Preparation, chemical characterization and antioxidant activity of crude polysaccharide and oligosaccharide extracted from the ascidian *Styela clava*

Xiaoling Zhang[1, #], Liming Liu[1, #], Minglv Sun[2], Bin Chen[2], Aili Jiang[3, *]

[1]Ocean School, Yantai University, Yantai Shandong 264005, China. [2]Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai Shandong 264003, China. [3]College of Life Sciences, Yantai University, Yantai Shandong 264005, China

Received: May 01, 2018; accepted: June 15, 2018.

The crude polysaccharide (PS) and oligosaccharide (OS) were isolated from the ascidian *Styela clava*. The chemical and physical characteristics of the two saccharides were determined by IR spectrophotometer and several chemical methods, and their antioxidant activities were appraised by various *in vitro* assay systems, including the scavenging activity of free and hydroxyl radicals and reducing power. The EC_{50} of PS and OS against hydroxyl radical was 4.35 and 5.82 mg/mL, while EC_{50} on DPPH radicals was 4.95 and 0.84 mg/mL, respectively. The results indicated that both crude polysaccharide and oligosaccharide extracted from *Styela clava* had antioxidant activities and could be explored as novel potential antioxidants.

Keywords: Ascidian; *Styela clava*; Polysaccharide; Oligosaccharide; Antioxidant activity.

Abbreviations: ROS: reactive oxygen species; BHA: butylated hydroxyanisole; BHT: butylated hydroxytoluene; TBHQ: *tert*-butylhydroquinone; PG: propyl gallate; VCAM-1: vascular cell adhesion molecule 1; iNOS: inducible nitricoxide synthase; DPPH: 1, 1-diphenyl-2-picrylhydrazil; PS: polysaccharide; OS: oligosaccharide;

[*]**Corresponding author:** Aili Jiang, College of Life Sciences, Yantai University, Yantai Shandong 264005, China. Phone: +86 535 6902638. Fax: +86 535 6902638. E-mail: jal9035@163.com.

[#]These authors contributed equally to this manuscript.

Introduction

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{\cdot-}$) and hydroxyl radical ($OH^{\cdot}$), play a critical role in many diseases, such as gastric, atherosclerosis, cancer, and cardiovascular disorders [1, 2]. Antioxidants are indispensable and effective in delaying or preventing the oxidation of cellular oxidable substrates. They exert their effects by scavenging ROS, activating a battery of detoxifying proteins, or preventing the generation of ROS. The most commonly used antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ), and propyl gallate (PG), are now doubted toxicologically [3, 4]. Some studies have also implicated the use of these synthetic antioxidants in prompting the development of cancerous cells in rats [5]. Therefore, much attention has been focused on the research for the development of alternative antioxidants from natural origins.

Many natural antioxidants have already been extracted from terrestrial resources [6]. In recent years, many marine resources have attracted attention in the search for bioactive compounds to develop new drugs and health foods. The antioxidant activities of several

polysaccharides from many marine resources have been described [7, 8]. Up to now, the studies on marine natural antioxidants are mainly limited to marine algae polysaccharides [9, 10]. There is some lack of knowledge about polysaccharides from marine animals [11, 12]. Oligosaccharides are known to exhibit several biological and physiological activities, but only a few literatures have demonstrated the antioxidant activity of oligosaccharides [13, 14]. Previous studies mainly dealt with the isolation of oligosaccharides by treatment with a mixture of polysaccharide hydrolyzing enzymes, which may have high randomness and low repetition. There are few studies on natural oligosaccharides with direct extraction. Researches mainly on polysaccharides and oligosaccharides from marine animal are also deficient [11, 15].

Styela clava, the solitary ascidian which is commonly known as the clubbed tunicate, distributed widely in Bohai and western Yellow seas. It is an efficient filter-feeder capable of depleting the water volume of suspended particles [16]. Previous researchers found that *Styela clava* contained substantial amounts of antimicrobial peptides such as clavanins and stylins which played significant roles in host defense against microbes [17, 18]. And the chondroitin sulfate extracted from *Styela clava* significantly inhibited NF-κB driven expressions of vascular cell adhesion molecule 1 (VCAM-1) and inducible nitricoxide synthase (iNOS) [11]. However, to the best of our knowledge, there are no reports about the antioxidant activity of polysaccharide and oligosaccharide from *Styela clava*.

