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Composition and cytotoxicity of a novel polysaccharide from brown alga (*Laminaria japonica*)

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ABSTRACT

A novel polysaccharide WPS-2-1, with an average molecular weight of 80 kDa, was purified from aqueous extracts of *Laminaria japonica*. Monosaccharides analysis revealed that WPS-2-1 was composed of mannose, rhamnose and fucose with a molar ratio of 1.0:2.3:1.2. Analysis by periodate oxidation-Smith degradation indicated that WPS-2-1 had a backbone of array by (1 → 4)-glycosidic linkages. Cytotoxicity assay showed that WPS-2-1 presented significantly higher antitumor activities against A375 and BGC823 cells with a dose-dependent manner, and exhibited lower cytotoxicity to vascular smooth muscle cells. The results suggested that WPS-2-1 should be explored as a potential antitumor agent with low toxicity.

1. Introduction

For thousands of years, seaweeds have been valued as an edible and health-enhancing resource in oriental countries like China, Japan and Korea. Seaweeds contain high amounts of essential minerals, vitamins, dietary fibers and polysaccharides (Christine, Rainer, & Gerhard, 2007). Furthermore, many polysaccharides isolated from seaweeds have attracted a great deal of attention in the biomedical field because of their diverse biological activities, including anticoagulant activities (Athukorala, Lee, Kim, & Jeon, 2007), antioxidant effects (Qi et al., 2005), antitumor activities (Sheng et al., 2007; Ye, Wang, Zhou, Liu, & Zeng, 2008) and anti-inflammatory properties (Kanga et al., 2011; Subash et al., 2010), antiviral activities (Karmakar, Pujolb, Damonte, Ghosh, & Ray, 2010). Therefore, seaweed polysaccharides have been exploited as the functional foods and sources of new pharmaceutical products.

Laminaria japonica, a member of brown alga, is the most important economic seaweed cultured in China. In traditional Chinese medicine, it has been documented as a drug for over a thousand years. Recent studies demonstrated that polysaccharides isolated from *L. japonica* could protect endogenous antioxidant enzymes (Zhao et al., 2004), inhibit platelet aggregation (Zhao et al., 2011), and scavenge free radicals (Wang, Zhang, Zhang, & Li, 2008). Studies on the relationship between structure and activity of *L. japonica* polysaccharides suggested that several parameters were associated with their biological functions, such as molecular weight, degree of sulfation, glycoside branching, monosaccharide composition and type of sugar (Melo, Pereira, Foguel, & Mourao, 2004; Patankar, Oehninger, Barnett, Williams, & Clark, 1993). However, to date, no investigation has been reported about the cytotoxicity of polysaccharides from *L. japoniaca.* Therefore, the aim of the present investigation was to characterize the purified water-soluble polysaccharide from *L. japonica* and evaluate the cytotoxicity of polysaccharide on both carcinomatous and normal cells in vitro.

2. Materials and methods

2.1. Materials and reagents

Dried *L. japonica* was obtained from Fuzhou, Fujian province of China.

The human gastric carcinoma cell line BGC823, human melanoma cell line A375 and aortic vascular smooth muscle cells (VSMCs) were obtained from College of Biological Science and Technology of Fuzhou University. Dulbecco's Modified Eagle's Medium (DMEM, low glucose) and RPMI1640 medium were purchased from GIBCO-BRL (Grand Island, New York, USA). Fetal bovine serum (FBS) was purchased from Sangong (Shanghai, China). Dimethylsulphoxide (DMSO), 3-methyl-1-phenyl-2-pyrazole-5-one (PMP)

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and T-series dextrans of different molecular weights were purchased from Sigma. (St. Louis, USA). Thiazolyl blue (MTT) was purchased from Fluka Chemie (Buchs, Switzerland). All chemicals used in this study have a purity of 95% or greater.

2.2. Extraction and purification polysaccharide

The powdered *L. japonica* (100.0 g) was defatted with anhydrous ethanol at 60 °C for 3 h under stirring, then dipped in 5 L distilled water at 100 °C for 2 times and 3 h for each time. After centrifugation, the supernatant was concentrated to a quarter of the original volume by evaporation and precipitated with 20% ethanol containing 0.2% $CaCl_2$ at 4 °C overnight. After separating the precipitate by filtrating, the ethanol was added to the supernatant to yield a 75% ethanol solution, and the suspension was kept at 4 °C overnight. The precipitate was the crude polysaccharide, named as WPS.

