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Structure and chain conformation of a (1 → 6)-α-D-glucan from the root of *Pueraria lobata* (Willd.) Ohwi and the antioxidant activity of its sulfated derivative

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ABSTRACT

A water soluble glucan, PLB-2C, was isolated from the water extract of the root of *Pueraria lobata* (Willd) Ohwi using anion-exchange and gel permeation chromatography. Its structure was investigated by gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS), infrared (IR) spectra, and nuclear magnetic resonance (NMR) spectroscopy of heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) techniques. The results indicated that PLB-2C was a linear glucan composed of (1 → 6)-α-D-Glc*p*. Chain conformation study showed that the polysaccharide took random coil compact conformation. *In vitro* cell viability assay by MTT method, its sulfated derivative PLB-2CS which was substituted at 2-O, 3-O, 4-O positions, at 0.1, 1, and 5 mg/ml, could attenuate PC12 cell damage significantly caused by hydrogen peroxide.

1. Introduction

As a brain attack, stroke is the third leading cause of death in the Western world. Nearly in all instances, stroke involves brain cells death and dysfunction, and neurological deficits that ultimately reflect the location and size of the compromised brain area. There are at least three fundamental mechanisms leading to cells death during ischemic brain injury: excitotoxicity and ionic imbalance, oxidative/nitrosative stress, and apoptotic-like cell death (Eng, Turgay, & Michael, 2003). Thus, one of the putative ways to deal with this disease is to develop antioxidants. Though many synthetic chemicals, such as phenolic compounds, are found to be strong radical scavengers, they usually have some severe adverse effects (Heilmann, Merfort, & Weiss, 1995; Zhou & Zheng, 1991). Accordingly biotechnological natural products are receiving growing attention from the viewpoint of antioxidation (Hou, Hsu, & Lee, 2002).

The root of *Pueraria lobata*, a wild creeping leguminous plant, is one of the earliest and also important crude herbs used in Chinese medicine for various medicinal purposes. It has been widely employed in the clinic for the management of various diseases including hypertension, diabetes, cardiovascular disorders and ischemic stroke (China Pharmacopoeia Committee, 2005). The material mainly contains isoflavonoids and triterpene glycosides. The former constituents include 3′-hydroxypuerarin, puerarin, 3′-methoxypuerarin, daidzin, genistin, formononetin-7-glucoside and daidzein (Sibao, Dajian, Shilin, Hongx, & Chan, 2007). Puerarin was confirmed as a major isoflavonoid in *P. lobata* (Cherdshewasart, Subtang, & Dahlan, 2007; Kirakosyan et al., 2003) and in starch samples derived from Kudzu roots (Kirakosyan et al., 2003). The latter contain at least six saponins (Arao, Kinjo, Nohara, & Isobe, 1997). To date, the effects of daidzin against alcohol abuse and alcoholism, which have been confirmed by *in vivo* and *in vitro* experiments, are particularly being focused on in the Western countries (Keung, Lazo, Kunze, & Vallee, 1996).

It is also reported that the ethanol extract from *P. lobata* presents the protective effect from the oxidative damage induced by hydrogen peroxide (H_2O_2) (Bo, Ming, Gang, Lei, & Jia, 2005). *In vitro* test, the antioxidant activity of isoflavonoids from *P. lobata* significantly correlated only with puerarin, though puerarin and daidzein exhibited the same level of antioxidant activity as α-tocopherol (Cherdshewasart & Sutjit, 2008). In most cases, *P. lobata* was extracted with water and administrated orally to treat ischemic stroke. Yet, puerarin is slightly soluble in water while extracts with water from *P. lobata* at least contain soluble polysaccharides. This inspires us that except puerarin, some polysaccharides might also exert antioxidant effects which contributed to ischemic stroke therapy. However, the biological activities of water soluble polysaccharides from *P. lobata* have not been investigated yet. In recent years, sulfated polysaccharides from marine brown algae had been discovered to be antioxidants. Using the ferric

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reducing antioxidant power assay, Ruperez, Ahrazem, and Leal (2002) found that sulfated polysaccharides from *Fucus vesiculosus* had antioxidant activities. Similarly, the sulfated polysaccharides from *Laminaria japonica* and *Ecklonia kurome* also showed free radical scavenging activities (Hu, Geng, Zhang, & Jiang, 2001; Xue et al., 2001). All these prompt us to find out whether polysaccharides from *P. lobata* or their derivatives have the antioxidant activities. If so, this may help to understand the mechanism underlying the treatment of stroke with herbs.

In this communication, we described a (1 → 6)-α-D-glucan, designated as PLB-2C, which was isolated from the roots of *P. lobata*. Besides the structure elucidation, its chain conformation in aqueous was also determined. In addition, we evaluated the protection effects of PLB-2C and its sulfated derivative on PC12 cells from hydrogen peroxide induced damage.

