Biological Activities of Fucose-Containing Polysaccharide Ascophyllan Isolated from the Brown Alga Ascophyllum nodosum

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A fucose-containing, sulfated polysaccharide ascophyllan was isolated from the brown alga Ascophyllum nodosum. Composition analysis demonstrated that ascophyllan mainly contains uronic acid, xylose, fucose, and sulfate half ester in approximately equimolecular proportions, which are evidently distinct from those of alginate and fucoidan. Ascophyllan inhibited the proliferation of U937 cells in a concentration-dependent manner, and DNA-fragmentation and typical apoptotic nuclear morphological changes were observed in the ascophyllan-treated cells. Furthermore, ascophyllan induced the secretion of tumor necrosis factor-α (TNF-α) and granulocyte colony-stimulating factor (G-CSF) from mouse macrophage cell line RAW264.7.

Key words: Ascophyllum nodosum; ascophyllan; fucoidan; apoptosis; cytokine

Various useful polysaccharides are available from several species of marine algae, and it is well known that some polysaccharides have a wide variety of biological activities. Laminaran and especially fucoidan are drawing increasing attention from medical and food supplement areas for their potential biological activities. Although the composition of these polysaccharides, molecular sizes, and entire structures vary depending on the algal species, the extraction procedure, and even the season of harvest and local climate conditions, the polysaccharides have a common structural feature. Fucoidan is basically a sulphated fucan containing fucose as a main component and sometimes containing uronic acids, galactose, and xylose as minor compositions. Laminaran is composed of (1,3)-β-glucan with β(1,6) branching, and alginate is composed of mannuronic and guluronic acid with β(1,4)-linkages. In addition to the well-known anticoagulant and antithrombotic activity, fucoidans act on the inflammation and immune systems and have antitumor and antiviral activities and antiproliferative and antiadhesive effects on cells, and can interfere with the mechanisms involved in fertilization. These activities and the structural aspects of fucoidans have been reviewed in recent papers. Ascophyllan is a fucan, and has similar but distinct structure from fucoidans based on a backbone of uronic acid (mannuronic acid) with fucose containing branches (3-O-α-D-xylolyl-L-fucose-4-sulfate). In spite of increasing study on the various biological activities of fucoidans, there is no detailed study on the biological activities of ascophyllan. Therefore, in this study, we prepared ascophyllan from powdered A. nodosum based on a method reported previously, and explored its biological activities on two cultured cell lines. To our knowledge, this is the first time that the biological activities of ascophyllan have been reported.

Fucoidan (from Fucus vesiculosus) was purchased from Sigma (St. Louis, MO). Ascophyllum nodosum collected on the coast of Norway was obtained from KAISEI (Shimonoseki, Japan). For the preparation of ascophyllan, fucoidan, and alginate fractions from A. nodosum, 40 g of milled A. nodosum was pretreated with diluted HCl (final 0.2N) to remove inorganic cations. After filtration, the residue was put into 1,000 ml of distilled water, then neutralized with aqueous NaOH. This solution was gently stirred at 20 °C for 20 h, then filtrated. After the residue was again put into 1,000 ml of distilled water, this solution was stirred at 100 °C for 2 h, then filtrated. The filtrates were combined, and the solution was adjusted to pH 1.3 with HCl. The precipitated alginate fraction was removed by centrifugation, and the supernatant was subsequently filtrated. The precipitate was dissolved in water, dialyzed, and lyophilized to give the alginate fraction. The filtrate was neutralized with NaOH. The neutralized solution was concentrated by evaporation under reduced pressure, then dialyzed extensively against distilled water to remove generated salt by neutralization. Subsequently, alginate lyase was added to the solution to degrade residual alginate. After incubation at 30 °C for 20 h, the solution was dialyzed extensively against distilled water. Five parts of this solution were then mixed with 1 part of 1 M NaCl solution and 6 parts of ethanol. This process of dissolution of the macromolecules in water and their precipitation with ethanol was repeated twice. The precipitated ascophyllan fraction was collected by centrifugation and the pellet was dissolved in water, dialyzed extensively against water, and lyophilized. After precipitation of the ascophyllan fraction, the supernatant was concentrated, dialyzed...
extensively against water, and lyophilized to give the fucoidan fraction. For sugar composition analysis, the p-aminobenzoic ethyl ester (ABEE)-labeling procedure was employed, as previously reported.\textsuperscript{10} Uronic acid was determined by a modified carbazole-sulfuric acid method, and was calculated as glucuronic acid.\textsuperscript{11} Ester sulfate was determined by the method of Dodgson-Price’s turbidimetry.\textsuperscript{12}

A human myeloid leukemia U937 cell line was obtained from the Riken Cell Bank (Tsukuba, Japan).

