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Ultrasonic degradation, purification and analysis of structure and antioxidant activity of polysaccharide from *Porphyra yezoensis* Udea

Cunshan Zhou[a,b,1], Xiaojie Yu[b,*,1], Youzuo Zhang[b], Ronghai He[a,c], Haile Ma[a,c,**]

[a] School of Food and Biological Engineering, Jiangsu University, ZhenJiang 212013, China
[b] School of Agriculture and Food Science, Zhejiang A & F University, Lin'an 311300, China
[c] Jiangsu Provincial Research Center of Agricultural Products Bio-processing and Bio-separation Engineering, Zhenjiang 212013, China

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ABSTRACT

The chemical structure and antioxidant of natural and ultrasonic degraded polysaccharides from *Porphyra yezoensis* Udea was investigated. The degraded polysaccharide ($PYPS_{UD}$) was purified, and F2 (a homogeneous fraction) was obtained. FT-IR, ^{1}H and ^{13}C NMR spectral analysis revealed that F2 have typical porphyran structure. It has a backbone of alternating (1 → 4)-3,6-anhydro-α-L-galactopyranose) units and (1 → 3)-linked β-D-galactose or (1 → 4)-linked α-L-galactose 6-sulfate units. The result ascertained ultrasound degradation did not change the main structure of polysaccharides in the test conditions. Antioxidant proved that the activity of scavenging superoxide and hydroxyl radical is $F_2 > V_C > PSPY_{UD} > PSPY$. It was possible that ultrasonic treatment is an effective way for enhancing PSPY's antioxidant activity ascribing to decreasing molecular weight of polysaccharides.

1. Introduction

Porphyra, commonly known as laver, is a wide consumed seaweed in the world. Polysaccharide from *Porphyra* has many physiological activities (Gao & Shan, 2004) including antiviral (Peat & Rees, 1961; Xiao et al., 2003), immunoregulatory (Yoshizawa, Enomoto, Todoh, Ametani, & Kaminogawa, 1993; Yoshizawa et al., 1995; Zhang et al., 2001), anticancer (Zhang, Zhou, Chen, Ling, & Hou, 2002), anticoagulant and antihyperlipidemic (Zhou & Chen, 1990). Many investigations of the structure and function of the polysaccharides isolated from different *Porphyra* species have been undertaken (Sato, Kanno, & Sato, 1987; Yoshizawa et al., 1995; Zhang et al., 2004). In recent years, it has been demonstrated that algal polysaccharide as a kind of free-radical scavenger and antioxidant plays an important role in preventing damage of oxidation on living organisms (Zhang et al., 2003, 2004). In addition, the chemical properties of polysaccharides from different species show great variations (Cyrille & Marc, 1989). On the one hand, many properties of polysaccharides depend on their molecular weights. They refer to both physical characteristics, such as solution viscosity, and more complex properties, such as biological activity. For example, it was proved that digesting polysaccharides from *Porphyra yezoensis* Udea with β-agarase showed higher macrophage stimulation activity and solubility (Yoshizawa et al., 1995). Similarly, antithrombotic activity and stimulating plant growth also depend on its molecular weight (Inui, Tsujikubo, & Hirano, 1995; Kim, Ihm, Choi, Nah, & Cho, 2003). On the other hand, many functional activities have been studied and these works were mainly focused on *Porphyra haitanensis* and the native polysaccharides of *Porphyra* (Chen, 1992; Xiao et al., 2003; Zhang et al., 2003).

Ultrasonic irradiation has been recently looked upon as a new technique for degradation of polymer compounds, mainly due to the fact that the reduction in the molecular weight is simply by splitting the most susceptible chemical bond without causing any changes in the chemical nature of the polymer (Li, Li, Guo, & Li, 2005; Vaibhav, Mohan, & Parag, 2008). Partially degrading polysaccharides of *P. yezoensis* with utilizing ultrasound to enhance its physicochemical properties and ever to change its activity function has been reported (Zhou & Ma, 2006; Zhou, Wang, Ma, & He, 2008). However the effect of chemical structure in ultrasonic degradation of polysaccharides has not yet been investigated. In this study, the polysaccharide from *P. yezoensis* was isolated by traditional method of hot-water extraction and ethanol precipitation. The polysaccharide was partially degraded by ultrasound wave in water phase. Degree of the degradation was indirectly judged by intrinsic viscosity. Chemical structure of a homogeneity polysaccharide fraction separated from the degraded polysaccharide was affirmed by Fourier-transformed infrared (FT-IR) and nuclear magnetic resonance (NMR) spectral analysis. In vitro antioxidant activity was

