Carbohydrate Polymers 113 (2014) 325–335

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Synthesis and characterization of phosphorylated galactomannan: The effect of DS on solution conformation and antioxidant activities

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ARTICLE INFO

Article history:
Received 19 June 2014
Received in revised form 14 July 2014
Accepted 14 July 2014
Available online 21 July 2014

Keywords:
Galactomannan
Guar gum
Phosphorylation
Solution conformation
Antioxidant activities

ABSTRACT

Phosphorylated derivatives of galactomannan from guar gum (GG) with the degree of substitution (DS) of 0.35–0.52 were synthesized using $POCl_3$/pyridine. FT-IR, ^{13}C NMR and XPS results revealed that phosphorylation had occurred and C-6 substitution was predominant in phosphorylated guar gum (PGG). PGG showed an increase in M_w and more broad molar mass distribution in size exclusion chromatography (SEC) analysis. Higher reaction temperature (above 60 °C) resulted in a higher M_W value in PGG. It might be due to the cross-linking of polysaccharide chains by $POCl_3$ via di-ester which was also supported by monosaccharide composition result. Results of $M_W - \langle S^2 \rangle_z^{1/2}$ showed a decrease in fractal dimension (d_f) values. DS had greater influence on its conformation in aqueous solution. The introduction of -PO_3H_2 groups improved significantly the stiffness of the chains due to the electrostatic effect. Furthermore, antioxidant experiments revealed that high DS could enhance the scavenging activities of radicals of PGG in vitro

1. Introduction

The study of the correlation between chemical structure and biological activities of polysaccharides is one of the most intriguing and challenging scientific endeavors (Tao, Zhang, Yan, & Wu, 2007). The structure-activity relationships show that the activities of modified polysaccharides depend upon the chemical structure of the carbohydrate biopolymer, such as monosaccharide composition, average molecular weight, degree and pattern of substituting groups and molecular conformation of the polysaccharides (Zhang et al., 2010; Zhang, Li, Wang, Zhang, & Peter, 2011). Considerable attention has been paid to the solution conformation of natural polysaccharides (Wu, Li, Cui, Eskin, & Goff, 2012; Yi et al., 2012; Tao et al., 2007; Yang & Zhang, 2009). It is reported that the extended chain conformation were beneficial to enhance the anti-tumor activity, as a result of increasing the interaction between polysaccharide and immune system (Chen, Xu, Zhang, & Zeng, 2009).

Among the natural and synthetic biopolymers, phosphorylated polysaccharides represent a class of macromolecules of particular interest. It has been reported that monosaccharide, oligosaccharide and polysaccharide phosphate exhibit good antitumor (Chen et al., 2009), immunomodulatory (Zhang, Nie, Huang, Li, & Xie, 2013) and antioxidant activities (Chen et al., 2011; Wang, Zhang, Yao, Zhao, & Qi, 2013). The synthesis of phosphorylated polysaccharide such as cellulose is effectuated by different systems of reactions: phosphoric acid, phosphoric anhydride, phosphorus trichloride or dimethylphosphate (Suflet, Chitanu, & Popa, 2006). H_3PO_4 and H_3PO_3 is commonly used as phosphorylated reagent at higher temperature, which usually lead to the low degree of substitution (DS of 0.26, phosphate content of 4.05%) and degradation of polysaccharides (Wang et al., 2013). However, studies aimed at investigating the effects of synthetic polysaccharide phosphate with high DS are limited. Therefore, it is crucial to develop suitable synthetic strategy for phosphorylated polysaccharides.

It is well known that the structure of chemical modified polysaccharides is depended on the reaction conditions. This suggests that upon interaction with reaction reagent, modified polysaccharide under-goes complex conformational changes that subsequently modify the biological activities. The purpose of the present investigation is to explore systematically the solution conformation of

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http://dx.doi.org/10.1016/j.carbpol.2014.07.028
0144-8617/

phosphorylated polysaccharides with different structure features. Guar gum (GG) is a widely applied polysaccharide in industry (Wu, Cui, Eskin, & Goff, 2009). In our previous studies, sulfated derivatives of galactomannan from guar gum (SGG) were prepared. Antioxidant assays showed that SGG had better antioxidant activities (Wang, Zhao, Wang, Yao, & Zhang, 2012). However, early results were not related to any aspects on synthesis, solution conformation and antioxidant activities of phosphorylated GG.

The present study is focused on the mechanisms governing the relations between chain conformation and structure features. For that purpose, phosphorylated derivatives of GG (PGG) are prepared by $POCl_3$. FT-IR, ^{13}C NMR, X-ray photoelectron spectroscopy (XPS), monosaccharide composition analysis are employed to characterize the chemical structure of PGG. We also attempt to study the solution properties of GG and PGG using size exclusion chromatography combined with laser light scattering (SEC-LLS). Moreover, we evaluate the effect of DS on the antioxidant activities of PGG in vitro and offer theoretical evidence for the synthesis of phosphorylated polysaccharides with different structure features. Antioxidant properties are assayed in terms of antioxidant activities in vitro, by testing the scavenging abilities on superoxide radicals, hydroxyl radicals, 1,1-diphenyl-2-picrylhydrazyl (DPPH), reducing power and chelating ability on ferrous ions.

2. Materials and methods

2.1. Purification of GG

The protein of commercial GG (protein content was 5.02%, number average molecular weights was 1.46×10^4 g/mol) was removed by Sevage method joined papain according to the reported method. The carbohydrate content of purified GG was 91.20%, determined by phenol sulfuric acid procedure (Wang et al., 2010a).

2.2. Synthesis of phosphorylated GG

2.2.1. Preparation of reaction reagent

Phosphorus oxychloride ($POCl_3$) was dropped one by one in anhydrous pyridine filled in three-necked flask, under agitating and cooling in ice water bath. The ratio of $POCl_3$ to pyridine was 1:10. All determinations were completed in 30 min.

2.2.2. Phosphorylated reaction

GG (500 mg) was suspended in 20 mL anhydrous *N*,*N*-dimethylformamide (DMF) at room temperature with stirring for 60 min, and the reaction reagents (20 mL) were added dropwise. The mixture was stirred under N_2 atmosphere (The reaction conditions were shown in Table 1). After the reaction, the mixture was cooled to room temperature and the pH value was adjusted to 7–8 with 2 mol/L NaOH solution. The mixtures were precipitated with ethanol (95%), washed and then dialyzed (molecular weight cutoff 8–12 kDa) against tap water for 48 h and distilled water for 24 h to remove pyridine and salt. Phosphorylated GG with different DS were collected (PGG_1–PGG_8). The samples were stored in a dry box under room temperature till use.

The phosphorus contents (*P*%) of PGG were determined with ICP-AES (Cheng, 2011). The DS was calculated according to the equation:

$$DS = \frac{1.62 \times P\%}{31 - 0.84 \times P\%} \quad (1)$$

2.3. Characterization of phosphorylated GG

2.3.1. FT-IR analysis

The FT-IR spectrum of each sample was measured by using a method as reported by Wang et al. (2013). A known weight of dried sample of each polysaccharide was homogenized with KBr powder and then pressed into pellets for FT-IR spectrum (Thermo Nicolet iS10) measurement in the frequency range of 400–4000 cm^{-1} at a resolution of 4 cm^{-1}. The total number of scans recorded was 16.

