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ORIGINAL PAPER

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Two fucoidans in the holdfast of cultivated *Laminaria japonica*

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Abstract Two fucoidans have been isolated from the holdfast of cultivated *Laminaira japonica*. One (L-fucoidan) is rich in fucose and sulfate; the other (GA-fucoidan) is rich in uronate. L-fucoidan was found in the fronds of *L. japonica* (cultivated and wild), *L. angustata*, and *Kjellmaniella crassifolia* whereas GA-fucoidan was not detected in these fronds and may be a fucoidan specific to the holdfast. These two fucoidans were proved to have anti-tumor activity against Adenocarcinoma 755-transplanted mice by i.p. and p.o. administration.

Keywords *Laminaira japonica* · Cultivated · Holdfast · Fucoidan · Anti-tumor activity

Introduction

Kombu, made from the frond of *Laminaria* sp., is a traditional foodstuff in Japan and has been used as a Chinese medicine to treat thyroid disease and hypertension. In China, the holdfast of *Laminaria japonica*, as "hai dai gen", is also used to treat hypertension and other diseases [1]. We have been studying the holdfast of cultivated *L. japonica* to evaluate its use it as a functional food resource. The holdfast of cultivated *L. japonica* is attached to rope, so is easy to collect on a large scale and it is free from rock and sand, unlike that of the wild plant. Our studies have revealed that the potassium content of the holdfast is remarkably high and that K/Na ratios for holdfasts are greater than those for fronds [2]. We have also reported the presence of oxygenated sterols derived from fucosterol and 24-methylenecholesterol, which are cytotoxic to MCF-7 breast cancer cells [3].

Fucoidans are a family of polysaccharides mainly composed of sulfated fucose [3], and are reported to have a variety of biological activity, for example antithrombotic [4, 5], anti-inflammatory [4], and anti-tumor [6–11] effects. In this paper we report the isolation, characterization, and anti-tumor activity of two fucoidans from the holdfast of cultivated *L. japonica*.

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Results and discussion

Two polysaccharide fractions were isolated from an acidic water extract of the holdfast of cultivated *L. japonica* by column chromatography on DEAE-Toyopearl 650 M. One was eluted with 0.5 mol L^{-1} NaCl (GA-fucoidan) the other with 1.0 mol L^{-1} NaCl (L-fucoidan). The molecular weights of these fucoidans were 79,000 and 221,000, respectively. The ^1H NMR spectra of these fucoidans contained signals from methyl groups and complicated signals from sugar moieties. The sugar composition, uronate content, and sulfated content of these two fucoidans are shown in Table 1. The fucose and sulfate content of GA-fucoidan (18.2 and 20.9%, respectively) were less than those of L-fucoidan (30.9 and 38.0%). The uronate content of GA-fucoidan was higher than that of L-fucoidan, and galactose, mannose, xylose, and glucose were detected in GA-fucoidan. Kitamura et al. [12] studied the sugar composition of a

Table 1 Chemical analysis of fucoidans in the holdfast of cultivated *Laminaria japonica*

Sample	Composition (%)[a]						
	Fucose	Galactose	Xylose	Mannose	Glucose	Uronic acid	SO_4^{2-}
L-fucoidan	30.9	3.3	–	–	–	1.2	38.0
GA-fucoidan	18.2	4.3	1.5	4.7	1.2	18.0	20.9

[a]Composition was estimated as percentage content on a dry-weight basis

fucoidan isolated from *L. angustata* var. longissima, and reported the ratio of fucose to galactose was 9:1; no other sugar was detected. The ratio of fucose to galactose in L-fucoidan was 9.36:1.00. The composition of L-fucoidan was similar to that of the fucoidan from *L. angustata* var. *longissima*. GA-fucoidan was shown to be rich in uronate and contained sugars other than fucose and galactose. There has been no report of a fucoidan corresponding to GA-fucoidan in *Laminaria* sp.

We, therefore, investigated the presence of GA-fucoidan in fronds of other *Laminaria* sp. and of *Kjellmaniella crassifolia*. As shown in Table 2, none of these samples contained a fucoidan corresponding to GA-fucoidan. GA-fucoidan might, therefore, be a polysaccharide specific to the holdfast (Table 2).

