JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Chemical Composition and Antioxidant Activity of an Acidic Polysaccharide Extracted from *Cucurbita moschata* Duchesne ex Poiret

XINGBIN YANG,*,† YAN ZHAO,‡ AND YOU LV†

College of Life Sciences, Key Laboratory of Ministry of Education for Medicinal Plant Resource and Natural Pharmaceutical Chemistry, Shaanxi Normal University, Xi'an 710062, China, and Faculty of Pharmaceutical Sciences, Fourth Military Medical University, Xi'an 710032, China

A simple and sensitive high-performance capillary electrophoresis (HPCE) method was designed for quantitative analysis of the component monosaccharides of an acidic polysaccharide extracted from pumpkin. In this method, the polysaccharide was hydrolyzed into component monosaccharides with 2.0 M trifluoroacetic acid at 100 °C for 6 h and then labeled with 1-phenyl-3-methyl-5-pyrazolone, and subsequently the labeled monosaccharide derivatives were separated by HPCE. As a result, glucose (21.7%) and glucuronic acid (18.9%) were identified to be the main component monosaccharides, followed by galactose (11.5%), arabinose (9.8%), xylose (4.4%), and rhamnose (2.8%). Furthermore, the pumpkin polysaccharide was also demonstrated to effectively inhibit the H_2O_2-caused decrease of cell viability, lactate dehydrogenase leakage, and malondialdehyde formation, and also reduced the H_2O_2-caused decline of superoxide dismutase activity and glutathione depletion in cultured mouse peritoneal macrophages, indicating that pumpkin polysaccharide possessed significant cytoprotective effect and antioxidative activity.

KEYWORDS: Pumpkin polysaccharide; monosaccharide composition; capillary electrophoresis; macrophages; antioxidant activity

INTRODUCTION

Pumpkin (*Cucurbita moschata* Duchesne ex Poiret), which belongs to the *Cucurbitaceae* family, is widely grown and consumed as a dietary constituent and agricultural product among peasants in China and Korea. It is rich in polysaccharides, carotene, mineral salts, vitamins, and other substances beneficial to health, resulting in various processed food products being developed (*1*, *2*). Pumpkin has received considerable attention in recent years because of the nutritional and health protective value of the seeds as well as the polysaccharides from the fruits (*1*−*3*). Preliminary investigations showed that a pumpkin-rich diet could reduce blood glucose (*1*, *4*, *5*), and the active polysaccharides from the pumpkin fruit could obviously increase the levels of serum insulin, reduce the blood glucose levels, improve tolerance of glucose, and hence could be developed as new antidiabetic agent (*6*, *7*). Accordingly, the polysaccharide rich in the pumpkin fruits has drawn the attention of researchers and consumers, and an understanding of the chemical and pharmacological properties of pumpkin polysaccharide (PPS) should be of importance from scientific points of view.

Methods and technologies that are available for the analysis of complex polysaccharides are mainly based on determination of their constituent sugar residues, obtained after chemical hydrolysis of the native polymers (*8*). The disadvantages of quantitative analysis of the liberated sugars from polysaccharides originated mainly from incomplete hydrolysis and the necessary derivatization of the monosaccharide (*9*−*11*). Acidic hydrolysis of polysaccharides into uronic acid and neutral sugar has typically been performed using hydrochloric, sulfuric, or trifluoroacetic acid (TFA) at high temperatures. TFA has become the preferred acid because of its high effectiveness in hydrolyzing glycosidic bonds of most polysaccharides without causing extensive destruction of the resulting monosaccharides, and TFA is also easily removed from the hydrolysate because of the high volatility (*11*). The differences in analytical results suggest that hydrolytic techniques should be significantly improved to increase the accuracy for composition analysis of polysaccharides.

In addition, a considerable improvement in the sensitivity of monosaccharide analysis was obtained by the development of high-performance liquid chromatography (HPLC), high-performance capillary electrophoresis (HPCE) based on traditional paper chromatography (PC), thin layer chromatography (TLC), gas chromatography (GC), and GC−MS combination (*12*). Monosaccharides generally have low intrinsic UV spectral activity, and therefore the derivatization of monosaccharides is indispensable to obtain highly sensitive detection (*13*). The reagent 1-phenyl-3-methyl-5-pyrazolone (PMP) is one of the

* To whom correspondence should be addressed. Telephone: +86-29-85310266. Fax: +86-29-85303736. E-mail: xbyang@snnu.edu.cn.
† Shaanxi Normal University.
‡ Fourth Military Medical University.

