

## Breast Cancer Chemopreventive Activity of Polysaccharides from Starfish *In Vitro*

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**Abstract** Polysaccharides from the starfish *Asterina pectinifera* were assessed *in vitro* for their chemopreventive potential in human breast cancer. The polysaccharides from *A. pectinifera* inhibited cell proliferation in the estrogen receptor-positive (MCF-7) and estrogen receptor-negative (MDA-MB-231) human breast carcinoma cell lines. In addition, the polysaccharides were found to be an inhibitor of cytochrome P450 1A1-mediated ethoxyresorufin *O*-deethylase activity, and caused a dose-dependent inhibition of aromatase activity in microsomes isolated from a human placenta. There was a significant reduction in the ornithine decarboxylase activity to 30.7% of the control in the polysaccharide-treated MCF-7 breast cancer cells. Therefore, the polysaccharides from *A. pectinifera* merit further investigation with respect to breast cancer chemoprevention.

**Key words:** Aromatase, *Asterina pectinifera*, breast cancer, cytochrome P450 1A1, ornithine decarboxylase

Breast cancer is the most common cancer in women worldwide and continues to be a major health problem [7]. Thus, breast cancer chemoprevention has become the subject of significant research efforts to improve the health of women. Chemoprevention is defined as the systemic use of natural or synthetic chemical agents to reverse or suppress the progression of a premalignant lesion to an invasive carcinoma.

Carcinogen-metabolizing enzymes are one target of chemoprevention, as the downregulation of activating enzymes can reduce the activity of carcinogenic compounds at the target sites [19]. The marked enzymatic activity of cytochrome P450 1A1 has been demonstrated in the human mammary epithelium [9]. Since estrogen has a

major effect on the development of breast cancer, and aromatase is the last enzyme responsible for estrogen biosynthesis, the overexpression of aromatase in breast cancer cells may significantly influence breast cancer progression and maintenance. The expression of aromatase in breast cancer tissue has already been reported, and was at a higher level than that in noncancer breast tissue [8, 10]. Polyamines and the enzymes responsible for their biosynthesis also play a significant role in carcinogenesis, tumor promotion, and cellular hyperplasia. The activity of ornithine decarboxylase (ODC), the first and rate-limiting enzyme in polyamine synthesis, is higher in tumor tissue than in non-tumor tissue, and an elevated ODC activity and polyamine levels are biomarkers for human breast carcinoma [3, 12].

Many marine flora and fauna contain substances that have anticancer, antiviral, antimicrobial, anticoagulant, cardioactive, or neurophysiologic properties. These highly active compounds can serve as models in the development of new drugs, and over the past decade, about 3,000 new classes of anticancer agents from marine sources have been described and some have entered preclinical and clinical trials [4]. Therefore, these developments suggest that, in the future, the ocean will become a valuable source of novel chemical classes not found in a terrestrial environment.

Accordingly, the present study investigated the effect of polysaccharides from the starfish *Asterina pectinifera* on the proliferation of breast cancer cells and activities of cytochrome P450 1A1, aromatase, and ornithine decarboxylase.

