Preparation, analysis and antioxidant evaluation of the controlled product of polysaccharide from *Macrta veneriformis* by mild acid hydrolysis

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Abstract

The polysaccharides from *M. veneriformis* (MVPS) were degraded by controlled mild acid hydrolysis to produce active oligosaccharides. MVPS can easily be hydrolyzed by H₂SO₄ or HCl. The hydrolyzing process was investigated in acid addition and reaction time by evaluating the producing content of reducing sugar. Hydrolysis with 1M HCl to MVPS can generate a time-depended behavior that is mild and controllable. HPLC analysis monitored the change of oligosaccharides composition in hydrolyzing. Total nine oligosaccharides are recognizable in the HPLC profile, and their content showed a regular transformation in hydrolysis. Those nine ingredients were identified as glucooligosaccharides with DP from 1 to 7 by MS analysis. Antioxidant activities of the typical hydrolyzates as well as MVPs were further tested in assays of DPPH and hydroxyl radicals scavenging, and reducing power. It was found that HCl hydrolyzate exhibited stronger antioxidant effects than MVPS and H₂SO₄ hydrolyzate due to its higher content of oligosaccharides.

Keywords: *M. veneriformis*; Polysaccharide; Hydrolyzation; Oligosaccharide; Antioxidant activity

Chemical compounds studied in this article

Alpha-D-glucose (PubChem CID: 79025); Maltose (PubChem CID: 109991481); Kojibiose (PubChem CID: 164939); Maltotriose (PubChem CID: 109991481)

1. Introduction

M. veneriformis, a typical marine bivalve mollusk, is a delicious sea food and a traditional Chinese medicine, while is low-cost, ubiquitous and abundant in Chinese coastal areas, especially in the coastal shoals of Jiangsu province. This marine clam was found containing high content of polysaccharide (Kasai, Horie, & Sakamoto, 2004; Luan, Wang, Wu, Jin, & Ji, 2011). By boiling its flesh in hot water and precipitating the decoction with ethanol, a crude polysaccharide extract with about 3.2% of yield can be acquired (Wang, Zhang, Di, Liu, & Wu, 2011). The crude polysaccharide extract has about 25% of protein impurities, which can be dislodged by trichloroacetic acid (TCA) disposal. After deproteinization, the crude polysaccharide was refined to pure *M. veneriformis* polysaccharide (MVPS) with total sugar content higher than 95% and protein content lower than 1.0%. At least three homogeneous polysaccharides, named MVPS-1, MVPS-2 and MVPS-3, can be isolated from MVPS by DEAE-52 column separation and all of them showed about 450 kDa of average molecular mass in GPC analysis. However, both MVPS and its three subdivided ingredients contain only D-glucose in monosaccharide composition. The D-glucose units in polysaccharide are linked each other by α -(1 $\rightarrow$ 4) or α -(1 $\rightarrow$ 2) glycosidic bonds and they appeared in the molecule with 6:1 of mole ratio. The sequence structures of MVPS are linear and no branches formation, in which six α -(1 $\rightarrow$ 4) linked glucoses connected with one α -(1 $\rightarrow$ 2) linked glucose are continuously repeated. Thus, the special molecular chain structure of MVPS can be vividly expressed with $[\rightarrow 4\text{Glc1}\rightarrow 4\text{Glc1}\rightarrow 4\text{Glc1}\rightarrow 2\text{Glc1}\rightarrow 4\text{Glc1}\rightarrow 4\text{Glc1}\rightarrow 4\text{Glc1}]_n$.

Additionally, MVPS has been proved having huge exploring potentials as functional foods and nutritional supplements. Early studies demonstrated that MVPS has hyperglycemia inhibiting, immunomodulatory, hepatic protecting and antioxidant activities *in vitro* or *in vivo* (Wang, Wu, Chang, & Zhang, 2011; Zheng, Wang, Wu, & Luan, 2011). Animal experimental tests showed that the crude and refining MVPS exhibited properly activities in glycemia inhibition and 300 mg/kg-weight has the optimal effect on hyperglycemia ICR mice that induced by alloxan injection. Oral administration of MVPS can increase the relative spleen and thymus weight of immunodeficiency rat, promote secretion of tumor necrosis factor alpha (TNF- α), stimulate lymphocyte proliferation, and augment phagocytosis and secretion of NO and TNF- α in peritoneal

macrophages.

