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# Antioxidant Effect of Enzymatic Hydrolyzate from a Kelp, *Ecklonia cava*

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The potential antioxidative activity of water-soluble enzymatic hydrolyzates from a kelp, *Ecklonia cava* was evaluated by free radical scavenging and lipid peroxidation assays. To prepare water-soluble hydrolyzates from *E. cava* the seaweed was enzymatically hydrolyzed by five carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo) and five proteases (Protamex, Kojizyme, Neutrase, Flavourzyme and Alcalase). Among all the hydrolyzates, Celluclast hydrolyzate effectively scavenged free radicals released from DPPH (1,1-diphenyl-2-pricrylhydrazyl) and recorded around 73% scavenging activity at the concentration of 4 $mg \cdot ml^{-1}$. This hydrolyzate was thermally stable and DPPH radical scavenging activity remained 80% or higher at heating temperatures of 40 and 60°C up to 12 h and around 80% at 100°C up to 8 h. AMG and Ultraflo hydrolyzate inhibited the lipid peroxidation of fish oil as that of $\alpha$-tocopherol. These results suggested that an enzymatic extraction will be an effective way for the production of a potential antioxidant from seaweeds.

**Key Words:** antioxidant, *Ecklonia cava*, fishoil, lipid peroxidation, radical scavenging activity

## INTRODUCTION

The kelp, *Ecklonia cava* is distributed in the temperate coastal zone of Korean peninsula and generally forms highly persistent populations in clear waters (Kang *et al.* 2001). *E. cava* has been widely used as a source for products of fucoidan which has been well-known as an antitumor, anticoagulant and antithrombin polysaccharides (Takashi *et al.* 1999; Takashi *et al.* 2000; Satoru *et al.* 2002). In our previous study (Heo *et al.* 2003), *E. cava* hydrolyzates showed positive effect on scavenging reactive oxygen species (ROS) including superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen which are by-products of normal metabolism and attack biological molecules, leading to cell or tissue injury in human body (Sies 1986; Wagner *et al.* 1992).

Recently many phytochemical researches including seaweeds have tried to find natural antioxidants strongly scavenging these free radicals that are powerful oxidants and contain unpaired electrons. Free radicals-mediated modification of DNA, proteins, lipids, and small cellular molecules is associated with a number of pathological processes, including atherosclerosis, arthritis, diabetes, pulmonary dysfunction, ischemia-reperfusion tissue damage and neurological disorders such as Alzheimer's disease (Steinberg *et al.* 1989; Frlich and Riederer 1995). Although 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 *in vivo*. This fact implies that their cells have protective antioxidative mechanisms and compounds (Dykens *et al.* 1992; Sukenik *et al.* 1993; Matsukawa *et al.* 1997). Plant phenolic compounds are known to possess antioxidant ability to scavenge reactive oxygen species and free radicals (Bernard *et al.* 1997; Bhagavathi *et al.* 2002; Owan *et al.* 2003; Cheng *et al.* 2003). Traditional extraction techniques to obtain bioactive materials from seaweeds have used different organic solvents or water. However, there have been several controversial points such as extremely low recovery rate and strict regulations in use of organic solvents in the food industry, and limited yield in the recovery of water-soluble components in water extractions.

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**Table 1.** Optimum hydrolyzation conditions of particular enzymes.

| Enzyme | Optimum conditions | | Buffer used[1)] | Enzyme composition |
|---|---|---|---|---|
| | pH | Temperature | | |
| Viscozyme | 4.5 | 50 | 0.1N AB[2)] | arabanase, cellulase, beta-glucanase, hemi-cellulase and xylanase |
| Celluclast | 4.5 | 50 | 0.1N AB | beta-glucanases |
| AMG | 4.5 | 60 | 0.1N AB | amyloglucosidase |
| Termamyl | 6.0 | 60 | 0.2M PB[3)] | alpha-amylases |
| Ultraflo | 7.0 | 60 | 0.2M PB | beta-glucanases |
| Protamex | 6.0 | 40 | 0.2M PB | endo-proteases |
| Kojizyme | 6.0 | 40 | 0.2M PB | endo / exopeptidase |
| Neutrase | 6.0 | 50 | 0.2M PB | neutral part of *B. amylolique-liquefaciens* proteases |
| Flavourzyme | 7.0 | 50 | 0.2M PB | endo / exopeptidase |
| Alcalase | 8.0 | 50 | 0.2M PB | alcalase |

