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Effect of ultrasonic treatment on the degradation and inhibition cancer cell lines of polysaccharides from *Porphyra yezoensis*

Xiaojie Yu[a,b,c,1], Cunshan Zhou[a,b,c,e,1], Hua Yang[d,e], Xingyi Huang[a], Haile Ma[a,b,c,*], Xiaopei Qin[a], Jiali Hu[a]

[a] School of Food and Biological Engineering, Jiangsu University, No. 301 Xuefu Road, Zhenjiang 212013, China
[b] Jiangsu Provincial Key Laboratory for Physical Processing of Agricultural Products, Jiangsu University, No. 301 Xuefu Road, Zhenjiang 212013, China
[c] Jiangsu Provincial Research Center of Bio-process and Separation Engineering of Agri-products, Jiangsu University, No. 301 Xuefu Road, Zhenjiang 212013, China
[d] Zhejiang Provincial Top Key Discipline of Biological Engineering, No. 8 South Qian Hu Road, Ningbo 315100, China
[e] College of Biological and Environmental Sciences, Zhejiang Wanli University, No. 8 South Qian Hu Road, Ningbo 315100, China

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ABSTRACT

The exposure of polysaccharides solutions to high-energy ultrasound produces a permanent reduction in viscosity and change in activity. However, the exact mechanism which occurs in the process is still not clear. In this work, degradation of polysaccharides from *Porphyra yezoensis* (PP) was indirectly and directly judged by intrinsic viscosity and high performance gel permeation chromatography. The degradation process was established with dynamics and affirmed by theoretical derivation. Inhibition of cancer cell lines (SGC-7901, 95D) was also investigated by assays of tetrazolium colorimetric. The intrinsic viscosity of the degraded PP decreased exponentially with increase in ultrasonic time, and theoretical derivation was established and confirmed well. The distribution and new fraction of degraded polysaccharides was found. Ultrasound degraded preferentially large PP molecules and cleavage took place roughly at the centre of the molecules. During ultrasound degradation the molecular weight distribution was narrowed. The inhibition activities of SGC7901 with ultrasound degraded polysaccharides were increased.
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1. Introduction

Ultrasound can mainly be classified into two application fields based on frequency: (1) high frequency low power diagnostic ultrasound in the MHz range, and (2) low frequency high power treatment ultrasound in kHz range (Zhou et al., 2012a; Fig. 1). The frequency of ultrasound in MHz range is usually used as an analytical technique for quality assurance, process control and non-destructive inspection, and this has been applied to determine food properties, to inspect food packages, etc. The frequency of ultrasound in kHz range has been explored in the food industry for the last 10 years. Various areas have been shown to be of great potential for future development, e.g. microorganisms and enzymes inactivation, crystallization, drying, degassing, extraction, filtration, homogenization, meat tenderization, oxidation, sterilization, etc. (Ma et al., 2011). The beneficial use of ultrasound is realized through its chemical, mechanical, or physical effects on the process or product (Zhou et al., 2013b). In recent years, there has been increasing interest in the field of degradation of organic matter with ultrasonic treatment. These may include substances from organic (kaempferol, polyethylene, benzene chlorobenzene phenol, nitrobenzene, aniline, methamidophos, dyes and so on) which gradually develop to the biological macromolecules (biomass cellulose, chitosan, starch, carrageenan; Zhou et al., 2014). It is well established that prolonged exposure of solutions of macromolecules to high-energy ultrasonic waves produces a permanent reduction in viscosity (Zhou & Ma, 2006; Zhou, Wang, Ma, & He, 2008; Zhou et al., 2012a, 2013a). The exact mechanism by which degradation occurs is still open to discussion. It is believed, that ultrasonic degradation, unlike chemical or thermal decomposition, is a non-random process, with cleavage taking place roughly at the centre of the molecule and with larger molecules degrading the fastest. In general, for any polysaccharides degradation process to become acceptable to industry, it is important to be able to specify the sonication conditions, which lead to a particular relative molar

* Corresponding author at: School of Food and Biological Engineering, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, China. Tel.: +86 511 88780201; fax: +86 511 88780201.
E-mail address: mhl@ujs.edu.cn (H. Ma).
[1] The authors contributed equally to this work.

