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Antioxidant and TNF-α Inducing Ability of *Styela plicata* Extracts

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After preparing extracts from fresh *Styela plicata* using methanol, ethanol, acetone, and water, the antioxidant and tumor necrosis factor (TNF)-α inducing ability of the extracts were evaluated. Most of the extracts showed more than 50% inhibitory activity against malonedialdehyde formation, and methanol extract exhibited the highest activity with values of 59.3%. The water and acetone extracts contained glutathione contents of 18.5 and 12.3 μM, respectively, however, these were not detected in the other extracts. TNF-α inducing abilities of the extracts were determined with RAW 264.7 cells. The water extract increased TNF-α production with correlation to increase of the added amount. However, the other extracts could not induce TNF- α production in RAW 264.7 cells. The results indicated that the antioxidant activities of *S. plicata* extracts were different depending on extracting solvents, and the water extract showed significant TNF-α inducing activity.

Key words : Antioxidant, immunostimulatory, *Styela plicata*, TNF-α

Introduction

Reactive oxygen species (ROS) and free radicals are continuously generated under normal physiological conditions but effectively eliminated by existing antioxidative defense mechanisms such as antioxidative enzymes [5]. Due to many environmental, lifestyle, and pathological situations, however, excess radicals can accumulate and result in oxidative stress [24]. The oxidative stress is believed to be a primary factor in various diseases such as cancer, cardiovascular disease, diabetes, neurodegenerative disease, and osteoporosis as well as in the normal process of aging [9]. Though some synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene are very effective to scavenge ROS, carcinogenic and toxic properties of them also have been reported [1]. Thus, researches on the potential applications of natural antioxidants have recently received much attention [14,25].

The sea, covering more than 70% of the surface of earth, contains an exceptional biological diversity, accounting for more than 95% of the whole biosphere [8]. Therefore, the marine environment is a sensational resource for the development of novel natural antioxidant. So many antioxidant researchers have largely focused on the antioxidant effects of crude extracts from marine organism such as tunicate, sponge, bacteria, coral, and alga [20]. Among various marine organisms, ascidians are marine animals with a high ability to synthesize bioactive substances [11]. *Styela plicata* belongs to *Ascidiacea* class, which commonly known as a sea squirt. Several researches such as antithrombotic properties of a heparin [2], antimicrobial peptide [23], and antiviral activity [7] of *S. plicata* have been reported. In this paper, the antioxidative and tumor necrosis factor (TNF)-α inducing ability of the extracts of *S. plicata* with four different solvents were evaluated.

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Meterials and Methods

Materials

Styela plicata was purchased in September, 2006, at the fish market of Masan City, Korea. Dimethyl sulfoxide (DMSO), potassium chloride, potassium phosphate, fish oil, and reduced glutathione (GSH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Malonedialdehyde (MDA) assay kit and GSH assay kit were purchased by OXIS Health Products, Inc. (Portland, OR, USA). All organic solvents and other chemicals were all of analytical grade and use as received.

Preparation of extracts from *Styela plicata*

Fresh *S. plicata* (100 g) was extracted with 1 l of solvents (methanol, ethanol, acetone, and water, respectively) in a shaking incubator (100 rpm) for overnight at room

temperature. The extract was filtered through a Whatman No. 1 filter paper (Advantec, Tokyo, Japan), and then the filtrate was evaporated to dryness under reduced pressure on a rotary evaporator at 40°C. The dried extract was dissolved in DMSO with concentration of 50 mg/ml for further experiments, and diluted with DMSO when needed.

MDA formation inhibitory activity

One ml of the solvent extract in DMSO (5 mg/ml) was mixed with 2.5 g of fish oil. The mixture was oxidized using an automated Metrohm 793 Rancimat (Herisan, Switzerland) at 100°C for 5 min. The airflow rate through the sample was adjusted to 20 ml/hr. After oxidization, the mixture was cooled by fan for 5 min. at room temperature. The amount of malonedialdehyde (MDA) in the mixture was measured by using Bioxytech MDA-586 Kit according to the manufacture's instructions. MDA formation inhibitory activity was expressed as a percentage inhibition, and it was calculated by using the following formula:

% MDA formation inhibitory activity=

[1-(MDA amount of sample/MDA amount of control)]×100

Determination of glutathione content

Glutathione content of *S. plicata* extract was determined by using GSH assay Kit. The extract dissolved in DMSO was diluted with potassium phosphate buffer (final concentration of 10 mg/ml). The diluted sample (900 μl) was added to 50 μl of chromogenic reagent (4-chloro-1-methyl-7-trifluromethyl-quinolium methylsulfate) in HCl, and 50 μl of 30% NaOH was added to the mixture. The mixture were incubated at 25°C for 10 min in the dark, and then the absorbance was measured at 400 nm. The standard curve was drawn using 0-100 μM of reduced glutathione.

