J Food Sci Nutr
Vol 12, p 191 ~196 (2007)
DOI: 10.3746/jfn.2007.12.4.191

J Food Science and Nutrition

Protective Activity of Fucoidan and Alginic Acid against Free Radical-Induced Oxidative Stress under *in Vitro* and Cellular System

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Abstract

We investigated radical scavenging effects and protective activities of fucoidan and alginic acid, active polysaccharide components from brown seaweeds, against peroxyl radical-induced oxidative stress under *in vitro* and cellular system. Fucoidan exerted strong radical scavenging effects against nitric oxide (NO) and superoxide anion (O_2^-). On the other hand, alginic acid did not show inhibitory activity against NO and relatively weak O_2^- scavenging effect. Additionally, alginic acid exhibited higher hydroxyl scavenging activity than fucoidan. Both fucoidan and alginic acid significantly enhanced cell viability against oxidative stress induced by 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH). At 1000 μg/mL concentration of fucoidan and alginic acid, the viability was increased from 16.4% to 85.9% and 67.7%, respectively. In addition, fucoidan and alginic acid ameliorated the lipid peroxidation in LLC-PK1 cell induced by AAPH in a dose-dependent manner. In particular, fucoidan showed stronger inhibitory effect than alginic acid in the cellular system. The present study suggests that fucoidan and alginic acid may be promising antioxidants against oxidative stress induced by free radicals.

Key words: fucoidan, alginic acid, antioxidative activity, free radical, oxidative stress

INTRODUCTION

Reactive oxygen species (ROS)-induced oxidative stress leads to the modification of DNA, cellular proteins and membrane lipids; therefore, it plays a crucial role in a wide range of diseases and age-related degenerative conditions including cardiovascular diseases, inflammatory conditions and neurodegenerative diseases such as Alzheimer's disease and cancer (1,2). This relationship has led to considerable interest in searching for antioxidants to scavenge free radicals and elevate defense activity in biological systems. Although several synthetic antioxidants have been suggested for the prevention and treatment of diseases, the various kinds of side effects and toxicities have become an issue. Therefore, dietary natural antioxidants have attracted much attention as preventive and therapeutic agents attenuating oxidative damage because of their safety and effectiveness.

Edible seaweeds are rich sources of dietary fibers, minerals, vitamins, proteins and antioxidants (3,4). In particular, brown seaweeds such as *Undaria stolonifera* and *Laminaria japonica* have been studied for their various biological effects. Although the active components from brown seaweeds have not been clearly elucidated, they contain abundant alginate and fucoidan which are soluble dietary polysaccharide fibers (5). Alginic acid consists of alternating units of mannuronic and guluronic acids. Several reports have shown biological functions of alginic acid such as hypocholesterolemic, antidiabetic, antimutagenic, anticancer and antioxidative activities (6-10). Fucoidan contains a number of fucose and sulfate ester groups and it has been reported to exhibit various biological activities, such as antioxidant (5), antitumor (11), anticoagulant (12) and antiviral (13) activities. However, the combined and separate effects of fucoidan and alginic acid on radical scavenging activity and protective potential from free radical-induced oxidative stress have not been clearly elucidated. In particular, under the oxidative cellular system using cells vulnerable to oxidative stress, the antioxidative activity of fucodian and alginic acid has not been investigated. In this study, we investigated radical scavenging effect of fucoidan and alginic acid using *in vitro* superoxide anion (O_2^-), nitric oxide (NO), hydroxyl radical (·OH) scavenging assays, and evaluated their protective activity against peroxyl radical-induced oxidative damage in a cellular system.

MATERIALS AND METHODS

Materials

Fucoidan and alginic acid were purchased from Sigma

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Chemical Co. (St. Louis, MO, USA). 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH), sodium nitroprusside (SNP) and 3-(4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2*H* tetrazolium bromide (MTT) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The LLC-PK1 porcine renal epithelial cells were obtained from ATCC (Manassas, VA, USA). Dulbecco's modified Eagal medium/nutrient mixture F-12 (DMEM/F-12) and fetal bovine serum (FBS) were purchased from Invitrogen Co. (Grand Island, NY, USA).

