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Structural elucidation of polysaccharide fractions from brown seaweed *Sargassum pallidum*

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ABSTRACT

The structural characteristics of two purified fractions of polysaccharides from *Sargassum pallidum* (SPS) were investigated in the present study. As results, the molecular weights of the two polysaccharide fractions, SPS-3-1 and SPS-3-2, were determined to be 5.87 and 7.25 kDa, respectively. SPS-3-1 was composed of glucose, mannose and galactose in a molar ratio of 11.18:1.00:0.96, while SPS-3-2 was composed of fucose, xylose, mannose, glucose and galactose in a molar ratio of 2.53:0.61:1.00:0.46:0.92. Both SPS-3-1 and SPS-3-2 exhibited the characteristics of polysaccharide in the frequency range of 4000–400 cm^{-1} based on their Fourier-transform infrared spectra. Furthermore, the results of periodic acid oxidation, Smith degradation, methylation analysis and nuclear magnetic resonance spectroscopic analysis suggested that SPS-3-2 was composed of (1→4)-linked fucopyranosyl backbone and (1→3)-linked galactopyranosyl, (1→3)-linked mannopyranosyl, (1→2)-linked xylopyranosyl and (1→6)-linked glucopyranosyl branch chains.

1. Introduction

Seaweeds have caused an emerging interest in the biomedical area, mainly due to the presence of bioactive substances which show great potential as anti-inflammatory, antimicrobial, antiviral and anti-tumor drugs (Gupta & Abu-Ghannam, 2011; Vo & Kim, 2013; Wijesekara, Pangestuti, & Kim, 2011). Indeed, several species of algae have been found to be the sources of polysaccharides with antioxidant, immune-stimulant, antitumor, anti-inflammatory and antiviral effects (de Sousa et al., 2007; Dore et al., 2013; Nishino, Yokoyama, Dobashi, Fujihara, & Nagumo, 1989; Smit, 2004; Vo & Kim, 2013; Wijesekara et al., 2011). *Sargassum pallidum*, one kind of brown seaweeds extensively distributed in China Yellow Sea and East China Sea, is rich in vitamins, amino acids, dihomogammalinolenic acid, trace elements and polysaccharides (Khomenko & Ovodov, 1975; Wang et al., 2010; Ye, Wang, Zhou, Liu, & Zeng, 2008; Zhukova & Svetashev, 1999). In our previous study, we have demonstrated that the polysaccharides from *S. pallidum* (SPS) have significant antitumor activities against HepG2 cells, A549 cells and MGC-803 cells *in vitro* (Ye et al., 2008).

It has been reported that biological activity of polysaccharide depends on its chemical structure, molecular weight and chain conformation (Duarte, Cardoso, Noseda, & Cerezo, 2001; Sinha, Astani, Ghosh, Schnitzler, & Ray, 2010). Accordingly, chemical structures of polysaccharides from seaweeds have been investigated extensively in the past (Barros et al., 2013; Chevolot et al., 1999; Chevolot, Mulloy, Ratiskol, Foucault, & Colliec-Jouault, 2001; Chizhov et al., 1999; Maciel et al., 2008; Souza, Cerqueira, Bourbon, Pinheiro, Martins, & Teixeira, 2012). However, little information about the chemical structure of SPS is available compared to those of other seaweeds, such as *Sargassum stenophyllum*, *Fucus vesiculosus*, *Gracilaria birdiae* and *Saccharina longicruris* (Bilan et al., 2002; Dias et al., 2005; Duarte et al., 2001; Rioux, Turgeon, & Beaulieu, 2007; Souza et al., 2012). In order to better exploit and utilize this rich resource, it is essential to further investigate the structural property of SPS with commercial interest. Therefore, the chemical structures of the purified fractions of SPS (SPS-3-1 and SPS-3-2), having relative higher antitumor activity against HepG2 cells, A549 cells and MGC-803 cells *in vitro*, were characterized in the present study using gas chromatography (GC), high performance liquid chromatography (HPLC), Fourier-transform infrared (FT-IR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, periodic acid oxidation, Smith degradation and methylationan analysis.

