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Preparation of low molecular weight fucoidan by gamma-irradiation and its anticancer activity

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ABSTRACT

Fucoidan is a marine sulfated polysaccharide with a wide variety of biological activities. Recently, it has been reported that low molecular weight fucoidan has the enhanced antioxidant and anticoagulative activities. However, degradation techniques such as enzymolysis and acid hydrolysis for obtaining low molecular weight fucoidan, have the disadvantages such as narrow substrate specificity and unfavorable hydrolysis of side groups, respectively. In this study, low molecular weight fucoidan was prepared by gamma-irradiation. When fucoidan was gamma-irradiated, the molecular weight rapidly dropped to 38 kDa when the sample was irradiated at 10 kGy, then gradually dropped to 7 kDa without the significant elimination of the sulfate groups. Low molecular weight fucoidan had higher cytotoxicity than native fucoidan in cancer cells, such as AGS, MCF-7, and HepG-2. In addition, low molecular weight fucoidan showed higher inhibitory activity of cell transformation, which resulted in higher anticarcinogenicity. This result suggests that low molecular weight fucoidan with enhanced biological activities can be produced by a simple irradiation method without changing the functional groups.

1. Introduction

Fucoidan, a sulfated polysaccharide containing a substantial percentage of fucose and small amounts of galactose, xylose, mannose, and uronic acid, is a constituent of brown seaweed. For the past decades, fucoidans isolated from different species have been extensively studied because of their various biological activities, such as anticoagulant and antithrombotic, antiviral, and gastric protective properties (Choi et al., 2010; Li, Lu, Wei, & Zhao, 2008). A number of studies have shown that fucoidan suppressed the growth of tumor cells in *in vitro* and *in vivo* models. It has been suggested that the anticancer activity of fucoidan is attributable to the activation of the immune system or the inhibition of tumor growth by suppressing angiogenesis.

The biological activities of fucoidans are closely related to their molecular structures, which include fucose linkage, the sugar type, sulfate content, and molecular weight. Among these, molecular weight is one of the most important factors determining the biological activities of polysaccharides. High molecular weight polysaccharides may cause low solubility and processability, thereby hampering their penetration into the cell to perform a function. On the contrary, low molecular weight sulfated cellulose shows higher antioxidative and anticoagulation activities (Wang, Zhang, Zhang, Song, & Li, 2010a; Wang, Xiao, Li, & Wu, 2010b). In addition, low molecular weight fucoidan promotes revascularization of hindlimb ischemia in rats (Luyt et al., 2003), boosts osteoblast proliferation for bone regeneration (Igondjo Tchen Changotade et al., 2008), and enhances human endothelial cell formation (Chabut et al., 2003; Lake et al., 2006).

Low molecular weight fucoidan can be produced by acidolysis and enzymolysis, however, acidolysis is difficult to obtain the proper size of fucoidan and the sulfate groups in fucoidan can be removed because of the hydrolysis properties with acid. Pomin et al. (2005) reported that acid hydrolysis tends to selectively desulfate at the 2 position of the first fucose unit and then the glycosidic linkage between nonsulfated fucose residue and the subsequent 4-sulfated residue is preferentially cleaved by acid hydrolysis. Fucoidanase has been found in marine bacteria including *Pseudomonas*, *Bacillus*, and *Vibrio*, and marine fungi (Bakunina et al., 2000). However, the activity of fucoidanase is quite variable depending on the molecular structure of fucoidan, and no conventional fucoidanse degrades all the different structures of fucoidan. In addition, the production of low molecular weight fucoidan less than 10 kDa, needs a complex enzymatic process (Wu et al., 2010). There are several reports showing that the low molecular weight polysaccharides degraded by gamma-irradiation enhance biological activities. When β-glucan is degraded by irradiation, its antioxidant and immune-enhancing activities increase, depending on the irradiation dose (Byun et al., 2008; Sung et al., 2009). The antioxidative activity of laminarin, another polysaccharide in

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seaweed, also increases after gamma-irradiation (Choi, Kim, & Lee, 2011). The benefits of radiation degradation include a short process period, with no addition of acid and enzyme, unnecessary purification after degradation or possible degradation of powder form, and simultaneous sterilization.

Therefore, in this study, fucoidan was degraded by gamma-irradiation and its biological activity was investigated on inhibitory activity in different types of cancer cells and its anticarcinogenic activity.

