

## Note

### Effects of Fucoidan from Mozuku on Human Stomach Cell Lines

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*Cladosiphon okamuranus* (Okinawamozuku) is a species of Mozuku known to contain the most fucoidan of any brown algae. Fucoidan is a sulfated hetero polysaccharide with various biological activities. We isolated and purified fucoidan from *C. okamuranus*. The yield of fucoidan was 1.0% (w/w) based on wet algae from *C. okamuranus*. The sulfate content and molecular weight of the fucoidan were 9.8% (w/w) and approximately 3,200,000, respectively. We examined the effects of fucoidan on human stomach cell lines. Fucoidan from *C. okamuranus* showed an inhibitory effect on anticancer agent 5-fluorouracil (5-FU) in Hs 677.st normal stomach cells with minimum inhibition of the original effect of 5-FU in cancer cells. Furthermore, fucoidan from *C. okamuranus* showed growth inhibition of stomach cancer cells but did not show any effects on normal cells. Thus, fucoidan from Mozuku, especially *C. okamuranus*, may be useful for cancer therapy.

Keywords: fucoidan, Mozuku, *Cladosiphon okamuranus*

#### Introduction

Mozuku are classified as brown algae, and most Mozuku are produced by artificially seeded culture nets in Okinawa, Japan. Among the various Mozuku, *C. okamuranus* and *N. decipiens* are edible seaweeds, and are typically served in salads with vinegar (Sunomono). Brown algae possess fucoidan and alginate in their wall matrix (Kloareg *et al.*, 1986). Mozuku contains the most fucoidan of any-brown seaweed. Fucoidan is a fucose-containing sulfated hetero polysaccharide, but details of their structure remain unknown. Fucoidan is not uniform, and their structure differ depending on the species they are derived from (Chevolot *et al.*, 2001; Sakai *et al.*, 2002). Fucoidan has many biological activities, including anticoagulation (Kuznetsova *et al.*, 2003; Nishino *et al.*, 1991), antiviral (Lee *et al.*, 2004; Preeprame *et al.*, 2001) and antitumor activities (Aisa *et al.*, 2005; Koyanagi *et al.*, 2003). The anticoagulant activity of high molecular weight fucoidan extracted from *Ecklonia kurome* was reported to be dependent on the sulfate content and molecular weight, as in numerous other sulfated polysaccharides. Lee *et al.* reported that the fucoidan from sporophyll of *Undaria pinnatifida* (Mekabu) showed potent antiviral activities against herpes simplex virus and human cytomegalovirus. Regarding the antitumor effects of fucoidan, many studies reported effects on leukemia cells and lung cancer cells both *in vitro* and *in*

*vivo*. Recently, Aisa *et al.* reported that the fucoidan from *Fucus vesiculosus* induced apoptosis in human lymphoma HS-Sultan cell lines. In human beings, fucoidan is ingested with food. However, only a few studies have identified fucoidan in food, or have used digestive organ cells. Therefore, in the present research, we studied the effects of fucoidan on stomach cell lines with or without an anticancer agent.

#### Materials and Methods

*Cell lines and culture conditions* The stomach cell lines used in this study were obtained from the American Type Culture Collection (ATCC) and Riken Cell Bank (Saitama, Japan). According to ATCC, Hs 677.st (CRL-7407) cells were obtained from stomach fibroblasts in a 62-year-old Caucasian woman at 16 weeks gestation. The stomach cancer cell line MKN45 (RCB1001) were a gastric adenocarcinoma (Motoyama *et al.*, 1986).

Both cell lines were cultured in a 5% CO<sub>2</sub> humidified incubator at 37°C. Hs 677.st cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) containing 4.5 µg/ml glucose on collagen-coated culture plates. MKN45 cells were cultured in RPMI 1640 (Nihon Pharmaceutical Co., Ltd.). All media contained 10% cell growth promoting factor Daigo's GF21 (Wako Pure Chemical Industries, Osaka, Japan), 60 µg/ml streptomycin (Sigma Chemical Co., Tokyo, Japan), and 50 µg/ml kanamycin (Wako Pure Chemical Industries, Osaka, Japan).