1) In enzymatic hydrolysis; 2)Acetate buffer; 3) Phosphate buffer

In our previous study (Heo *et al*. 2003), a novel extraction technique using digestive enzymes such as carbohydrases and proteases was employed in order to degrade seaweed tissues and to help releasing a variety of bioactive compounds from the seaweeds. These kinds of enzymes can convert water-insoluble seaweeds into water-soluble materials. Additionally, it has a high yield (around 50%) and higher radical scavenging activity than organic solvent extracts.

In the present study, *E. cava*, a marine brown macroalga, was enzymatically hydrolyzed by carbohydrases and proteases to prepare water-soluble seaweed hydrolyzates, and then the resulting enzymatic hydrolyzates of *E. cava* were evaluated for antioxidant effects related to the free radical scavenging activity and lipid peroxidation inhibitory effect.

## MATERIALS AND METHODS

### Materials

*Ecklonia cava*, a marine brown macroalga, was collected along the Jeju Island coast of Korea from October 2002 to March 2003. Salt, epiphytes and sand were removed using tap water. Finally the seaweed was rinsed carefully in deionised water and stored in a medical refrigerator at –20°C for further experiments. The frozen sample was lyophilized and homogenized with a grinder before extraction. Five carbohydrases such as Viscozyme L, Celluclast 1.5 L FG, AMG 300 L, Termamyl 120 L, Ultraflo L, and five proteases such as Protamex, Kojizyme 500 MG, Neutrase 0.8 L, Flavourzyme 500 MG, Alcalase 2.4 L FG were donated from Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark). The optimum pHs and temperature, and characterizations of those enzymes were summarized in Table 1. 1,1-Diphenyl-2-pricrylhydrazyl (DPPH), fish oil, $\alpha$-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.

### Preparation of enzymatic hydrolyzates from seaweeds

The enzymatic hydrolyzates were obtained according to the method used by Heo *et al.* (2003). The seaweed sample was pulverized into powder using a grinder. A hundred m*l* of a buffer solution was added to one gram of dried alga, and then enzyme was mixed. The enzymatic hydrolysis reactions were performed under optimum conditions of the respective enzymes, as shown in Table, during 12 h. Each sample was clarified by centrifugation at 3,000 rpm for 20 min to remove the residue unhydrolysed. Enzymatic hydrolyzate 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 hydrolyzates were adjusted to 4 $mg \cdot ml^{-1}$.

### Radical scavenging activity

This assay was based on the scavenging of stable DPPH radicals by radical scavenging components in *E. cava* hydrolyzates. Free radical scavenging activity of the enzymatic hydrolyzate of seaweed was determined according to the modified method of Blois (1958). DPPH

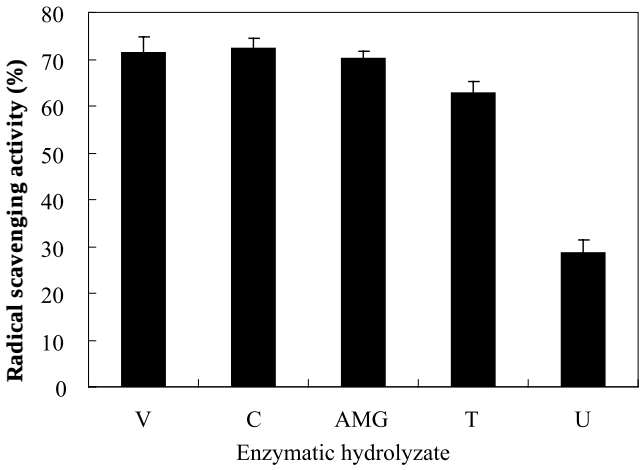


**Fig. 1.** Radical scavenging activity of *E. cava* carbohydrase hydrolyzates. Mean ± SE of determinations was made in triplicate experiments. The enzymatic hydrolyzates were prepared using the five carbohydrases (V, Viscozyme ext.; C, Celluclast ext.; AMG, AMG ext.; T, Termamyl ext.; U, Ultraflo ext.).

