Available online at www.sciencedirect.com
SCIENCE DIRECT
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 2441–2445

In vitro antioxidant activity of acetylated and benzoylated derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta)

Huimin Qi,[a,b] Quanbin Zhang,[a,*] Tingting Zhao,[a,b] Rugui Hu,[a,b] Kun Zhang[c] and Zhien Li[a]

[a]*Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, PR China*
[b]*Graduate School of the Chinese Academy of Sciences, Beijing 100039, PR China*
[c]*Department of Life Sciences and Technology, Ocean University of China, No.5 Yushan Road, Qingdao 266003, PR China*

Received 26 October 2005; revised 8 January 2006; accepted 24 January 2006
Available online 14 February 2006

Abstract—The antioxidant activity of natural ulvan and its derivatives (acetylated and benzoylated ulvans) in vitro was determined, including scavenging activity against superoxide and hydroxyl radicals, reducing power, and chelating ability. Obvious differences in antioxidant activity between natural ulvan and its derivatives were observed, moreover, the antioxidant activity of acetylated and benzoylated ulvans was stronger than that of natural ulvan.

A vast amount of circumstantial evidence implicates oxygen-derived free radicals (especially superoxide and hydroxyl radicals) as mediators of inflammation, shock, and ischemia/reperfusion injury. Furthermore the radicals also play a role in the process of aging and carcinogenesis.[1] In order to reduce damage of free radicals, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and *tert*-butylhydroquinone (TBHQ) are used. However, BHA and BHT have been suspected of being responsible for liver damage and carcinogenesis.[2] Thus, it is essential to develop and utilize effective and natural antioxidants so that they can protect the human body from free radicals and retard the progress of many chronic diseases.[3] In the search of new natural antioxidants, exploration of aquatic habitats has led to the discovery that marine plants also contain antioxidants. Marine plants characteristically contain sulfated polysaccharides that are not found in land plants. In recent years, sulfated polysaccharides from marine algae have been reported to have antioxidant activity. Hu et al.[4] found that sulfated polysaccharides from *Laminaria japonica* and *Ecklonia kurome* showed free radical scavenging activities; sulfated polysaccharides from *Ulva pertusa* were also demonstrated to have antioxidant activity including scavenging activity against superoxide and hydroxyl radicals, chelating ability, and reducing power.[5]

The green alga, *U. pertusa*, is distributed in China in the intertidal zone of the Yellow Sea and the Bohai Sea. *U. pertusa* is nutritious with low calorie and abundant vitamins, trace elements, and dietary fibers.[6] The polysaccharide extracted from *U. pertusa* is a group of sulfated heteropolysaccharides and the main disaccharide units are [β-D-Glcp A-(1 → 4)-α-L-Rhap 3s] and [α-L-Idop A-(1 → 4)-α-L-Rhap 3s] (Fig. 1).[7] For simplicity, the sulfated polysaccharide is referred to as ulvan in this paper. In our previous study, ulvan showed antioxidant activity, but the activity was weak.[5] The activity of polysaccharide depends on several structural parameters such as the degree of sulfation (DS), the molecular weight, the sulfation

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

Keywords: Ulvan; Antioxidant activity; Acetylated and benzoylated ulvans.

* Corresponding author. Tel./fax: +86 532 8289 8703; e-mail addresses: qihuimin@ms.qdio.ac.cn; qbzhang@ms.qdio.ac.cn

0960-894X/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2006.01.076

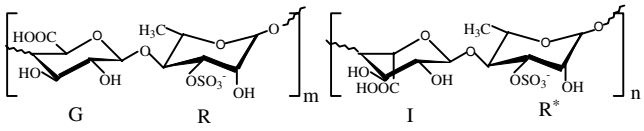

Figure 1. The structure of ulvan, the main disaccharide units [β-D-Glcp A-(1 → 4)-α-L-Rhap 3s] and [α-L-Idop A-(1 → 4)-α-L-Rhap 3s], G: (1 → 4)-linked β-D-glucuronic acid; R: (1 → 4)-linked α-L-rhamnose-3-sulfate (linked with β-D-glucuronic acid); I: (1 → 4)-linked α-L-iduronic acid; R*: (1 → 4)-linked α-L-rhamnose-3-sulfate (linked with α-L-iduronic acid).

position, type of sugar, and glycosidic branching.[8] Thus, chemical modifications of polysaccharides provided an opportunity to obtain new agents with possible therapeutic uses.[9] In the present study, acetylated and benzoylated derivatives of ulvan were prepared and their antioxidant activity in vitro was determined. To our knowledge, the preparation and antioxidant activity of acetylated and benzoylated ulvans have not been reported.