2. Materials and methods

2.1. Materials

Fucoidan was purchased from Sigma Chemical Co. (St. Louis, MO), which was originated from *Fucus vesiculosus*. Samples were dissolved at 10 mg/mL (w/v) in double distilled water for the gamma-irradiation. Other constituents such as proteins and uronic acid in fucoidan samples were determined because these possibly involve in highly biological response. Uronic acid content was shown to be 11.6 (mass%) determined by carbazole reaction (Bitter & Muir, 1962), Also, protein concentration was about 1.2% quantified by Bradford method (1976). The contents of uronic acid and proteins in fucoidan samples used in this study was almost same as in other purified fucoidan samples (You, Yang, Lee, & Lee, 2010).

Eagle's minimal essential medium (MEM), Roswell Park Memorial Institute (RPMI-1640) medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was purchased from Calbiochem-Novabiochem (San Diego, CA, USA).

2.2. Preparation of degraded fucoidan with gamma-irradiation

The dissolved fucoidan (10 mg/mL) was gamma-irradiated in a ^{60}cobalt gamma-irradiator (IR-221, Nordion International Ltd., Ontario, Canada) with a strength of 11.1 petabecquerel (pBq) at $22 \pm 2\,^{\circ}C$ at a dose rate of 10 kGy/h. The applied dose levels were 10 kGy, 30 kGy, 50 kGy, and 100 kGy, respectively. Dosimetry was performed with 5 mm-diameter alanine dosimeters (Bruker Instruments, Rheinstetten, Germany). After the gamma-irradiation process, the samples were stored at 4 °C for further experiments.

2.3. Gel permeation chromatography (GPC) analysis

GPC was performed using a following system; separation module (Waters 2690, Waters Co., Milford, MA), refractive index detector (RI, Waters 2410, Waters Co.), Empower software (System Software, Empower option GPC, Waters Co.), and PL aquagel-OH -60, -40, and -30 columns (300 mm × 7.5 mm, 8 μm, Polymer laboratories Ltd., UK). The mobile phase was 0.1 M sodium nitrate at the flow rate of 1 mL/min, and the analyses were performed at 40 °C. The injection volume was 200 μL (10 mg/mL fucoidan), and calibration was carried out using pullulan standard (Showa Denko K.K., Tokyo, Japan) (Choi et al., 2011).

2.4. Determination of sulfate contents

The content of sulfate in fucoidan was determined by the method using 1,9-dimethyl-methylene blue (DMMB) (Farndale, Buttle, & Barrett, 1986). The sample solution (10 mg/mL fucoidan, 0.1 mL) was added to 0.2 mL of DMMB reagent (16 mg of DMMB, 3.04 g of glycine, and 2.37 g of sodium chloride per 1 L). The reaction mixture was vortexed for thorough mixing. The absorbance was measured using a spectrophotometer (UV-1601PC, Shimadzu Co., Tokyo, Japan) at 525 nm. Quantification was done based on a standard curve generated with chondroitin-6-sulfate (Sigma Chemical Co.).

2.5. Cytotoxicity in cancer cells

Human breast cancer cells (MCF-7), human stomach cancer cells (AGS), and human liver cancer cells (HepG-2) were purchased from the Korean Cell Line Bank (Seoul, South Korea) and adapted in RPMI-1640 medium containing 10% FBS, 100 unit/mL penicillin and 100 unit/mL streptomycin and then cultured at 37 °C in 5% CO_2. Types of cancer cells were seeded into 96-well-plate with 3×10^4/well, respectively, and fucoidan samples were added in the plate at different doses. After 24 h incubation, cancer cell cytotoxicity was detected by the MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide] (Sigma Chemical Co.) assay method. Briefly, 30 μL of 5 mg/mL MTT reagent dissolved in PBS was added to each well and the plate was incubated at 37 °C. After 2 h, each plate was then centrifuged and the medium was removed. One hundred microliters of dimehylsulfoxide (Sigma Chemical Co.) was then added. After incubation at 37 °C for 5 min, the absorbance from fucoidan treated and non-treated cells was measured at 517 nm by micro-plate reader (Zenyth 3100, Anthos Labtec Instrumentrs GmbH, Salzburg, Austria). The percentage of cell cytotoxicity was calculated the following equation,

$$\text{Cytotoxicity} = 100 \times [1 - (A_s/A_c)],$$

where A_s is the absorbance with fucoidan sample and A_c is the absorbance of control for which H_2O was used instead of sample solution.