As far as the researches on natural active polysaccharide are concerned, structural modification is one important method to enhance or transform the activities of polysaccharide. Recent studies demonstrated that natural polysaccharides exhibiting their physiological functions by oral administration to animals are correlated to the oligosaccharide derivatives produced by gastric acid or enzymes in digestive tract (Beveridge, & Richards, 1973; Agbagla-Dohnani, Cornu, & Broudiscou, 2012). It is well known that treatment of polysaccharide with acid hydrolysis can interrupt the polymer chain, generating the less molecular mass of carbohydrates (oligosaccharides) (Du, Song, Hu, Liao, Ni, & Li, 2011). The produced oligosaccharides exhibited improving physiological activities in contrast with their initial polysaccharides because they are easier to be absorbed into blood circulations and have higher bioavailability in organisms (Al Jamal, Roughley, & Ludwig, 2001; Juskiewicz, Zdunczyk, Jankowski, Krol, & Milala, 2008). Sometimes, the derived oligosaccharides might bring some novel biological effect due to their different structures and physicochemical properties when compared with their parent polysaccharide, such as chitooligosaccharide and chitosan (Aam, Heggset, Norberg, Sorlie, Varum, & Eijsink, 2010), agaro-oligosaccharide and agar (Chen, Zheng, Lin, & Yan, 2004), fructooligosaccharide and fructan (Apolinario, Goulart de Lima Damasceno, de Macedo Beltrao, Pessoa, Converti, & da Silva, 2014), and so on (de Moura, Macagnan, & da Silva, 2015). Those beneficial reports would support our study on the hydrolysis of MVPS. However, rare research had been performed on hydrolysis of MVPS and the bioactive degrading products never been reported before.

Therefore, MVPS was regularly processed with partial acid hydrolysis in this study. By this way, some active oligosaccharides might be desired at controlled hydrolysis extent by adjusting acid usage and hydrolyzing time. The active oligosaccharides from MVPS will be subsequently analyzed by HPLC and LC-MS like some previous reports (Chen, Zheng, Lin, & Yan, 2004; Vismeh, Humpala, Chundawat, Balan, Dale, & Jones, 2013; Barroso, Dijkstra, Geerts, Lagerwerf, van Veelen, & de Ru, 2002). Meanwhile, the *in vitro* antioxidant activity of the oligosaccharide product compared with the initial polysaccharide was further investigated.

2 Materials and method

2.1 Materials

MVPS used as the initial polysaccharides in this study were prepared by our lab as described by previous report (Wang, Zhang, Di, Liu, & Wu, 2011). It is opalescent powders with higher than 97.3% of total sugar content that referenced as standard glucose and measured by anthrone-sulfuric acid method (Laurentin, & Edwards, 2003).

The standard D-glucose, 3,5-dinitrosalicylic acid (DNS), sodium hydroxide (NaOH), concentrated hydrochloric acid (HCl) and sulfuric acid (H₂SO₄) were of analytical grade from Sinopharm (Shanghai, China). Chromatographic grade acetonitrile (CH₃CN) and methanol (MeOH) were obtained from Thermo Fisher Scientific (Waltham, USA). MilliQ deionized water was used in this study. Ascorbic acid (Vc), 1,1-diphenyl-2-picrylhydrazyl (DPPH), safranin T, ferrous sulfate, potassium ferricyanide, hydrogen peroxide (H₂O₂), ethylenediamine tetraacetic acid (EDTA) was purchased from Fluka Chemie (Buchs, Switzerland). Phosphate buffer saline with pH=7.4 were purchased from Sigma Chemicals (St. Louis, USA).

2.2 Mild acid hydrolysis of MVPS

The hydrolysis action was investigated between two types of acid, HCl and H₂SO₄. Accordingly, four concentrations (0.2, 2, 4, and 6 M) of solution of HCl and H₂SO₄ were prepared as the reaction media. About 2.0 g MVPS was solubilized in 100 mL distilled water to prepared 20 mg/mL stock solution. Every 10 mL stock solution was added into 10 mL of reaction media in one 50 mL conical flask under ice-bath condition. Then conical flasks were moved to water bath (60 °C) to start the acidic hydrolyzing reaction without agitation. During the reaction (from 0 to 300 min), hydrolyzate (200 µL) was withdrawn at set time and neutralized with NaOH, then was measured its reducing sugar, or analyzed by HPLC and MS, or evaluated with antioxidant activities. The effect of acid usage on the hydrolysis was compared between HCl and H₂SO₄ at 0.1, 1, 2, and 3 M of concentration, which was analyzed through an Arrhenius-type plot of the kinetic behavior.

