. This is probably due to the occurrence of a slight degradation, especially in the high molecular weight fractions. When heated for 5 and 10 min, two distinct peaks were observed at 62 and 70 min and 15 min-treated fucoidan polymers showed additional two peaks at 78 and 84 min in the HPSEC chromatogram, indicating the occurrence of significant polymer degradation by the acid and heat treatments. The polymeric degradation of fucoidans is also shown in Table 1 that the weight average molecular weight (M_w) calculated from entire HPSEC fractions markedly decreased from 5100 to 2200 kDa after 1 min heating in boiling water, significantly decreased to 490 kDa with the increase of heating time

Table 1
The weight average molecular weight (M_w) of partially hydrolyzed fucoidans obtained by heating in boiling water or in a microwave at different times in the presence of 0.01N HCl.

Heating in boiling water		Heating in a microwave	
Heating time (min)	M_w (kDa)	Heating time (s)	M_w (kDa)
0	5100 ± 102	0	5100 ± 102
1	2200 ± 44	30	2200 ± 44
5	490 ± 9.8	60	500 ± 10
10	390 ± 7.8	90	300 ± 6
15	260 ± 5.2	120	30 ± 0.6

to 5 min, and again drastically decreased to 260 kDa after 15 min heating.

The HPSEC chromatograms of partially hydrolyzed fucoidan polymers obtained with 0.01N HCl and microwave treatment at different times from 0 to 120 s are shown in Fig. 1b. The elution time of major fucoidan fractions was not significantly changed by the acid and microwave treatment for 30 s but a slight degradation also occurred especially in the high molecular weight fractions similar to the fucoidans treated with the acid and heating in boiling water for 1 min. With increased heating times of 60 and 90 s, fucoidan polymers showed more than two peaks in the HPSEC chromatograms and major fucoidan fractions were eluted at higher elution times of 72 and 78 min, respectively. When heated for 120 s, fucoidan polymers were mostly eluted in a high and narrow peak at 88 min, indicating the occurrence of considerable polymer degradation and the formation of relatively homogeneous fucoidan chains. The considerable degradation of fucoidans is also shown in Table 1 that the M_w calculated from entire HPSEC fractions markedly decreased from 5100 to 2200 kDa after 30 s microwave heating, significantly decreased to 500 and 300 kDa with increased heating times of 60 and 90 s, respectively, and again drastically decreased to 30 kDa after heating for 120 s. Microwave heating caused more drastic degradation of fucoidan polymers than boiling water heating. This is probably due to the high temperature and pressure generated inside the microwave vessel, as reported by Bello-Perez et al. [25] that the temperature of a polysaccharide solution in the microwave vessel approached to 143 and 211 °C after 35 and 90 s microwave heating, respectively, using a 900 W-microwave oven.

3.2. Anticancer activity of partially hydrolyzed fucoidans

The anticancer activity of the native fucoidan polymers, expressed as a percentage of the growth inhibition of cancer cell line A549, is shown in Fig. 2. The native fucoidans inhibited the growth of the cancer cell lines in a dose-dependent manner and showed the anticancer activity from about 14.9 to 37.6% in the concentrations from 0.2 to 1.0 mg/mL. The anticancer activity of the native fucoidans was considerably lower than those of fucoidan polymers from *Fucus vesiculosus* and *Laminaria japonica* which showed more than 89% of inhibition activity on the growth of HS-Sultan and MCF-7 cells, respectively [26,27]. These considerable variations in the anticancer activity between fucoidans are probably because of the various chemical compositions of fucoidan polymers originated from differences in species, anatomical regions and growing conditions of brown seaweeds and extraction and purification procedures as well as the use of different cancer cell lines.

The anticancer activity of partially hydrolyzed fucoidan polymers (1.0 mg/mL), obtained by 0.01N HCl and heat treatment in boiling water for different times from 0 to 15 min, is shown in Fig. 3a. The fucoidan polymers having the M_w of 2200 kDa obtained

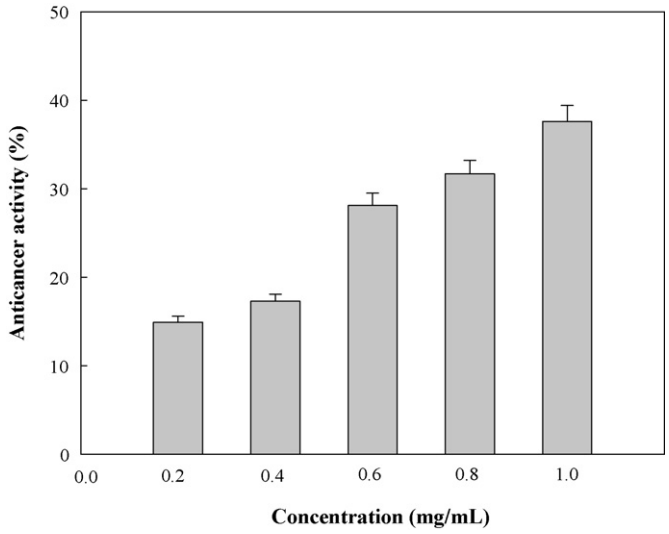

Fig. 2. The growth inhibition of cancer cell lines (A549) by native fucoidans in the various concentrations (0.2–1.0 mg/mL).