2.6. Anchorage-independent transformation assay

JB6 Cl41 mouse epidermal cells were cultured in MEM supplemented with 5% FBS. TPA-induced cell transformation was investigated in JB6 Cl41 cells. In brief, cells (8×10^3/mL) were exposed to TPA (10 ng/mL) with or without fucoidan (1 μg/mL or 10 μg/mL) in 1 mL of 0.3% Basal Medium Eagle (BME) agar containing 10% FBS, 2 mM L-glutamine, and 25 μg/mL gentamicin. The cultures were maintained at 37 °C in a 5% CO_2 incubator for 10 days, and the cell colonies were scored using a microscope and the Image-Pro PLUS computer software program (Media Cybernetics, Silver Spring, MD) as described by Lee et al. (2008a, 2008b).

2.7. Statistical analysis

All of the experiments were carried out 3 times. For molecular weight and cytotoxicity experiments, one-way analysis of variance was carried out using the SPSS software system, and the Duncan's multiple-range test was used to compare the differences among the mean values. For the comparison of cytotoxicity and carcinogenetic inhibitory effect of fucoidan, student's *t*-test was performed with $p < 0.05$ for statistical significance.

3. Results and discussion

3.1. Degradation of fucoidan with gamma-irradiation

The change in the molecular weight of fucoidan by the gamma-irradiation is shown in Fig. 1. The weight average molecular weight decreased as the irradiation dose increased. When the irradiation dose was 10 kGy, the molecular weight of fucoidan decreased to 38 kDa from 217 kDa of the non-irradiated fucoidan. The molecular weights of fucoidan after irradiation at the doses of 30 kGy, 50 kGy, and 100 kGy were 16 kDa, 10 kDa, and 7 kDa, respectively. The molecular weight loss of fucoidan by irradiation was similar to

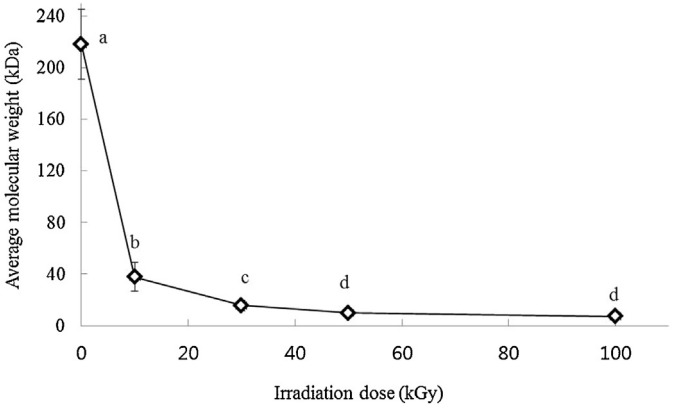

Fig. 1. Average molecular weight of fucoidan degraded by gamma-irradiation at different doses. Values with different letters are statistically different according to Duncan's multiple-range test.

that of β-glucan. The molecular weight of β-glucan, with a native molecular weight of 178 kDa, decreased to 25 kDa after irradiation at the absorbed dose of 50 kGy (Byun et al., 2008). The extent of degradation of the fucoidan was not linearly proportional with the dosage of gamma-radiation. The slope of degradation in the molecular weight was steeper at the low dose range than at the high dose range. It was proposed that fucoidan with a higher molecular weight degraded more severely because of the higher possibility of scission by the free radicals generated by irradiation, whereas the degradation rate became retarded when the molecular weight decreased. Similar degradation patterns, following irradiation, were observed in the degradation of carboxymethylcellulose (Choi et al., 2008) and laminarin (Choi et al., 2011). The difference between the molecular weight of native fucoidan samples in this study and previous report was attributable to the different seaweed sources and preparation method.