## MATERIALS AND METHODS

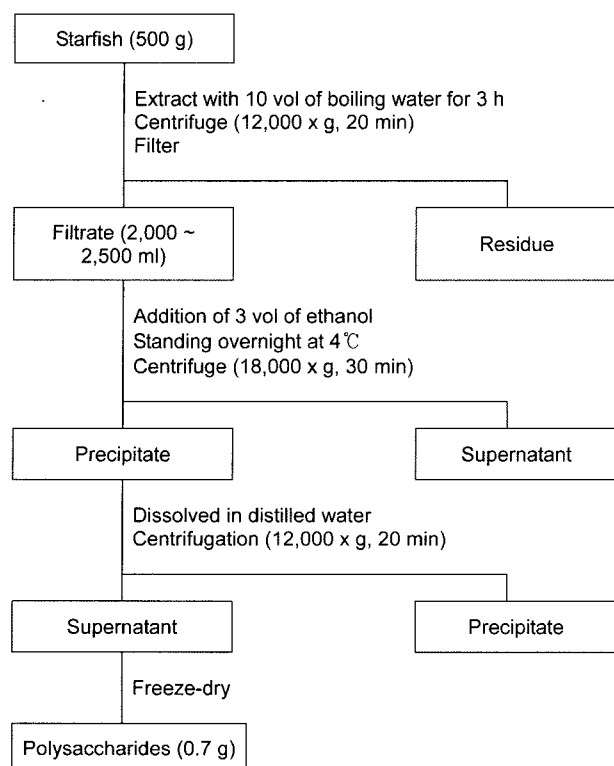
### Animal Material

The *A. pectinifera* was collected in August 2004, off the coast of Pohang, Korea. A voucher specimen (A0014-3) of

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**Fig. 1.** Procedure used to extract polysaccharides from *A. pectinifera*.

the starfish has been deposited in the Intractable Disease Research Center, Dongguk University, Gyeongju, Korea. After collection, the starfish was washed with distilled water and stored at  $-20^{\circ}\text{C}$  until used.

### Extraction of Polysaccharides

The *A. pectinifera* was cut into small pieces, and the extraction of the polysaccharides followed the procedure shown in Fig. 1. [1, 16]. The samples were extracted with 10 volumes of water for 3 h at  $100^{\circ}\text{C}$ . After filtration, the filtrates were precipitated with 3 volumes of 95% (v/v) ethanol overnight at  $4^{\circ}\text{C}$ , and then collected by centrifugation ( $18,000 \times g$  for 30 min). Next, the precipitates were dissolved in distilled water and centrifuged at  $12,000 \times g$  for 20 min. Finally, the supernatant (polysaccharides) was freeze-dried. The carbohydrate content was determined using a phenol-sulfuric acid reaction, and the lyophilized polysaccharides dissolved in a cell culture medium, potassium phosphate buffer, or Tris-HCl buffer, and then filtered through a  $0.22\text{-}\mu\text{m}$  sterile membrane filter disc before being subjected to the experiments.

### Cell Proliferation Assay

Estrogen receptor-positive MCF-7 and estrogen receptor-negative MDA-MB-231 cells were plated at an initial cell concentration of  $0.5 \times 10^4$  cells per well in RPMI 1640

medium supplemented with 10% fetal bovine serum for 24 h, and then treated with the polysaccharides from *A. pectinifera* for 4 days at various doses. The cell proliferation was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described [13, 14].

### Placental Microsome Aromatase Assay

The aromatase activity was determined using a modified version of the tritiated water method of Thompson & Siiteri [22]. The assay mixture (500  $\mu\text{l}$ ) contained the substrate [ $1\beta\text{-}^3\text{H}(\text{N})$ ] androst-4-ene-3,17-dione (100 nM), human placental microsomes (40  $\mu\text{g}$ ), progesterone (10  $\mu\text{M}$ ), bovine serum albumin (0.1%), and a potassium phosphate buffer (67 mM, pH 7.4). After 10 min of preincubation at room temperature, 50  $\mu\text{l}$  NADPH (12 mM) was introduced and the reaction continued at  $37^{\circ}\text{C}$  for an additional 20 min. The enzyme activity was terminated with 5% trichloroacetic acid. After 10 min of centrifugation at  $1,000 \times g$ , the supernatant containing the aromatase product, [ $^3\text{H}$ ]H<sub>2</sub>O, was extracted with chloroform and dextran-treated charcoal. The charcoal mixtures were then vortexed and subsequently pelleted by centrifugation ( $15,000 \times g$  for 5 min), and an aliquot of the supernatant counted for radioactivity. The aromatase activity was calculated as pmol [ $^3\text{H}$ ]H<sub>2</sub>O formed/mg protein/h.