Ulva pertusa was collected on the coast of Qingdao China. Algae were washed, air-dried, and kept in plastic bags at room temperature before using. Ulvans were obtained according to the method of Yu et al.[10] The acetylated ulvan was prepared by the method of Tosh et al.[11] with minor modification. Briefly, a mixture of ulvan (8 g) and DMAc (*N*,*N*-dimethylacetamide) (300 mL) was heated to 150 °C for 26 min. Then, LiCl (lithium chloride) (6.5 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 100 mL of 1% ulvan solution, 7.0 g of *p*-TsCl (*p*-toluenesulfonyl chloride) was added, followed by dropwise addition of 30 mL of Ac_2O (acetic anhydride). After reaction for 10 h at 60 °C, the mixture was terminated 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 100 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 ulvan (yield, 75.6%). The benzoylated ulvan was prepared according to a similar procedure, except that Ac_2O was replaced with phthalic acid anhydride (yield, 65.3%). Infrared spectra were recorded from polysaccharide powders in KBr pellet on a Nicolet-360 FTIR spectrometer. The symbols and IR spectrum data of natural ulvan and its derivatives are shown in Table 1. The peaks at 1747 and 1716 cm^{-1} were assigned to the characteristic absorbance of C=O (ester) stretching vibration, which showed that the acetylated and benzoylated ulvans were obtained.[10,11] Both the derivatives of ulvan (AU and BU) gave broad signals in the ^1H NMR and ^{13}C NMR spectra recorded in D_2O with poor resolution. The substituted positions needs to be further studied.

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

Samples	IR (KBr) (cm^{-1})
U[a]	1641, 1260, 850
AU[b]	1747 (C=O)[d], 1626, 1246, 835
BU[c]	3028 (=C–H)[e], 1716 (C=O)[d], 1621, 1568 (C=C)[f], 1447 (C=C)[f], 1264, 845

[a] Ulvan, the natural polysaccharide extracted from *Ulva pertusa*.
[b] Acetylated ulvan.
[c] Benzoylated ulvan.
[d] C=O of ester.
[e] =C–H of phenyl group.
[f] C=C of phenyl group.

The superoxide radical scavenging ability of natural ulvan and its derivatives was assessed by the method of Nishimiki et al.[12] The reaction mixture, containing samples (0.006–0.2 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 superoxide radical was calculated using the following equation:

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

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

According to the reference,[13] the reaction mixture, total volume 4.5 mL, containing the samples (0.25–1.52 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 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 hydroxyl radical was calculated using the following equation:

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

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

The reducing power of natural ulvan and its derivatives was quantified by the method described earlier by Yamaguchi et al.[14] with minor modification. Briefly, 4 mL of reaction mixture, containing different concentration of samples (0.39–1.01 mg/mL) in phosphate buffer (0.2 M, pH 6.6), was incubated with potassium ferricyanide (1% w/v) at 50 °C 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 1 mM solution of Vitamin C.

The ferrous ion chelating ability of natural ulvan and its derivatives was investigated according to the method of Lopes et al.[15] Briefly, the reaction mixture, containing samples (0.25–1.90 mg/mL), $FeCl_2$ (0.1 mL, 2 mM), and ferroizine (0.4 mL, 5 mM), was shaken well and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm against a blank. The ability of all samples to chelated ferrous ion was calculated using the following equation:

$$\text{Chelating ability } (\%) = (A_{\text{control 562}} - A_{\text{sample 562}})/A_{\text{control 562}} \times 100,$$

where $A_{\text{control 562}}$ is the absorbance of the control (distilled water, instead of sample).