NO scavenging activity

NO was generated from SNP and measured by the Griess reaction (14) according to the method of Sreejayan and Rao (15). SNP (5 mM) in phosphate buffered saline was mixed with different concentrations of samples and incubated at 25°C for 150 min. The amount of NO produced by SNP was assayed by measuring the accumulation of nitrite, using a microplate assay method based on the Griess reaction.

O_2^- scavenging activity

O_2^- level was measured following the method as described by Ewing and Janero (16). For the assay, fucoidan or alginic acid were added to microplate wells containing 200 μL freshly prepared 0.125 mM EDTA, 62 μM nitro blue tetrazolium (NBT) and 98 μM NADH in 50 mM phosphate buffer (pH 7.4). The reaction was initiated with the addition of 25 μL freshly prepared 33 μM 5-methylphenazium methyl sulfate (PMS) in 50 mM phosphate buffer (pH 7.4). The absorbance at 540 nm was continuously monitored over 5 min as an index of NBT reduction using a microplate reader (model SPECTRAmax 340PC, Molecular Devices, Sunnyvale, CA, USA).

·OH scavenging activity

The reaction mixture contained 0.45 mL of 0.2 M sodium phosphate buffer (pH 7.0), 0.15 mL of 10 mM 2-deoxyribose, 0.15 mL of 10 mM $FeSO_4$-EDTA, 0.15 mL of 10 mM H_2O_2, 0.525 mL of H_2O and 0.075 mL of sample solution. The reaction was started by the addition of H_2O_2. After incubation at 37°C for 4 hr, the reaction was stopped by adding 0.75 mL of 2.8% trichloroacetic acid and 0.75 mL of 1.0% of 2-thiobarbituric acid in 50 mL 0.05 N NaOH. The solution was boiled for 10 min, and then cooled in water. The absorbance was measured at 520 nm. ·OH scavenging activity was evaluated as the inhibition rate of 2-deoxyribose oxidation by ·OH (17).

Cell culture

Commercially available LLC-PK1 cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in culture plates with 5% FBS-supplemented DMEM/F-12 medium. The cells were subcultured weekly with 0.05% trypsin-EDTA in calcium- and magnesium-free phosphate buffer.

Treatment of peroxyl radical generator

To investigate the protective activity from oxidative damage, we employed the AAPH-induced cellular oxidative model (18,19). After confluence had been reached, the cells were seeded into 96-well culture plates at 10^4 cells/mL. Two hours later, 1.0 mM of AAPH was added for 24 hr, and then treated with fucoidan or alginic acid for 24 hr in the test wells.

MTT assay

Cell viability was determined using an MTT colorimetric assay (20). Fifty microliters of MTT (1 mg/mL) solution was added to the each well. After incubation for 4 hr at 37°C, the MTT solution was removed from the medium. The resultant formazan crystals in the renal cells were solubilized with 100 μL of dimethylsulfoxide (DMSO). The absorbance of each well was then read at 540 nm using a microplate reader (model SPECTRAmax 340PC, Molecular Devices, Sunnyvale, CA, USA).

Thiobarbituric acid reactive substances (TBARS)

The level of lipid peroxidant released from the cultured cells was estimated as TBARS according to the methods of Yagi (21) and Yokode et al. (22) with a slight modification. One aliquot of medium was mixed with 1.5 mL of 0.67% TBA aqueous solution and 1.5 mL of 20% trichloroacetic acid, and boiled at 95～100°C for 45 min. The mixture was cooled with water and shaken vigorously with 3.0 mL of *n*-butanol. After the mixture was centrifuged at 4000×*g* for 10 min, the *n*-butanol layer was removed, and the absorbance was measured on a fluorescence spectrophotometer (Model FR-550, SHIMADZU, Kyoto, Japan).

Statistical analysis

The results for each group were expressed as mean ±SD values. Data were analyzed by one way ANOVA between control and sample treated groups using SAS software (SAS Institute Inc., Cary, NC, USA). Significant differences were determined among groups at $p<0.05$.