2.3 Total and reducing sugars quantification

The MVPS were quantified by measuring the reducing sugars after total acid hydrolysis, which were carried out in 1.0 mL reactions containing 10.0 mg of MVPS and 6 M of HCl at 60 °C for 12 h. Reducing sugars were quantified by the 3,5-dinitrosalicylic acid (DNS) method (Kai, Pengju, & Guangye, 2008) using a standard curve of D-glucose. Hydrolysis degree was expressed as glucose equivalent (GE), defined as the percentage of total sugars converted into reducing sugars during the HCl or H₂SO₄ hydrolysis (Avila-Fernandez, Galicia-Lagunas,

Rodriguez-Alegria, Olvera, & Lopez-Munguia, 2011; Allerdings, Ralph, Steinhart, & Bunzel, 2006).

2.4 Composition analysis of oligosaccharides with HPLC-ELSD

The oligomers analysis of the MVPS hydrolyzate was performed by high performance liquid chromatography (HPLC) with a method adapted from Chen, Zheng, Lin, & Yan (2004). HPLC analysis was performed on a WatersTM Alliance 2695 HPLC Separation Module (Waltham, MA, USA) equipped with a Shodex Asahipak NH₂P-4E column (4.6 × 250 mm) in combination with a Dionex PA-1 guard column (4.0 mm × 50 mm). Elution was performed with a flow rate of 1.0 mL/min and a column oven temperature of 30 °C. The injection volume was set to 10 µL. The acetonitrile (A) and Millipore water (B) were used as eluents. The following elution profile was used: 0 – 5 min, isocratic 25% (v/v) of B; 5 – 35 min, linear gradient from 25% to 50% of B. The NH₂P-4E column (NP) was coupled to an evaporative light scattering detector (ELSD, WatersTM 2424). The optimized parameters of ELSD were set at sprayer heating mode (80% power, 40 °C) as 90 °C for drift tube temperature, 40 psi for nitrogen gas flow pressure, and gains to 50. The peak area from the HPLC profile was obtained using Empower software (Waters). A semi-quantitative estimation of oligosaccharides concentration was obtained through the total area of the carbohydrates eluted in the range of 8-28 min.

2.6 Oligosaccharides identification with LC-ESI-TOF-MS

Identification of the oligosaccharides in MVPS hydrolyzate was performed on a HPLC-ESI-TOF-MS system. The system consisted of two successive coupled apparatus, a Waters 2695 HPLC Separation Module equipped with a Shodex Asahipak NH₂P-4E column (4.6 × 250 mm), and a Time-of-Flight Mass Spectrometer (QTOF-MS, Waters QTOF Premier) equipped with an electrospray source (run in). The HPLC separation was performed by the method of HPLC-ELSD analysis that mentioned above, i.e., 30 °C of column oven temperature, 10 µL of injection volume, 1.0 mL/min of flow rate and gradient eluent with acetonitrile (A)-water (B) (25% of B held for 5 min and linear gradient elution for next 30 min from 25% to 50% B). After the HPLC separation, elutes were then introduced into the ESI-TOF-MS device for analysis at positive ionization mode [M+Na]⁺. Mass spectrometric data was acquired in MS scan mode. Data acquisition and analysis were performed using Waters MassLynx (version 4.1). Mass spectrometer settings for MS analysis were as follows: capillary voltage 3.0 kV, cone voltage 45 V, source

temperature 120 °C, desolvation temperature 280 °C, collision energy 5.0 V, scan range 80-2000 Da. Gas (N₂) flow used for cone and desolvation were, respectively, 50 and 350 L/h.

2.7 The in vitro antioxidant activity assay

MVPS and its two hydrolyzates were used as test samples in antioxidant assays. The two hydrolyzates were recorded as HCl hydrolyzate and H₂SO₄ hydrolyzate because they were obtained from processing with 1h of 1M HCl and H₂SO₄ hydrolysis, respectively. The withdrawn hydrolysates were wiped off the residual acids by NaOH neutralization before using, and then were tested their antioxidant activity by some conventional methods involving DPPH, hydroxyl radicals scavenging and reducing power assay.