2.3.2. NMR spectroscopy

NMR experiments were obtained using a 400 MHz Bruker model Avance spectrometer (DPX-400) incorporating Fourier transform. ^{13}C NMR (100.59 MHz) analyzes were performed at 70 °C, with the samples being dissolved in D_2O. The chemical shifts were expressed in ppm relative to the resonance of the internal standard Me_4Si. The sample (10 mg of each) was heated (at 80 °C for 30 min) with water (1 mL), centrifuged and the resulting supernatant lyophilized. The freeze-dried sample was deuterium-exchanged by lyophilisation with D_2O and then examined in 99.9% D_2O.

2.3.3. X-ray photoelectron spectroscopy (XPS)

For chemical composition analysis, samples were characterized using XPS (PHI 5702, USA) with a focused monochromatic Al-*K*a source (1486.7 eV) for excitation. The pressure in the chamber was below 2.666×10^{-4} kPa before the data were taken, the voltage and power of the anode were 15 kV and 200 W, respectively. The take-off angle was set at 45°. The pass energies for survey and multiplex scans were 117.40 and 23.50 eV, respectively. Data analysis was carried out with Multipak software provided by the manufacturer. For quantitative chemical composition analysis, two samples in each group were acquired and the survey scans were performed in two different areas on each sample.

2.3.4. Components analysis

The monosaccharide composition was analyzed according to the earlier report from our laboratory (Wang et al., 2010b). Briefly, 10 mL of sample was dissolved in 4 mL of 4 M trifuoroacetic acid acetate (TFA) in a test tube and then hydrolyzed at 120 °C for 10 h under airtight condition. TFA was then evaporated through decompression and distillation. When the tube was dry, 10 mg of ammonium hydrochloride and 0.5 mg pyridine were added and allowed to react in a 90 °C water bath for 30 min. Then 0.5 mL cold (kept at 4 °C in a refrigerator) acetic anhydride was added to the test tube and the mixture was incubated in the 90 °C water bath for another 30 min to allow the acetylation reaction to occur. The end product was decompressed and distilled to dryness. The acetate derivatives were analyzed by GC–MS (Thermo Polaris-Q) with a HP-5 capillary column. The temperature program was set to increase from 120 °C to 250 °C at an increment of 5 °C/min and N_2 was the carrier gas. The standard monosaccharides were measured following the same procedure.

2.3.5. Molecular weight determination

SEC-LLS measurements were carried out on size-exclusion chromatograph combined with multi-angle laser photometer (MALLS, $\lambda = 690$ nm; DAWN EOS, Wyatt Technology Co., USA). Ultrahydrogel™ column (7.8 × 300 mm, Waters, USA) was used. An optilab refractometer (Dawn, Wyatt Technology Co., USA) was simultaneously connected. GG and PGG with desired concentrations (0.01 mg/mL) were prepared (dissolved in ultrapure water) and optical clarification of these samples was achieved by filtration into a scattering cell. The injection volume was 50 μL and the flow rate was 0.5 mL/min (mobile phase of ultrapure water). The refractive index increment (dn/dc) value of the sample was

Table 1
Reaction conditions and molecular characterization of GG and its phosphorylated derivatives.

Samples	Reaction Time (h)	Temperature (°C)	Yield (%)	DS	$M_W \times 10^4$	$M_n \times 10^4$	PD [b]	$\langle S^2 \rangle_z^{1/2}$ (nm)	Relation equation	d_f	Conformation
GG	–	–	–	nd[a]	5.57	1.46	3.83	31.7	$\langle S^2 \rangle_z^{1/2} = 0.403 M_w^{0.28\pm0.031}$	3.57	Hard sphere
PGG_1	1	25	91.00	0.37	5.49	2.06	2.66	41.7	$\langle S^2 \rangle_z^{1/2} = 0.177 M_w^{0.31\pm0.045}$	3.22	Hard sphere
PGG_2	2	25	120.31	0.43	5.64	1.90	2.97	43.6	$\langle S^2 \rangle_z^{1/2} = 0.231 M_w^{0.29\pm0.03}$	3.44	Hard sphere
PGG_3	4	25	100.82	0.52	6.07	2.16	2.81	38.8	$\langle S^2 \rangle_z^{1/2} = -0.205 M_w^{0.38\pm0.04}$	2.63	Between hard sphere and random coil (fully swollen)
PGG_4	5	25	112.60	0.49	6.19	1.68	3.67	40.2	$\langle S^2 \rangle_z^{1/2} = 0.162 M_w^{0.34\pm0.035}$	2.94	Between hard sphere and random coil (fully swollen)
PGG_5	6	25	103.71	0.50	6.30	2.01	3.13	39.6	$\langle S^2 \rangle_z^{1/2} = 0.566 M_w^{0.35\pm0.038}$	2.85	Between hard sphere and random coil (fully swollen)
PGG_6	1	0	111.20	0.35	6.25	1.55	4.03	43.5	$\langle S^2 \rangle_z^{1/2} = 0.467 M_w^{0.30\pm0.014}$	3.33	Hard sphere
PGG_7	1	40	88.23	0.47	8.13	2.74	2.97	44.5	$\langle S^2 \rangle_z^{1/2} = 0.158 M_w^{0.34\pm0.038}$	2.88	Between hard sphere and random coil (fully swollen)
PGG_8	1	60	72.61	0.39	10.10	3.49	2.89	53.3	$\langle S^2 \rangle_z^{1/2} = 0.866 M_w^{0.27\pm0.02}$	3.70	Hard sphere

[a] Not detected.
[b] $PD = M_W/M_n$.