2. Materials and methods

2.1. Materials and reagents

The purified fractions of SPS-3-1 and SPS-3-2 were prepared from *S. pallidum* according to our reported method (Ye et al., 2008).

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Standards of arabinose, fructose, rhamnose, fucose, galactose, glucose, mannose and xylose were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Standards of pullulan P-800, P-400, P-200, P-100, P-20, P-10 and P-5 were purchased from Showa Denko K.K (Tokyo, Japan). All other chemicals were of analytical grade.

2.2. Determination of molecular weight

The molecular weights of SPS-3-1 and SPS-3-2 were determined by an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a refractive index detector (RID) and a TSK-GEL G3000SW$_{xl}$ column (7.5 mm × 300 mm, Tosoh Corp., Tokyo, Japan). The temperature of column oven was set at 25 °C, and the column was eluted with 0.1 M Na_2SO_4 solution (dissolved in 0.01 M phosphate buffer, pH 6.8) at a flow rate of 0.8 ml/min. The injection volume of sample was 20 μl at a concentration of 1.0 mg/ml. Pullulans from Showa Denko K.K were used as the standards for the molecular weight measurement.

2.3. Analysis of monosaccharide composition

Analysis of monosaccharide composition was carried out using the reported methods (Li et al., 2008; Qiao et al., 2010) with minor modifications. SPS-3-1 and SPS-3-2 (5 mg, each) were hydrolyzed with 4 ml of 4 M trifluoroacetic acid (TFA) at 120 °C for 2.5 h. The hydrolyzate was repeatedly co-concentrated with methanol to dryness and the resulting residue was derivatized using the following procedure. Hydroxylammonium chloride (10 mg), inositol (5 mg, used as the internal reference) and pyridine (0.5 ml) were added to the hydrolyzed sample, and the mixture was kept in a water bath of 90 °C for 30 min. After cooling to room temperature, acetic anhydride (0.5 ml) was added, and the reaction mixture was placed in the water bath of 90 °C for 30 min again. The derivatives of standard monosaccharides were prepared in the same way. After cooling, all the samples were analyzed by GC (Agilent GC 6890) equipped with a flame ionization detector (FID) and a HP-5 fused silica capillary column (30 m × 0.32 mm × 0.25 μm). Nitrogen gas was used as carrier gas at a flow rate of 1 ml/min. The temperatures of injector and detector were set at 250 °C and 280 °C, respectively. The initial column temperature was held at 120 °C for 3 min, then programmed at a rate of 3 °C/min to 210 °C and held at 210 °C for 4 min.

2.4. FT-IR spectrometric analysis

The FT-IR spectra of SPS-3-1 and SPS-3-2 were recorded with a Tensor-27 FT-IR Spectrometer in the frequency range of 4000–400 cm^{-1} (Maciel et al., 2008). The dried sample was grinded with potassium bromide (KBr) powder and pressed into pellet for spectrometric measurement.

