

Immunological Properties of the Marine Brown Alga *Endarachne binghamiae* (Phaeophyceae)

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Abstract: Seaweeds contain a variety of biologically active substances, which are useful for therapy and medicinal purposes. One of the potent uses has been found in its immune functions. In the present report, we describe immunological properties of the marine brown alga *Endarachne binghamiae* against murine macrophage and human T cells *in vitro*. It was observed that various extracts from the alga effectively stimulated cell proliferation and that the stimulation activity of active substances varied with growth habitat of the alga assayed. The reported alga was found rich in polysaccharides. Of which sodium alginate exhibited strong stimulation activity for macrophage and T cell proliferation, and also alginic acid but to a lesser extent. A glycoprotein isolated from the reported alga was also a strong proliferation stimulant. Additionally, it significantly induced the production of TNF- α and nitric oxide by macrophages and IFN- γ by T cells in a concentration-dependent manner. These assay results suggested that alginate and protein of the reported alga could be promising immune stimulants and modulators.

Keywords: marine algae; sodium alginate; protein; proliferation stimulation; Cytokines.

1. Introduction

Marine algae have been traditionally used as food and medicine. It is because these marine algae contain the essential amino acids and polyunsaturated fatty acids, necessary vitamins and minerals, and larger amounts of dietary fibers. Additionally, these contain a variety of biologically active substances, which possess antibacterial (Liao et al., 2003), antiviral (Witvrouw and De Clercq, 1997; Hudson et al., 1999), agglutinating, and anti-tumor (Suzuki et al., 1980; Yamamoto et al., 1982; Okai et al., 1997; Nika et al., 2003) activities. Other reports indicated that marine algal polysaccharides and proteinaceous substances have valuable functions in immune

modulation and stimulation (Hori et al., 1988; Otterlei et al., 1991; Yoshizawa et al., 1993; Shan et al., 1999; Son et al., 2001), as well as in lowering blood pressure, cholesterol, and glucose level (Hoppe, 1979). Thus, marine algae can be a suitable source of materials for development and utilization of health foods and drugs.

The marine brown alga *Endarachne binghamiae* (Phaeophyceae) is widely distributed in temperate and tropical waters. It occurs abundantly on the northeastern coast of Taiwan during February and March. It looks like a tiny kelp with about 10 cm height. *E. binghamiae* is a common sea vegetable and also a good prey for fishing lembus rudder fish by local fishermen. However, its economic value

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in health protection and pharmaceutical purpose is not yet known. In this report, we have described the immunopotential properties of this brown alga based on the *in vitro* assay of polysaccharides and protein against murine macrophages and human T lymphocytes.

2. Materials and methods

2.1. Algal material and pretreatment

The brown alga *Endarachne binghamiae* was collected from different localities of the northeastern coast of Taiwan in March 2004. The harvested thalli were first washed thoroughly in cold seawater, then cleaned in previously distilled Milli Q ion exchanged water to remove epibionts and other contaminants using ultrasonic cleaner, and finally freeze-dried, ground into powder under liquid nitrogen and maintained at -20°C until used for chemical composition analysis and extraction.

2.2. Chemical composition of thalli

1. Water and ash contents

Water content was determined on the average loss of weight of fresh thalli at 105°C for 24 h. For ash content, the thalli were heated at 600°C for 6 h, and then weighted at room temperature.

2. Total carbohydrate, crude protein and fat

Total carbohydrate was measured after reaction of the powdered alga with phenol and sulfuric acid (Dubois et al., 1956), using glucose as a standard. Crude protein and fat in the thalli were determined quantitatively according to the methods of A.O.A.C. (1995). The former was determined after reduction of nitrogenous compounds to nitrogen by sulfuric acid at $380\text{-}400^{\circ}\text{C}$, while the latter was obtained by treatment with ether at 45°C for 16 h and then determined on the loss of weight in dry powder after extraction.