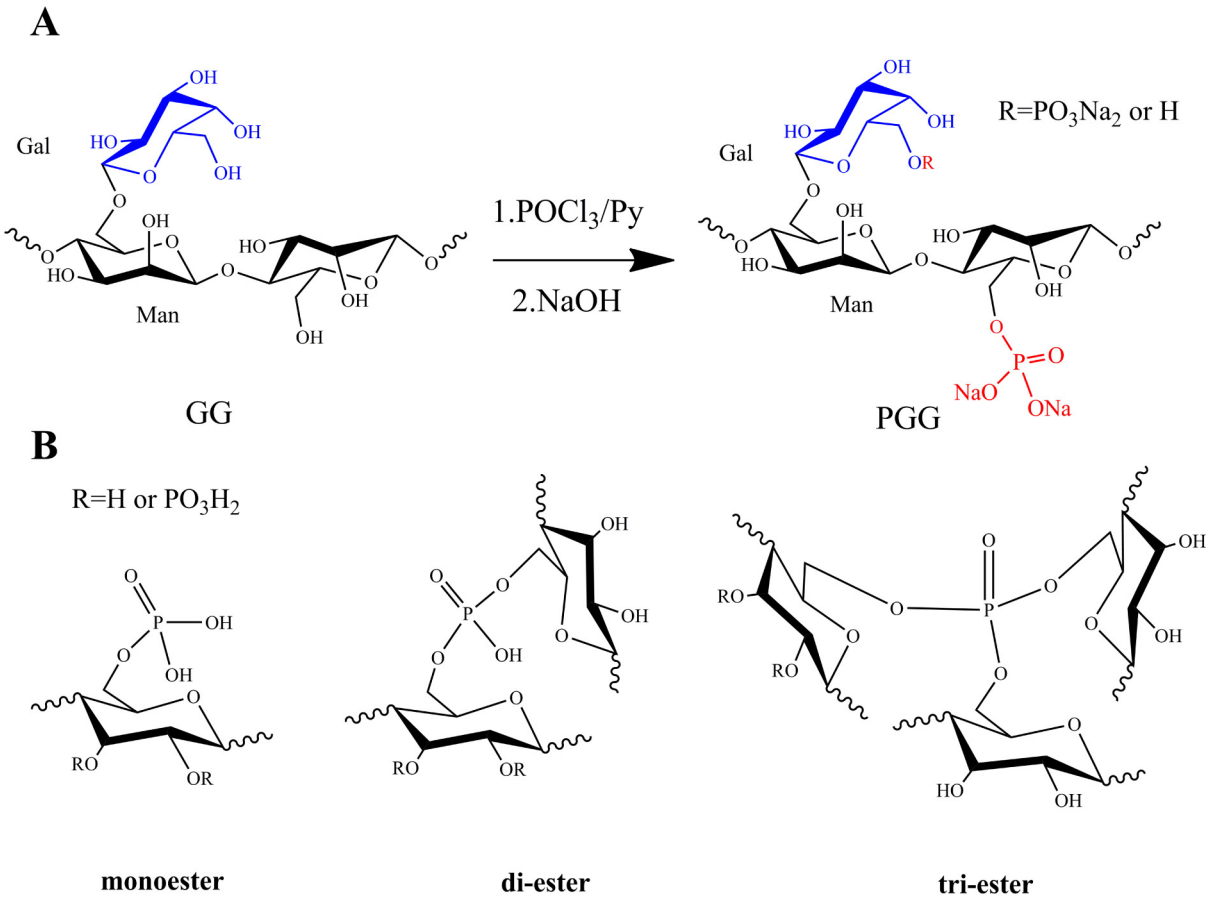

Fig. 1. Reaction of GG with $POCl_3$/Py (A) and formation of monoester, di-ester and tri-ester by $POCl_3$ (B).

determined by using an optilab refractometer at 690 nm (25 °C) to be 0.145 mL/g. The basic light scattering equation was as follows

$$\frac{KC}{R_\theta} = \frac{1}{M_w}\left(1 + \frac{16\pi^2\langle S^2\rangle_z}{3\lambda^2} \times \sin^2\left(\frac{\theta}{2}\right)\right) + 2A_2C \quad (2)$$

where K was an optical constant equal to $[4\pi^2 n_0^2 (dn/dc)^2]/(\lambda^4 N_A)$; C, the polysaccharide concentration in mg/mL; R_θ, the Rayleigh ratio of the scattered to incident light intensity; M_W, the average molecule weight; λ, the wavelength of incident light; n_0, the refractive index of the solvent; dn/dc, the refractive index increment; N_A, the Avogadro' number; A_2, the second virial coefficient; $\langle S^2\rangle_z$, the mean square radius of gyration; θ, the scattering angle. As the column separated the polymer according to molecular size, each fraction was led to the light scattering detector for instantaneous measurement of the scattering intensities. The polymer concentration was calculated based on the peak area determined by the refractive index detector, according to glucan standards.

2.4. Assay for antioxidant activities

The free radical scavenging activity of the modified polysaccharides was measured by DPPH test according to the method of Shimada (Shimada, Fujikawa, Yahara, & Nakamura, 1992). The hydroxyl radical assay was measured by the method of Ghiselli, Nardini, Baldi, and Scaccini (1998). The superoxide radical scavenging assay was measured according to the method of Qi et al. (2005). The ferrous ion-chelating ability of the samples was investigated with slightly modified method of Li, Li, and Zhou (2007). The reducing power was determined according to the method of Qi et al. (2005).

All the data were repeated 3 times and presented as mean ± S.D. P value of less than 0.05 was considered significant. Statistical evaluations and EC_{50} values in all experiments were performed by SPSS software 17.0.

3. Results and discussion

3.1. Synthesis of phosphorylated GG

As shown in Table 1, eight phosphorylated derivatives of GG (PGG_1–PGG_8) were obtained by varying the reaction conditions (Fig. 1A). The DS of the samples varied from 0.35 to 0.52. PGG_3 had the highest DS with the yield of 100.80%. It was clearly indicated that the DS of PGG increased with the increasing of reaction time (from 1 h to 4 h). However, further increasing in the reaction time from 4 h to 6 h did not lead to substantial increments in DS. Phosphorylated dextran, cellulose and curdlan with high DS (1.78, 1.11 and 1.0, respectively) was synthesized by Suflet et al. (2006), Suflet, Chitanua, and Desbrieresb (2010) and Suflet, Nicolescu, Popescu, and Chitanu (2011) at very high temperatures (140–150 °C). Phosphorylated polysaccharide with low P content (0.09%) from *Portulaca oleracea* was reported by Chen et al. (2011). Wang et al. (2013) reported the synthesis of phosphorylated polysaccharide (P content of 4.05%) from *Enteromorpha linza* with low-molecular-weight at room temperature. The reaction time was 20 h. The references regarding the synthesis of phosphorylated derivatives were characterized by high temperature and long reaction time. In the present study, $POCl_3$/Py could improve the efficiency of phosphorylated polysaccharide in the current reaction system.

In addition, with the increase of the reaction temperature from 0 to 60 °C, the yields of the corresponding derivatives decreased and the samples were more viscous. It could be concluded that phosphorylation and cross-linking occurred simultaneously in the reaction process due to the highly reactive of —OH groups at high temperature (Fig. 1B). It was also confirmed by the results of molecular weight determination in Section 3.5. In order to prove unambiguously that DS was an important parameter influencing bioactivity, it was worthwhile evaluating the effect of DS on antioxidant activities. In that regard, antioxidant experiments of PGG with different DS were employed. The effect of DS on antioxidant activities was shown in Section 3.6.

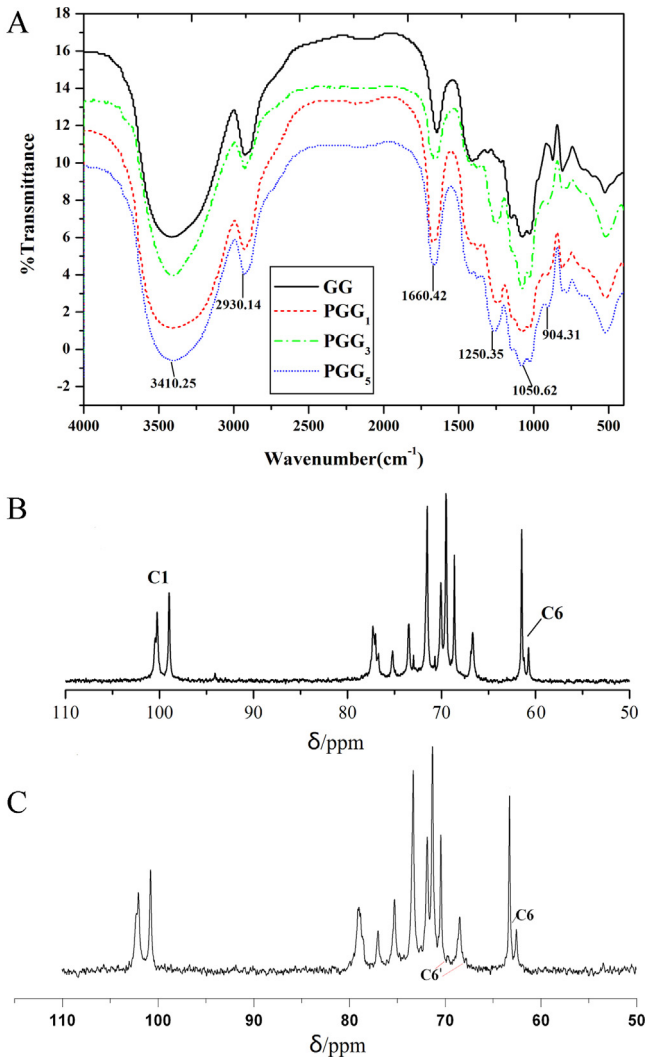

Fig. 2. FT-IR spectra of GG and its phosphorylated derivatives (A). ^{13}C NMR (125 MHz, D_2O, 25 °C) spectra of GG (B) and PGG_3 (C).

