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Full Length Research Paper

In vitro antioxidant activity of acetylated derivatives of polysaccharide extracted from *Ulva pertusa* (Cholorophta)

Huimin Qi[1,2], Xiaolei Liu[3], Juan Ma[1], Quanbin Zhang[2]* and Zhien Li[2]

[1]Weifang Medical University, No.7166 Baotong Road, Weifang, 261053, P.R.China.
[2]Institute of Oceanology, Chinese Academy of Sciences, Qingdao, 266071, P.R.China.
[3]Weifang Preschool and Special Education Normal School, No.1979 Youai Road, Weifang, 261021, P.R.China.

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Polysaccharides obtained from *Ulva pertusa* (Chlorophyta) contained a group of sulfated heteropolysaccharide referred to as ulvan in this paper. In this study, the acetylated ulvans with different substitution degree were prepared with acetic anhydride/ *p*-toluenesulfonyl chloride in *N*, *N*-dimethylacetamide, and their antioxidant activities were investigated including scavenging activity on superoxide, hydroxyl radicals and reducing power. The natural ulvan and its derivatives exhibited much higher scavenging activity on superoxide radical than vitamin C. Compared with natural ulvan, the acetylated ulvans exhibited stronger reducing power. The antioxidant activity of acetylated ulvans did not increase with increasing substitution degree. Interestingly, the ulvan (AU4) with highest degree of substitution showed weakest scavenging effect on hydroxyl radical.

Key words: Polysaccharides, *Ulva pertusa*, antioxidant activity, acetylated ulvans.

INTRODUCTION

The green alga, *Ulva pertusa* (Chlorophyta), is an important food source in the world. *U. pertusa* is nutritious with low calorie and abundant vitamins, trace elements and dietary fibers (Lahaye and Jegon, 1993). Moreover, it has been used as a drug in traditional Chinese medicine for hyperlipidemia, sunstroke and urinary diseases, etc (Li, 907 - 960, Tang Dynasty). Polysaccharides extracted from *U. pertusa* showed a number of biological effects, such as antihyperlipidemic, antioxidant activity and immune function (Bian et al., 2006; Yu. et al., 2003a and b).

*Corresponding author. E-mail: qihuimin78@yahoo.com.cn. Tel: +86 532 82898708.

Abbreviations: NBT, nitro blue tetrazolium; **PMS,** phenazine methosulfate; **NADH,** nicotinamide adenine dinucleotide-reduced; **EDTA,** ethylene diamine tetra-acetic acid; **H_2O_2,** hydrogen peroxide; **TCA,** trichloroacetic acid; ***p*-TsCl,** *p*-toluenesulfonyl chloride; **DMAc, *N*,** *N*-dimethylacetamide; **LiCl,** lithium chloride; **Ac_2O,** acetic anhydride.

The polysaccharides obtained from *U. pertusa* contained a group of sulfated heteropolysaccharides and the main disaccharide units are [ß-D-Glcp A- (1->4) -α-L-Rhap 3s] and [α-L-Idop A- (1->4) -α -L-Rhap 3s] (Figure 1) (Lahaye, 1998). For simplicity, the sulfated polysaccharides are referred to ulvan in this paper. The activity of polysaccharides depends on several structure parameters such as the molecular weight, the type of sugar, the glycosidic branching, the degree of sulfation and acetylation, and the sulfation or acetylation position (Melo et al., 2002). For example, Zhao et al. (2006) reported that all the degraded porphyrans showed strong antioxidant activity in both assay systems and it increased with reduction of molecular weight. The oversulfated (SD), lowly (LAD), and highly acetylated derivatives (HAD) in reducing power assay exhibited antioxidant activity higher than that of κ-carrageenan oligosaccharides (Yuan et al., 2005). The results indicated that the chemical modification of polysaccharides or oligosaccharides can enhance their antioxidant activity *in vitro*. The aim of this work was to prepare the acetylated ulvans with different substitution degree and determine their

