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Chromatographically Purified Porphyran from *Porphyra yezoensis* Effectively Inhibits Proliferation of Human Cancer Cells

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Abstract In this study, we isolated porphyran was isolated from the red seaweed *Porphyra yezoensis* and assessed in terms of *in vitro* anti-proliferative activity. Sequential anion-exchange and gel-filtration chromatography led to purification of 3 porphyrans of different molecular masses, which contained <50 μg/mL protein and >10 μg/mL porphyran. Crude porphyran inhibited cell growth in a dosĕ-dependent manner (0-5 mg/mL). When HT-29 colon cancer cells and AGS gastric cancer cells were cultured with various concentrations of the purified porphyran, cancer cell growth was inhibited by 50% at a low concentration (5 or 10 μg/mL). Furthermore, the polysaccharide portion of the porphyran preparation, rather than the protein portion, is the most effective at inhibiting cancer cell proliferation via apoptosis, as indicated by increased caspase-3 activity. Our results indicate that purified porphyran has significant *in vitro* anti-proliferative activity ($p<0.05$).

Keywords: porphyran, anion-exchange, gel filteration chromatography, anti-proliferative activity, *porphyra yezoensis*

Introduction

Large amounts of fresh or dried seaweed are consumed as part of the typical Asian diet. A number of studies have shown that seaweeds have significant nutritional value and other beneficial properties. Certain edible seaweeds contain abundant amounts of essential proteins, vitamins, and minerals. In addition, dietary seaweed has been reported to decrease serum and liver triglyceride concentrations in rats, suggesting that it may be useful for the prevention and treatment of hypertriglycerolemia (1, 2). Furthermore, seaweed polysaccharides exhibit diverse biological activities that include effects on the immune system and on cancer cells (3-6). Yamamoto *et al.* (7, 8) reported that the oral administration of several varieties of seaweed significantly decreased the incidence of carcinogenesis *in vivo*.

Porphyran and agarose are closely related, but only porphyran has galactose-6-sulfate moieties (9). Porphyran mainly contains about 40-50% carbohydrates, 25-35% proteins, lipids, and some vitamins also. It is therefore thought to be rich in dietary fiber. Because porphyran is a major dietary fiber that usually constitutes nearly 40-50% of the total seaweed components, the nutritional and physiological functions of porphyrans isolated from different *Porphyra* species have been examined in many structural and functional studies. Using agarase I, Morrice *et al.* (9) showed that the primary structure of porphyran consists of alternating 1,4-linked 3,6-anhydro-L-galactose units and 1,3-linked β-D-galactose with and without methylation. They have also reported that a high molecular porphyran remained after agarase treatment, suggesting that it is difficult to make a low molecular weight porphyran as well as to make pure porphyran without proteins. Matsuo *et al.* (10) found that desulfation of saccharide-6-sulfate results in porphyran gelation, suggesting that porphyran might function as a new polysaccharide surfactant. Kayama *et al.* (11) reported that dried, low-quality *nori*, inexpensive seaweed of no practical value, has high carbohydrate content, and thus high porphyran content. These reports show that porphyran can easily be extracted with hot water, or with alkaline or acid treatment (12). However, the structures of basic porphyran might be changed to unexpected products by such treatment. Therefore, several researchers have found agarases produced by various marine microorganisms (13-18) since porphyran is hard to absorb and tends not to affect physiological functions of the body such as prebiotic activity (19), antitumor (5, 6) or antihyperlipidemic activity (20), but applications have not been reported in detail. Therefore, many studies have also reported the development of a key purification procedure that includes the application of proteinase and agarase. Hence, a simple procedure is needed to prepare more effective porphyran. In particular, our concern is how to reduce the amount of protein, a common contaminant in porphyran preparations. In the present study, we prepared crude porphyran (CP) from hot water extract and isolated 3 different chromatographic fractions of purified porphyran which contained <50 μg/mL protein and >10 μg/mL porphyran. In addition, we found it had more effective anti-proliferative activity in carcinoma cells.

Materials and Methods

Materials RPMI 1640 medium, fetal bovine serum (FBS), and antibiotics were obtained from Gibco BRL (Gaithersburg, MD, USA). Sephadex G-75 was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and 2-diethylaminoethanol (DEAE) anion-exchange resin was purchased from Whatman. All chemicals were of analytical grade or the highest purity available.

Preparation of crude porphyran (CP) *Porphyra*

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yezoensis was harvested from the Korean seashore, and a 250 g dried sample was steeped in 2.5 L of distilled water for 3 hr at 90°C. The resulting extract was subjected to Millipore filtration and lyophilized for use in subsequent experiments.