* Corresponding author. Tel.: +86 571 63741276; fax: +86 571 63741276.
** Corresponding author at: School of Food and Biological Engineering, Jiangsu University, ZhenJiang 212013, China. Tel.: +86 511 88790958; fax: +86 511 88790958.
E-mail addresses: cunshanzhou@163.com (X. Yu), mhl@ujs.edu.cn (H. Ma).
[1] Equal contributions to this paper.

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investigated by assays of scavenging capacity of samples on superoxide anion and hydroxyl radicals.

2. Materials and methods

2.1. Materials and chemicals

P. yezoensis, being cultured near the coast of Nantong City, Jiangsu Province, China, was collected in October 2004. The *P. yezoensis* powder (40 mesh sieved) was kindly provided by Nantong Lanbo Industry Co. Ltd. (Jiangsu, China). The polysaccharide was extracted by the method of hot-water extraction and ethanol precipitation.

Briefly, *P. yezoensis* powder was defatted for 5 h with supercritical CO_2 under the conditions as follows: extraction: 45 °C, 30 MPa; separation: 35 °C, 5 MPa; and CO_2 flow rate: 10 L/h. A 116 g amount of the defatted *P. yezoensis* was extracted with 10 L of distilled water at 100 °C for 4.7 h. The extract was subjected to the Sevag method (Whistler, 1965) to remove free proteins and ultrafiltered (Mw of cutoff 10^4 g/mol, PLCC 10K Regenerated Cellulose, Millipore), and the retentate re-dissolved in water and precipitated by 4 times volume 95% ethanol. Then the precipitate collected by centrifugation and washed successively with 80% ethanol and acetone, and finally dried under reduced pressure (≥0.095 MPa) at 40 °C, so the polysaccharide of *P. yezoensis* (PYPS) was obtained (Zhou & Ma, 2006; Zhou et al., 2008).

2.2. Ultrasonic degradation

Ultrasonic treatment under 20 kHz and 800 W was carried out by using a HF-20B ultrasonic reactor (Beijing Hong Xiang Long Biotechnology Developing Co., Ltd.). A 50 mL amount of the PYPS solution (1.0%, w/v) was placed into a reaction vessel (a 50 mL rosette cell) and kept at a stable temperature 30 °C by a water bath (HH-S2, ChangZhou JinTan Co., Ltd.) for 4 h, thus the solution of ultrasonic degraded PYPS ($PYPS_{UD}$) was obtained. To reduce the experimental errors caused by uneven power transfer, the sample was located just under the ultrasound source and dipped into the solution about 20 mm. Irradiated samples were carried out in three replicates.

2.3. Purification of fractions from $PYPS_{UD}$

A 200 mg amount of $PYPS_{UD}$ solution was put into a DEAE-52 column (2.6 cm × 60 cm) that had been equilibrated with distilled water. After the column was washed with the distilled water, the absorbed materials were eluted by a gradient of NaCl solutions (0, 0.05, 0.1, 0.2 and 0.4 mol/L) at a flow rate of 0.4 mL/min. Fractions (F1, F2, F3, F4 and F5) containing polysaccharides were collected, and the content of their total carbohydrate was detected by the phenol-sulfuric acid method (Dubois, Gillis, Hamilton, Rebers, & Smith, 1956).

2.4. General analytical methods

Total carbohydrate content was analyzed by the phenol-sulfuric acid method (Dubois et al., 1956), and D-galactose was used as a standard sample. 3,6-Anhydrogalactose content was determined according to the method of Yaphe (Yaphe & Arsenault, 1965). Sulfate content was determined as described previously (Kawai, Seno, & Anno, 1969).

