Extraction and antioxidant activities of oligosaccharides from different parts of the ascidian *Styela clava*

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***Abstract*—The crude water-soluble oligosaccharides, coded as TOS, VOS and BOS, were isolated from tunica, viscera and whole body of the ascidian *Styela clava*, which chemical and physical characteristics were determined by several chemical methods, as well as their antioxidant activities by various *in vitro* assay systems. TOS with the lowest yield had higher sulfate and uronic acid content than the other two samples. The EC_{50} of TOS, VOS and BOS were 6.32, 3.45 and 5.55 mg/mL against hydroxyl radical and 1.35, 0.77 and 0.90 mg/mL against DPPH radicals, respectively. VOS showed the highest antioxidant activities among three samples. The results indicated that crude oligosaccharide extracted from different parts of *Styela clava* had antioxidant activities, which could be explored as novel potential antioxidants.**

Keywords- Ascidian; Styela clava; Oligosaccharide; Antioxidant activity

I. INTRODUCTION

Reactive oxygen species (ROS) play a critical role in many diseases, such as gastric, atherosclerosis, cancer and cardiovascular disorders [1, 2]. Therefore, there is a growing interest in the use of antioxidants. The most commonly used antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butylhydroquinone (TBHQ). However, BHA and BHT have been suspected of being responsible for liver damage and carcinogenesis [3, 4]. Thus, much attention has been focused on the research for the development of alternative antioxidants from natural resources.

Many natural antioxidants have already been extracted from natural resources [5]. In recent years, many marine resources have attracted attention in the search for bioactive compounds, especially the polysaccharides [3, 6]. However, there is limited information on oligosaccharides from marine animals. Oligosaccharides are known to exhibit several biological and physiological activities [4, 7-9]. But only a few literatures have demonstrated the antioxidant activity of oligosaccharides.

Styela clava, the solitary ascidian which is commonly known as the clubbed tunicate, distributed widely in Bohai and western Yellow seas. Previous researchers found that *Styela clava* contained amounts of antimicrobial peptides and chondroitin sulfate with high biological activities [10-13]. However, to the best of our knowledge, there are few reports on the antioxidant activity of oligosaccharides extracted from *Styela clava*.

In this study, the crude water-soluble oligosaccharides, coded as TOS, VOS and BOS were isolated from tunica, viscera and whole body of the ascidian *Styela clava*. And their antioxidant activities were investigated using various *in vitro* assay systems.

II. METERIALS AND METHODS

A. Materials and chemicals

Specimens of *Styela clava* were collectned from Yantai, Shandong Province, China and stored under 0 °C. Dialysis membranes were produced by Spectrum Co., and molecular weight 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.

B. Extraction of sulfated oligosaccharide from different parts of Styela clava.

Tunicae and viscera of *Styela clava* were separated, lyophilized and powdered. Each sample (300 g) was degreased by ethyl acetate first and then 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. The proteins were removed using the Sevage reagent three times. After the removal of the Sevage reagent, the condensation was dialyzed in cellulose membrane tubing against distilled water for two successive days. The retained fraction was recovered, concentrated under reduced pressure,

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frozen at -20 °C and precipitated by addition of 3 folds volume of 95% (v/v) ethanol. After removing the precipitate, the supernatant was concentrated and extracted by n-butanol to remove glycosides. The crude oligosaccharides which were coded as TOS and VOS in the water-solution were obtained by concentrating to a small volume under vacuum at 58 °C. The oligosaccharides extracted from the whole body of *Styela clava*, BOS, were also obtained by using upper method. All the samples were dried and stored at -20 °C.

C. Chemical analysis

Sulfate content in oligosaccharides was determined by the barium chloride-gelatin method [14]. A standard curve was made as follows: 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.16, 0.18 and 0.20 mL K_2SO_4 standard solution (0.6 mg/mL) were accurately put into tubes; hydrochloric acid was compensated to 0.2 mL solution. Then 3.8 mL of trichloroacetic acid and 1.0 mL of barium chloride-gelatin solution were added, and absorbance of A1 was measured at 360 nm after incubation for 15 min at room temperature, and 0.2 mL hydrochloric acid solution was used as a blank. Absorption of A2 was measured under similar conditions, except that the barium chloride solution was replaced by gelatin solution. A standard curve was made with mass of sulfate (mg) for abscissa and the absorption of (A1-A2) for ordinate. Uronic acid was estimated in a modified carbazole method [15, 16]. The curve was made using D-glucuronic acid as standard.

