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Antioxidant activity of enzymatic extracts from brown seaweeds

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국문초록

제주도는 사면이 바다라는 지리적인 조건과 청정해역이라는 천해의 조건을 두루 갖추고 있는 지역으로서 특히 다양한 해조류들이 풍부하게 서식하고 있다. 해조류는 육상식물에 비해 생육하는 환경이 현저한 차이가 있어 구성성분이 다를 뿐만 아니라 미네랄과 비타민이 매우 풍부하게 함유하고 있고, 또 어떤 특정적인 해조류의 성분은 항균, 항산화, 항바이러스 활성을 비롯하여 혈압 및 혈중 콜레스테롤의 조정, 항종양활성, 면역활성 자극효과 등을 나타내는 것으로 알려져 있다. 해조류를 이용한 항산화제의 연구는 해조류를 많이 섭취하는 일본에서 특히 많은 연구가 이루어져 있으며 국내에서도 꾸준히 이루어지고 있다. 최근 들어 해조류를 비롯한 여러 천연물에서 항산화 물질을 탐색하는 이유는, 국민소득이 증대됨에 따라 식생활이 서구화되면서 고혈압, 당뇨병, 고지혈증, 비만 및 대장암 등 각종 성인병이 유발되고, 사회가 점차 고도화됨에 따라 고령화인구가 늘어나게 되면서 건강문제에 대한 인식이 바뀌게 되었기 때문이다. 또한 합성 항산화제를 다량 섭취했을 때의 독성문제가 야기되면서 인체에 무해하고 활성이 우수한 천연 항산화제를 찾기 위한 노력이 계속되고 있다. 지금까지 연구되었던 대부분의 연구결과는 유기용매 추출물을 이용해 항산화 활성을 검색하였다. 하지만 이것은 수율이 아주 낮고, 그들이 원래 내재하고 있는 유독성에 의한 인체독성이나 혹은 양적, 경제적인 이유로 인해 효과적인 항산화제를 개발한 경우는 극히 드물다.

따라서 이 연구에서는 해조류로부터 수용성이면서 인체에 무해한 천연 항산화물질을 개발하기 위한 목적으로 지금까지 시도된 적이 없는 효소적 가수분해방법을 이용하여 각종 효소적 추출물을 조제해 내고, 그것들의 라디칼 소거활성, 활성산소종 소거활성, 과산화지질 억제활성 및 DNA 손상 억제활성에 대하여 검토하였다. 그 결과 감태의 효소추출물에서 가장 높은 라디칼 소거활성을 나타내었고, 괭생이 모자반의 효소추출물이 우수한 과산화수소 소거활성을 나타내었다. 항산화 작용에 관련된 여러 가지 활성 검색 중에 과산화수소 소거활성이 가장 높은 효과를 나타내었는데 특히 괭생이 모자반과 지충이의 Ultaflo와 Alcalase 추출물은

90% 이상의 높은 소거활성을 나타내었다. 이러한 결과를 바탕으로 하여 DNA 손상 억제활성을 human lymphocytes를 이용해 확인해 본 결과 화학적 실험에서 뿐만 아니라 cell상에서도 우수한 항산화 효과가 나타난 것을 확인할 수 있었다.

이러한 결과를 종합해 볼 때, 해조류의 효소적 가수분해물은 각종 질병을 유발시키는 활성 산소종들을 효과적으로 억제시킬 수 있을 뿐만 아니라, 수용성의 천연 항산화물질이라는 점 때문에 산업적 용도가 매우 다양하며 또한 기존의 항산화제가 갖고 있던 인체 안전성문제도 충분히 극복할 수 있을 것으로 판단된다. 따라서 이 연구에서 수행된 해조류 유래 수용성 항산화물질을 효과적으로 추출해 낸다면 잠재적 의약품으로서 혹은 식품분야에도 광범위하게 이용될 수 있을 것이다.

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INTRODUCTION

Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) are physiological metabolites formed during aerobic life as a result of the metabolism of oxygen (Fridovich, 1974). DNA, cell membranes, proteins and other cellular constituents are target site of the degradation processes, and consequently induce different kinds of serious human diseases including atherosclerosis, rheumatoid arthritis, muscular dystrophy, cataracts, some neurological disorders and some types of cancer as well as aging (Kovatcheva *et al*., 2001; Ruberto *et al*., 2001; Lim *et al*., 2002). Moreover, ROS are predominant causes of qualitative decay of foods which lead to rancidity, toxicity and destruction of biomolecules important in physiologic metabolism. During last few decades, various synthetic [BHT (butylated hydroxytoluene), BHA (butylated hydroxyanisol) and TBHQ (tert-butylhydroquinone)] and natural antioxidants (vitamin C and E, carotenoids, flavonoids, phenolic compounds) have been used to prevent or to retard the lipid oxidation by ROS. Due to some side effects and toxicity of the synthetic antioxidants, however, much higher attention of investigators and consumers are focusing on natural antioxidants. But there are some disadvantages of natural antioxidants. Specially, Vitamin E, carotenoids, and phenolic compounds are water-insoluble. Moreover, vitamin C as well as E are greatly heat-sensitive and denatured easily.

Over the past several decades, seaweeds or their extracts have been studied as novel sources which have been shown to produce a variety of compounds and some of them have been reported to possess biological activity of potential medicinal value (Moore, 1978; Anggadiredja *et al*., 1997; Tutour *et al*., 1998; Konig *et al*., 1994; Satoru *et al*., 2003; Damonte *et al*., 1994; David and Victor, 2000). Recently, much attention has been paid on the anti-tumor activity, anticholesterolemic activity and antioxidant activity of seaweed

constituents. Especially, antioxidant activity is intensively focused due to the currently growing demand from the pharmaceutical industry where they are interested in anti-aging and anticarcinogenic natural bioactive compounds, which posses health benefits. Almost all photosynthesizing plants including seaweeds are exposed to a combination of light and high oxygen concentrations, which lead to the formation of free radicals and other strong oxidizing agents, but they do not affect from any photodynamic damage during living. This fact implies that their cells have protective antioxidative mechanisms and compounds (Dykens *et al*., 1992; Sukenik *et al*., 1993; Matsukawa *et al*., 1997). Additionally seaweeds are rich in vitamins, minerals, natural bioactive compounds and various functional polysaccharides. In particular, the polysaccharides present in brown seaweeds, such as alginates, fucans and laminarans are water-soluble dietary fibers and possess various bioactive properties. These polysaccharides are little or non-digested by digestive enzymes produced by human intestine. Instead the polysaccharides are considered to be digested by some types of carbohydrases derived from microorganisms. Those kinds of enzymes can convert water-insoluble seaweed materials into water-soluble components. It is thought that a variety of bioactive effects including antioxidant activities will be expected from the resulting such components. Despite of the urgent need to screen seaweeds having the antioxidant activities no report had dealt with producing enzymatic extracts of seaweeds and investigating their bioactivities.

In the present study, some edible seaweeds were enzymatically hydrolyzed with various types of carbohydrases and proteases derived from microorganisms. Those enzymatic extracts from seaweeds have water-solubility and safety because of no use of organic solvent or toxic chemicals during extraction. Additionally, it has a high yield and high radical scavenging activity compared to organic solvent extracts. The objective of this study is to evaluate the antioxidative effect from enzymatic extracts of seaweeds using different assays. And then investigate a potential as a water-soluble antioxidant.

Part Ⅰ.

Antioxidant activity of enzymatic extracts from brown seaweeds

Part Ⅰ.

Antioxidant activity of enzymatic extracts from brown seaweeds

1. ABSTRACT

This work was carried out to find water-soluble natural antioxidants from seaweeds. Seven species of brown seaweeds (*Ecklonia cava, Ishige okamurae, Sargassum fulvellum, Sargassum horneri, Sargassum coreanum, Sargassum thunbergii,* and *Scytosiphon lomentaria*) growing along Jeju Island coasts were collected and enzymatically hydrolyzed to prepare water-soluble products. Enzymes used were five carbohydrases (Viscozyme, Celluclast, AMG, Termamyl, and Ultraflo) and five proteases (Protamex, Kojizyme, Neutrase, Flavourzyme, and Alcalase) commercially available from Novozyme Co. Antioxidant activities of enzymatic extracts from seaweeds were determined using two different assays, free radical scavenging activity by 1,1-diphenyl-2-pricrylhydrazyl (DPPH) decolorization assay and inhibitory capacity of lipid peroxidation in linoleic acid. Among all the enzymatic extracts enzymatically produced from the seven species of seaweeds, the extracts of *E. cava* scavenged most effectively free radicals released from DPPH and especially Cellulast extract of *E. cava* caused around 80% scavenging activity at the extract concentration of 8 mg/mL. The antioxidant assay in terms of inhibitory capacity of lipid peroxidation revealed that Ultraflo and Alcalase extracts of *E. cava* and Neutrase extract of *S. lomentaria* had the highest inhibitory capacity. In particular it was noteworthy that the Neutrase extract of *S. lomentaria* had completely suppressed the lipid peroxidation during five days of the incubation period. In contrast to this result observed in the lipid peroxidation those extracts were shown to have poor radical scavenging activity. These results indicate that a large difference exists between

the two antioxidant activity assays mentioned above. We demonstrated in the study that enzymatic extracts of seaweeds possess a potent antioxidant activity.

2. MATERIALS AND METHODS

2. 1. Materials

Seaweeds were collected along Jeju Island coast of Korea during a period from October 2001 to March 2002. Of the Jeju coastal seaweeds, seven species of brown algae samples (Table 1-1) were collected, and then salt, epiphytes and sand, that had been attached to the surface of the samples, were removed using tap water. Finally the seaweeds were rinsed carefully in freshwater and stored in a medical refrigerator at -20℃. The frozen samples were lyophilized with a grinder to carry out the enzymatic extraction. Carbohydrase (Viscozyme L, Celluclast 1.5L FG, AMG 300L, Termamyl 120L, Ultraflo L) and Protease (Protamex, Kojizyme 500 MG, Neutrase 0.8L, Flavourzyme 500 MG, Alcalase 2.4L FG) were donated from Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark). 1,1-Diphenyl-2-pricrylhydrazyl (DPPH), thiobarbituric acid (TBA), trichloroacetic acid (TCA), butylated hydroxyanysol (BHA), butylated hydroxytoluene (BHT), α-tocopherol and linoleic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). And other chemicals used were 99% or greater purity.

2. 2. Approximate chemical compositions of seaweeds

Approximate chemical compositions of the seaweeds were determined according to AOAC method (1990). Crude carbohydrate was determined by phenol-sulfuric acid reaction (absorbance at 480nm, using glucose as the calibration standard), crude lipid was performed by Soxhlet method and crude ash was prepared at 550℃ in the dry-type furnace. The amount of crude protein was determined by Lowry

Table 1-1. The list of brown seaweeds and collection site

Scientific name	Selection site*
Ecklonia cava	Hamdeok
Ishige okamurae	Seongsan
Sargassum fulvellum	Hamdeok
Sargassum horneri	Samyang
Sargassum coreanum	Samyang
Sargassum thunbergii	Hamdeok
Scytosiphon lomentaria	Seongsan

* Seaweeds were collected at the above places of Jeju Island in Korea.

method, measuring absorbance at 540 nm using bovine serum albumin as the calibration standard (Lowry *et al.*, 1951).

2. 3. Preparation of enzymatic extracts from seaweeds

The seaweed samples were pulverized into powder using a grinder. A hundreds mL of buffer solution was added to one gram of dried alga, and then 100㎕ (or mg) of enzyme was mixed. The enzymatic hydrolysis reactions were performed for 12 h to achieve optimum hydrolysis. The hydrolysates were clarified by centrifugation at 3,000 rpm for 20 min to remove the residue unhydrolyzed. The degree of enzymatic hydrolysis was determined by subtracting the dried weight of the residue from one gram of seaweed samples dried and was expressed as a percentage. Enzymatic extracts of seaweeds was obtained after filtering the supernatant and were used for two assays of antioxidant activity, composed of radical scavenging activity and inhibitory capacity of lipid peroxidation. Concentrations of all the extracts were adjusted to 4 mg/mL.

2. 4. Free radical scavenging activity by DPPH decolorization

Free radical scavenging activity (RSA) of the enzymatic extracts of seaweeds was determined by using stable free radical, DPPH, according to the modified method of Blois (1958). DPPH solution was prepared at the concentration of 4×10^{-4}M in ethyl alcohol. During the assay, the enzymatic extract of 0.1 mL was mixed with 2.9 mL DPPH solution. The mixture was incubated in the room temperature for 30 min. After standing for 30 min, absorbance was read at 516 nm, and the percentage of inhibition was defined by the absorbance at 516 nm in the absence of enzymatic extract to that measured with the sample. Commercial antioxidants such as α-tocopherol, BHA and BHT, whose concentrations were all 2 mg/mL, respectively, were assayed together as controls.

2. 5. Total phenolic assay

Phenolic contents were determined using the protocol similar to Chandler and Dodds (1983) described by Shetty *et al.* (1995). Each 1.0 mL of seaweed enzymatic extracts, 1.0 mL of 95% EtOH, 5.0 mL of distilled water, and 0.5 mL of 50% Folin-Ciocalteu reagent (Sigma Chemical, St. Louis, MO) were mixed and then were allowed to react for 5 min. 1.0 mL of 5% Na_2CO_3 was added, and the mixture was vortexed and placed in the dark for 1 h. Absorbance was measured at 725 nm using gallic acid as the calibration standard.

2. 6. Inhibitory capacity of lipid peroxidation in linoleic acid

The assay for inhibition of lipid peroxidation in linoleic acid was carried out in linoleic acid system with and without (control group) added antioxidants according to the modified method of Mitsuda *et al.* (1996) and Sidwell *et al.* (1954). The substrate solution was made by mixing 0.1M phosphate buffer (pH 7.0) and ethanol (4:1, v/v). Then 20 mL of substrate solution, 0.8 mL sample solution and 0.1M phosphate buffer 19.2 mL were mixed and incubated in 40℃ for 5 days. During incubation, 2 mL of each mixture was taken in every 24 h interval. Then each 2 mL of mixture, 1 mL of 35% TCA and 2 mL of 0.75% TBA were mixed and placed in a boiling water bath for 40 min. The tubes were cooled and 1 mL of acetic acid and 2 mL of chloroform were added. Then the mixture was throughly mixed and centrifuged at 4,000 rpm for 5 min. Absorbance of the supernatant was measured at 532 nm and TBA values were calculated.

3. RESULTS

3. 1. Approximate chemical compositions

Approximate chemical compositions of seven species of brown seaweeds, *Ecklonia cava, Ishige okamurae, Sargassum fulvellum,*

Table 1-2. Chemical compositions of brown seaweeds (%)

Scientific name	Moisture	Ash	Protein	Carbohydrate	Lipid
Ecklonia cava	4.29	15.41	10.55	68.42	1.33
Ishige okamurae	4.49	13.26	18.9	60.69	2.66
Sargassum fulvellum	5.13	17.85	14.2	62.49	0.33
Sargassum horneri	5.19	20.83	17.2	55.45	1.33
Sargassum coreanum	4.3	12.77	14.4	67.2	1.33
Sargassum thunbergii	8.9	13.3	13.85	63.62	0.33
Scytosiphon lomentaria	4.62	24.37	16.85	53.49	0.67

Sargassum horneri, Sargassum coreanum, Sargassum thunbergii and *Scytosiphon lomentaria*, collected along Jeju-Do coasts were indicated in Table 1-2. The major chemical component of the seaweeds tested was found to be carbohydrate whose content occupied over 60% of the total dry weight, except for *S. lomentaria* (53.49%). Protein contents determined from the seaweeds were between 10.6% and 18.9% showing *I. okamurae* has the highest protein content. Ash contents of most of the tested seaweeds were around 15% but *S. horneri* and *S. lomentaria* showed the value of 20.8% and 24.4%, respectively.

3. 2. Preparation of enzymatic extracts from seaweeds

The seaweeds were enzymatically hydrolyzed by using the five types of carbohydrases (Viscozyme, Celluclast, AMG, Termamyl, and Ultraflo) and five proteases (Protamex, Kojizyme, Neutrase, Flavourzyme, and Alcalase) to produce enzymatic extracts as potential natural water-soluble antioxidants. As shown in Table 1-3, most of the seaweeds showed much higher susceptibility to the action of carbohydrases than proteases. Among the five kinds of carbohydrases, particularly, Viscozyme, Celluclast and AMG exhibited more than 30% of the hydrolytic degree for most seaweeds, except *S. lomentaria*. And these enzymes were able to hydrolyze around 40% for *E. cava*. The higher degree of enzymatic hydrolyses of *E. cava* seemed to reflect the higher content of carbohydrate in this seaweed. In contrast to this, *S. lomentaria* had the lowest content of carbohydrate. Enzymatic hydrolyses of the seaweeds by the proteases were less efficient than those by the carbohydrases, even though Alcalase showed similar hydrolytic levels compared to the three types of carbohydrases (Viscozyme, Celluclast and AMG). *E. cava* showed the highest susceptibility to the enzymes treated, which was followed by *S. horneri* and *S. coreanum*.