RESULTS

NO scavenging activity

Table 1 shows the *in vitro* NO scavenging effects of fucoidan and alginic acid. Fucoidan scavenged NO in

Table 1. NO scavenging activity of fucoidan and alginic acid

Concentration (μg/mL)	NO scavenging effect (%)	
	Fucoidan	Alginic acid
50	–	–
100	6.6±1.3[d]	–
250	18.4±1.3[c]	–
500	39.5±4.7[b]	–
1000	72.4±6.8[a]	–
IC_{50} (μg/mL)	580.3±81.2	–

Values are mean±SD.
[a-c]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

a dose-dependent manner and showed NO scavenging activity by 18.4%, 39.5% and 72.4% at the concentrations of 250, 500 and 1000 μg/mL, respectively. However, alginic acid did not exert NO scavenging effect at these concentrations.

O_2^- scavenging activity

The effect of fucoidan and alginic acid against O_2^- is shown in Table 2. Fucoidan had stronger scavenging activity of O_2^- than alginic acid. At the concentrations of 100 and 250 μg/mL, fucoidan scavenged O_2^- by 36.8% and 63.9%, respectively. On the other hand, alginic acid exhibited relatively weak activity; 7.3% and 15.3% of O_2^- scavenging effect at the same concentrations.

Table 2. O_2^- scavenging activity of fucoidan and alginic acid

Concentration (μg/mL)	O_2^- scavenging effect (%)	
	Fucoidan	Alginic acid
50	5.3±1.2[c]	7.7±1.3[c]
100	36.8±1.2[b]	7.3±1.5[c]
250	63.9±1.2[a]	15.3±1.3[ab]
500	61.0±1.0[a]	19.1±3.9[a]
1000	61.6±5.3[a]	8.7±1.8[bc]
IC_{50} (μg/mL)	271.8±1.4	1737.3±1127.2

Values are mean±SD.
[a-c]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

Table 3. ·OH scavenging activity of fucoidan and alginic acid

Concentration (μg/mL)	O_2^- scavenging effect (%)	
	Fucoidan	Alginic acid
50	–	5.2±0.4[d]
100	5.5±0.1[d]	17.9±0.2[c]
250	9.9±0.5[c]	16.6±0.2[c]
500	14.6±0.6[b]	31.7±0.1[b]
1000	27.1±0.2[a]	48.9±0.8[a]
IC_{50} (μg/mL)	11833.0±705.4	1311.6±53.5

Values are mean±SD.
[a-d]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

·OH scavenging activity

As shown in Table 3, alginic acid exhibited a higher ·OH scavenging effect than fucoidan. At the concentrations of 250, 500 and 1000 μg/mL, fucoidan showed 9.9%, 14.6% and 27.1%, and alginic acid exerted 16.6%, 31.7% and 48.9% of ·OH scavenging activity, respectively. In contrast with NO and O_2^-, alginic acid showed strong antioxidative activity against ·OH.

Protective activity against peroxyl radical-induced oxidative stress

Figure 1 shows the effect of fucoidan and alginic acid on the viability of LLC-PK1 renal epithelial cells treated with AAPH, a well-known peroxyl radical generator. The viability of LLC-PK1 cells was reduced to 16.4% by treatment with 1 mM AAPH for 24 hr. However, the treatment of fucoidan and alginic acid exerted protective activity against AAPH-induced cellular damage. When fucoidan and alginic acid were treated at the dose of 100 μg/mL, the cell viability was elevated from from 16.4% to 85.9% and 67.7%, respectively.

Inhibition of lipid peroxidation against peroxyl radical-induced oxidative stress

As shown in Fig. 2, AAPH led to an increase in lipid peroxidation in LLC-PK1 renal tubular epithelial cells, whereas fucoidan and alginic acid significantly decreased the formation of TBARS in a concentration-dependent manner. Although 0.795 nmol/mg protein of