In present study, the major polysaccharide and oligosaccharide were extracted from *Styela clava*. Their chemical and physical characteristics were determined by chemical methods and IR spectrophotometry. Hydroxyl radical scavenging, DPPH radical scavenging, and reducing power of these two samples were investigated using various *in vitro* assay systems in order to discover the potential value of *Styela clava*.

Materials and methods

Materials and chemicals

Specimens of *Styela clava* were collected from Yantai, Shandong Province, China and stored under 0°C.

Dialysis membranes were produced by Spectrum Co., and molecular weight (MW) was cut off at 3,500 Da. 1, 1-diphenyl-2-picrylhydrazil (DPPH) and D-glucuronic acid were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). All other chemicals used were of analytical grade.

Extraction of sulfated polysaccharide from *Styela clava*

The lyophilized and powdered *Styela clava* (300 g) was degreased by ethyl acetate and extracted twice in 3 L boiling water for 2 h. After cooling, the solution was separated from the residues by successive filtration through gauze and concentrated using a rotary evaporator at 58°C under reduced pressure, followed by adding 60 mL papain (5 U/mL). The solution was heated in 55°C water bath for 4 h. Next, a portion of trichloroacetic acid (5%) was dropped into the condensation solution. As a result, the proteins including papain were precipitated and removed by centrifugation. This step was repeated until no precipitation was separated.

After the removal of proteins, the supernatant was collected and dialyzed in cellulose membrane tubing against distilled water for two successive days to remove the impurities with MW<3,500 Da. The retained fraction was recovered, concentrated under reduced pressure, frozen at -20°C overnight and precipitated by adding triple volume of 95% (v/v) ethanol. The extract was centrifuged and crude polysaccharide (PS) was obtained as the precipitate, which was lyophilized and stored at -20°C.

Extraction of sulfated oligosaccharide from *Styela clava*.

After removing the precipitate of PS from the extract as illustrated above, the supernatant was concentrated using a rotary evaporator at 40°C. The condensation was extracted by n-butanol to remove glycosides. Then, the water-solution was concentrated to a small volume under vacuum at 58°C and dried to obtain oligosaccharide (OS). The sample was also stored at -20°C.

Chemical analysis

The carbohydrates were determined by the phenol-sulfuric acid method, using D-glucose as standard. Sulfate content was analyzed by the barium chloride-gelatin method. Uronic acid was estimated in a modified carbazole method using D-glucuronic acid as standard [19].

IR spectroscopy analysis

The IR spectrum of PS and OS was determined using an infrared spectrophotometer (Shimadzu IR-400, Shimadzu Co., Japan). The samples were ground with KBr powder and then pressed into pellets for IR measurement in the 4,000-400/cm frequency range.

Antioxidant activity

(1) Hydroxyl radical assay:

Hydroxyl radical-scavenging activity was measured by the method of Smirnoff & Cumbes [20] with a minor modification. Samples (PS and OS) were substituted with distilled water at 0 (blank), 0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 mg/mL. The reaction mixture contained 0.5 mL of safranine O (360 µg/mL), 0.5 mL of EDTA-Fe (2 mM), 1.0 mL of H_2O_2 (3.0%) and 1 mL samples of varying concentrations in 4.5 mL sodium phosphate buffer (150 mM, pH 7.4). After incubated for 30 min at 37°C, the absorbance of the mixture was measured at 520 nm. Sodium phosphate buffer replaced H_2O_2 in the mixture served as control. The capability of scavenging hydroxyl radical was calculated using the following equation:

$$\text{Scavenging effect (\%)} = [(A_1 - A_0')/A_0] \times 100$$

Where A_0 is the absorbance of the control, A_1 is the absorbance of the sample, and A_0' is the absorbance of blank.