3.2. FT-IR analysis

Fig. 2A showed the FT-IR spectra of GG and its phosphorylated derivatives. The bands around 3410 cm^{-1} and 2930 cm^{-1} were due to the hydroxyl stretching vibration and C—H stretching vibration, respectively. New bands around 1250 cm^{-1} describing an asymmetrical P=O stretching vibration in PGG samples. A shoulder at 1050 cm^{-1} attributable to the P—OH bond (Suflet et al., 2006, 2011). An increase in DS from 0.37 (PGG_1) to 0.52 (PGG_3) led to an increment in the intensity of the bands at 1250 cm^{-1} for P=O stretching vibration. It could be concluded that $-PO_3H_2$ existed in PGG.

3.3. ^{13}C NMR

The ^{13}C NMR spectrum of GG was presented in Fig. 2B, where the signal positions were compared to that of PGG_3 (Fig. 2C). In

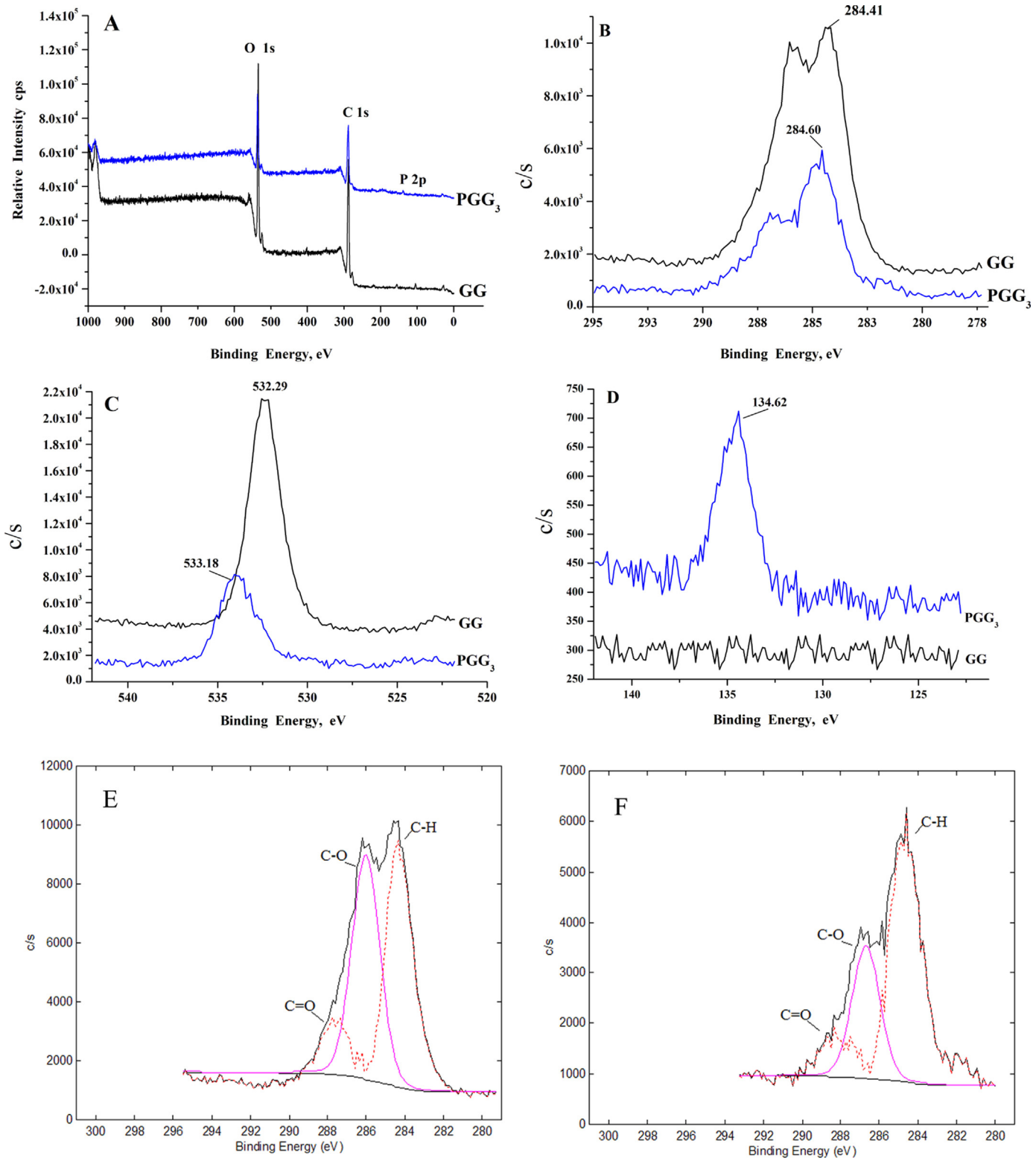

Fig. 3. XPS spectra and curve-fitting of C 1s spectra (A) survey spectra. (B) C 1s spectra. (C) O 1s spectra. (D) P 2p spectra. (E) curve-fitting of GG (F) curve-fitting of PGG_3.

the ^{13}C NMR spectrum, GG revealed two signals in the anomeric region at 99.5 ppm (α-D-galactose) and 98.2 ppm (β-D-mannose). Peak at 76.5 ppm corresponded to continuous substituted β-D-mannose units which were very similar to the results of the ^{13}C NMR spectra of galactomannan from *Dimorphandra gardneriana* (Cunha, Vieira, Arriaga, Paula, & Feitosa, 2009). Signal at 60.49 ppm was assigned to C-6 signal of unsubstituted β-D-mannopyranosyl of GG backbone. The downfield displacement of 65.9 ppm was characteristic of 6-O substitution of β-D-mannose main chain linked to galactose (Fig. 1A) (Melo, Feitosa, Feitosa, Freitas, & Paula, 2002).

In phosphorylated sample, the new peaks at 67.4 ppm and 65.2 ppm of PGG_3 were assigned to the C-6 substitution of β-D-mannose and α-D-galactose by phosphate groups, respectively (Melo et al., 2002; Liu et al., 2009). In addition, the appearance of

C-6 signals near δ 60 ppm indicated that the OH group of C-6 was not completely substituted. From the results of ^{13}C NMR spectra, the phosphorylation of GG occurred. The intensity of the signals of the O-substitution carbons and the appearance of C-6 signals near 60 ppm denoted that C-6 substitution was predominant in PGG_3 compared with other positions, probably owing to steric hindrance as reported elsewhere (Yang, Gao, Han, & Tan, 2005).

3.4. XPS analysis

XPS is a surface-sensitive analysis technique, which is capable of providing both qualitative and quantitative information about the presence of different elements of the samples. Fig. 3A showed the qualitative XPS spectra of purified GG and its phosphorylated derivatives. GG had presented three main elements. The binding energy were 288.3 eV for C, 535.1 eV for O and 402.6 eV for N. Carbon and oxygen originated from the well known carbohydrate composition on polysaccharide samples. The presence of nitrogen originated from protein or glycoprotein complex, which was unavoidable contamination in GG. Fig. 3B–D also showed the XPS spectra of GG after phosphorylated modification. Apart from C and O, additional elements, P (binding energy of 134.6 eV) was observed in PGG_3 when compared with the original GG. This newly emerged peak resulted from the abundant $-PO_3H_2$ groups that were widely present in PGG molecules.