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

Superoxide radical was generated in a PMS/NADH system for being assayed in the reduction of NBT. Figure 2a depicts the inhibitory effect on the superoxide radical of U, AU, and BU. The inhibitory effect of all samples was marked and concentration related. As shown in Figure 2a, for U, AU, and BU, at the concentration below 0.05 mg/mL, the scavenging effect significantly increased with increasing concentration, at the concentration higher than 0.08 mg/mL, the scavenging effect was about 90% and increased slowly. On the other hand, at the concentration below 0.03 mg/mL, U showed stronger scavenging activity against superoxide radicals than AU and BU. At the concentration over 0.08 mg/mL, scavenging activity of U was weaker than that of AU and BU, but the difference was not significant. The IC_{50} values of U, AU, and BU were 9.17, 12.1, and 22.7 μg/mL, respectively. We also studied the scavenging activity of Vitamin C against superoxide radical using the above-mentioned model. As shown in Figure 2b, the IC_{50} value of Vitamin C was 1.6 mg/mL. Compared with this result, natural ulvan and its derivatives showed stronger scavenging activity for superoxide radicals than Vitamin C ($P < 0.05$). Although superoxide is a relatively weak oxidant, it decomposes to form stronger, reactive oxidative species, such as singlet oxygen and hydroxyl radicals, which initiate peroxidation of lipids. Furthermore, superoxides are also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals.[16] These results clearly suggested that the antioxidant activities of all samples were related to their abilities to scavenge superoxides.

The hydroxyl radicals, generated by the Fenton reaction 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. Among the three samples and Vitamin C, AU exhibited the strongest scavenging activity against hydroxyl radical. Moreover, the three samples showed higher scavenging activity against hydroxyl radical than Vitamin C, and that the scavenging effect of all samples and Vitamin C increased with increasing the concentration. At a concentration of 0.25–1.52 mg/mL, the scavenging effect was 3.3–37% for U, 30.8–70.2% for AU, 10.4–46.9% for BU, and 2.7–36.2% for Vitamin C. The IC_{50} value of AU was 0.88 mg/mL, however, the IC_{50} values of U, BU, and Vitamin C could not be read in Figure 3.

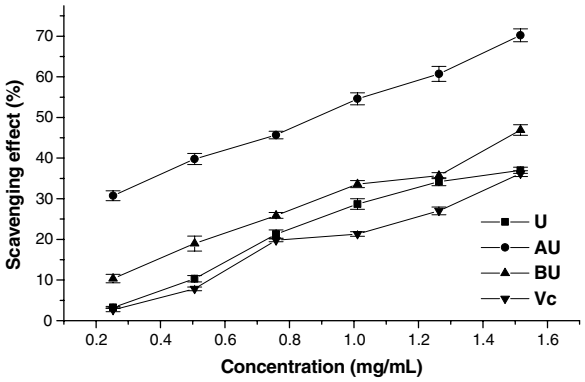

Figure 3. Scavenging effect of natural ulvan, its derivatives and Vitamin C on hydroxyl radicals. Values are means ± SD ($n = 3$).

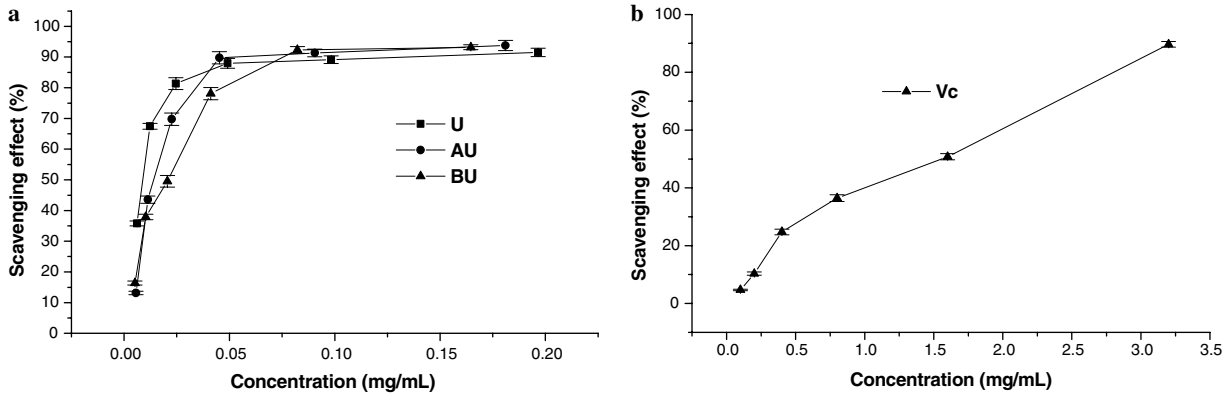

Figure 2. (a) Scavenging effect of natural ulvan and its derivatives on superoxide radicals. Values are means ± SD ($n = 3$). (b) Scavenging effect of Vitamin C on superoxide radicals. Values are means ± SD ($n = 3$).