2.3. Preparation of algal extracts

The powdered alga was extracted separately at a 1: 100 (w: v) ratio in phosphate buffered saline (PBS, pH 7.4, containing 0.1 M NaCl), 20 % ethanol and hot water by autoclaving for 20 min. Extracts were centrifuged, and the supernatants were used in MTT assays.

2.4. Preparation of polysaccharides and mitogenic protein

The reported alga contains a large amount of sodium alginate and alginic acid. These polysaccharides were extracted in sodium carbonate solution after pretreatment of algal powder with hot calcium chloride solution, following the procedures described by Whyte (1988). Isolation of mitogenic protein was carried out after extraction. The extracted proteins from *E. binghamiae* were precipitated by mixing gradually with 30- 70% saturated ammonium sulfate. The protein, which showed positive in mitogenesis assay, was then isolated and purified in dual column chromatography, i.e. gel filtration and ion exchange. Detailed extraction and isolation procedures for the mitogenesis-active protein have been described in the following sections:

2.5. In vitro assay for mitogenic activity

Murine (BALB/c) macrophage (RAW 264.7) and human T cells (CCRF-CEM) were used for assaying with a 96-well flat-bottomed microtiter plate. Macrophages were cultured in D-MEM medium supplemented with 10% FBS, while T cells were grown in RPMI-1640 medium containing 20% FBS. Both cell lines were allowed to grow in an incubator supplied with 5% CO_2 at 37°C . After being cultured in the presence of extract, polysaccharide or protein in a serial twofold dilutions for 72 h, cells viability and density were measured based on the absorp-

tion at 540 nm in an ELISA Reader, using MTT method (Mossman 1983). The culture without treatment with algal substance was used as a control. Mitogenic activity of algal substances for macrophages and T cells was expressed in stimulation index (SI), which was calculated by dividing the absorbance at 540 nm of the treated culture by the untreated culture.

2.6. Measurement of TNF- α , IFN- γ and nitric oxide production

The cytokines TNF- α and IFN- γ produced by murine macrophage and human T cells, respectively, in the presence or absence of the isolated protein were measured quantitatively by sandwich ELISA, using Biosource Mouse TNF- α and Human IFN- γ kits. Nitric oxide released by macrophages in the presence of isolated protein was also measured based on the concentration of nitrite (NO₂⁻) in cell-free supernatants using BIOXYTECH Non-enzymatic Assay. The released nitrite oxide was thereafter measured for absorbance at 540 nm after reaction with color reagents. The amounts of TNF- α , IFN- γ and NO were calibrated with standard solutions at various concentrations.

3. Results

3.1. Chemical composition

A chemical analysis was performed on the fresh thalli and powder of *Endarachne binghamiae*. The results are shown in Table 1 as percentage of wet and dry matter. Carbohydrate accounted for nearly half of the thalli in dry weight, revealed that the major components were polysaccharides. In addition, the reported alga had relatively high mineral and low fat content, explaining approximately 25 % and 0.8 % of the dry weight, respectively. These figures are common to marine macroalgae.

Table 1. Chemical composition of *E. binghamiae*.

Component	Wet weight (%)	Dry weight (%)
Water	87.00	—
Crude protein	2.12	16.30
Carbohydrate	5.87	45.15
Crude fat	<0.10	0.77
Crude fiber	1.69	13.00
Ash	3.23	24.78

3.2. Mitogenic activity of crude extracts

Saline, ethanolic and hot-water extracts of the reported alga could significantly stimulate murine macrophages to proliferate. Of which saline extract had higher stimulation index than the other two extracts (Figure 1a), with maximal index of 1.62 at the concentration of 24.4 μg (dry wt) mL^{-1} . However, stimulation activity was variable with locality for algal sampling; the thalli collected from the intertidal zone of Lai-Lai coast had greater activity than that from other three coasts (Figure 1b).

3.3. Mitogenic activity of alginate and alginic acid

Both sodium alginate and alginic acid stimulated macrophages and T cells to proliferate. However, alginate appeared to be more active than alginic acid in mitogenesis, with maximal stimulation index of 2.0 for T cells at 0.25 μg glucose mL^{-1} (Figure 2a, Figure 2b). Beside, alginate stimulated cell proliferation in a concentration- dependent manner.

