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***In vitro* promoting activity of human interferon β production by extracts of marine algae from Japan**

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Abstract

Two hundreds species of marine algae were investigated for *in vitro* promoting activity of human interferon β (IFN- β) production by poly(I:C)-induced human osteosarcoma cell line, MG-63. A brown alga, *Sargassum hemiphyllum* promoted most its activity, showing more than 11-fold. When we attempted to partially purify the active substances by particular two-step chromatography, two peaks of active fractions were obtained. These fractioned materials exhibited the heat-stable and non-cytotoxic characters with the molecular weight less than 3000.

Introduction

Marine algae form huge biocenosis and diversity with other micro- or macro-organisms in the sea. The extensive reports on screening and preparation of bioactive compounds from marine algae have been reviewed for such as antibacterial, antifungal, antitumor and antiviral activities (Rinehart, 1981). These data indicate that marine algae may be more advantageous for developing biologically useful substances (Richard, 1993; Harada *et al.*, 1997). However, the research on the natural products like marine algae have not been substantially conducted as terrestrial microorganisms such as actinomycetes groups.

In this study, we investigated for capability of *in vitro* promoting activity of IFN- β production in poly(I:C)-induced osteosarcoma line, MG-63 by marine algae extracts. Furthermore, the active substances were attempted to partially purify and characterize.

Materials and methods

Interferon β production

Two hundreds species (77 of brown, 81 of red, 38

of green and 4 of other seaweeds) of marine algae were successively extracted with phosphate-buffered saline (PBS) and MeOH (Harada *et al.*, 1997). One % each filter-sterilized PBS- or MeOH-extract was assayed for IFN- β production promoting activity by super-induction method (Tan *et al.*, 1970). MG-63 cells (ATCC-CRL-1427) were plated at a density of 1×10^5 cells per well in a 48-well plate and incubated overnight. Developed monolayer cells were induced IFN- β production by $0.2 \mu\text{g ml}^{-1}$ of poly(I:C) (Sigma) supplemented 2% fetal bovine serum and $5 \mu\text{g ml}^{-1}$ of cycloheximide (Sigma) with test algal extracts. After 4 h-incubation, $4 \mu\text{g ml}^{-1}$ actinomycin D (Wako chemicals) was added, incubated for 1 h, and then drugs were removed and cells were cultured with fresh medium for 24 h.

Enzyme-linked immunosorbent assay

96-well microplate was coated with mouse anti-human INF- β monoclonal antibody (YAMASA). Then, the culture fluids were added and incubated for 3 h. First and second antibodies were used rabbit anti-human IFN- β polyclonal antibody (Paesel) and peroxidase-conjugated goat anti-rabbit IgG antibody (Bio-Rad), respectively. P-2,2' azino-di-(3-ethylbenzothiazoline-

Figure 1. Sephadex LH-20 chromatogram of IFN- β production promoting active fraction from *S. hemiphyllum*.

6-sulfonic acid) diammonium salt (ABTS) was used as a substrate. The developed pigment was read at A_{405} – A_{490} .

MTT assay

Six human cell lines, HDL, WI-38, HL-60, MG-63, MOLT-4 and WIL-2NS were employed. These cells were plated at a density of 5×10^4 cells per well in a 48-well plate and incubated for 3 days with test algal extracts. The growth was measured at $A_{570} - A_{655}$ with formazan after treated with 0.5 mg ml^{-1} of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 h at 37°C .

Partially purification and characterization

S. hemiphyllum MeOH-extract was separated twice with EtOAc/H₂O (2:1) and BuOH/H₂O (1:1). The concentrated water layer was chromatographed on Sephadex LH-20. Subsequently, the pooled active fraction was applied to sephadex G-25 chromatography. These partially purified substances were heated at 100°C for 20 min and ultrafiltered with 3000 MW-cut off membrane filter (Milipore).