For the DPPH radical scavenging tests, a reliable reaction system was used as the description of Braca, De Tommasi, Di Bari, Pizza, Politi, & Morelli (2001). One milliliter of all test samples (Vc, MVPS, HCl and H₂SO₄ hydrolyzates) with different concentrations (from 10 to 2000 µg/mL) was added to 3 mL of a 0.004% (wt./v) MeOH solution of DPPH. Absorbance at 517 nm was measured after 30 min, and the percent scavenging activity was calculated by absorbance difference between control (only DPPH solution) and test sample (DPPH solution plus sample) take part in the absorbance of control (only DPPH solution).

Hydroxyl radical-scavenging activity of the test samples (Vc, MVPS, HCl and H₂SO₄ hydrolyzates) was determined according to the safranin T fading assay (Li et al., 2013). The reaction system contained 1 mL sodium phosphate buffer (15 mM, pH 7.4), 1 mL 360 g/mL safranin T, 0.5 mL 2 mM EDTA-FeSO₄, 1 mL 3% H₂O₂ and 1 mL tested samples solution (concentration ranged from 10 to 2000 µg/mL). After incubation at 37°C for 30 min, hydroxyl radical was detected by monitoring the absorbance at 520 nm against a blank. In the control, test sample was substituted with distill water and the H₂O₂ was substituted with sodium phosphate buffer. The hydroxyl radical-scavenging ratio was calculated as the proportion of the absorbance difference between control and sample in the absorbance of the control.

The reducing power of MVPS hydrolyzate was determined referring to the method (Yuan, Carrington, & Walsh, 2005) with some modifications. The different concentrations of samples (from 100 to 1600 µg/mL) 1 mL were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (2.5 mL, 1%). The mixture was incubated at 50 °C for 20 min. A portion (2.5 mL) of TCA (10%) was added to the mixture which was then centrifuged for

10 min at 3000 rpm. The upper layer of solution (2.5 mL) was mixed with water (2.5 mL) and FeCl₃ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm.

2.8 Statistical analysis

All data were shown as mean $\pm$ standard deviation (S.D.). Statistical differences between the experimental groups were determined by one way ANOVA, and differences were considered to be statistically significant if $P < 0.05$.

3. Result and Discussion

3.1. Acid hydrolysis of MVPS and the glucose equivalent (GE) index

For our study, HCl and H₂SO₄ were used to hydrolyze MVPS in aqueous solution system, and the effects acids type and usage on degraded behavior were investigated in detail. The hydrolyzing isotherms were drawn by measuring the relative content of reducing sugar (expressed with GE) in hydrolyzate following with hydrolyzing time. The measured GE of hydrolyzate for both HCl and H₂SO₄ exhibited first increasing then stable trends when hydrolysis prolonged. The platform of GE can be used to evaluate the final hydrolyzing degree and the first time reaching platform can assess the hydrolyzing speed. In such experiments, some important results can be visualized from **Fig. 1**. Firstly, HCl as well as H₂SO₄ can effectively degrade the MVPS. MVPS are amorphous polysaccharides and linear polymers with flexible chain due to containing α -type glycosidic linkages (Wang, Zhang, Di, Liu, & Wu, 2011; Wang, Di, Liu, & Wu, 2013), and can be easily dissolved in aqueous solvents. In aqueous media, the stretched molecular induce the sugar unit linkages contact well with catalyzer (acids), therefore, hydrolysis reaction is more easily happen. Secondly, increasing acid usage can accelerate degradation of MVPS. For example, MVPS disposed with 0.1, 1.0, 2.0, and 3.0 M of HCl hydrolysis for 300 min can result in 5.2, 40.8, 66.5, and 96.1% of GE producing, respectively. Similarly, such conclusion can also be resulted by observing the H₂SO₄ hydrolysis to MVPS. Thirdly, MVPS were more easily degraded by H₂SO₄ other than HCl in such hydrolyzing system ($P < 0.05$). H₂SO₄ disposal exhibited with the higher GE value and shorter time to GE platform than HCl disposal under same condition. Within 60 min (a very short term) of H₂SO₄ disposal (at 3 mol/L of excitation), almost all resultants in MVPS hydrolyzate have become monosaccharides because the GE nearly reached 90%. In contrast, HCl hydrolysis only arrived at 56.7% of GE level. This might be attributed that H₂SO₄ is a polybasic acid and possesses stronger acidity and oxidibility than HCl. However, from the viewpoint on

efficient oligosaccharides production, HCl is more suitable to hydrolyze MVPS because the reaction is mild and its process is controllable. The optimal reaction time and acid usage will thus depend on the desired oligomer composition and yield. Finally, a suitable hydrolysis can be achieved from an initial GE of 5.8% up to a GE of 30.5% within 180 min by using 1M of HCl at 60 °C condition. The GE of 30.5 % at 180 min is a vital point in kinetic curve for 1M HCl hydrolysis of MVPS. Before this point, the hydrolysis basically keeps at a constant rate. From this point onwards, the rate decreases rapidly until maximum hydrolysis with platform GE of 40.8% is reached at 300 min.