### Inhibition of DMBA-Induced Cytochrome P450 1A1 Activity

Female Sprague Dawley rats (43 days old) were treated with a single (intragastric) dose of 7,12-dimethylbenz[*a*]anthracene (15 mg), and the liver microsomes isolated through differential centrifugation. The cytochrome P450 1A1 activity was monitored by assessing the ethoxyresorufin *O*-deethylase activity [2, 18], where the reaction mixture contained microsomal protein, a 0.05 M Tris-HCl buffer (pH 7.5), bovine serum albumin, 0.25 M  $\text{MgCl}_2$ , NADPH-generating system (NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase), ethoxyresorufin, and various sample concentrations. After incubating at  $37^{\circ}\text{C}$  for 4 min, the reaction was terminated by the addition of 2 ml of methanol. The formation of resorufin was determined fluorometrically using a BIO-TEK SFM25 spectrofluorometer (550 nm excitation and 585 nm emission) (BIO-TEK Vermont, U.S.A.). The assays were all conducted in triplicate and the percent of inhibition was calculated as:

$$[1 - (\text{sample A} - \text{blank}) / (\text{solvent A} - \text{blank})] \times 100.$$

### Ornithine Decarboxylase Activity

The MCF-7 breast cancer cells were plated at a density of  $1 \times 10^5$  cells/well in 24-well tissue culture plates. After incubation at  $37^{\circ}\text{C}$  for 18 h in a 5% (v/v)  $\text{CO}_2$  atmosphere, the medium was removed and replaced with

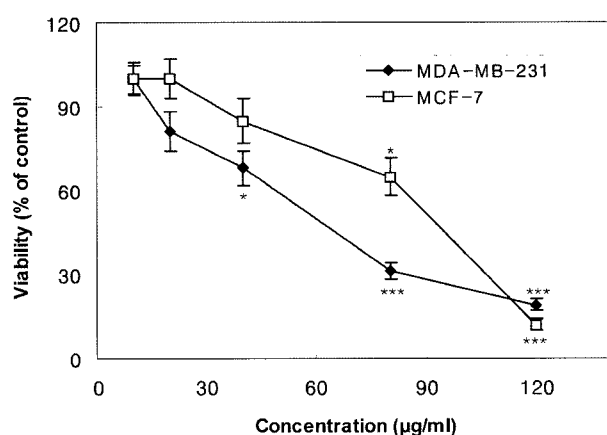
media containing TPA (200 nM) alone, TPA plus the polysaccharides from *A. pectinifera* (10–120 µg/ml), or 0.01 mM difluoromethylornithine as a positive inhibitor of ornithine decarboxylase (ODC). After an additional 6 h of incubation at 37°C, the cells were washed twice with Ca<sup>2+</sup>, Mg<sup>2+</sup>-free phosphate-buffered saline (pH 7.4) and subjected to three freeze-thaw cycles. The ODC activity was assayed directly in the 24-well plates by measuring the release of <sup>14</sup>CO<sub>2</sub> from L-[1-<sup>14</sup>C]ornithine [17]. A reaction mixture of 200 µl containing 4 µl of L-[1-<sup>14</sup>C]ornithine (200 nCi, 56 mCi/mmol, 50 µCi/ml), 48 µl of a 0.2 M sodium phosphate buffer (pH 7.2), 16 µl of 12.5 mM EDTA, 10 µl of 50 mM dithiothreitol, 4 µl of 5 mM pyridoxal phosphate, and 118 µl of unlabeled 620 µM L-ornithine was added to each well. The release of <sup>14</sup>CO<sub>2</sub> gas was then captured by a paper disk during incubation of the plates at 37°C for 1 h while shaking. The amount of radioactivity absorbed by the paper disks was measured using liquid scintillation counting (Beckman LS 6500, Fullerton, U.S.A.).