Table 1-3. The degree of enzymatic hydrolysis of the brown seaweeds (%)

		Ecklonia cava	*Ishige okamurae*	*Sargassum fulvellum*	*Sargassum horneri*	*Sargassum coreanum*	*Sargassum thunbergii*	*Scytosiphon lomentaria*
Carbohydrases	Viscozyme	39.59	33.79	24.51	29.99	30.3	31.48	15.01
	Celludast	40.66	23.17	26.45	35.4	30.64	30.83	15.12
	AMG	41.52	17.48	27.94	22.63	33.24	33.87	9.47
	Termamyl	23.29	6.32	22.12	19.15	17.98	19.18	16.5
	Ultraflo	28.26	12.6	26.82	20.01	29.03	26.47	17.82
Proteases	Protamex	28.12	9.62	16.97	27.43	21.3	24.97	18.37
	Kojizyme	20.94	9.51	14.06	25.33	15.46	13.07	2.8
	Neutrase	26.69	14.3	10.93	30.64	26.72	15.03	14.42
	Flavourzyme	32.81	17.01	19.07	35.51	19.51	15.3	14.26
	Alcalase	42.72	28.93	11.65	40.04	40.68	30.51	30.91

3. 3. Free radical scavenging activity by DPPH decolorization

Antioxidant activities of various enzymatic extracts from different seaweed species were estimated by measuring RSA with DPPH decolorization (Table 1-4). Great variations in the activities among different seaweed species and among different enzymatic extracts were observed. The extracts prepared from enzymatic hydrolyses of *E. cava* other than the extracts with Ultraflo, Flavourzyme and Alcalase indicated very strong RSA, showing around 70% activity. Table 1-3 and 1-4 showed that the enzymatic extracts prepared with carbohydrases had a hydrolytic level-dependent increase in the RSA activities. In case of the hydrolyses by proteases, however, the results were quite contrasted to those obtained by carbohydrases. The extracts obtained by treating proteases, namely Protamex, Kojizyme and Neutrase exhibited relatively high values of RSA, in spite of a lower degree of hydrolysis, which ranged between 20% and 30%. Meanwhile, the extract prepared with Alcalase, a protease, showed very low level of RSA (2.6%), even though relatively high degree of hydrolysis was achieved (42.7%).

Table 1-4. RSA (Radical scavenging activity) for enzymatic extracts of the seaweeds (%)

		Ecklonia cava	*Ishige okamurae*	*Sargassum fulvellum*	*Sargassum horneri*	*Sargassum coreanum*	*Sargassum thunbergii*	*Scytosiphon lomentaria*
Carbohydrases	Viscozyme	71.49	15.98	4.4	ND	38.41	9.7	ND
	Celluclast	72.46	12.77	11.62	ND	33.79	6.48	ND
	AMG	70.17	12.4	3.34	ND	32.56	8.41	ND
	Termamyl	62.9	23.03	19.91	11.18	37.15	16.78	16
	Ultraflo	28.66	ND	ND	ND	7.97	ND	ND
Proteases	Protamex	68.16	9.7	20.14	9.44	37.98	23.12	17.84
	Kojizyme	66.49	24.21	20.45	8.26	40.62	20.71	19.33
	Neutrase	66.64	25.42	20.42	11.7	40.56	20.14	18.7
	Flavourzyme	32.64	ND	ND	ND	11.56	ND	ND
	Alcalase	2.61	ND	ND	ND	ND	ND	ND

N.D : Not Detected

Activity of commercial antioxidants (Tocopherol 89.64%; Ascorbic acid 94.58%; BHA 87.38%; BHT 56.05%)

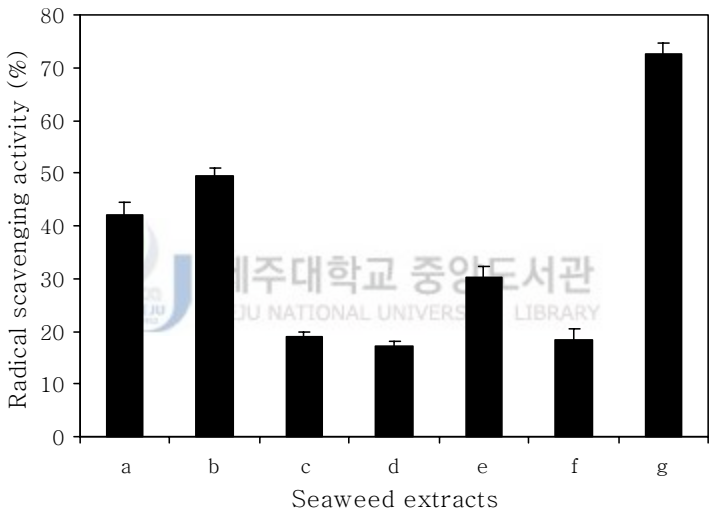

Fig. 1-1. Comparisons on radical scavenging activities between various organic solvent extracts and enzymatic extract of *E. cava*.

Mean ± SE determinations was made in triplicate experiments.

a: EtOH ext., b: MeOH ext., c: Ether ext., d: Chloroform ext.,

e: Aceton ext., f: Hexane ext., g: Celluclast ext.

The seaweed which possessed the second highest levels of RSA next to *E. cava* was *S. coreanum*, whose activities were quite comparable to those of *E. cava*. The activity values were at the range of 30% to 40% of RSA for those with protease other than Ultraflo, Flavourzyme and Alcalase, whose activities were less than 12%. The enzymatic extracts from the other seaweed species listed in Table 1-4 did not show any activity, regardless of enzyme types used for enzymatic hydrolysis. The results indicated that the enzymatic extracts of different seaweeds prepared with different enzymes exhibited quite different value of RSA and that the enzymatic extracts of *E. cava* obtained by Viscozyme, Celluclast, and AMG was the most effective antioxidants in terms of RSA. These values were by about 20% lower than those of representative natural antioxidants, namely, tocopherol and ascorbic acid.

The Celluclast extract of *E. cava*, which was found to be the most effective enzymatic extract of *E. cava*, was compared to the extracts produced with various organic solvents in terms of their RSA (Fig. 1-1). Among them the methanol extract of *E. cava* exhibited RSA value as much as almost 50%. This value is around 20% lower than Celluclast extract of *E. cava*.

Fig. 1-2 shows the change of RSA with increase in the degree of hydrolysis. RSA values of Celluclast extract of *E. cava* considerably increased in parallel with the increased within 2 h of incubation time. No further increase in RSA was observed during the incubation time period when the hydrolysis rate achieved a steady rate. RSA levels of the enzymatic extract were dependent on the concentration of the enzymes reaching around 80% level of the activity at 8 mg/mL of the extract concentration.

Further analysis was carried out in terms of phenolic content of the extracts to clarify their effectiveness in the antioxidant activity (Table 1-5). *E. cava, I. okamurae, S. fulvellum S. coreanum*, and *S. thunbergii* unhydrolyzed had phenolic compounds at a higher level than 10 mg/g, especially *E. cava, S. coreanum* and *S. thunbergii* having the content of the compounds higher than 15 mg/g. The enzymatic extracts

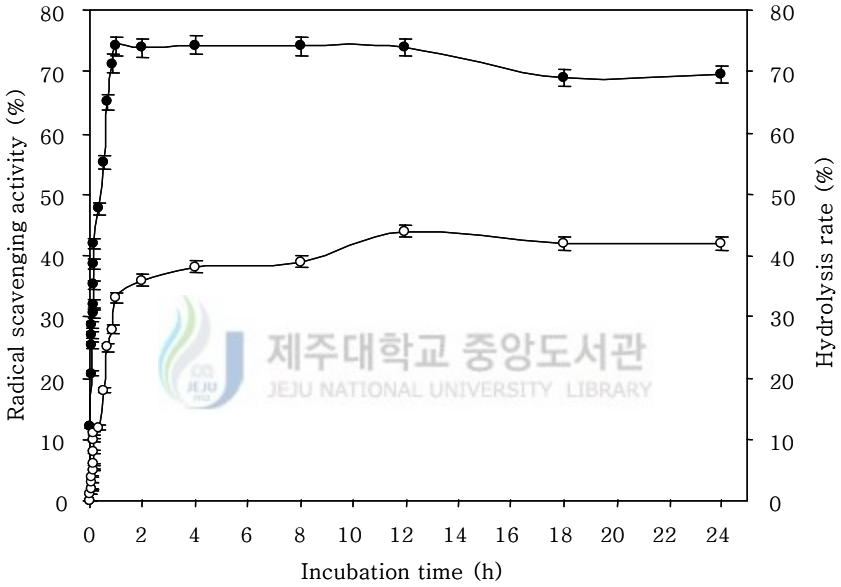

Fig. 1-2. Change in hydrolysis rate and radical scavenging activity of *E. cava* Celluclast extract during enzymatic hydrolysis at 50℃. Mean ± SE determinations was made in triplicate experiments.

(—●— Radical scavenging activity, —○— Degree of hydrolysis)

Table 1-5. Phenolic contents in enzymatic extracts of seaweeds

		Ecklonia cava	*Ishige okamurae*	*Sargassum fulvellum*	*Sargassum horneri*	*Sargassum coreanum*	*Sargassum thunbergii*	*Scytosiphon lomentaria*
	Unhydrolysate	15.6	14.1	10.7	3.3	15.6	15.2	2.1
Carbohydrases	Viscozyme	13.5	2.7	2.5	1.8	11.2	3.8	1.4
	Celluclast	13.5	2.3	2.3	1.6	10.6	3.1	1.1
	AMG	13.4	2.2	2.6	1.6	10.9	3.5	1.1
	Termamyl	11.6	2.1	2.4	2.7	9.4	2.6	1.1
	Ultraflo	10.3	2.7	3.1	3.8	7.4	2.6	1.4
Proteases	Protamex	12.4	2.3	2.3	2.4	9.9	2.9	1.2
	Kojizyme	12.1	1.9	1.9	1.9	9.9	2.3	1.1
	Neutrase	12.3	2.4	2.2	2.5	9.5	2.7	1.3
	Flavourzyme	10.8	2.8	2.2	3.1	8.1	2.5	1.4
	Alcalase	10.9	4.2	3.6	5.3	9.6	4.1	2

(mg phenolic compound / g seaweed)

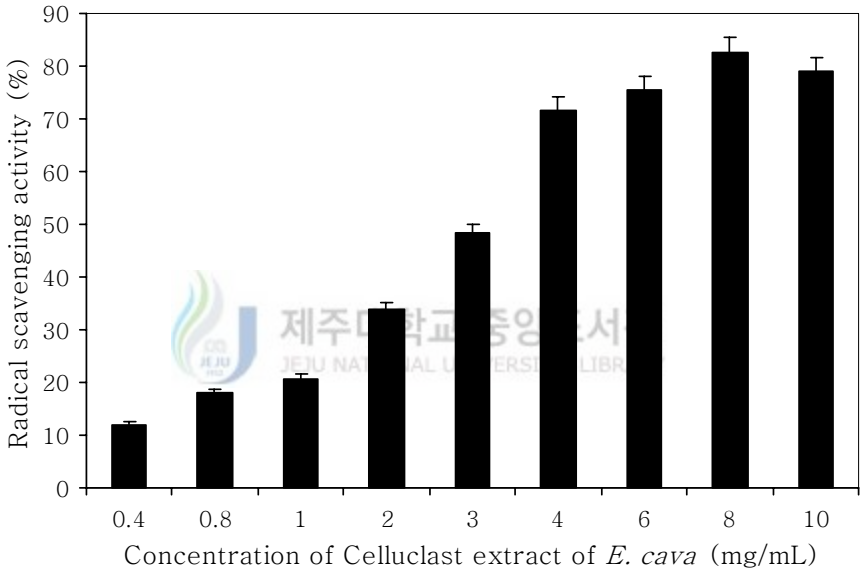

Fig. 1-3. Changes in radical scavenging activity of Celluclast extract from *E. cava* as affected by concentration of the extract.
Mean ± SE determinations was made in triplicate experiments.

having higher values of RSA contained distinctly larger amounts of phenolic compounds. In particular Viscozyme, Celluclast and AMG, which are carbohydrases, extracted approximately 90% of the phenolic compounds present in the original seaweeds when the hydrolysis was completed. Ultraflo, Flavourzyme and Alcalase, which are protease, also extracted around 60% of the phenolic compounds, although they showed lower activities of RSA. This fact suggested that phenolic compounds of seaweeds might not be a sole factor influencing the level of RSA, even thought they have a significant effect on the activity. Fig. 1-3 revealed that RSA of Celluclast extract of *E. cava* was dependent on the increased concentration of samples and reached around 85% at a concentration of 8 mg/mL.

3. 4. Inhibitory capacity of lipid peroxidation in linoleic acid

Another assay for the antioxidant activity assay was performed in linoleic acid system to evaluate the capacity of the enzymatic extracts to inhibit lipid peroxidation in linoleic acid and the amount of lipid peroxides generated from linoleic acid was expressed as TBA value which is considered to the level of antioxidant activity.

Fig. 1-4 shows the TBA values of lipid peroxidation against linoleic acid by the enzymatic extracts prepared using the carbohydrases. Fig. 1-5 shows the TBA values obtained by applying the extracts produced with the proteases. The control group in which no seaweed extract or synthetic antioxidant was added rapidly oxidized to produce lipid peroxides during the incubation period of 5 days, while the respective commercial antioxidants such as tocopherol, BHA and BHT completely inhibited the oxidation. Among the enzymatic extracts prepared from seven species of seaweeds, Ultraflo and Alcalase extract of *E. cava* and Celluclast extract of *S. horneri* and Neutrase extract of *S. lomentaria* could effectively suppress the lipid peroxidation. In particular Neutrase extract of *S. lomentaria* almost completely inhibited the lipid peroxidation to a comparable level with tocoperol, BHA or BHT.

Apart from this observation, a remarkable difference was found between the both assay methodologies for the antioxidant activity; the extracts which were found to possess antioxidant activities when measured by the TBA-based assay showed no or very little RSA levels, when were measured by the method of DPPH decolorization. For examples, although Ultraflo or Alcalase extract exhibited lower levels of RSA, their inhibitory capacity of lipid peroxidation displayed higher activities. The result suggest that there seems to be some contradictions in the evaluation of the antioxidant activity of seaweeds between the assay of TBA value and RSA.

4. DISCUSSION

Recently many researchers are interested in finding any natural antioxidants having safety and effectiveness, which can be substituted for current commercial synthetic antioxidants, BHA and BHT. Seaweeds have become good candidates for the source of natural antioxidants due to a number of studies recently revealed (Fujimoto and Kaneda 1984; Cahyana *et al.*, 1992; Lee, B. H. *et al.*, 1996; Yan *et al.*, 1996; Matsukawa *et al.*, 1997; Yan *et al.,* 1998; Yan *et al.*, 1999; Rupérez *et al.*, 2002; Lim *et al.*, 2002).

In this study, seven species of brown seaweeds inhabiting the costal area of Jeju Island coasts were collected and enzymatically hydrolyzed to produce water-soluble seaweed extracts having effective antioxidant activities. Most seaweeds tested showed a possibility to enzymatically produce the water-soluble extract from seaweed, with enzymatic extracts of *E. cava* being the most effective (the hydrolytic levels between about 20% and 40%). This fact implies that seaweed extracts will be able to be produced in commercial scale. Most of reports so far have dealt with organic solvent extracts of seaweeds which are water-insoluble materials in a small amount.