Chemical analysis of CP The protein and sugar contents of the CP preparation were determined using the Kjeldahl and phenol/sulfonic acid methods, respectively (21). Protein and sugar comprised 12.1 and 59.6%, respectively, of the CP preparation.

Fractionation of CP using ion-exchange column chromatography For analytical fractionation, a column (1.6×50 cm) was filled with 40 g of DEAE resin previously equilibrated in H_2O. The lyophilized CP was dissolved in H_2O and applied to the column. The column was then eluted with a stepwise gradient of 0-2 M NaCl in H_2O. Fractions (5 mL) were collected, and aliquots were assayed for sugars using the phenol-sulfuric acid method. The fractions were dialyzed against water in a 3,500 Da-cutoff dialysis membrane and then lyophilized. The protein and carbohydrate contents were analyzed, and the column fractions with the highest carbohydrate contents (0.5 M NaCl) were mixed together to yield one pooled fraction (F1). The pooled DEAE fraction represented a 50% yield from the CP. The other column fractions were not investigated further.

Gel-filtration column chromatography on Sephacryl S-200 resin F1 (45 mL) fraction was applied to a Sephacryl S-200 column (1.8×50 cm, Sigma Chemical Co.) equilibrated with H_2O. The column was eluted with 20 mM NaCl at a flow rate of 3 mL/min, and 37 mL fractions were collected. The protein was measured by the bicinchonic acid (BCA) method (Pierce Chemical Co., Rockford, IL, USA) according to the manufacturer's instructions. The total sugar content was determined using the phenol-sulfonic acid method (21) and the optical density at 490 nm.

Gel filteration column chromatography on Sephadex G-75 resin The pooled Sephacryl fraction was applied to a Sephadex G-75 column and eluted with distilled H_2O at a flow rate 2 mL/min. The saccharide-containing column fractions were pooled into 3 different fractions and lyophilized. These fractions contained porphyrans of different molecular masses. F2 fraction was pooled for use in cell viability assays; the resulting preparation of purified porphyran was designated CP Sep.

Composition of CP Sep The sulfate content of CP Sep was determined according to the method of Dodgson (22). 3,6-Anhydrogalactose content was determined as described previously (23).

Cell culture Human HT-29 colon cancer cells (ATCC HTB-38) and AGS gastric adenocarcinoma cells (ATCC CRL-1739) were obtained from the American Type Culture Collection (Rockville, CT, USA). The cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS (HyClone), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cell cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Cell proliferation assay AGS and HT-29 cells were cultured in RPMI-1640 media containing 10% FBS and then seeded into 96-well plates at a density of 5×10^3 cells per well. The cells were then treated with various concentrations of CP or CP Sep with serum free medium for 24 hr. Measurement of cell proliferation was performed colorimetrically using the 3-(4,5-dimethythiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay with CellTiter96 Aqueous One Solution Reagent (Promega, Madison, WI, USA). After cells were exposed to MTS for 3 hr, the absorbance at 490 nm was measured using a microplate reader. The percentage of cell survival as a function of CP or CP Sep concentration was then plotted to determine the drug concentration at which cell proliferation was decreased by 50% (IC_{50}).

Caspase-3 activity assay The activity of caspase-3 was measured in a 96-well plate using a colorimetric caspase-3 assay kit (Promega) according to the manufacturer's protocol. Reaction mixtures contained 30 L of cell lysate and 10 μL of caspase-3 substrate (Ac-DEVD-pNA, final concentration 200 μM) in assay buffer. To control for non-specific hydrolysis of the substrate, a control reaction mixture contained 30 μL of cell lysate and 10 μL of the specific caspase-3 inhibitor (final concentration 20 M) and substrate in assay buffer. After reaction mixtures were incubated for 90 min at 37°C, the absorbance at 405 nm was measured. Caspase-3 activities are expressed as OD_{405} values.

Statistical analysis Student's *t*-tests were used to compared data from the control and test conditions ($p< 0.05$).

Results and Discussion

Polysaccharides can be obtained using many methods, including aqueous extraction (24-26), ethanol extraction (26-28), acid hydrolysis (2), and enzyme hydrolysis (10, 13, 15); each method produces different products with different activities. In addition, protein is a common contaminant in polysaccharide preparations. Therefore, high-quality polysaccharides can only be obtained through improvements in commercial and laboratory-scale processes.