3.2. Analysis of oligosaccharides produced during the acid hydrolysis of MVPS

Although the acid hydrolysis of MVPS has been roughly described in the above section, the evolution on the composition and distribution of carbohydrate profile for hydrolysis products has not yet been deeply considered. The carbohydrate profile of hydrolyzate at different hydrolysis time was obtained by high-performance chromatography with an evaporative light scattering detection (HPLC-ELSD). HPLC-ELSD has been widely used for the analysis of oligosaccharide because it allows the observation of short and longer linear oligosaccharide up to a degree of polymerization (DP) of about 30. This technique has been previously applied to describe the carbohydrate profile of hydrolysis products of other polysaccharides (Arrizon, Morel, Gschaedler, & Monsan, 2010). Thereby, it is also used to analysis the distribution of short oligosaccharide of MVPS hydrolyzate.

As shown in **Fig. 2**, fingerprint composed of serial separate eluting peaks in the chromatogram from retention time 8 to 35 min expressed the carbohydrate profile of the reaction liquors prescribed with different acid hydrolysis for suitable periods. Each component peak is appointed to a specific low-molecular-weight oligosaccharide with different DP. Theoretically, peaks eluted later through the separation column are oligosaccharides with higher DP than that eluted early (Chen, Zheng, Lin, & Yan, 2004). Total nine eluting peaks from HCl hydrolyzates and seven eluting peaks from H₂SO₄ hydrolyzates are recognizable in the carbohydrate profile. These notable peaks are marked with consecutive numbers (1-9) according to their retention time. However, there are no available standards for their identification. It can be seen from **Fig. 2** that the long chain molecules of MVPS are rapidly hydrolyzed to low molecule weight oligomer as the acidic hydrolysis proceeds. With regard to the H₂SO₄ hydrolysis, peaks 1, 2, 4, 6, 7 are detected in

hydrolyzate withdrawn at 0.5 h; peaks 3 and 5 started to appear while peaks 6 and 7 are rapidly decayed at 1h; after that, all compounds peaks except peak 1 in H₂SO₄ hydrolyzate are mainly disappeared, indicating that oligosaccharides with higher DP were degraded into monose. However, in HCl hydrolyzate, peaks 2, 4, 6-9 obviously decreased (even to undetectable extent) with hydrolyzing time, but peak 1 and 5 notably increased with time, peak 3 exhibited first increased and then decreased trend in hydrolyzate during the hydrolysis. At end of HCl hydrolysis (300 min), a small proportion of oligosaccharides, indicated with peaks 1-3, 5, are yet observed on HPLC profile. Semi-quantification for the relative content of each oligomer in HCl hydrolyzate was expressed with area percentage of every peak accounting for the total peaks, and its change with hydrolyzing time can be seen in **Fig. 3**. It is shown that treatment of MVPS with HCl can produce various hydrolyzates, whose oligosaccharides composition and compounds content were largely depended on time control.

3.3. Oligosaccharides identification of the MVPS hydrolyzate

HPLC chromatography coupled with mass spectrometry is a very powerful analytical technique, due to its high sensitivity, and the structural information that can be obtained about the analytes (Vismeh, Humpula, Chundawat, Balan, Dale, & Jones, 2013; Wang, Yuan, Sun, Cao, Tian, & Wang, 2009). In this study, HPLC-ESI-TOF-MS was used to directly identify the oligomers in MVPS hydrolyzate. The hydrolyzate produced by 1M HCl hydrolysis for 1h was selected as representative analytes for HPLC-ESI-TOF-MS analysis, because it contains abundant producing oligosaccharides and show total characteristic compound peaks in HPLC-ELSD profile. The result of total ion chromatogram at positive model ($[M+Na]^+$) for oligosaccharides in MVPS hydrolyzate eluted from NH₂P-4E (NP) column by HPLC separation was shown in **Fig. 4a**, which combined with extracted molecules mass ion chromatograms for all oligosaccharides (**Fig. 4b, c, d, e, f, g, h**). In the total ion chromatogram (**Fig. 4a**), nine compound structures are co-eluted from NH₂P-4E column in the periods of 9.58-28.00 min, the relative retention time of the major populations agree well with those obtained from HPLC profiles, thus were labeled with same number signals (1-9). These nine ion peaks were respectively extracted with molecule mass from the total ion chromatogram. Extracted ion of compound 1 eluted at 10.58 min in LC-MS chromatogram shows the $[M+Na]^+$, $[2M+Na]^+$ and $[3M+Na]^+$ adduct ions peaks with $m/z=203.06$, 707.29 and 1071.43

