ABSTRACT: One fractional polysaccharide (HSSEP2) was isolated from the Umbilicaria esculenta grown in Huangshan Mountain. The structural and rheological properties of HSSEP2 were investigated. Methylation and NMR analysis showed that HSSEP2 had a β-D-(1 → 6)-glucan backbones without side chains. The intrinsic viscosity was 47.7 cm$^3$g$^{-1}$ at 25°C. The rheological studies indicated that the HSSEP2 solution was pseudoplastic fluid and presented the properties of gels. The HSSEP2 solutions exhibited certain temperature stability when the temperature was under 40°C. The metal ions had a significant impact on the rheological properties of HSSEP2 solution. Bivalent cation had more significant effect on the modulus of HSSEP2 solutions than the monovalent cation. Low concentration of NaOH solution could improve the viscosity and modulus but destroyed the structure of HSSEP2 with high concentration. HSSEP2 exhibited gel properties suggesting that it might be a potential supplement in food industry.

KEY WORDS: Polysaccharide, Rheological properties, Structural properties, Umbilicaria esculenta

INTRODUCTION

Polysaccharide, protein, nucleic acid and lipid are the most basic life substance. Polysaccharides can be derived from plant, animal and microbial (Jia et al., 2015; Mierczyńska et al., 2015). It is generally recognized that polysaccharides usually are non-polluting renewable sources and highly stable, safe for the sustainable supply of food products (Ding et al., 2012; He et al., 2015). The published research papers indicated that polysaccharides have many healthy functions, such as promoting the body’s immune, delaying aging, preventing cardiovascular disease, and promoting good bacteria in digestive system (Ding et al., 2012; Li and Nie, 2016; Wang et al., 2016; You et al., 2013). Thus, polysaccharides had shown a great application in food industry (Ding et al., 2012; Wei and Prasad, 2015).

According to the previous reports, the functional properties of polysaccharides were closely related to their structural features and physicochemical properties (You et al., 2013; Scoparo et al., 2013). It has been found that polysaccharides express functional properties prefer to occupy specific solution state and conformation (You et al., 2013; Bohn and Bemiller, 1995). In addition to the structural of polysaccharides, research and analysis of the physicochemical properties was also essential for developing its applications (Bao et al., 2016). Moreover, the study of rheological properties was an important means to ascertain structural and physicochemical properties of polysaccharides (Shi et al., 2016; Timilsena et al., 2015). However, more detailed knowledge of structure characteristics and rheological properties have not been clearly elucidated. To investigate the rheological properties of polysaccharides is of practical value for favoring them to the applications (Liu et al., 2016a). For example, in food systems, polysaccharides were used as thickening agents, preservatives, stabilizers, gelling agents, water-binding agents, emulsifiers (Zhu et al., 2009; Wang et al., 2015c).
**Umbilicaria esculenta** was one kind of lichen-forming fungi and mainly distributed in the Far East such as China, Korea and Japan (Cao et al., 2014; Kim and Lee, 2006). Polysaccharides derived from *U. esculenta* have shown biological activities. Previous studies have demonstrated that GE-3-S, the polysaccharide sulfate from *U. esculenta*, have significant effect of against the HIV virus (Hirabayashi et al., 1989). Furthermore, polysaccharides extracted from *U. esculenta* display activities for thromboxane, melanin formation, cholesterol synthesis, antitumor (Kim and Lee, 2006; Lee and Kim, 2000; Müller, 2001; Wang et al., 2014). But the report of structural and rheological properties of *U. esculenta* polysaccharides is still rare.