Several other natural polysaccharides have been investigated for their anti-tumor activity *in vitro* and *in vivo* (26, 29). For example, mushrooms are a promising source of polysaccharides with anti-tumor activity (30), some of which are now used as adjuvants in the treatment of cancers (24, 28). Most polysaccharides with anti-tumor effects have a basic (1→3)-β-D-glucan structure, but α-D-glucan polysaccharides, polysaccharide-protein complexes, and heteropolysaccharides also exhibit anti-tumor activity. In general, the biological activity of sulfated oligosaccharides is closely related to their molecular weight, carbohydrate structure, and content and linking position of sulfur groups. Sulfated polysaccharides from marine algae exhibit antioxidant activity. Sulfated polysaccharides from

Fucus vesiculosus, Laminaria japonica, and *Ecklonia kurome* are antioxidants and free-radical scavengers (25, 31).

Porphyran, which was extracted with hot water, is also a sulfated polysaccharide. Porphyran is similar to agarose in that it contains disaccharide units consisting of 3-linked β-D-galactosyl residues alternating with 4-linked 3,6-anhydro-α-L-galactose (9), but it differs in that some residues occur as the 6-sulfate. In addition, the chemical components and structures of porphyrans isolated from different species vary greatly.

To determine the cytotoxicity of CP, we previously conducted a dose-response study of its effect on the proliferation of IEC-6 normal intestinal cells (14). The IC_{50} could not be determined, suggesting that CP is not cytotoxic to normal cells. However, when HT-29 colon cancer cells in monolayer culture were treated with CP (0-5 mg/mL) for 24 hr in serum-free medium, the number of viable cells decreased in a dose-dependent manner (Fig. 1A). In addition, CP markedly decreased the viability of AGS gastric carcinoma cells (Fig. 1B). These results suggest that the anti-proliferative activity of CP is related to the induction of apoptosis in cancer cells.

The CP was fractionated using DEAE anion-exchange chromatography with a stepwise NaCl gradient (Fig. 2A). The isolated fractions were analyzed for their total sugar content to determine the optimal NaCl concentration. One major fraction (F1) with high sugar content was eluted with 0.5 M NaCl. However, its high A_{562} value by the BCA method and its positive H_2SO_4-phenol reaction indicated that this preparation contained protein in addition to polysaccharide. Therefore, we further fractionated F1 using sequential Sephacryl S-200 and Sephadex G-75 gel filteration column chromatography, resulting in 3 different final fractions (Fig. 2B). The lack of protein content and the positive H_2SO_4-phenol reaction indicated that these porphyran fractions (CP-Sep) contained less protein than DEAE column fractions (Fig. 2C).

Purified porphyran was obtained by first fractionating the CP using DEAE anion-exchange chromatography with a stepwise NaCl gradient. The resulting high-porphyran fraction was then further purified using sequential Sephacryl S-200 and Sephadex G-75 gel filteration column

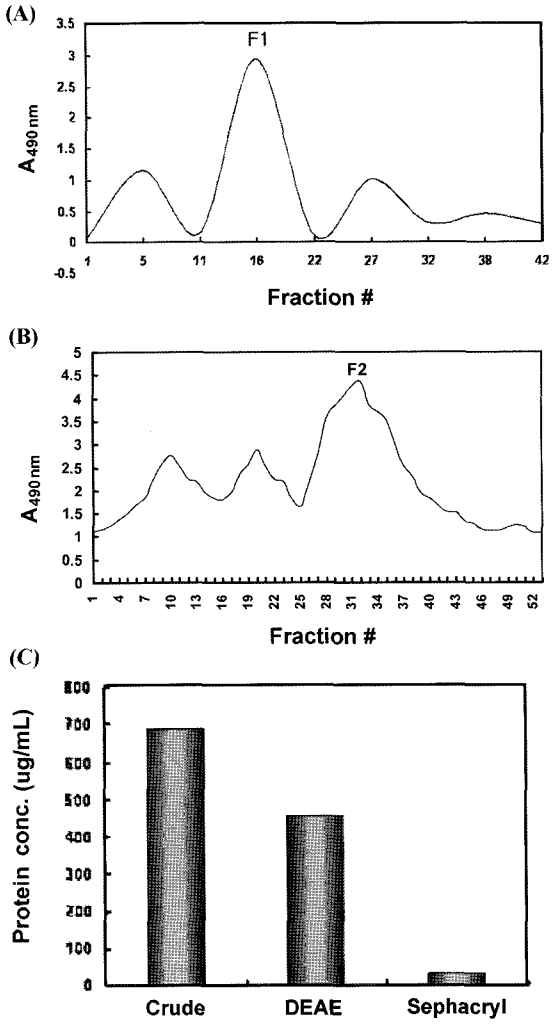

Fig. 2. (A) Fractionation of CP by DEAE anion-exchange column chromatography. (B) Separation of CP on a Sephadex G-75 column. After removal of contaminating protein on a Sephacryl S-200 column, the porphyran sample was applied to a Sephadex G-75 column. **(C) Comparison of protein concentrations.** The protein concentrations were estimated spectrophoto-metrically by measuring the absorbance at 562 nm using the BCA method.