For hydroxyl radical, there are two types of antioxidation mechanism; one suppresses the generation of the hydroxyl radical, and the other scavenges the hydroxyl radicals generated. In the former, the antioxidant activity may ligate to the metal ions which react with H_2O_2 to give the metal complexes. The metal complexes thus formed cannot further react with H_2O_2 to give hydroxyl radicals.[17] In this study, in another assay system, we demonstrated the iron chelating ability of all samples. U and BU samples exhibited weak chelating ability. It was likely that both of the two mechanisms might be responsible for the inhibition of hydroxyl radical. However, AU showed strong chelating ability. It was likely that the radical scavenging activity of AU might be attributed to the chelating ability. The mechanism of natural ulvan and its derivatives on the hydroxyl radicals need to be further investigated.

In the reducing power assay, the yellow color of test solution changes into 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+}/ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm.[18] Figure 4 depicts the reducing power of U, AU, and BU. The reducing power of AU and BU correlated well with increasing concentration. In addition, the reducing power of ulvan derivatives was more pronounced than that of natural ulvan, and that of BU was the most pronounced ($P < 0.05$, compared with U). The orders of reducing power were BU > AU > U. However, for all the samples, the reducing power was weaker than that of Vitamin C. Tanaka et al.[19] reported that the antioxidant activity was concomitant with the reducing power. Our data on the reducing power of all samples, especially AU and BU, suggested that it was likely to contribute toward the observed antioxidant effect.

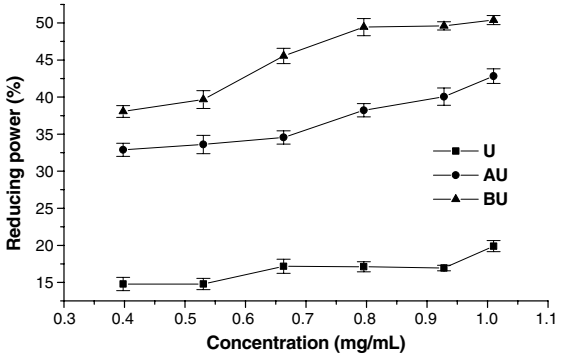

Figure 4. Reducing power of natural ulvan and its derivatives. Values are means ± SD ($n = 3$). Reducing power was expressed as a percentage of the activity shown by a 1 mM solution of Vitamin C.

Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxidase to reactive free radicals via the Fenton type reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$). Fe^{3+} ion also produces radicals from peroxides, although the rate is tenfold less than that of Fe^{2+} ion.[20] Fe^{2+} ion is the most powerful pro-oxidant among various species of metal ions.[21] Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complexes decreases. Measurement of color reduction therefore allows estimating the metal chelating activity.[22,23] Figure 5a shows that the ferrous ion chelating ability of AU was concentration related and that of U and BU was not concentration dependent. The chelating ability of U and BU was weak. At a concentration of 0.31–1.88 mg/mL, the chelating ability ranged from 10% to 20% for U and BU. AU showed the strongest chelating ability among the three samples. At a concentration of 0.31–1.88 mg/mL, the chelating ability ranged from 37% to 78% for AU. However, as shown in Figure 5b, compared with EDTA, the chelating ability of all samples, especially U and BU on ferrous ion, was weaker. Gordon reported that chelating agents, which form σ bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion.[24] In addition, it was reported that the