The WPS was dissolved in Tris–HCl (20 mmol/L, pH 7.4) buffer and loaded onto a DEAE-A25 chromatography column (26 mm × 300 mm) equilibrated with Tris–HCl buffer. After loading with sample, the column was eluted with 0–2.0 mol/L NaCl in a successive manner. The fractions were detected by the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebes, & Smith, 1956), using glucose as standard. Three fractions were collected and the second fraction (WPS-2) was further purified by anion-exchange chromatography on a column of DEAE-A25 (16 mm × 200 mm), followed by gradient elution with 0.7 and 0.8 mol/L NaCl. The main fraction was collected and then dialyzed. Finally, a purified polysaccharide named WPS-2-1 was obtained by lyophilization.

2.3. Characterization of polysaccharide fraction

2.3.1. Measurement of carbohydrate and protein contents

Total carbohydrate content of the polysaccharide was determined by phenol-sulfuric acid colorimetric method. Protein in the polysaccharide was quantified according to the Bradford's method using bovine serum albumin (BSA) as the standard (Bradford, 1976). Sulfate content was measured by barium chloride-gelatin method (Kawai, Seno, & Anno, 1969).

2.3.2. Infrared analysis

The IR spectra of the polysaccharides were determined using a Fourier transform infrared spectroscopy (FT-IR) spectrophotometer. The purified polysaccharide of WPS-2-1 was ground with KBr powder and then pressed into pellets for FT-IR measurement in the frequency range of 4000–500 cm^{-1} (Kumar, Joo, Choi, Koo, & Chang, 2004).

2.3.3. Homogeneity and molecular weight

The homogeneity and average molecular weight of WPS-2-1 were identified by high performance gel permeation chromatography (HPGPC). The sample solution was applied to Agilent 1100 HPLC system equipped with a TSK-GEL G3000 PWXL column (7.8 mm × 300 mm), eluted with distilled water at a flow rate of 0.5 mL/min. Peaks were detected by ELSD800 Detector (Alltech, USA). The molecular weight was estimated by reference to the calibration curve made from a Dextran T-series standard of known molecular weight (150,000, 70,000, 3500, 1500 Da).

2.3.4. Analysis of monosaccharide composition

The polysaccharides of WPS, WPS-2 and WPS-2-1 were hydrolyzed with 150 μL of 2 mol/L trifluoroacetic acid (TFA) at 110 °C for 4 h. After removing the residual acid, the standard sugars and hydrolysates were pre-column derivated with PMP at 70 °C for 100 min. The resulting solutions were extracted with 1.0 mL chloroform for 3 times. Then the aqueous layer was subjected to high performance liquid chromatography (HPLC) fitted with RP-C_{18} column.

2.4. Periodate oxidation and Smith degradation

WPS-2-1 (25 mg) dissolved in 12.5 mL of distilled water was mixed with 12.5 mL of 30 mmol/L $NaIO_4$, and the mixture was kept in the dark at 4 °C. About 0.1 mL aliquots were withdrawn from the mixture at every 6 h intervals, diluted to 25 mL with distilled water and read in a spectrophotometer at 223 nm. The reaction stopped when absorbency did not descend. Ethylene glycol (2.0 mL) was added to terminate the reaction. About 2.0 mL periodate-oxidized product was used to calculate the yield of formic acid by 0.007 mol/L sodium hydroxide, and the rest was extensively dialyzed against tap water and distilled water for 24 h, respectively. The content inside was concentrated and reduced with $NaBH_4$, neutralized with 50% acetic acid, and dialyzed as described above. One-third of solution mentioned above was freeze-dried and fully hydrolyzed for GC analysis, others were added to the same volume of 1.0 mol/L sulfuric acid, kept for 40 h at 25 °C, neutralized to pH 6.0 with $BaCO_3$, and filtered. The filtrate was dialyzed, and the content out of sack was desiccated for GC analysis. The content inside was diluted with ethanol, and the supernatant and precipitate were also dried out for GC analysis after centrifugation.