Fig. 3E and F showed the detailed C 1s spectra of GG before and after modification. It was composed of three components centered at binding energies of 284.4, 286.1 and 287.4 eV, which correspond to C—C, C—O and C=O, respectively (Wang et al., 2006). As shown in Fig. 3F, lower intensity signal at 287.4 eV corresponded to the reduction of the carboxyl after the phosphorylated modification of GG. In addition, the C/O ratio reflected the chemical composition of the polysaccharides. For PGG_3, the C/O ratio was determined as 0.403, which was different from the value of 0.447 for GG. The decrease in C/O ratio suggested an increase in oxygen content after modification.

Fig. 3C showed O 1s spectra of both GG and PGG_3. The main component at high binding energy (533.1 eV) was due to the electron-withdrawing groups. Furthermore, a broadening of the O 1s peak upon phosphorylation was observed in PGG_3 which in agreement with the work of Essayem, Martin, Riondel, and Vedrine (2007). In the case of the O 1s peak presented a different shape possibly due to a much larger component attributable to O—P. The P 2p spectra of GG and PGG_3 were shown in Fig. 3D. No P 2p signal was detected in GG. In PGG_3, it could be found that data from P 2p was fitted by a Gaussian function centered at 134.6 eV, which in agreement with the binding energies of P 2p in $-PO_3H_2$. The high binding energy of the P 2p revealed that P was only presented as P^{5+}. The result was also consistent with the results of FT-IR analysis.

3.5. Monosaccharide composition analysis

Monosaccharide composition analysis showed that GG consist of mannose and galactose (Fig. 4A and B). The ratio of mannose to galactose was 2.62. The monosaccharide composition of PGG_3 was showed in Fig. 4C. In PGG_3, mannose and galactose were the dominant monosaccharide. In contrast, PGG_3 had a higher ratio (2.86) of mannose/galactose. Based on the results, it could be concluded that no degradation was occurred in the phosphorylation process. It was proposed that such an increase in the ratio of mannose/galactose reflected the increase in mannose backbone. Therefore, both monosaccharide composition and molecular weight (Section 3.5) data strongly suggested that the cross-link of two polysaccharide chains by $POCl_3$ was determined.

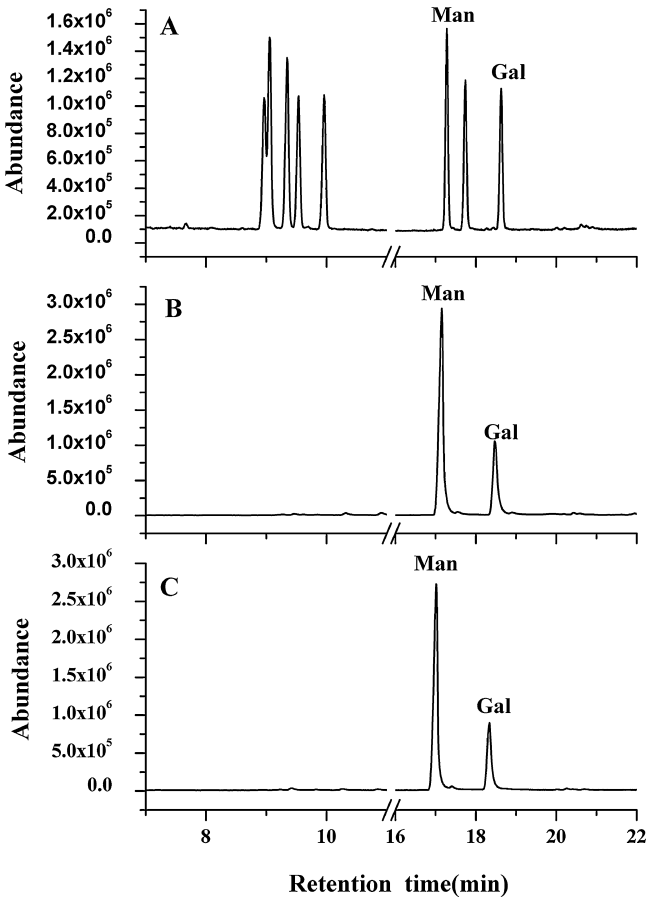

Fig. 4. Monosaccharide composition analysis of standard monosaccharides (A), GG (B) and PGG_3 (C) by GC-MS.

3.6. Analysis of molecular properties

In the present study, we used phosphorylated samples with different molecular weight and DS to study how molecular weight and DS affected conformation in aqueous solution. The average molecular weight and molecular weight distribution of GG and its derivates were determined with SEC-LLS. The weight average molar mass (M_w), polydispersity (PD, M_W/M_n) and Z-average radius of gyration ($\langle S^2 \rangle_z^{1/2}$) were shown in Table 1 for all the samples. The radius of gyration, M_w, and PD for GG were measured to be 31.7 nm, 5.57×10^4, and 3.830, respectively. Our results showed that phosphorylated derivates with different DS and molecular weight were obtained by changing the reaction conditions (Fig. 5A–D). Compared to the native one, all the samples showed an increase in M_w and more broad molar mass distribution due to the anhydrous conditions of the polysaccharides during the phosphorylation, which was in accordance with previous results (Suflet et al., 2010).

Furthermore, too much higher temperature resulted in the highest M_w value in PGG_8 (Fig. 5C and D). This might be due to the cross-linking of polysaccharides by $POCl_3$ via di-ester and/or tri-ester formation. Theoretically, the formation of monoester, di-ester (cross-link of two polysaccharide chains) and tri-ester (cross-link of three polysaccharide chains) by $POCl_3$ is possible. However, due to the steric-hindrance, it is hard to observe tri-ester in phosphorylation reaction (Figs. Fig. 1B and Fig. 6A). The highly reactive of —OH groups at high temperature would cause the formation of di-ester and we also got the result of higher M_W and M_n in PGG_8. Based on the results, it could be concluded that cross-linking of polysaccharide chains occurred at high temperature in the process. The