respectively, indicating that its molecule mass is 180 Da and recognized as α -D-glucose. The appearance of polymolecule ion peaks might be attributed to the higher concentration of compounds in analyte, which was usually reported in other research (Hsu, Chang, & Franz, 2006). Oligosaccharides 2 and 3 that co-eluted at 14.09 and 15.13 min are two dimers of glucose with same molecule mass 342 Da because they were found having $[M+Na]^+$ adduct ions with $m/z=365.13$ in MS spectra. Similarly, Oligosaccharides 4 and 5 co-eluted at 17.34 and 18.14 min are two trimers of glucose with same molecule mass 504 Da (m/z of $[M+Na]^+$ are 527.19 and 527.20). The existence of these geometric isomers was caused by two different glycosidic linkages between units of α -D-glucose, one is $-(1\rightarrow4)-$ and the other is $-(1\rightarrow2)-$, they existed in MVPS molecules with 6:1 of mole ratio (Wang, Zhang, Di, Liu, & Wu, 2011). As the hydrolyzing products of MVPS, the oligosaccharides 2 and 3 are here identified as Glu- α -(1 $\rightarrow$ 4)-Glu dimer (Maltose) and Glu- α -(1 $\rightarrow$ 2)-Glu dimer (Kojibiose), respectively. The oligosaccharides 4 and 5 with DP=3 are hence confirmed as Glu- α -(1 $\rightarrow$ 4)-Glu- α -(1 $\rightarrow$ 4)-Glu trimer (Maltotriose) and Glu- α -(1 $\rightarrow$ 4)-Glu- α -(1 $\rightarrow$ 2)-Glu trimer, respectively. Their completely chemical structures were drawn on the corresponding MS spectra and shown in the **Fig. 5**. Accordingly, via recognition of the molecules ion by ESI-TOF-MS analysis, the adduct ion peaks eluted at 20.18, 22.48, 24.12 and 25.53 min were identified as glucooligosaccharides with DP=4, 5, 6 and 7, respectively (**Fig. 6**). All these glucooligosaccharides are still confirmed as isomer mixtures, containing two or more types of molecules due to the co-existence of two types of glycosidic linkage (e.g., Glu- α -(1 $\rightarrow$ 2)-Glu structures with $[\alpha$ -(1 $\rightarrow$ 4)-Glu]_n). The positional isomers of oligosaccharide with higher DP (≥ 4) are very difficult to distinguish by TOF-MS analysis because the NP-HPLC profiling was unable to separate them. This limitation resulted in the shouldering peak appearance for the higher DP oligosaccharides in the profile. Thus complicated fragment ions presented on the MS spectrums for some higher DP of oligosaccharides and caused the problem in precisely elucidating. Considering this, the peaks eluted from HPLC profile at different retention time in the range of 8-28 min was confirmed as homogeneous oligosaccharides and thereby expressed with various DP (DP=1 for peak1, DP=2 for peaks 2 and 3, DP=3 for peaks 4 and 5, DP=4 for peak 6, DP=5 for peak 7, DP=6 for peak 8, and DP=7 for peak 9). All of them are rational products of MVPS under the mild acid hydrolysis.