Seven kinds of enzymatic extracts from *E. cava*, that showed higher hydrolytic degrees, exhibited higher antioxidant activities (around

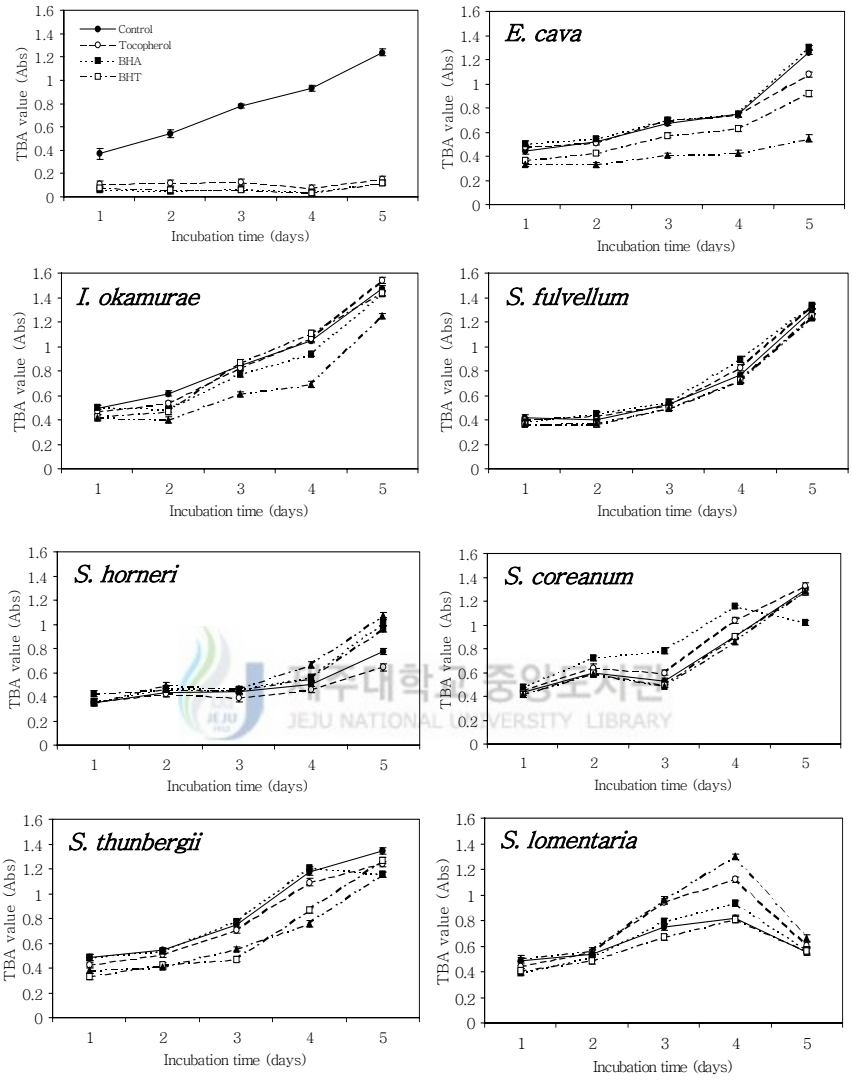

Fig. 1-4. Antioxidant effect of carbohydrase extracts from seaweeds on inhibition of lipid peroxidation in linoleic acid with and without (control) antioxidants. The activity was carried out by TBA assay at 532 nm.

Mean ± SE determinations was made in triplicate experiments.

(—●— Viscozyme ext., —○— Celluclast ext., —■— AMG ext., —□— Termamyl ext., —▲— Ultraflo ext.)

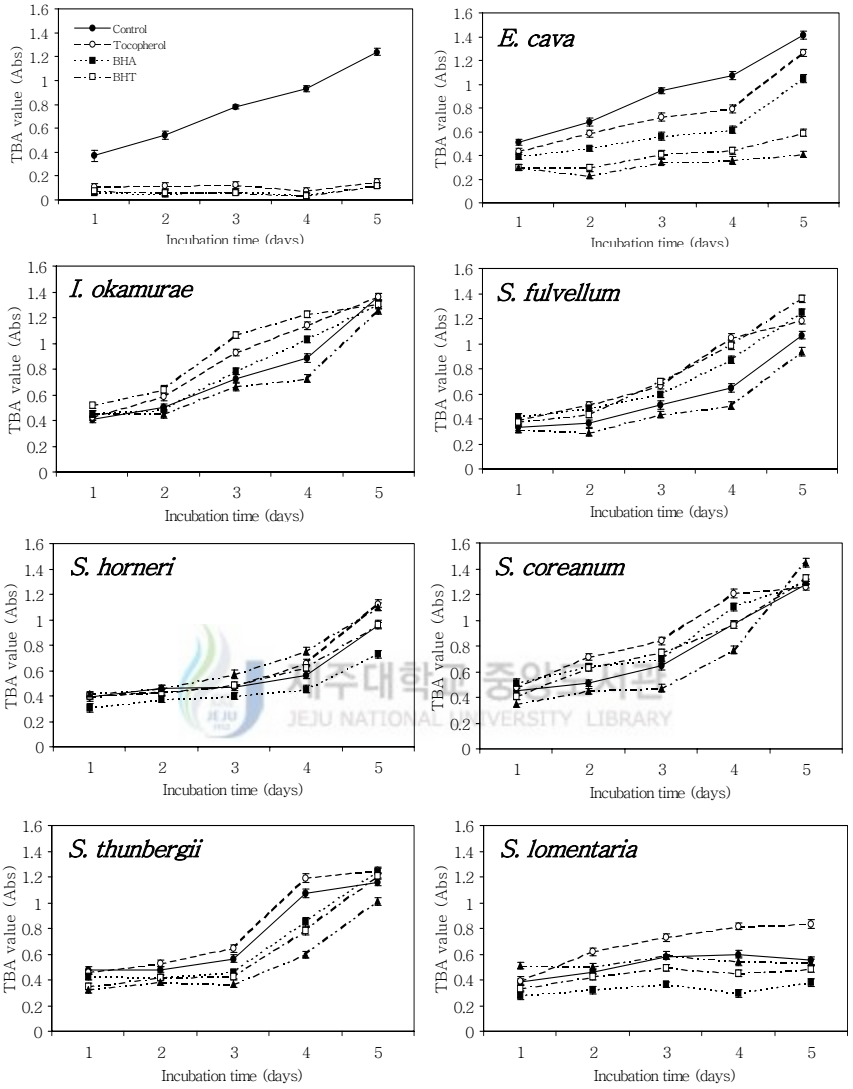

Fig. 1-5. Antioxidant effect of protease extracts from seaweeds on inhibition of lipid peroxidation in linoleic acid with and without (control) antioxidants. The activity was carried out by TBA assay at 532 nm.

Mean ± SE determinations was made in triplicate experiments.

(—●— Protamex ext., —○— Kojizyme ext., —■— Neutrase ext., —□— Flavourzyme ext., —▲— Alcalase ext.)

70%) in RSA assay by DPPH decolorization. The results implied that higher the degree of hydrolysis induced higher antioxidant activity, except for the Alcalase extract.

Above all Celluclast extract had the highest and superior antioxidant activity to various organic extracts of *E. cava*, whose activities was less than 50%. The content of phenolic compounds in the enzymatic extracts as shown in Table 1-5 suggested that phenolic compound could be a positive effector of the antioxidant activity when examined by RSA assay. The enzymatic extracts of *E. cava* were the richest in phenolic compounds and those of *S. coreanum* ranked the second most abundant. The results are consistent with the observation that the extracts of *E. cava* showed the highest activity of anti-oxidation, followed by those of *S. coreanum*. Previous reports proposed that phytophenolic compounds are closely associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (Rice-Evans *et al*., 1995; Jorgensen *et al*., 1999; Duval *et al*., 2000). Considering the fact that some enzymatic extracts of *E. cava* and *S. coreanum*, especially Alcalase extracts, did not have any antioxidant activity, despite they contain as much phenolic compound as the other extracts of *E. cava*, it is noted that the amount of phenolic compounds might not play a sole role in determining the activity. It is thought that unknown compounds which might be produced from seaweeds during enzymatic reaction, such as small molecular weight polysaccharides, proteins or some organic compounds, probably influence the activity.

When the antioxidant activity was assayed in terms of inhibitory effect of lipid peroxidation in linoleic acid, Ultraflo and Alcalase extract of *E. cava* were the most excellent. The results were quite contrasting to the RSA assay for these extract, in which no or only a little activity of antioxidation was observed. Additionally not only Neutrase extract of *S. lomentaria* completely inhibited lipid peroxidation, but some enzymatic extracts prepared with proteases such as Flavourzyme, Protamex and Alcalase also displayed considerably excellent effects. However, it was quite remarkable that these extracts did not show any antioxidant

activity when determined by RSA assay. These results suggest that there is no correlation between the evaluation system of antioxidant activity based on RSA assay and that based on the inhibitory effect of lipid peroxidation in linoleic acid. Matsukawa *et al.* (1997) also pointed out a lack correlation between lipoxygenase inhibition, which corresponds to inhibition of lipid peroxidation formation discussed in the current study, and RSA assay, noting that the two types of assays for determining antioxidant activities of seaweed enzymatic extracts were carried out in different media system, with RSA in polar system and inhibition of lipid peroxidation in non-polar system, respectively. The extracts in RSA assay act as electron or hydrogen donors for DPPH and those in lipid peroxidation assay act as inhibitors for the formation of lipid peroxides.

The present study demonstrated that the enzymatic extracts of seaweeds, especially carbohydrase extracts of *E. cava* showed positive effect for RSA by DPPH decolorization assay and that protease extracts of *S. lomentaria* and *E. cava* showed the strongest activity of antioxidant when measured by inhibition of lipid peroxidation in linoleic acid. There is no report so far on the study of antioxidant activities with enzymatic extracts of seaweeds. Enzymatic extraction of seaweeds for the purpose of obtaining natural antioxidant substances would provide several potential advantages: water solubility, and simple and large scale production process of antioxidant extracts from seaweeds. Further study is required for identification of antioxidant active compounds from enzymatic extracts of seaweeds.

Part Ⅱ.

Reactive oxygen scavenging effect of enzymatic extracts from brown seaweeds

Part Ⅱ.

Reactive oxygen scavenging effect of enzymatic extracts from brown seaweeds

1. ABSTRACT

Potential antioxidative activity of the enzymatic extracts from seven brown seaweeds (*Ecklonia cava, Ishige okamurae, Sargassum fulvellum, Sargassum horneri, Sargassum coreanum, Sargassum thunbergii,* and *Scytosiphon lomentaria*) was evaluated using four different scavenging assays involving superoxide anion, hydroxyl radical, hydrogen peroxide and free radical scavenging. Among all the enzymatic extracts of the seven seaweeds, only *E. cava* extracts could effectively scavenge free radicals released from DPPH, around 70%. In superoxide anion assay, *E. cava* Termamyl extract indicated the highest activity (68%) and *S. fulvellum* Alcalase extract indicated the highest hydroxyl radical scavenging activity (47%). Many seaweed enzymatic extracts indicated the most distinct antioxidative effect in hydrogen peroxide scavenging assay compared to the other three different scavenging assays. Some enzymatic extracts from *I. okamurae*, *S. horneri* and *S. thunbergii* recorded over 90% scavenging effects. In comparison of total phenolic content and the result, of ROS scavenging assays, only the DPPH assay showed a marked correlation with phenolic contents. This fact implies that there is a large difference among the four ROS scavenging activity assays. According to the results, the enzymatic extracts of seaweeds appear to possess a potential antioxidant activity. Therefore, further work will be performed for identification and purification of antioxidant compounds from enzymatic extracts of seaweeds.

2. MATERIALS AND METHODS

2. 1. Materials

Seaweeds were collected along Jeju Island coast of Korea during a period from October 2002 to March 2003. Of the Jeju coastal seaweeds, seven species of brown algae samples were collected, and then salt, epiphytes and sand were removed using tap water. Finally the seaweeds were rinsed carefully in freshwater and stored in a medical refrigerator at -20℃. The frozen samples were lyophilized and homogenized with a grinder before extraction. Carbohydrases (Viscozyme L, Celluclast 1.5L FG, AMG 300L, Termamyl 120L, Ultraflo L) and Proteases (Protamex, Kojizyme 500 MG, Neutrase 0.8L, Flavourzyme 500 MG, Alcalase 2.4L FG) were donated from Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark). 1,1-Diphenyl-2-pricrylhydrazyl (DPPH), pyrogallol, 2-deoxyribose, peroxidase, ABTS (2,2′-azino-bis-(3-ethylbenzthiazoline)-6-sulfonic acid), thiobarbituric acid (TBA), trichloroacetic acid (TCA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). And other chemicals used were 99% or greater purity.

2. 2. Preparation of enzymatic extracts from seaweeds

The enzymatic extracts were obtained according to the method used by Heo *et al.* (2003). The seaweed samples were pulverized into powder using a grinder. A hundred mL of buffer solution was added to one gram of dried alga, and then 100 μL (or mg) of enzyme was mixed. The enzymatic hydrolytic reactions were performed for 12 h to achieve optimum degree of the hydrolysis. Each samples were clarified by centrifugation at 3,000 rpm for 20 min to remove the residue unhydrolysed. The degree of enzymatic hydrolysis was determined by subtracting the dried weight of the residue from one gram of seaweed samples dried and was expressed as a percentage. Enzymatic extract of seaweed was obtained after filtering the supernatant and was used for four antioxidant activity assays including free radical scavenging

activity, superoxide anion scavenging activity, hydroxyl radical scavenging activity and hydrogen peroxide scavenging activity. Concentrations of all the extracts were adjusted to 4 mg/mL.

2. 3. Free radical scavenging activity by DPPH decolorization

Free radical scavenging activity of the enzymatic extracts from seaweeds was determined by using a stable free radical, DPPH, according to the modified method of Blois (1958). DPPH solution was prepared at the concentration of 4×10^{-4}M in ethanol. During the assay, an enzymatic extract of 0.1 mL was mixed with 2.9 mL DPPH solution. The mixture was incubated in the room temperature for 30 min. After standing for 30 min, absorbance was read at 516 nm by UV-VIS spectrophotometer (Opron 3000 Hanson Tech. Co. Ltd., Korea). The percentage inhibition was defined by the absorbance at 516 nm in the absence of enzymatic extract to that measured with the sample.

2. 4. Superoxide anion scavenging activity

Superoxide anion scavenging activity was determined by measuring the inhibition of the auto-oxidation of pyrogallol using a modified method of Marklund and Marklund (1974). A sample solution (0.3 mL) and 2.61 mL of 50 mM phosphate buffer (pH 8.24) were added into freshly prepared 90 μL of 3 mM pyrogallol (dissolved in 10 mM HCl). The inhibition rate of pyrogallol auto-oxidation was measured at 325 nm. Absorbance of each extract was recorded at every 1 min interval for 10 min and the increment of absorbance was calculated by the difference (the absorbance at 10 min - the absorbance at the starting time).

2. 5. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was determined according to the modified method of the 2-deoxyribose oxidation method. Hydroxyl

radical was generated by Fenton reaction in the presence of $FeSO_4 \cdot 7H_2O$. A reaction mixture containing each 0.2 mL of 10 mM $FeSO_4 \cdot 7H_2O$, 10 mM EDTA and 10 mM 2-deoxyribose was mixed with 0.2 mL of the extract solution and 0.1 M phosphate buffer (pH 7.4) was added into the reaction mixture until the total volume reached to 1.8 mL. Then 0.2 mL of 10 mM H_2O_2 was finally added to the reaction mixture and incubated at 37℃ for 4 h. After incubation, each 1 mL of 2.8 % TCA (trichloroacetic acid) and 1.0 % TBA (thiobarbituric acid) were added. Then, the mixture was placed in a boiling water bath for 10 min. Absorbance was measured at 532 nm.

2. 6. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was determined according to the method of Müller *et al.* (1985). A hundred μL of 0.1 M phosphate buffer (pH 5.0) and the sample solution were mixed in a 96 microwell plate. And 20 μL of hydrogen peroxide was added to the mixture, and then incubated at 37℃ for 5 min. After the incubation, 30 μL of 1.25 mM ABTS and 30 μL of peroxidase (1 unit/mL) were added to the mixture, then incubated at 37℃ for 10 min. The absorbance was read with an ELISA reader at 405 nm.

2. 7. Determination of total phenolic compound

Phenolic contents were determined using a protocol similar to Chandler and Dodds (1983) described by Shetty *et al.* (1995). Each 1.0 mL of seaweed enzymatic extracts, 1.0 mL of 95% EtOH, 5.0 mL of distilled water, and 0.5 mL of 50% Folin-Ciocalteu reagent (Sigma Chemical, St. Louis, MO) were mixed. The mixtures were allowed to react for 5 min, and then 1.0 mL of 5% Na_2CO_3 was added, and the mixture was throughly mixed and placed in the dark for 1 h. Absorbance was measured at 725 nm and a gallic acid standard curve was obtained for the calibration of phenolic content.