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Nomenclature

M	molecular weight
M_t	average molecular weight at time t
M_∞	limiting molecular weight
M_0	initial average molecular weight
$[\eta]$	intrinsic viscosity
$[\eta]_0$	initial intrinsic viscosity
$[\eta]_t$	intrinsic viscosity at time t
$[\eta]_\infty$	limiting intrinsic viscosity
k	the rate constant of degradation reaction
r	the correlation coefficient
c	is concentration of PP solution in g L^{-1}

weight distribution, and even the effects of the ultrasonic degraded on activities.

Sulfated polysaccharides from *Porphyra yezoensis* has many physiological and nutraceutical activities including antiviral, immunoregulatory, anticancer, anticoagulant and antihyperlipidemic (Guo et al., 2007; Bhatia et al., 2013; Jiang, 2014; Qian, Zhou, & Ma, 2014). Many investigations on the structure and function of the polysaccharides isolated from *Pophyra* species have been undertaken (Suetsuna, 1998; Yoshimura et al., 2006). Porphyrans, the sulfated polysaccharides comprising the hot-water fraction, are the main components of *Porphyra*. Porphyran (Fig. 2) is similar to agarose in its basic structure (a linear backbone of alternating 3-linked β-D-galactose and 4-linked 3,6-anhydro-α-L-galactose units), while it is very different in terms of having L-galactose-6-sulfate (Zhang et al., 2005). Recently, it has been demonstrated that the algal polysaccharide as a kind of free-radical scavenger and antioxidant plays an important role in preventing oxidative damage to living organisms. In addition, the chemical properties of polysaccharides from different species show great variations. Furthermore, many properties of polysaccharides depend on their molecular weights. These properties range from physical characteristics, such as solution viscosity, to more complex properties, such as biological activity. For example, it was proved that digesting polysaccharides from *P. yezoensis* with β-agarase led to higher macrophage stimulation activity and solubility. Similarly, antithrombotic activity and plant growth stimulation also depend on its molecular weight (Yoshizawa et al., 1995). Also, many functional activities of *Porphyra haitanensis* and native polysaccharides of *Porphyra* have been studied. We have used ultrasonic treatment to enhance the antioxidant activity of partially degrading polysaccharides of *P. yezoensis* (Zhou et al., 2008; Zhou et al., 2012a), but the degradation mechanism on its molecular weight, theoretical derivation of the process and the change of cancer cell line lines inhibition has not yet been published.

In this study, the polysaccharides from *P. yezoensis* was isolated using the hot-water extraction method, and precipitated with ethanol. The polysaccharides were degraded with ultrasonic treatment in water medium, and the degree of degradation was indirectly and directly judged by intrinsic viscosity and high performance gel permeation chromatography (HPGPC). The degradation process was established with dynamics and affirmed by theoretical derivation. In vitro inhibition of cancer cell lines (SGC-7901, 95D) was investigated by assays of tetrazolium (MTT) colorimetric assay.

2. Material and methods

2.1. Material and chemicals

P. yezoensis, was cultured near the coast of Nantong City, Jiangsu Province, China. The *P. yezoensis* powder (sieved with 40 meshes)

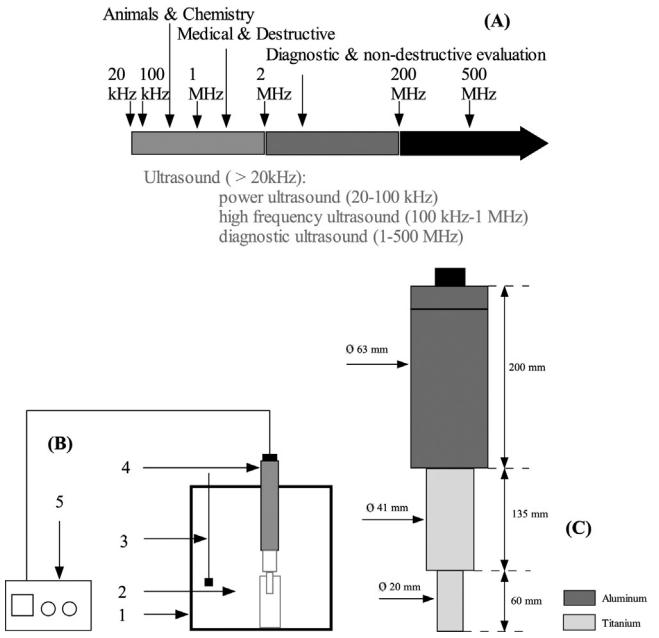

Fig. 1. (A) Diagram of ultrasound range and application fields. (B) Schematic diagram of ultrasonic treatment. (1) Thermostatic water bath, (2) sample pretreatment vessel, (3) thermometer, (4) ultrasound, (5) ultrasonic generator. (C) Schematics of the sonotrode.