D. IR spectroscopy analysis

The IR spectrum of each sample was determined using an infrared spectrophotometer (Shimadzu IR-400, Japan). The samples were ground with KBr powder and then pressed into pellets for IR measurement in the 4,000-400 cm^{-1} frequency range [17].

E. Hydroxyl radical assay

Hydroxyl radical-scavenging activity was measured by the method of Smirnoff and Cumbes with a minor modification [18, 19]. The reaction mixture, containing different samples, was incubated with 2 mM EDTA-Fe (0.5 mL), 3% H_2O_2 (1 mL), and 360 μg/mL crocus (0.5 mL) in 4.5 mL sodium phosphate buffer (150 mM, pH 7.4) for 30min at 37 °C, and hydroxyl radical was detected by monitoring absorbance at 520 nm. Sample was substituted with distilled water, and sodium phosphate buffer replaced H_2O_2 served as control. A blank, containing distilled water instead of sample solution, was prepared. 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.

F. DPPH free-radical assay

The free radical scavenging activity was assayed according to the method of Shimada et al using 1, 1-diphenyl-2-picryl-hydrazil (DPPH) [20]. 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 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the sample.

G. Reducing power assay

The reductive potential of extracts was determined according to the method of Oyaizu [21]. Different concentrations of each sample in 1 mL distilled water were 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

H. 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 every antioxidant attribute. Differences at $P < 0.05$ (95% confidence level) were considered to be significant.

III. RESULTS AND DISCUSSION

A. Extraction and chemical analysis

Data of extraction and main chemical composition of different samples were given in Table 1. Yields of TOS, VOS and BOS were 1.74%, 2.73% and 2.12%, respectively. The sulfate contents of the samples were 20.04% (TOS), 1.01% (VOS) and 10.68% (BOS). The sulfate content of TOS was about 20 times that of VOS, which indicated that the chemical composition of TOS may significantly different from VOS. The uronic acid content in TOS, VOS and BOS was 7.60%, 5.55% and 6.74%, respectively. Oligosaccharides extracted from viscera of *Styela clava* with the highest yields had lower sulfate and uronic acid content than the other two samples.

Table 1 Yield and chemical composition of the samples (% w/w of dry weight).

Sample	Yield	Sulfate	Uronic acid
TOS	1.74	20.04	7.60
VOS	2.73	1.01	5.55
BOS	2.12	10.68	6.74

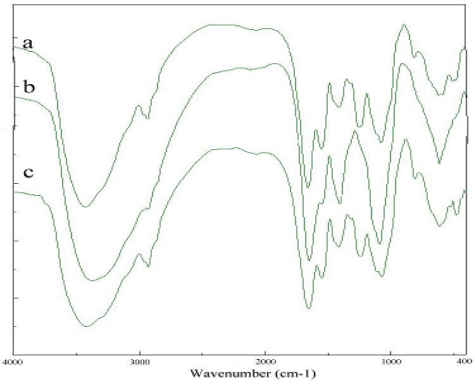

Figure 1. The IR spectra of TOS (a), VOS (b) and BOS (c).

B. IR spectra

IR spectra of the samples from *Styela clava* are shown in Fig. 1. The IR spectrum of TOS was much similar with that of BOS. Signals around 3428, 1635, 1410 and 1072 cm^{-1} were clear for all samples. The intensity of bands around 3428 cm^{-1} was assigned to υOH stretching frequency which was existed in the hydrogen bond of the molecules, and as expected it was broad. VOS showed no obvious absorption around 1240 cm^{-1}, which was considered to be the signal of S=O stretching vibration, while VOS and BOS demonstrated the obvious absorption around 1240 cm^{-1} [22]. The results indicated VOS, which was extracted from tunicae of *Styela clava*, was quite different from the others.

C. Hydroxyl radical scavenging activity

Hydroxyl radical-scavenging activity of the samples was measured according to the method of Smirnoff and Cumbes with a minor modification [18, 19]. As shown in Fig. 2, all crude oligosaccharides were found to exhibit the ability to scavenge hydroxyl radicals in a concentration-dependent manner. Scavenging effect of VOS on hydroxyl radicals was the highest among three samples ($P < 0.05$). The EC_{50} of TOS, VOS and BOS against hydroxyl radical was 6.32, 3.45 and 5.55 mg/mL, respectively. At 5 mg/mL, the scavenging effect of TOS, VOS and BOS was 39.67%, 72.51% and 45.04%, respectively. Scavenging effect of oligosaccharides extracted from different parts of *Styela clava* were all obviously higher than fractions of the low molecular weight fucoidan (DFPS) extracted from brown seaweed *Laminaria japonica* which showed less than 30% hydroxyl radical scavenging activity at 5 mg/mL [16].