Determination of TNF-α in macrophage cell

RAW 264.7 mouse macrophage cell line was obtained from the American Type Culture Collection. Cells were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL) and maintained at 37°C in a humidified incubator containing 5% CO_2. The amount of TNF-α in the cell line was determined by using mouse TNF-α (Mono/Poly) ELISA Kit (BD OptEIA, BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacture's instructions. Briefly, the RAW 264.7 cells were seeded onto a 96-well plate (5×10^4 cells/well) for experiments. The cells were pretreated with 0.1, 0.5, 1, 5, and 10 μg/ml of *S. plicata* extract dissolved in 0.01% DMSO, respectively. After 24 hr of incubation, supernatant was used for TNF-α determination. For the control group, RAW cells were cultured with 0.01% DMSO alone.

Statistical analysis

All measurements were done in triplicate, and analysis of variance was conducted according to the procedure of the General Linear Model using SAS software [19]. Student-Newman-Keul's multiple-range tests were used to compare the significant differences of the mean value among treatments ($P<0.05$).

Results and Discussion

Malondialdehyde (MDA) formation inhibitory activity of *Styela plicata* extracts

Oxidatively damaged lipids have been linked to cancer, coronary heart disease, and the degenerative diseases of aging. The extent of lipid peroxidation has been quantified by measuring various analytes, including LOOH, conjugated dienes, expired hydrocarbons, and carbonyls. Carbonyls have generated biochemical interest because of their intrinsic potential for toxicity. MDA is a well known carbonyl in rancid foods. In biological systems, it is a product of pathologic lipid oxidation and normal prostaglandin biosynthesis.

MDA formation inhibitory activity of each *S. plicata* extract was shown in Table 1. The results indicated that *S. plicata* extracts could significantly inhibit the formation of MDA. Methanol extract of *S. plicata* showed the strongest inhibitory activity with value of 59.3%. Ethanol and water extracts of *S. plicata* also showed more than 50% of inhibitory activity against MDA formation. This result coincides with that antioxidant activity of *S. plicata* determined by reducing

Table 1. MDA formation inhibitory activity and glutathione contents of *Styela plicata* extracts

	Extraction solvent			
	Methanol	Ethanol	Acetone	Water
MDA formation inhibitory activity (%)	59.3[a]	53.9[b]	33.3[c]	54.0[b]
Glutathione Contents (μM)	ND	ND	12.3[b]	18.5[a]

ND means not detected. All measurements were done in triplicate, and analysis of variance was conducted by of the General Linear Model using SAS software. Different letters (a-c) within a row are significantly different ($P<0.005$).

power was the greatest in methanol extract [6]. Antioxidant activity of natural extracts strongly depends upon the solvent used for extraction [13] because each compound has different polarity [10]. In case of *S. plicata,* antioxidant materials for MDA formation inhibitory activity might prefer to be dissolved in methanol.

S. plicata belongs to Urochordata Subphylum of Chordate Phylum, and its biological characteristics were very similar to those of *Halocynthia roretzi. H. roretzi* contains variety of carotenoids, which show strong antioxidant activity [3,4]. Carotenoid isolated from cartotenoprotein of *S. plicata* has been identified as astaxantnin [15]. Catotenoids including astaxanthin of *S. plicata* extract might play important role in MDA formation inhibitory activity.