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*Extraction and purification of fucoïdan from Mozuku* Wet and salted Mozuku (*C. okamuranus*), which was commercially cultured on nets in the sea around Iheya Island in Okinawa, was used in this study. Mozuku was mixed with same doses of purified water. The mixture was boiled for 1 h and filtered. The filtrate was desalted using an electro-dialysis system (Micro Acilyzer S3, Asahi Kasei Corporation, Tokyo, Japan). Then, the desalted extract was freeze-dried using a lyophilizer, and was used as the crude fucoïdan sample. 0.02 M or less sodium sulfate was added to the filtrate, and CTAB was added at 3 times the volume of the crude fucoïdan (Fujikawa *et al.*, 1975). The precipitate was washed using 30 mg/ml KCl solution. Some of the fucoïdan was eluted with different concentrations of KCl solution. The eluted solution was precipitated using ethanol, and dried. This was used as the purified fucoïdan sample.

Molecular weights (MWs) were determined using high-performance steric exclusion chromatography (HPSEC) in 0.15 M NaCl, 0.05 M NaH<sub>2</sub>PO<sub>4</sub> buffer at pH 7.0, using pullulans (Shodex STANDARD P-82; Showa Denko K.K., Tokyo, Japan) as standards (Nardella *et al.*, 1996; Haroun-Bouhedja *et al.*, 2000). An Asahipak GF-710 (Showa Denko K.K., Tokyo, Japan) column was used. Polysaccharide content was calculated according to the rate of high molecular side peak area against all peaks area. Sulfate content of fucoïdan was determined by the Dodgson's method (Dodgson *et al.*, 1961).

*Assay of the effects on stomach cells with or without 5-FU* Fucoïdan from *Fucus vesiculosus* (Sigma Chemical Co., Tokyo, Japan) and sodium alginate (Kimitsu Chemical Industries) were used for comparison. Phosphate buffered saline (PBS) was used as a control in order to investigate the effects of 5-FU only.

Hs 677.st and MKN45 cells were cultured in each medium, treated with trypsin, and washed with the appropriate medium. 90 µL culture solution of the 2,000 cells/well cells were in 96-well microplates. 10 µL of each polysaccharide solution were added to microplate wells to a concentration of 1.0 mg/mL. For each cell line, the same concentration was used for eight wells per experiment.

After a 4 h pre-incubation period, 5-FU was added to the appropriate wells at 50 µg/mL, and plates were incubated for 4 days at 37°C. The growth of each cell line cultured in the presence or absence of polysaccharides with 5-FU was monitored using a modified MTT assay (Mosmann *et al.*, 1983). After culture, 10 µL MTT (5.0 mg/mL) solution in PBS was added to each well, and incubated for 4 h at 37°C. Then, 100 µL 10% SDS solution was added to wells to dissolve the MTT crystals. We measured absorbance at 570 nm, and subtracted absorbance at 630 nm. The number of each type of stomach cells grown in culture medium only was defined as 100% relative growth. In the experiment of the effects of fucoïdan on stomach cells on the presence of 5-FU, recovery rate or inhibitory rate were calculated according to the rate of increasing relative growth with each polysaccharides compared to that of the control.

*Statistical analysis* Comparison of two means were made using Student's *t*-test (Fisher, 1958).

## Results and Discussion

*Extraction and purification of fucoïdan from Mozuku* We prepared crude fucoïdan from Mozuku, *C. okamuranus*. Molecular weights and sulfate content of the fucoïdan are shown in Table 1. The polysaccharide content of crude fucoïdan from *C. okamuranus* was 65%. We further purified and isolated one kind of fucoïdan from Mozuku. At first, fucoïdan from *C. okamuranus* was purified with an estimated yield of 1.0% (w/w) based on wet algae. The sulfate content and molecular weight of the fucoïdan were 9.8% (w/w) and approximately 3,200,000, respectively. The molecular weights of fucoïdan prepared in the present study are the highest reported to date, when compared with previous studies (Tako *et al.*, 1996) that reported values under 500,000. In previous studies, these fucoïdan were extracted in acidic conditions using hydrochloric acid for the purpose of high yield. However, in the present study, fucoïdan were extracted in neutral conditions. These results show that fucoïdan prepared in the present study are closer to native fucoïdan in Mozuku. This indicates that we are able to estimate the biological activity of