2.5. Intrinsic viscosity

Intrinsic viscosity [η] was detected by an Ubbelohde capillary (type ⌀ 0.5–0.6 mm, 0.01187 mm^2/s^2) at 25 ± 0.1 °C. The [η] value was determined by the mean intercept of Huggins and Kraemer plots (Young & Lovell, 1991).

2.6. Sugar analysis

About 10 mg amount of polysaccharide sample was hydrolyzed using 2 mol/L trifluoroacetic acid for 8 h at 100 °C. The trifluoroacetic acid was removed under the reduced pressure (≥0.095 MPa) at 40 °C, then the hydrolysates were converted into the acetylated aldononitrile derivatives according to the converted procedure and derivatives were analyzed by GC (HP5890, HP Company) with an capillary column (30 m × 0.32 mm). As above method, the following neutral sugars were also converted to their acetylated aldononitrile derivatives and analyzed: rhamnose, arabinose, xylose, mannose, fucose, galactose and glucose.

2.7. Homogeneity and molecular weight of fractions

Homogeneity and molecular weights of the fractions were determined by HPSEC with a column of TSK-3000. The column was pre-calibrated with T-series Dextran T-10, T-40, T-70, T110, T500, T-2000 (Sigma). NaAc (3 mM) was used as eluant and the flow rate was 0.5 mL/min, and 20 μL of aliquot was injected for each run.

2.8. Spectroscopic methods

FT-IR spectrum was recorded from the polysaccharide powder in KBr pellets on a Nicolet Nexus 670 FT-IR spectrophotometer. For NMR spectroscopic analysis, the freeze-dry sample was dissolved in D_2O, and ^1H and ^{13}C nuclear magnetic resonance spectra were acquired from a Bruker DRX-300 NMR spectrometer. ^1H chemical shifts were measured relative to external DSS, and ^{13}C NMR chemical shifts were measured in ppm relative to internal dimethyl sulfoxide at 39.5.

2.9. In vitro antioxidant activity

2.9.1. Assay of scavenging capacity on hydroxyl radical

Hydroxyl radical produced by H_2O_2/Fe^{2+} (Fenton's reaction) was determined by the method of 1,10-phenanthroline-Fe^{2+} oxidative assay (Jin, Cai, Li, & Zhao, 1996). Briefly, the reaction mixtures contained 1 mL of 1,10-phenanthroline solution (0.75 mmol/L), 1.5 mL of PBS solution (150 mmol/L, pH 7.4), 1 mL of $FeSO_4$ solution (0.75 mmol/L), 2 mL of various sample concentrations and 1 mL of H_2O_2 solution (0.01%). A spectrophotometer (UV-754, Shanghai NO.3 Analysis Equipment LTD) was used, and the absorbance at 536 nm was measured after the reaction mixtures were incubated for 1 h at 37 °C. Mean values were obtained from triplicate experiments. Inhibition percent was calculated using the equation as follows:

$$\text{Inhibition}\% = \frac{A_2 - A_1}{A_0 - A_1} \times 100$$

where A_0 is the control absorbance of the absent of H_2O_2 and sample, A_1 is the control absorbance of the absent of sample but presence of H_2O_2, A_2 is the absorbance of the sample.

2.9.2. Assay of scavenging activity on superoxide anion radical

The assay was performed according to the method of AP-TEMED (Ammonium Persulfate-N,N,N′N′-tetramethylenediamine) system (Xiao, He, Fu, Cao, & Fan, 1999). Reaction mixtures contained, in a final volume of 8.4 mL, the following reagents at final concentrations: HCl (2.29% (v/v)), Tris (0.14 mmol/L), TEMED (N,N,N′N′-tetramethylenediamine) (0.1 mmol/L), Ammonium persulfate (1.17 μmol/L), ρ-aminobenzene sulphonic acid