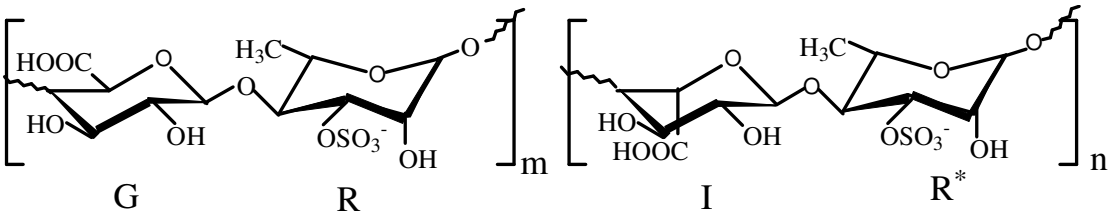

Figure 1. The structure of ulvan, the main disaccharide units [ß-D-Glcp A- (1->4) -α-L-Rhap 3s] and [α-L-Idop A- (1->4) -α -L-Rhap 3s], G: (1→4)- linked ß- D-Glucuronic acid; R: (1→4)- linked α- L-Rhamnose-3-sulfate (linked with ß- D-Glucuronic acid); I: (1→4)- linked α- L- Iduronic acid; R^*: (1→4)- linked α- L-Rhamnose-3-sulfate (linked with α- L- Iduronic acid).

antioxidant activity.

MATERIALS AND METHODS

Chemicals

Nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), nicotinamide adenine dinucleotide-reduced (NADH), ethylene diamine tetra-acetic acid (EDTA), hydrogen peroxide (H_2O_2), and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co (Shanghai, Chinese). All other reagents were of analytical grade. Dialysis membranes were obtained from Spectrum Co (Qingdao, Chinese) and molecular weight was cut off at 3600 Da.

Plant materials

U. pertusa was collected on the coast of Qingdao China (Lu Baoren, Research Associate, Institute of Oceanology, Chinese Academy of Sciences). Algae were washed; air dried and kept in plastic bags at room temperature before using.

Preparation of ulvan

Ulvan were obtained according to the method of Yu et al. (2003b). Dry algae (100 g) were cut roughly and autoclaved in 4000 mL of water at 125°C for 4 h. The hot aqueous solution was separated from the algae residues by successive filtration with gauze and siliceous earth. The solution was dialyzed against tap water for 48 h and against distilled water for 48 h, and then the solution was concentrated to about 1000 mL under reduced pressure. The polysaccharides were precipitated by the addition of 4000 mL of 95% (v/v) ethanol. The resultant was washed three times with dry ethanol, and then dried at 80°C (mean yield, 22.5%).

Preparation of acetylated ulvans

The acetylated ulvan was prepared by the method of Tosh et al. (2000) with minor modification. Briefly, a mixture of ulvan (10 g) and DMAc (*N*, *N*-dimethylacetamide) (375 mL) was heated to 150°C for 26 min. Then, LiCl (8.125 g) was added and the mixture was heated to 166°C for 8 min. And then the reaction mixture was cooled to room temperature and stirred overnight for dissolution. The ulvan solution prepared above was diluted to 1% by further addition of DMAc solvent. To 200 mL of 1% ulvan solution, 14.0 g of *p*-TsCl (*p*-toluenesulfonyl chloride) was added, followed by drop-wise addition of 15 mL or 75 mL of Ac_2O (acetic anhydride). After reaction for 10 h at different temperature, the mixture was termina-ted by pouring 50 mL of distilled water, cooled to room temperature, and precipitated with 85% ethanol for 24 h. The precipitate was filtered off and washed three times with ethanol, and then dissolved in 200 mL distilled water. The solution was dialyzed against tap water for 48 h and distilled water for 48 h using 3600 Da Mw cutoff dialysis membranes. The product was then concentrated and lyophilized to give acetylated ulvans.