3.4 Antioxidant activities

3.4.1 DPPH radical-scavenging activity

DPPH is a useful reagent for investigating the free radical-scavenging activities of materials. Antioxidants transfer either electrons or hydrogen atoms to DPPH and thus neutralize free radical (Naik et al., 2003). It is noticeable by eye that there is a discolouration from purple to yellow induced by antioxidants. **Fig. 7a** illustrated the results on scavenging activity of the tested samples to DPPH radicals. It suggested that all samples had inhibitory effects on DPPH radical in a concentration-dependent manner from 10 to 2000 $\mu\text{g/mL}$. Among the three tested samples, HCl hydrolyzate has the strongest ability in DPPH radical-scavenging and its concentration inhibiting 50% radical generation (IC_{50}) is 482 $\mu\text{g/mL}$. MVPS has a moderate DPPH radical-scavenging ability with IC_{50} = 709 $\mu\text{g/mL}$. However, inhibitory effect of H_2SO_4 hydrolyzate is weakest with IC_{50} =1246 $\mu\text{g/mL}$ and just 69% of inhibitory can be reached even at as high as 2000 $\mu\text{g/mL}$ of concentration. The difference in ability to quench DPPH free radical among HCl hydrolyzate, MVPS and H_2SO_4 hydrolyzate might be caused by amount of some special oligosaccharide contains. Those oligosaccharides might have stronger activities than the long chain polysaccharide and monose forms, which have been confirmed by many reports (Sun, Wang, Shi, & Ma, 2009; Wu, & Yu, 2015). The H_2SO_4 hydrolyzate contains fewer long chain molecules and oligosaccharides, thus have the lowest DPPH scavenging activity.

3.4.2 Inhibitory effects on hydroxyl radical

Comparing with other reactive oxygen species, hydroxyl radical has the highest activity which could induce severe damage to organism. Previous studies had reported that suppression against hydroxyl radical generation and cleaning the hydroxyl radical generated might be responsible for the antioxidative effects of natural polysaccharides (Shon, Kim, & Sung, 2003, Xing, Liu, Yu, Zhang, Li, & Li, 2004). The $\text{EDTANa}_2\text{-Fe (II)-H}_2\text{O}_2$ system was used to generate hydroxyl radical ($\bullet\text{OH}$) which would discolor safranin T. Added hydroxyl radical inhibitor could inhibit the bleaching. The above mentioned model was used to measure inhibitory effects of the three tested samples on hydroxyl radical and compared with that of Vc. The results indicated that inhibitory effects of the samples were concentration-dependent in the range of 10–2000 $\mu\text{g/mL}$ and samples with higher concentration induce better effect (see **Fig. 7b**). The IC_{50} of MVPS, HCl hydrolyzate and H_2SO_4 hydrolyzate was 309, 485, and 1489 $\mu\text{g/mL}$ respectively. Inhibitory of vitamin C to hydroxyl radical are obviously higher than the three test samples, which was between

80% and 90% at 100 $\mu\text{g/mL}$ and accorded with the previous reports (Li et al., 2013). The results also showed the inhibitory effects of HCl hydrolyzate on hydroxyl radical were keeping on higher levels than MVPS and H_2SO_4 hydrolyzate. It was suggested that containing more oligosaccharides would enhance the hydroxyl radical inhibitory effect, because sugar contents were almost equal between HCl and H_2SO_4 hydrolyzates. However, the antioxidants of the test samples were not depended on single factor but a combination of several factors, varying from contents composition to spatial structure and groups (Song, Zhang, Zhang, & Wang, 2010).

3.4.3 Reducing power

In the reaction system, antioxidant substances in samples cause reduction of Fe^{3+} /ferricyanide complex to the Fe^{2+} form. Therefore, Fe^{2+} can be monitored by measuring the formation of Prussian blue at 700 nm. Higher absorbance value means stronger reducing power of sample. **Fig. 7c** depicts the reducing powers of the tested samples that exhibited concentration-dependent increasing manner. At 400 $\mu\text{g/mL}$, the reducing powers were 0.23, 0.20, and 0.11 for MVPS, HCl hydrolyzate, and H_2SO_4 hydrolyzate, respectively. At 1200 $\mu\text{g/mL}$, the reducing powers were 0.89, 0.95, and 0.58 for MVPS, HCl hydrolyzate, and H_2SO_4 hydrolyzate, respectively. HCl hydrolyzate showed slightly higher activity than that of the other two samples. The reducing power was generally associated with the presence of reductones in oligosaccharides and polysaccharides, which had been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. Reductones were also reported to react with certain precursors of peroxide, thus preventing peroxide formation (Li et al., 2013; Song, Zhang, Zhang, & Wang, 2010). The results suggest that all the samples might supply reducing agents or groups to enhance their antioxidant activities.