2.5. Cytotoxicity assay

The cytotoxicity of polysaccharides (WPS, WPS-2 and WPS-2-1) on A375, BGC823 cells and VSMCs cells were evaluated in vitro by MTT assay. Briefly, cells (1×10^5 cells/mL) were incubated in 96-well plates. Each well was incubated with 100 μL culture medium containing 10% FBS at 37 °C in a humidified atmosphere with 5% CO_2. After 24 h, cells were treated with different concentrations of polysaccharides (0.03125, 0.125, 0.5 and 2.0 mg/mL). The wells were further incubated for 20 h at 37 °C. Then 5 mg/mL MTT stock solution (100 μg/well) was added and cells were incubated for another 4 h. After removing the medium, 100 μL DMSO were added to terminate reaction. The amount of purple formazan was determined by measuring the absorbance at 578 nm. For treated cells, cytotoxicity was expressed as follow:

$$\text{Growth inhibition rate}\,(\%) = \left(1 - \frac{A_{\text{treated}}}{A_{\text{negative control}}}\right) \times 100$$

where A_{treated} is the absorbance of cells with sample treated, $A_{\text{negative control}}$ is the absorbance of cells without sample treated.

2.6. Statistical analysis

The data were presented as means ± SD of six determinations. Statistical analyses were performed using Student's *t*-test and one way analysis of variance. Multiple comparisons of means were done by the least significance difference test. All computations were done by employing the statistical software (SAS, version 8.0).

3. Results and discussion

3.1. Isolation and purification of polysaccharide fractions

The water-soluble crude polysaccharide (WPS) was isolated from *L. japonica* by hot water extraction, ethanol precipitation and lyophilization. The final yield of WPS was about 2.5% of the dried material. The WPS was purified by anion-exchange chromatography with a successive elution manner. Three main fractions of WPS were obtained from NaCl eluent, namely WPS-1, WPS-2 and WPS-3 (Fig. 1a). Antitumor activities against A375 cells of these three

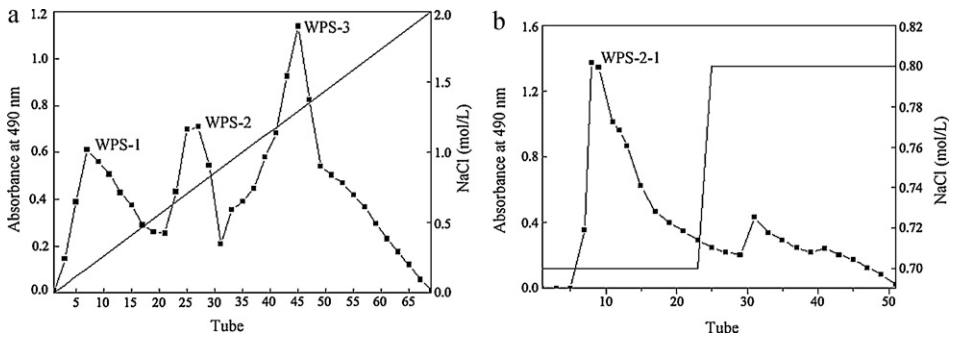

Fig. 1. Elution profiles of *Laminaria japonica* polysaccharide by DEAE-A25 anion-exchange chromatograph. (a) Elution profiles of WPS on DEAE-A25 anion exchange chromatography (26 mm × 300 mm) and (b) elution profiles of WPS-2 on DEAE-A25 anion-exchange chromatography (16 mm × 200 mm).

fractions were evaluated and the fraction of WPS-2 showed higher antitumor activity. Therefore, the WPS-2 was further purified on DEAE-A25 column in a stepwise manner and the main fraction of WPS-2 was obtained from 0.7 mol/L NaCl eluent, namely WPS-2-1, for further analysis of chemical composition and cytotoxicity (Fig. 1b).