was kindly provided by Nantong Lanbo Industry Co. Ltd (Jiangsu, China). The polysaccharides were extracted with hot-water and precipitated by ethanol (Zhou & Ma, 2006). In brief, *P. yezoensis* powder was defatted for 5 h with supercritical CO_2 under the following conditions: extraction: 45 °C, 30 MPa; separation: 35 °C, 5 MPa; and CO_2 flow rate: 10 L h^{-1}. 116 g 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 to remove free proteins and ultrafiltered (MW of cutoff 10 kDa, PLCC 10 K REGENERATED CELLULOSE, MILLIPORE), and the permeat was precipitated using 4 times volume 95% ethanol. Then the precipitate was 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 polysaccharides from *P. yezoensis* (PP) was obtained, and the component analysis of the obtained polysaccharides sample is shown in Table 1.

2.2. Ultrasonic pretreatment

Ultrasonic treatment of 20 kHz, 400–1200 W and pulsed as 2–8 s/2 s (on/off) was carried out by using a HF-20B ultrasonic reactor (Beijing Hong Xiang Long Biotechnology Developing Co., LTD). 50 mL of the PP 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 Tanjin Co., Ltd.) for 4 h, thus the solution of ultrasonic degraded PP (U-PP) was obtained. To reduce the experimental errors caused by uneven power transfer, the sample was located just under the ultrasound source and dipped into water about 20 mm. Irradiation of samples was carried out in three replicates.

2.3. Intrinsic viscosity

Intrinsic viscosity is an important index for studying degradation of large biological molecules, because it is relative to the change of molecular weight and molecular configuration. Intrinsic viscosity $[\eta]$ was detected by an Ubbelohde capillary (type ⌀ 0.5–0.6 mm, 0.01187 mm^2 s^{-2}) at (25 ± 0.1) °C (Zhou, Yu, Zhang, He,

Table 1
Component analysis the polysaccharides from *P. yezoensis*.

Yield (%)	Total sugar (%)	Intrinsic viscosity (dL g^{-1})	Sulfate (%)	3,6-AG[a,b] (%)	Monosaccharide[c]/molar ratio			
					Galactose	Xylose	Fucose	Arabinose
6.24	94.5	3.28	7.2	12.3	46	2	1	1

[a] Percentage of the dry weight of PP (%, w/w).
[b] 3,6-Anhydro-α-L-galactose.
[c] The monosaccharide composition was detected by GC analysis (Zhou et al., 2012b).

& Ma, 2012b). The [η] value was determined by the mean intercept of Huggins and Kraemer plots (Young & Lovell, 1991).

2.4. Homogeneity and molecular weight of fractions

Homogeneity and molecular weights of the fractions were determined by HPGPC (Varian Prostar 210 with refractive index detector Shodex RI-71) with a column of TSK-4000. The column was calibrated with T-series Dextran (lgM = −0.3246 t + 9.6188, r = 0.9948) T-10, T-40, T-70, T110, T500, T-2000 (Sigma). NaAc (3 mM) was used as eluent and the flow rate was 0.5 mL min^{-1}. 20 μL of aliquot was injected for each run (Zhou & Ma, 2006).

2.5. Inhibition of cancer cell lines

Human gastric cancer cell lines SGC7901 or human lung cancer cell line 95D (provided by the Cell Bank of Chinese Academy of Sciences, Shanghai, China) were suspended in RPMI 1640 medium (GIBCO, New York, USA) containing 10% foetal bovine serum (FBS), 100 U mL^{-1} streptomycin, and 100 U mL^{-1} penicillin, and were grown in an incubator in a humidified atmosphere at 37°, with 5% CO_2. For all experiments, cells were seeded at a density of 1×10^5 cells mL^{-1}. The test compound was added after 24 h of seeding (Zhou et al., 2013b).

SGC7901 or 95D cells were seeded into 96-well plates at a density of 1×10^5 cells mL^{-1}. After 24 h incubation, cells were treated with different concentrations of samples for 24 h, and media alone was used as control. Proliferation of cancer cells was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to determine whether there was anti-proliferative activity. Following the addition of 20 μL of MTT solution (5 mg mL^{-1}) the mixture was incubated at 37° for 3 h; then the MTT solution was discarded, and 150 μL of dimethyl sulphoxide (DMSO) was added to dissolve the formazan crystals. The amount of formazan was determined by measuring the absorbance at 492 nm using Multiskan GO (THERMO, Boston, USA). The percentage of cell viability (IR%) was calculated as a ratio of OD value of sample to the OD value of control.