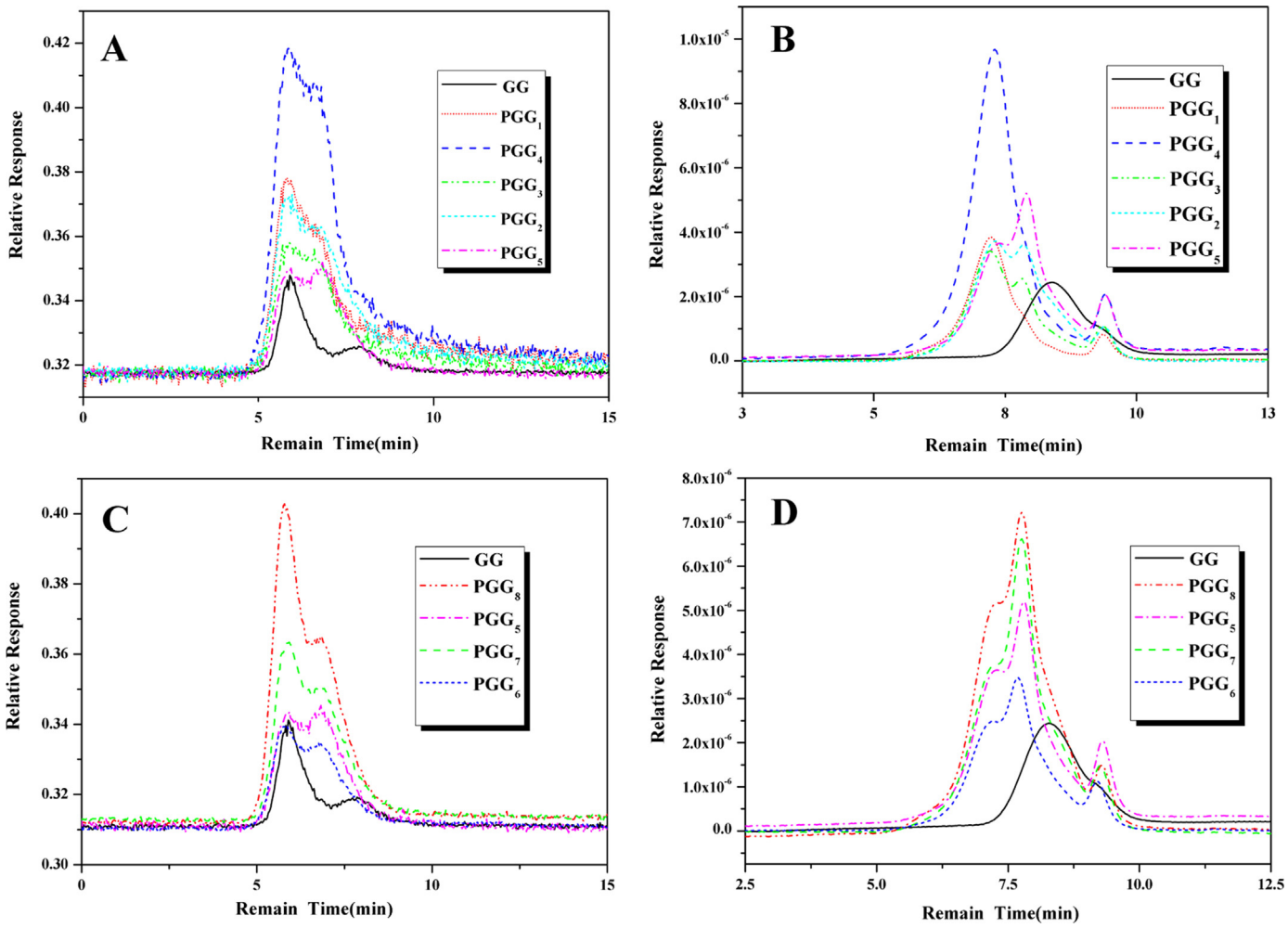

Fig. 5. Evolution of molecular weight of PGG with different reaction conditions. (A) Laser light scattering photometry and (B) Differential refractive signals of PGG_1- PGG_5 with different reaction times. (C) Laser light scattering photometry and (D) Differential refractive signals of PGG_5-PGG_8 with different reaction temperatures.

mechanism of cross-linking was due to the high reaction activities of $POCl_3$. The reaction temperature became an essential step in the phosphorylation of GG.

Relevant structural information and further insight into the nature of the polysaccharide could be obtained by investigating the fractal dimension (d_f) (Tao, Zhang, & Peter, 2006). The d_f value could be determined from the M_w dependence of $\langle S^2 \rangle_z^{1/2}$, and was defined as the inverse of the exponent ν:

$$\langle S^2 \rangle_z^{1/2} = fM^{\nu} \tag{3}$$

$$df = \frac{1}{\nu} \tag{4}$$

On the basis of the theory of polymer solutions, the value of d_f was 1 for a rigid rod, and linear polymers with Gaussian coil nature had d_f value ranging from 5/3 to 2. A three-dimensional object with a homogeneous density had a mass fractal dimension of 3. The d_f value of monodisperse polymers could be extracted directly from the angular dependence of the scattered light or neutron intensity. This approach had been successfully applied to highly branched polysaccharides with high molecular size such as amylopectin and other synthetic branched polymers (Tao & Xu, 2008; Wang et al., 2011).

The straight line fitting of the experimental data points was shown in Table 1 and Fig. 6B. The ν value of 0.28 suggested that GG molecules in aqueous solution were in the state of hard sphere. Furthermore, the value of d_f was calculated to be 3.57 according to Eq. (4). The result confirmed that GG existed as a sphere conformation in aqueous solution. In phosphorylated samples, a decrease in d_f values was observed. The d_f values of PGG_1, PGG_2, PGG_3, PGG_4 and PGG_5 were 3.22, 3.44, 2.63, 2.94 and 2.85, respectively. A d_f value of 2.63–2.94 was characteristic of a particle having an internal structure between the hard sphere ($d_f = 3.0$) and the fully swollen branched macromolecule in a thermodynamically good solvent ($d_f = 2.0$). In addition, an increase in the ν values of high DS samples was observed (Table 1). The d_f value of 2.63 indicated that PGG_3 with the highest DS exhibited as a hard sphere and the fully swollen branched macromolecule in aqueous solution. This value was lower than that of PGG_4 with similar molecular weight but lower DS. In addition, with the increase of M_W (PGG_6–PGG_8), a decrease of ν value was observed. It might be due to the cross-linking of polysaccharide chains and more flexible conformation in solution, which was in accordance with molecular weight results. However, PGG_7 showed a more expanded conformation (d_f value of 2.88) compared to PGG_6 (d_f value of 3.33) with lower molecular weight and DS.

The result indicated that under the same conditions, PGG with higher DS had a lower ν value. It suggested that DS had greater influence on its conformation in aqueous solution. Our results indicated that phosphorylated polysaccharides showed less flexibility compared with the native one. It was confirmed by the results of Tao et al. A d_f value (1.79) in sulfated polysaccharide showed