**Table 1.** Components and yields of various polysaccharides.

Polysaccharides	M.w. <sup>a)</sup>	Content <sup>b)</sup> (%)	Sulfate <sup>c)</sup> (%)	Yield <sup>d)</sup> (%)
Fucoïdan from <i>F. vesiculosus</i>	104,000	84	18.8	-
Sodium alginate	700,000	94	0	-
Crude fucoïdan from <i>C. okamuranus</i>	3,080,000	65	5.6	1.30
Purified fucoïdan from <i>C. okamuranus</i>	3,190,000	94	9.8	0.99

a) Determined by HPSEC using pullulans as standards. b) Content of each polysaccharide was calculated using the rate of two peak areas detected at HPSEC. c) Sulfate content is expressed as a percentage of SO<sub>4</sub> content per polysaccharide weight. d) Based on wet algae.

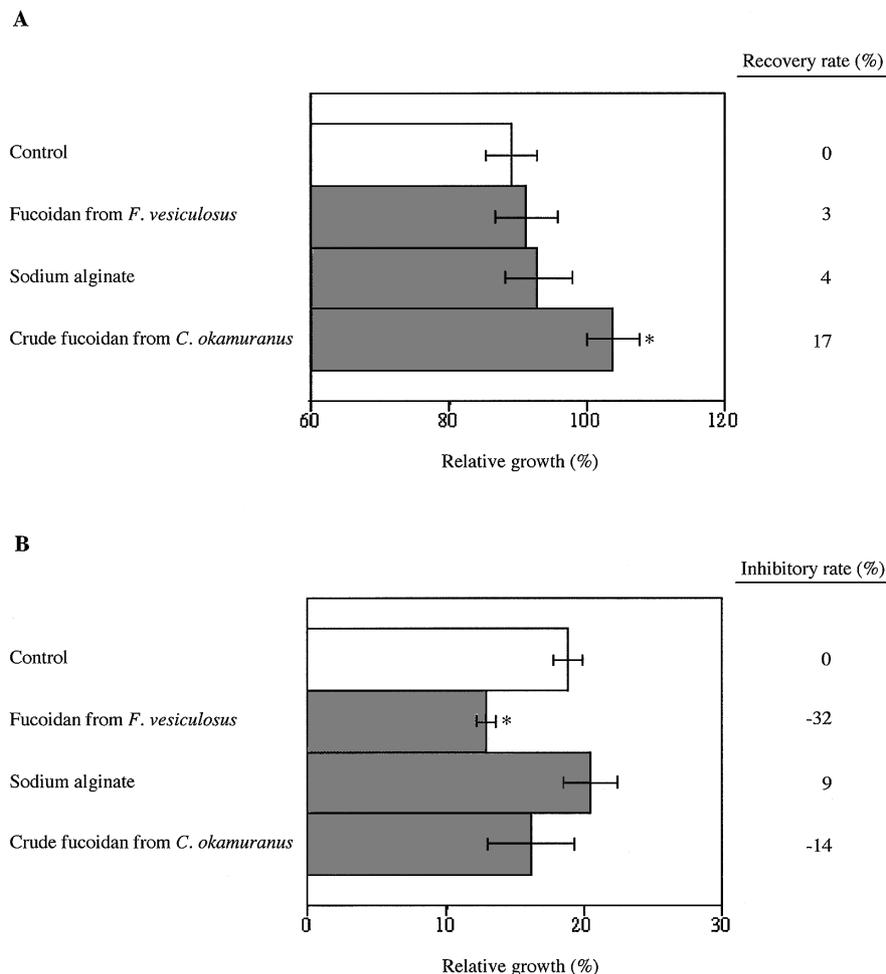
fucoidan in conditions similar to those in food.