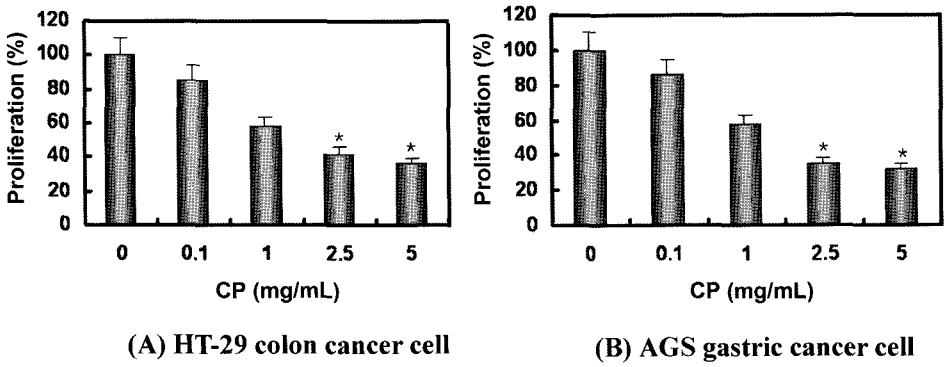

Fig. 1. Effect of crude porphyran (CP) on proliferation of human cancer cells. After serum starvation, the cells were incubated in serum-free medium in the absence or presence of various concentrations of CP as indicated. Cell numbers were estimated using the MTS assay. Values shown are means±SE; n=6 (*p<0.05).

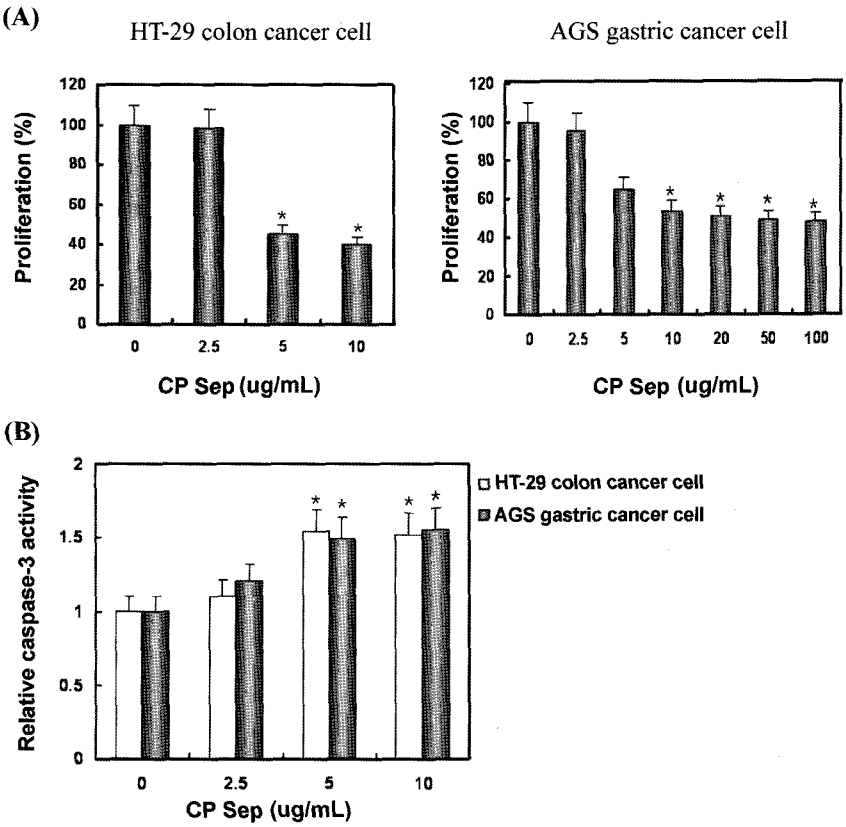

Fig. 3. (A) Effect of CP Sep at various concentrations on proliferation of human cancer cells. The cells were plated, cultured, and serum-starved. They were then incubated for 2 days in serum-free medium containing CP Sep, and cell numbers were estimated using the MTS assay. **(B) Caspase-3 activity was measured after treatment with or without CP Sep; activity is indicated by $OD_{405\ nm}$ values.** Values shown are means±SE; n=6 (*p<0.05).

chromatography. The final product, CP-Sep, contained less protein than other column fraction, as shown by the positive H_2SO_4-phenol reaction. Zhang *et al.* (32) prepared three sulfated polysaccharide fractions from *P. haitanesis* that exhibited significant antioxidant effects. They reported that, similar to sulfated polysaccharides from brown algae, sulfated galactans from red algae are effective antioxidants. Investigations of the structure and function of the sulfated polysaccharides (i.e., porphyran) isolated from different *Porphyra* species have shown that they have some physiological effects, including improvement of the cecum microflora environment (33), anti-tumor activity (27), antihypertensive and antihyperlipidemic effects (20), and macrophage stimulation activity (5, 6).