2. Experimental

2.1. Materials

The slices of dried tuberous roots of *P. lobata* were bought from Shanghai Xuhui Herb Slices Co., Ltd., Shanghai, China. The tuberous samples were collected by villagers in Henan Province, China in December, 2005. *P. lobata* samples were identified by Prof. Ping Li, Department of Pharmacognosy, China Pharmaceutical University, Nanjing, People's Republic of China. The voucher herbarium specimen of *P. lobata* (No. 20060320) was deposited in the Herbarium of Shanghai Institute of Materia Medica, Chinese Academy of Sciences. DEAE-Cellulose 32 was from Whatman Co., U.K. Monosaccharide standards were all from Fluka, Switzerland. Dextran T-2000, T-700, T-580, T-500, T-80, T-70, T-40, T-11, and T-9.3 were purchased from Pharmacia Co., Sweden. Sodium borohydride, trifluoroacetic acid (TFA), and DC-Alufolien cellulose TLC plates were from Merck-Schuchardt Co., Germany. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) was from Sigma–Aldrich, USA. Dimethyl sulfoxide was from E. Merck, Germany. Hydrogen peroxide was from Sinopharm Chemical Reagent Co., Ltd., China. EtOAc, pyridine, HOAc, and *o*-phthalic acid were analytical grade reagent from Sinopharm Chemical Reagent Co., Ltd, China. Other reagents were analytical grade unless otherwise claimed.

2.2. General methods

The optical rotation was determined on a Perkin-Elmer 241M digital polarimeter (PerkinElmer, USA) in water at 20 ± 1 °C. IR spectra were determined using a Perkin-Elmer 591B spectrophotometer (PerkinElmer, USA) with a KBr pellet (native polysaccharide) or Nujol film (permethylated polysaccharide). GC analysis was carried out with a Shimadzu-14B apparatus (Shimadzu, Japan) equipped with a 3% OV-225/AW-DMCS-Chromosorb W column (2.5 m × 3 mm) and an FID detector. GC–MS was performed on a Shimadzu QP-5050 apparatus (Shimadzu, Japan). Evaporation under vacuum was performed on a Büchi 461 rotary evaporator (Büchi, Switzerland) with a working temperature of <40 °C. TLC analysis was performed on a PEI-cellulose plate (E. Merck, Germany), developed with EtOAc–pyridine–HOAc–water (5:5:1:3). The plate was visualized by spraying with *o*-phthalic acid reagent and heating at 100 °C for 5 min.

2.3. Isolation and purification

The dried roots (1.2 kg) of *P. lobata* were soaked with 95% EtOH for 6 h to remove lipids, followed by filtration. The residue was dried in air and then extracted with boiling water for three times (6 h for each). The filtrate was concentrated, dialyzed, and centrifuged to remove insoluble material and small molecular compounds. The supernatant was added with 3 volumes of 95% EtOH to precipitate crude polysaccharides. The precipitate was recovered by centrifugation and washed successively with absolute EtOH and acetone, followed by drying in vacuo at 45 °C, yielding the crude polysaccharide PLB (48.1 g, recovery 4.0%). PLB (8 g) was subjected to a DEAE–cellulose column (4.5 cm × 50 cm, Cl^-) and eluted stepwise with distilled water, 0.1, 0.2, 0.4, and 1.0 M NaCl. The eluate was monitored by the phenol–sulfuric acid method. The 0.1 M NaCl eluation was concentrated, dialyzed, and lyophilized. The resulting polysaccharide was purified on a Sephadex G-150 column (2.6 cm × 60 cm) and eluted with 0.1 M NaCl. The yield rate of PLB-2C was 0.16% (0.324 g) for the starting material.

2.4. Purity and molecular weight determination

High performance gel permeation chromatography (HPGPC) was carried out with a Waters 515 pump equipped with a Waters UltrahydrogelTM 1000 column and a Waters 2410 RI detector. The column was calibrated with standard T-series Dextran (T-500, T-110, T-80, T-70, T-40, and T-9.3) with 0.003 M NaOAc as the mobile phase at a flow rate of 0.5 mL/min. The sample was prepared as 0.4% (w/v) solutions followed by analysis with 20 μL of solution. The data were processed with Waters GPC (Millennium^{32} software).

2.5. Monosaccharide composition analysis

The polysaccharide (3 mg) was hydrolyzed with 2 M TFA at 110 °C for 3 h, followed by evaporation to dryness. The residue was redissolved in water (0.2 mL). Five microliters of the solution was used for TLC analysis as described previously (Liu, Dong, & Fang, 2001). The other portion was successively reduced with sodium borohydride, acetylated with Ac_2O at 100 °C for 1 h. The resulting alditol acetates were examined by gas chromatography (Dong, Zhang, Lin, & Fang, 1995).

2.6. Methylation analysis

PLB-2C (9 mg) was methylated for four times according to the Ciucanu–Kerek method (Ciucanu & Kerek, 1984). After complete methylation as shown by IR (Nujol), the permethylated polysaccharide was depolymerized in 90% HCO_2H at 100 °C for 3 h followed by hydrolysis in 2 M TFA at 100 °C for another 6 h. After $NaBH_4$ reduction and acetylation, the partially methylated alditol acetates were analyzed by GC–MS.