3. RESULTS

3. 1. Free radical scavenging activity by DPPH decolorization

Free radical scavenging ability of various enzymatic extracts was evaluated with the change of absorbance caused by the reduction of DPPH. The percentage scavenging activity of each extract against DPPH was shown in Table 2-1 and 2-2. Significant differences in the activities among different seaweed species and among different enzymatic extracts were observed. The extracts prepared by enzymatic hydrolysis of *E. cava* indicated strong free radical scavenging effects, except for Ultraflo, Flavourzyme and Alcalase extracts and particular activity levels of the enzymatic extracts reached around 70%. Most enzymatic extracts from the seaweeds, except the extracts of *E. cava*, showed very poor free radical scavenging effects. The enzymatic extracts from *E. cava* having around 70% scavenging activities were inferior to the commercial antioxidants such as α-tocopherol, BHA and little superior to BHT.

3. 2. Superoxide anion scavenging activity

Superoxide anion scavenging activity of the seaweed enzymatic extracts was measured using the pyrogallol auto-oxidation system and these results are indicated as inhibitory rate the superoxide productivity. As shown in Table 2-1 and 2-2, each seaweed enzymatic extracts were exhibited different activities and some extracts showed relatively higher activity compared with the commercial antioxidant. *E. cava* Termamyl extract indicated the highest scavenging activity of 68% over the other seaweed enzymatic extracts. Of the brown seaweeds used for this experiment, all the enzymatic extracts from *S. horneri* exhibited the highest inhibitory rate and were superior to the commercial anti-oxidants. *S. fulvellum, S. thunbergii, Scytosiphon lomentaria* enzymatic extracts also indicated relatively prominent superoxide scavenging effects.

From the result, most Termamyl extracts from all the seaweeds showed the positive effect (except *S. coreanum*), while most Alcalase extracts showed the negative effect (except *E. cava*).

3. 3. Hydroxyl radical scavenging activity

Scavenging activity of the enzymatic extracts from seaweeds against hydroxyl radical was investigated using Fenton reaction (Fe^{2+} + H_2O_2 → Fe^{3+} + OH^- + $^{\cdot}OH$) and these results were expressed as inhibition rates. Most seaweed enzymatic extracts tested possessed week hydroxyl radical scavenging effects. Only *S. fulvellum* Alcalase extract recorded around 50% of hydroxyl radical scavenging activity. This inhibitory rate was inferior to those of the commercial antioxidants such as α-tocopherol and BHA and similar to that of BHT.

3. 4. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity of the seaweed enzymatic extracts were showed in Table 2-1 and 2-2. As shown in the results, remarkable scavenging effects of the seaweed enzymatic extracts were observed in hydrogen peroxide scavenging assay, comparing to the other scavenging assays. Several extracts indicated around 90% scavenging activities. Especially most enzymatic extracts prepared by proteolytic hydrolyses of *Ishige okamurae* indicated the most prominent hydrogen peroxide scavenging activities, except Neutrase extract (43%) and the activity levels of the enzymatic extracts reached over 90%. Of them *I. okamurae* Kojizyme extract recorded the highest activity (around 96%). And respective Ultraflo and Alcalase extracts of *S. horneri* and *S. thunbergii* also exhibited relatively higher scavenging activities (around 90%) and all the enzymatic extracts of *E. cava* by hydrolyses of the proteases and carbohydrases were in the range of 60% to 90% scavenging activity. These scavenging effects suggested to be superior to those of the commercial antioxidants such as α-tocopherol, BHA and BHT (around 64.11, 67.37 and 50.32 %, respectively).

Table 2-1. Scavenging activities of reactive oxygen species and total phenolic contents of the carbohydrase extracts of the brown seaweeds

Scientific name	Treated enzyme	% Inhibition				Total Phenolics
		O_2^-	·OH	H_2O_2	DPPH	
Ecklonia cava	V	−7.14±0.23	16.45±2.25	87.94±0.04	71.49±0.24	1352±0.7
	C	1.43±0.55	16.51±0.06	88.74±0.14	72.46±0.04	1350±0.1
	AMG	−12.86±0.49	15.33±0.18	88.41±0.22	70.17±0.24	1341±0.5
	T	67.14±1.26	16.33±0.36	72.97±0.63	62.90±0.20	1162±0.2
	U	18.57±0.34	17.87±1.89	69.62±0.87	28.66±1.62	1032±0.1
Ishige okamurae	V	28.57±0.78	20.06±0.06	39.52±5.41	15.98±1.03	273±0.1
	C	17.14±0.43	19.35±2.07	36.93±1.17	12.77±0.65	230±0.3
	AMG	28.57±0.23	19.35±1.60	40.46±5.73	12.40±1.29	226±0.1
	T	37.14±0.54	25.33±0.12	18.01±0.83	23.03±0.39	213±0.1
	U	32.86±0.25	25.92±0.12	53.88±0.63	−2.60±0.96	275±0.2
Sargassum fulvellum	V	44.29±1.12	29.05±1.72	33.76±0.61	4.40±2.12	257±0.2
	C	41.43±1.03	25.33±0.24	24.62±0.20	11.62±1.03	234±0.3
	AMG	41.43±0.48	27.57±0.59	28.89±0.23	3.34±2.48	263±0.3
	T	50.00±0.16	23.96±2.43	61.14±0.18	19.91±0.07	240±0.2
	U	38.57±0.43	22.72±1.42	64.78±5.89	−12.11±1.43	313±0.2
Sargassum horneri	V	55.71±1.42	23.43±1.66	15.67±3.37	−9.91±3.89	182±0.2
	C	51.43±0.59	21.83±1.24	14.55±0.49	−9.58±2.26	163±0.1
	AMG	51.43±0.94	16.98±0.30	16.44±1.17	−6.50±3.20	160±0.2
	T	58.57±0.31	24.08±3.02	90.88±0.38	11.18±0.87	272±0.3
	U	48.57±0.76	23.67±0.36	92.69±0.65	−13.58±1.16	384±0.2
Sargassum coreanum	V	8.57±0.34	12.37±0.53	76.90±0.72	38.41±2.17	1123±0.7
	C	7.14±0.32	8.40±2.72	74.03±2.52	33.79±0.95	1063±0.5
	AMG	−1.43±0.12	9.94±0.24	72.19±0.11	32.56±0.69	1097±0.3
	T	5.71±0.22	13.20±3.24	68.48±2.25	37.15±0.75	945±0.4
	U	18.57±0.32	20.89±1.84	75.86±2.43	7.97±1.08	746±0.1
Sargassum thunbergii	V	41.43±0.49	33.37±1.66	40.59±2.36	9.70±1.36	386±0.5
	C	42.86±1.01	32.72±1.83	32.66±2.00	6.48±2.05	310±0.1
	AMG	40.00±0.06	26.98±1.54	32.84±0.70	8.41±1.75	352±0.4
	T	47.14±0.46	26.80±3.74	30.03±0.58	16.78±1.08	261±0.1
	U	42.86±0.12	29.94±2.25	91.49±0.29	−13.83±0.92	263±0.1
Scytosipon lomentaria	V	45.71±0.48	26.57±1.83	9.39±2.13	−1.15±0.34	140±0.2
	C	45.71±1.21	23.31±0.36	7.62±0.29	−1.20±0.92	115±0.1
	AMG	37.14±0.49	25.03±0.65	7.16±0.23	−0.55±0.20	114±0.1
	T	47.14±0.96	26.09±3.14	10.79±2.18	16.00±1.13	115±0.1
	U	41.43±1.04	31.07±1.72	14.84±3.73	−14.29±0.49	149±0.1
α−Tocopherol		41.14±0.87	78.89±0.43	64.11±0.49	89.64±0.27	
BHA		34.84±0.13	56.36±1.12	67.37±1.08	87.38±1.32	
BHT		24.74±0.98	46.87±0.61	50.32±0.49	56.05±0.19	

(V : Viscozyme ext., C : Celluclast ext., AMG : AMG ext., T : Termamyl ext., U : Ultraflo ext.)

Table 2-2. Scavenging activities of reactive oxygen species and total phenolic contents of the protease extracts of the brown seaweeds

Scientific name	Treated enzyme	% Inhibition				Total Phenolics
		$^{-}O_2$	·OH	H_2O_2	DPPH	
Ecklonia cava	P	31.43±0.98	15.09±0.41	77.99±0.49	68.16±0.09	1245±0.5
	K	−12.86±0.49	13.37±0.59	71.03±0.47	66.49±0.47	1216±0.6
	N	−4.29±0.25	12.43±1.78	76.09±0.59	66.64±0.36	1234±0.3
	F	15.71±0.43	17.28±1.54	62.75±0.02	32.64±0.43	1087±0.3
	A	1.43±0.17	17.16±0.47	64.31±1.14	2.61±0.56	1095±0.4
Ishige okamurae	P	34.29±0.78	29.47±3.20	93.70±0.29	9.70±0.69	236±0.1
	K	37.14±0.46	28.99±1.78	96.27±0.22	24.21±0.26	196±0.1
	N	24.29±0.12	25.15±0.53	43.13±6.11	25.42±0.50	248±0.3
	F	5.71±0.23	31.83±0.59	93.41±0.11	−5.06±2.85	283±0.2
	A	−8.57±0.03	32.96±0.30	91.62±0.13	−13.50±1.25	420±0.5
Sargassum fulvellum	P	42.86±0.49	23.91±0.36	91.02±0.11	20.14±1.25	235±0.3
	K	47.14±0.34	22.54±0.77	30.49±1.24	20.45±0.39	193±0.1
	N	48.57±1.09	24.32±0.30	33.20±1.68	20.42±0.56	225±0.2
	F	51.43±0.89	38.88±1.12	79.86±2.49	−10.90±3.84	224±0.3
	A	31.43±0.34	46.92±0.35	75.36±4.96	−12.87±1.88	366±0.4
Sargassum horneri	P	54.29±0.16	23.55±1.66	31.14±3.70	9.44±1.02	245±0.5
	K	52.86±0.73	18.28±1.95	28.91±1.15	8.26±2.05	199±0.1
	N	52.86±0.11	19.41±1.78	30.90±3.68	11.70±0.77	256±0.2
	F	52.86±0.45	19.70±0.53	60.47±7.30	−13.68±1.06	317±0.4
	A	37.14±0.32	20.71±2.13	88.09±0.87	−10.41±3.82	533±0.3
Sargassum coreanum	P	1.43±0.11	15.03±1.07	68.53±1.80	37.98±0.95	996±0.3
	K	4.29±0.23	17.51±2.01	68.55±1.60	40.62±0.82	994±0.3
	N	2.86±0.13	12.43±1.66	69.16±2.40	40.56±0.77	957±0.6
	F	−20.00±0.45	21.83±3.37	76.19±1.64	11.56±0.30	815±0.1
	A	−58.57±0.81	21.54±1.78	77.64±0.27	−14.59±0.19	961±0.8
Sargassum thunbergii	P	47.14±0.75	30.30±1.42	75.39±0.31	23.12±2.32	296±0.1
	K	45.71±0.65	28.93±2.07	76.55±1.50	20.71±0.19	238±0.3
	N	47.14±0.97	29.70±2.49	79.97±1.77	20.14±0.59	272±0.4
	F	47.14±0.78	30.41±1.89	92.37±0.25	−14.54±0.20	253±0.1
	A	28.57±0.65	29.11±2.01	93.58±0.04	−12.92±1.82	416±0.2
Scytosipon lomentaria	P	47.14±0.97	27.46±1.54	6.75±3.68	17.84±0.36	125±0.1
	K	48.57±0.31	26.21±2.74	7.07±3.14	19.33±0.49	112±0.2
	N	47.14±0.36	23.91±0.83	14.24±1.44	18.70±0.55	138±0.1
	F	34.29±0.42	28.88±0.83	30.34±4.06	−13.60±1.15	140±0.2
	A	34.29±0.12	26.57±2.90	39.90±5.53	−9.14±3.23	207±0.2
α−Tocopherol		41.14±0.87	78.89±0.43	64.11±0.49	89.64±0.27	
BHA		34.84±0.13	56.36±1.12	67.37±1.08	87.38±1.32	
BHT		24.74±0.98	46.87±0.61	50.32±0.49	56.05±0.19	

(P : Protamex ext., K : Kojizyme ext., N : Neutrase ext., F : Flavourzyme ext. A : Alcalase ext.)

4. DISCUSSION

Seaweeds have been recognized to possess antioxidant activities. Because most photosynthesizing plants including seaweeds are exposed to a combination of light and high oxygen concentrations, which lead to the formation of free radicals and other strong oxidizing agents, they seldom suffer from any serious photodynamic damage. The observation suggests that their cells have protective antioxidative mechanisms as well as antioxidative compound such as pigments, phenolic compounds and some of polysaccharides (Dykens *et al.*, 1992; Matsukawa *et al.*, 1997; Yan *et al.*, 1999; Hirata *et al.*, 2000; Rupérez *et al.* 2002).

In the present study, based on resent attentions to develop natural water-soluble antioxidant materials were prepared by enzymatic hydrolyses using different carbohydrases and proteases and their antioxidant effects were evaluated through the three different reactive oxygen species and free radical scavenging assays.

DPPH is a free radical donor which has been widely used to test the free radical scavenging effect of natural antioxidants (Jao and Ko, 2002; Matsukawa *et al.*, 1997; Yan *et al.*, 1998; Hatano *et al.*, 1997; Yoshida *et al.*, 1989). Some enzymatic extract from *E. cava* exhibited higher radical scavenging activities, especially Celluclast extract among of the five carbohydrases and Protamex extract among of the five proteases indicated about 70%, and those were even higher than the activity of BHT. Many researchers have been reported positive correlation between free radical scavenging activities and total phenolics. Oki *et al.* (2002) observed that the radical scavenging activity increased with the increase of phenolic compound content. The two studies conducted by Lu and Foo (2000a) and Kim and Chung (2002) reported higher correlations between DPPH radical scavenging activities and total polyphenolics. In this study, some enzymatic extracts of *E. cava* and *S. coreanum* (especially Alcalase extract) did not have any antioxidant activity, despite they contain phenolic compounds as much as the other extracts of *E. cava*. It is thought that another materials in seaweed extracts, such as small molecular weight polysaccharide, protein or

pigments, probably influence the activity.

Superoxide anion (O_2^-) are formed in living cells during several biochemical reaction (Fridovich, 1974) and its effects can be magnified because it produces other kinds of free radicals and oxidizing agent inducing cell damage (Lui and Ng., 1999). In this study, some enzymatic extracts showed higher superoxide anion scavenging activity over the commercial antioxidant, such as α-tocopherol, BHA and BHT. Especially *E. cava* Termamyl extract indicated the highest superoxide anion scavenging activity of 68%.

Hydroxyl radical scavenging activity of enzymatic extracts from seaweed were measured as the percentage of inhibition of hydroxyl radicals generated in the Fenton reaction mixture. Most of seaweed enzymatic extracts indicated less activities than 40%, except for *S. fullvelum* Alcalase extract (around 47%). The cell-damaging action of hydroxyl radical is well known as it is the strongest among free radicals. However enzymatic extracts of seaweed have little effect for scavenge of hydroxyl radical.

Hydrogen peroxide is a relatively unstable metabolic product being responsible for the generation of hydroxyl radical and singlet oxygen, which formed by fenton reaction and initiate lipid peroxidation or be toxic to cells. The seaweed enzymatic extracts showed the most effective antioxidant activity in hydrogen peroxide scavenging assay compared to the other reactive oxygen species scavenging activities (superoxide anion, hydroxyl radical and free radical scavenging activity). Especially *I. okamurae* Kojizyme extract reached around 96%, whose value is significantly higher than the commercial antioxidants. The measurement of hydrogen peroxide scavenging activity can be one of the useful methods determining the ability of antioxidants to decrease the level of pro-oxidants such as H_2O_2 (Czochra and Widensk, 2002). Therefore it was expected that enzymatic extracts of seaweed can be effectively used in pharmaceutics and food filed as a natural anti-oxidative source.

In this work, we dealed with the further investigation of antioxidative characteristics which were examined in our first research

(Heo *et al.*, 2003). To obtain natural water-soluble antioxidant substances tried enzymatic extraction of seaweeds would provide several potential advantages: water solubility, safety, simple and large scale production processes of antioxidant extracts from seaweeds. Further work is required for identification and purification of anti-oxidative compounds from enzymatic extracts of seaweeds.

Part Ⅲ.

Hydrogen peroxide scavenging effect of enzymatic extracts from *Sargassum horneri* and *Sargassum thunbergii*

Part Ⅲ.

Hydrogen peroxide scavenging effect of enzymatic extracts from *S. horneri* and *S. thunbergii*

1. ABSTRACT

The antioxidant activity of enzymatic extracts from *S. horneri* and *S. thunbergii* was investigated by examining the hydrogen peroxide scavenging activity and inhibitory effect of DNA damage. Among all the enzymatic extracts, Ultraflo and Alcalase extract showed strong hydrogen peroxide scavenging activity (around 90%) and the activities were dose-dependent. Moreover these extracts were thermal stable for hydrogen peroxide scavenging activity and the activity remained 90% or higher at 100℃ up to 24 h. Also in the present study, enzymatic extracts showed strong inhibitory effect of DNA damage and the inhibitory effect increased with increased concentrations of the extracts. The highest inhibitory effect (around 80%) was obtained from *S. thunbergii* Ultraflo extract at 50 ㎍/mL.