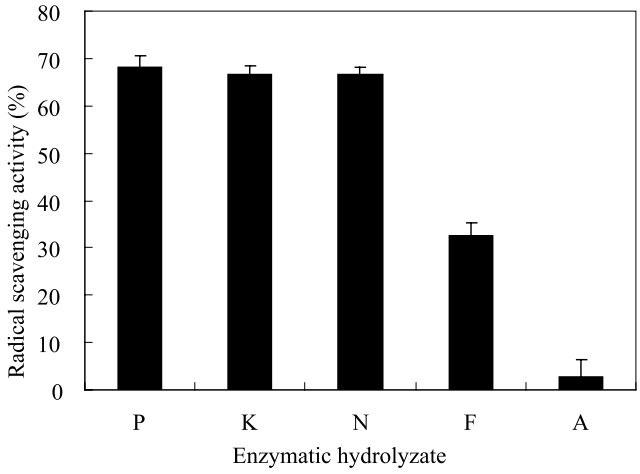


**Fig. 2.** Radical scavenging activity of *E. cava* protease hydrolyzates. Mean ± SE of determinations was made in triplicate experiments. The enzymatic hydrolyzates were prepared using the five proteases (P, Protamex ext.; K, Kojizyme ext.; N, Neutrase ext.; F, Flavourzyme ext.; A, Alcalase ext.).

solution was prepared at the concentration of 4 x $10^{-4}$ M in ethanol. During the assay, an enzymatic hydrolyzate of 0.1 m*l* was mixed with 2.9 m*l* DPPH solution. The mixture was incubated in the room temperature for 30 min. After 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 hydrolyzate to that measured with the sample.

### Inhibition effect of the kelp hydrolyzates on 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 described in AOAC (1995) 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 m*l* of water for 2 min at 9,000 rpm. Then water soluble enzymatic hydrolyzate 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°C for 12 days. During each storage day, a 20 m*l* of sample mixture was removed and mixed with 20 m*l* of dichloromethane, and then mixed with 5 g sodium chloride (Abdalla and Roozen 1999). The dichloromethane extract was evaporated under nitrogen and analysed for peroxide value.

## RESULTS

### Radical scavenging activity

Free radical scavenging ability of various enzymatic hydrolyzates 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 hydrolyzates were observed (Figs 1, 2). Viscozyme, Celluclast and AMG hydrolyzates showed relatively high radical scavenging activities with 70% or greater in the five carbohydrase hydrolyzates, while in the protease hydrolyzates Protamex, Kojizyme and Neutrase hydrolyzates showed relatively higher activities with around 65%. Most enzymatic hydrolyzates, except Ultraflo, Flavourzyme and Alcalase hydrolyzates, indicated over 60% radical scavenging activities and the commercial antioxidants also exhibited strong scavenging activities. The activities of the commercial antioxidants ($\alpha$-Tocopherol, Ascorbic acid, BHA, BHT) were 89.64, 94.58, 87.38, 56.05%, respectively (data not shown). Fig. 3 revealed that the activities of Celluclast hydrolyzate among the carbohydrase hydrolyzates and Protamex hydrolyzate among the protease hydrolyzates were dependent on the increased concentration of the hydrolyzates and reached about 80% at the concentration of 8 mg·m$l^{-1}$. Thermal stabilities of Celluclast and Protamex hydrolyzate were inves-