### Statistical Analysis

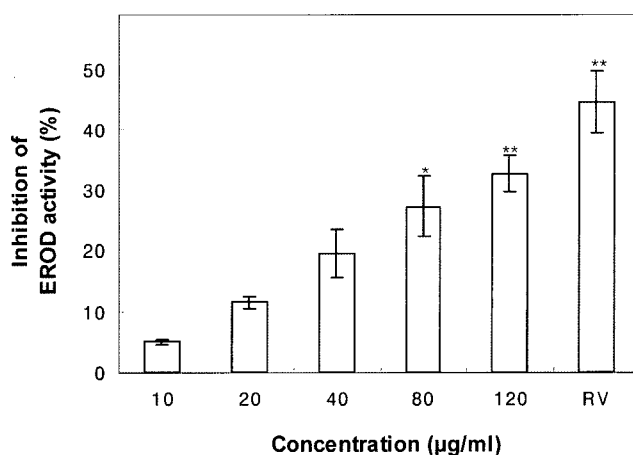
The data were analyzed for statistical significance using Student's *t*-test. *p*-Values less than 0.05 were considered to be significant.

## RESULTS AND DISCUSSION

The polysaccharides from *A. pectinifera* inhibited the growth of the estrogen receptor-negative MDA-MB-231 and estrogen receptor-positive MCF-7 cells with IC<sub>50</sub> values of 61 and 93 µg/ml, respectively (Fig. 2). The growth of the human breast cancer cell line MCF-7 was less susceptible to inhibition by the polysaccharides from *A. pectinifera*



**Fig. 2.** Effect of various concentrations of polysaccharides from *A. pectinifera* on MCF-7 and MDA-MB-231 human breast cancer cell growth measured after 4 days of treatment. Data shown are mean values with bars indicating the SD of the mean (n=3). \**p*<0.05, \*\*\**p*<0.005 compared with the control.



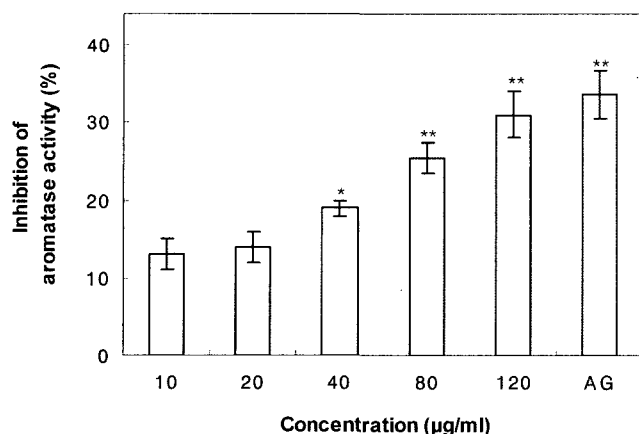
**Fig. 3.** Inhibition of cytochrome P450 1A1-mediated ethoxyresorufin *O*-deethylase (EROD) activity using liver microsomes derived from 7,12-dimethylbenz[a]anthracene-treated rats.

The EROD activity was assessed with the indicated concentrations of polysaccharides from *A. pectinifera*. RV, 0.05 nmole resveratrol. Data shown are mean values with bars indicating the SD of the mean (n=3). \**p*<0.05, \*\**p*<0.01 compared with the control.

(Fig. 2). However, the polysaccharides from *A. pectinifera* exhibited similar growth-inhibitory patterns in both estrogen receptor-positive and estrogen receptor-negative cells, indicating a lack of estrogen receptor-induced sensitivity to the polysaccharides.