3.4. Mitogenic activity of the isolated protein

The isolated protein EBP from the reported alga was a 69 kDa glycoprotein, containing a small amount of carbohydrate (14%). As shown in the assay results, it strongly stimulated both macrophages and T cells to proliferate (Figure 3). The stimulating activity of

the protein was also concentration-dependent, reaching maximal index approximately 2.2 as protein increased to 0.125 and 0.025 $\mu\text{g mL}^{-1}$ for macrophages and T cells, respectively.

While the index decreased markedly, as the protein further increased beyond the optimum concentration.

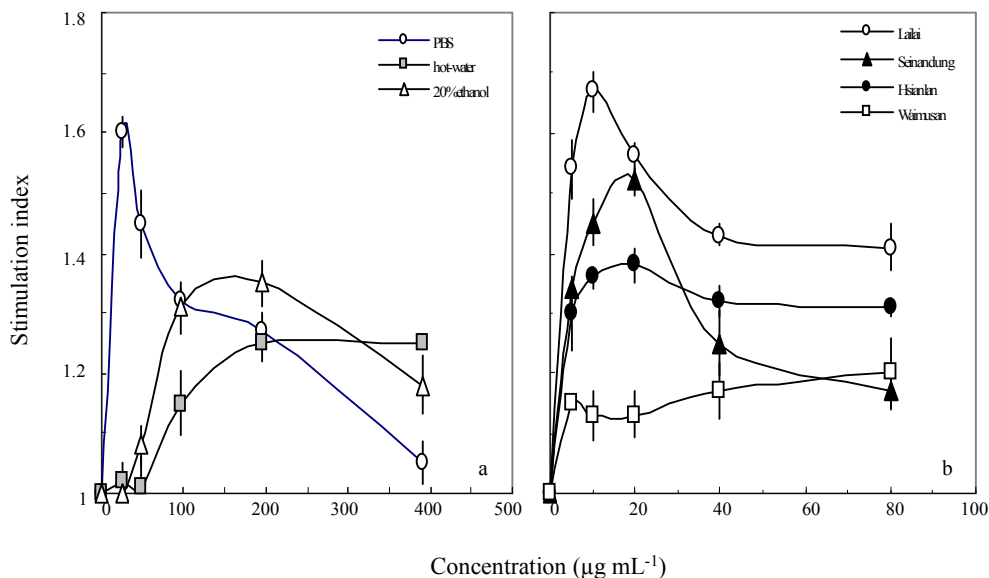


Figure 1. Stimulation indices of *E. binghamiae* extracts (a) and PBS extracts of the thalli collected from different localities (b) for murine macrophages.