(2) DPPH free-radical assay:

The free radical scavenging activity of PS and OS was assayed according to the method of Shimada *et al*. [21] using 1, 1-diphenyl-2-picryl-hydrazil (DPPH). Briefly, 1 mL 0.1 mM ethanol solution of DPPH was added in 3 mL water solution of extracts with different concentrations. The mixture was shaken vigorously and incubated at room temperature for 30 min in the dark. Then the absorbance was measured at 517 nm in a spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The DPPH radical concentration was calculated using the following equation:

$$\text{Scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample.

(3) Reducing power assay:

The reductive potential of extracts was determined according to the method of Oyaizu [22]. Different concentrations of each sample in 1 mL distilled water was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture to terminate the reaction. Then 2.5 mL of the solution was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%). The absorbance was measured at 700 nm in a spectrophotometer. Higher absorbance of the reaction mixture indicated greater reducing power.

Statistical analysis

All bioassay results were expressed as means ± standard deviation (SD). The experimental data were subjected to an analysis of variance (ANOVA) for a completely random design. For

each concentration, three parallel measurements were prepared for assays of each antioxidant attribute. Differences at P<0.05 (95% confidence level) were significant.

Results and discussion

Extraction and chemical analysis

Data of extraction and chemical compositions of two samples isolated from *Styela clava* were given in table 1. The results showed that the yield of OS was more than twice of PS. It was reported that ethyl alcohol precipitation was the most convenient method. The yields of samples were controlled by volume ratio of material to solvent, pH of the solution and extraction temperature *et al*. The sulfate contents of samples were 13.42% (PS) and 18.30% (OS), respectively. OS with a lower MW had higher sulfate content than PS. The uronic acid content in PS and OS was 6.74% and 4.27%, respectively.

Table 1. Extraction yield and chemical compositions of two samples isolated from *Styela clava*.

Sample	Yield	Sulfate	Uronic acid
PS	1.92%	13.42%	6.74%
OS	4.37%	18.30%	4.27%

PS and OS were crude polysaccharide and oligosaccharide extracted from *S. clava*, respectively.

IR spectra

IR spectra of two samples from *Styela clava* are shown in figure 1. Typical signals at 3,428, 1,635, 1,415, and 1,072/cm were clear for both samples. The intensity of bands around 3,428/ cm was assigned to υOH stretching frequency which was existed in the hydrogen bond of the molecules, and as expected it was broad. Signals at 2,930/cm or so were from the stretch vibration of C-H group. The peak at 1,635/cm of OS, attributed to the asymmetric stretch vibration of COO^- of uronic acid, was weaker than that of PS, which means the content of uronic acid was lower. And signals at 1,425/cm was due to the symmetric stretch vibrations of COO^- and the stretch vibration of C-O within -COOH. Absorption at 1,249/cm was possibly due to non-symmetric C-O-C stretching vibration. In addition, the band around 1,060/cm was dominated by the glycosidic linkage υ(C-O-C) stretching vibration contribution [23, 24].

Hydroxyl radical scavenging activity of PS and OS from *Styela clava*

The hydroxyl radical, generated in the system by the Fenton reaction, is known to be a highly potent oxidant, which can react with all biomacromolecules functioning in living cells [25]. As shown in figure 2, both samples were found to exhibit the ability to scavenge hydroxyl radicals in a concentration-dependent manner. PS and OS showed stronger hydroxyl radical scavenging activities than Vitamin C. At 5 mg/mL, the scavenging effect of PS, OS, and Vitamin C was 57.61%, 43.75% and 30.5%, respectively. Scavenging effect of PS was higher than OS (P<0.05). EC_{50} (50% effective concentration) of PS, OS, and Vitamin C against hydroxyl radical was 4.35, 5.82, and 8.16 mg/mL, respectively.