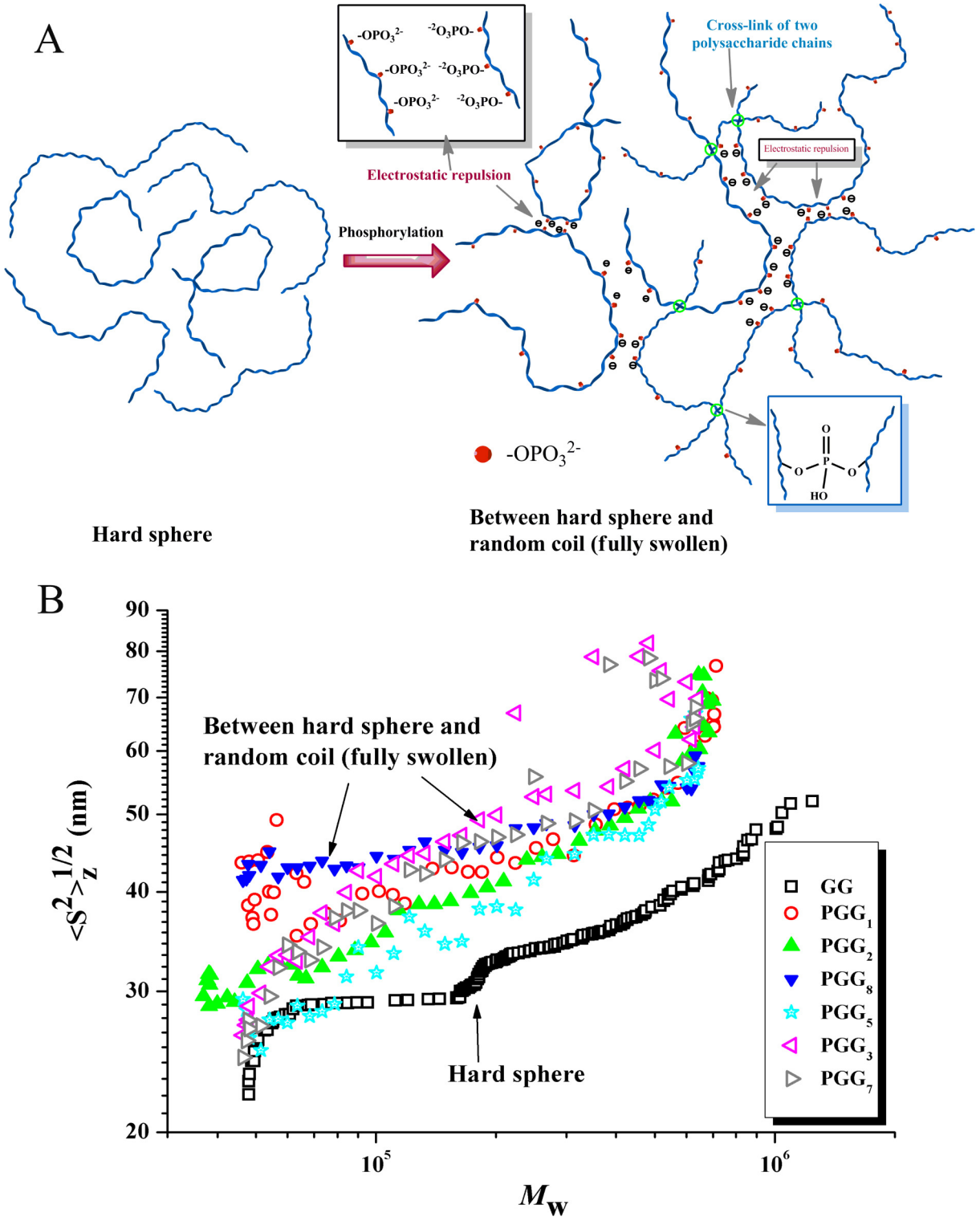

Fig. 6. Schematic representation of conformation transition after modification (B) and plot of log M_w versus $\langle S^2 \rangle_z^{1/2}$ for GG and PGG at 25 °C (B).

that the $-SO_3H$ groups in the derivatives enhance the steric hindrance between the polymer chains (Tao et al., 2006). The major driving force for the conformation in aqueous solution was due to the intramolecular hydrogen bonding (Zhang et al., 2011). Electrostatic effect of $-PO_3H_2$ groups leading to the relatively expanded conformation of the phosphorylated derivatives with totally different intramolecular hydrogen bonding compared to the native one (Fig. 6A). Similar result was reported that the introduction of $-SO_3H$ groups strengthens the effect of electrostatic repulsion, resulting in chain expansion and increased stiffness (Zhang, Zhang, Wang, & Cheung, 2003). Chen et al. also found that the introduction of the phosphate groups into the β-glucan improved significantly the stiffness of the chains (Chen et al., 2009).

Based on the results, it could be concluded that phosphorylation and cross-linking occurred simultaneously in the process. Such a feature could be assigned to the fact that intra-molecular repulsive

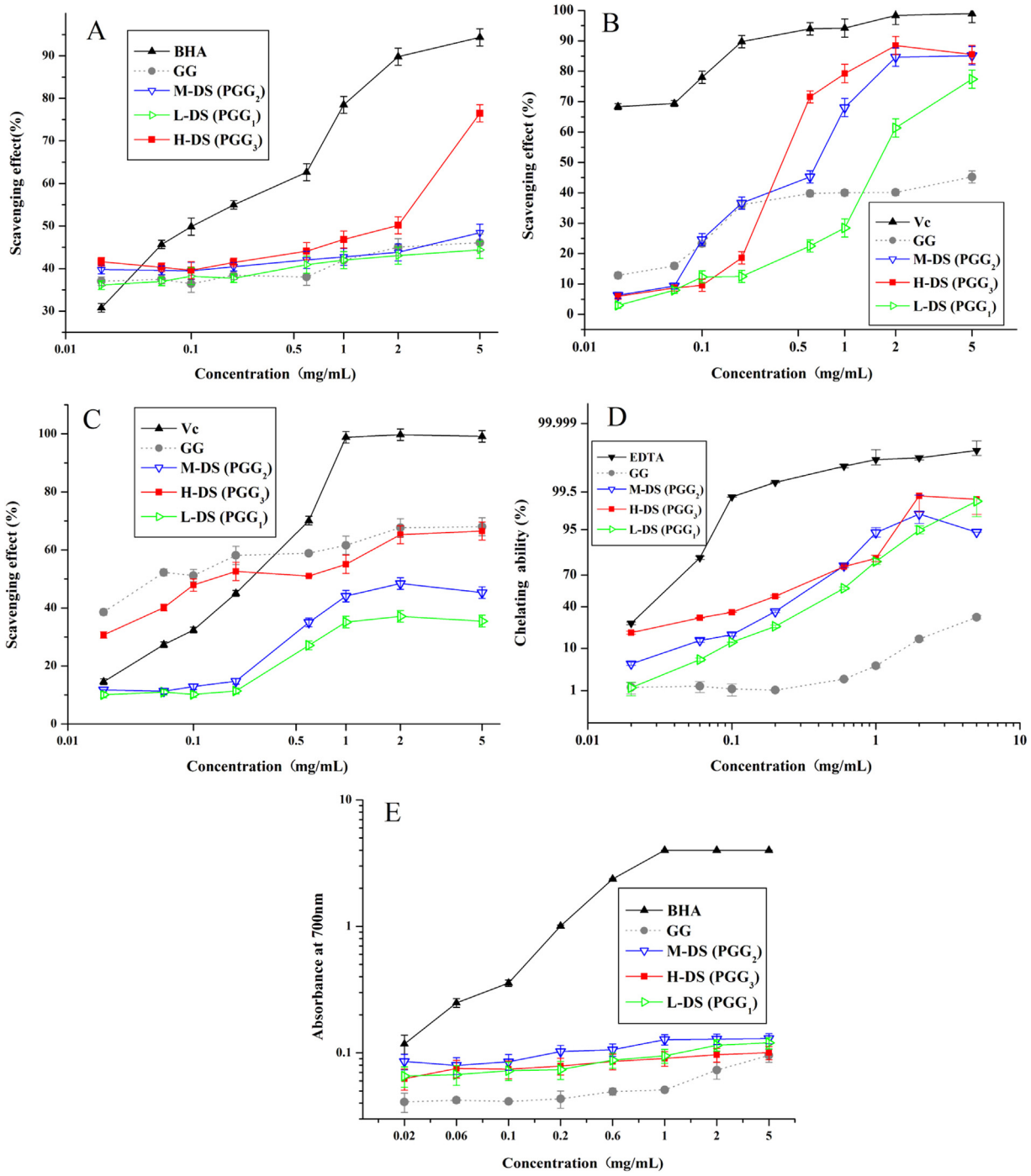

Fig. 7. Antioxidant effect of GG and its phosphorylated derivatives: (A) scavenging activity of DPPH radicals; (B) scavenging activity of superoxide radicals; (C) scavenging activity of hydroxyl radicals; (D) chelating effect on ferrous ions; (E) reducing power; data are presented as mean values ($n = 3$).

interactions were unneglectable in phosphorylated polysaccharides (Zou et al., 2008). Higher DS might lead to an expanded conformation. It suggested that DS had greater influence on its conformation in aqueous solution. In order to prove unambiguously that DS was an important parameter influencing bioactivity, it was worthwhile evaluating the effect of DS on antioxidant activities. In that regard, antioxidant experiments of PGG with different DS were employed. The effect of DS on antioxidant activities was shown in Section 3.6.