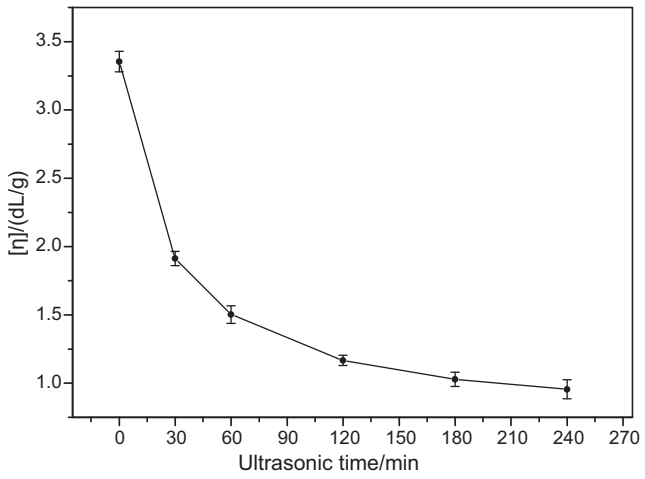

Fig. 1. Plot of intrinsic viscosity ([η]) against ultrasonic treatment time for PYPS. Concentration and volume of PYPS solution: 1.0% (w/v) and 50 mL; parameters of ultrasonic treatment: 20 kHz, 800 W, 30 ± 0.5 °C and 4 h. The results are expressed as the mean ± SD (n = 3).

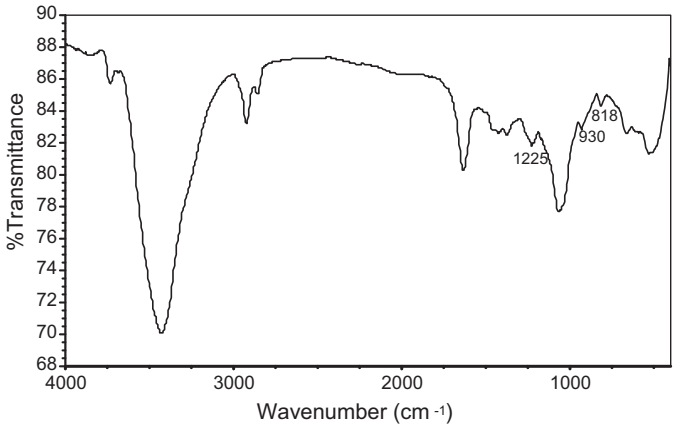

Fig. 2. FT-IR spectrum of fraction F2 from *P. yezoensis*.

anhydrous (4.0 μmol/L), α-naphthylamine (1.67 mmol/L), hydroxylamine hydrochloride (0.47 μmol/L) and various concentrations of sample. After the mixtures were incubated for 25 min at 25 °C, 8.4 mL of *n*-butanol was added to the mixtures respectively, and then the mixtures were centrifuged for 10 min at 3000 rpm. Absorbance of the upper layer of solution was measured at 530 nm. Mean absorbance values were obtained from triplicate experiments. The percentage inhibition of scavenging activity on superoxide anion radical was calculated using the following formulas:

$$\text{Inhibition}\% = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

3. Results and discussion

3.1. Ultrasonic degradation

The yield of PYPS is 6.24% (w/w) of the *P. yezoensis* dry weight. Fig. 1 shows that the [η], being taken as an index for indirectly elucidating the degree of degradation, of the degraded PYPS solution decreased exponentially with increase of ultrasonic treatment time. The function relation of regression analysis from the tested point is as follows:

$$[\eta] = 2.27e^{t/33.19} + 1.06, \quad R^2 = 0.9917$$

The rule of result is in agreement with the case of synthetic polymers (Ebert, Muller, & Suppanz, 1970; Mason & Lorimer, 1988) and similar to the molecular weight plots of other polysaccharides (Aliyu & Hepher, 2000; Heusinger, 1987). Effect of ultrasound irradiation on the degradation of PYPS may be explained by cavitation action (mechanical effect) (Psillakis, Goula, Kalogerakis, & Mantzavinos, 2004). The intrinsic viscosity of PYPS decreases very rapidly in the early stage of treatment time and gradually tends to a limiting value with treatment time. Therefore, a hypothesis comes into being that polysaccharide with higher molecular weight, especially that with its molecular weight exceeds a specific value, might be degraded first, and the breakpoint of glycosidic bonds exists in or near the middle part of the molecule (Zhou & Ma, 2006; Zhou et al., 2008). Differentiating from lower frequency (20 kHz of this study), responsible effect of higher frequencies (>500 kHz) on degradation are radical reactions (Portenanger & Heusinger, 1997). So the degradation of PYPS may also be explained by more for mechanical (cavitation action) effect and less for radical effect.