2.7. NMR spectra

Thirty milligrams of PLB-2C was deuterium-exchanged overnight and redissolved in 0.5 mL D_2O. ^1H and ^{13}C NMR spectra were measured using a Brüker AM-400 NMR instrument equipped with a dual probe in the FT mode at room temperature. Acetone was used as the internal standard. All chemical shifts are expressed in reference to tetramethylsilane.

2.8. Characterization with SEC-LLS

SEC-LLS measurements were carried out on a size exclusion chromatograph combined with multiangle laser photometer. AP100 pump (Thermo separation products, San Jose, CA, USA) equipped with columns of G4000PWXL (MicroPak, TSK) and G6000PWXL (MicroPak, TSK) in 0.1 M aqueous NaCl at 25 °C was used as the SEC instrument. A differential refractive index detector (RI-150, Thermo Separation Products, San Jose, CA, USA) was simultaneously connected. The carrier solutions were 0.1 M aqueous NaCl. The sample was dissolved in 0.1 M aqueous NaCl and set

aside overnight with stirring. The solvents and polysaccharide solutions were purified with a 0.2 μm filter and degassed before use. The injection volume was 200 μL with a concentration of 3 mg/mL for the sample, and the flow rate was 1.0 mL/min in 0.15 M aqueous NaCl. Astra software (version 4.90.07) was utilized for data acquisition and analysis.

2.9. Preparation of sulfate derivatives from PLB-2C

PLB-2C (100 mg) was suspended in dry formamide (10 mL) by stirring at room temperature for 15 min followed by addition of 2 ml sulfating reagents (0.66 mL chlorosulfonic acid and 1.33 mL pyridine). The mixture was maintained at room temperature for 2 h with continuous stirring. The reaction solution was incubated at 40 °C for 4 h before 10 mL methyl alcohol was added. Its pH was adjusted to 7.0 by 2.5 M NaOH solution. Eventually, the reaction solution was dialyzed, concentrated, and lyophilized to achieve sulfated PLB-2C. The degree of substitution (DS) was measured by the $BaCl_2$–gelatin method (Chaidedgumjorn et al., 2002). DS is calculated as $162 \times \%W/(96 - 80 \times \%W)$; %W is the content of SO_4^{2-}.

2.10. Cell culture

PC12 cells (ATCC, American Type Culture Collection, USA) were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were seeded into 96-well plates (Greiner, Germany) at a density of 1×10^5 cells/ml in Dulbcco's Modifed Eagle Medium (Gibco category: 31600-034, USA), which contained 10% heat-inactivated horse serum, 5% fetal bovine serum and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin).

2.11. Antioxidant activity assay

All experiments were carried out 24 h after cells were seeded. Hydrogen peroxide (H_2O_2, 8.8 M solution) was stored at 4 °C until 300 mM stock solution was prepared in phosphate-buffered saline (PBS) on the day of application to the cultures. Cell viability was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide, Sigma–Aldrich, USA) method (Hansen, Nielsen, & Berg, 1989).

Briefly, 10^4 PC12 Cells were pretreated with PLB-2C and PLB-2CS for 2 h before H_2O_2 exposure for 1 h. After the H_2O_2 was withdrawn, cells were maintained in the fresh medium for another 6 h. Then MTT solution was added reaching a final concentration of 0.5 mg/mL and the incubation was maintained for 4 h. Finally, 100 μL dimethyl sulfoxide was added followed by the absorbance measurement at 570 nm using the Universal Microplate Reader (Bio-Tek).

2.12. Statistical analysis

In MTT experiments, two independent experiments were carried out in triplicate. All data were expressed as percent of control value ± *SD*. Statistical analysis was performed using Student's *t*-test. Data were from triplicate cultures for each condition.

3. Results and discussion

3.1. Isolation and purification of PLB-2C

The dried roots of *P. lobata* were defatted in 95% EtOH for three times. The residue was dried in air followed by extraction with boiling water. The concentrated supernatant was precipitated with 3 volumes of 95% EtOH to achieve crude polysaccharide PLB. Then PLB (8 g) was subjected to a DEAE–cellulose chromatography and eluted stepwise with distilled water, 0.1, 0.2, 0.4, and 1.0 M NaCl, respectively. The fraction of 0.1 M NaCl elution was lyophilized. The resulting polysaccharide was purified successively on a Sephadex G-150 column equilibrated and eluted with 0.1 M NaCl, giving PLB-2C (0.324 g). On high-performance gel-permeation chromatography (HPGPC), PLB-2C showed a symmetrical peak (data not shown), indicating a homogenous polysaccharide.