Antioxidant activity

Superoxide radical assay

The superoxide radical scavenging ability of natural ulvan and its derivatives was assessed by the method of Nishimiki et al. (1972). The reaction mixture, containing samples (0.006 - 0.21 mg/mL), Tris-HCl (16 mM, pH 8.0), NADH (338 µM), NBT (72 µM) and PMS (30 µM)), was incubated at room temperature for 5 min and the absorbance was read at 560 nm against a blank. The capability of scavenging to superoxide radical was calculated using the following equation:

$$\text{Scavenging effect (\%)} = (1 - A_{\text{sample 560 nm}} / A_{\text{control 560 nm}}) \times 100$$

Where $A_{\text{control 560 nm}}$ is the absorbance of the control (Tris-HCl buffer, instead of sample).

Hydroxyl radical assay

According to the reference of Wang et al. (1994), the reaction mixture, total volume 4.5 mL, containing the samples (0.25 - 1.61 mg/mL), EDTA-Fe^{2+} (220 µM), safranine O (0.23 µM) and H_2O_2 (60 µM) in potassium phosphate buffer (150 mM, pH 7.4), was incubated with for 30 min at 37°C and the absorbance was read at 520 nm against a blank. Hydroxyl radical bleached the safranine O, so decreased absorbance of the reaction mixture indicated a decrease in hydroxyl radical scavenging ability. The capability of scavenging to hydroxyl radical was calculated using the following equation:

$$\text{Scavenging effect (\%)} = [(A_{\text{sample 520 nm}} - A_{\text{blank 520 nm}}) / (A_{\text{control 520 nm}} - A_{\text{blank 520 nm}})] \times 100$$

Where $A_{\text{blank 520 nm}}$ is the absorbance of the blank (distilled water, instead of the samples) and $A_{\text{control 520 nm}}$ is the absorbance of the control (phosphate buffer, instead of H_2O_2).

Reducing power

The reducing power of natural ulvan and its derivatives was

Table 1. The symbols and IR spectrum of ulvan and its derivatives.

Sample	IR (KBr) (cm^{-1})
U	1645, 1256, 844
AU1	1748 (C=O), 1634, 1243, 845
AU2	1742 (C=O), 1643, 1250, 850
AU3	1747 (C=O), 1626, 1248, 851
AU4	1735 (C=O), 1640, 1255, 845

U: natural ulvan; AU1, AU2, AU3, AU4: acetylated ulvans with different substitution degree.

Table 2. Symbols of different degree substitution ulvans, the condition of preparation and the amount of anhydride reacted (mmol).

Sample	Temperature (℃)	Time (h)	Amount of anhydride(mol)	Amount of anhydride reacted (mmol)
AU1	30	10	0.16	9.81
AU2	30	10	0.80	11.84
AU3	50	10	0.80	21.72
AU4	70	10	0.80	30.01

quantified by the method described earlier by Yamaguchi et al. (1998) with minor modification. Briefly, 4 mL of reaction mixture, containing different concentration of samples (0.35 - 0.96 mg/mL) in phosphate buffer (0.2 M, pH 6.6), was incubated with potassium ferricyanide (1% w/v) at 50℃ for 20 min. The reaction was terminated by TCA solution (10% w/v). And then the solution was mixed with distilled water and ferric chloride (0.1% w/v) solution and the absorbance was measured at 700 nm. Reducing power was expressed as a percentage of the activity shown by a 1mmol/L solution of vitamin C.

Statistical analysis

The data presented are means ± S.D. of three determinations, and followed by the Student's t-test. Differences were considered to be statistically significant if $P < 0.05$.

RESULTS

Infrared spectra and NMR spectra

Infrared spectra were recorded from polysaccharide powders in KBr pellet on a Nicolet-360 FTIR spectrometer. The peak at 1735 - 1748 cm^{-1} (Table 1) assigned to the characteristic absorbance of C = O (ester) stretching vibration, which showed that the acetylated ulvan was obtained. The derivative of ulvan gave broad signals in the ^{1}H NMR and ^{13}C NMR spectra recorded in D_2O with poor resolution. The substituted positions need to be further studied. The amount of anhydride consumed in the reaction was determined by titrated against 0.1 M NaOH. The data was shown in Table 2.