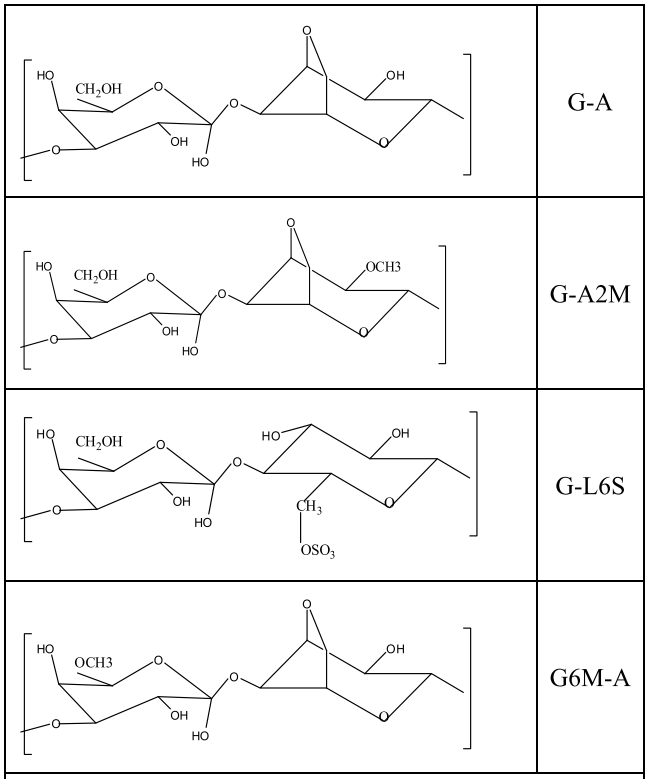

Fig. 2. The typical repetitive structures in polysaccharides from *P. yezoensis*.

2.6. Statistical analysis

All experiments were performed in triplicate and statistical analysis was performed using SPSS 16.0. Data were expressed as mean ± SD. Comparisons among all groups were performed with the one-way ANOVA analysis of variance test. Differences were considered significant at $P < 0.05$.

3. Results and discussion

3.1. Effect of ultrasonic pulse and H_2O_2 on the degradation of polysaccharides

Numerous properties of polysaccharides depend on their molecular weight. These range from simple physical characteristics such as solution viscosity to more complex properties like biological activity. Since the average chain length of natural polysaccharides is determined by their source of origin and cannot be influenced, in most cases, there is a need for fast, efficient and inexpensive methods of processing the native polysaccharide substrates in order to achieve the desired average molecular weight (or, in same cases, also a desired molecular weight distribution) (Czechowska-Biskup, Rokita, Lotfy, Ulanski, & Rosiak, 2005). Degradation with ultrasound seems to be a promising alternative. Fig. 3 shows that the [η], i.e. as an index for indirectly elucidating the degree of degradation, of PP degradated at 1.0% (w/v) generally decreased exponentially with increasing ultrasonic time, with the reduction of [η], for PP solution. The result is in agreement with the case for synthetic polymers and similar to the molecular weight plots of other polysaccharides. Effect of ultrasound radiation on the degradation of PP may be explained by cavitational action. Higher ultrasonic power (Fig. 3B) or longer on time of ultrasonic pulses (Fig. 3A) increases the number of cavitational events and this leads to the opportunities for free radicals to be generated, thus enhancing degradation. Use of additives of H_2O_2 can help in increasing the extent of viscosity