10.1021/jf070241r CCC: $37.00
Published on Web 05/19/2007

popular labels that react with reducing carbohydrates under mild conditions, requiring no acid catalyst and causing no desialylation and isomerization, and the resulting PMP derivatives can yield strong UV absorbance at 250 nm (*14*−*16*). The derivatization method was first developed for the analysis of monosaccharides by HPLC and later successfully applied to HPCE (*13*, *17*).

Moreover, polysaccharides isolated from natural plants have been regarded as an important class of biological response modifiers. Although many studies on these antidiabetic activities of pumpkin and its polysaccharide ingredient have been carried out (*1*, *4*−*7*), little is known about the antioxidant efficacy of the pumpkin polysaccharide against oxidative stress-induced cell damage. To the best of our knowledge, an excessive amount of reactive oxygen species (ROS) will induce oxidative damage to macromolecules and finally impair functions of organs. This process is especially evident in the immune cells, which exert their biofunctions through free radicals and suffer senescent deterioration probably linked to oxygen stress (*18*).

In view of the above, the present work was for the first time carried out to determine the relative efficacy of pumpkin polysaccharide against hydrogen peroxide (H_2O_2)-induced oxidative stress in isolated mouse peritoneal macrophages. In the second part of this work, the hydrolysis efficiency of the polysaccharide was investigated by performing a PMP procolumn derivatization HPCE analysis for the released monosaccharides from pumpkin polysaccharide, and finally the developed HPCE method was applied for the quantification of component sugar of the pumpkin polysaccharide. Such information would facilitate the use of the polysaccharides in food, pharmaceutical, and other technical applications, which would contribute to the sustainable use of pumpkin agricultural resource.

MATERIALS AND METHODS

Plant Materials. The fruits of pumpkin (*C. moschata* Duchesne ex Poiret) were harvested from the region of Shaanxi (northeast of China), and their mesocarp tissue was separated from the raw pumpkin. The samples were thoroughly washed with tap water, air-dried, and finely powdered.

Chemicals and Reference Compounds. D-Mannose, L-rhamnose, D-glucose, D-galactose, l-arabinose, D-xylose, D-glucuronic acid, D-galacturonic acid, and TFA were purchased from Sigma (St. Louis, MO). Triton X-100 was obtained from Amersco Inc. (Solon, OH). MTT (Thiazolyl blue), dimethyl sulfoxide (DMSO), RPMI1640, phosphate buffer saline (PBS), and fetal bovine serum (FBS) were products of Gibco BRL (Gaithersburg, MD). PMP, purchased from Beijing Reagent Plant (Beijing, China), was recrystallized twice from chromatographic-grade methanol before use. Other chemicals used in the study were of analytic grade.

Extraction of Pumpkin Polysaccharide. The acidic polysaccharide was extracted and purified as previously described (*19*). Briefly, the powder (300 g) from the pumpkin fruits was extracted thrice with 2500 mL of distilled water at 80 °C for 2 h. The filtrate of the obtained extract was condensed in vacuo to a syrup (ca. 500 mL) and precipitated with three volumes of ice cold 95% ethanol (approximate 1500 mL). The crude polysaccharide part was precipitated from the alcoholic liquor during its subsequent standing at 4 °C overnight. The precipitate was collected after centrifugation and then washed sequentially with smaller amounts of ethanol, acetone, and ether, respectively. The refined crude polysaccharide (2.5 g) was redissolved in distilled water (250 mL) at a concentration of 1% (w/v) and intensively dialyzed (MW > 10 000) against distilled water. The retentate portion was deproteinized by the freeze−thaw process (BenchTOP, Virtis Co., Gardiner, NY) for repeating eight times followed by filtration. The filtrate was lyophilized to obtain the polysaccharide-enriched fraction.

Acidic Hydrolysis and PMP Derivatization. The monosaccharide compositions of pumpkin polysaccharide were analyzed according to the following procedure: the pumpkin polysaccharide sample (10 mg) was hydrolyzed with 10 mL of 2.0 M TFA at 100 °C for 6 h to release component monosaccharides. Then, released monosaccharides were derivatized by PMP as described previously (*13*). Briefly, eight monosaccharide standards or the hydrolyzed samples of pumpkin polysaccharide were dissolved in 0.3 M aqueous NaOH (50 μL), and a 0.5 M methanol solution (50 μL) of PMP was added to each. Mannose as an internal standard was added to each sample before the derivatization. Each mixture was allowed to react for 30 min at 70 °C, then cooled to room temperature and neutralized with 50 μL of 0.3 M HCl. The resulting solution was dissolved in chloroform (1 mL). After being shaken vigorously and centrifuged, the organic phase (under aqueous layer) was carefully discarded to remove the excess reagents. The extraction process was repeated three times, and then the aqueous layer was filtered through a 0.45 μm membrane and diluted with water before HPCE analysis.