Scavenging activity on superoxide radical by natural ulvan and its derivatives

Superoxide radical scavenging activity was determined by the PMS-NADH superoxide generating system. Figure 2a shows the inhibitory effect on the superoxide radical of natural ulvan and its derivatives. The inhibitory effect of all samples was marked and concentration related, moreover, the effect increased with increasing concentration. At the concentration below 0.10 mg/mL, the scavenging activity increased with the concentration increasing significantly, at the concentration higher than 0.10 mg/mL, the scavenging activity was above 90% and increased slowly, while for AU2, the correlation between scavenging activity and concentration was strong, at the concentration 0.006 - 0.052 mg/mL, the scavenging activity was from 23.4 to 93.8%.

At all concentration, AU3 showed the weakest scavenging ability. Furthermore, we studied Vc to scavenging activity on superoxide radical using above-mentioned model, as shown in Figure 2b. The result showed scavenging activity of Vc on superoxide radical was only 4.5 - 89.7% at a concentration of 0.10 - 3.20 mg/mL. Compared to this result, the natural ulvan and its derivatives exhibited much higher scavenging activity on superoxide radical than Vc.

Scavenging hydroxyl radical by natural ulvan and its derivatives

The hydroxyl radicals, generated by the Fenton reaction

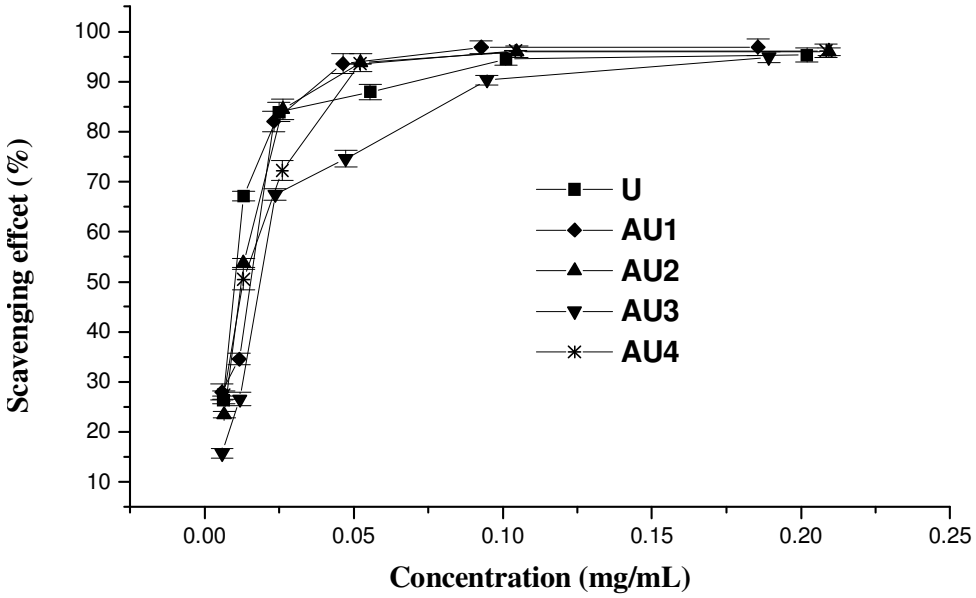

Figure 2a. Scavenging effect of natural ulvan and its derivatives on superoxide radicals. Values are means ± S.D. (n = 3).