### Table 1. Composition (%) of Ascophyllan, Fucoidan, Alginate Fractions, and Commercially Available Fucoidan (Sigma) and Molar Ratios of the Compositions

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Composition of neutral sugars\textsuperscript{a}</th>
<th></th>
<th></th>
<th></th>
<th>UA\textsuperscript{b}</th>
<th>SO\textsubscript{4}2\textsuperscript{−} \textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fuc</td>
<td>Xyl</td>
<td>Glu</td>
<td>Man</td>
<td>Gal</td>
<td></td>
</tr>
<tr>
<td>Ascophyllan</td>
<td>15.5 (1)</td>
<td>13.4 (0.95)</td>
<td>0.3 (0.02)</td>
<td>3.4 (0.2)</td>
<td>0.6 (0.04)</td>
<td>21.4 (1.17)</td>
</tr>
<tr>
<td>Fucoidan</td>
<td>28.4 (1)</td>
<td>4.3 (0.16)</td>
<td>2.0 (0.06)</td>
<td>0.8 (0.03)</td>
<td>5.3 (0.17)</td>
<td>5.8 (0.17)</td>
</tr>
<tr>
<td>Alginate</td>
<td>0.5 (1)</td>
<td>0.4 (0.79)</td>
<td>0.1 (0.16)</td>
<td>0.6 (1.07)</td>
<td>1.6 (2.99)</td>
<td>27.8 (47.4)</td>
</tr>
<tr>
<td>Sigma fucoidan\textsuperscript{d}</td>
<td>24.8 (1)</td>
<td>1.9 (0.09)</td>
<td>0.8 (0.03)</td>
<td>1.0 (0.04)</td>
<td>3.1 (0.11)</td>
<td>9.6 (0.33)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Determined by HPLC assay after acid hydrolysis.

\textsuperscript{b}Determined by carbazole method and calculated as glucuronic acid equivalent.

\textsuperscript{c}Determined by turbidimetric assay after acid hydrolysis.

\textsuperscript{d}Purified from Fucus vesiculosus.

The numbers in the brackets indicate the molar ratios of the compositions of the polysaccharides, calculated by taking fucose content as 1.
The cells were cultured at 37 °C in RPMI1640 medium (Gibco Grand Isle, NY) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂ and 95% air. RAW264.7 (mouse macrophage) cells were obtained from the American Type Culture Collection (Rockville, MD), and cultured in Dulbecco’s modified Eagle’s minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin G sodium (100 units/ml), and streptomycin sulfate (100 µg/ml). The cytotoxicity of each polysaccharide fraction with various concentrations (0–1,000 µg/ml) on U937 cells was measured by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) assay. Ascophyllan-induced DNA fragmentation in U937 cells was examined as described previously.13,14) Nuclear morphological changes were examined by staining with the DNA-binding fluorochrom bisbenzimide (Hoechst 33258) as previously described.14) The levels of TNF-α and G-CSF in the supernatants of RAW264.7 cells cultured with ascophyllan were measured by sandwich enzyme-linked immunosorbent assay (ELISA) with two antibodies to two different epitopes on each cytokine molecule by similar methods, as described previously.15)