*U. esculenta* grown in Huangshan Mountain (HSSE) was one of traditional health food in China. In our previous studies, the effects of different extraction factors on the extraction yield of polysaccharide (HSSEP) from HSSE were investigated. Under optimal extraction conditions, the yield of HSSEP was 27.46%. Additionally, one major fraction, which was termed HSSEP2, was separated by a freeze-thawing step from HSSEP. The monosaccharide composition of HSSEP2 was glucose (Wang et al., 2015a). The process of obtain HSSEP2 was similar to the procedure of forming physical cryogels based on polysaccharides. Cryogels, which were formed in moderately frozen solutions, exhibited special features in the processing of food production (Lozinsky et al., 2003). Therefore, HSSEP2 was a potential food additive due to its ease to obtain and non-toxicity. It has significance to study the structure and rheological properties of HSSEP2. In this study, structural and rheological properties of HSSEP2 were discussed. The structure of HSSEP2 was evaluated by a series of analytical technologies including methylation analysis, Fourier transform-infrared spectroscopy (FT-IR), gas chromatography-mass spectrum (GC-MS), and nuclear magnetic resonance (NMR) spectroscopy. The effects of concentration, temperature, metal ion and pH on the rheology of HSSEP2 were investigated. The results may provide a reference for the development of *U. esculenta* which would be significant to developing more healthy food.

**MATERIALS AND METHODS**

Materials and chemicals

HSSE was obtained from Huangshan city in the Anhui province of China. Standard monosaccharides (L-arabinose, D-xylose, L-rhamnose, D-mannose, D-glucose, and L-galactose) and DMSO-$_6^6$(0.03%, v/v) were purchased from Sigma–Aldrich Co., Ltd. (St. Louis, MO, USA). All other reagents were analytical grade.

Isolation and purification of polysaccharides

The isolation and purification of polysaccharides were according to the previous method (Wang et al., 2015a). Simply, the HSSE powder was extracted twice with hot distilled water. The supernatant was collected and concentrated. Then it was mixed with 95% ethanol and kept overnight. Subsequently, the precipitate was obtained and dissolved in water again. The solution was removed proteins by Sevag method (Jia et al., 2015). The deproteinated product was dialyzed and fractionated through freeze-thawing procedure. The precipitate and the supernatant were separated by filtration. The precipitate fraction was lyophilized to obtain the polysaccharides, coded as HSSEP2.

**Methylation analysis**

NaH was dissolved into 50 mL DMSO. After incubating at 25–60°C for 12 h, green sodium methyl sodium (SMSM) was produced. HSSEP2 (25 mg) vacuum-dried over P$_2$O$_5$ was dissolved in 5 mL DMSO and SMSM (1.5 mL) was added and stirred at room temperature for the night away from light after placed in the ultrasonic for about 30 min. The mixture was continually stirred and cooled on ice and iodomethane (CH$_3$I) about 1.5 mL was added dropwise in 30 min and stirred at room temperature for the night away from light (He et al., 2015; Wang et al., 2015b). After the mixture solution was dialyzed against running water of two days and ultra-pure water of one day and collected and freeze-dried.

The methylated HSSEP2 (HSSEP2D) was depolymerized in 4 mL formic acid (90%, v/v) at 100°C for 6 h. After formic acid treated by reduced pressure distillation, the residue was transformed into partially methylated alditol acetate according to the method of Zhang et al (Zhang et al., 2016). The end product was analyzed by GC-MS equipped with HP-5 capillary column (Zhang et al., 2016). Helium was the carrier gas with a flow rate of 1.0 mL/min.

**NMR analysis**

HSSEP2 (25 mg) vacuum-dried with P$_2$O$_5$ was dissolved in 0.5 mL DMSO-$_6^6$(0.03%, v/v), and then examined with NMR spectrometer to get 1$^H$ NMR, 13$^C$NMR spectra (Wang et al., 2015b).

**X-ray diffraction analysis**

X-ray diffraction (XRD) measurements were performed on an X-ray diffractometer with CuKα radiation with scattering angles (2θ) of 4–50° at a scan rate of 102 °/min (Liu et al., 2015). The current and voltage were maintained at 40 mA and 40 kV, respectively.

**Atomic force microscope analysis**

HSSEP2 was dissolved in anhydrous DMSO by drastically stirring for 24 h in room temperature and diluted to the concentration of 10 μg/mL. 10μL solution was took to drop onto freshly cleaved mica and dry in room temperature (Liu et al., 2016b). The specimen was examined using atomic force microscopy, with a scanning frequency of 1Hz. The obtained image was stored as 512 point arrays.