The sulfate group is the main characteristic of fucoidan and is responsible for the anticoagulant and antiproliferative effects of fucoidan (Haroun-Bouhedja et al., 2000; Kayanagi, Tanigawa, Nakagawa, Soeda, & Shimeno, 2003). The sulfate contents in the fucoidan degraded by irradiation are measured. The initial sulfate content was 820 μg/mL in 0.1% fucoidan solution and was approximately the same in fucoidan samples irradiated up to 50 kGy. When fucoidan was irradiated at the dose of 100 kGy, sulfate content was decreased to 760 μg/mL; however, the content difference from that of native fucoidan was statistically insignificant ($p > 0.05$). To confirm that free sulfate groups which might be eliminated by irradiation would not be accounted for the constant sulfate content, sample irradiated at 100 kGy was washed following the method by Yang et al. (2008). It was shown that the sulfate content per gram of fucoidan was not changed by additional washing step. It has been demonstrated that the negative charges of the sulfate group are important in determining its activities because of its contribution to the recognition of biological targets (Haroun-Bouhedja et al., 2000). Teruya, Konishi, Uechi, Tamaki, and Tako (2007) reported that oversulfated fucoidan showed enhanced anti-proliferative activity in leukemia cells through the induction of apoptosis. Park, Kim, Kim, Suh, and Choi (2002) examined the anticancer activity of fucoidan and reported that the fucoidan sulfate group affects DNA replication of cancer cells through the binding of replication protein. This study shows that the degradation of fucoidan by irradiation does not cause significant change in sulfate content, and degraded fucoidan can sustain its biological activities or have even higher activities owing to its low molecular weight.

3.2. *Effect of gamma-irradiation of fucoidan on cytotoxicity in cancer cells*

The cytotoxicity of fucoidan in a cancer cell line is shown in Fig. 2. In the case of MCF-7 human breast cancer cell line, the proliferation inhibition increased depending on the concentration of fucoidan (Fig. 2(a)). At 4 mg fucoidan/mL, the cytotoxicity was 46% of the native fucoidan. The cytotoxicities of the degraded fucoidan at different absorbed doses are shown in Fig. 2(a). The cytotoxicity of the degraded fucoidan increased depending on its concentration. Furthermore, when fucoidan was irradiated at the absorbed doses of 100 kGy, the cytotoxicity of the degraded fucoidan appeared to be increased. At a low concentration of fucoidan (0.5 mg/mL), cytotoxicity was not significantly different between native and degraded samples. However, the 42% cytotoxicity of native fucoidan significantly increased to 50% and 66% of samples irradiated at 50 kGy and 100 kGy, respectively, at a concentration of 2 mg/mL (Fig. 2(b)). When the concentration increased to 4 mg/mL (Fig 2(a)), the difference in cytotoxicity became more pronounced. Cytotoxicity of sample irradiated at 30 kGy was also statistically increased because of the possible dose effect. To compare gamma irradiation hydrolysis and acid-decomposed preparation, lower molecular weight fucoidan was prepared following the acid-decomposed method by You et al. (2010). The average molecular weight of acid-hydrolyzed fucoidan was about 8 kDa with the sulfate content of 520 μg/mL. The cytotoxicity of the acid-decomposed fucoidan was 45%, which was significantly lower than the 66% cytotoxicity of fucoidan irradiated at 100 kGy.

The effects of radiation on the cytotoxicity of fucoidan in other cancer cells including AGS and HepG-2 are also shown in Fig. 3(a) and (b), respectively. The concentration of native and radiation-degraded fucoidan was at 2 mg/mL. In the case of AGS (human stomach cancer cell line), the cytotoxicity of non-irradiated fucoidan was 35% and those of fucoidans irradiated at the doses of 10 kGy, 30 kGy, 50 kGy, and 100 kGy were 36%, 39%, 38%, and 47% respectively (Fig. 3(a)). The cytotoxicity appeared to increase