2.5. Periodic acid oxidation, Smith degradation and GC assay

Periodic acid oxidation, Smith degradation and GC analysis were performed according to the reported methods (Li et al., 2008; Qiao et al., 2010; Rout, Mondal, Chakraborty, & Islam, 2008) with some modifications. Firstly, the absorbance of mixed solution of sodium metaperiodate ($NaIO_4$, 0.015 M) and sodium iodate ($NaIO_3$, 0.015 M) at different volume ratio (5:0, 4:1, 3:2, 2:3, 1:4 and 0:5), diluted from 0.1 ml to 25 ml, was examined by spectrophotometer at 223 nm. The standard curve of $NaIO_4$ was produced using concentration of $NaIO_4$ as *X*-axis and absorbance at 223 nm as *Y*-axis. Then, polysaccharide sample (7.0 mg) was oxidized with 0.015 M $NaIO_4$ solution (40 ml) at 4 °C in the dark with interval stirring. After 4 h, 0.1 ml of the reaction mixture was diluted to 25 ml and the absorbance of dilution was detected at 223 nm. The absorbance of the diluted reaction mixture (from 0.1 ml to 25 ml) was monitored at 223 nm every 24 h until the absorbance unchanging. Ethylene glycol (4.0 ml) was then added to the reaction system with stirring to decompose the excess $NaIO_4$ at room temperature for 30 min. The consumption amount of $NaIO_4$ was calculated according to the $NaIO_4$ standard curve and the 223 nm absorption. The reaction mixture was reduced with sodium borohydride ($NaBH_4$, 50 mg) at room temperature for 24 h in the dark, and then it was adjusted to pH 5.5 by the addition of acetic acid solution (0.1 M). The reaction solution was dialyzed against distilled water for 48 h. The dialyzed product was dried and hydrolyzed with 4 ml of 4 M TFA at 120 °C for 2 h. The hydrolyzate was repeatedly co-concentrated with methanol to dryness, and the residue was converted to its trimethylsilyl (TMS) derivative by adding 1.0 ml pyridine, 0.4 ml hexamethyldisilazane and 0.2 ml trimethylchlorosilane and reacting at room temperature for 5 min. The TMS derivatives of monosaccharide standards, glycerol and erythritol were prepared in the same way. All the samples were analyzed by GC as described in Section 2.3.

The amount of formic acid (HCOOH) produced was determined by titration. In brief, the reaction product (1.0 ml) was mixed with phenolphthalein solution (0.5%, 50 μl) and then NaOH solution (0.5 mM) was added drop by drop until the color of reaction system changed from colorless to purple red. The amount of HCOOH was calculated based on the consumption amount of NaOH.

2.6. Methylation and GC–MS analysis

Methylation and GC–MS analysis were carried out according to the reported methods (Nie et al., 2011; Qiao et al., 2010) with slight modifications. Polysaccharides sample (10 mg) was dissolved in dimethylsulfoxide (2 ml), and then dried NaOH power (50 mg) was added to the polysaccharide solution with interval vibration under the protection of N_2. One hour later, 1.0 ml of methyl iodide was added to the reaction system with interval vibration under the protection of N_2. After 1 h, the reaction was stopped by adding 0.5 ml of water and dialyzed against running water for 24 h and distilled water for 24 h respectively. The dialyzed product was freeze-dried to afford partially methylated polysaccharide, and completely methylated polysaccharide was obtained by repeating above procedure for three times. Complete methylation was confirmed by the lack of hydroxyl peak in FT-IR spectrum. The permethylated polysaccharide was hydrolyzed with 4 ml of 4 M TFA at 100 °C for 2 h, reduced with $NaBH_4$ and acetylated with acetic anhydride. The resulting partially methylated alditol acetate derivatives were analyzed by GC–MS. The GC–MS analysis was performed on a Varian CP-3800 GC coupled with a Saturn 2000 ion trap mass spectrometer (Walnut Creek, CA, USA). A DB-5 fused silica capillary column (30 m × 0.25 mm × 0.25 μm, J & W Scientific, CA, USA) was used with Helium as the carrier gas. The oven temperature was initially set at 100 °C, programmed at 6 °C/min to 250 °C and held at 250 °C for 5 min.

2.7. NMR spectral analysis

NMR spectral analysis was carried out according to the reported method (Leone et al., 2008; Serrato et al., 2008). Before analysis, dried SPS-3-2 sample (10 mg) was exchanged with deuterium by lyophilizing with D_2O for three times. The deuterium-exchanged sample was dissolved with D_2O and both ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX-500 NMR spectrometer, and etramethylsilane (TMS) was used as the internal standard.