3.7. Antioxidant activity analysis

3.7.1. Scavenging activity of DPPH radicals

The scavenging ability of the samples on DPPH radical was shown in Fig. 7A and compared with BHA. Compared to GG (EC_{50} of 4.97 mg/mL), all phosphorylated polysaccharides showed better scavenging effects. PGG_3 had strong antioxidant activity with EC_{50} value of 1.32 mg/mL. The scavenging effects of PGG_1 and PGG_2 were weaker than PGG_3, the EC_{50} values were 1.74 mg/mL and

1.45 mg/mL, respectively. It suggested that the DS of PGG had great influence on its scavenging effects of DPPH radicals. It was reported that the effect of antioxidant activities was due to their hydrogen donating ability (Wang, Zhang, Zhang, Zhang, & Li, 2009). The presence of $-OPO_3H_2$ groups in the PGG molecules could activate the hydrogen atom of the anomeric carbon. The higher activated capacity of the group, the stronger hydrogen atom-donating capacity. It was concluded that PGG_3 with highest DS showed the best scavenging activity of DPPH radicals.

3.7.2. Scavenging activity of superoxide radicals

Fig. 7B showed that the inhibitory effect of all samples (with different DS) on superoxide radicals was significant at all tested concentrations in a concentration dependent manner. The EC_{50} values of PGG_1, PGG_2 and PGG_3 were 1.48, 1.27 and 1.17 mg/mL, which were lower than V_c (0.077 mg/mL) but higher than GG (1.99 mg/mL). According to the results, all phosphorylated samples showed stronger scavenging effect than the native one. This demonstrated that DS affected the antioxidant activity, which was in accordance with Wang et al. (2013) that electron-withdrawing groups in polysaccharide showed greater scavenging effect of superoxide radical.

3.7.3. Scavenging activity of hydroxyl radicals

As shown in Fig. 7C, phosphorylated derivatives were found to have the scavenging ability on hydroxyl radicals in a DS-dependent fashion. The EC_{50} values of PGG_1, PGG_2 and PGG_3 were 3.65, 1.13 and 0.92 mg/mL, respectively. Relationship between EC_{50} value and DS was obvious. The result indicated that the $-OPO_3H_2$ groups played an important role in the scavenging of hydroxyl radicals. However, all the phosphorylated samples were found to have less ability to scavenge hydroxyl radicals compared to GG with EC_{50} value of 0.84 mg/mL. This could be due to the high molecule weight of phosphorylated samples. Zou et al. (2008) confirmed that low M_W showed the best antioxidant capacities. The result indicated that molecule weight and DS played an important role in the scavenging of hydroxyl radicals.

3.7.4. Chelating effect on ferrous ions

All samples were found to have the chelating ability on ferrous ions in a concentration-dependent fashion as shown in Fig. 7D. Relationship between chelating effect and DS was obvious. At the concentration of 0.02 mg/mL, PGG_3 showed the highest chelating ability of 18.50%, while the chelating ability was 25.24% of EDTA. At the concentration above 0.06 mg/mL, the effects of phosphorylated derivates were more pronounced. It was reported that the chelating effect of phosphorylated *Portulaca oleracea* polysaccharides was 36.10% at the concentration of 5 mg/mL (Chen et al., 2011). Compared with the results, PGG with higher DS showed stronger chelating ability on ferrous ions. The present study was in accordance with previous results that the substitution of —OH with $-OPO_3H_2$ groups would enhance the ferrous chelating ability (Yang et al., 2005).

3.7.5. Reducing power

The reducing activities were usually related to the development of reductones, which terminate free radical chain reactions by donating a hydrogen atom (Qi et al., 2005). Fig. 7E depicted the reducing power of the samples and BHA. The reducing power values of PGG_2 and GG were 0.127 and 0.051 at 1 mg/mL, which were weaker than BHA. Moreover, the reducing power of PGG were relating more pronounced than that of the native one. Reductones such as polysaccharides were also reported to react with precursors of peroxide, thus preventing peroxide formation. Wang et al. also reported that the reducing power of phosphorylated polysaccharides from *Enteromorpha linza* was between 0.125 and 0.153 at the concentration of 1 mg/mL (Wang et al., 2013). The reducing power of sulfated lacquer polysaccharides was 1.2 at 1.0 mg/mL (Zou et al., 2008). Compared to the results, PGG had reducing capacity and the relation of reducing power to $-OPO_3H_2$ groups was significant.

Antioxidant activities of modified polysaccharides had been reported previously, but few investigations had been studied the effect of DS as an independent variable. It was interesting to note that the M_W of both PGG_2 and PGG_3 was similar but with different DS (Table 1). PGG_3 could achieve a significantly higher antioxidant activities than PGG_2 with lower DS. The possible reasons might be due to the supply of hydrogen by phosphorylated derivatives, which combined with radicals and formed a stable radical to terminate the radical chain reaction. This would be a new developmental direction for the synthesis of polysaccharide derivatives.

4. Conclusion

In the present study, phosphorylated derivatives of GG with the DS of 0.35–0.52 were synthesized using $POCl_3$/Py as reaction reagent. New elements, P (binding energy of 134.6 eV) was observed in XPS analysis. The high binding energy of the P 2p revealed that $-PO_3H_2$ groups were widely present in PGG molecules. According to the results of FT-IR and ^{13}C NMR, the phosphorylation reaction had occurred. The intensity of the signals of the O-substitution carbons denoted that C-6 substitution was shown in phosphorylated samples. Compared with GG, an increase in M_w was observed in all samples according to SEC-LLS results. Monosaccharide composition analysis also supported the result of cross-linking between polysaccharide chains by $POCl_3$ via di-ester.

Furthermore, molecular parameters of GG and PGG were calculated successfully from the experimental data of M_W and $\langle S^2 \rangle_z^{1/2}$. PGG_3 with the highest DS of 0.52 exhibited as a hard sphere and the fully swollen branched macromolecule in aqueous solution. The results revealed that the introduction of $-PO_3H_2$ groups improved significantly the stiffness of the chains due to the electrostatic effect. In addition, antioxidant experiments revealed that high DS could enhance the scavenging activities of radicals of PGG in vitro. In summary, this work would give an insight that PGG possessed better bioactivity, which will extend the biological applications of this natural polysaccharide.

Acknowledgments

Financial support of this research from National Natural Science Foundation of China (21204073) is gratefully acknowledged. Financial supports of this research from Natural Science Foundation of Gansu Province (1308RJZA200) and Promotion Plan of Scientific Research Ability for Young Teachers from Northwest Normal University (Grant No. NWNU-LKQN-11-16) are also gratefully acknowledged. We would also like to thank Prof. Fengyiny Jing (Chang Chun Institute of Applied Chemistry, Chinese Academy of Science) for the measurement of NMR and Prof. Ruibin Guo (The Key Laboratory of Ecological Environment Related Polymer Materials of Ministry of Education, Northwest Normal University) for the use of SEC-LLS.

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