Qi *et al*. [25] reported high sulfate content ulvans exhibited stronger antioxidant activity than that of natural ulvan *in vitro*. Similar results were observed by Hu *et al*. [23], which implying a positive correlation between sulfate content and antioxidant activity. But the result in this paper was contrary to them. OS with higher sulfate content exhibited weaker hydroxyl radical scavenging activity than that of PS. Therefore, in addition to sulfate content, some other chemical components may be responsible for the scavenging activity as well. According to another report by Qi *et al*. [25], the results indicated that MW markedly affected the antioxidant activities of ulvans. This phenomenon was also confirmed by Kong *et al*. [26], who isolated three fractions of polysaccharide from pulp tissue of litchi (*Litchi chinensis* Sonn.) and found that the fraction with lowest MW possessed the strongest scavenging effect of hydroxyl radical. However, the result in this paper is different with these.

Moreover, earlier researchers suggested that two mechanisms might be responsible for the

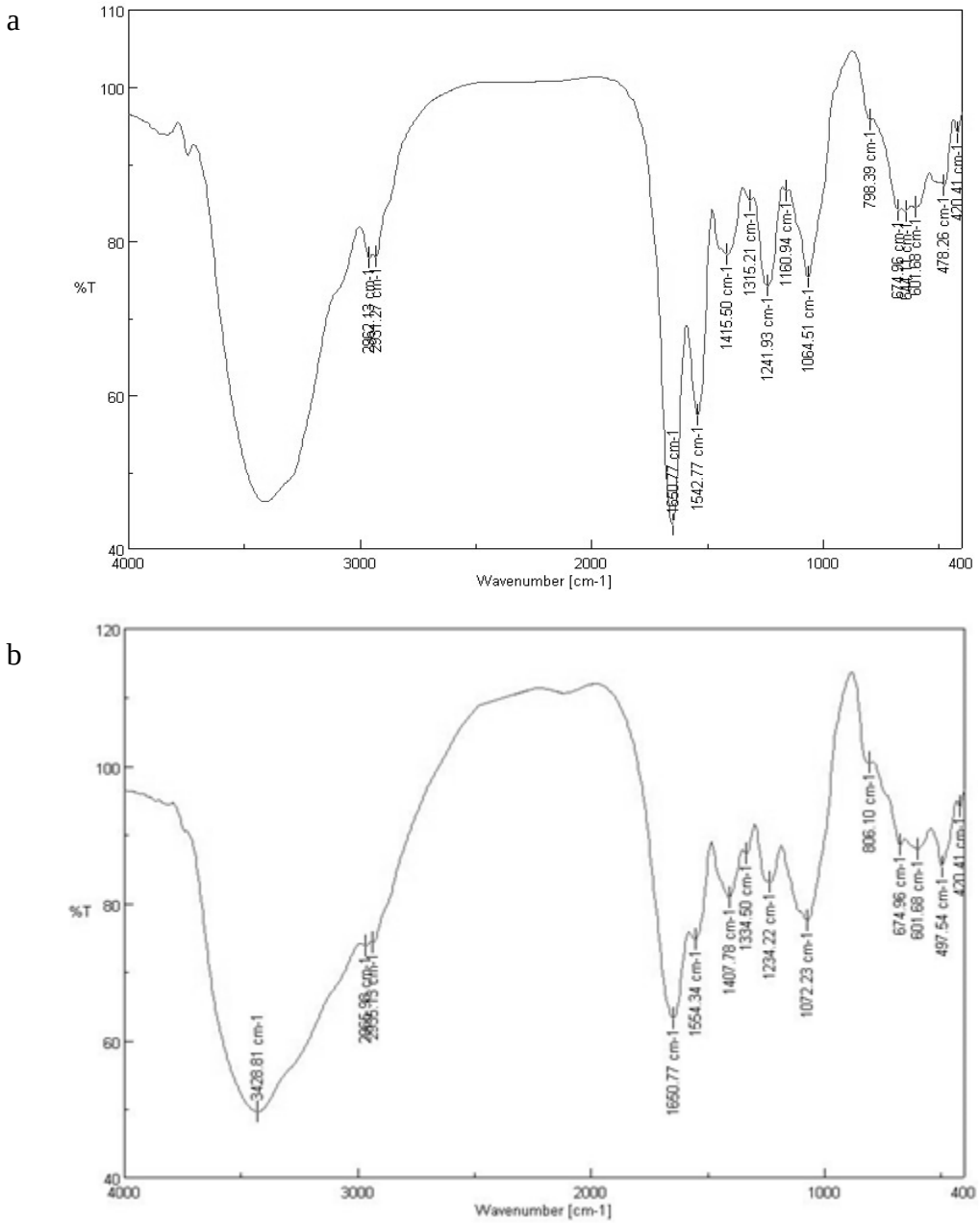

Figure 1. The IR spectra of samples PS (a) and OS (b).