3.2. Structure elucidation of PLB-2C

The average molecular weight of PLB-2C was estimated to be 1.4×10^5. Its specific rotation $[\alpha]_D^{20}$ is +175.7° (*c* 0.5, H_2O). After complete hydrolysis with 2 M trifluoroacetic acid (TFA), TLC analysis showed that the polysaccharide contained no uronic acid (data not shown) (Liu et al., 2001). Monosaccharide composition analysis indicated that it was composed of glucose exclusively. The IR spectrum of PLB-2C was shown in Fig. 1. The bands 3423.1, 2925.5, and 1646.9 cm^{-1} are due to the hydroxyl stretching vibration, C—H stretching vibration and associated water, respectively (Ashoda, Prabha, & Tharanathan, 2005). Absorption at 918.0 cm^{-1} is typical signal for D-Glc in the pyranose form (Barker, Bourne, Stacey, & Whiffen, 1954). Absorption signal at 848.5 cm^{-1} in IR indicates a possible α-D-glucan (Wu & Tu, 2005).

After methylation by the modified Ciucanu–Kerek method (Ciucanu & Kerek, 1984) for four times, the hydroxyl group absorption at 3600–3200 cm^{-1} in IR disappeared (date not shown), indicating the completeness of methylation. The methylated polysaccharide was depolymerized and converted into partially methylated alditol acetates. The analysis of the methylated sugars was conducted by GC–MS (Sweet & Shapiro, 1975). The methylation analysis of the PLB-2C gave only one peak of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D- glucitol (*m*/*z*: 43, 58, 71, 87, 101, 117, 129, 161, 173, 189, 233) (Needs & Selvandran, 1993). Therefore, the results indicated that PLB-2C was a linear α-D-glucan and only contained (1 → 6)-linked-glucopyranosyl residues.

The anomeric proton signal at δ 5.015 ppm in the ^1H NMR (400 MHz) spectrum of PLB-2C showed that the polysaccharide was α-glycosidically linked glucan (Chakraborty, Mondal, Pramanik, Rout, & Islam, 2004; Kim et al., 2000), which was in good agreement with the presence of an IR band at 848.5 cm^{-1}.

In ^{13}C NMR (600 MHz) spectrum of PLB-2C (Fig. 2), the anomeric carbon signal for the 1,6-linked-Glc residue was assigned at δ 100.145 ppm. This also confirmed that the sugar residues were α-glycosidically linked (Chakraborty et al., 2004). The signal at δ 67.964 ppm was assigned to C-6 (Bao, Wang, Dong, Fang, & Li, 2002). The signals at δ 75.838, 73.844, 72.618, and 71.963 ppm corresponded to C-3, C-2, C-5, and C-4 of (1 → 6)-α-D-Glc*p*, respectively (Funane et al., 2001; Kim et al., 2000; Seymour, Knapp, & Bishop, 1976, 1979; Tylianakis, Spyros, Dais, Taravel, & Perico, 1999; Uzochukwu, Balogh, Loefler, & Ngoddy, 2002). These assignments were also corroborated by HSQC experiment (Fig. 3). All the chemical shifts of PLB-2C in the ^{13}C NMR and ^1H NMR spectra were assigned with the help of the HSQC spectrum (Fig. 3) and were summarized in Table 1.

HMBC (Fig. 4) experiment was used to deduce the sequence of glucopyranosyl residues of PLB-2C and to further confirm the assignments made from the HSQC spectrum. In the HMBC spectrum, cross peaks of the H1—C3, H1—C2, H1—C5, H1—C6, C1—H3, C1—H6a, C1—H6b, C6—H4 were assigned (Fig. 4).

According to all these data, the structure of PLB-2C was confirmed unambiguously as:

$$[\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow]_n$$

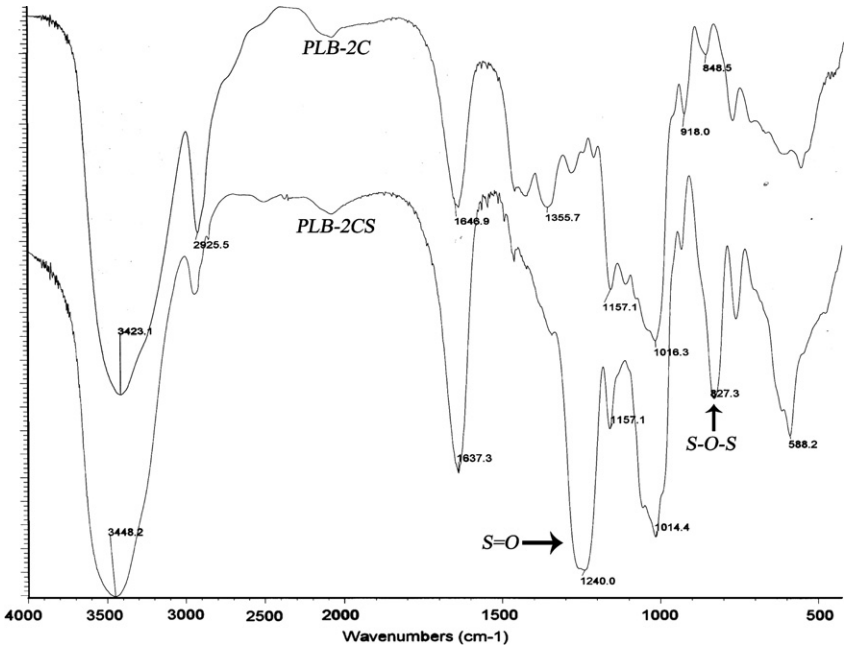

Fig. 1. FTIR spectra of PLB-2C and PLB-2CS.