**Effects of fucoidan on stomach cells in the presence of 5-FU** Initially, relative growth of Hs 677.st cells in the presence of both polysaccharides and 5-FU was compared to conditions with the addition of buffer as control. Relative growth of Hs 677.st cells in conditions with addition of buffer as control was approximately 90%, and relative growth in the presence of both 5-FU and fucoidan from *F. vesiculosus* or sodium alginate were almost identical. In addition, the recovery rate of fucoidan from *F. vesiculosus* or sodium alginate was only a single-digit% level. However, relative growth with both 5-FU and fucoidan from *C. okamuranus* was increased to 100% (Fig. 1A). Furthermore, the recovery rate of fucoidan from Mozuku was 17%. These results indicate that fucoidan from *C. okamuranus* reduced the damage of anticancer agent 5-FU in normal cells.

Subsequently, the relative growth of MKN45 stomach

cancer cells in the presence of polysaccharides was determined (Fig. 1B). Relative growth in the presence of both 5-FU and fucoidan from *F. vesiculosus* or *C. okamuranus* was slightly lower than relative growth with 5-FU only. The inhibitory rates of fucoidan from *F. vesiculosus* or *C. okamuranus* were -32% and -14%. These results indicate that fucoidan from *F. vesiculosus* or *C. okamuranus* hardly inhibited the original effect of anticancer agent 5-FU in cancer cells. Relative growth in the presence of both 5-FU and sodium alginate was similar to Relative growth with 5-FU only. An inhibitory rate of only 9% was observed.

These results indicate that fucoidan from *C. okamuranus* protect human stomach cells from growth inhibition by 5-FU. In other words, fucoidan from *C. okamuranus* inhibited the anticancer agent 5-FU in Hs 677.st normal stomach cells, but not in MKN45 stomach cancer cells. We suspect that high molecular weight fucoidan from *C.*



**Fig. 1.** Effect of polysaccharides on human stomach cell lines with 5-FU.

A: Hs 677.st, B: MKN45. 90  $\mu$ l culture solution of the 2,000 cells/well cells were in 96-well microplates. 10  $\mu$ l of each polysaccharide solution were added to microplate wells to a concentration of 1.0 mg/ml. After 4 h pre-incubation, 5-FU was added to the appropriate wells at 50  $\mu$ g/ml, and plates were incubated for 4 days at 37°C. Growth of each cell line was monitored using a modified MTT assay. The number of stomach cells grown in culture medium only is defined as 100% relative growth. PBS with 5-FU was used as a control (white bar) without any polysaccharide. Recovery rate was calculated according to the rate of increase in the number of cells for each polysaccharide compared to that of the control. Values represent the average  $\pm$ SD for relative growth (%). \* $P$ <0.01 compared with the control (Fisher, 1986).

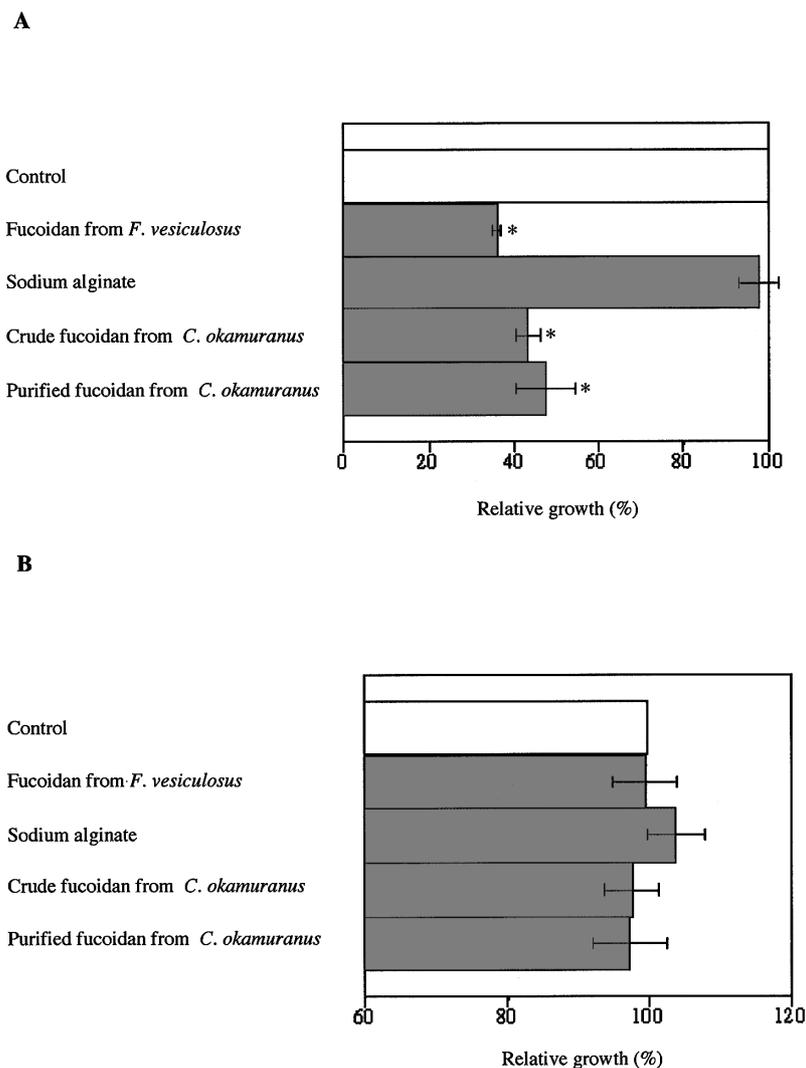
*okamuranus* interacts with some substances on the surface of stomach cells, and therefore protects the stomach cells. Mechanisms of the effects on stomach cells in the presence of the anticancer agent 5-FU may be related to mechanisms involved in the inhibition of fucoidan in the adhesion of *Helicobacter pylori* (Shibata *et al.*, 1999; Shibata *et al.*, 2000; Nagaoka *et al.*, 2000).