**The intrinsic viscosity analysis**

The intrinsic viscosity of HSSEP2 solutions was measured by an Ubbelohde viscometer at 25 °C (Timilsena et al., 2015; Liu et al., 2016a). Viscometer was selected that pure solvent spent
120 s on through capillary, which kinetic energy correction can be ignored. Using dilution method step by step according to the following equation dilution concentration step by step to zero, thus get \([\eta]\).

\[
\eta sp/c = [\eta] + k[\eta]2c
\]

\((ln[\eta])/c = [\eta] – k''[\eta] 2c\)

Where \(k\) and \(k''\) are constant.

**Rheological measurements**

**Effect of concentration**

HSSEP2 solutions were prepared at concentrations of 0.60 mg/mL, 0.70 mg/mL, 0.80 mg/mL, 0.90 mg/mL and 1.00 mg/mL in ultrapure water, and stored at 4 °C for further analysis. The dependence of viscosity on shear rate for HSSEP2 with different concentrations (0.6%, 0.7%, 0.8%, 0.9%, and 1.0%) at 25 °C was measured by Rheometer at a shear rate of 0.01–1000 s⁻¹ and frequency sweep of 1 rad/s using cone and plate geometry (40mm² steel cone, 59μm gap) (Nwokocha and Williams, 2016).

In the small oscillation procedure, frequency sweep test was performed to analysis storage modulus \(G'\) and loss modulus \(G''\) varies with the change of angular frequency. The test was in the condition of frequency sweep of 0.1–100 rad/s and using cone and plate geometry (40mm² steel cone, 59μm gap) and 25°C. All the tests were carried out in the linear viscoelastic region.

**Effect of temperature**

0.70 mg/mL and 0.80 mg/mL HSSEP2 solution were formed of ultrapure water and HSSEP2 powder, and stored at 4 °C for further analysis. The variety of the modulus concern to temperature was measured by Rheometer at a stepped rise procedure in the temperature at 2 °C/min interval.

**Effect of metal ion**

1% HSSEP2 solutions contained one of Na⁺, K⁺, Ca²⁺ and Mg²⁺ at concentrations of 0.01 M, 0.05 M, 0.10 M and 0.20 M were obtained by mixing different concentrations of NaCl, KCl, CaCl₂ and MgCl₂ solution and 2% HSSEP2 solution in equal volumes. Rheometer was used to assay viscosity change and frequency sweep curve of polysaccharide solutions possess different concentrations of metal ions.

**Effect of sodium hydroxide**

1% HSSEP2 solutions contained concentrations of 0.01 M, 0.05 M and 0.10 M NaOH were prepared, and stored at 4 °C for further utilized. Rheometer was used to assay viscosity change and frequency sweep curve of polysaccharide solutions possess different concentrations of NaOH.

**RESULTS AND DISCUSSION**

**Methylation analysis and NMR analysis**

The physicochemical property and preliminary characterization of the polysaccharide HSSEP2 were studied in our previous research (Wang et al., 2015a). The total sugar content of HSSEP2 was 98.80% and it had 0.56% uronic acid without nucleic acids and proteins. HSSEP2 was mainly composed of glucose. However, detailed information of structure of HSSEP2 was not clear.

**FIGURE 1. FT-IR spectra of methylated HSSEP2.**

Methylation analysis was a commonly used method to understand the linkage types of polysaccharides (Ding et al., 2012). The methylated HSSEP2 was ground with dry KBr powder and then pressed into sheets in a mold for IR measurement in the range of 4000–400 cm⁻¹. The FT-IR spectrum was shown in Fig. 1. As shown in Fig. 1, the broad peak at 3400–3500 cm⁻¹ disappeared, indicating that the O–H was removed. The methylated polysaccharides were further analyzed by GC-MS, revealing one partially methylated alditol acetate 1, 5, 6-tri-acyt-2, 3, 4-O-methyl glucitol with the peak time of 26.89 min only. Accordingly, the HSSEP2 had only one linkage (1 → 6)-linked glucosyl. Compared to the FI-IR spectrum of HSSEP2, the residue was β-D-(1 → 6)-linked glucosyl.