hydroxyl radical scavenging ability of sulfated saccharide. One suppresses the generation of hydroxyl radical, and the other scavenges hydroxyl radicals generated [1]. The free-radical scavenging activity is partially related to monosaccharide constitutions [27]. It is likely that the antioxidant activities of PS and OS were combination of several factors. The mechanisms of the samples need to be further studied.

DPPH radical scavenging activity of PS and OS from *Styela clava*

The scavenging effect of antioxidants on DPPH radical was thought to be due to their hydrogen donating ability. Since DPPH is a stable radical, the model of scavenging DPPH radical is widely accepted as a method to evaluate the free radical-scavenging activities of antioxidants. It accepts an electron or hydrogen radical to

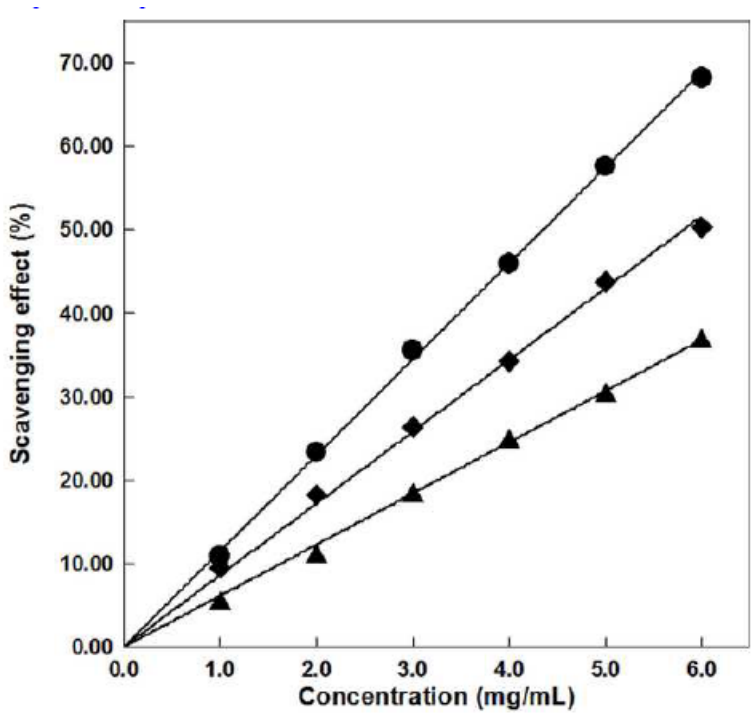

Figure 2. The scavenging effect of PS (●), OS (♦) and Vitamin C (▲) on hydroxyl radical.

become a stable diamagnetic molecule [28]. Lower absorbance of the reaction mixture at 517 nm indicated higher free radical scavenging activity. As shown in figure 3, the scavenging abilities of both PS and OS were concentration related. Both samples showed obvious scavenging effect on DPPH radical. The effect of OS was significantly higher than that of PS ($P<0.05$) at all the experimental concentrations. EC_{50} of PS and OS against DPPH radicals were 4.95 and 0.84 mg/mL, respectively. At 1 mg/mL, the scavenging ability was 10.24% for PS and 60.01% for OS. At a concentration of 1.5 mg/mL, the scavenging ability increased to 15.04 and 87.27% for PS and OS, respectively. However, the two samples showed weaker DPPH scavenging effect than Vitamin C, which exhibited 44.22% scavenging ability even at 10 µg/mL (data not shown).