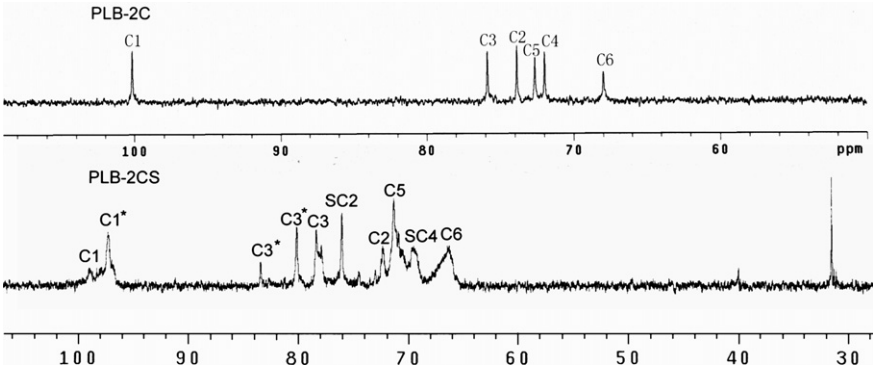

Fig. 2. ^{13}C NMR spectra of polysaccharide PLB-2C and PLB-2CS. C1* peak was a high field shifted resonance of C1 due to the sulphation at C-2. C3* was down-field shifted resonance of C3 resulting from the sulphation at C-2, C-3, and C-4. SC2 and SC4 corresponded to the sulphation at C-2 and C4, respectively.

3.3. Conformation investigation of PLB-2C

The characterization of natural polymers having various chemical components, molar mass, and chain conformation is important because of their critical effect on end-use structure–property relations. The molecular weight and chain conformation of PLB-2C were determined with size exclusion chromatography combined with laser light scattering (SEC-LLS). The SEC-LLS chromatogram patterns of PLB-2C in 0.1 M aqueous NaCl at 25 °C are shown in Fig. 5. The PLB-2C sample exhibits a single peak detected both by LLS and by refractometer. This suggests that there is no aggregation of the PLB-2C polysaccharide in the aqueous solution.

The radius of gyration ($\langle S^2\rangle_z^{1/2}$), weight average molecular weight (M_w), *and polydispersity index* (M_w/M_n) in 0.1 M aqueous NaCl were measured to be 17.9 nm, 1.37×10^5, and 7.8, respectively. More information on chain confirmation could be obtained from SEC chromatograms. The power law of $\langle S^2\rangle^{1/2} = f(M_w)$ can be estimated from many experimental points in the SEC chromatogram (Tao & Zhang, 2006). On the basis of the theory of polymer solution, there are many power laws, such as Mark-Houwink equation ($[\eta] = kM_w^{\alpha}$), and $\langle S^2\rangle_z^{1/2} = k'M_w^{v}$. The exponents of the power laws can be used to describe the molecular conformation of the polymers in solution (Teraoka, 2002, chap. 1). Here, the power law represents radius of gyration ($\langle S^2\rangle_z^{1/2}$) dependences on the weight average molecular weight (M_w) for PLB-2C in aqueous solution, namely $\langle S^2\rangle_z^{1/2} = k'M_w^{v}$.

Fig. 6 shows log–log plots of $\langle S^2\rangle^{1/2}$ vs M_w for PLB-2C in 0.1 M aqueous NaCl at 25 °C. The straight line fitting the experimental points from SEC chromatogram is represented by

$$\langle S^2\rangle^{1/2} = 1.12 \times 10^{-2} M_w^{0.56\pm0.008}$$

The exponent may provide additional insights into polymer solution conformation. Usually, the exponents of 0.33, 0.50–0.60, and 1.0 reflect the chain shape in adapting for sphere, random coil, and rigid rod, respectively (Tao & Zhang, 2006). The low exponent values indicated that the PLB-2C sample exhibited as a random coil conformation in aqueous solution.