3.2. Purification and chemical features

The $PYPS_{UD}$ was purified into five fractions by DEAE-52 column, and the absorbed fractions were eluted with gradient NaCl solutions. Fraction F2 (the main and homogeneous fraction of $PYPS_{UD}$), along with four fractions, was colleted for further study here. F2 shows single symmetrical peak on high performance size-exclusion chromatography (HPSEC) with a mean molecular weight (Mw) of 2.1×10^5 Da, which also indicates F2 is homogeneous. No absorption at 280 nm and a negative response to the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) confirm that F2 do not contain protein. Chemical analysis indicated that the content of 3, 6-Anhydrogalactose in F2 was 12.1%, and the sulfate content was 7.6% (Table 1). GC analysis of acetylated aldononitrile derivatives of acid hydrolysates indicates that F2's the majority is galactose, it contains trace of arabinose and fucose, and in the molar ratio of galactose, arabinose and fucose is 48.5:1.2:1.

Infrared spectroscopic analysis indicates that F2 shows typical signals of porphyran, including signals at 3429, 1634, 1424, 1225, 1065, 930 and 818 cm^{-1} (Fig. 2). The signal at 1225 cm^{-1} was attributed to the asymmetric stretching vibration of sulfate group. The band at 930 cm^{-1} was attributed to the absorption of 3,6-anhydrogalactose and at 818 cm^{-1} was assigned to the absorption of the sulfate group attached at the C-6 position of galactose (Brasch, Chang, Chuah, & Melton, 1981).

The ^{1}H NMR spectrum is shown in Fig. 3. In the ^{1}H NMR spectrum of F2, the anomeric resonances at 5.14 and 5.28 ppm originated from (1 → 4)-linked α-L-galacto-pyranose units (Lahaye, Revol, Rochas, Mclachlan, & Yahphe, 1988; Murano et al., 1993). The signal at 5.28 ppm in the ^{1}H NMR spectrum was due to the anomeric proton of the 4-*O*-linked α-L-galactose-6-sulfate units, and that at 5.14 ppm was attributed to the anomeric proton of the 3,6-anhydro-α-L-galactose units.

The ^{13}C NMR spectrum for F2 is shown in Fig. 4. The signal assignment of F2 was achieved by comparing with previously model compounds (Table 2) (Lahaye, Yahphe, Viet, & Rochas, 1989). The alternating units of the agarose and the agarose biological precursor were detected from the corresponding signals.

It can be concluded from the above analysis that F2 has a typical porphyran structure, and has a backbone of alternating (1 → 4)-3,6-anhydro-α-L-galactopyranose units and (1 → 3)-linked β-D-galactose or (1 → 4)-linked α-L-galactose 6-sulfate units. It can

Table 1
Chemical analysis of F2 from *P. yezoensis*.

Molecular weight ($\times 10^3$ g/mol)[a]	Total sugar (%)[b]	Sulfate (%)[b]	3,6-AG (%)[b,c]	Monosaccharide composition (molar ratio)[d]		
				Galactose	Arabinose	Fucose
21	91.4	7.6	12.1	48.5	1.2	1

[a] Evaluated by HPSEC as a component.
[b] Percentage of the dry weight of F2 (%, w/w).
[c] 3,6-Anhydro-α-L-galactose.
[d] The monosaccharide composition was detected by GC analysis (molar ratio).

Table 2
Chemical shift assignment for ^{13}C NMR spectrum of F2.