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 [23]. Earlier researchers suggested that two mechanisms might be responsible for the hydroxyl radical scavenging ability of sulfated saccharide. One suppresses the generation of hydroxyl radical, and the other scavenges hydroxyl radicals generated [1]. Previous studies had reported the sulfate content and molecular weight may affect the antioxidant activities [3, 19, 24, and 25]. Results in this paper implied the antioxidant activities of the tested samples are not a function of a single factor.

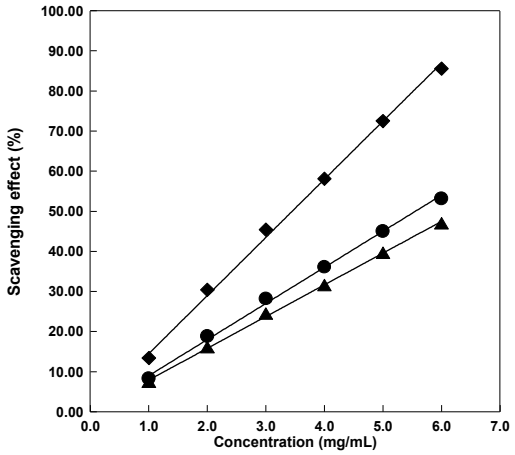

Figure 2. The scavenging effect of TOS (▲), VOS (◆) and BOS (●) on hydroxyl radical.

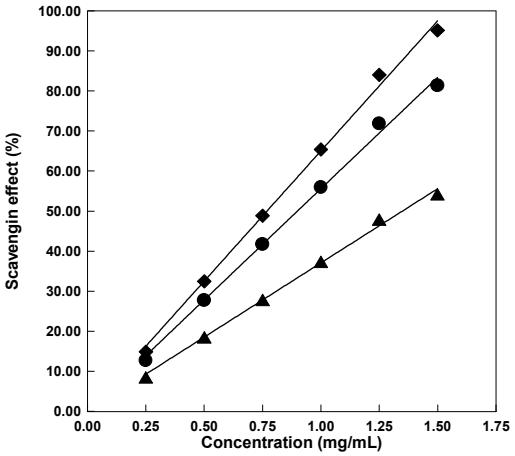

Figure 3. The scavenging effect of TOS (▲), VOS (◆) and BOS (●) on DPPH radical.

D. DPPH radical scavenging activity

The DPPH radical scavenging activity of antioxidants was thought to be due to their hydrogen donating ability [26]. Lower absorbance of the reaction mixture at 517 nm indicated higher free radical scavenging activity. As shown in Fig. 3, the scavenging abilities of all samples were concentration related. All the three samples showed obvious scavenging effect on DPPH radical. EC_{50} of TOS, VOS and BOS against DPPH radicals were 1.35, 0.77 and 0.90 mg/mL, respectively. At 1 mg/mL, the scavenging ability was 37.29%, 65.42% and 55.94%, respectively. Significantly, VOS, which was extracted from viscera of *Styela clava*, had the best DPPH radicals scavenging effect among these three samples ($P < 0.05$).

It was reported 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 [27]. All the results indicated oligosaccharides, especially VOS, exhibited high DPPH radicals scavenging activities, and could probably act as primary antioxidants.

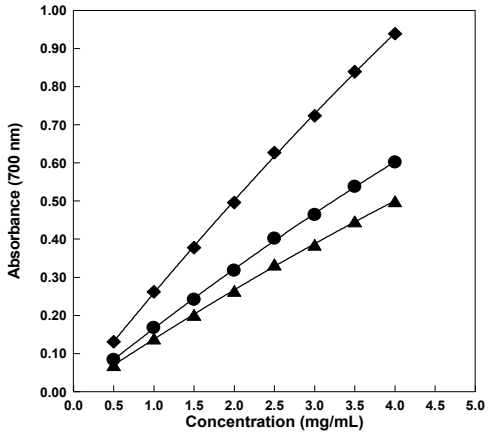

Figure 4. The reducing power of TOS (▲), VOS (◆) and BOS (●)

E. Reducing power

The reducing power serves as a significant indicator of potential antioxidant activity [28]. Higher absorbance value equals to stronger reducing power ($P < 0.05$). Fig. 4 depicted the reducing power of TOS, VOS and BOS. Among the three samples, VOS showed the highest reducing power. All the reducing power of the samples was concentration related. Data implied that reducing power of these samples probably play a role in the observed antioxidant effect.

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