The anti-tumor activity of these fucoidans were tested using adenocarcinoma 755-transplanted mice. As shown in Tables 3 and 4, these fucoidans had anti-tumor activity after both intraperitoneal (i.p.) and oral (p.o.) administration.

After i.p. administration GA-fucoidans had dose-dependent anti-tumor activity and tumor weights were reduced to 35.1, 52.4 and 68.8% of control weights by doses of 10, 30, and 100 mg kg^{-1}, respectively. L-Fucoidan also reduced tumor weight by 68.8% after a dose of 30 mg kg^{-1}. Seven mice receiving 100 mg kg^{-1} L-fucoidan died on the second day of the experiment, however (Table 3), possibly as a result of over-administration of purified fucoidan. Reported doses of purified fucoidans in anti-tumor experiments are in the range 20–50 mg kg^{-1} [6–11].

Table 2 Comparison of the presence of fucoidans in *Laminanaira* sp. and *Kjellmaniella crassifolia*

	GA-fucoidan (%)[a]	L-fucoidan (%)
Laminaria japonica (cultivated)		
Holdfast	0.55	1.04
Frond	–[b]	0.98
Nekonbu[c]	–	1.42
Laminaria japonica (wild)		
Frond	–	1.37
Nekonbu	–	1.42
L. angustata		
Frond	–	0.53
Kjellmaniella crassifolia		
Frond	–	2.52

[a]Isolated yield on a dry-weight basis
[b]Not detected
[c]Nekombu is the commercial name of a product prepared from the lowest part of the frond

These two fucoidans also had dose-dependent anti-tumor activity after p.o. administration of doses of 30–300 mg kg^{-1}. The activity of the 100 mg kg^{-1} dose was almost the same as that of the 30 mg kg^{-1} dose after i.p. administration. The antitumor activity of these fucoidans after p.o. administration was almost one third that after i.p. administration. The anti-tumor activity of L- and GA-fucoidan was almost the same as in previous reports [6–11].

Takahashi et al. [13] studied the anti-tumor activity of crude fucoidan from *Eisenia bicyclis* after p.o. administration and reported that the activity might depend on activation of the reticuloendothelial system, which increased NK activity. Noda et al., using Ehrlich carcinoma and p.o. administration, reported anti-tumor effects of fucoidan fractions from *Undaria pinnatifida* and *Sargassum ringgoldianum*, among 17 kinds of polysaccharide preparation from algae [8]. Fucoidan fractions from *Sargassum thumbergii* also had anti-tumor activity after i.p. administration [10]. Fucoidan extracted from sporophyll of *U. pinnatifida* has recently been shown to have anti-tumor activity against P-388 leukemia after i.p. administration; it was suggested the activity was related to NK cell activation by IFN-γ [11]. The anti-tumor activity of fucoidans from the holdfast of *L. japonica* might be because of these effects.

As mentioned above, GA-fucoidan was shown to be a specific fucoidan in the holdfast of cultivated *L. japonica* with anti-tumor activity.

Experimental

Materials and chemicals

Holdfasts of cultivated *L. japonica* and other brown algae were obtained from Minamikayabe, Hokkaido, in August 2001. All chemicals were obtained from Wako Pure Chemicals (Osaka, Japan).

General

GPC-HPLC was performed with an Hitachi L-6100 pump and a Shodex RI-71 RI detector. Polysaccharides were separated on an Asahipak GS-520HQ column (Showa Denko, Tokyo, Japan), at 60°C, with 0.3 mol L^{-1} $NaNO_3$ as mobile phase at a flow rate of 0.8 mL min^{-1}. Sugar composition was determined by

Table 3 Antitumor effect of fucoidans from the holdfast of *L. japonica* against adenocarcinoma-transplanted mice after intraperitoneal administration