by 1 min hydrolysis showed a significantly enhanced anticancer activity (71.3%) compared with the native fucoidans (37.6%). Further increase in the anticancer activity (75.9%) was observed with the fucoidan polymers having the M_w of 490 kDa produced by 5 min hydrolysis. The mechanism why low molecular weight fucoidan polymers increase the anticancer activity has not been clearly elucidated. Based on the above results, it is suggested that low molecular weight fucoidans may have an increased molar concentration induced by the depolymerization as well as may allow greater molecular mobility and diffusivity than high molecular weight fucoidans. Thus, both effects of increased molar concentrations and improved interactions with cancer cell lines seemed to be responsible for the enhanced anticancer activity of partially hydrolyzed fucoidan polymers. It was also reported that low molecular weight fucoidans (18.6 kDa) from *Ascophyllum nodosum* at the concentration of 1.0 mg/mL showed almost 100% inhibition activity on CCL39 cell growth [19]. When the acid and heat treatment was applied for 15 min, the anticancer activity of fucoidan polymers (260 kDa) decreased to 61.7%. It seemed that the slight decrease in the anticancer activity was probably due to the partial removal of sulfate groups from the fucoidan polymers during the prolonged acid and heat treatment (15 min). The occurrence of desulfation was also observed in the fucoidan polymers from *Strongylocentrotus pallidus* when they were heated at 60 °C for 20 min in the presence of 0.01N HCl [28]. In addition, according to Haroun-Bouhedja et al. [19], the partial removal of sulfate groups from the fucoidan polymers from 27 to 12.5% drastically lowered their inhibition activity on the growth of CCL39 cells from 100 to 12%.

The anticancer activity of partially hydrolyzed fucoidan polymers (1.0 mg/mL), obtained by the acid and microwave heating at different times from 0 to 120 s, is shown in Fig. 3b. The fucoidan polymers having the M_w of 2200 kDa produced by 30 s microwave heating showed a slight increase in the anticancer activity (45.8%) compared with the native fucoidans (37.6%). The inhibition activity on the growth of cancer cells decreased to 40.6% with 60 s-treated fucoidan polymers having the M_w of 500 kDa. With further heat treatment of 90 and 120 s, the fucoidan polymers having the M_w lower than 300 kDa showed less than 30% of the anticancer activity. This reduction of anticancer activity may also be because of the partial removal of the sulfate groups from the fucoidan polymers by the harsh microwave treatment with the acid for longer times.