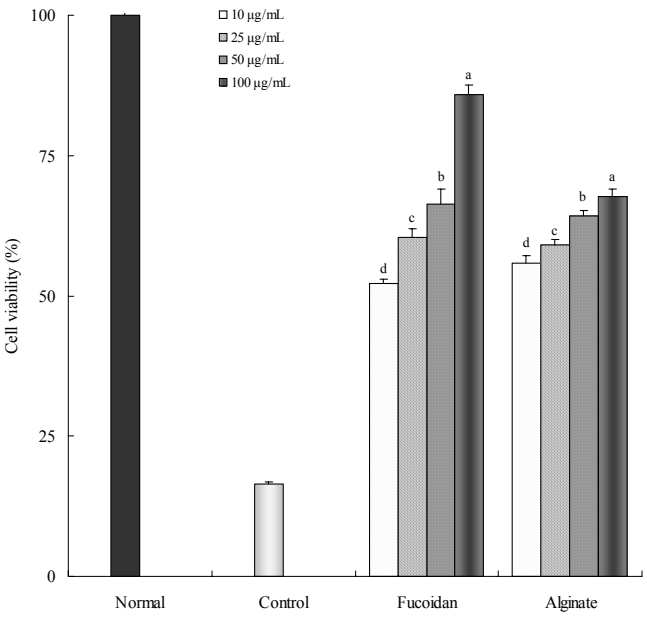

Fig. 1. Effect of fucoidan and alginic acid on viability of LLC-PK_1 cells treated with AAPH.
Values are mean±SD.
[a-d]Means with different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

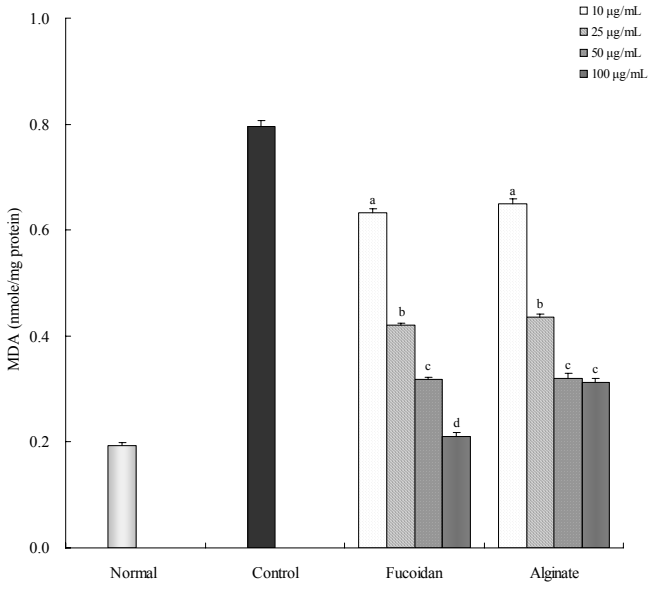

Fig. 2. Effect of fucoidan and alginic acid on lipid peroxidation of LLC-PK1 cells treated with AAPH.
Values are mean±SD.
[a-d]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

TBARS was produced in wells treated only with AAPH, lipid peroxidation was decreased by the treatment of fucoidan and alginic acid at the 100 μg/mL to 0.211 nmol/mg protein (73.5% decrease) and 0.313 nmol/mg protein (60.6% decrease), respectively.

DISCUSSION

Free radical-mediated oxidative stress results in a variety of pathological conditions (1,2). Therefore, antioxidants that prevent damage caused by free radicals are considered to be worthy of study. Several studies demonstrated that brown seaweeds have a protective role against pathological phenomena. Although active components have to be clearly elucidated, the biological activities are mainly attributed to fucoidan and alginic acid. Therefore, the present investigation was focused on the protective activity of fucoidan and alginic acid from free radical-induced oxidative stress under *in vitro* and cellular system.

We found that fucoidan showed a strong radical scavenging effect against NO and O_2^-. On the other hand, alginic acid did not show NO scavenging activity and exhibited relatively weak inhibitory effect against O_2^-. NO is a free radical with a single unpaired electron and it plays both useful and harmful effects. Although useful physiological roles of NO have been identified, overproduction of NO causes tissue damage and contributes to pathological conditions (23,24). In addition, reaction of O_2^- with NO forms the short-lived peroxynitrite ($ONOO^-$) which is far more reactive and toxic than its precursors (25,26). This reaction is extremely rapid, and it can generate the most toxic radical, ·OH (27). Against NO and O_2^-, fucoidan exerted greater scavenging effect than alginic acid. Fucoidan, the sulfated polysaccharide extracted from brown algae, contains substantial percentages of L-fucose and sulphate ester groups (28). Several reports demonstrated that fucoidan exhibits various biological activities; anticoagulant, antithrombotic, anti-inflammatory, antitumor and antiviral functions (29-34). The present results support an antioxidative role of fucoidan through scavenging NO and O_2^-.