Previous studies showed that the scavenging ability of three main crude polysaccharides extracted from brown seaweed *Sargassum pallidum* on DPPH radicals were no more than 20% at the concentration of 3.8 mg/mL [29], while data of PS in this paper was more than 30% at the same concentration. OS showed an obviously better DPPH scavenging ability than the fractions of the low MW fucoidan (DFPS) extracted from *Laminaria japonica*, which EC_{50} were much higher than 3 mg/mL [19]. Therefore, though the scavenging effects of two samples on DPPH radicals were all relatively lower than that of ascorbic acid, they may act as primary antioxidants, especially the OS.

Reducing power

The reducing capacity of a compound serves 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, and Fe^{2+} can be monitored at 700 nm [30]. The color of test solution changed from yellow to various shades of green and blue depending on the reducing power of samples. Higher absorbance value equals to stronger reducing power. Figure 4 depicted the reducing power of PS and OS using the potassium ferricyanide

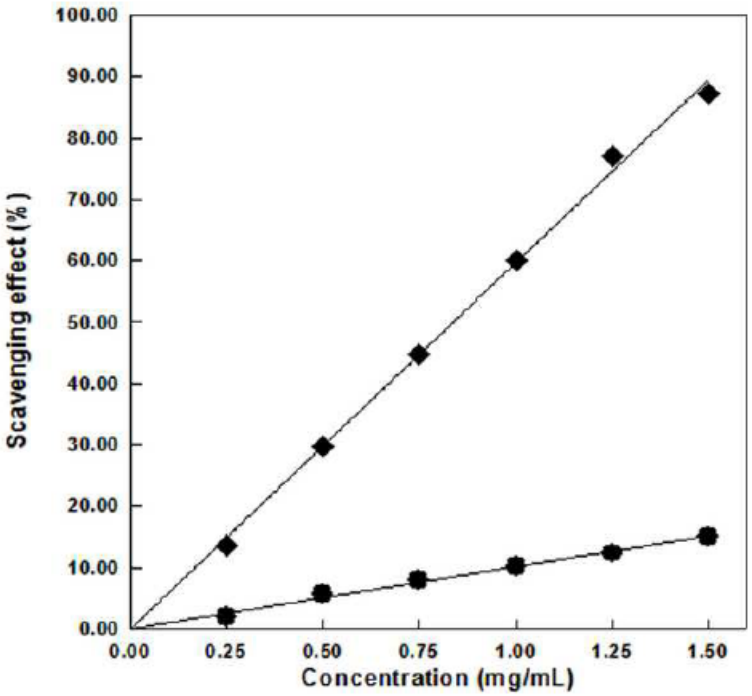

Figure 3. The scavenging effect of PS (●) and OS (◆) on DPPH radical.

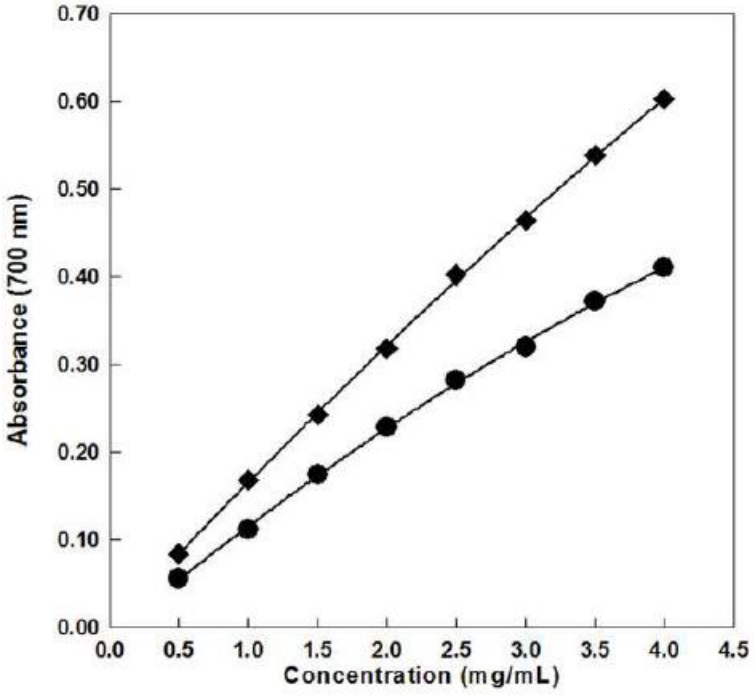

Figure 4. The reducing power of PS (●) and OS (◆) on hydroxyl radical.

reduction method. The reducing power of the samples correlated well with the increasing concentration. The reducing power of OS was significantly higher than that of PS ($P<0.05$) when the experimental concentrations was higher than 1.0 mg/mL. Data indicated that reducing power of PS and OS probably play a role in the observed antioxidant effect.