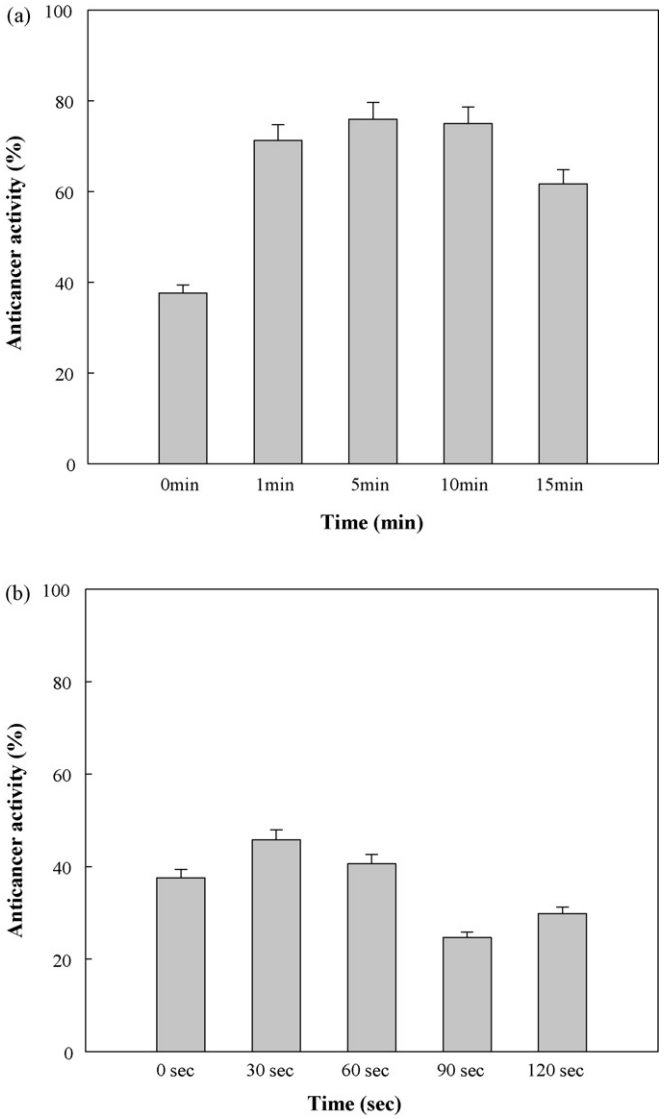

Fig. 3. Effect of partially hydrolyzed fucoidans (1 mg/mL) obtained by heating in boiling water (a) with 0.01N HCl at different times (0, 1, 5, 10, or 15 min) and by heating in a microwave (b) with 0.01N HCl at different times (0, 30, 60, 90, or 120 s) on the growth of cancer cell lines (A549).

3.3. Effects of M_w and hydrolysis conditions on anticancer activity

Fig. 4 is a plot of the anticancer activities against the M_w for partially hydrolyzed fucoidans obtained by two different hydrolysis methods but having similar M_w. It was clearly shown that fucoidan polymers hydrolyzed in boiling water had significantly greater anticancer activity than those hydrolyzed by microwaving in the corresponding M_w values. These variations in the anticancer activities between two hydrolyzed fucoidan polymers may be due to their structural differences, especially their sulfate content rather than their M_w. Therefore, the results suggest that hydrolysis method could significantly influence the anticancer activity of depolymerized fucoidans through the removal of sulfate groups. Thus, the boiling water heating appeared to cause less desulfation from fucoidan polymers during the hydrolysis than the microwave treatment. It is, therefore, suggested that the microwaving with an acid is not a suitable method for producing depolymerized fucoidans. On the other hand, the boiling water heating with an acid up to 10 min could be an effective method to produce partially

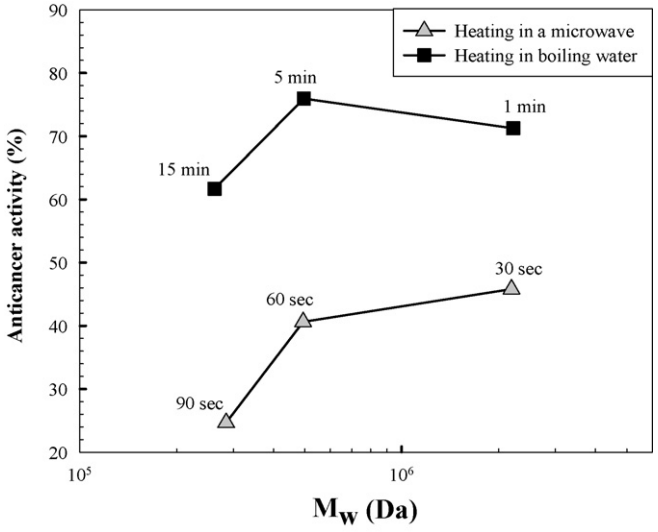

Fig. 4. Effect of the M_w and hydrolysis conditions of partially hydrolyzed fucoidans on the growth of cancer lines (A549).

hydrolyzed fucoidan polymers with significantly enhanced anticancer activity.

4. Conclusions

Partially depolymerized fucoidans, extracted from the sporophyll of *U. pinnatifida* and then obtained by different hydrolysis methods, were used to investigate the effects of M_w and hydrolysis conditions on the anticancer activity. The native fucoidans (5100 kDa) showed the anticancer activity of 37.6% with a concentration of 1.0 mg/mL. When hydrolyzed in boiling water for 5 min with the acid, the fucoidan polymers (490 kDa, 1 mg/mL) significantly enhanced the anticancer activity up to 75.9%. This may be because of the increased molar concentration and the enhanced mobility and diffusivity of the partially hydrolyzed fucoidans. On the other hand, the microwave-hydrolyzed fucoidans (30–2200 kDa, 1 mg/mL) were found to be less effective in improving the anticancer activity and even showed lower than 30% of inhibition activity against the cancer cells when hydrolyzed more than 90 s. This is probably due to the partial removal of sulfate groups from fucoidans by the harsh microwave treatment. The current study suggests that the anticancer activity of fucoidans could be significantly improved by lowering their molecular weight when they are depolymerized by mild hydrolysis conditions without causing considerable desulfation.

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