Sample	Dose (mg kg^{-1})	Average tumor wet weight (mg)	Inhibition ratio (%)	Toxicity or death
Control	–	2774 ± 629	–	0/8
L-fucoidan	10	1958 ± 533	29.6*	0/8
	30	837 ± 239	69.8**	0/8
	100	–	–	7/8
GA-fucoidan	10	1799 ± 337	35.1*	0/8
	30	1320 ± 350	52.4**	0/8
	100	867 ± 131	68.8**	0/8

Animals: BDF_1 mice; vehicle: sterilized distilled water
Significantly different from control—at least $^*P < 0.05$ and $^{**}P < 0.01$

Table 4 Antitumor effect of fucoidans from the holdfast of *L. japonica* against adenocarcinoma-transplanted mice after oral administration

Sample	Dose (mg kg^{-1})	Average tumor wet weight (mg)	Inhibition ratio (%)	Toxicity or death
Control	–	4,633 ± 189	–	0/8
L-fucoidan	30	2,636 ± 469	43.1**	0/8
	100	1,865 ± 250	59.7**	0/8
	300	1,513 ± 334	67.3**	0/8
GA-fucoidan	30	3,215 ± 531	30.6*	0/8
	100	2,523 ± 476	45.5**	0/8
	300	1,332 ± 225	71.2**	0/8

Animals: BDF_1 mice; vehicle: sterilized distilled water
Significantly different from control—at least $^*P < 0.05$ and $^{**}P < 0.01$

use of the same equipment and a Shodex NH2P-50 column (Showa Denko), at 30°C, with 75% CH_3CN as mobile phase at a flow rate of 1.0 mL min^{-1}.

Preparation of crude fucoidan fraction

Pulverized holdfast of cultivated *L. japonica* (2.5 kg) was extracted, at 75°C, with 3×25 L distilled water adjusted pH 3.0 by addition of 2.0 mol L^{-1} HCl, each time for 5 h. The combined extract was concentrated to approximately one-tenth volume and twice the final volume of ethanol was added to the solution. The resulting precipitate was collected by centrifugation (3,000 rpm, 20 min), dissolved in distilled water, and the pH of the solution was adjusted to 2.0 with 2.0 mol L^{-1} HCl. The solution was left for 1 h and the insoluble material was then removed by vacuum filtration. The filtrate was concentrated to one-tenth volume and twice the final volume of ethanol was added. The precipitate collected by centrifugation (3,000 rpm, 20 min) was washed with ethanol and was dried at 60°C for 24 h to furnish crude fucoidan fraction (56.7 g).

Purification of fucoidans

The crude fucoidan fraction (3.15 g) was dissolved in distilled water (100 mL) and the solution was applied to a 5.0 cm i.d.×60 cm DEAE-Toyopearl 650 M column (Tosoh, Tokyo, Japan). The column was eluted, in sequence, with distilled water (1,000 mL), 0.2 mol L^{-1} NaCl (700 mL), 0.5 mol L^{-1} NaCl (900 mL), and 1.0 mol L^{-1} NaCl (700 mL) and the eluate was collected in 20-mL fractions. The fractions were analyzed by GPC-HPLC to detect polysaccharides. One polysaccharide, GA-fucoidan, was eluted with 0.5 mol L^{-1} NaCl; another, L-fucoidan, was eluted with 1.0 mol L^{-1} NaCl. Both fucoidans were eluted as single peaks. The fractions containing these two fucoidans were separately combined and concentrated to one-tenth volume. The concentrates were dialyzed three times, against distilled water, using a Spectra/por membrane (MWCO 1000; Spectrum Laboratories, CA, USA). The non-dialyzed parts were concentrated and lyophilized to furnish GA-fucoidan (1.09 g) and L-fucoidan (2.06 g). The molecular weights of these fucoidans were determined by use of the HPLC method reported elsewhere [14].

Chemical analysis of fucoidans

Acid hydrolysis of each fucoidan was performed with sulfuric acid at 110°C for 1 h in an autoclave. Neutral sugars in the acid hydrolysate were determined by HPLC on a Shodex NH2P-50 column. The total inorganic sulfate liberated by HCl hydrolysis was determined by the Dodgson method [15]. Total uronic acid was determined by the carbazole–sulfuric acid method, using glucuronic acid as standard.