3. Results and discussion

The molecular weights of SPS-3-1 and SPS-3-2 were detected by HPLC with size exclusion column. As shown in Fig. 1, both SPS-3-1

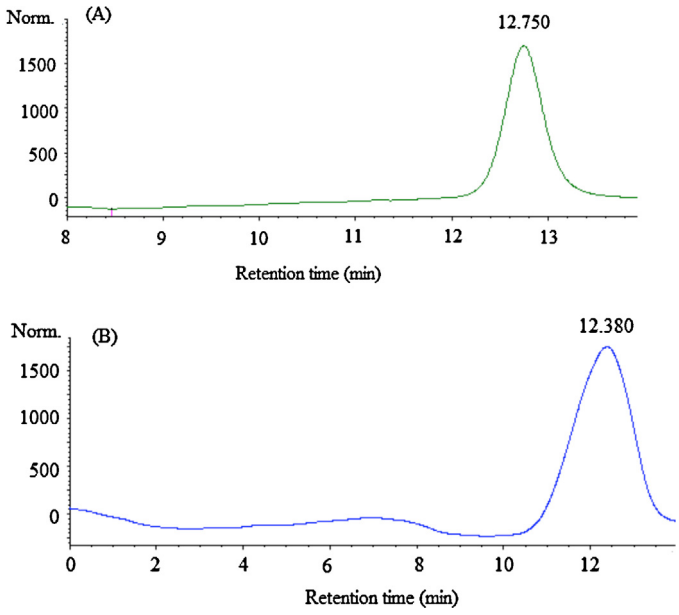

Fig. 1. HPLC chromatograms of SPS-3-1 (A) and SPS-3-2 (B) from *S. pallidum* on TSK-Gel G3000SW$_{xl}$ column.

and SPS-3-2 showed only one symmetrical peak on HPLC, indicating that no other polysaccharide was present in the sample. In addition, the molecular weights of SPS-3-1 and SPS-3-2 were estimated to be 5.87 and 7.25 kDa, respectively, according to the calibration curve of the elution times of standards. The results demonstrated that SPS were relatively lower molecular weight polysaccharides, and the results are consistent with that of our previous report that SPS-3 was the fraction with molecular weight less than 50 kDa from the hot water extract of *S. pallidum* by ultrafiltration (Ye et al., 2008).

The GC spectra of derivatives from monosaccharide standards, SPS-3-1 and SPS-3-2 are shown in Fig. 2. Table 1 presents the monosaccharide compositions and molar ratios of SPS-3-1 and SPS-3-2. Obviously, SPS-3-1 was composed of glucose, mannose and galactose, and glucose was the predominant monosaccharide in the monosaccharide composition of SPS-3-1. SPS-3-2 was composed of fucose, xylose, mannose, glucose and galactose, while fucose was the predominant monosaccharide in the monosaccharide composition of SPS-3-2. Accordingly, the monosaccharide composition of SPS-3-2 was quite different from that of SPS-3-1 and more complicated than that of SPS-3-1.

In order to investigate the functional groups in SPS-3-1 and SPS-3-2, their FT-IR spectra were measured in KBr pellets. As shown in Fig. 3, SPS-3-1 displayed a broad stretching intense characteristic peak at 3419 cm^{-1} for the hydroxyl group, and a weak C—H stretching band at around 2900 cm^{-1}. Two characteristic absorptions, a band at 1648 cm^{-1} (C=O asymmetric stretching vibration) and a band of 1409 cm^{-1} (C=O symmetric stretching vibration), indicated that there were carboxyl groups in SPS-3-1. The weak band at around 1200 cm^{-1} was due to S=O symmetric stretching vibration, indicating the existence of sulfuric acid radicals in SPS-3-1. It has been reported that SPS-3 exhibited the highest sulfate content (22.62%) in the three fractions of SPS (Ye et al., 2008),

Table 1
Monosaccharide compositions and molar ratios of SPS-3-1 and SPS-3-2 from *S. pallidum*.

Fraction	Fucose	Xylose	Mannose	Glucose	Galactose
SPS-3-1	–[a]	–	1.00	11.18	0.96
SPS-3-2	2.53	0.61	1.00	0.46	0.92

[a] Not detected.