3.2. Characterization of polysaccharide WPS-2-1

The WPS-2-1 had a negative response to the Bradford's method, indicating the absence of protein. The total carbohydrate content and sulfate content in WPS-2-1 were 93.5% and 13.9%, respectively (Table 1). The average molecular weight of WPS-2-1 was determined by HPGPC. According to the calibration curve with standard dextran, the average molecular weight of WPS-2-1 was calculated as 80 kDa. The HPGPC profile also suggested that WPS-2-1 had a single symmetrical peak, revealing that WPS-2-1 was a homogeneous polysaccharide.

3.3. Monosaccharide analysis

According to HPLC analysis, fucose was detected in WPS, WPS-2 and WPS-2-1 (Table 1). WPS-2-1 primarily consisted of mannose, rhamnose and fucose with a molar ratio of 1.0:2.3:1.2. In particular, rhamnose and mannose were the predominant monosaccharides of WPS-2-1, accounting for 36.4% and 15.7%, respectively. Compared with WPS-2-1, WPS-2 and WPS had high content of galactose and low contents of both mannose and rhamnose. Generally, the biological activities of polysaccharide would be related to mannose and rhamnose contents (Andres et al., 2006; Lee, Cho, & Hong, 2009).

3.4. Structural analysis

The characteristic absorption of WPS-2-1 was identified by the FT-IR spectra (Fig. 2). The infrared spectrum of WPS-2-1 displayed a broad stretching intense characteristic peak at 3354 cm^{-1} for the

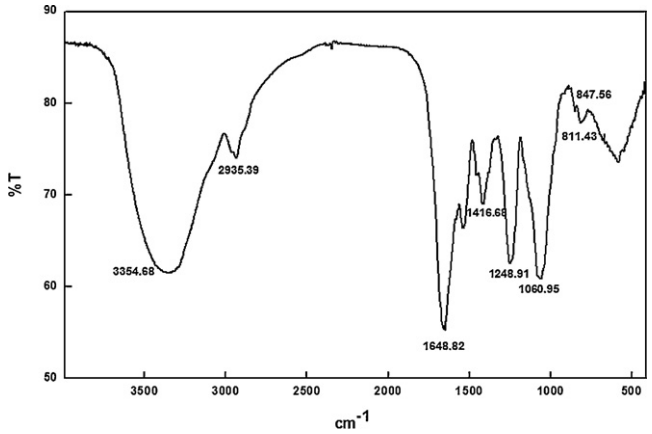

Fig. 2. FT-IR spectra of WPS-2-1.

hydroxyl group. A weak band at 2935 cm^{-1} was attributed to the C—H stretching and bending vibration. The absorption band centered at 1648.82 cm^{-1} was caused by C=O asymmetric stretching vibration. A characteristic band at 847.56 cm^{-1} was due to α-type glycosidic linkages. The peak at 811.4 cm^{-1} was the characteristic of mannan, which had been demonstrated in monosaccharide analysis. An intense band at 1248.91 cm^{-1} assigned to sulfate esters, which were in accordance with the elution curve on DEAE-A25 anion-exchange column, indicating that WPS-2-1 was a negatively charged polysaccharide.

Results of periodate oxidation of WPS-2-1 showed that $NaIO_4$ consumption was 2.96 mol/mol sugar residue, then formic acid production was 0.0245 mol/mol sugar residue. The consumption of $NaIO_4$ was more than double of formic acid production, indicating that there were 1→ and 1 → 6 linkaged glycosidic bonds existed in WPS-2-1. The periodate-oxidized product was hydrolyzed and analyzed by GC chromatography. The existence of large amounts of glycerol revealed that sugar residues were not (1 → 3)-linked

Table 1
Chemical composition of polysaccharides.

	Carbohydrate (%)	Sulfated (%)	Contents of the sugar residues (%)						
			Man [a]	Rha [b]	Glu [c]	Gal [d]	Xyl [e]	Fuc [f]	Unknown
WPS-2-1	93.5	13.9	15.7	36.4	3.9	8.3	8.4	18.4	8.9
WPS-2	50.3	12.7	10.2	20.0	nd	20.9	nd	45.1	3.8
WPS	23.4	12.5	2.56	2.97	5.10	56.25	6.04	25.74	1.32

nd, not detectable below the limit at 0.01.
[a] Mannose.
[b] Rhamnose.
[c] Glucose.
[d] Galactose.
[e] Xylose.
[f] Fucose.