Treatment with the polysaccharides from *A. pectinifera* resulted in a concentration-dependent inhibition of 7,12-dimethylbenz[a]anthracene-induced cytochrome P450 1A1 activity (Fig. 3). The influence of the cytochrome P450 enzyme system on the biological effect of carcinogens, including estrogen, makes this enzyme a predictor of breast cancer development, as well as a potential target for chemoprevention. Cytochrome P450 1A1 encodes aryl hydrocarbon hydroxylase activity, which catalyzes the first step in the metabolism of a number of polycyclic aromatic hydrocarbons to their ultimate DNA-binding forms [11, 15]. Polycyclic aromatic hydrocarbons are already known to be powerful mammary carcinogens in mice [23]. Cytochrome P450 1A1 is also involved in estrogen metabolism, catalyzing the conversion of estradiol to hydroxylated estrogen [5]. Thus, reduced estrogen exposure can protect against the development of breast cancer, whereas increased estrogen exposure can enhance the risk of developing breast cancer. Therefore, altering the activity of aryl hydrocarbon hydroxylase could plausibly lead to a change in the levels of estrogen, thereby affecting the risk of breast cancer.

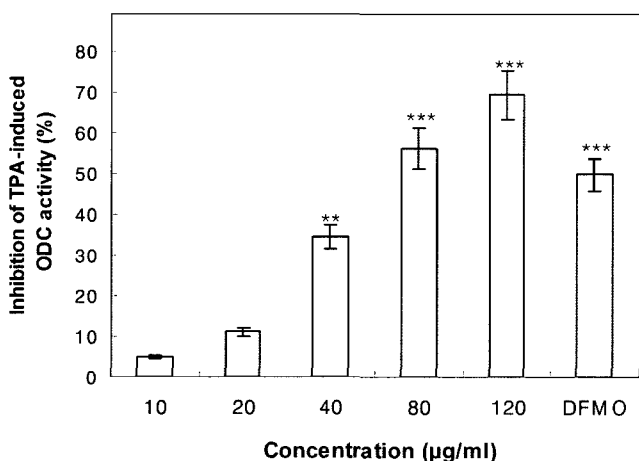
The incubation of 40 µg of microsomes with a 100 nM aromatase substrate and the cofactor NADPH for 30 min resulted in an aromatase specific activity of 54.3±3.7 pmol/mg protein/h. However, the addition of the polysaccharides from *A. pectinifera* to the reaction caused a dose-dependent



**Fig. 4.** Effect of polysaccharides from *A. pectinifera* on microsomal aromatase activity. AG, 5 µM aminoglutethimide. Data shown are mean values with bars indicating the SD of the mean (n=3). \* $p < 0.05$ , \*\* $p < 0.01$  compared with the control.

decrease in the aromatase activity (Fig. 4). This inhibited aromatase activity could then lead to a decrease in the availability of the highly active endogenous estrogen.

The polysaccharides exhibited a suppressive effect on the TPA-induced ODC activity of the human breast cancer cells in a dose-dependent manner. At 80 and 120 µg/ml, the polysaccharides were a more potent inhibitor of ODC activity than difluoromethylornithine, a suicide inhibitor of ornithine decarboxylase (Fig. 5). A high expression of ODC characterizes breast cancer. Furthermore, polyamines have been shown to be essential mediators of estrogen-



**Fig. 5.** Effect of polysaccharides from *A. pectinifera* on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ornithine decarboxylase (ODC) activity in MCF-7 human breast cancer cells.

The ODC activity of the control was  $397 \pm 29$  pmol  $^{14}\text{CO}_2/\text{h}/\text{mg}$  protein. DFMO, 0.01 mM difluoromethylornithine. Data shown are mean values with bars indicating the SD of the mean (n=3). \*\* $p < 0.01$ , \*\*\* $p < 0.005$  compared with the control.

stimulated breast cancer growth, as they interface with estrogen action at multiple levels, including the association kinetics of the estrogen receptor to specific DNA sequences [20], the synthesis of estradiol-regulated cell-cycle-specific genes [21], and the synthesis and/or action of estradiol-modulated growth factors [6].

Since more than one compound is implicated in breast cancer, the chemopreventive activity of the polysaccharide extract cannot be ruled out, requiring further isolation to unravel the probable mode of action. Nonetheless, the present results still provide sufficient information for the further development of the extract from *A. pectinifera* as a breast cancer chemopreventive agent in animal studies and later in human clinical trials.

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