2. MATERIALS AND METHODS

2. 1. Materials

S. horneri and *S. thunbergii*, marine brown macroalgae, were collected along Jeju Island coast of Korea during a period from October 2002 to March 2003. Salt, epiphytes and sand were removed using tap water. Finally the seaweeds were rinsed carefully in deionized water and stored in a medical refrigerator at -20℃ for further experiments. The frozen samples were lyophilized and homogenized with a grinder before extraction. Carbohydrases such as Viscozyme L (a multi-enzyme complex containing a wide range of carbohydrases, including arabanase,

cellulase, beta-glucanase, hemicellulase and xylanase), Celluclast 1.5L FG (catalyzing the breakdown of cellulose into glucose, cellobiose and higher glucose polymers), AMG 300L (an exo-1,4-alpha-D-glucosidase), Termamyl 120L (a heat-stable alpha-amylases), Ultraflo L (a heat-stable multi-active beta-glucanase) and Proteases such as Protamex (hydrolysis of food proteins), Kojizyme 500 MG (boosting of the soya sauce fermentation), Neutrase 0.8L (an endoprotease), Flavourzyme 500 MG (containing both endoprotease and exopeptidase activities), Alcalase 2.4L FG (a endo protease) were donated from Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark). Peroxidase, ABTS (2,2′-azion-bis-(3-ethylbenzthiazoline)-6-sulfonic acid), ㅁ-tocopherol, BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisol) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). And other chemicals used were of 99% or greater purity.

2. 2. Preparation of enzymatic extracts from seaweed

The enzymatic extracts were obtained according to the method used by Heo *et al.* (2003). Seaweed was pulverized into powder using a grinder. A hundred mL of a buffer solution was added to one gram of dried alga, and then enzyme was mixed. The enzymatic hydrolysis reactions were performed for 12 h to achieve optimum hydrolysis. Each sample was clarified by centrifugation at 3,000 rpm for 20 min to remove the residue unhydrolysed. Enzymatic extract of seaweeds was obtained after filtering the supernatant and was used for hydrogen peroxide scavenging activity. Concentration of all the extracts were adjusted to 4 mg/mL.

2. 3. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was determined according to the method of Müller *et al.* (1985). 100 μL of 0.1 M phosphate buffer (pH 5.0) and sample solution were mixed in a 96 microwell plate. And 20 μL of hydrogen peroxide was added to the mixture, and then

incubated at 37℃ for 5 min. After incubation, 30 μL of 1.25 mM ABTS and 30 μL of peroxidase (1 unit/mL) were added to the mixture, then incubated at 37℃ for 10 min. The absorbance was read with an ELISA reader at 405 nm.

2. 4. Isolation and cryoconservation of human peripheral lymphocytes

Blood samples were obtained from two healthy male volunteers (non-smokers, 27 and 35 years old, respectively). A 5 mL of fresh whole blood was added to 5 mL of phosphorous buffered saline (PBS) and layered onto 5 mL of Histopaque 1077. After centrifugation for 30 min at 400g at room temperature, the lymphocytes were collected from just above the boundary with the Histopaque 1077, washed with 5 mL PBS, Finally, they were resuspended in freezing medium (90% fetal calf serum, 10% dimethyl sulfoxide) at 6×10^6 cells/mL. The cells were frozen to -80℃ using a Nalgene Cryo 1℃ freezing container and then stored in liquid nitrogen. The cells were thawed rapidly prior to each experiment in a water bath at 37℃.

2. 5. Incubation of lymphocytes

Each lyophilized extract was dissolved in PBS and diluted into concentrations 0, 1, 10, 25 and 50 ㎍/mL. Diluted extract aliquots of 1 mL with a lymphocyte suspension containing 2×10^4 cell/mL were incubated for 60 min at 37℃ in a dark incubator together with untreated control sample. After preincubation, samples were centrifuged at 2000 rpm for 5 min on 4℃. The incubated cells were resuspended in PBS with 50μM hydrogen peroxide (H_2O_2) for 5 min on ice. The untreated control sample was resuspended only in PBS without H_2O_2. Cells were centrifuged as described above and then washed with 1 mL PBS. All the experiments were repeated twice with lymphocytes from each of two donors.

2. 6. Determination of DNA damage (Comet assay)

The alkaline comet assay was conducted according to Singh *et al.* with a little modification. The cell suspension was mixed with 75 μL of 0.5% low melting agarose (LMA), and added to the slides precoated with 1.0% normal melting agarose (NMA). After solidification of the agarose, the slides were covered with another 75 μL of 0.5% LMA and then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% sodium laurylasarcosine; 1% Triton X-100 and 10% DMSO) for 1 h at 4℃. The slides were next placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 min for DNA unwinding. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 min at 4 ℃. The slides were washed three times with a neutralizing buffer (0.4M Tris, pH 7.5) for 5 min at 4℃, and then treated with ethanol for another 5 min before staining with 50 μL of ethidium bromide (20 ㎍ /mL). Measurements were made by image analysis (Kinetic Imaging, Komet 5.0, U.K) and fluorescence microscope (LEICA DMLB, Germany), determining the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of two replicate slides).

2. 7. Statistical Analysis

Data were analysed using the SPSS package for Windows (Version 10). Values were expressed as mean±standard error (SE). The mean values of the tail intensity from each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. *P*-value of less than 0.05 was considered significant.

3. Results

3. 1. Hydrogen peroxide scavenging activity

Table 3-1. Hydrogen peroxide scavenging activity by *S. horneri* and *S. thunbergii* enzymatic extracts

		S. horneri	*S. thunbergii*
Carbohydrases	V	15.67±3.37	40.59±2.36
	C	14.55±0.49	32.66±2.00
	AMG	16.44±1.17	32.64±0.70
	T	90.88±0.38	30.03±0.58
	U	92.69±0.65	91.49±0.66
Proteases	P	31.14±3.70	75.39±0.31
	K	28.91±1.15	76.55±1.50
	N	30.90±3.68	79.97±1.77
	F	60.47±7.30	92.58±0.25
	A	88.09±0.87	93.37±0.55

Mean ± SE determinations was made in triplicate experiments. The enzymatic extracts were prepared using the five carbohydrases and proteases. (V : Viscozyme ext., C : Celluclast ext., AMG : AMG ext., T : Termamyl ext., U : Ultraflo ext., P : Protamex ext., K : Kojizyme ext., N : Neutrase ext., F : Flavourzyme ext., A : Alcalase ext.)

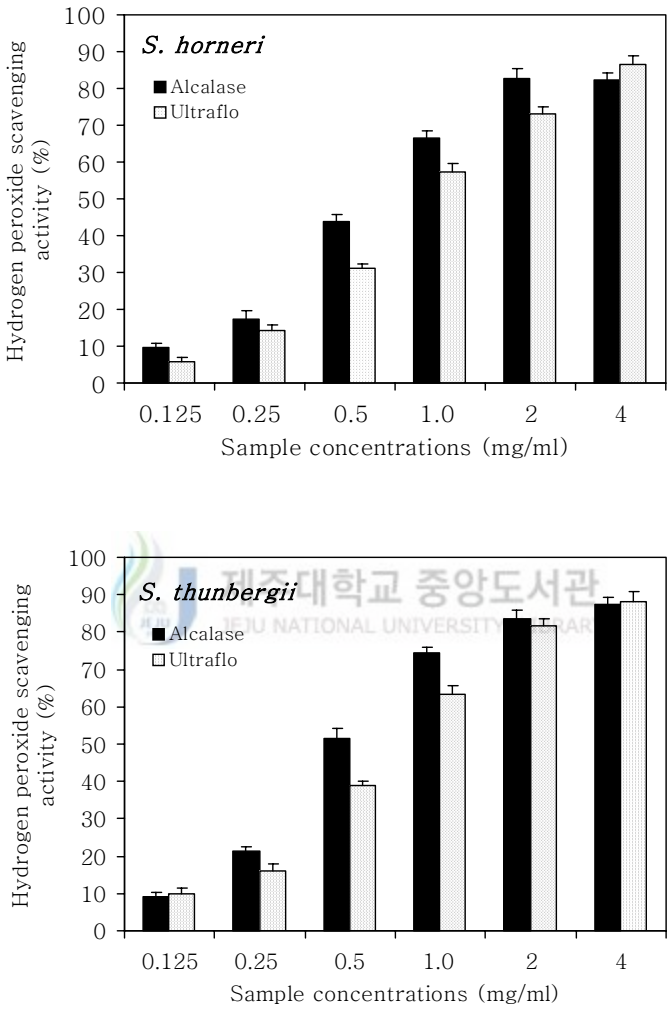

Fig. 3-1. Change in radical scavenging activity of Ultraflo and Alcalase extract from *S. horneri* and *S. thunbergii* as affected by concentration of the extracts.
Mean ± SE determinations was made in triplicate experiments.

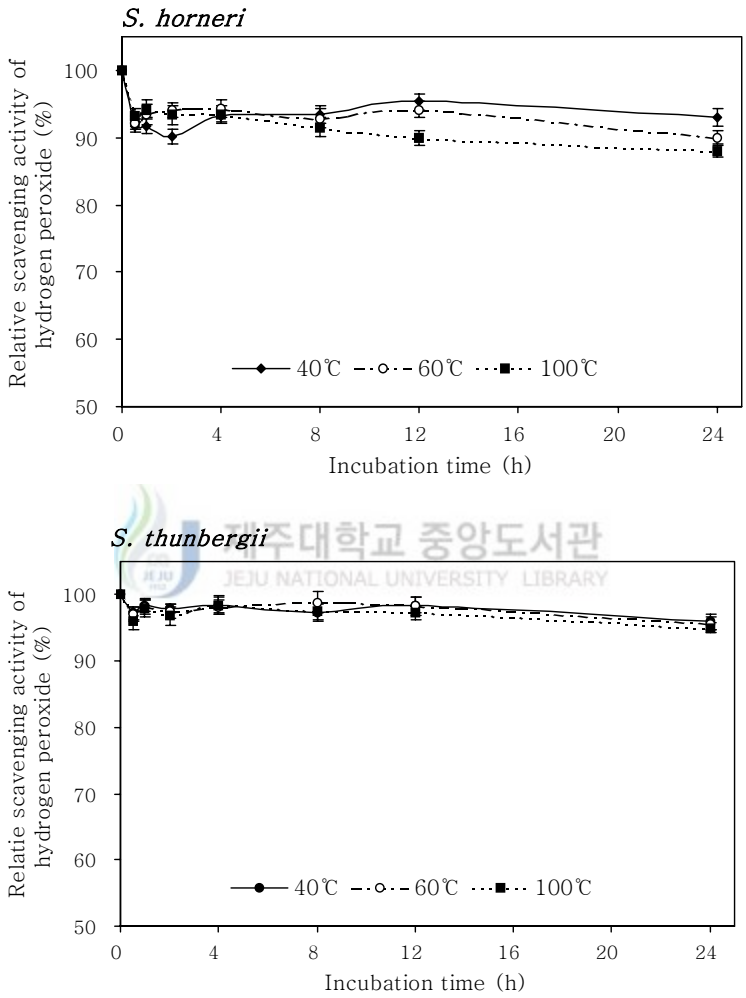

Fig. 3-2. Thermal stability of Ultraflo extract in hydrogen peroxide scavenging activity.
Mean ± SE determinations was made in triplicate experiments.

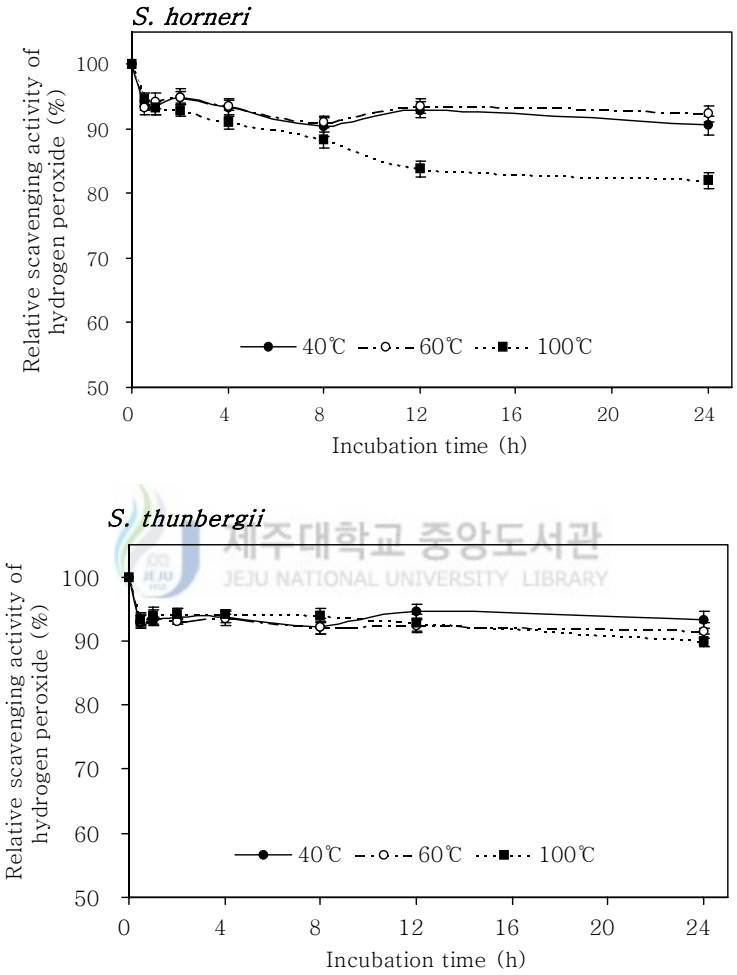

Fig. 3-3. Thermal stability of Alcalas extract in hydrogen peroxide scavenging activity.
Mean ± SE determinations was made in triplicate experiments.

H_2O_2 scavenging activities by *S. horneri* and *S. thunbergii* enzymatic extracts were showed in Table 3-1. The proteases extracts of *S. thunbergii* exhibited higher hydrogen peroxide scavenging activities than the other enzymatic extracts, all the values are over 75%. Especially Alcalase extract of the seaweed showed the highest hydrogen peroxide scavenging activity (around 93%). Both Ultraflo extracts from *S. horneri* and *S. thunbergii* showed remarkable hydrogen peroxide scavenging activities, 92.69 and 91.49%, respectively. From the result of Table 3-1, in general, all the Ultraflo and Alcalase extracts were excellent in the scavenging effect. However Termamyl extract of *S. horneri* and Flavourzyme extract of *S. thunbergii* exceptionally recorded over 90%. Fig 3-1 revealed that the activities of both Ultraflo extract among the carbohydrase extracts and Alcalase extract among the protease extracts were dose-dependent and reach about 90% at the concentration of 4 mg/mL. Thermally stabilities of Alcalase and Ultraflo extracts of *S. horneri* and *S. thunbergii* were investigated at 40, 60 and 100℃ according to the heating time (Fig 3-2 and 3-3). Those extracts remained their activity about 90% after 24 h heating at 100℃. However Alcalase extract of *S. horneri* indicated a slight reduction (about 20% after 24 h at 100℃).

3. 2. Determination of DNA damage

The DNA damage inhibitory effect of respective Ultraflo and Alcalase extract of *S. horneri* and *S. thunbergii*, which were selected from the results of colorimetric assay by chemical reaction was investigated using comet assay (Fig. 3-4, 5, 6 and 7). All the samples showed good inhibitory effects against DNA damage not only at high concentrations (50 ㎍/mL) but also at low concentrations (1 ㎍/mL). In DNA damage inhibitory effect of *S. horneri* extracts both Ultraflo and Alcalase extracts significantly inhibited the damage at additions of 25 ㎍/mL concentration or higher and indicated the inhibitory activities more than at least 50%. The both extracts from *S. thunbergii* exerted better inhibitory effects than *S. horneri*. Additionally, when the

concentration is higher than 25 ㎍/mL, the activities were over 50%. Moreover, the highest inhibitory (about 80%) effect was observed at 50 ㎍/mL of *S. thunbergii* Ultraflo extract. Fig. 3-8 and 3-9 showed photomicrographs of different DNA migration profiles obtained from human lymphocytes, when treated with different concentrations of the extracts. In the group treated with only hydrogen peroxide, the DNA was completely damaged but the addition of *S. horneri* and *S. thunbergii* enzymatic extracts with hydrogen peroxide reduced the damage caused by hydrogen peroxide. In the application of samples at different concentrations the DNA migration changed according to the concentrations. Especially, at the highest concentration (50 ㎍/mL) indicated strong inhibitory effect of DNA damage compared to the control group.