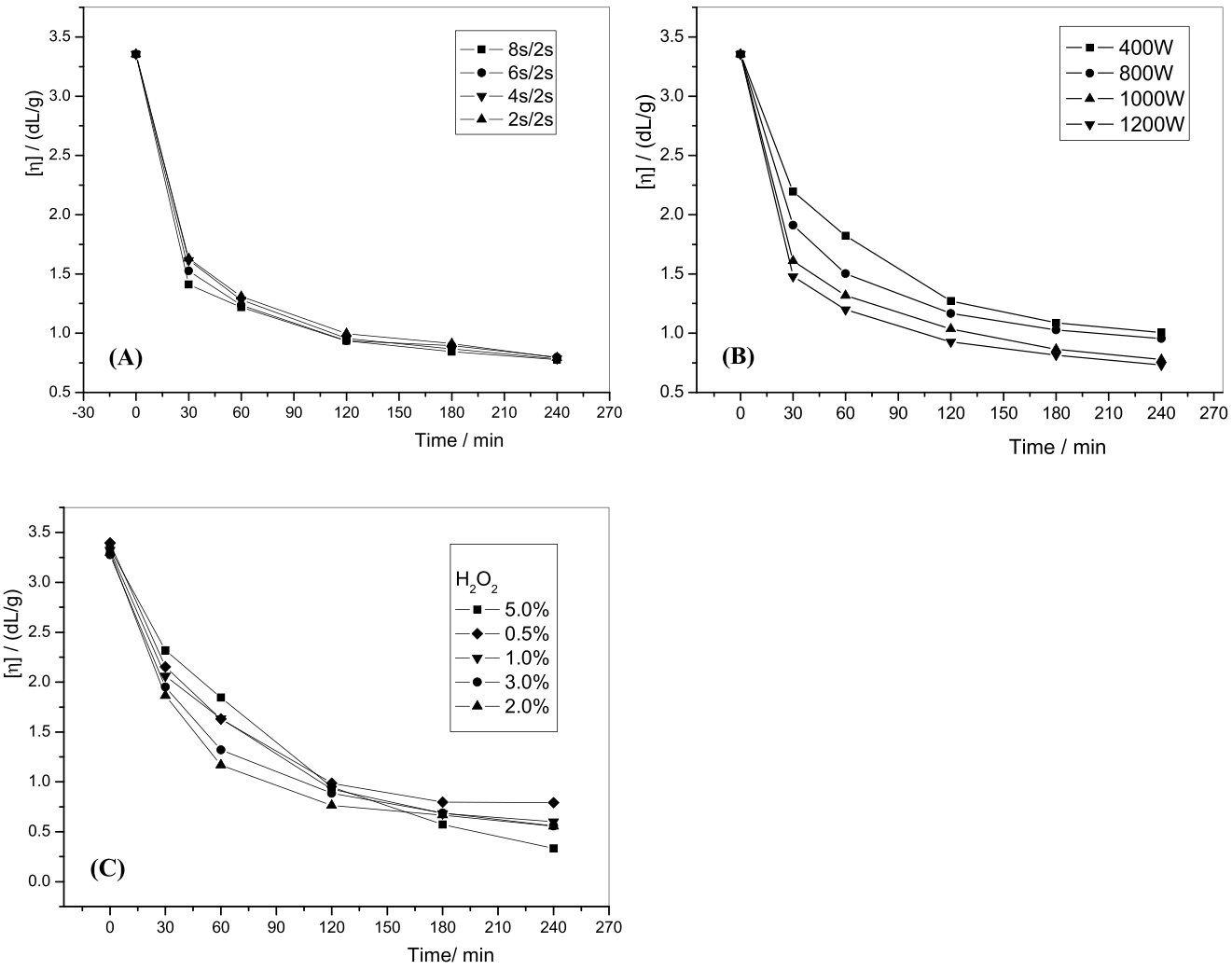

Fig. 3. Effect of ultrasonic pulsed (A), power (B) and the adding H_2O_2 concentration (C) on intrinsic viscosity of polysaccharides from *P. yezoensis*. (A) Conditions: PP 1.0%, 50 mL, 20 kHz, 800 W, 2–8 s/2 s, 30 ± 0.5 °C, 4 h. (B) Conditions: PP 1.0%, 50 mL, 20 kHz, 400–1200 W, 2 s/2 s, 30 ± 0.5 °C, 4 h. (C) Conditions: PP 1.0%, H2O2 0.5–5.0%, 50 mL, 20 kHz, 800 W, 2 s/2 s, 30 ± 0.5 °C, 4 h.

reduction, and a concentration of 2.0% H_2O_2 resulted in the highest viscosity reduction in the range of 0.5–5.0% (Fig. 3C). As H_2O_2 is the most common strong oxidizing agent, it affects the formation of hydroxyl radicals due to oxidizing effects. It has been found that the maximum extent of degradation is observed at 2.0% H_2O_2. This might be due to the increase in OH radical formation when H_2O_2 is first added, but at higher H_2O_2 concentrations OH might be consumed by various mechanisms, including the scavenging effects of H_2O_2 and the recombination of OH radical (Ma, Sung, & Lin, 2010).