3.4. Preparation of sulfate derivatives from PLB-2C

The sulfation derivative of PLB-2C, designed as PLB-2CS, was prepared by the chlorosulfonic acid-pyridine method (Inoue et al., 1983) at 40 °C for 4 h. The ratio between chlorosulfonic acid and pyridine was 1:2. After the reaction, the mixture was adjusted to pH 7.0 and dialyzed against 2 L water for three times followed by lyophilization. In the IR spectra (Fig. 1), the appearance of a

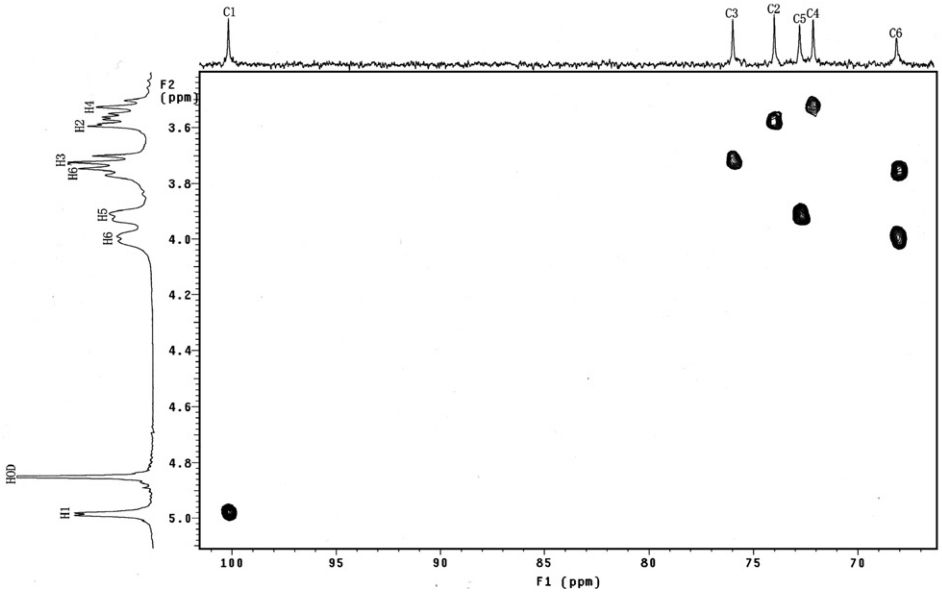

Fig. 3. HSQC spectrum (600 Hz) of PLB-2C.

Table 1
Summary of ^{1}H NMR and ^{13}C NMR chemical shifts for PLB-2C

δ	Position					
	1	2	3	4	5	6
δH	5.015	3.621	3.751	3.529	3.934	4.031[a] 3.773[b]
δC	100.145	73.844	75.838	71.963	72.618	67.964

[a,b] Interchangeable.

S═O signal near 1250 cm^{-1} and a S—O—S signal near 820 cm^{-1} indicated that PLB-2CS was sulfated successfully (Zhang et al., 2005). The degree of substitution (DS) was measured to be 1.18 by the $BaCl_2$–gelatin method (Chaidedgumjorn et al., 2002). The molecular weight of sulfated PLB-2CS was estimated as 1.8×10^4. It was noted that the M_w of the sulfated derivative was lower than that of the original as a result of the degradation of macromolecule in the sulfation process.

In the ^{13}C NMR spectra (Fig. 2) of PLB-2CS, the signals at δ 99.037 and δ 66.263 ppm corresponded to C-1 and C-6, respectively. The signal at δ 78.410 ppm was assigned to C-3. There are other two signals at which δ 83.429 and δ 80.204 ppm were also assigned to C-3. The signal appeared at δ 83.429 ppm was due to the sulfation at C-3, while signal at δ 80.204 ppm was caused by the sulfation at C-2 or C-4 (Mähner, Lechner, & Nordmeier, 2001). There were a high field C-2 resonance at δ 72.394 ppm, and a down field shifted signal of the sulfated C-2 at δ 76.092 ppm. The signals at δ 71.369 and δ 70.945 ppm corresponded to the C-5. The double split might result from a high field shift because of the sulfation at the C-4. The signal at δ 69.538 ppm was probably a high-field shifted resonance of the sulfated C-4 that

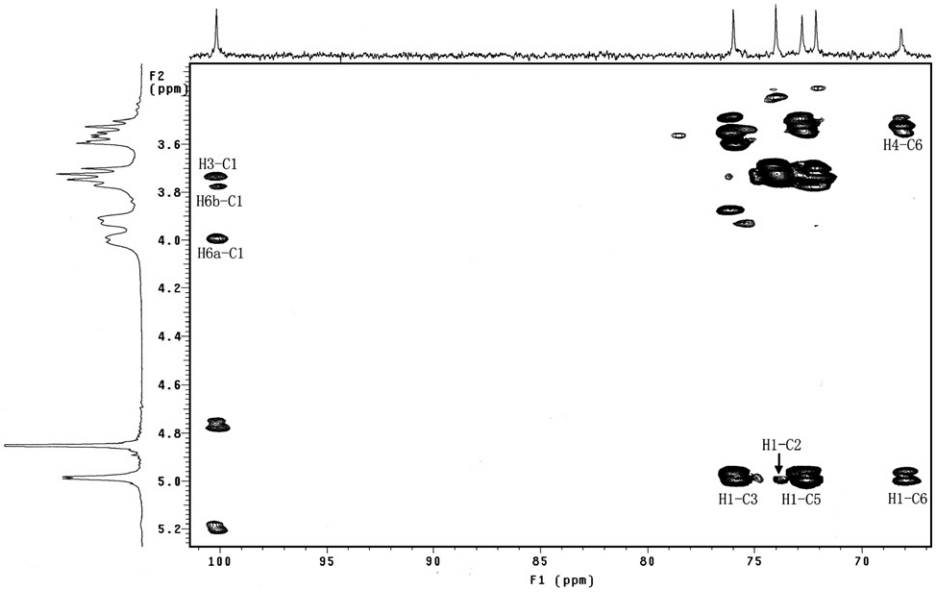

Fig. 4. HMBC spectrum (600 Hz) of PLB-2C.