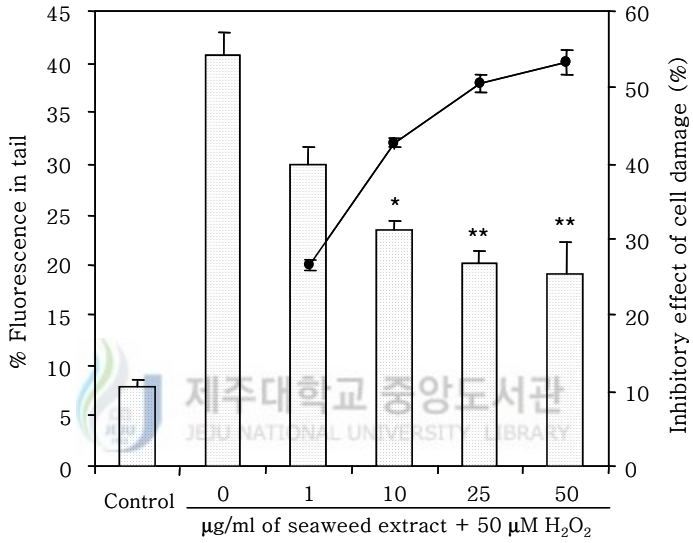

Fig. 3-4. The effect of supplementation in vitro with different concentration of *S. horneri* Ultraflo extract on H_2O_2-induced human lymphocytes DNA damage. Values are mean with standard error of duplicate experiments with lymphocytes from each of two different donors. Significant different to values for samples treated with 50μM H_2O_2 only using LSD: *, $p<0.05$, **, $p<0.01$.
(▨ % Fluorescence in tail, —●— Inhibitory effect of cell damage)

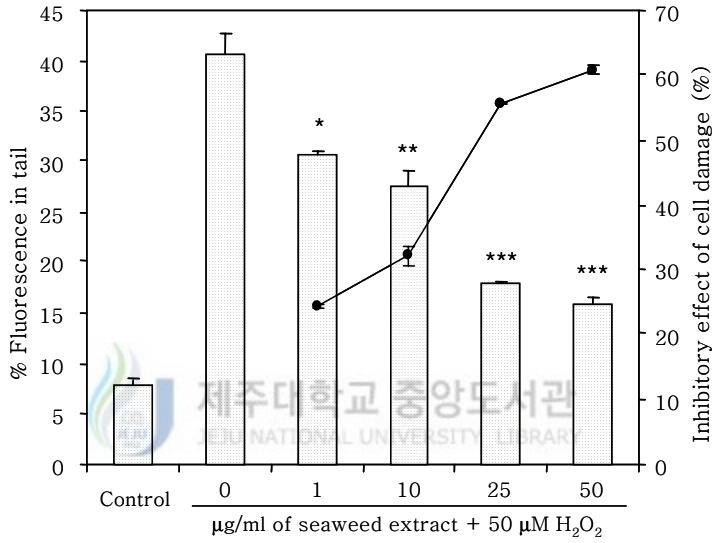

Fig. 3-5. The effect of supplementation in vitro with different concentration of *S. horneri* Alcalase extract on H_2O_2-induced human lymphocytes DNA damage. Values are mean with standard error of duplicate experiments with lymphocytes from each of two different donors. Significant different to values for samples treated with 50μM H_2O_2 only using LSD: *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

(▨ % Fluorescence in tail, —●— Inhibitory effect of cell damage)

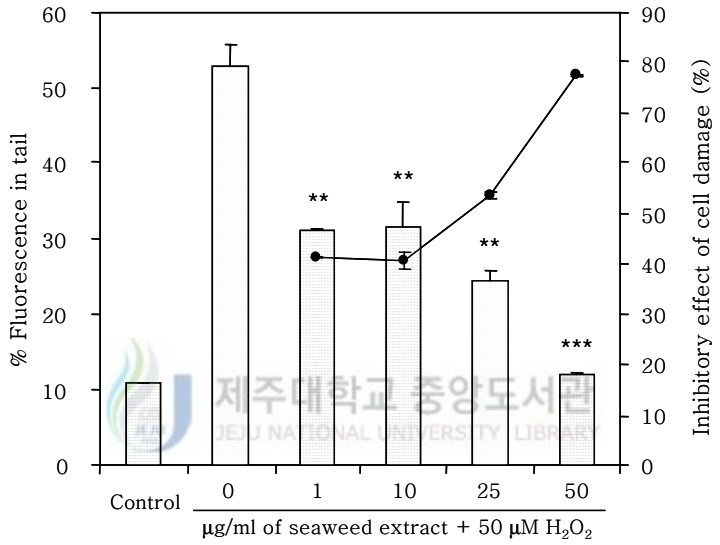

Fig. 3-6. The effect of supplementation in vitro with different concentration of *S. thunbergii* Ultraflo extract on H_2O_2-induced human lymphocytes DNA damage. Values are mean with standard error of duplicate experiments with lymphocytes from each of two different donors. Significant different to values for samples treated with 50μM H_2O_2 only using LSD: **, $p<0.01$, ***, $p<0.001$.
(▨ % Fluorescence in tail, —●— Inhibitory effect of cell damage)

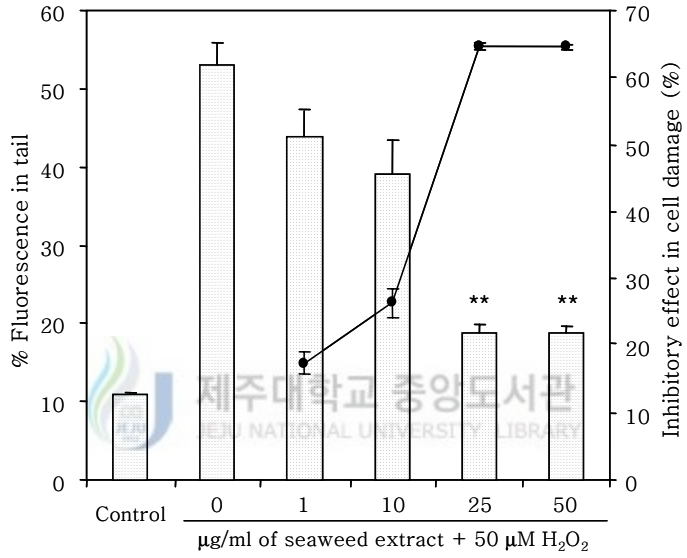

Fig. 3-7. The effect of supplementation in vitro with different concentration of *S. thunbergii* Alcalase extract on H_2O_2-induced human lymphocytes DNA damage. Values are mean with standard error of duplicate experiments with lymphocytes from each of two different donors. Significant different to values for samples treated with 50μM H_2O_2 only using LSD: **, $p<0.01$.
(▨ % Fluorescence in tail, —●— Inhibitory effect of cell damage)

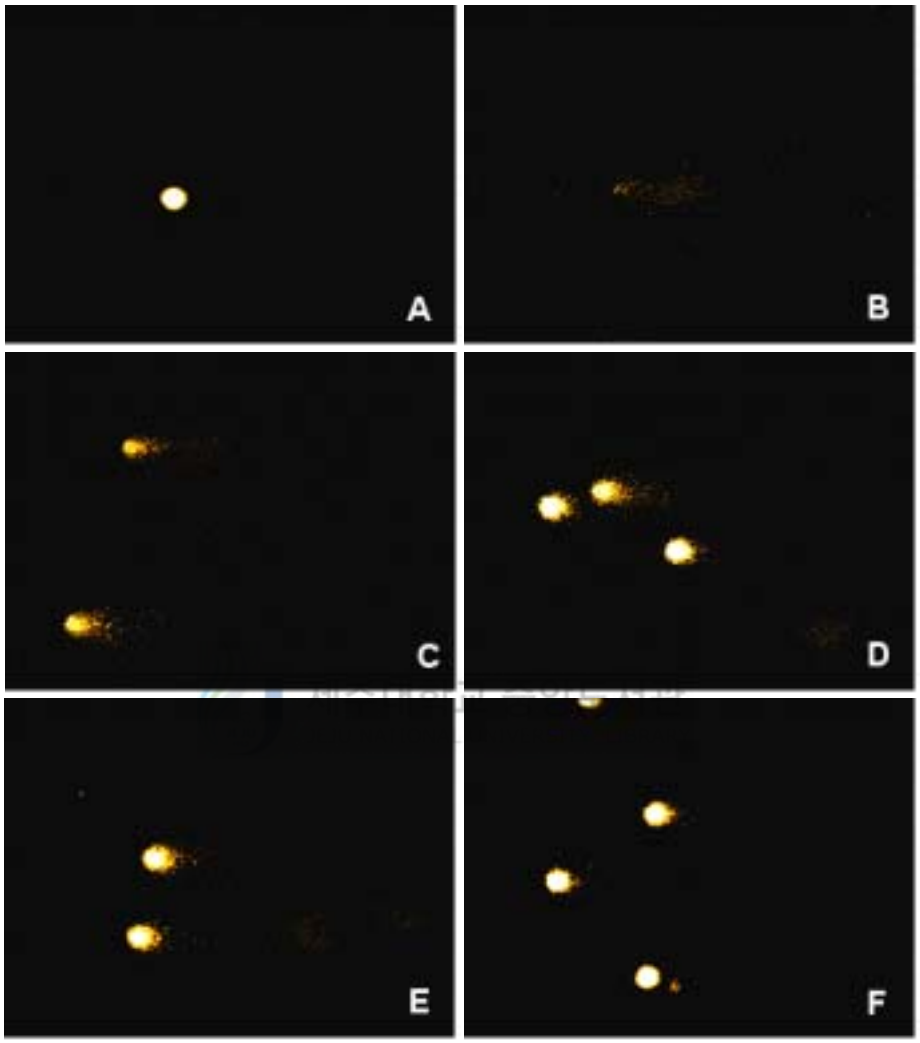

Fig. 3-8. Comet images of human lymphocytes.
A: (A) negative control; (B) lymphocytes treated with 50μM H_2O_2; (C) lymphocytes treated with 1μg/ml *S. horneri* Alcalase ext. + 50μM H_2O_2; (D) lymphocytes treated with 10μg/ml *S. horneri* Alcalase ext. + 50μM H_2O_2; (E) lymphocytes treated with 25μg/ml *S. horneri* Alcalase ext. + 50μM H_2O_2; (F) lymphocytes treated with 50μg/ml *S. horneri* Alcalase ext. + 50μM H_2O_2

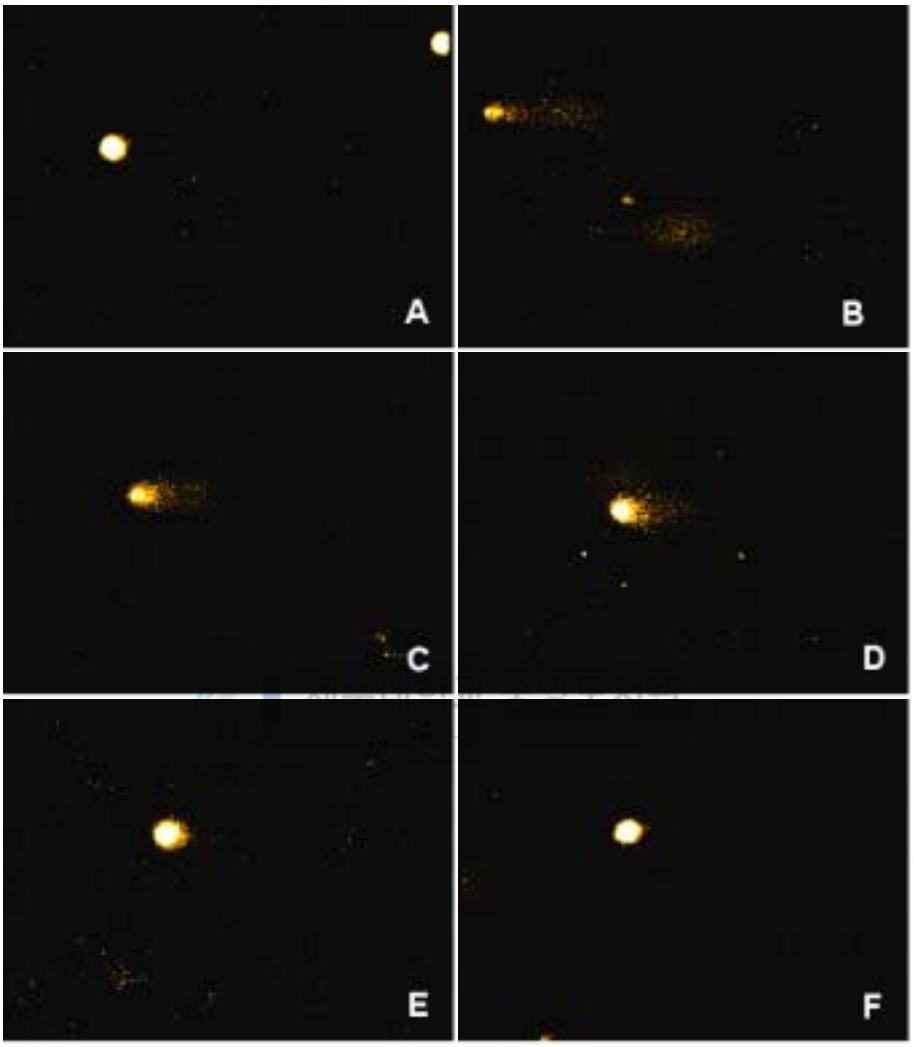

Fig. 3-9. Comet images of human lymphocytes.
(A) negative control; (B) lymphocytes treated with 50μM H_2O_2; (C) lymphocytes treated with 1μg/ml *S. thunbergii* Alcalase ext. + 50μM H_2O_2; (D) lymphocytes treated with 10μg/ml *S. thunbergii* Alcalase ext. + 50μM H_2O_2; (E) lymphocytes treated with 25μg/ml *S. thunbergii* Alcalase ext. + 50μM H_2O_2; (F) lymphocytes treated with 50μg/ml *S. thunbergii* Alcalase ext. + 50 μM H_2O_2

4. Discussion

Hydrogen peroxide together with reactive oxygen species (ROS) can damage many cellular component. Hydrogen peroxide is relatively an unstable metabolic product being responsible for the generation of hydroxyl radical which is formed by fenton reaction and initiate lipid peroxidation, and subsequently might be toxic to cells. In this investigation, respective Ultraflo and Alcalase extracts of the both seaweeds showed higher hydrogen peroxide scavenging activity, especially extract of *S. thunbergii* indicated strong scavenging activity compared to the commercial antioxidants. In different dosages applied, each samples increased their scavenging activity with the increased dosage. Also the scavenging activities rapidly increased up to 2 mg/mL concentration but slowly increased in the concentration range of 2 mg/mL or higher (Fig. 3-1). Especially these enzymatic extracts have very good heat stabilities (Fig. 3-2, 3-3). At 100℃ only a little reduction was observed in the remaining activity and the other heat treatments with temperatures less than 100℃ showed very similar scavenging activities to the activity of the sample without heating. These results suggest that the enzymatic extracts consist of very good heat stable antioxidant compound. The measurement of H_2O_2 scavenging activity is one of the useful methods determining the ability of antioxidants to decrease the level of prooxidants such as H_2O_2 (Czochra *et al.*, 2002). The hydrogen peroxide scavenging effect and thermal stability of the seaweed antioxidants suggests their potential as a good antioxidative source.