The 1H and 13C NMR spectrum of HSSEP2 in DMSO-d₆ was shown in Fig. 2. The chemical shifts at 106.3(C1), 76.5(C2), 79.6(C3), 72.9(C4), 78.6(C5) and 71.5(C6) ppm were assigned to the carbon atoms of the β-D-(1 → 6)-glucan backbones (Wang et al., 2016; Zhang et al., 2014). The chemical shifts at 4.9(H1), 3.5(H2), 3.2(H3), 4.2(H4), 3.1(H5), 4.2(H6) ppm were assigned to the hydrogen atoms of the backbone accordingly (Iorio et al., 2008; Imbs et al., 2015). The chemical shifts at 173.6 ppm in 13C NMR spectrum and 2.1 ppm in 1H NMR spectrum were attributed to the existence of acetyl groups (He et al., 2015), respectively.

Combining the methylation and NMR spectrum analysis of HSSEP2, it could be concluded that the HSSEP2 had a β-D-(1 → 6)-glucan backbones and there were no side chains in HSSEP2. There were many varieties of β-D-glucans separated from different lichens (Bohn and Bemiller, 1995). Previous studies have demonstrated that the major chain of MD-fraction extracted from *Grifola frondosa* is made up of β-1,6-linked glucose residues with branches of β-1,3-linked glucose.
A gel polysaccharide from *umbilicaria esculenta* (Nanba et al., 1987). The primary structure of HEEP2 was more concise than the MD-fraction.

**X-ray diffraction and Atomic force microscope analysis**

Usually, many polysaccharides didn’t contain crystallizations due to the complex structure and lack of powerful and regular interaction force (Wu et al., 2014). X-ray diffraction (XRD) can be used to study the crystal structure of polysaccharides. In general, crystalline substances show sharp narrow diffraction peaks when it was analyzed by X-ray diffraction (Liu et al., 2015). As shown in Fig. 3a, there were two peaks in the X-ray diffraction spectrogram of HSSEP2, indicating the existence of the crystallizations. The Atomic force microscope (AFM) can be used to observe the shape of polysaccharides in solution directly. The HSEEP2 was dissolved in DMSO and its images in AFM were shown in Fig. 3b. Visually, the polysaccharides presented a shape of globular aggregates in DMSO. Similarly, polysaccharides derived from fungi such as *Polyporus rhinoceros*, *Lactarius deliciosus Gray*, and *Lentinus edodes* were displayed in globular aggregates like (Ding et al., 2012; Chen et al., 2016). This phenomenon could be caused by strong intramolecular and intermolecular interaction provided by hydroxyl groups (Liu et al., 2016b).

**Intrinsic viscosity analysis**

Intrinsic viscosity can reflect the basic character of materials in that it ignores the interaction between particles (Timilsena et al., 2015). The intrinsic viscosities of polysaccharides...
were closely related to the molecular weight, molecular conformation and molecular interactions (Timilsena et al., 2015; Bae et al., 2008; Herlina et al., 2016). The intrinsic viscosity of HSSEP2 was determined using an Ubbelohde viscometer at 25 °C. As shown in Fig. 4a, the intrinsic viscosity of HSSEP2 was 47.7 cm³g⁻¹. It was higher than the water-soluble polysaccharide from Microbacterium laevaniformans, which was 38.0 cm³g⁻¹ (Bae et al., 2008). Fig. 4b showed the change of intrinsic viscosities of HSSEP2 with different storage times. The intrinsic viscosity increased with the extension of storage time. Molecular conformation and molecular interactions of HSSEP2 may be changed in the process of storage, which caused the increase of intrinsic viscosity (Herlina et al., 2016).