The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [25]. Polysaccharide chains have one reducing and one non-reducing end. Lower MW samples show higher number of reducing and non-reducing ends [25]. Therefore, the reducing power may be affected by MW of samples. This phenomenon has been confirmed in this paper for OS with lower MW and higher antioxidant activity.

Conclusions

The results clearly indicated that both crude polysaccharide and oligosaccharide extracted from marine animal *Styela clava* possesses antioxidative ability. The scavenging effect of PS on hydroxyl radical was significantly higher than that of OS. The reducing power and DPPH radical scavenging ability of OS were higher than PS. The present findings appear useful in further studies on the purification and identification of specific fractions that are responsible for the higher antioxidant activities. These experiments are now in progress. The results may shed the light on a better understanding on the polysaccharide and oligosaccharide extracted from *Styela clava* as potential functional antioxidants for their antioxidant activity.

Acknowledgements

This work was supported by grant 31070368 from the China Natural Science Foundation and Collaborative Innovation Center of Quality and Safety Controlling of Modern Mariculture and Food Processing.

Reference

1. Shon MY, Kim TH, Sung NJ. 2003. Antioxidants and free radical scavenging activity of *Phellinus baumii* (*Phellinus* of Hymenochaetaceae) extracts. Food Chem. 82(4):593–597.
2. Duan XJ, Zhang WW, Li XM & Wang BG. 2006. Evaluation of antioxidant property of extract and fractions obtained from a red alga, *Polysiphonia urceolata*. Food Chem. 95(1):37–43.
3. Grice HC. 1986. Safety evaluation of butylated hydroxytulene (BHT) in the liver, lung and gastrointestinal tract. Food Chem Toxicol. 24:1127–1130.
4. Buck DF. 1991. Antioxidants in food additives, users handbook. Glagow: Academic Publishers. pp. 5.
5. Huang HL, Wang BG. 2004. Antioxidant capacity and lipophilic content of seaweeds collected from the Qingdao coastline. J. Agric. Food Chem. 52(16):4993–4997.
6. Moure A, Cruz JM, Franco D, Domínguez JM, Sineiro J, Domínguez H, Núñez MJ, Parajó JC. 2001. Natural antioxidants from residual sources. Food Chem. 72(2):145–171.
7. Liqin S, Ling W, Jing L, Honghui L. 2014. Characterization and antioxidant activities of degraded polysaccharides from two marine Chrysophyta. Food Chem. 160(3):1–7.
8. Liqin S, Ling W, Yan Z. 2012. Immunomodulation and antitumor activities of different molecular weight polysaccharides from *Porphyridium cruentum*. Carbohyd Polym. 87(2):1206–1210.
9. Nagai T, Yukimoto T. 2003. Preparation and functional properties of beverages made from sea algae. Food Chem. 81(3):327–332.
10. Qi HM, Zhang QB, Zhao TT, Chen R, Zhang H, Niu XZ, Li ZE. 2005. Antioxidant activity of different sulfate content derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta) *in vitro*. Int. J. Biol. Macromol. 37(4):195–199.
11. Xu CX, Jin H, Chung YS, Shin JY, Woo MA, Lee KH, Palmos GN, Choi BD, Cho MH. 2008. Chondroitin sulfate extracted from the *Styela clava* tunic suppresses TNF-α-induced expression of inflammatory factors, VCAM-1 and iNOS by blocking Akt/NF-κB signal in JB6 cells. Cancer Lett. 264(1):93–100.
12. Lee YE, Kim H, Seo C. 2017. Marine polysaccharides: therapeutic efficacy and biomedical applications. Archives of Pharm Res. 40(9):1006–1020.
13. Sun T, Yao Q, Zhou DX, Mao F. 2008. Antioxidant activity of N-carboxymethyl chitosan oligosaccharides. Bioorg. Med. Chem. Lett. 18(21):5774–5776.
14. Yuan XP, Wang J, Yao HY. 2005. Antioxidant activity of feruloylated oligosaccharides from wheat bran. Food Chem. 90(4):759–764.
15. Matias JC, Rui RC, João FM. 2016. Marine origin polysaccharides in drug delivery systems. Mar Drug. 14(34):1–27.
16. Jiang AL, Lin J, Wang CH. 2008. Physiological energetics of the ascidian *Styela clava* in relation to body size and temperature. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 149(2):129–136.
17. Löfgren SE, Miletti LC, Steindel M, Bachère E, Barracco MA. 2008. Trypanocidal and leishmanicidal activities of different antimicrobial peptides (AMPs) isolated from aquatic animals. Exp. Parasitol. 118(2):197–202.
18. Menzel LP, Lee IH, Sjostrand B, Lehrer RI. 2002. Immunolocalization of clavanins in *Styela clava* hemocytes. Dev. Comp. Immunol. 26(6):505–515.