**Effects of fucoidan on stomach cells** To further assess the effects of fucoidan itself on stomach cancer cells, the relative growth of MKN45 cells in the presence of crude or purified fucoidan was examined (Fig. 2A). While sodium alginate did not show any effect on the growth of stomach cancer cells, the relative growth of cancer cells with fucoidan from *F. vesiculosus* was reduced to approximately 30%. Fucoidan from *F. vesiculosus* showed inhibitory effects on the growth of stomach cancer cells. The relative growth of MKN45 cancer cells in the presence of crude

fucoidan from *C. okamuranus* was reduced to approximately 40%. The relative growth of cancer cells with purified fucoidan from *C. okamuranus* was approximately 50%. In contrast, the relative growth of Hs 677.st cells in the presence of all fucoidan was similar to the control, indicating that fucoidan did not affect normal cell growth (Fig. 2B), but only affected cancer cell lines.

When we observed the morphological features of stomach cells treated with polysaccharides, MKN45 and Hs 677.st cells treated with all varieties of polysaccharides were similar to the control (data not shown).

These results indicated that fucoidan from *C. okamuranus* show growth inhibitory effects on stomach cancer cells. Many studies reported that fucoidan induce the apoptosis of cancer cells. However, apoptosis induction by fucoidan from Mozuku was not clearly observed in the present study. We suspect that growth inhibition by



**Fig. 2.** Effect of polysaccharides on human stomach cell lines without 5-FU.

A: MKN45, B: Hs 677.st. 90  $\mu$ l culture solution of the 2,000 cells/well cells were in 96-well microplates. 10  $\mu$ l of each polysaccharide solution were added to microplate wells to a concentration of 1.0 mg/ml. The plates were incubated for 4 days at 37°C and the growth of each cell line was monitored using a modified MTT assay. The number of stomach cells grown in culture medium only is defined as 100% relative growth. Values represent the average  $\pm$  SD for relative growth (%). \*  $P < 0.01$ , compared with the control (Fisher, 1986).

fucoidan from *C. okamuranus* on stomach cancer cells may relate not only to apoptosis induction but also to inhibitory effects of adhesion (Liu *et al.*, 2000) on stomach cancer cells.

We consider that fucoidan from *C. okamuranus* may be suitable for cancer therapy, and that it could be applied to inhibitory medicine, in order to treat side effects of 5-FU anticancer agent in the future. We expect that fucoidan from Mozuku could be developed into a health food, a medicine, and an applied product. Therefore, we anticipate further research using other anticancer reagents and cell lines.

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