Anti-tumor activity

Adenocarcinoma 755 (1×10^5 cells/mouse) was subcutaneously transplanted into ventral subcutaneous tissue of 6-week-old BDF1 mice. Each fucoidan was dissolved in sterilized water and administered i.p. or p.o. for 14 days. Doses were 10, 30, and 100 mg kg^{-1} for i.p. administration, and 30, 100 and 300 mg kg^{-1} for p.o. administration, and administration was started 24 h after transplantation. Control mice received sterilized water, and eight mice were tested in each group. All the mice were sacrificed 24 h after final administration and anti-tumor activity was evaluated by determination of the decrease of tumor weight, compared with that of the control group.

Statistical analysis

Data are expressed as the mean ± S.E.M. and were analyzed by one-way ANOVA followed by Duncan's post hoc test using SPSS. The difference considered statistically significant was $P < 0.05$.

References

1. Chuyaku Daijiten (1985) Shanhai Kagaku-gijutsu shuppansha and Shogakkan, pp 218–219
2. Funaki M, Nishizawa M, Sawaya T, Inoue S, Yamagishi T (2001) Mineral composition in the holdfast of three algae of the genus *Laminaria*. Fish Sci 67:265–300
3. Nishizawa M, Takahashi N, Shimozawa K, Aoyama T, Jinbow K, Noguchi Y, Horita K, Bando H, Yamagishi T (2003) Cytotoxic constituents in the holdfast of cultivated *Laminaria japonica*. Fish Sci 69:639–643
4. Berteau O, Mulloy B (2003) Sulfated fucans, fresh perspective: structure, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. Glycobiology 13:29R–40R
5. Nishino T, Nagumo T (1991) Structure characterization of a new anticoagulant fucan sulfate from the brown seaweed *Ecklonia kurome*. Carbohydr Res 211:77–90
6. Usui T, Asari K, Mizuno T (1980) Isolation of highly purified "fucoidan" from *Eisenia bicyclis* and its anticoagulant and antitumor activities. Agric Biol Chem 44:1965–1966
7. Yamamoto I, Takahashi M, Tamura E, Murayama H, Mori H (1984) Antitumor activity of edible marine algae: Effect of crude fucoidan fractions prepared from edible brown seaweeds against L1210 leukemia. Hydrobiologia 116/117:145–148
8. Noda H, Amano H, Arashima K, Hashimoto S, Nisizawa K (1989) Antitumor activity of polysaccharides and lipids from marine algae. Nippon Suisan Gakkai Shi 55:1265–1271
9. Nod H, Amano H, Arashima K, Nisizawa K (1990) Antitumor activity of marine algae. Hydrobiologia 204/205:577–584
10. Zhung C, Itoh H, Mizuno T, Ito H (1995) Antitumor active fucoidan from the brown seaweed, umitoranoo (*Sargassum thunbergii*). Biosci Biotech Biochem 59:563–567
11. Maruyama H, Tamauchi H, Hashimoto M, Nakano T (2003) Antitumor activity and immune response of mekabu fucoidan extracted from sporophylla of *Undaria pinnatifida*. In vitro 17:245–250
12. Kitamura K, Matsuo M, Yasui T (1991) Fucoidan from brown seaweed *Laminaria angustata* var. *longissima*. Agric Biol Chem 55:615–616
13. Takahashi M (1983) Studies on the mechanism of host-mediated antitumor action of crude fucoidan from a brown alga *Eisenia bicylis* (in Japanese). J Jpn Soc Reticuloendothel Sys 22:269–283
14. Shinohara M, Kamono H, Aoyama T, Bando H, Nishizawa M (1999) Relationships between guluronate contents in alginates determined by ^{1}H-NMR spectroscopy ant their average molecular weights. Fish Sci 65:909–913
15. Dodgson KS (1961) Determination of inorganic sulphate in studies on the enzymic and non-enzymatic hydrolysis of carbohydrate and other sulphate esters. Biochem J 78:312–318