·OH is the most reactive and toxic radical, and it can react with unsaturated fatty acids of membrane phospholipids to generate free radicals, which in turn react quickly with oxygen to form peroxides. Peroxides themselves then act as free radicals, initiating an autocatalytic chain reaction, resulting in further loss of unsaturated fatty acids and extensive membrane damage. Although alginic acid did not exhibit antioxidative activity against NO and O_2^-, it showed protective potential against ·OH. The present results suggest that alginic acid would play the protective role against free radical-induced oxidative stress.

The reactions of free radicals in biological systems are complicated. To study these reactions, a well-designed *in vitro* model system is required. Thermal decompositions of free radical initiators, including peroxides, hyponitrites, and azo compounds induce oxidative stress. To generate free radicals at a known, constant and well-defined rate, thermal decomposition of free radical initiators is preferred. AAPH, one of the hydrophilic azo compounds, generates free radicals at a constant and measurable rate by its thermal decomposition without biotransformation (35). The free radicals generated from AAPH react with oxygen molecules rapidly to yield peroxyl radicals. The lipid peroxyl radicals attack other lipid molecules to form lipid hydroperoxide and new lipid radicals. This reaction takes place repeatedly with resultant attacks upon various biological molecules, and induces physiochemical alterations and cellular damage (36). Finally, AAPH causes a diverse array of pathological changes. Therefore, an AAPH-intoxication experiments are useful for evaluating biological activities of antioxidants.

To investigate the antioxidative activity of fucoidan and alginic acid in a cellular system using LLC-PK1 renal tubular epithelial cells that are susceptible to oxidative stress, we employed such an AAPH model system. Several reports documented that AAPH decreased the

viability of hepatic and neuron cells (37,38). The treatment with AAPH induces apoptosis in the cell, causing loss of viability. The present study also shows that AAPH leads to the decline in viability of LLC-PK1 renal epithelial cells. However, our results demonstrated that fucoidan and alginic acid exerted a protective effect against oxidative damage by AAPH to LLC-PK1 cells, resulting in increased cell viability in a dose dependent manner. In particular, fucoidan showed stronger activity against AAPH-induced oxidative damage than alginic acid. This activity of fucoidan may be positively correlated with NO and O_2^- scavenging activity.

It is well accepted that lipid peroxidation in biological systems is toxicological phenomenon, resulting in pathological consequences (39). Therefore, measurement of lipid peroxidation end products such as TBARS provides a good index of cell destruction. To determine cellular damage induced by AAPH, we investigated the lipid peroxidation products, TBARS. In this study, the result revealed that AAPH treatment increased the formation of TBARS by LLC-PK1 renal epithelial cells. However, the treatment of fucoidan and alginic acid, particularly fucoidan, significantly inhibited TBARS formation in AAPH damaged LLC-PK1 cells. These results indicate that fucoidan and alginic acid, in particular fucoidan, exerted protective activity from AAPH-induced cell injury and lipid peroxidation by scavenging peroxyl radicals generated from AAPH, suggesting their roles as promising antioxidants.

In conclusion, fucoidan and alginic acid, which are bioactive compounds from brown seaweeds, exhibit radical scavenging activity *in vitro* and antioxidative activity against oxidative stress in cellular model. In particular, fucoidan showed the stronger activity than alginic acid. Although the further study on the protective mechanisms also has to be carried out, the present study supports the promising role of fucoidan and alginic acid as antioxidative agents against free radical-induced oxidative stress.

ACKNOWLEDGEMENTS

This research was supported by a project grant (YSG-RF0605) from Yeongnam Sea Grant, Korea.

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(Received October 4, 2007; Accepted November 12, 2007)