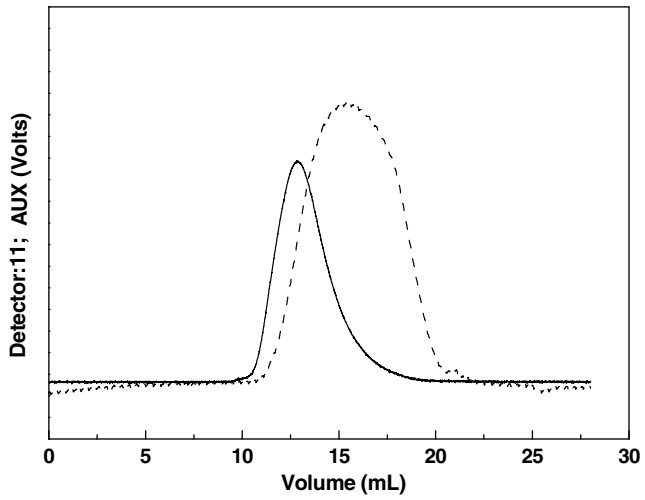

Fig. 5. SEC-LLS chromatograms of PLB-2C. SEC and LLS chromatograms for PLB-2C was detected in 0.1 M aqueous NaCl at 25 °C by laser light scattering photometry (—) and differential refractometer (·····). The detector 11 and AUX values were signals from the LLS at 90° and the refractive index detector.

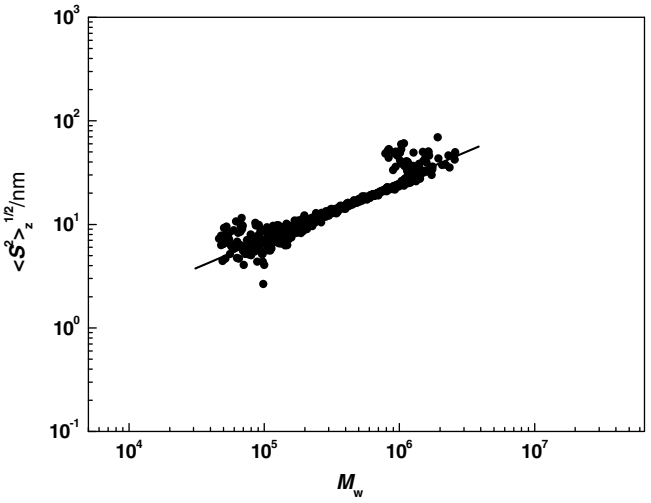

Fig. 6. Plot of $\langle S^2\rangle^{1/2}$ vs *Mw* for PLB-2C in 0.1 M aqueous NaCl at 25 °C.

occurred when a sulfation group was attached at the C-3. In general, the substitution position was deduced to C-2, C-3, and C-4 (Mähner et al., 2001).

3.5. Antioxidant activity analysis of PLB-2C and PLB-2CS by MTT

MTT experiments were employed to test protection effect of PLB-2C and PLB-2CS on PC12 cell from hydrogen peroxide (H_2O_2) induced damage. Results (shown in Fig. 7) indicated PLB-2CS may attenuate the damage on PC12 cells induced by H_2O_2. Fig. 7 showed that pretreatment of the cells with 0.01, 0.1, 1, and 5 mg/mL of PLB-2CS could protect the PC12 from H_2O_2 (150 μM) damage in a dose dependent way, with the cell viability rate of 23.9%, 35.5%, 44.4%, and 51.9%, respectively, comparing to 21.6% of H_2O_2 treatment group. However, pretreatment of the cells with PLB-2C which was not sulfated did not cause significant protection from H_2O_2 injury (Fig. 7).

Heparan sulfate (HS) glycosaminoglycans distributed on cell surface and extracellular matrix (ECM) are complex polysaccharides that play a vital role in many biological processes. It has been demonstrated that heparan sulfate regulates the activity of enzymes such as antithrombin III and superoxide dismutase to confer anticoagulant and antioxidant properties (Rosenberg, Shworak, Liu, Schwartz, & Zhang, 1997; Salmivirta, Lidholt, & Lindahl, 1996; Stringer & Gallagher, 1997). Designing novel drugs (HS-mimetics) that mimic HS sugar sequence is probably a promising approach for some disease therapeutics (Freeman et al., 2005; Funane et al., 2001; Sasisekharan, Shriver, Venkataraman, & Narayanasami, 2002).