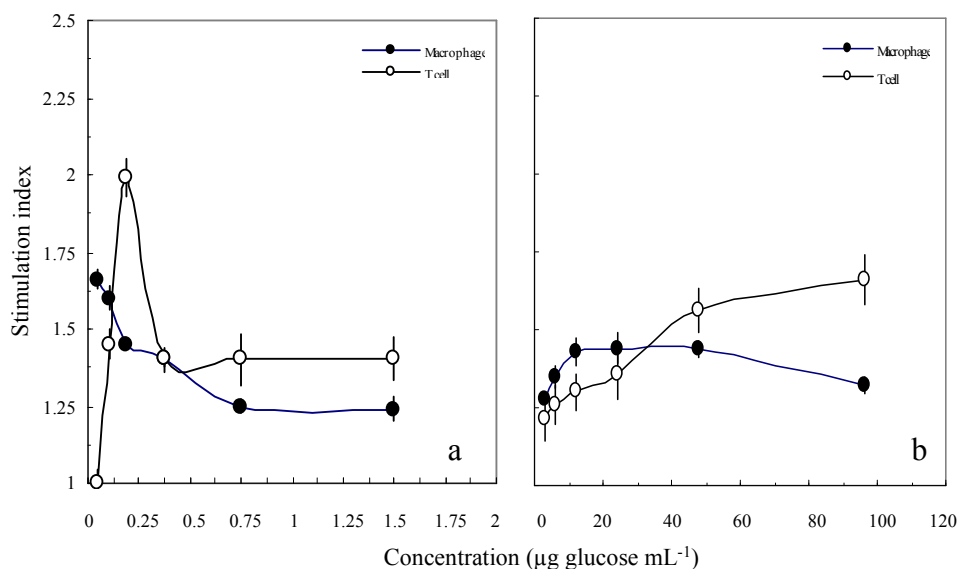


Figure 2. Stimulation indices of alginate (a) and alginic acid (b) for murine macrophages and human T cells.

3.5. Protein-induced $TNF-\alpha$, $IFN-\gamma$ and NO production

Compared to the control, the release of the

cytokine $TNF-\alpha$ and nitric oxide (NO) from macrophages was induced markedly in the presence of the isolated protein, with maximal increase of 70% and 45% for $TNF-\alpha$ and NO,

respectively, at the protein concentration of 0.15-0.16 $\mu\text{g}/\text{mL}^{-1}$ (Figure 4a). A rapid increase of IFN- γ , which was excreted from T cells, was also observed in the cultures following addition of the isolated protein. The maximum increase (90%) in contrast to the control, occurred at protein concentration of 0.15-0.2 $\mu\text{g}/\text{mL}^{-1}$, similar to macrophages (Figure 4b). This indicates that the induction of cytokines and NO was concentration dependent.

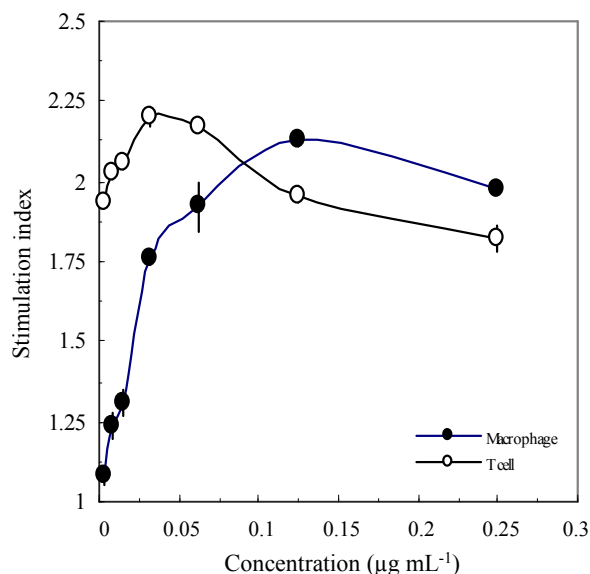


Figure 3. Mitogenic activity of the isolated protein against murine macrophages and human T cells.

4. Discussion

Marine algae have been shown to possess mitogenic activity both *in vitro* and *in vivo* as reported in previous studies (Yoshizawa et al., 1993; Liu et al., 1997; Shan et al., 1999; Son et al., 2001). The extracts of *Endarachne binghamiae* in buffered saline, hot water and aqueous ethanol effectively stimulated murine macrophages to mitosis, revealing that the reported alga has heterogeneous immune-active substances. However, such biological activity was variable with growth habitat of the alga assayed.

Marine algae are rich in sulfated polysaccharides which enhance glucose consumption

and cytokine production, such as Ig, IL-1, IL-6 and TNF- α and NO in macrophages and lymphocytes (Yoshizawa et al., 1993; Okai et al., 1997; Shan et al., 1999). Alginic acid and alginate are major matrix components of brown algal cell wall. Both are carboxylated polysaccharides, made of β -1,4 linked D-mannuronic acid residues with variable amounts of α -1,4-linked L-guluronic acid Mannuronic acid, but not guluronic acid, appears to regulate proliferation stimulation and cytokine production in macrophages and monocytes (Seljelid et al., 1989; Otterlei et al., 1991). A high mannuronic acid-containing alginate from the brown alga *Macrocystis pyrifera* has been reported to induce significant murine peritoneal macrophage proliferation and phagocytosis, as well as TNF- α , NO and H_2O_2 production (Son et al., 2001). Although we did not determine mannuronic acid and guluronic acid in this study; the obtained alginate extract exhibited strong mitogenic activity against murine macrophages, comparable with that of laminarin and fucoidan in previous study (Yoshizawa et al. 1993). Alginic acid also stimulated macrophage proliferation, but to a lesser extent. This implied that Na^+ binding to the polymer was necessary for a high mitogenic activity.