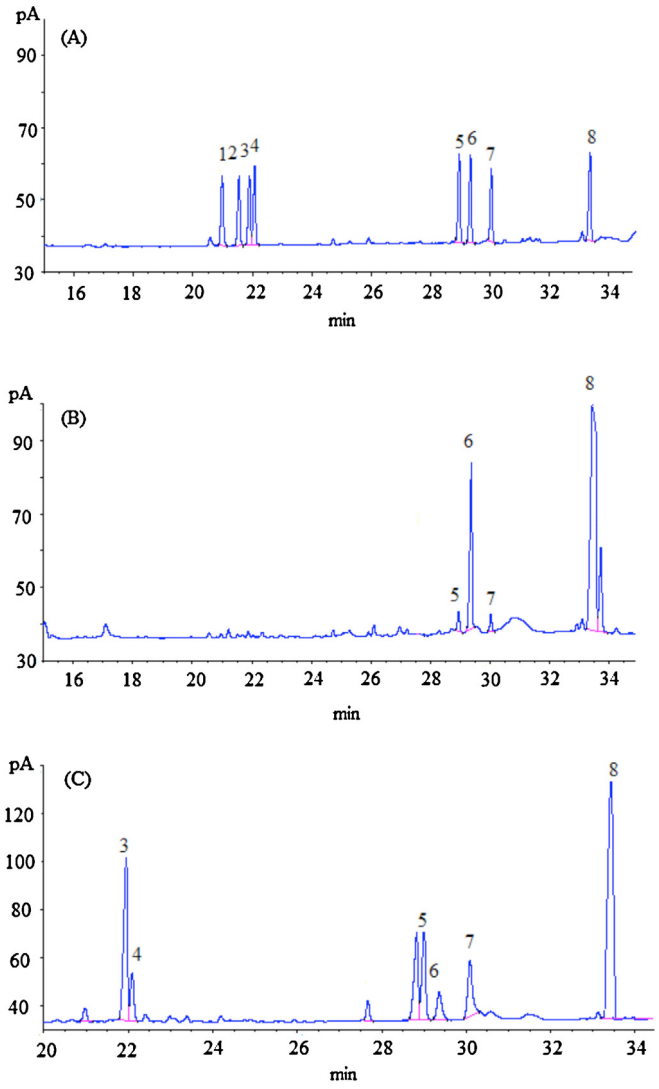

Fig. 2. GC chromatograms of derivatives from standard monosaccharide (A), SPS-3-1 (B) and SPS-3-2 (C) from *S. pallidum*. 1, rhamnose; 2, arabinose; 3, fucose; 4, xylose; 5, mannose; 6, glucose; 7, galactose; 8, inositol.

indicating that the result is consistent with that of our previous report. The band at 1030 cm^{-1} corresponded to C—O—H deformation vibration. SPS-3-2 revealed similar spectral characteristics to SPS-3-1, indicating they had similar functional groups (Wu, Wu, Zhou, & Pan, 2009; Zhang, 1999, chap 2–4).

Glycosidic linkage location of polysaccharides could be preliminarily determined by consumption of $NaIO_4$ and production of HCOOH in periodic acid oxidation (Zhang, 1999, chap 2–4). In the periodic acid oxidation of SPS-3-2, 1 mol glycosyl residues consumed 0.628 mol of $NaIO_4$ and produced 0.231 mol of HCOOH. The production of HCOOH indicated that the non-reducing terminal residues or 1→6 linked glycosidic bonds existed in SPS-3-2. Consumption of $NaIO_4$ was less than that of glycosyl residues and more than double of HCOOH production. The results meant that plenty of 1→3 linked glycosidic bonds which did not consume $NaIO_4$ existed in SPS-3-2. 1→2 or 1→4 linked glycosidic bonds also existed in SPS-3-2 which consumed $NaIO_4$ and did not produce HCOOH. To obtain more information about glycosidic linkage location, the product of periodic acid oxidation was further degradated and analyzed by GC (Zhang, 1999, chap 2–4).