The degradation of the polysaccharides is affected by the ultrasonic condition chosen and the characteristics of polysaccharides itself (The characteristics of the present samples are listed in Table 1 and the molecular weight distribution is shown in Fig. 4). Use of ultrasound can yield polysaccharides as indexed by significant reduction in intrinsic viscosity and the molecular weight. For the investigations of direct effect of molecular weight of PP, experiments were carried out.

According to HPGPC examinations, degradation had the effect of narrowing the molecular weight distribution (Fig. 4), and the new fraction (molecular weight near 300,000 Da) of degraded polysaccharides was found. After sonication, the solution contained an increase in new fractions and some long chain lengths could not be shortened, resulting in a narrower molecular weight distribution. Ultrasound degraded preferentially large molecules (molecular weight near 600,000 Da) and cleavage took place roughly at the centre of the PP molecules (molecular weight near 600,000 Da). This observation strengthens the claim that ultrasonic degradation, unlike chemical or thermal decomposition, is a non-random process with cleavage taking place roughly at the centre of the molecule and with larger molecules degrading the fastest.

3.2. Theoretical analysis of the ultrasonic degradation process

Baramboim (1964) and Mohod and Gogate (2011) suggested that the kinetics of polymerdegradation under stress could be described as in Eq. (1), and this assumption is based on the glycosidic linkages α (1 → 3) and β (1 → 4) in these polysaccarides show similar susceptibility to breakage by ultrasound. In this study, the polysaccharides from *P. yezoensis* were assumed to be an ideal mixture of polymers and the intrinsic viscosity of the polysaccharides was assumed to be the molecular weight of polymers.

From this assumption, the mathematical model and kinetic for the ultrasonic degradation could be expressed as Eqs. (2) and (3),

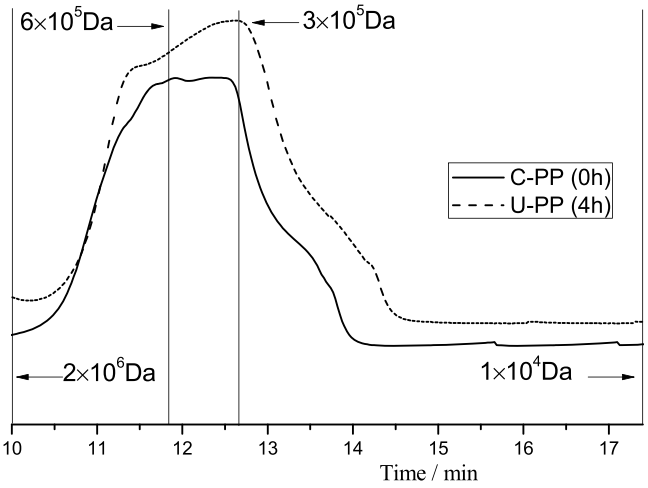

Fig. 4. Chromatograms of molecular weight distribution affecting on ultasonic time (C-PP at time $t = 0$, U-PP at time $t = 4$ h) dependence in process of PP ultrasonic degradation. Conditions: PP 1.0%, 50 mL, 20 kHz, 800 W, 2 s/2 s, 30 ± 0.5 °C, 4 h.

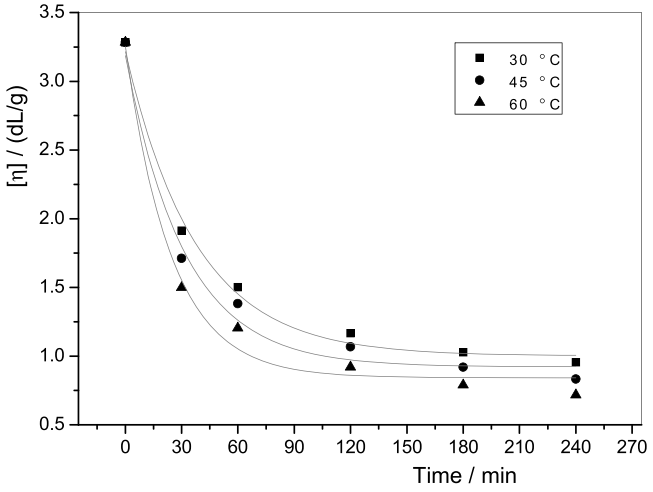

Fig. 5. Regression analysis on effect of reaction temperature on intrinsic viscosity of PP. Conditions: PP 1.0%, 50 mL, 20 kHz, 800 W, 2 s/2 s, 30–60 °C, 4 h.