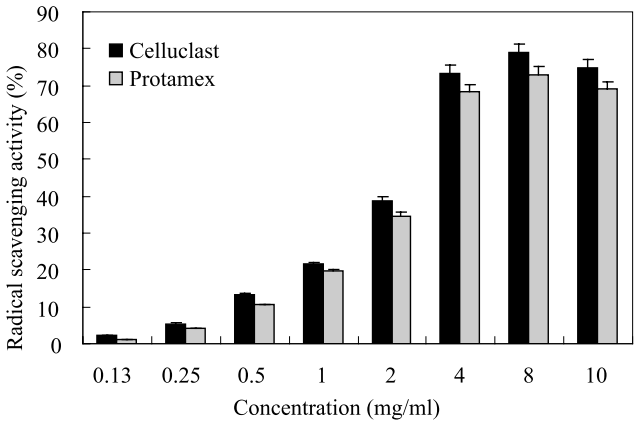


**Fig. 3.** Change in radical scavenging activity of Celluclast and Protamex hydrolyzate from *E. cava* affected by concentration of the extracts. Mean ± SE of determinations was made in triplicate experiments.

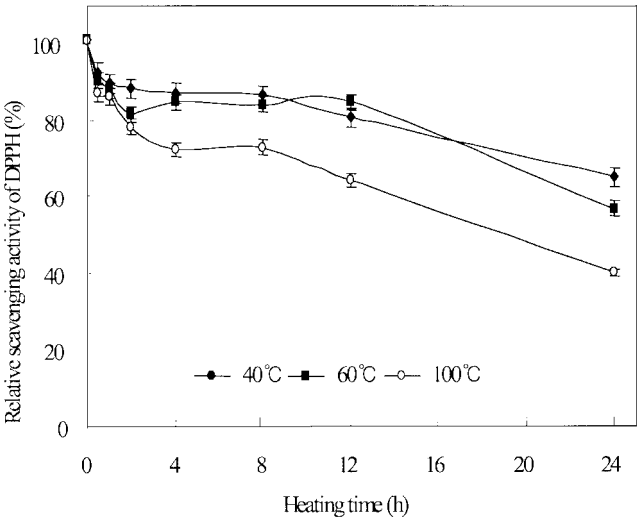


**Fig. 4.** Thermal stability of Celluclast hydrolyzate in DPPH radical scavenging activity.

tigated at 40, 60 and 100°C according to the heating time (0-24 h) (Figs. 4, 5). Those hydrolyzates were found to be thermally stable, although rapid decreases were observed after 12 h of heating time at 100°C. At 40 and 60°C those hydrolyzates 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°C.

### Inhibition effect of the kelp hydrolyzates on lipid peroxidation in fish oil

*Ecklonia cava* enzymatic hydrolyzates and the commercial antioxidants (BHT and α-Tocopherol) retarded lipid oxidation in the fish oil-in-water emulsion (Figs. 6, 7).