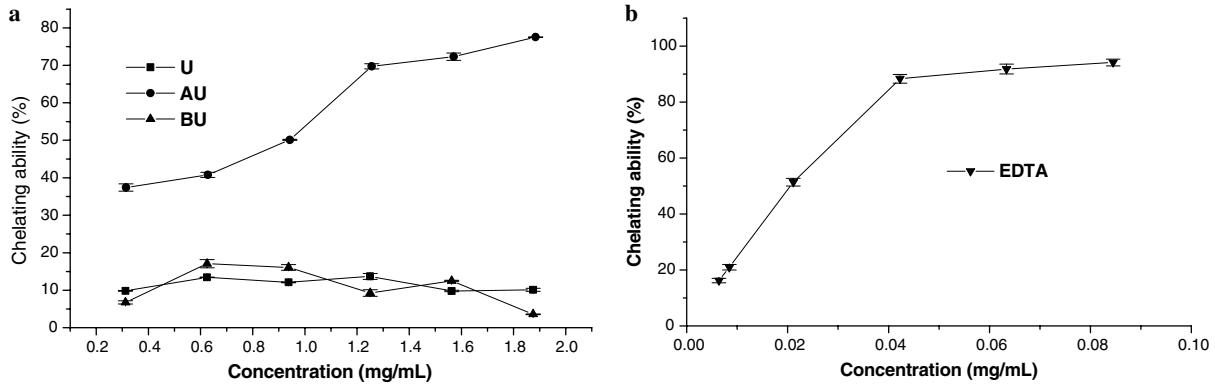

Figure 5. (a) Chelating ability of natural ulvan and its derivatives. Values are means ± SD ($n = 3$). (b) Chelating ability of EDTA. Values are means ± SD ($n = 3$).

compounds with structures containing two or more of the following functional groups: –OH, –SH, –COOH, $–PO_3H_2$, C═O, $–NR_2$, –S–, and –O– in a favorable structure–function configuration can show metal chelating activity.[25,26]

The acetylated and benzoylated ulvans exhibited higher antioxidant activity than natural ulvan in certain antioxidant systems in vitro, which indicated that the chemical modification of ulvan could enhance their antioxidant activity. In our previous study, different sulfate content ulvans showed different antioxidant activity, furthermore, high sulfate content ulvans showed stronger antioxidant activity than natural ulvan.[5] In addition, the acetylated ulvan (AU) exhibited stronger chelating ability compared with high sulfate ulvan (HU5, sulfate content 32.8%). At a concentration of 1.85 mg/mL, the chelating ability was 36% and 75%, for HU5 and AU, respectively. Yuan et al.[27] reported that oversulfated and acetylated κ-carrageenan oligosaccharides showed higher scavenging activity on superoxide radical than κ-carrageenan oligosaccharides. These results proved that chemical structure modification of polysaccharide could enhance their antioxidant activity.

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.[28,29] However, Yanagimoto et al.[30] reported that addition of electron-withdrawing groups (acetyl) to the pyrrole enhanced antioxidant activity. 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.[30] One of the mechanisms involved in antioxidant activity is the ability of a molecule to donate a hydrogen atom to a radical and the propensity of hydrogen donation is the critical factor that involves free radical scavenging.[31] In our opinion, the antioxidant activity may have originated from their hydrogen atom-donating capacity. The acetyl and benzoyl groups, which might substitute in C-2 and (or) C-3 of ulvan, could activate the hydrogen atom of the anomeric carbon. The higher activated capacity of the group, the stronger hydrogen atom-donating capacity the derivatives have. The acetylated and benzoylated ulvans appear to function as good hydrogen atom donors and therefore should be able to terminate radical chain reactions by converting free radicals to more stale products. Hence, acetylated and benzoylated ulvans showed more pronounced antioxidant activity than natural ulvan.

In conclusion, the antioxidant activity of natural ulvan, acetylated and benzoylated ulvans in vitro was determined, including scavenging activity against superoxide and hydroxyl radicals, reducing power, and chelating ability. Among the three samples, acetylated ulvan showed the strongest scavenging activity against hydroxyl radical and chelating ability, however, benzoylated ulvan exhibited the strongest reducing power.

Acknowledgments

This work was supported by 04-2-HH-68 of Scientific and Technical Bureau of Qingdao, and 'The Research of New Marine Drug'.