DNA damage is known to be one of the most sensitive biological markers for evaluating oxidative stress representing the imbalance between free radical generation and efficiencies of the antioxidant system (Kassie *et al.,* 2000; Donelly, 1999). Presently, a more useful approach called comet assay (single cell gel electrophoresis), which is a rapid and sensitive fluorescence microscopic method for detection of primary DNA damage on the individual cell level, is increasingly used to evaluate the genotoxicity of test substances (Fairbairn *et al.,* 1995;

Olive *et al.,* 1990). In this investigation, *S. horneri* and *S. thunbergii* Ultraflo and Alcalase extract showed strong hydrogen peroxide scavenging activities. The higher H_2O_2 scavenging ability of our study conformed the notable result recorded in comet assay due to strong H_2O_2 scavenging ability of *S. horneri* and *S. thunbergii* enzymatic extracts. All the extracts with higher concentrations than 25 ㎍/mL inhibited at least more than 50% of DNA damage, caused by an addition of 50 μM H_2O_2 to human lymphocyte cells. Several studies have shown that increasing materials (environmental pollutants, radiation, dietary habits and various chemicals) induced DNA damage which led to the diseases such as cancer and heart disease (Betii *et al.,* 1995; Binkova *et al.,* 1996; Hartmann *et al,.* 1995; Singh *et al.,* 1995). Also many researchers have investigated on inhibition of DNA damage by food materials such as tea (Zhang *et al.,* 2002; Wei *et al.,* 1999), juice (Park *et al.,* 2003), plant extract (Yen *et al,.* 2001; Zhu and Loft 2001), flavonoid (Senthilmohan *et al.,* 2003) and aquatic animals (Janssens *et al.,* 2002; Lee and Steinert 2003). The cells of the human body are continuously attacked by physical agent (like solar radiation), a variety of chemical compounds and reactive oxygen species which arise as natural byproducts of metabolic process. These substances can induce the DNA damage. If those DNA lesions are not repaired, those can initiate cascade of biological consequences at the population and also can promote cancer development via several mechanism (Bagchi *et al.,* 2000).

In the present study, we examined enzymatic extracts from the seaweeds (*S. horneri* and *S. thunbergii*) as natural water-soluble antioxidative sources, and observed a significant hydrogen peroxide scavenging activity and inhibition of DNA damage on comet assay. Therefore enzymatic extracts from seaweeds can be applied in food and medicinal industry as a natural and water-soluble antioxidant. Further studies are required for the identification of antioxidant compounds responsible for the recorded potential effects.

Part Ⅳ.

Antioxidant effect of enzymatic extracts from *Ecklonia cava*

Part Ⅳ.

Antioxidant effect of enzymatic extracts from *Ecklonia cava*

1. ABSTRACT

The potential antioxidative activity of water-soluble enzymatic extracts from *Ecklonia cava* was evaluated by free radical scavenging and lipid peroxidation assays. The brown seaweed, *E. cava* was enzymatically hydrolyzed to prepare water-soluble extracts by five carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo) and five proteases (Protamex, Kojizyme, Neurtase, Flavourzyme and Alcalase). Among all the extracts, Celluclast extract could effectively scavenge free radicals released from DPPH (1,1-diphenyl-2-pricrylhydrazyl) and recorded around 73% scavenging activity at the concentration of 4 mg/mL. These extract was thermally stable and DPPH radical scavenging activity remained 80% or higher with the heating temperatures of 40 and 60℃ up to 12 h and around 80% with 100℃ up to 8 h. AMG and Ultraflo inhibited the lipid peroxidation of fish oil as great as that of α-tocopherol. These results suggest that an enzymatic extract will be can effective technique for the production of potential antioxidants from seaweeds.

2. MATERIALS AND METHODS

2. 1. Materials

E. cava, a marine brown macroalga, was collected along Jeju Island coast of Korea during a period from October 2001 to March 2002. Salt, epiphytes and sand were removed using tap water. Finally the seaweeds were rinsed carefully in deionized water and stored in a

medical refrigerator at -20℃ for further experiments. The frozen samples were lyophilized and homogenized with a grinder before extraction. Carbohydrases such as Viscozyme L (a multi-enzyme complex containing a wide range of carbohydrases, including arabanase, cellulase, beta-glucanase, hemicellulase and xylanase), Celluclast 1.5L FG (catalyzing the breakdown of cellulose into glucose, cellobiose and higher glucose polymers), AMG 300L (an exo-1,4-alpha-D-glucosidase), Termamyl 120L (a heat-stable alpha-amylases), Ultraflo L (a heat-stable multi-active beta-glucanase) and Proteases such as Protamex (hydrolysis of food proteins), Kojizyme 500 MG (boosting of the soya sauce fermentation), Neutrase 0.8L (an endoprotease), Flavourzyme 500 MG (containing both endoprotease and exopeptidase activities), Alcalase 2.4L FG (a endo protease) were donated from Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark). 1,1-Diphenyl-2-pricrylhydrazyl (DPPH), fish oil, α-tocopherol, potassium iodide (KI), potassium dichromate ($K_2Cr_2O_7$), sodium thiosulfate ($Na_2S_2O_3$), BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisol) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). And other chemicals used were of 99% or greater purity.

2. 2. Preparation of enzymatic extracts from seaweeds

The enzymatic extracts were obtained according to the method used by Heo *et al.* (2003). The seaweed samples were pulverized into powder using a grinder. A hundred mL of a buffer solution was added to one gram of dried alga, and then enzyme was mixed. The enzymatic hydrolysis reactions were performed for 12 h to achieve optimum hydrolysis. Each sample was clarified by centrifugation at 3,000 rpm for 20 min to remove the residue unhydrolysed. Enzymatic extract of seaweed was obtained after filtering the supernatant and was used for two assays of antioxidant activity, composed of free radical scavenging activity and inhibitory effect of lipid peroxidation. Concentration of all the extracts were adjusted to 4 mg/mL.

2. 3. Free radical scavenging activity by DPPH decolorization

This assay was based on the scavenging of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals by radical scavenging components in *E. cava* extracts. Free radical scavenging activity of the enzymatic extract of seaweed was determined according to the modified method of Blois (1958). DPPH solution was prepared at the concentration of 4×10^{-4}M in ethanol. During the assay, an enzymatic extract of 0.1 mL was mixed with 2.9 mL DPPH solution. The mixture was incubated in the room temperature for 30 min. After standing for 30 min, absorbance was read at 516 nm by UV-vis spectrophotometer. The percentage inhibition was defined by the absorbance at 516 nm in the absence of enzymatic extract to that measured with the sample.

2. 4. DPPH radical scavenging assay by ESR

DPPH radical scavenging activity was measured using the method described by Nanjo *et al.* (1996). An ethanol solution of 60 μL each sample (or ethanol itself as control) was added to 60 μL of DPPH (60 μmol/L) in ethanol. After mixing vigorously for 10 s, the solutions were transferred into a 100 μL Teflon capillary tube and fitted into the cavity of the ESR spectrometer. The spin adduct was measured on an ESR spectrometer (JES-FA machine, JOEL, Tokyo, Japan) exactly 2 min later. A central field of 3475 G, modulation frequency of 100 kHz, modulation amplitude of 2 G, microwave power of 5 mW, gain of 6.3×10^5 and a temperature of 298 K were used. The scavenging effects of samples on DPPH radical were calculated by the ESR signal intensities of samples with and without samples, respectively.

2. 5. Inhibition effect of lipid peroxidation in fish oil

The assay for inhibitory effect of lipid peroxidation in fish oil-in-water emulsion was carried out according to the peroxide value measurement discribed in AOAC (1990) method. Fish oil-in-water

emulsion was prepared as follows. A 40 g of fish oil sample was mixed with 2 g of Tween 80 and homogenized with a 200 mL of water for 2 min at 9,000 rpm. Then water soluble enzymatic extract was introduced into the homogenized emulsion. Thereafter the emulsion was again homogenized by digital homogenizer (HMZ-20DN, Young Ji Co. Ltd., Korea) at 15,000 rpm for 8 min and stored in the dark at 60℃ for 12 days. During each storage day, a 20 mL of sample mixture was removed and mixed with 20 mL of dichloromethane, and then mixed with 5 g sodium chloride (Abdalla and Roozen, 1999). The resulted dichloromethane extract was evaporated under nitrogen and analysed for peroxide value.

3. RESULTS

3. 1. Free radical scavenging activity by DPPH decolorization

Free radical scavenging ability of various enzymatic extracts prepared from *E. cava* was evaluated with the change of absorbance caused by the reduction of DPPH radicals these results are shown as relative activities against the control. Significant differences of the activities among the enzymatic extracts were observed (Fig. 4-1 and 4-2). Viscozyme, Celluclast and AMG extract showed relatively higher radical scavenging activities with 70% or greater in the five carbohydrase extracts, while in the protease extracts Protamex, Kojizyme and Neutrase extract showed relatively higher activities with around 65%. Most enzymatic extracts, except Ultraflo, Flavourzyme and Alcalase extract, indicated over 60% radical scavenging activities and the commercial antioxidants also exhibited strong scavenging activities. The activities of the commercial antioxidants (α-Tocopherol, Ascorbic acid, BHA and BHT) were 89.64, 94.58, 87.38 and 56.05%, respectively (data not shown). Fig. 4-3 revealed that the activities of Celluclast extract among the carbohydrase extracts and Protamex extract among the protease extracts were dependent on the increased concentration of the extracts and reached about 70% at the concentration of 4 mg/mL.

Thermal stabilities of Celluclast and Protamex extract were investigated at 40, 60 and 100℃ according to the heating time (0～24 h) (Fig. 4-4 and 4-5). Those extracts were found to be thermally stable, although rapid decreases were observed after 12 h of heating time at 100℃. At 40 and 60℃ those extracts indicated the remaining activity ranging from 80% to 90% up to 12 h heating time, and ranging from 70% to 80% up to 8 h heating time at 100℃.

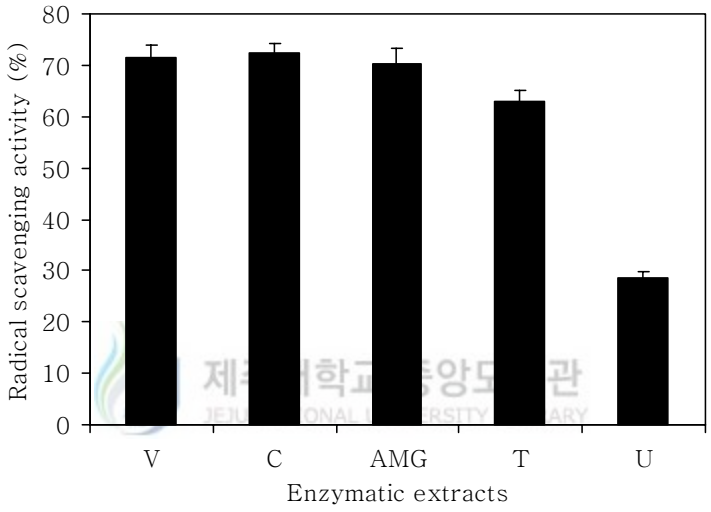

Fig. 4-1. Radical scavenging activity by *E. cava* carbohydrases extracts.
Mean ± SE determinations was made in triplicate experiments.

The enzymatic extracts were prepared using the five carbohydrases. (V : Viscozyme ext., C : Celluclast ext., AMG : AMG ext., T : Termamyl ext., U : Ultraflo ext.)

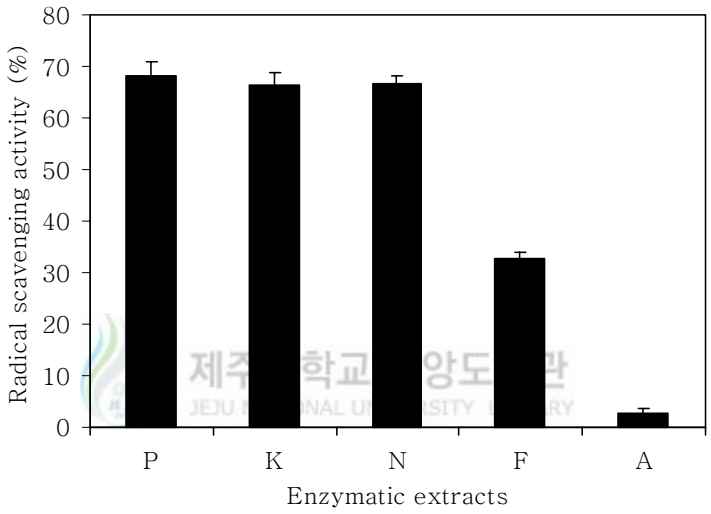

Fig. 4-2. Radical scavenging activity by *E. cava* proteases extracts. Mean ± SE determinations was made in triplicate experiments. The enzymatic extracts were prepared using the five proteases. (P : Protamex ext., K : Kojizyme ext., N : Neutrase ext., F : Flavourzyme ext. A : Alcalase ext.)

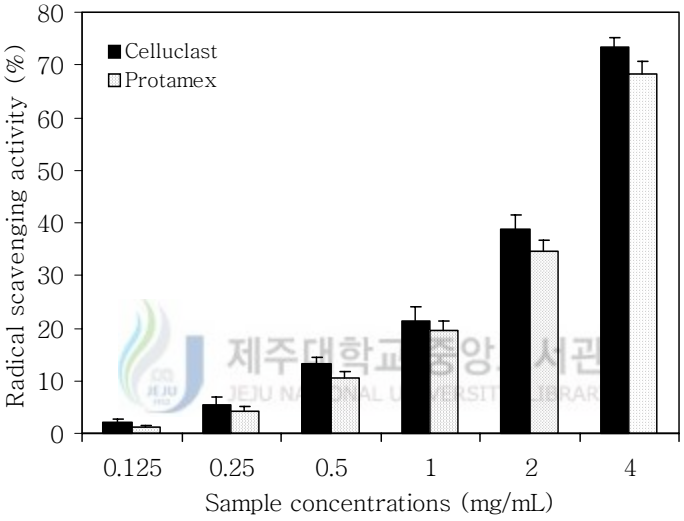

Fig. 4-3. Change in radical scavenging activity of Celluclast and Protamex extract from *E. cava* as affected by concentration of the extracts.
Mean ± SE determinations was made in triplicate experiments.

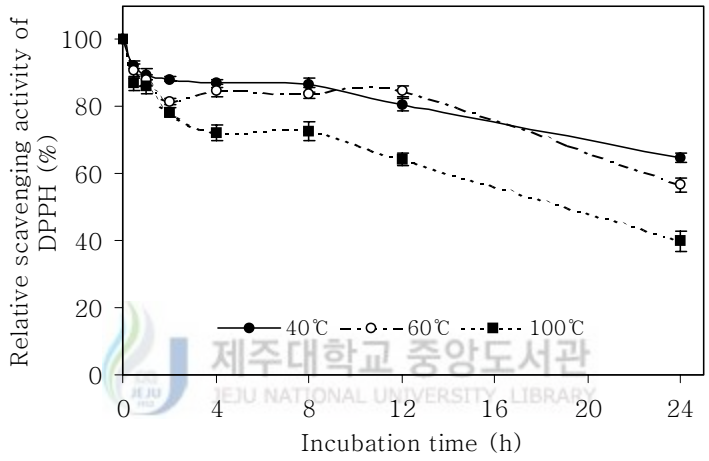

Fig. 4-4. Thermal stability of Carbohydrase (Celluclast) extract in DPPH radical scavenging activity.
Mean ± SE determinations was made in triplicate experiments.

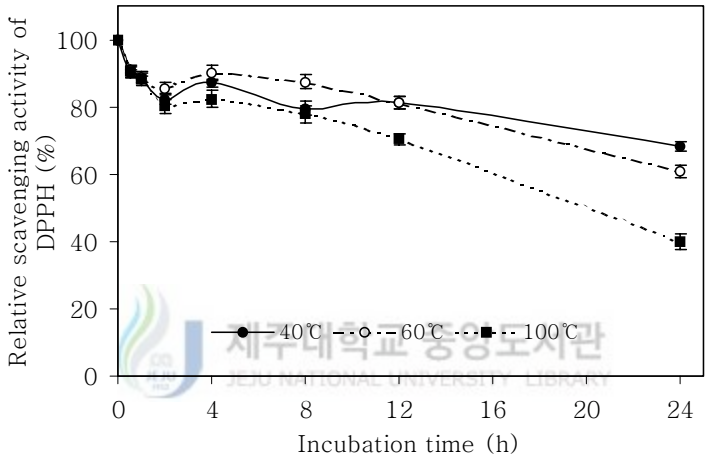

Fig. 4-5. Thermal stability of Ptotease (Protamex) extract in DPPH radical scavenging activity.
Mean ± SE determinations was made in triplicate experiments.

3. 2. DPPH radical scavenging assay using an ESR spectrometer

DPPH is a stable free radical which has been used to evaluate free radical scavenging activity of natural antioxidants. In this study, *E. cava* enzymatic extracts of five carbohydrases and five proteases were investigated on ESR DPPH scavenging assay (Fig. 4-6 and 4-7). The DPPH radical scavenging activities of 2.5 ㎍/mL Viscozyme, Celluclast, AMG, Termamyl and Ultraflo extract were 92.94, 96.75, 93.67, 85.34 and 87.76%, respectively. Moreover, the scavenging activities of 2.5 ㎍/mL protease extracts (Protamex, Kojizyme, Neutrase, Flavourzyme and Alcalase extract) were 87.67, 89.08, 85.74, 83.5 and 87.85%, respectively (Fig. 4-7). The typical ESR signal adducts and the radical scavenging activities of Celluclast and Kojizyme extract on DPPH was observed as shown in Fig. 4-8. According to the results, a great variation existed in the activities among different alga extracts and the radical scavenging activity was dose-dependent.