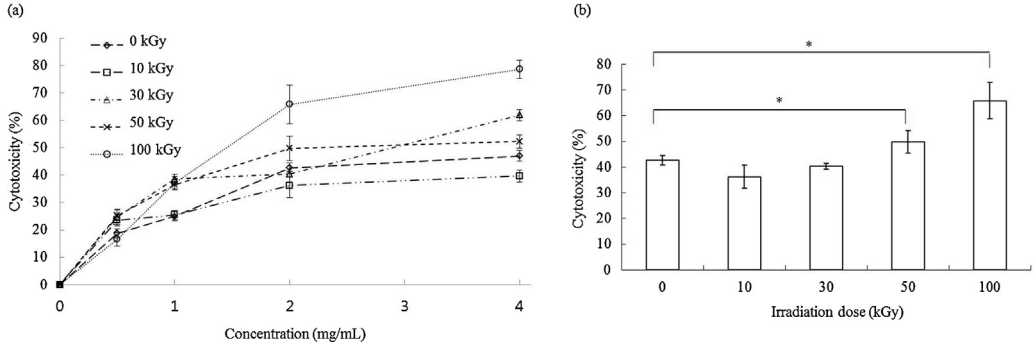

Fig. 2. (a) Cytotoxicity of fucoidan degraded by gamma-irradiation at different doses in human breast cancer cells (MCF-7) at various concentrations. (b) Cytotoxicity of fucoidan (at 2 mg/mL) degraded by gamma-irradiation at different doses in MCF-7. * Indicates that two values are statistically different, with $p < 0.05$.

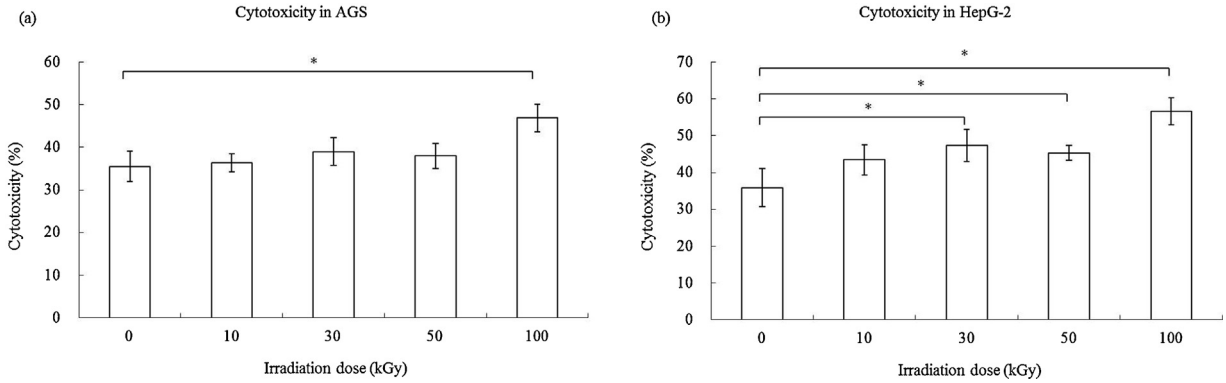

Fig. 3. Cytotoxicity of fucoidan (at 2 mg/mL) degraded by gamma-irradiation at different doses in (a) human stomach cancer cells (AGS) and (b) human liver cancer cells (HepG-2). * Indicates that two values are statistically different, with $p < 0.05$.

depending on the absorbed dose of fucoidan, and it significantly increased ($p < 0.05$) from that of native fucoidan when irradiated at 100 kGy.

The cytotoxicity of fucoidan in HepG-2 human liver cancer cell line is shown in Fig. 3(b). The cytotoxicity of native fucoidan against HepG-2 was 35% and that of irradiated fucoidan at 100 kGy increased up to 57%. When fucoidan was irradiated at a dose higher than 30 kGy, the cytotoxicity of irradiated fucoidan significantly increased ($p < 0.05$). This result shows that higher cytotoxicity was obtained with a fucoidan of less than 16 kDa molecular weight. Fucoidan from *Undaria pinnatifida* sporophyll was fractionated into >30 kDa, 30–5 kDa, and <5 kDa; fucoidan molecular weight ranging between 5 kDa and 30 kDa showed the highest inhibitory activity (You et al., 2010).

When fucoidan was hydrolyzed from 5100 kDa to 490 kDa by heating in boiling water with acid, the inhibition activity against A549 cancer cell increased significantly. However, further hydrolysis caused a slight decrease in the inhibition activity. It was also interesting that inhibition activity of fucoidan slightly increased compared to native fucoidan activity when degraded to the same molecular weight of 50 kDa by microwave heating in acid solution, but this inhibition activity decreased upon further degradation (Yang et al., 2008). In their study, the decrease in the inhibitory activity of degraded fucoidan might have occurred because of the partial removal of the sulfate group. Furthermore, Cho et al. (2011) reported that over-sulfation of low molecular weight fucoidan (5-30 kDa) increased its inhibitory activity in the cancer cell line more than in the case of high molecular weight fucoidan (>30 kDa).