**FIGURE 4.** (a) Intrinsic viscosity of HSSEP2, (b) Intrinsic viscosities of HSSEP2 with different storage times.

**FIGURE 5.** Effect of different factors on viscosity and modulus of HSSEP2. (a) effect of shear rate on viscosity of different HSSEP2 concentrations (0.6–1.0%), (b) effect of angular frequency on modulus of different HSSEP2 concentrations (0.6–1.0%), (c) effect of temperature on modulus of 0.70 mg/mL HSSEP2 solution, (d) effect of temperature on modulus of 0.80 mg/mL HSSEP2 solution.

**Rheological properties of HSSEP2**

**Influence of the concentration**

The dependence of viscosity on shear rate for HSSEP2 with different concentrations (0.6%, 0.7%, 0.8%, 0.9%, and 1.0%) at 25 °C was shown in Fig. 5a. The viscosity increased...
with the increasing of concentration. Moreover, the viscosity of all solutions decreased with the increasing of shear rate, which exhibiting shear-thinning behavior, indicating that the HSSEP2 solutions were pseudoplastic fluid (Bae et al., 2008).

The dependence of the storage modulus \( G' \) and the loss modulus \( G'' \) on the angular frequency for different concentrations of HSSEP2 solutions was shown in Fig. 5b. With the increasing of concentration, the \( G' \) and \( G'' \) increased. The values of \( G' \) were higher than \( G'' \) in most of the angular frequency range, in other words, the solutions mainly exhibited elastic properties (Nwokocha and Williams, 2016). Moreover, the \( G' \) and \( G'' \) had little dependence on angular frequency, which was the typical properties of gels (Bao et al., 2016). The values of \( G' \) increased and the values of \( G'' \) decreased when the angular frequency reached high frequency region. This tendency was also detected in the guar gum aqueous solutions (Zhu et al., 2009). It may be because the gel structure was damaged in larger angular frequency (Zhu et al., 2009; Xu et al., 2015).

**Influence of the temperature**

Temperature is a very important factor affecting the rheological properties of polysaccharides (Shi et al., 2016; Bae et al., 2008). Polysaccharides can present a different structure and conformation at different temperatures, due to the change of intramolecular or intermolecular forces (Timilsena et al., 2015). To examine the dependence of modulus on the temperature, the temperature sweep was conducted for 0.7% and 0.8% HSSEP2 solutions. As shown in Fig. 5c and Fig. 5d, the values of \( G' \) of two kinds of solutions began to drop at about 40 °C. Compared to the \( G' \), the change of \( G'' \) was slight in the selected range of temperatures. The \( G' \) of 0.8% HSSEP2 solution dropped sharply when the temperature was higher than 50 °C was presented in Fig. 5d. These results showed that the HSSEP2 solutions had a certain temperature stability when the temperature below 40 °C.

**Influence of the metal ions**

The type and concentration of metal ions could affect the rheological properties of polysaccharides in solutions (Mierczyńska et al., 2015; Bao et al., 2016; Shi et al., 2016; Samanta et al., 2010). Hence, the rheological properties of HSSEP2 were tested at 25 °C with four metal ions (Na\(^+\), K\(^+\), Mg\(^{2+}\), and Ca\(^{2+}\)).

The dependence of viscosity on shear rate was shown in Fig. 6. It could see that the influence of four ions on viscosity was little except Ca\(^{2+}\). The viscosity increased slightly with the increasing of concentration of Ca\(^{2+}\). The added calcium ions could cause the increase of cross-linking and influence the rheological behavior of HSSEP2 (Mierczyńska et al., 2015;...
A gel polysaccharide from *umbilicaria esculenta* (Samanta et al., 2010). This effect could also be detected for apple pomace polysaccharide in its aqueous solution in which viscosity was increased by addition of CaCl$_2$.

**FIGURE 7. Effect of different metal ions on modulus of HSSEP2.** (a) effect of different Na$^+$ concentrations on modulus of HSSEP2 solution, (b) effect of different K$^+$ concentrations on modulus of HSSEP2 solution, (c) effect of different Mg$^{2+}$ concentrations on modulus of HSSEP2 solution, (d) effect of different Ca$^{2+}$ concentrations on modulus of HSSEP2 solution.