It is well known that starch and glycogen, as energy source, which is among versatile roles of carbohydrates in numerous biological settings, both contain (1 → 6)-α-D-glucose branches. Dextran, another glucan with (1 → 6)-α-D-linkage backbone, already has found application in a wide range of processes. Recently, some work also showed that (1 → 6)-α-D-glucan exhibited immunostimulatory activity *in vivo* and *in vitro* (Zhao, Kana, Li, & Chen, 2005). Previous studies also demonstrated that sulfated polysaccharides are potent antioxidant substance (Ruperez et al., 2002; Xue et al., 2001). In this study, we obtained a (1 → 6)-α-D-glucan and its sulfate derivative. Its sulfated derivative PLB-2CS attenuated PC12 cell damage significantly caused by hydrogen peroxide. Recent studies have demonstrated that NF-*kappa*B is involved in cell survival. Overexpression of NF-*kappa*B increases cell viability by suppress-

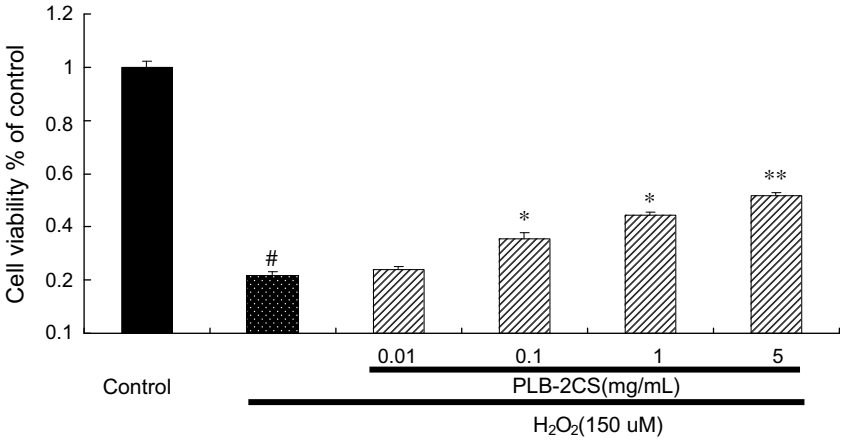

Fig. 7. PLB-2CS attenuated PC12 cell damage induced by hydrogen peroxide.PLB-2CS was added to the medium for 2 h prior to H_2O_2 addition. After the H_2O_2 was withdrawn, cells were maintained in the fresh medium for another 6 h. Cell viability was evaluated by the MTT assay. Two independent experiments were carried out in triplicates. All data were expressed as percent of control value ± *SD*. Statistical comparison was made using Student's *t*-test. $^{\#}p < .05$ vs control; $^{*}p < .05$ vs H_2O_2 group; $^{**}p < .01$ vs H_2O_2 group.

ing induction of apoptosis in various cell types. Cells that become resistant to oxidative cell death exhibit constitutive activation of NF-*kappa*B as an adaptive defense mechanism (Jang & Surh, 2004). We have ever used HEK293 cells which were transfected with NF-*kappa*B luciferase reporter plasmid (pNF-*kappa*B-luc) to screen the sulfated derivative. Results showed that luciferase activity was elevated notably by 1 mg/ml of PLB-2CS (date not shown). Base on this result, we presume that the sulfated derivative might induce cell protection from hydrogen peroxide damage via the NF-*kappaB* activation (Jang & Surh, 2004; Lezoualc'h, Sagara, Holsboer, & Behl, 1998). The sulfation position and density of heparan sulfate occur most frequently as *N*-sulfates but are also present as sulfate esters at the 6-O- or 3-O- position (less commonly) of glucosamine or the 2-O-position of iduronic acid (Gallagher, Lyon, & Steward, 1986). It was demonstrated that Heparan sulfate may function as an antioxidant in the glomerular basement membrane (GBM), such that other molecules are protected from degradation by reactive oxygen species (ROS) (Raats, Bakker, van den Born, & Berden, 1997). From the point of structure–activity relationships (SAR), the structure of PLB-2CS, which was substituted at 2-O, 3-O, 4-O positions and similar to that of Heparan sulfate, might contribute to its activity.

In this report, we obtained one homogeneous polysaccharide from the root of *P. lobata*, namely PLB-2C. Structure study demonstrated that PLB-2C was a (1 → 6)-α-D-glucan with a random coil compact conformation in 0.1 M aqueous NaCl. By antioxidant activity assay, we found that the sulfated derivative PLB-2CS, could significantly attenuate PC12 cell damage caused by hydrogen peroxide. Overall, *P. lobata* may be one of an ideal sources of antioxidants development for both isoflavonoids, such as puerarin and daidzin (Cherdshewasart & Sutjit, 2008), and the sulfate derivatives of glucan, although the glucan in the plant showed no antioxidant activity.

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