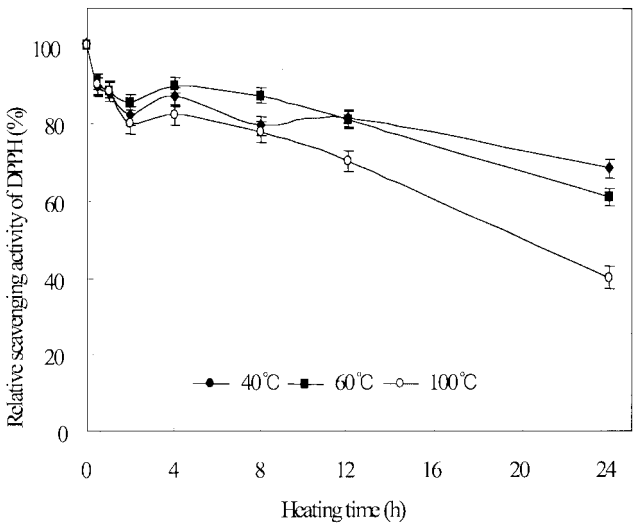


**Fig. 5.** Thermal stability of Protamex hydrolyzate in DPPH radical scavenging activity.

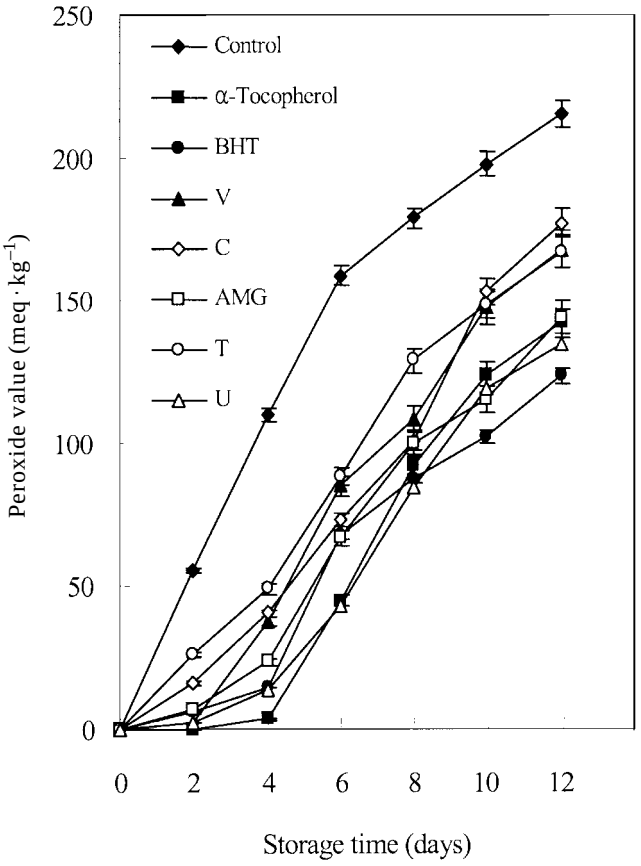


**Fig. 6.** Inhibitory effect on lipid peroxidation of the carbohydrases hydrolyzates of *E. cava* in fish oil-in-water emulsion stored at 60°C. The degree of lipid peroxidation was measured by peroxide value.

During 12 days of storage at 60°C, the treated emulsion samples showed significantly lower peroxide value than

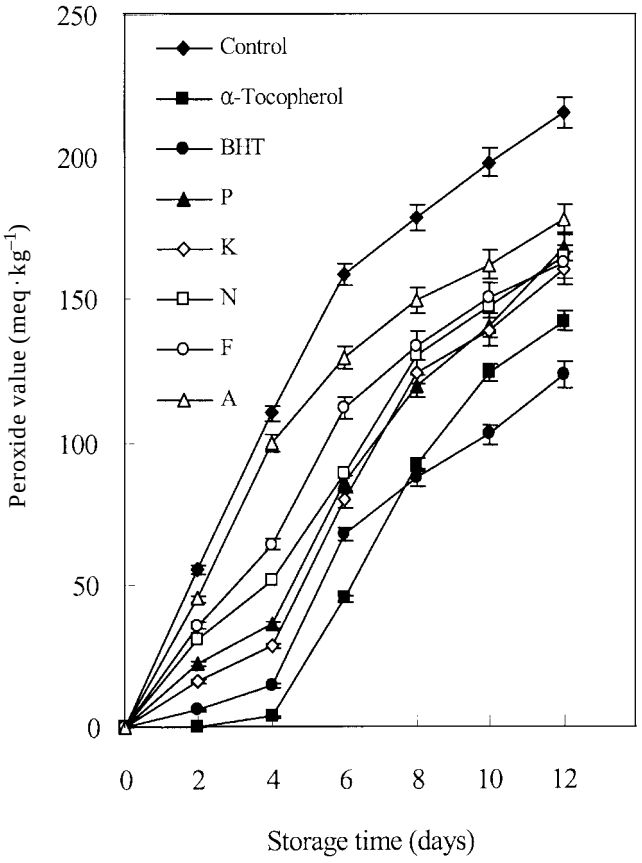


**Fig. 7.** Inhibitory effect on lipid peroxidation of the proteases hydrolyzates of *E. cava* in fish oil-in-water emulsion stored at 60°C. The degree of lipid peroxidation was measured by peroxide value.

that of control. According to Fig. 6 and 7 the peroxide values in the emulsion samples with antioxidants indicated relatively a small increment during 4 days of the storage but rapid increment after that storage periods. BHT showed the highest efficiency in the fish oil peroxidation. AMG and Ultraflo hydrolyzate in the carbohydrase hydrolyzates inhibited the lipid peroxidation as great as that of tocopherol, but all the protease hydrolyzates did not effectively inhibit the peroxidation.