19. Wang J, Zhang QB, Zhang ZS, Song HF, Li PC. 2010. Potential antioxidant and anticoagulant capacity of low molecular weight fucoidan fractions extracted from *Laminaria japonica*. Int. J. Biol. Macromol. 46(1):6–12.
20. Smirnoff N, Cumbes QJ. 1989. Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28(4):1057–1060.
21. Shimada K, Fujikawa K, Yahara Y, Nakamura T. 1992. Antioxidative properties of xanthan on autoxidation of soybean oil in cyclodextrin emulsion. J. Agric. Food Chem. 40(6):945–948.
22. Oyaizu M. 1986. Studies on products of browning reaction: antioxidative activities of products of browning reaction prepared from glucosamine. Jpn. J. Nutr. Diet. 44(6):307–315.
23. Hu TT, Liu D, Chen Y, Wu J, Wang SS. 2010. Antioxidant activity of sulfated polysaccharide fractions extracted from *Undaria pinnitafida in vitro*. Int. J. Biol. Macromol. 46(2):193–198.
24. Liu CH, Wang CH, Xu ZL, Wang Y. 2007. Isolation, chemical characterization and antioxidant activities of two polysaccharides from the gel and the skin of *Aloe barbadensis* Miller irrigated with sea water. Process Biochem. 42(6):961–970.
25. Qi HM, Zhang QB, Zhao TT, Li ZE, Zhao ZQ. 2005. Antioxidant activity of different molecular weight sulfated polysaccharides from *Ulva pertusa* Kjellm (Chlorophyta). J. Appl. Phycol. 17(6):527–534.
26. Kong FL, Zhang MW, Kuang RB, Yu SJ, Chi JW, Wei ZC. 2010. Antioxidant activities of different fractions of polysaccharide purified from pulp tissue of litchi (*Litchi chinensis* Sonn.). Carbohyd. Polym. 81(3):612–616.
27. Tsiapali E, Whaley S, Kalbfleisch J, Ensley HE, Browder IW, Williams DL. 2001. Glucans exhibit weak antioxidant activity, but stimulate macrophage free radical activity. Free Radic. Biol. Med. 30(4):393–402.
28. Soares JR, Dins TCP, Cunha AP, Almeida LM. 1997. Antioxidant activity of some extracts of *Thymus zygis*. Free Rad. Res. 26(5):469–478.
29. Ye H, Wang KQ, Zhou CH, Liu J, Zeng XX. 2008. Purification, antitumor and antioxidant activities *in vitro* of polysaccharides from the brown seaweed *Sargassum pallidum*. Food Chem. 111(2):428–432.
30. Qi HM, Zhang QB, Zhao TT, Hu RG, Zhang K, Li ZE. 2006. *In vitro* antioxidant activity of acetylated and benzoylated derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta). Bioorg. Med. Chem. Lett. 16(9):2441–2445.