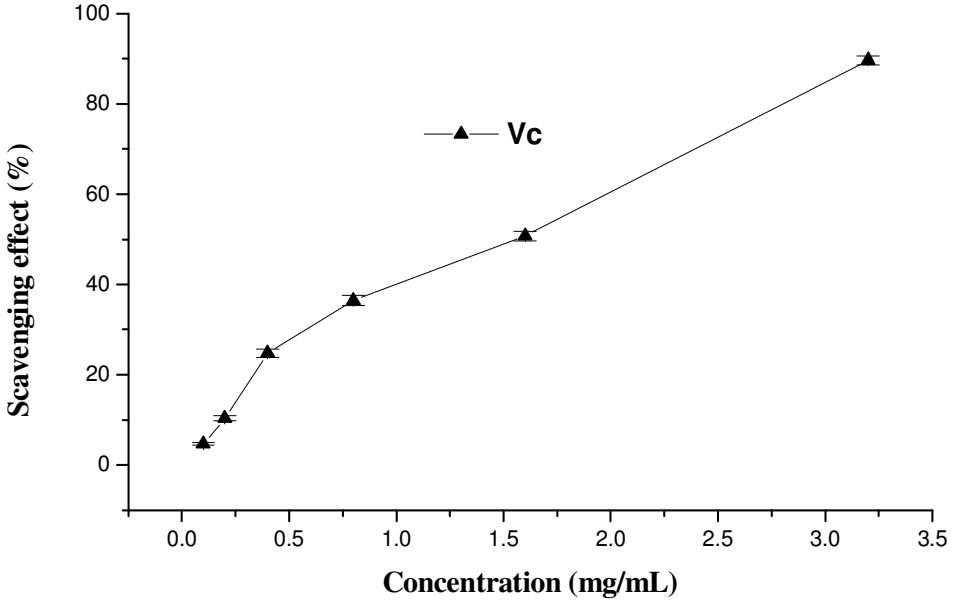

Figure 2b. Scavenging effect of Vc (vitamin C) on superoxide radicals. Values are means ± S.D. (n = 3).

in the system, were scavenged by natural ulvan and its derivatives. The scavenging effects of all samples and Vitamin C are shown in Figure 3. At a concentration of 1.05 mg/mL, the scavenging effects for U, AU1, AU2, AU3, AU4 and vitamin C were determined as 28.8, 42.5, 67.5, 63.1, 11.3 and 22.5%, respectively. This result indicated that all samples except for AU4 had stronger scavenging effect than Vc on hydroxyl radical.

Reducing power of natural ulvan and its derivative

Both natural ulvan and its derivatives exhibited reducing capacity and the results are shown in Figure 4. The reducing capacity of all samples increased with increasing concentration, furthermore, the reducing capacity of ulvan derivatives was stronger than that of natural ulvan, and that of AU4 was the most pronounced. The reducing