The influence of four metal ions on the modulus of HSSEP2 in solutions was shown in Fig. 7. The Na$^+$ had no effect on the modulus basically. This type of behavior was different with polysaccharides from *Guar* (Wang et al., 2015c), *Enteromorpha prolifera* (Qiao et al., 2016), *Ponpy seed dreg* (Shi et al., 2016), and *Auricularia auricular-judae* (Bao et al., 2016). On the contrary, the K$^+$ had significant effect on the modulus. The storage modulus $G'$ and the loss modulus $G''$ increased when the concentration of K$^+$ reached 0.01M. But the $G'$ and $G''$ decrease continuously with the increasing of concentration of K$^+$ (0.05M, 0.10M, 0.20M). In the HSSEP2 solutions with Ca$^{2+}$, the $G'$ and $G''$ increased gradually with the increasing of concentration of Ca$^{2+}$. In the groups with Mg$^{2+}$, the modulus increased when the concentration of Mg$^{2+}$ increased to 0.05M, then fell when the ion concentration reached 0.10M. But the modulus increased again when the ion concentration increased to 0.20M. On the whole, the bivalent cation had more significant effect on the modulus of HSEEP2 solutions than the monovalent cation. The rheological properties of HSEEP2 were very different in different ion solutions.

**Influence of the sodium hydroxide**

Sodium hydroxide (NaOH) can destroy the intramolecular or intermolecular hydrogen bonds in polysaccharides and the rheological properties may have a certain degree of change (Wang et al., 2016; Qiao et al., 2016).

As shown in Fig. 8, the NaOH had significant effect on the viscosity and modulus of HSSEP2 in solutions. The viscosity and modulus had the maximum values when the concentration of NaOH reached 0.01M. It may because the addition of NaOH led to the lack of acetyl groups and the water solubility of HSSEP2 declined (Qiao et al., 2016). With the increasing of concentration of NaOH, the viscosity and modulus decreased gradually. When the concentration of NaOH reached 0.10M, the viscosity and modulus were smaller than the pure HSSEP2 solutions, which had no NaOH. The structure of the HSSEP2 had changed with the addition of NaOH and high concentration of NaOH solution destroyed the hydrogen bonds in polysaccharides (Bao et al., 2016; Samanta et al., 2010).
The HSSEP2 aqueous solution has exhibited gel properties at low concentrations. The rheological behavior of HSSEP2 aqueous solutions was little influenced by the presence of NaCl. The pH effect on viscosity and modulus of HSSEP2 aqueous solutions was evaluated. The values of viscosity and modulus were both increased when the added sodium hydroxide was less than 0.05M. These results suggested that HSSEP2 as an easily available colloid was suitable for most foods. It was particularly applicable to high-sodium food due to sodium has little effect on its viscosity and modulus. In addition, it performed colloidal properties in the pH range of alkaline foods. The above results indicated that HSSEP2 a potential supplement in food industry.

CONCLUSIONS

HSSEP2 was extracted from U. esculenta grown in Huangshan Mountain. Its structure was composed with a repeating unit (1 → 6) linking β-D-Glc as backbone and there were no side chains in it. The intrinsic viscosity of HSSEP2 was 47.7 cm²g⁻¹ at 25 °C and it was increased with the extension of storage time. The XRD test indicated the existence of the crystallizations in HSSEP2. The observation in AFM showed that the HSSEP2 presented a shape of globular aggregates in DMSO. The viscosity of HSSEP2 solutions exhibited shear-thinning behavior at 25 °C and increased slightly with the addition of Ca²⁺. HSSEP2 dispersion behaved as gel when the temperature below 40 °C. The viscosity and modulus of HSSEP2 dispersion were improved by low concentration of NaOH solution and decreased gradually with the increasing of concentration of NaOH. These results provided fundamental and useful data for the application of HSEEP2 in food industry.

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