GC chromatograms of derivatives from references and SPS-3-2 after periodic acid oxidation and Smith degradation are presented

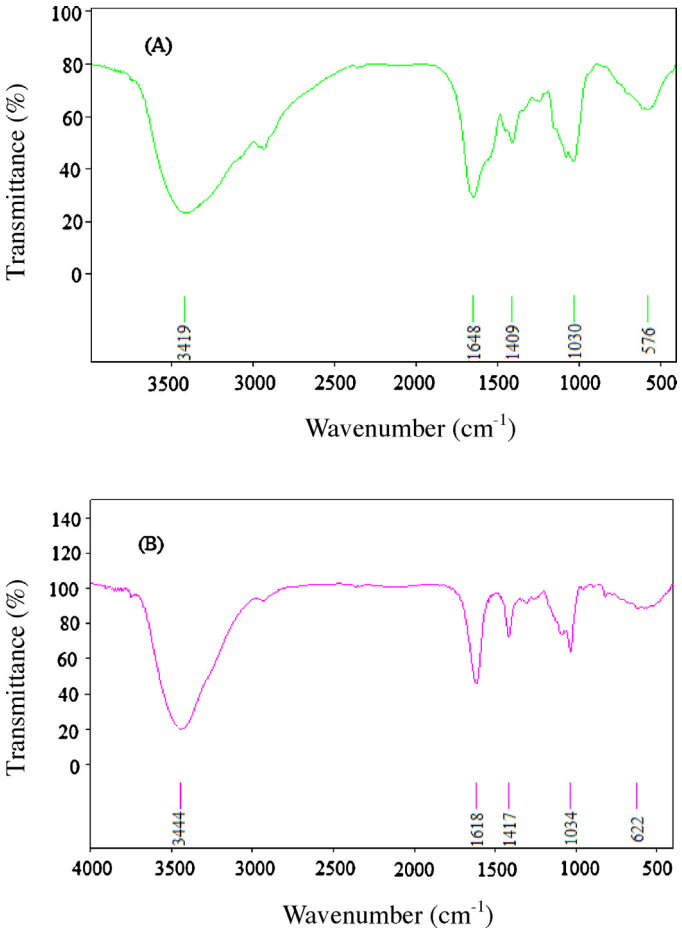

Fig. 3. FT-IR spectra of SPS-3-1 (A) and SPS-3-2 (B) from *S. pallidum*.

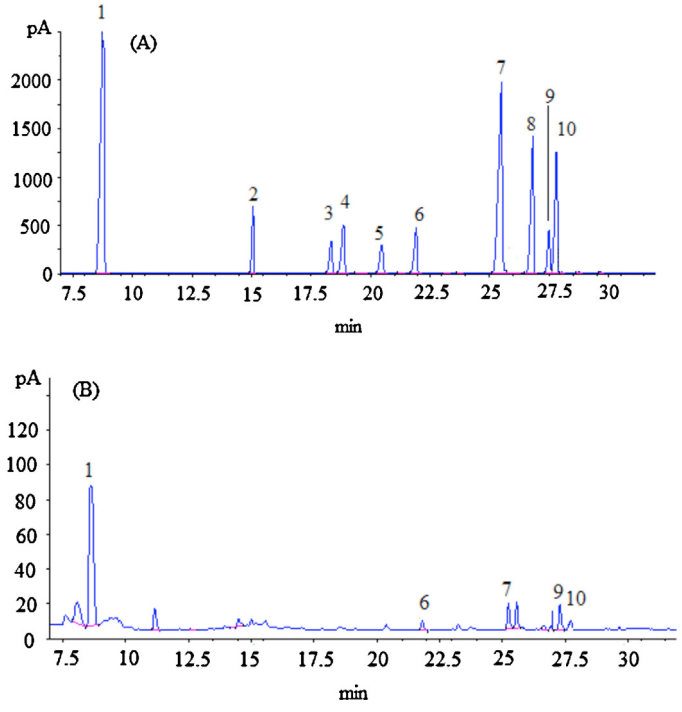

Fig. 4. GC chromatograms of derivatives from references (A) and SPS-3-2 (B) from *S. pallidum* after periodic acid oxidation and Smith degradation. 1, glycerol; 2, erythritol; 3, rhamnose; 4, arabinose; 5, fucose; 6, xylose; 7, mannose; 8, Fructose; 9, galactose; 10, glucose.