By taking advantage of the solubility of fucans in acidic medium and use of alginic lyase, algamines were removed from the fucan fractions. Subsequently, using the different solubility between ascophyllan and fucoidan in aqueous ethanol, ascophyllan was separated from the fucoidan fraction. The yields of alginic, fucoidan, and ascophyllan fractions obtained from 40 g of milled A. nodosum were 4.7 g, 0.50 g, and 0.76 g, respectively. Composition analysis of the three polysaccharide fractions showed that each of them had a characteristic composition feature (Table 1). Ascophyllan had fucose and xylose in about equimolecular proportion, whereas fucoidan had much higher ratio of fucose than of xylose, and the contents of these monosaccharides in the alginic were very low. The monosaccharide composition profile of fucoidan fraction obtained in this study was similar to that of a commercially available fucoidan. Significant levels of sulphate half-ester groups were detected in the ascophyllan and fucoidan fractions, but no level was detectable in the alginate fraction. The level of uronic acid in ascophyllan fraction was much higher than that of fucoidan. Based on the molar ratios calculated by taking fucose content as 1 (Table 1), ascophyllan is suggested to be a heteropolysaccharide (xylofucoglycuronan) consisting of fucose, xylose, uronic acid, and sulphate half-ester in approximately equimolecular proportion, consistently with previous reports,7,9) and the composition was obviously distinct from those of fucoidan and alginic fractions.

To examine the cytotoxic effects of the fucoidan, ascophyllan, and alginic fractions on U937 cells, WST-8 assay was carried out. As shown in Fig. 1A, fucoidan and ascophyllan showed similar cytotoxic effects on U937 cells, while alginic had no toxic effect. The cytotoxicity of ascophyllan was concentration-dependent, and even at 62.5 µg/ml, more than 50% of cells died after 72 h incubation. DNA-fragmentation assay and fluorescent nuclear staining suggested that the cytotoxicity of ascophyllan was accompanied by induction of apoptosis. To our knowledge, this is the first report suggesting that ascophyllan induces apoptosis in U937 cells. Since ascophyllan-induced DNA fragmentation was strongly inhibited by the caspase family inhibitor, perhaps the caspase-activation pathway was involved. Recent study has demonstrated that a commercially available fucoidan (Sigma, St. Louis, MO) purified from Fucus vesiculosus induced apoptosis in human myeloma and leukemia cells through activation of the caspase-3 and down-regulation of ERK pathways.16) In addition, Riou et al. reported that fucoidan extract obtained from Ascophyllum nodosum directly inhibited the proliferation of a human non-small cell lung cancer cell line (NSLC-N6).17) Thus, it seems likely that sulfated fucans like fucoidans and ascophyllan are able to cause direct cytotoxic effects on target cells, including tumor cells, through induction of apoptosis. Regarding antitumor activity in in vivo systems, it has been reported that fucoidan fractions extracted from Sargassum thunbergii and Sargassum kjellmannianum showed antitumor effects for Eherlich carcinoma-bearing mice and L1210 leukemia-bearing mice, respectively.18,19) In these in vivo experimental models, perhaps fucoidan-induced enhancement of immune responses is involved in the anti-cancer effects of fucoidan. Previous studies have pointed out that fucoidans or sulfated fucans can act as immunological response modifiers.20) To gain insight into this aspect of the biological activity of ascophyllan, we investigated the effects of ascophyllan on mouse macrophage cell

**Fig. 2.** Ascophyllan-Induced Cytokine Secretion from RAW264.7 Cells.
Adherent cells (2 × 10⁴ cells/well) in 96-well plates were incubated with the indicated concentrations of ascophyllan in DMEM containing 10% FBS at 37 °C. After 24 h, the cytokine levels in the supernatant were measured by the sandwich ELISA method, as described in the text. Columns represent averages of triplicate measurements and bars indicate the standard deviations.
line RAW264.7. The results clearly indicated that ascophyllan induced the secretion of TNF-α and G-CSF from RAW264.7 cells in a concentration-dependent manner (Fig. 2). Similarly to ascophyllan, it has been shown that fucoidan can stimulate TNF-α release from monocytes.\textsuperscript{21} Therefore, ascophyllan may have various biological activities similar to fucoidan, and it is possible that ascophyllan can exert anti-tumor activity through activation of host immune systems in addition to the direct cytotoxic effects on target cells. To clarify this point, further studies are necessary to investigate the anti-tumor effects of ascophyllan using tumor-bearing mice models.

References