In this study, chromatographic purification of porphyran (CP-Sep) resulted in the removal of protein and yielded 3 porphyran fractions, each containing a porphyran of a different molecular mass (181,818, 100,365, and 705 Da). Gel-filtration chromatography caused little loss of yield but successfully reduced the protein content; the 3 final fractions contained <50 μg/mL protein and >10 μg/mL porphyran. The elution position of the fractions was independent of the porphyran molecular mass, and the purified porphyran content of the last fraction eluted (F2) was relatively high compared to that of other fractions. Chemical analysis indicated that F2 contained 27.1 and 18.4% sulfate and 3,6-anhydrogalactose, respectively, and gas chromatographic analysis of the acetylated aldononitrile derivatives of an F2 hydrolysate revealed that galactose was the predominant component. In addition to galactose, xylose was also present at low levels.

In this study, porphyran was prepared by sequential

Table 1. Chemical analysis of polysaccharide fraction F2 (CP Sep) from *P. yezoensis*

Total sugar[1)]	Sulfate[1)]	3,6-AG[1,2)]	N[1,3)]	Monosaccharide composition[4)] (mol %)	
				Galactose	Xylose
71.1	27.1	18.4	0.37	87	13

[1)]Percentage of the dry weight of F2.
[2)]3,6-Anhydro-α-L-galactose.
[3)]The nitrogen content was determined by elemental analysis.
[4)]The monosaccharide composition was detected by GPC analysis.

extraction with hot water at 90°C. After optimal filtration of the resulting precipitates, this procedure supplied CP at a total yield of 24% from dry seaweed. When cancer cells in monolayer culture were treated with CP (0-5 mg/mL) for 24 hr in serum-free medium, the number of viable cells decreased in a dose-dependent manner (Fig. 1). Other researchers have reported that CP inhibits cell proliferation in various cancer cell lines (5, 6) and has antioxidant activity for the prevention of oxidative damage (32, 34), which is an important contributor in carcinogenesis. Previously we have shown that the mechanism of CP action in human cancer cells by identifying the apoptotic signaling components (14). These results suggest that the anti-proliferative activity of CP is related to the induction of apoptosis in cancer cells.

Next, CP-Sep (F2) was used to treat HT-29 and AGS cells at concentrations ranging from 2.5 to 100 μg/mL for 24 hr. As shown in Fig. 3A, proliferation of the tumor cells was inhibited by CP Sep at various concentrations. The minimum concentration of CP Sep required to significantly inhibit proliferation of the cells was 5 (HT-29) or 10 μg/mL (AGS). To evaluate the apoptotic effects of CP Sep, we performed a caspase-3 colorimetric protease assay. Treatment with CP Sep induced caspase-3 activity in cancer cells (Fig. 3B), indicating that CP Sep has an anti-proliferative effect in cancer cells via apoptosis.

Apoptosis, a highly regulated process, involves caspase activation and subsequent nuclear events that cause cell death. Apoptotic cell death is characterized by cell shrinkage, membrane blebbing, chromatin condensation, and internucleosomal DNA cleavage. The cleavage of caspase-3 was detected if the cells were induced to undergo apoptosis (35). For example, fucoidan-induced apoptosis was accompanied by the activation of caspase-3 in the human HS-sultan cell (36) and immune cells (33). A caspase-3 colorimetric protease assay showed treatment with CP Sep induced caspase-3 activity in cancer cells (Fig. 3B), indicating that CP Sep has an anti-proliferative effect in cancer cells via apoptosis.

The inhibitory effect of CP Sep on the growth of human cancer cells indicates that column chromatography is an effective method for removal of contaminating proteins from porphyran. Although our results suggest that both crude and purified porphyran have significant *in vitro* anti-proliferative activity in human cancer cells, the purified porphyran (CP Sep) exhibited a significantly greater apoptotic effect than did the crude porphyran (CP). This implies that the porphyran polysaccharide moieties have significant bioactivities.

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