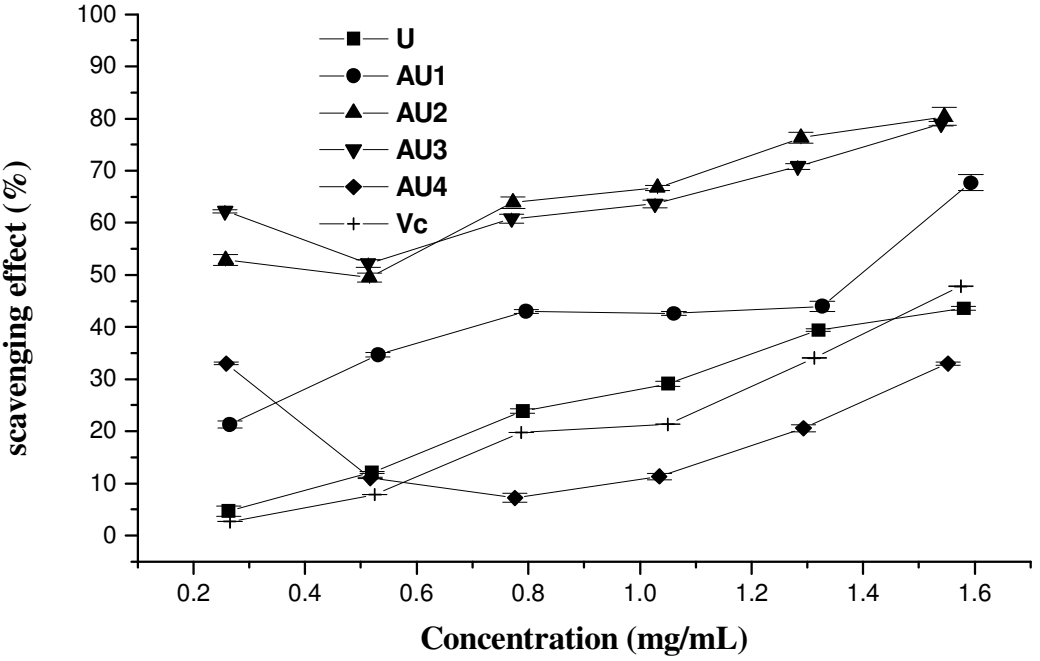

Figure 3. Scavenging effect of natural ulvan and its derivatives on hydroxyl radicals. Values are means ± S.D. (n=3).

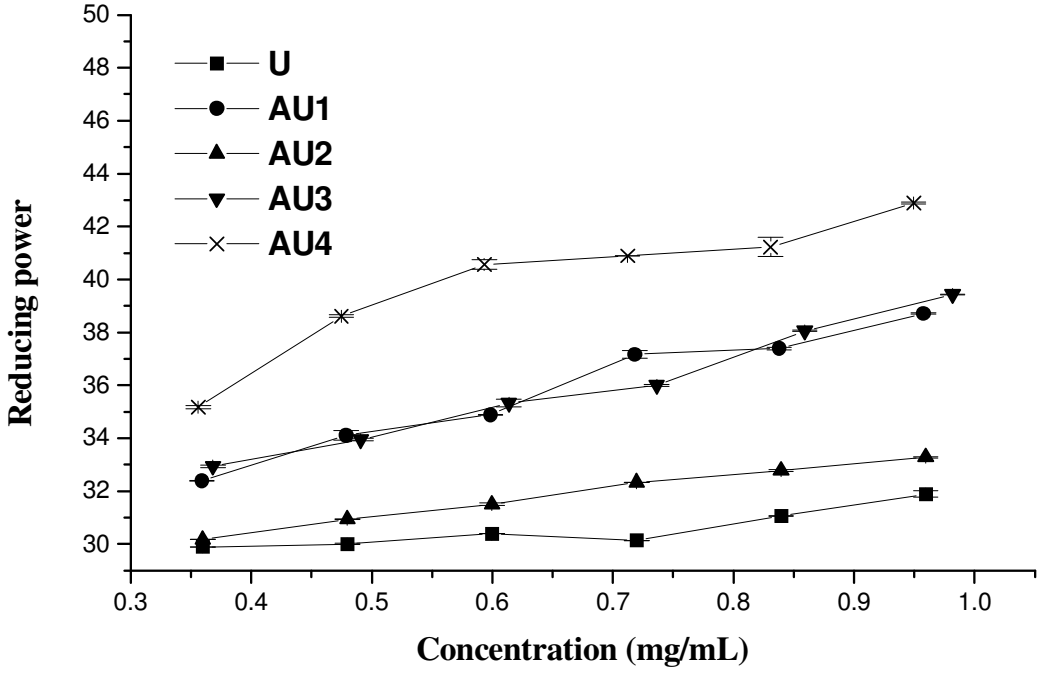

Figure 4. Reducing power of natural ulvan and its derivatives. Values are means ± S.D. (n = 3). Reducing power was expressed as a percentage of the activity shown by a 1 mmol/L solution of vitamin C.

capacities were determined as AU4 > AU1(AU3) > AU2 > U. However, for all the samples, the reducing power was weaker than that of Vc.

DISCUSSION

Superoxide plays an important role in the formation of

other reactive oxygen species, such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems (Lee et al., 2004). In the present study, all samples effectively scavenged superoxide in a concentration-dependant manner. At a concentration of 400 µg/mL, the inhibitory effects of the oversulfated, low-DS and high-DS acetylated derivatives were 92.8, 90.5 and 91.1%, respectively (Yuan et al., 2005). However, at a concentration of 52 µg/mL, the scavenging activity of AU2 was 93.8%. This result proved that the inhibitory effect of acetylated ulvan (AU2) was stronger than that of low-DS and high-DS acetylated derivatives of κ-carrageenan oligosaccharides (Yuan et al., 2005).