which is applied to describe the kinetics of ultrasound degradation in the present work:

$$\frac{\mathrm{d}\left(M_t - M_\infty/M_\infty\right)}{\mathrm{d}t} = -k\left(\frac{M_t - M_\infty}{M_\infty}\right) \quad (1)$$

$$\frac{\mathrm{d}\left(([\eta]_t - [\eta]_\infty)/[\eta]_\infty\right)}{([\eta]_t - [\eta]_\infty)/[\eta]_\infty} = -k\mathrm{d}t \quad (2)$$

$$\ln\frac{[\eta]_t - [\eta]_\infty}{[\eta]_\infty} = -kt + a \quad (3)$$

By integrating and considering that at $t = 0$, $[\eta]_t = [\eta]_0$, an in Eq. (3) could be expressed as:

$$a = \ln\frac{[\eta]_0 - [\eta]_\infty}{[\eta]_\infty} \quad (4)$$

Integrated Eqs. (3) and (4) as:

$$\ln\frac{[\eta]_t - [\eta]_\infty}{[\eta]_0 - [\eta]_\infty} = -kt \quad (5)$$

From Eq. (5), the following equation can be obtained.

$$[\eta]_t = ([\eta]_0 - [\eta]_\infty)e^{-kt} + [\eta]_\infty \quad (6)$$

According to Eq. (6), and $[\eta]_0 - [\eta]_\infty$ is a constant (A) for the polysaccharides

$$[\eta]_t = Ae^{-kt} + [\eta]_\infty \quad (7)$$

According to Eq. (5), the magnitude of the rate constant has been calculated for all the runs knowing the initial viscosity and the limiting intrinsic viscosity of the polysaccharide solution by plotting a graph of $\ln(B)$ against time where:

$$B = \frac{[\eta]_0 - [\eta]_\infty}{[\eta]_t - [\eta]_\infty} \quad (8)$$

3.3. Kinetic analysis of the ultrasonic degradation process

In order to confirm the theoretical analysis of ultrasonic degradation process, kinetic analysis at temperatures of 30, 45, and 60 °C were carried out with ultrasound degradation on PP. Fig. 5 shows that the $[\eta]$, being taken as an index for indirectly elucidating the degree of degradation, of the degraded PP solution decreased exponentially with increase in ultrasonic time. The functional relation of the regression analysis as follows:

$$[\eta] = 2.22e^{-t/37.63} + 1.00 \quad r = 0.9947 \quad 30\,^\circ\mathrm{C} \quad (9)$$

$$[\eta] = 2.27e^{-t/31.59} + 0.92 \quad r = 0.9864 \quad 45\,^\circ\mathrm{C} \quad (10)$$

$$[\eta] = 2.41e^{-t/24.57} + 0.84 \quad r = 0.9899 \quad 60\,^\circ\mathrm{C} \quad (11)$$

The rule of result is in agreement with the case of synthetic polymers (Eq. (7)) and similar to the molecular weight plots of other polysaccharides (Chen et al., 2014; Luo, Fang, & Smith, 2014). Similar sulfated polysaccharides from algae (agarose, *k*- and *i*-carrageenans) with ultrasonic degradation in water generally decreased exponentially with increasing ultrasonic time, and is dependent on molecular size (Lii, Chen, Yeh, & Lai, 1999). The effect of ultrasound irradiation on the degradation of PP may be explained by cavitation action. Higher acoustic power can increase the number of cavitational events and lead to the more opportunities of enhanced degradation. Different from lower frequency (20 kHz of this study), responsible effect of higher frequencies (>500 kHz) on degradation are radical reactions. So the effect of power on the sonolysis of PP may also be explained much more by mechanical than radical effects.

3.4. Antiproliferation of cancer cell lines

The potential antiproliferative effect of PP, U-PP on SGC7901, 95D was investigated by MTT. The samples exhibited a dose-dependent antiproliferative on SGC7901 and 95D cells, respectively (Fig. 6). The percentage of cell SGC7901 viability (IR%) was 32.34, 23.24, 22.71, 17.14 and 2.65 when treated with 1000, 500, 250, 125 and 62.5 μg mL^{-1} of U-PP, respectively. The results indicated that U-PP had higher antiproliferative effects than PP of the same dose. At the test concentrations, U-PP and PP exhibited an antiproliferative effect on 95D cells in a similar concentration-dependent manner which showed that U-PP had almost the same antiproliferative effect as PP of the same dose.