Residue		^{13}C chemical shift (ppm)					
		C-1	C-2	C-3	C-4	C-5	C-6
Lahaye et al. (1989)							
$(G–A)_n$	G	102.4	70.2	82.2	68.8	75.3	61.4
	A	98.3	69.9	80.1	77.4	75.7	69.4
$(G–L6S)_n$	G	103.7	69.8	81.2	69.1	75.9	61.8
	L6S	101.3	69.3	71.7	79.1	70.3	67.7
Observed spectra in Fig. 4							
$(G–A)_n$	G	102.4	70.3	82.3	68.8	75.5	61.2
	A	98.5	69.8	80.2	77.4	75.7	69.5
$(G–L6S)_n$	G	103.7	69.9	80.9	69.1	76.0	61.7
	L6S	101.3	69.5	72.3	79.2	70.1	69.9

G: (1 → 3)-linked β-D-galactose; A: (1 → 4)-linked 3,6-anhydro-α-L-galactose; L6S: (1 → 4)-linked α-L-galactose 6-sulfate.

be calculated from the data listed in Table 1 that the molar ratio of 3,6-anhydrogalactose and sulfate is 1.1:1.

3.3. Scavenging capacity on hydroxyl radical

Hydroxyl radical produced from H_2O_2/Fe^{2+} was determined by the absorbance at 536 nm, i.e. the peak absorbance of 1,10-phenanthroline-Fe^{2+} (Jin et al., 1996). Above-mentioned model was used to measure inhibitory capacity here. The results were plotted in Fig. 5. $PYPS_{UD}$ had stronger scavenging capacity on hydroxyl radical than PYPS. The correlations between the scavenging capacity and the concentration of V_C (ascorbic acid, the positive sample), $PYPS_{UD}$ and PYPS were analyzed by linear regression, and the results are as follows:

$$V_C : Y = 65.605X + 5.786, \quad R^2 = 0.9905$$

$$PYPS_{UD} : Y = 57.753X + 2.794, \quad R^2 = 0.9977$$

$$PYPS : Y = 48.102X + 1.472, \quad R^2 = 0.9974$$

As for F2, the correlation was logarithmic:

$$F2 : Y = 20.068 \ln X + 104.76, \quad R^2 = 0.9657$$

IC_{50} of F2, V_C, $PYPS_{UD}$ and PYPS were 0.065, 0.674, 0.817 and 1.009 mg/mL, respectively. The scavenging capacity of polysaccharide samples on hydroxyl radical and the control standard (V_C) followed the sequences: F2 > V_C > $PYPS_{UD}$ > PYPS. Compared chemical features of F2 with typical porphyran (alternating of (1 → 4)-linked 3,6-anhydro-L-galactose units and (1 → 3)-linked β-D-galactose units, which sometimes occur as the L-galactose-6-sulfate and 2 or 6-O-methyl derivative) (Morrice, Maclean, Long, & Williamson, 1983), along with the content of sulfate in $PYPS_{UD}$ and in PYPS are all about 15.5% (w/w), and from which could indirectly concluded that $PYPS_{UD}$ was different with PYPS only in molecular weight, i.e. $PYPS_{UD}$ had lower molecular weight than PYPS. For hydroxyl radical, there were two types of antioxidation mechanism; one suppresses the generation of the hydroxyl radical, and

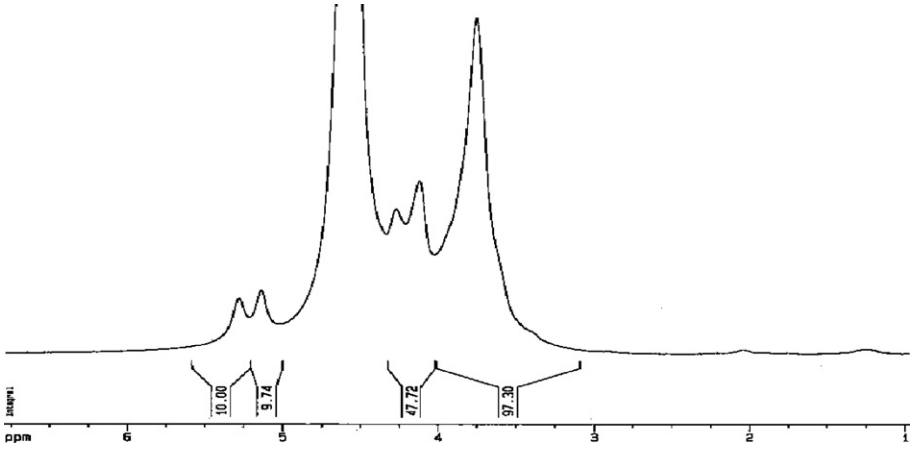

Fig. 3. ^{1}H NMR spectrum of fraction F2 from *P. yezoensis*.