Table 2
GC–MS data from methylation analysis of SPS-3-2 from *S. pallidum*.

Mode of linkage	Methylated sugar residue	Molar ratio
→2)-Xyl-(1→	3,4-Me_2-D-Xyl	1
→3)-Gal-(1→	2,4,6-Me_3-D-Gal	2
→6)-Glc-(1→	2,3,4-Me_3-D-Glc	1
→3)-Man-(1→	2,4,6-Me_3-D-Man	2
→4)-Fuc-(1→	2,3-Me_2-D-Fuc	5

in Fig. 4. There was no erythritol in SPS-3-2, while glycerol was produced in the Smith degradation of SPS-3-2. It can be determined that the three hexoses in SPS-3-2, namely mannose, glucose and galactose, were not linked by 1→4 glycosidic bonds. The contents of mannose and galactose residues were almost unchanged in GC chromatograms of Smith degradation compared to those without periodic acid oxidation, which meant that mannose and galactose residues were not oxidated in periodic acid oxidation and linked mainly by 1→3 glycosidic bonds in SPS-3-2. The content of glucose residues decreased, indicating that majority of glucose residues were oxidized and linked mainly by 1→6 or 1→2 glycosidic bonds. The content of xylose residues decreased, indicating that majority

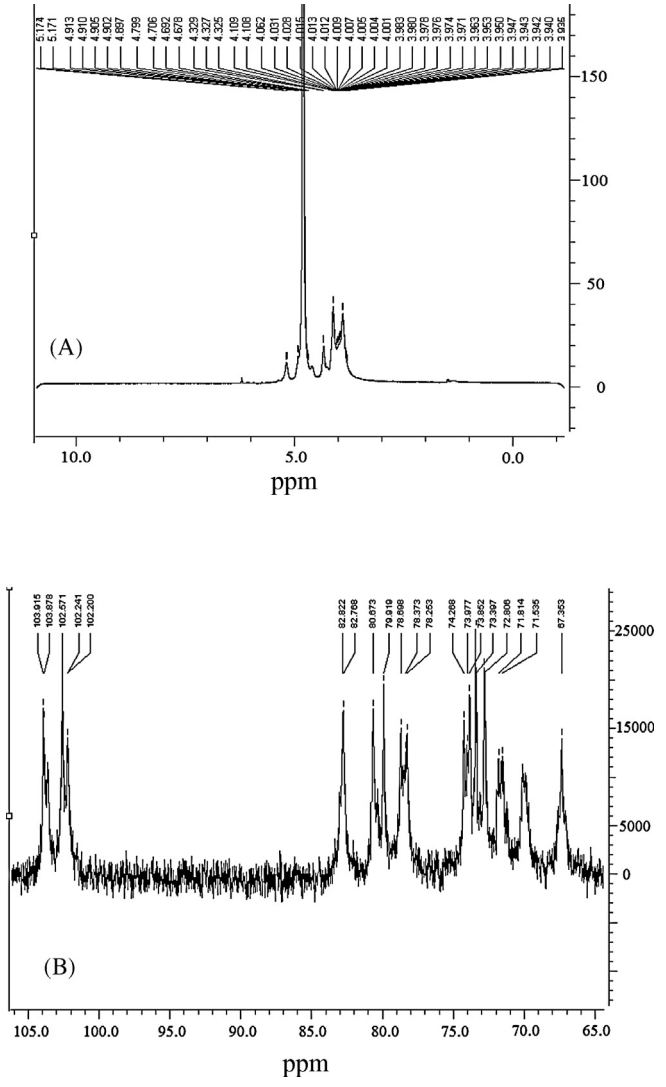

Fig. 5. ^{1}H NMR spectrum (A) and ^{13}C NMR spectrum (B) of SPS-3-2 from *S. pallidum*.

of them were oxidized and linked mainly by 1→2 or 1→4 glycosidic bonds. Fucose residues were not detected, which meant that they were completely oxidized and linked by 1→2 or 1→4 glycosidic bonds.