Table 2
GC analysis results from fractions of Smith degradation of WPS-2-1.

Farction	Glu [c]	Gly [d]	Ery [e]	Man [f]
Full acid hydrolysis	–[a]	+[b]	–[a]	–[a]
Smith degradation				
Out of sack	–[a]	+[b]	+[b]	–[a]
Precipitation in the sack	–[a]	–[a]	–[a]	–[a]
Supernatant in the sack	–[a]	–[a]	–[a]	–[a]

[a] Undetectable.
[b] Detectable.
[c] Glucose.
[d] Glycerol.
[e] Erythritol.
[f] Mann.

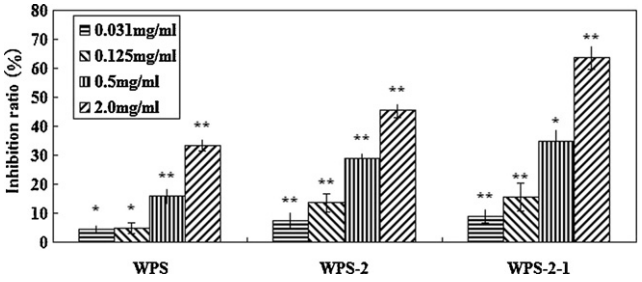

Fig. 3. Inhibition of proliferation of A375 cells by WPS, WPS-2 and WPS-2-1 at different concentrations. Values are mean ± SD of six separated experiments. Significant differences compared to control group are designated as $^{*}p < 0.05$ and $^{**}p < 0.01$.

and (1 → 2)-linked that could not be oxidized. As mannose was not observed in the hydrolyzed products, it indicated that mannose could be all linkages which could be oxidized by periodate.

The product of periodate oxidation was degraded and analyzed by GC chromatography for more information about glycosidic linkage location. Results of Smith degradation analysis of WPS-2-1 were summarized in Table 2. There was no precipitation in dialysis bag, which demonstrated that the backbone of WPS-2-1 could be completely degraded by HIO_4. The presence of glycerol and erythritol out of dialysis bag indicated that sugar residues were (1 → 2)-linked or (1 → 4)-linked. According to the results above, the linkages of backbone could be the arrays by (1 → 4)-glycosidic linkages.

3.5. Cytotoxicity of L. japonica polysaccharides

The antitumor activities of WPS, WPS-2 and WPS-2-1 were shown in Figs. 3 and 4. All polysaccharides presented higher growth inhibition than did blank control groups, and the inhibition abilities were dose-dependent. The growth of A375 cells could be inhibited by WPS, WPS-2 and WPS-2-1 at a concentration as low as 0.031 mg/mL, and the inhibition ratios were 4.45%, 7.53%

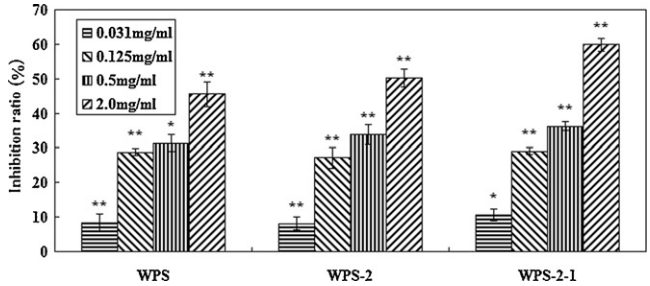

Fig. 4. Inhibition of proliferation of BGC823 cells by WPS, WPS-2 and WPS-2-1 at different concentrations. Values are mean ± SD of six separated experiments. Significant differences compared to control group are designated as $^{*}p < 0.05$ and $^{**}p < 0.01$.