Results and discussion

The MeOH-extracts from 12 *Sargassum* species exhibited prominent IFN- β production promoting activity (Table 1). In particular, two MeOH-extracts from *Agarum crathrum* and *Sargassum hemiphyllum* remarkably activated IFN- β production approximately 10 times higher than negative control. Thus, we attempted to partially purify the active substances from the crude

Table 1. Algal species showing IFN- β production promoting activity

Type	Algal species	Activity ^b
Green	<i>Caulerpa cupressoides</i>	3.32 ^a
	<i>Caulerpa racemosa</i>	3.86 ^a
	<i>Caulerpa wabbiana</i>	2.1
	<i>Chaetomorpha moniligera</i>	2.38
	<i>Halicoryne wrightii</i>	3.01
	<i>Halimeda incrassata</i>	2.21 ^a
	<i>Halimeda opuntia</i>	2.06
Red	<i>Eucheuma denticulatum</i>	3.85 ^a
	<i>Martensia fragilis</i>	3.18
	<i>Melanamansia glomerata</i>	2.64 ^a
	<i>Meodilsea yendoana</i>	2.71
	<i>Peyssonnelia caulifera</i>	3.70 ^a
	<i>Porhyra tenera</i>	2.63
	<i>Porphyra tenera</i>	2.63
Brown	<i>Agarum crathrum</i>	10.38
	<i>Agarum crathrum</i>	2.04 ^a
	<i>Alaria crassifolia</i>	2.09
	<i>Bornetella nitida</i>	2.42
	<i>Colpomenia sinuosa</i>	2.27 ^a
	<i>Dilophus okamurae</i>	2.11
	<i>Ecklonia kurome</i>	3.95
	<i>Eisenia bicyclis</i>	5.08
	<i>Hormophysa cuneiformis</i>	2.31
	<i>Laminaria angustata</i>	2.62
	<i>Laminaria japonica</i>	3.30
	<i>Leathesia difformis</i>	3.42
	<i>Myelophycus simplex</i>	3.04
	<i>Padina arborescens</i>	2.76
	<i>Sargassum confusum</i>	2.28
	<i>Sargassum crassifolium</i>	2.03
	<i>Sargassum crispifolium</i>	2.41
	<i>Sargassum duplicatum</i>	2.01
	<i>Sargassum fulvellum</i>	5.45
	<i>Sargassum hemiphyllum</i>	11.25
	<i>Sargassum macrocarpum</i>	2.28
	<i>Sargassum poluliferum</i>	2.33
	<i>Sargassum ringgoldianum</i>	3.41
	(ssp. ringgoldianum)	
	<i>Sargassum ringgoldanum</i>	2.38
	(ssp. coreanum)	
	<i>Sargassum sp.</i>	2.51
Other	<i>Zostera japonica</i>	2.76

^a are the promoting activity of PBS-extract and the others are that of MeOH-extract; ^b relative productivity.

MeOH-extracts from *S. hemiphyllum* because of high-stable activity of this alga. After two-step chromatographies with sephadex LH-20 and G-25, two promoting active fractions were detected (Figures 1 and 2). These

Figure 2. Sephadex G-25 chromatogram of IFN- β production promoting active fraction after LH-20 chromatography. Three mg of sample was applied to ϕ 25 mm x 300 mm column and eluted by H₂O/MeOH (4:1). IFN- β production promoting activity of each fraction was assayed at concentrations of 1 μ g ml⁻¹ (—●—) or 4 μ g ml⁻¹ (—○—), respectively.

Figure 3. Effect of two active peak fractions on IFN- β production.

two active fractions enhances dose-dependently IFN- β production more than 10-fold than negative control at 0.5–2 μ g ml⁻¹ and 4–8 μ g ml⁻¹, respectively (Figure 3), while sphingosine (Osada *et al.*, 1992) and poly-L-lysine (Yamamoto *et al.*, 1995) known as the active substances showed less than twice (data not shown). In addition, heat-treatment little affected on these IFN- β production promoting activities. The active substances might be less than 3000 molecular weight. Further cytotoxic study with six human cell lines indicated that cytotoxicity was not observed even at 200 μ g ml⁻¹ in all cell lines tested (data not shown). These results suggested that activation of IFN- β production by the extract of *S. hemiphyllum* is not presumably caused by accelerated synthesis of proteins such

as heat shock protein under the fatal culture conditions, indicating that the active substances in *S. hemiphyllum* might be novel compounds.

The IFNs are non-specific factor in vertebrates host defense system against viral and parasitic infections and certain tumors (Sen and Lengyel, 1992). Thus, it is considered the possibilities of IFNs as a drug for several diseases (Smuel *et al.*, 1991), although the administration is restricted by several problems such as rapid reduction of blood IFN level, side effects and high cost. The use of strong chemical IFN inducer, poly(I:C) (Gariglio, 1991; Wong *et al.*, 1995) is also prevented because of the cytotoxic property. In this point, the IFN- β production promoting substances from *S. hemiphyllum* may solve these chemotherapeutic problems and be useful for immunotherapy by spontaneously activating host immune defense systems.

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