## DISCUSSION

Recently many researches on phytochemicals in food materials and their effects on human body, especially natural antioxidants from mushroom (Liu *et al.* 1997), a red ginseng (Kim *et al.* 2002), an apple pomace (Lu *et al.* 2000), plants (Choi *et al.* 1992) and seaweeds (Lee *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 (Moore 1978; Konig *et al.* 1994). 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; Ruperez *et al.* 2002; Athukorala *et al.* 2003).

Free radical scavenging activity of natural antioxidants can be investigated by using stable DPPH radical scavenging ability of natural extracts. To measure scavenging of DPPH free radicals can be used to evaluate the antioxidant activity of specific compounds or extracts in a short time. Some kinds of enzymatic hydrolyzates from *E. cava* showed higher radical scavenging activities, especially Celluclast hydrolyzate among the five carbohydrase hydrolyzates and Protamex hydrolyzate among the five protease hydrolyzates indicated about 73 and 69% activity, which was even higher than BHT. Many reports have emphasized the fact that correlation between DPPH and phenolic compounds (Lu *et al.*, 2000; Kim *et al.*, 2002). Oki *et al.* (2002) observed that the radical scavenging activity increased with the increase of phenolic content. In our previous study, *E. cava* enzymatic hydrolyzates also reported correlations between DPPH radical scavenging activities and total polyphenolics (Heo *et al.*, 2003). However controversial results also existed in the previous (Heo *et al.* 2003) and the present study. Although some enzymatic hydrolyzates of *E. cava*, especially Alcalase hydrolyzate, contain phenol contents as much as the other hydrolyzates of *E. cava* which showed higher inhibitory effect of lipid peroxidation in linoleic acid, they did not indicate good DPPH radical scavenging activities. This fact implies that the amount of phenolic compounds might not significantly affect the antioxidant activity. It is thought that another materials in seaweed hydrolyzates such as small molecular weights of polysaccharides, proteins or some organic compounds, probably influence the activity.

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 leads 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 many surface active components and their interfacial effects in multi-phased food systems (Frankle 1996). In the present study the fish oil emulsion systems were prepared for testing the antioxidative efficacy of the enzymatic hydrolyzates of *E. cava*. The recorded peroxide value of AMG and Ultraflo hydrolyzate indicated good antioxidative activities during 12 days of incubation. The results were quite contrasting to the DPPH assay for these hydrolyzates, in which a little 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 lipoxygenase 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 hydrolyzates of *E. cava* are very useful in antioxidant activities regarding to the free radical scavenging activity and lipid peroxidation inhibitory effect. Although Celluclast and Protamex hydrolyzate were less effective in lipid peroxidation inhibition, they could effectively scavenge DPPH radicals (around 73% and 69%, respectively). In contrast, AMG and Ultraflo hydrolyzates strongly inhibited lipid peroxidation of fish oil, although their radical scavenging activities were weak (around 41% and 29%, respectively). Thermal stable antioxidative ability found in this work would be an advantage specially in food systems where the high cooking temperature were applied during processing. The enzymatic hydrolyzates were stable up to 8 h even at the heating temperature of 100°C. Enzymatic extraction of seaweeds to obtain natural water-soluble antioxidants would provide remarkable advantages of excellent water solubility, safety and convenient large scale production process of antioxidant preparations from seaweeds. Further studies are required for identification and purification of antioxidative compounds from the enzymatic hydrolyzates of *E. cava*.

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