Accumulated investigations have revealed algal polysaccharides could inhibit cancer proliferation in vivo and in vitro (Peng et al., 2013). They all have the ability to scavenge superoxide anion and hydroxyl radical, and lower molecular weight porphyrans exhibit stronger DPPH-radical scavenging activity and reducing power (Zhao et al., 2006; Zhang et al., 2003). It has been reported that the antiproliferative activities of polysaccharides depend on their molecular weight, chemical composition, structure of the polymeric backbone, and degree of branching, and as far as sulfated polysaccharides is concerned, the molecular weight is especially important (Ma et al., 2013). The change of PP molecular weight can increase the strength of interactions between sulfate groups

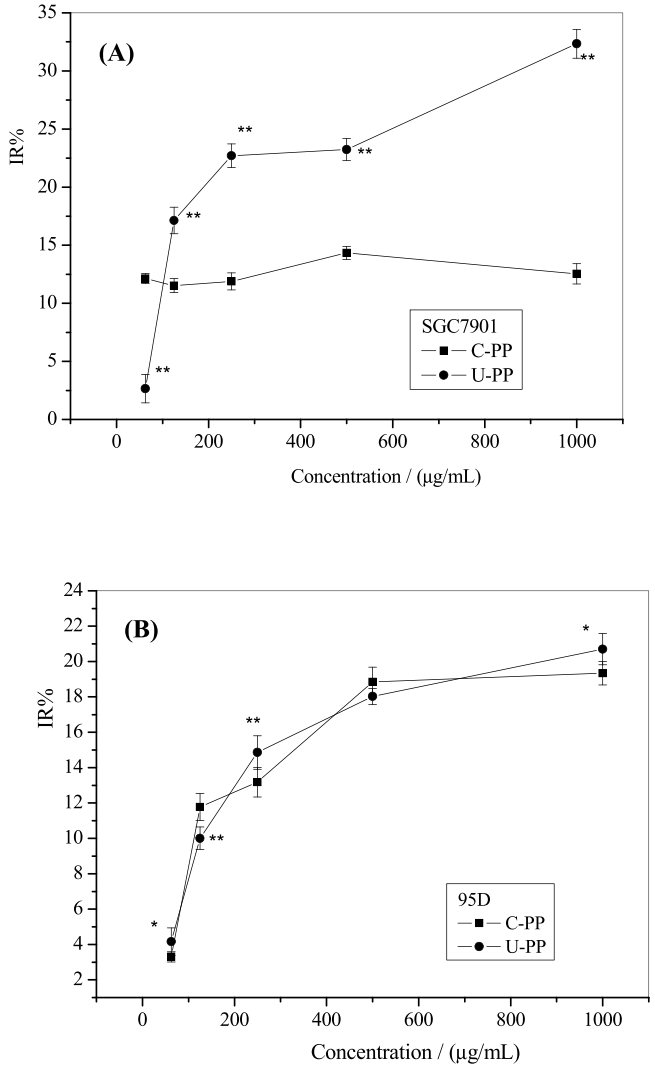

Fig. 6. Inhibition activity to SGC7901 (A) and 95D (B) cells of U-PP (C-PP as control) (mean ± SD, $n = 6$).

of PP and proteins of cancer cell lines, which could explain the enhancement of the antiproliferation of ultrasonic-modified sulfated polysaccharides. However, the specificity mechanism of the antiproliferative activity on SGC7901 and 95D exerted by PP and U-PP which resulted in ultrasonic degradation is still not clear, and so needs further study.

4. Conclusion

It has been demonstrated on PP that treatment with 20 kHz, 400–1200 W and pulsed as 2–8 s/2 s ultrasound in aqueous solution is an efficient procedure for reduction of molecular weight of polysaccharides. Sonochemical yield of this process has been shown to depend on polysaccharide molecular weight, ultrasound power and number of pulses, and also the addition of oxidizing agent. The intrinsic viscosity of the degraded PP decreased exponentially with increase in ultrasonic time, and this was confirmed with theoretical derivation analysis, which on the basis of the glycosidic linkages α (1 → 3) and β (1 → 4) in these polysaccharides, show similar susceptibility to breakage by ultrasound. The distribution of PP was narrowed, and new fraction (near 300,000 Da) of degraded polysaccharides was found. Ultrasound degraded preferentially large PP molecules and cleavage took place roughly at the centre of the molecules (near 600,000 Da). The antiproliferation of SGC7901 with ultrasound degraded polysaccharides was increased. It might serve as a promising modification for polysaccharides as antitumor agent. Studies on the chemical structures and further induced apotosis of ultrasound degradation PP are in progress.

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