Glutathione (GSH) contents of *Styela plicata* extracts

GSH is a naturally occurring tripeptide, which has been identified as an important role in antioxidant system [22]. GSH can directly scavenge free radicals or act as coenzyme with a variety of enzymes including glutathione peroxidase, glutathione S-transferase, and thiol transferase [18]. Table 1 showed GSH contents of four kinds of extracts from *S. plicata*. GSH contents of water and acetone extracts were 18.51 and 12.27 μM, respectively, however, it was not detected in methanol and ethanol water. Two points - solubility and stability of GSH - can be discussed from the results. In the view of the solubility of GSH dependent on solvent, GSH has been reported that it is freely soluble in water, diluted alcohol, liquid ammonia, and dimethylformamide [12]. This is one of the reasons why the water extract showed the highest GSH content, and no GSH in concentrated methanol or ethanol. Secondly, the stability of GSH can be discussed in point of peptide. GSH is a tripeptide (γ-glutamyl-L-cysteinylglycine), and two amino acids (L-glutamic acid and L-cysteine) of GSH have hydrophilic side chains. Therefore GSH of *S. plicata* prefers to be extracted in water.

Table 2. Effects of *Styela plicata* extracts on the secretion of TNF-α in RAW 264.7 cells (pg/ml)

Concentration (μg/ml)	Extraction solvent			
	Methanol	Ethanol	Acetone	Water
0	312.49^{aw}	276.87^{aw}	264.92^{aw}	285.38^{az}
0.1	273.43^{abw}	239.49^{bw}	248.59^{bw}	306.32^{az}
0.5	275.45^{bw}	352.62^{bw}	301.76^{bw}	1320.62^{ay}
1	223.15^{bw}	198.47^{bw}	246.67^{bw}	1367.99^{ay}
5	232.10^{bw}	258.28^{bw}	376.48^{bw}	4172.04^{ax}
10	312.67^{bw}	297.80^{bw}	225.92^{bw}	5940.46^{aw}

All measurements were done in duplicate, and analysis of variance was conducted by of the General Linear Model using SAS software. Different letters (a-b) within a row are significantly different (*P*<0.005), Different letters (w-z) within a column are significantly different (*P*<0.005).

TNF-α inducing ability of extracts from *Styela plicata*

TNF-α inducing ability of *S. plicata* extract was with RAW 264.7 cells. TNF-α is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. The primary role of TNF-α is in the regulation of immune cells. TNF-α is also able to induce apoptotic cell death, to induce inflammation, and to inhibit tumorigenesis and viral replication.

After stimulating RAW 264.7 cells with the *S. plicata* extracts for 24 hr, produced TNF-α was evaluated(Table 2). The water extract of *S. plicata* increased TNF-α production with increasing the added amount. In concentration of 10 μg/ml of the water extract, the amount of TNF-α in RAW 264.7 cells was 5,940.46 pg/ml, which was more than 20 times of that in non-added control cells. However, methanol, ethanol, and acetone extracts of *S. plicata* could not induce TNF-α production in RAW 264.7 cells. The results indicated that immunostimulatory activity of *S. plicata* was strongly restricted in the water extract. While Ohgami *et al.* [17] reported that astaxanthin, a carotenoid found in sea animals, inhibited TNF-α generation, water soluble compound of *S. plicata* showed TNF-α inducing effect. Marine omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been reported to suppress TNF-α production [21]. Though *S. plicata* contains high amount (32.5%) of omega-3 fatty acids among total fatty acids [16], water extracts might embrace water-soluble TNF-α inducing materials. The immunostimulatory compound should be studied further.

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초록 : 오만둥이의 항산화 및 TNF−α 유도 활성

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메탄올, 에탄올, 아세톤 그리고 물을 이용하여 신선한 오만둥이의 추출물을 제조한 후, 추출물의 항산화능 및 TNF-α 유도능을 조사하였다. 아세톤 추출물을 제외한 대부분의 추출물은 malonedialdehyde 생성을 50% 이상 저해하는 항산화능을 보였으며, 메탄올 추출물은 가장 높은 값인 59.3%의 malonedialdehyde 생성 저해능을 나타내었다. 물과 아세톤 추출물은 각각 18.5와 12.3 μM의 glutathione 함량을 보였지만 그 외의 추출물에서는 검출되지 않았다. 추출물들의 TNF-α 유도능을 RAW 264.7 세포주로 조사하였다. 오만둥이 물 추출물은 첨가량이 증가할수록 TNF-α 생성을 증가시켰다. 그러나 그 이외의 추출물들은 RAW 264.7 세포주에서의 증가할수록 TNF-α 생성을 유도하지 못하였다.