Therefore, the increase of the inhibitory activity of fucoidan by irradiation is based on its low molecular weight without the removal of the sulfate group. Along with the molecular weight and sulfate content, the degree of branching or chain conformation of fucoidan is very important to define biological activities. Clement et al. (2010) examined the structural heterogeneity and the random sulfation of fucoidan with relation to how they affected a biological system – in this case, complement binding and inhibition. Their results suggested that branching of fucoidan oligosaccharides is a major factor in their biological activity at the conformational level. This work was significant as it demonstrated that not only molecular weight, sulfation and sugar composition affect bioactivity, but also three dimensional structure.

3.3. Inhibitory effect of gamma-irradiated fucoidan on cell transformation

Even though fucoidan is structurally similar to heparin with anticoagulant and antithrombotic activities, only fucoidan is known to exhibit anticarcinogenic activities (Lee et al., 2008a, 2008b). These previous studies suggest that fucoidan could be used as a chemopreventive agent against carcinogenesis. Therefore, the anticarcinogenic activity of low molecular weight fucoidan prepared by gamma-irradiation was investigated.

TPA is a highly potent tumor promoter and is widely used for cell transformation. JB6 mouse epidermal cells were treated with TPA in the absence or presence of native and irradiated fucoidan samples. The result of this treatment showed that fucoidan inhibits the formation of TPA-promoted neoplastic cell transformation of JB6 cells in a concentration dependent manner (Fig. 4). When 1 μg/mL of native fucoidan was treated with TPA, there was no statistically significant difference between TPA and TPA-fucoidan treated groups. At 10 μg/mL of native fucoidan, cell transformation was inhibited by fucoidan with statistical significance ($p < 0.05$). However, degraded fucoidan irradiated at 100 kGy had a higher inhibitory effect on cell transformation. Even at 1 μg fucoidan/mL, cell transformation was decreased by the treatment of irradiated fucoidan with statistical significance ($p < 0.05$). The increased inhibitory activity was more pronounced at a higher treatment concentration of 10 μg/mL. Lee et al. (2008a, 2008b) reported that fucoidan exerted an inhibitory effect on epidermal growth factor (EGF)-induced phosphorylation of epidermal growth factor receptor (EGFR) and suppressed the phosphorylation of extracellular signal regulated kinase or c-Jun N-terminal kinase, resulting in the suppression of cell transformation.

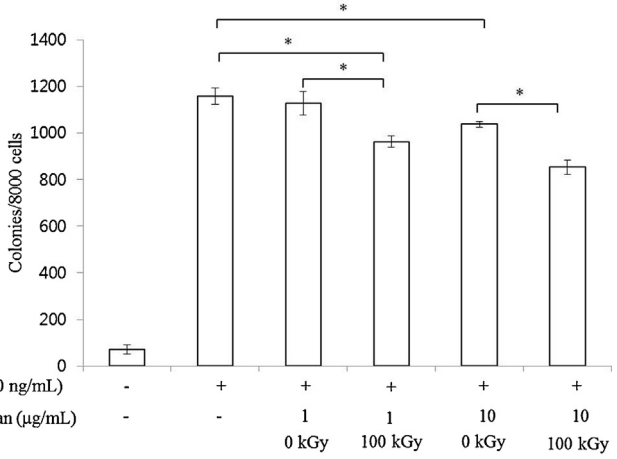

Fig. 4. Inhibitory activity of native and irradiated (at 100 kGy) fucoidan on TPA-induced neoplastic cell transformation in JB6 mouse epidermal cells. * Indicates that two values are statistically different, with $p < 0.05$.

4. Conclusion

In this study, fucoidan was efficiently degraded to a low molecular weight by gamma-irradiation. There have been several reports on the acidic hydrolysis of fucoidan, but the studies on the biological activities of low molecular weight fucoidan are not consistent because of the removal of the sulfate group. Different from enzymatic degradation and acidic hydrolysis, the gamma-irradiation method can be used to degrade structurally different polysaccharides without changing the sulfate groups. The low molecular weight fucoidan obtained after degradation by gamma-irradiation showed enhanced cytotoxicity in cancer cells and inhibitory activity in cell transformation. This increased inhibitory activity may be attributed to the easier access of small fucoidan into the target molecules.

Acknowledgments

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