Several glycoproteins isolated from red marine algae exhibited both mitogenic activities while assayed using [^3H]-thymidine incorporation and/or MTT methods, except haemagglutinating activity (Hori et al., 1988; Kawakubo et al., 1997; Lima, 1998). In our study, protein isolated from the reported alga strongly stimulated both murine macrophages and human T lymphocytes to mitosis and release TNF- α , IFN- γ and NO at very low concentration. Such induced effect is concentration-dependent, being inhibited at higher concentrations, not due to cytotoxic, but presumably due to the increased unidentified compound formed following immune reactions, as observed earlier (Hori et al., 1988; Lima et al., 1998). Additionally, the isolated

protein is a powerful stimulant, as compared with solins and amasin (also glycoproteins) from other marine red algae.

It is known that the enhanced immune system by algal substances can induce antitumoral activity in the host (Yoshizawa et al., 1993; Okai et al., 1997). Considering the strong stimulation activity for cell proliferation and the secretion of cytokines, alginate extract and the isolated protein in the present study could induce intracellular signal transduction in macrophages and T cells. Macrophages and T cells are important in immune

system, and their tumoricidal activity may be mediated by the production of TNF- α and INF- γ , as well as the accumulation of NO. Various sulphated polysaccharides of seaweed origin have been shown recently to modulate immune responses via binding with cytokines (Nika et al., 2003). Thus, our alga can be a promising source of active substances for immune modulation and stimulation. However, the complete mechanism of alginate and protein exerts remain to be elucidated.

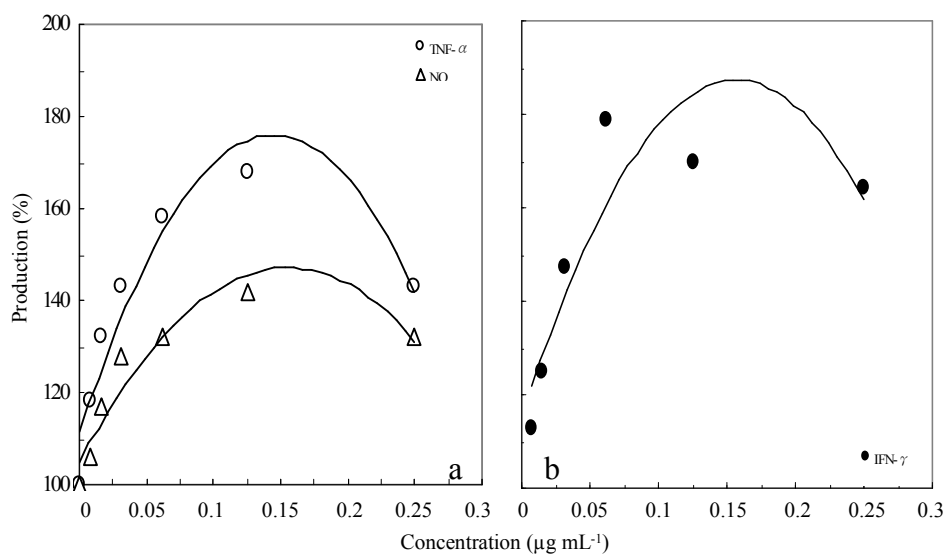


Figure 4. The induced production of TNF- α and NO by murine macrophages (a) and IFN- γ by human T cells (b) in presence of isolated protein EBP at various concentrations; the induced effect is expressed in percentage increase as compared with the production in the absence of EBP.

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