The hydroxyl radicals, generated by the Fenton reaction in the system, were scavenged by natural ulvan and its derivatives. The Fenton reaction, that is $Fe^{2+}+H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$ is a standard method employed in investigation of the capabilities if ${}^{\bullet}OH$ radical scavengers (Miller and Fry, 2001). In this research, all samples showed strong scavenging activity on hydroxyl radical, especially AU2 and AU3. Hydroxyl radical is the most reactive free radical and can be formed from superoxide anion and hydrogen peroxide, in the presence of metal ions, such as copper or iron. Hydroxyl radicals have the highest 1-eletron reduction potential (2310 mV) and can react with everything in living organisms at the second-order rate constants of 10^9 - 10^{10} mol/s, moreover, they react with lipid, polypeptides, proteins, and DNA, especially thiamine and guanosine (Hirata, 2004).

In the reducing power assay, the color of test solution was changed from yellow to various shades of green and blue colors depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of the Fe^{3+}/ferrcyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Chung et al., 2002). Tanaka et al. (1988) reported that the antioxidant activity was concomitant with the reducing power. The antioxidant activity has been reported to have a direct, positive correlation with the reducing power (Osman et al., 2004). Our data on the reducing power of all samples suggested that it was likely to contribute toward the observed antioxidant effect.

In this present study, the acetylated ulvans exhibited higher antioxidant activity than natural ulvan in certain antioxidant systems *in vitro*. AU1, AU2 and AU3 showed stronger scavenging activity than natural ulvan on hydroxyl radical, in addition, the reducing power of ulvan derivatives was more pronounced than that of natural ulvan. The results proved that chemical structure modification of polysaccharide could enhance their antioxidant activity. There are few reports on the structure-antioxidant activity relationship of saccharides. Xing et al. (2005) investigated the free radical scavenging activity of differently regioselective chitosan sulfates. They observed that sulfated chitosan of $C_{2,3,6}$ sulfation showed the highest scavenging activity on superoxide radical. Yuan et al. (2005) reported that oversulfated and acetylated κ-carrageenan oligosaccharides exhibited higher scavenging activity on superoxide radical than κ-carrageenan oligosaccharides. In our previous study, different sulfate content ulvans showed different antioxidant activity, furthermore, high sulfate content ulvans showed stronger antioxidant activity than natural ulvan. In our opinion, the antioxidant activity may have originated from their hydrogen atom donating capacity. According to previous studies, the addition of electron-donating substituents to a heterocyclic ring increased radical scavenging activity as a result of increasing electron density at carbon atoms in the heterocyclic ring. In contrast, the presence of electron-withdrawing substituents decreases electron density around the heterocyclic ring, hence decreasing its ability to scavenge free radicals (Eiserich et al., 1995; Eiserich and Shibamoto, 1994). However, Yanagimoto et al. (2002) reported that addition of electron-withdrawing groups (acetyl) to the pyrrole enhanced antioxidant activity. In our study, the antioxidant activity of acetylated ulvans was not increased with increasing degree of substitution, and the highest degree of substitution ulvan (AU4) showed the weakest scavenging effect on hydroxyl radical at high concentration. This suggests that only the electron density of carbon atoms on a heterocyclic ring may not determine the strength of antioxidant activity. Other properties of the compounds, such as polarity, may also be involved in their antioxidant activity. Thus, further investigation is necessary to clarify this point.

Conclusion

The antioxidant activity of natural ulvan and ulvans with different substitution degree *in vitro* was determined. Among these samples, AU2 and AU3 showed the strongest scavenging activity on hydroxyl radical. However, AU4 exhibited the weakest scavenging activity. On the other hand, AU4 showed the most pronounced reducing power, and U exhibited the weakest reducing power, but the difference was not significant. This result explained that chemical modification could enhance the antioxidant activity of ulvan. However, the antioxidant activity was not increased with increasing degree of substitution. Further *in vivo* experiments are planned to verify relation between chemical structure and properties and antioxidant activity.

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