References and notes

1. Cuzzccrea, S.; Riley, D.; Caputi, A. P.; Selvemini, D. *Pharmacol. Revs.* **2001**, *53*, 135.
2. Grice, H. C. *Food Chem. Toxicol.* **1988**, *26*, 717.
3. Nandita, S.; Rajini, P. S. *Food Chem.* **2004**, *85*, 611.
4. Hu, J. F.; Geng, M. Y.; Zhang, J. T.; Jiang, H. D. *J. Asian Nat. Prod. Reports* **2001**, *3*, 353.
5. Qi, H. M.; Zhang, Q. B.; Zhao, T. n. T.; Chen, R.; Zhang, H.; Niu, X. Z.; Li, Z. E. *Int. J. Biol. Macromol.* **2005**, *37*, 195.
6. Lahaye, M.; Jegon, D. *J. Appl. Phycol.* **1993**, *5*, 195.
7. Lahaye, M. *Carbohydr. Res.* **1998**, *314*, 1.
8. Melo, M. R. S.; Feitosa, J. P. A.; Freitas, A. L. P.; de Paula, R. C. M. *Carbohydr. Polym.* **2002**, *49*, 491.
9. Franz, G.; Alban, S. *Int. J. Biol. Macromol.* **1995**, *17*, 311.
10. Yu, P. Z.; Zhang, Q. B.; Li, N.; Xu, Z. H.; Wang, Y. M.; Li, Z. E. *J. Appl. Phycol.* **2003**, *15*, 21.
11. Tosh, B.; Saikia, C. N.; Dass, N. N. *Carbohydr. Res.* **2000**, *327*, 345.
12. Nishimiki, M.; Rao, N. A.; Yagi, K. *Biochem. Biophys. Res. Commun.* **1972**, *46*, 849.
13. Wang, J. C.; Xing, G. S.; Hu, W. D.; Zhu, T. L.; Wang, Q.; Zhao, H. *Chin. Pharm. J.* **1994**, *29*, 23.
14. Yamaguchi, T.; Takamura, H.; Matoba, T.; Terao, J. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 1201.
15. Lopes, G. K. B.; Schulman, H. M.; Hermeslima, M. *Biochim. Biophys. Acta* **1999**, *1472*, 142.
16. Dahl, M. K.; Richardson, T. *J. Dairy Sci.* **1978**, *61*, 400.
17. Ueda, J.-i.; Saito, N.; Shimazu, Y.; Ozawa, T. *Arch. Biochem. Biophys.* **1996**, *333*, 377.
18. Chung, Y. C.; Chang, C. T.; Chao, W. W.; Lin, C. F.; Chou, S. T. *J. Agric. Food Chem.* **2002**, *50*, 2454.
19. Tanaka, M.; Kuie, C. W.; Nagashima, Y.; Taguchi, T. *Nippon Suisan Gakkaishi* **1988**, *54*, 1409.
20. Miller, D. D. *Mineral*; Elsevier: New York, 1996, p 618.
21. Halliwell, B.; Gutteridge, J. M. C. *Biochem. J.* **1984**, *219*, 1.
22. Gülçin, İ.; Şat, İ. G.; Beydemir, Ş; Elmastaş, M.; Küfrevioğlu, O. İ. *Food Chem.* **2004**, *87*, 393.
23. Yamaguchi, F.; Ariga, T.; Yoshimira, Y.; Nakazawa, H. *J. Agric. Food Chem.* **2000**, *48*, 180.
24. Gordon, M. H. *The Mechanism of Antioxidant Action In Vitro*; Elsevier: London, 1990, p 1..
25. Lindsay, R. C. *Food additives*; Elsevier: New York, 1996, p 778 (chapter 12)..
26. Yuan, Y. V.; Bone, D. E.; Carrington, M. F. *Food Chem.* **2005**, *91*, 485.
27. Yuan, H. M.; Zhang, W. W.; Li, X. G.; Lü, X. X.; Li, N.; Gao, X. L.; Song, J. M. *Carbohydr. Res.* **2005**, *340*, 685.
28. Eiserich, J. P.; Wong, J. W.; Shibamoto, T. *J. Agric. Food Chem.* **1995**, *43*, 647.
29. Eiserich, J. P.; Shibamoto, T. *J. Agric. Food Chem.* **1994**, *42*, 1060.
30. Yanagimoto, K.; Lee, K. G.; Ochi, H.; Shibamoto, T. *J. Agric. Food Chem.* **2002**, *50*, 5480.
31. Hu, C.; Zhang, Y.; Kitts, D. D. *J. Agric. Food Chem.* **2000**, *48*, 3170.