4. Conclusion

This study investigated the degradable characteristics of water soluble polysaccharides from *M. veneriformis* (MVPS) with controlled acid hydrolysis method for the first time. Results confirmed that MVPS can easily be hydrolyzed by two types of mineral acids, HCl and H_2SO_4 , producing some MVPS fragments formed with oligosaccharides and glucose in hydrolyzate. The hydrolyzing reaction was investigated in three aspects, type of acid, acid addition amount and reaction time. The acid type and addition amount influence the hydrolyzing rate of MVPS. H_2SO_4

usage in hydrolysis can rapidly induce the MVPS degrading to monose, while HCl processing to MVPS behave a time-depended hydrolyzing manner, which is mild and controllable. During the acidic processing, high molecule weight of MVPS will be gradually hydrolyzed to low molecule weight of oligomers of polysaccharide. The change in oligomers compositions and relatively contents for carbohydrate profile of hydrolyzate with time is detailed by HPLC method. Total nine eluting peaks are recognizable in the carbohydrate profile of MVPS hydrolyzates, and then they are identified as glucooligosaccharides with DP from 1 to 7 by HPLC-ESI-TOF-MS analysis.

Representative hydrolyzates by HCl and H₂SO₄ processing were further investigated their antioxidant activities, comparing with the initial polysaccharide (MVPS) and positive control (Vc). In brief, initial polysaccharide and the polysaccharide hydrolyzates with different oligosaccharides contents all possessed antioxidant activities, performing with scavenging DPPH radicals, inhibiting the hydroxyl radicals and generating reducing power at relative low concentrations, and sample marked with HCl hydrolyzate exhibited stronger antioxidant effects. The study indicated the oligosaccharides content might be a significant factor to the antioxidant activity when sugar content and monosaccharide composition of the samples were similar. However, the mechanism of antioxidant activities in vivo and the effectiveness of HCl hydrolyzate from MVPS for human consumption should be further investigated.

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Figure Caption:

Fig. 1 The hydrolyzing isotherms of MVPS under the hydrolysis of HCl (a) and H₂SO₄ (b). The reaction was carried out with 10% (w/v) MVPS in 0.1, 1.0, 2.0 and 3.0 M acid at 60 °C. The degree of hydrolysis is expressed as GE and read as % (see Section 2.3 for definition).

Fig. 2 The evolution on HPLC-ELSD profiles of oligosaccharides in hydrolyzates of 1.0 M HCl (A) and H₂SO₄ (B) hydrolyzing for different time. Profiles (a), (b), (c), (d), (e), (f), (g) and (h) are the hydrolyzate samples that disposed with acids hydrolysis for 30, 60, 120, 180, 240, 300, and 360 min, respectively. Peaks 1-9 are denoted as oligosaccharides in hydrolyzate of MVPS.

Fig. 3 The relative content (peak areas) of the recognizable oligosaccharides from MVPS hydrolyzate at various disposal time of 1.0 M HCl.

Fig. 4 The total ionization chromatography (a) and extracted ion chromatograms ([M+Na]⁺) for various glucose oligomers in HCl hydrolyzate. (b) Extracted ion at m/z = 203.05 for peak 1; (c) extracted ion at m/z = 365.15 for peaks 2 and 3; (d) extracted ion at m/z = 517.20 for peaks 4 and 5; (e) extracted ion at m/z = 689.29 for peak 6; (f) extracted ion at m/z = 851.37 for peak 7; (g) extracted ion at m/z = 1013.44 for peak 8; (h) extracted ion at m/z = 1175.49 for peak 9.

Fig. 5 Structure elucidation and ESI-TOF-MS spectra (in positive mode) of the oligosaccharides in HCl hydrolyzate that retained at HPLC column from 10.58 to 18.15 min. MS spectra of (a), (b), (c), (d), and (e) are of the compound peaks of 1, 2, 3, 4, and 5, respectively.

Fig. 6 Structure elucidation and ESI-TOF-MS spectra (in positive mode) of the oligosaccharides in HCl hydrolyzate that retained at HPLC column from 20.18 to 25.13 min. MS spectra of (a), (b), (c), and (d) are of the compound peaks of 6, 7, 8, and 9, respectively.

Fig. 7 The DPPH radicals scavenging (a), hydroxyl radicals scavenging (b), and reducing power (c) for MVPS, and its HCl and H₂SO₄ hydrolyzates. Vc as the positive control. Each sample was assayed in triplicate for each concentration. **P* < 0.05 and ***P* < 0.001 vs. group of H₂SO₄ hydrolyzate.

Figure 1

Figure 2

Figure 3

Figure 5 Oligosaccharide 1 at 10.58 min

Figure 7