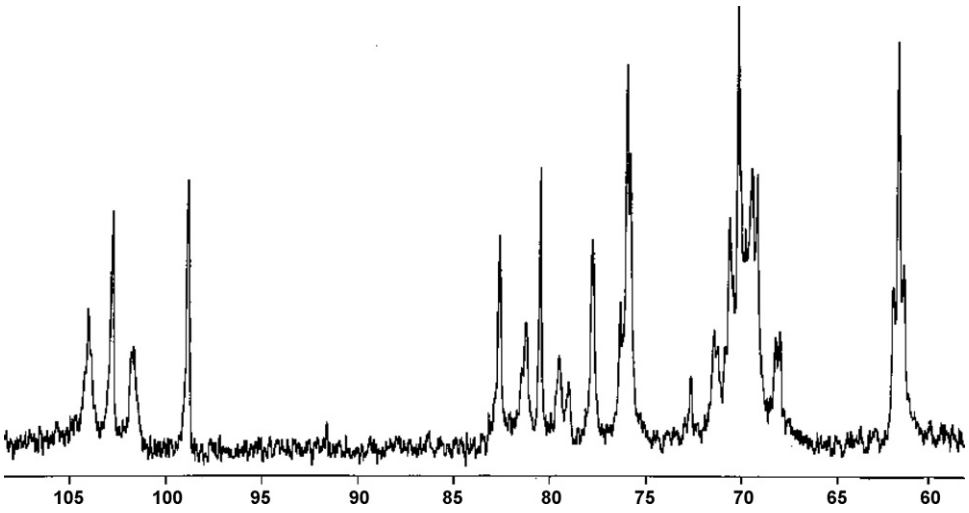

Fig. 4. ^{13}C NMR spectrum of fraction F2 from *P. yezoensis*.

the other scavenges the hydroxyl radicals generated. This result likely proved that molecular weight of PYPS had significant effect on scavenging hydroxyl radical (Zhang et al., 2009). It can also be found in previous studies that lower molecular weight of sulfated polysaccharides had more pronounced scavenging capacity on hydroxyl radical (Xing et al., 2005, 2008). However, the real mechanism of this result needs to be further researched. Furthermore, as scavenging capacity on hydroxyl radical of $PYPS_{UD}$ and PYPS be concerted, it was also concluded that ultrasonic degradation would be an effective way for the application of enhancing PYPS's antioxidant activity.

3.4. Scavenging activity on superoxide anion radical

A system of AP-TEMED was used here to assay the consumption of superoxide anion radicals. The reaction of hydroxylamine and superoxide anion produced NO_2^- and then it was developed by sulphanilic acid and α-naphthylamine (Xiao et al., 1999). When the sample was detected by spectrophotometer, the absorbance at 530 nm showed a quantitative relation to the concentration of superoxide anion radicals. Fig. 6 shows that the inhibitory activity is significant at all tested concentrations and in a concentration-dependent manner. The tests found that correlations between scavenging activities on superoxide anion and concentration of V_C, $PYPS_{UD}$ and PYPS were linear as follows:

$$V_C : Y = 65.885X + 4.913, \quad R^2 = 0.9492$$

$$PYPS_{UD} : Y = 57.969X + 3.719, \quad R^2 = 0.9952$$

$$PYPS : Y = 53.604X - 0.077, \quad R^2 = 0.9868$$

as for F2, the correlation was nonlinear:

$$F2 : Y = -309.93X^2 + 310.53X + 3.821, \quad R^2 = 0.9952$$

IC_{50} of F2, V_C, $PYPS_{UD}$ and PYPS were 0.182, 0.684, 0.798 and 0.934 mg/mL, respectively. Their order of scavenging activity on superoxide anion was: F2 > V_C > $PYPS_{UD}$ > PYPS. On the one hand, superoxide is a relatively weak oxidant; it is decomposed to form a stronger reactive oxidative species, which initiate peroxidation of lipids (Dahl & Richardson, 1978). F2 could effectively scavenge superoxide in a nonlinear concentration-dependant manner. On the other hand, superoxides are also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals (Oey, Van der Plancken, Van Loey, & Hendrickx,