3. 3. Inhibition effect of lipid peroxidation in fish oil

It was observed in peroxide values that *E. cava* enzymatic extracts and the commercial antioxidants (BHT and α-Tocopherol) retarded lipid oxidation in the fish oil-in-water emulsion (Fig. 4-9 and 4-10). During 12 days of storage at 60℃, the treated emulsion samples showed significantly lower peroxide value than the control counter part. According to Fig. 4-9 and 4-10 the peroxide values in the emulsion samples with antioxidants indicated almost static or a small increment during 4 days of the storage but rapid increment after that storage periods. BHT suppressed the most effectively the fish oil peroxidation. AMG and Ultraflo extract in the carbohydrase extracts inhibited the lipid peroxidation as greatly as that of tocopherol, but all the protease extracts did not effectively inhibit the peroxidation.

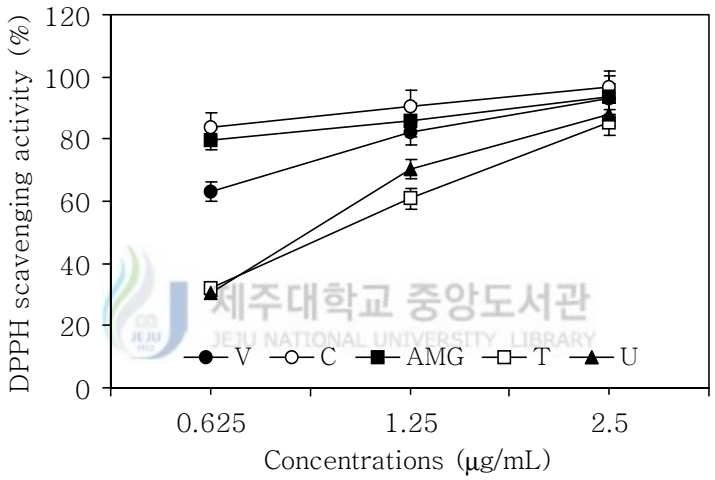

Fig. 4-6. DPPH radical scavenging activity of the extracts prepared with various carbohydrases from *E. cava* using ESR spectrometer. Mean ± SE determinations was made in triplicate experiments.

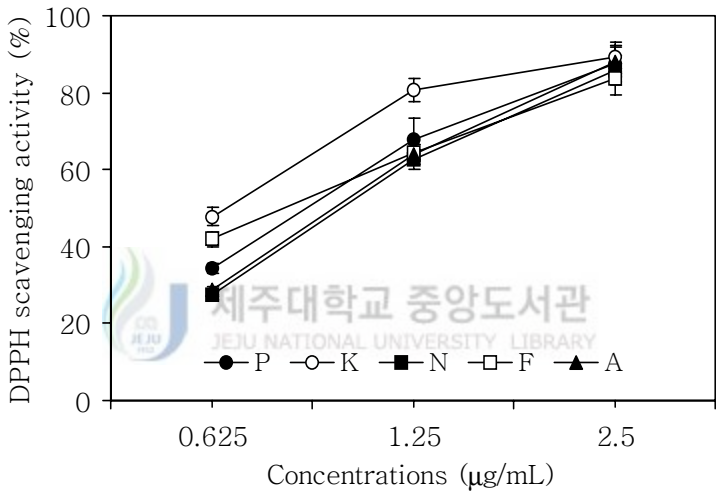

Fig. 4-7. DPPH radical scavenging activity of the extracts prepared with various proteases from *E. cava* using ESR spectrometer. Mean ± SE determinations was made in triplicate experiments.

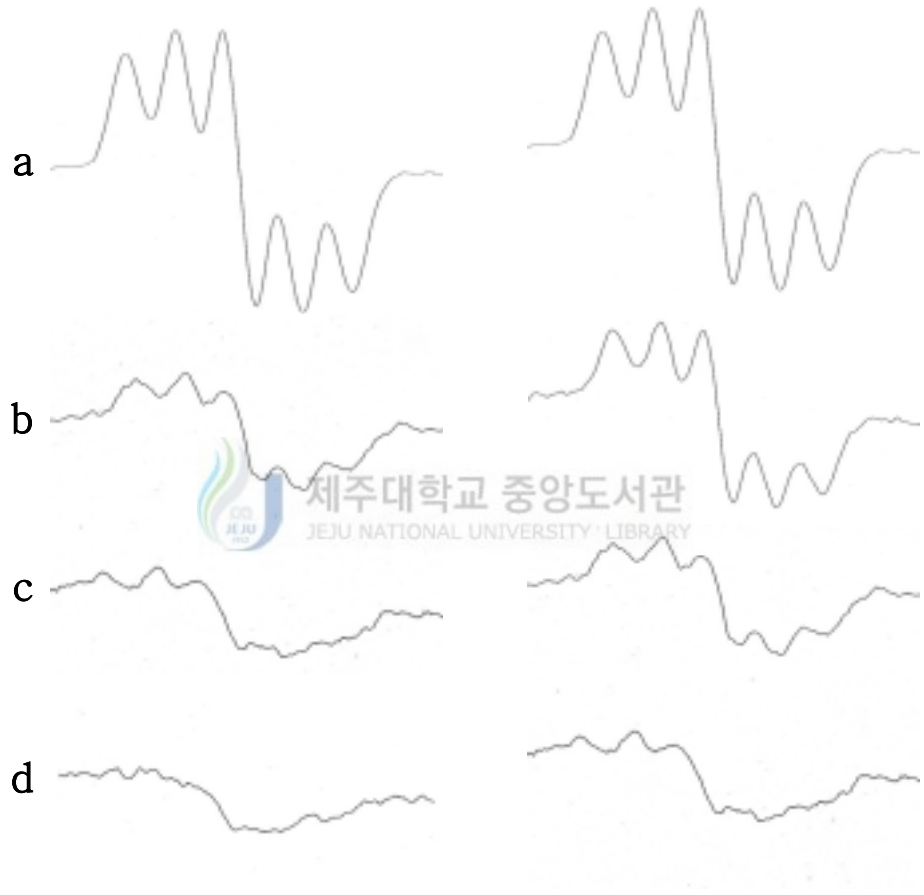

Fig. 4-8. ESR spectra of DPPH radical obtained in an ethanol solution of 30 μM/L DPPH at various concentration of Celluclast extract (left) and Kojizyme extract (right).
a: control, b: 0.625μg/mL, c: 1.25μg/mL, d: 2.5 μg/mL

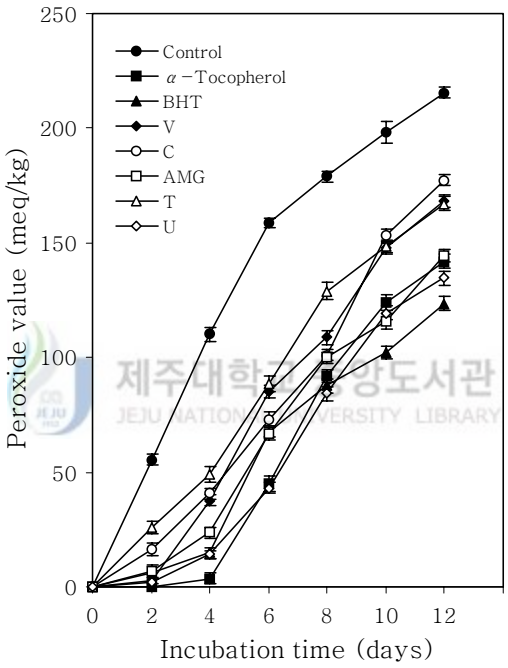

Fig. 4-9. Lipid peroxidation inhibitory effect of the carbohydrases extracts of *E. cava* in fish oil-in-water emulsion stored at 60℃. The amount of lipid peroxidation was measured by peroxide value. Mean ± SE determinations was made in triplicate experiments.

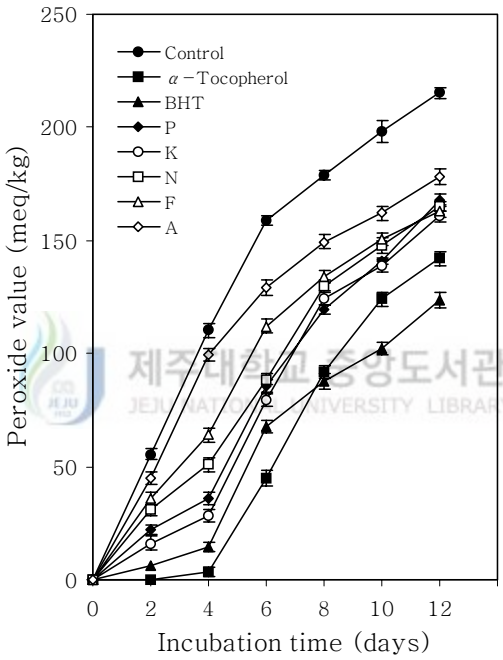

Fig. 4-10. Lipid peroxidation inhibitory effect of the proteases extracts of *E. cava* in fish oil-in-water emulsion stored at 60℃. The amount of lipid peroxidation was measured by peroxide value. Mean ± SE determinations was made in triplicate experiments.

4. DISCUSSION

Recently many researches on phytochemicals of food materials and their effect on human body, especially natural antioxidants from mushroom (Liu *et al*., 1997), red ginseng (Kim *et al*., 2002), an apple pomace (Lu and Foo 2000b), plants (Choi *et al.,* 1992) and seaweeds (Lee, J. H. *et al*., 1996; Heo *et al*., 2003) have been intensively studied. Among them, researches on natural products from seaweeds made significant advances during last two decades and marine algae have been shown to produce a variety of bioactive compounds and some of them have been shown to possess biological activities of potential medicinal value (Konig *et al.,* 1994; Moore, 1978). Seaweeds have become good candidates for the source of natural antioxidants due to a number of studies recently revealed (Fujimoto and Kaneda 1984; Cahyana *et al*., 1992; Yan *et al*., 1998; Rupérez *et al*., 2002; Athukorala *et al*., 2003).

The ability to scavenge free radicals generated by DPPH has been used to evaluate the antioxidant activity of natural compounds or extracts in a short time. Some enzymatic extracts from *E. cava* showed higher radical scavenging activities, especially Celluclast extract among of the five carbohydrases and Protamex extract among the five proteases indicated about 73 and 69% activities respectively, and those were even higher than the activity of BHT. Many researchers have been reported positive correlations between DPPH and phenolic compounds (Lu and Foo 2000b; Kim *et al*., 2002; Oki *et al.,* 2002). In our previous study, *E. cava* enzymatic extracts also reported correlations between DPPH radical scavenging activities and total polyphenolics (Heo *et al*., 2003). However, some controversial results existed in the pervious (Heo *et al.,* 2003) and the present study. Although some enzymatic extracts of *E. cava* (especially Alcalase extract) contained the same phenol contents as the other extracts of *E. cava,* which showed higher activities, they did not indicate higher DPPH radical scavenging activities. This fact implies that the amount of phenolic compounds might not take responsibility of a decisive role for

the activity. It is thought that another materials in seaweed extracts such as small molecular weight polysaccharides, proteins or some organic compounds, probably influence the activity.

Electron spin resonance (ESR) is quite sensitive and a direct method for measuring the presence of long-lived (stable) free radicals. For reactive or transient radicals with low steady-state concentrations, spinning is required in order to absorb the radiation and indicate the resonance from the stable spin adduct. The methodology of spin trapping involves the effect of a magnetic field which changes the frequency of free radical electron and result absorbtion of electromagnetic radiation giving absorbtion spectra. Generally, the absorbtion decreased when the odd electron of DPPH radical is paired. In the present results all the enzymatic extracts examined exerted DPPH radical scavenging activity by paring the odd electron of DPPH radicals. Especially Celluclast among the extracts by the five carbohydrase and Kojizyme extract among the extracts by the five protease showed strong scavenging activities and proteases that the values were 96.75 and 89.08% at 2.5 μg/mL, respectively. The pattern of DPPH radical scavenging by enzymatic extracts were almost similar in both DPPH assays (general UV method and ESR method). This occurrence confirmed the radical scavenging activity and further experiments are to be conducted in order to investigate the mechanism and specific antioxidative compounds.

Peroxide value is an important parameter of the primary oxidation products of an oil system. Lipid peroxidation is a critical problem affecting food quality and stability which lead to rancidity, toxicity and destruction of biochemical components important in physiological metabolism. Fats and oils are usually used as substrates for evaluating the antioxidant activity from natural sources (Duh and Yen 1997; Tian *et al.*, 1994). Lipid oxidation, however, is basically a surface phenomenon, and complex foods contain a multitude of surface active components and their interfacial effects in multi-phased food systems (Frankle, 1996). In the present study, thus the fish oil emulsion systems were prepared for testing the antioxidative efficacy of the enzymatic

extracts of *E. cava*. The recorded peroxide value of AMG and Ultraflo extract indicated higher antioxidative activities during 12 days of storage. The results were quit contrasting to the DPPH assay for these extracts, in which poor activities were observed. These results suggest that there is no correlation between DPPH assay and that based on the inhibitory effect of lipid peroxidation in fish oil. Matsukawa *et al*. (1997) also pointed out the lack of correlation between lipxygenase inhibition, which corresponds to inhibition of lipid peroxidation formation discussed in the current study, and DPPH assay, noting that the two assay systems had quite different antioxidative mechanisms.

In the present study, we found that the enzymatic extracts of *E. cava* are very useful in antioxidant activities regarding to free radical scavenging activity (using UV and ESR assay) and lipid peroxidation inhibitory effect. Although Celluclast, Protamex and Kojizyme extract were less effective in lipid peroxidation inhibition, they strongly scavenged DPPH radicals. In contrast AMG and Ultraflo extract strongly inhibited lipid peroxidation of fish oil, although their radical scavenging activity were weak. Thermal stable antioxidative ability found in this work would be an advantage specially in food systems where the high cooking temperature are being applied during processing. The enzymatic extracts were stable up to 8 h even at the heating temperature of 100℃. Enzymatic extraction of seaweeds in the purpose to obtain natural water-soluble antioxidants would provide remarkable advantages about excellent water solubility, safety and convenient large scale production process of antioxidant extract from seaweeds. Further studies are required for identification and purification of antioxidative compounds from the enzymatic extracts of *E. cava*.

SUMMARY

Special geographical conditions in Jeju costal provide optimum environmental conditions for a variety of marine species. Different compositions of bioactive materials (vitamin, mineral, polyphenols, polysaccharide etc.) can be expected in seaweeds, comparing to the land plants, because marine plants are growing under quite different environmental conditions over the land ones. Many researchers involved in seaweed works reported antimicrobial, antioxidant, antiviral, anti-cancer and immunosuppressant activities as well as control of blood pressure and cholesterol in blood. A number of researches on bioactivities of seaweed have been conducted to seek potential compounds for curing or control hypertension, diabetes, hyperlipemia, obesity and other adult diseases due to recent wrong food habit and the industrialization. Moreover, the use of the synthetic antioxidant can cause a variety of diseases, but natural antioxidants are safe and assured. Therefore researchers continuously pay their attention on such harmless and potential natural antioxidants. Researchers so far were based on screening antioxidants from organic extracts of seaweeds but organic solvent itself are toxic and non-effective in extraction process. Suspected toxicity, lower/specific extraction ability and economic problems are major factors stagnant development in natural bioactive industry.

The objective of this study was to develop water-soluble and harmless natural antioxidants from seaweed. Applying different hydrolytic enzymes to seaweeds could produce various antioxidative compounds which were evaluated through free radical scavenging activity, reactive oxygen species scavenging activity, and inhibitory effect of lipid peroxidation and DNA damage. Of all the enzymatic extracts, the extracts from *E. cava* showed the highest free radical scavenging activity and those from *S. thunbergii* showed the highest hydrogen peroxide scavenging activity. The seaweed enzymatic extracts showed the most effective antioxidant activity in hydrogen peroxide

scavenging assay among all the scavenging assays and in particular respective Ultraflo and Alcalase extract of *S. horneri* and *S. thunbergii* recorded over 90%. This result led to the study on the inhibitory effect of the DNA damage inducing by hydrogen peroxide in human lymphocyte cells and very excellent results were obtained.

Since the enzymatic extracts of seaweed are water-soluble and capable of scavenging reactive oxygen species, this enzymatic procedure could be used in the industry over the conventional extraction procedure. Moreover this enzymatic extraction will be a convenient and a safe method for water-soluble natural antioxidative compound in medicine or food industry.

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