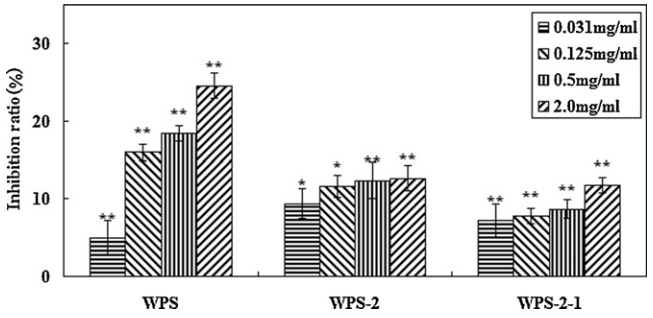

Fig. 5. Inhibition of proliferation of VSMCs by WPS, WPS-2 and WPS-2-1 at different concentrations. Values are mean ± SD of six separated experiments. Significant differences compared to control group are designated as $^{*}p < 0.05$ and $^{**}p < 0.01$.

and 8.91%, respectively (Fig. 3). In particular, WPS-2-1 exhibited strong inhibition ratio of more than 60% at the concentration of 2.0 mg/mL, significantly higher than that of WPS and WPS-2 (33.51% and 45.38%). Fig. 4 demonstrated antitumor activities of all polysaccharides against BGC823 cells. Similarly, WPS-2-1 had more obviously inhibitory proliferation activity on BGC823 cells than WPS and WPS-2, and could significantly inhibit cells growth in a dose-dependent manner. With the concentration increase from 0.031 to 2.0 mg/mL, the inhibition ratios of WPS-2-1 on BGC823 were significantly enhanced from 10.62% to 59.82%. The results suggested that WPS-2-1 could be a potential antitumor drug.

In vitro inhibition ratios of VSMCs growth by WPS, WPS-2 and WPS-2-1 at different concentrations were shown in Fig. 5. WPS-2-1 and WPS-2 showed low inhibition against the proliferation of VSMCs. The inhibition ratios of WPS-2 and WPS-2-1 on VSMCs at the concentrations from 0.031 to 2.0 mg/mL were no more than 13%. On the contrary, WPS exhibited relatively higher growth inhibition against VSMCs. At the concentration of 0.125 mg/mL, the inhibition ratio of WPS on VSMCs had reached 15.95%. And the highest inhibition ratio of WPS on VSMCs was 24.54% at the concentration of 2.0 mg/mL. The results showed that WPS-2 and its purified fraction of WPS-2-1 had selective cytotoxicity. The proliferation of A375 and BGC823 cells could be effectively inhibited by purified fraction, at the same time little obvious anti-proferative effect on VSMCs was observed. The results showed that WPS-2-1 had no negative side-effects and a relatively lower cytotoxicity on human normal cells.

Several investigators have found that some structural features are required for biological activities of polysaccharides, such as degree of sulfation, molecular weight, compositions, glycosidic linkages and type of sugar (Melo et al., 2004). The foregoing differences in cytotoxicity assay among polysaccharides were probably due to their different monosaccharide compositions. Compared with monosaccharide compositions of WPS and WPS-2, WPS-2-1 showed relatively high mannose and rhamnose contents, suggesting that high antitumor activities of WPS-2-1 were positively correlated with the amounts of mannose and rhamnose. Although the sulfate contents of all polysaccharides were similar, higher antitumor activities and lower cytotoxicity on VSMCs of WPS-2-1 were observed. Possibly, the presence of major sulfate monosaccharides units at the non-reducing terminal of branches found in WPS-2-1 induced to interaction with polysaccharide and various cells. A similar observation was reported for sulfated polysaccharides from tropical seaweeds (Costa et al., 2010). The results presented above indicated that the biological activities of purified polysaccharide fraction WPS-2-1 should be associated with monosaccharide compositions and sulfated position.

4. Conclusions

This study demonstrated that WPS-2-1 purified from *L. japonica* was a homogeneous polysaccharide with an average molecular weight of 80 kDa. Chemical analysis indicated that it was composed of mannose, rhamnose and fucose. The main skeleton of WPS-2-1 was α-(1 $\rightarrow$ 4)-glycosidic linkages. Cytotoxicity assay showed that WPS-2-1 exhibited significant antitumor activities against BGC823 and A375 carcinoma cells in vitro. No antiproliferative effects of WPS-2 and its purified fraction of WPS-2-1 on VSMCs were observed, implying that these polysaccharides had no direct cytotoxicity to non-cancer cells. The results suggested that WPS-2-1 could be considered as a potential candidate for antitumor agent. The correlation between structural and antitumor activity of WPS-2-1 would be further elucidated in future work.

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