In order to determine the linkage of monosaccharides in SPS-3-2, it was methylated, hydrolyzed and converted into their corresponding alditol acetates for GC–MS analysis (Zhang, 1999, chap 2–4). As summarized in Table 2, the major derivative for SPS-3-2 was 2,3-dimethylated fucose, indicating that SPS-3-2 was composed of (1→4)-linked fucopyranosyl (Fuc*p*) backbone. Branch chains were composed of (1→3)-linked galactopyranosyl (Gal*p*), (1→3)-linked mannopyranosyl (Man*p*), (1→2)-linked xylopyranosyl (Xyl*p*) and (1→6)-linked glucopyranosyl (Glc*p*) residues.

Finally, NMR spectroscopy was used to verify the linkages deduced from GC–MS. The ^{1}H NMR spectrum of SPS-3-2 is showed in Fig. 5A. Chemical shift at 4.6 ppm indicated the glycosyl residues of SPS-3-2 were linked by β-configuration glycosidic bonds. A group of signals at 3.8–4.4 ppm were produced by C_2–C_6 protons, and the signal at 6.2 ppm was assigned to sulfated group (Zhang, 1999, chap 2–4). According to the ^{13}C NMR spectrum of SPS-3-2 (Fig. 5B), the anomeric region of SPS-3-2 contained a set of resonances between 102.20 and 103.92 ppm, indicating that sugar rings of SPS-3-2 were pyranose rings and glycosyl residues of SPS-3-2 were linked by β-configuration glycosidic bond. These signals might be assigned to C-1 of (1→4)-linked β-L-Fuc*p* residues, C-1 of (1→2)-linked β-D-Xyl*p* residues, C-1 of (1→3)-linked β-D-Gal*p* residues, C-1 of (1→6)-linked β-D-Glc*p* residues and C-1 of (1→3)-linked β-D-Man*p* residues. Furthermore, the occurrence of (1→6)-linked β-D-Glc*p* residues was supported by the signal at 67.35 ppm, which was characteristic of 6-substitution. The signals at 78.25–82.82 ppm were produced by substituted C_2, C_3 and C_4, and signals at 71.54–74.27 ppm were assigned to un-substituted C_2, C_3 and C_4. These results are in accordance with those results about the glycosidic linkages of SPS-3-2 deduced by GC–MS (Han & Clarke, 1990; Zhang, 1999, chap 2–4).

4. Conclusion

In summary, the results of preliminary characterization for purified SPS-3 showed that the molecular weights of SPS-3-1 and SPS-3-2 were 5.87 and 7.25 kDa, respectively. For monosaccharide composition, SPS-3-1 was composed of glucose, mannose and galactose in a molar ratio of 11.18:1.00:0.96, while SPS-3-2 was composed of fucose, xylose, mannose, glucose and galactose in a molar ratio of 2.53:0.61:1.00:0.46:0.92. The FT-IR spectra of SPS-3-1 and SPS-3-2 showed the character of polysaccharide in 4000–400 cm^{-1}. Periodic acid oxidation, Smith degradation, methylation analysis and NMR spectroscopy showed that SPS-3-2 was composed of (1→4)-linked Fuc*p* backbone, and branch chains were composed of (1→3)-linked Gal*p*, (1→3)-linked Man*p*, (1→2)-linked Xyl*p* and (1→6)-linked Glc*p* residues. Therefore, some important structural features of SPS were established in this work. To better utilize this kind of resource, further works on physical and other biological activities of SPS is in progress.

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