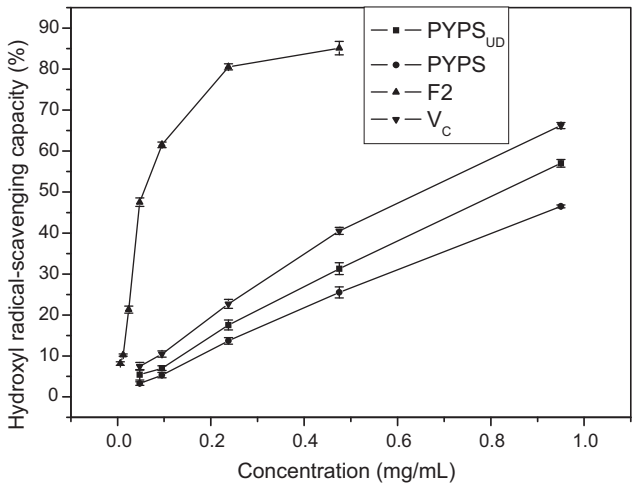

Fig. 5. Scavenging capacity of F2, $PYPS_{UD}$, PYPS and V_C on hydroxyl radical as detected by the 1,10-phenanthroline-Fe^{2+} oxidative assay. Values were mean ± SD of three detections.

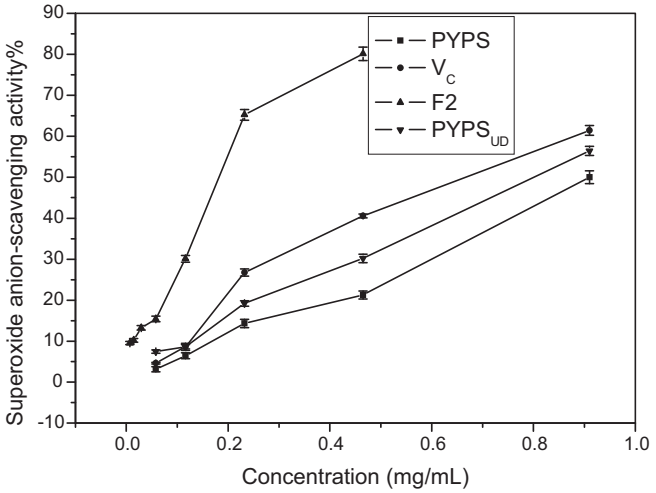

Fig. 6. Scavenging capacity of F2, $PYPS_{UD}$, PYPS and V_C on superoxide anion radical as determined by the AP-TEMED system. Values were mean ± SD of three determinations.

2008). As for the sulfated derivatives, the fully sulfated and 6-O-sulfated derivatives showed stronger scavenging activity than their raw material porphyran (Zhang et al., 2010). These results shows that F2 has strong scavenging activity on superoxide anion and also potentially suggested that lower molecular weight of sulfated polysaccharides has more strong scavenging activity on superoxide anion radical.

4. Conclusion

The results of the present work indicated that the ultrasonic degraded $PYPS_{UD}$ exhibited stronger antioxidant activity than that of the natural PYPS. The function of relation between intrinsic viscosity and treatment time is submitted to well exponential function. F2 has a backbone of alternating $(1\rightarrow4)$-3,6-anhydro -α-L-galactopyranose units and $(1\rightarrow3)$-linked β-D-galactose or $(1\rightarrow4)$-linked α-L-galactose 6-sulfate units. The result ascertained ultrasound degradation did not change the main structure of polysaccharides in the test conditions. It was likely that molecular weight had significant effect on the PYPS's antioxidant activity and that ultrasound degradation had important applications for food and pharmaceutical industries.

Acknowledgements

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