Analytical Methods of HPCE. The HPCE analysis procedure was performed as described previously with proper modification (*20*). A capillary electrophoresis instrument P/ACE MDQ (Beckman, Fullerton, CA) equipped with a diode array detector and an automatic injector was used. An integrated P/ACE 32 Karat Station (software version 4.0) was used to perform the data collection and to control the operational variables of the system. Separation was carried out in an unmodified fused silica capillary (58.5 cm × 75 μm i.d., effective length 48.5 cm). The cartridge temperature was maintained at 20 °C, and applied voltages were 15 kV. Injection was performed by pressure (0.5 psi for 5 s), and detection was done at 214 nm. The percentage of composition monosaccharides in the sample was calculated from the peak areas using response factors.

Macrophage Isolation and Culture. Peritoneal macrophages were isolated from BALB/c mice (6−8 weeks old, 17−20 g body weight) as described previously (*21*). Briefly, peritoneal macrophages were harvested from BALB/c mice, which had been injected intraperitoneally with 3 mL of thioglycollate 3 days before sterile peritoneal lavage with 10 mL of Hank's balanced salt solution. The collected cells were seeded and cultured in RPMI1640 containing 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at a density 2×10^6 cells/well. The cells were allowed to adhere for 3 h to a 96-well culture plate at 37 °C in a 5% CO_2 incubator. Then the cultures were washed twice with RPMI1640 to remove nonadherent cells prior to the addition of 1 mL of fresh RPMI1640 containing 10% FBS.

The adherent macrophages were pretreated with or without pumpkin polysaccharide for 24 h before exposure to H_2O_2. The control group received vehicle only, and there were three repetitions for each treatment. At the end of the designated treatment, the cultured media were quickly collected, frozen, and stored at −20 °C or immediately used for analyzing the release of extracellular lactate dehydrogenase (LDH). Furthermore, the cultured cells were quickly harvested, washed with ice-cold PBS (0.1 M, pH = 7.4), and centrifuged at 1000*g* to pellet cells. Cells were then suspended in an appropriate volume of buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTANa_2, 0.2 M NaCl, 1% Triton X-100) and subjected to four freeze−thaw cycles to lyse cells, and the yielded cell homogenate was immediately centrifuged at 4 °C for 20 min at 10 000*g*. The afforded supernatant was then divided into several tubes and stored at −20 °C prior to the assays of malondialdehyde (MDA) content, glutathione (GSH) level, and superoxide dismutase (SOD) activity.

Protective Potential of Pumpkin Polysaccharide against H_2O_2-Induced Cytotoxicity. Macrophages were pretreated with the indicated concentration of pumpkin polysaccharide and then exposed to H_2O_2. Colorimetric MTT assay was performed to assess cell viability (*22*). Briefly, 20 μL of MTT (5 mg/mL) in PBS solution was added to each well at a final concentration of 5 mg/mL, and then the plate was further incubated for 4 h. All remaining supernatant was removed, and 100 μL of DMSO was added to each well and mixed thoroughly to dissolve the formed crystal formazan. After 10 min of incubation to ensure all crystals were dissolved, the light absorption was measured at 570 nm. For treated cells, viability was expressed as a percentage of absorbance values to control cells. In addition, the leakage into the media of LDH,

an indicator of cell injury, was detected with an assay kit (Jiancheng BioEngineering, Nanjing, China) according to the manufacturer's instructions as described by Mi and Zhang (*23*). Briefly, at the end of the incubation, 30 μL of cell medium was taken out for the activity analysis of extracellular LDH, which could catalyze the conversion of lactate to pyruvate, and then reacted with 2,4-dinitrophenylhydrazine to give the brownish red color in basic solution. After reaction, each sample was detected, the absorbance was read at wavelength 440 nm, and the results were also expressed as the percentage of LDH leakage versus control cells.

Antioxidant Capacity of Pumpkin Polysaccharide against H_2O_2-Induced Injury. Lipid peroxidation was evaluated by measuring MDA concentration according to the thiobarbituric acid (TBA) method with a commercial kit (Jiancheng BioEngineering) as described previously (*24*). The method was based on the spectrophotometric measurement of the color produced during the reaction to TBA with MDA. MDA concentration was calculated by the absorbance of TBA reactive substances at 532 nm, which were expressed in nanomole/10^6 cells. SOD activity was examined according to a xanthine oxidase method provided by a standard assay kit (Jiancheng BioEngineering) with minor modifications (*25*). The assay used the xanthine−xanthine oxidase system to produce superoxide ions, which react with 2-(4-iodophenlyl)-3-(4-nitrophenol-5-phenlyltetrazoliom chloride) to form a red formazan dye, and the absorbance at 550 nm was determined. The values were expressed as units per 10^6 cells, where one unit of SOD was defined as the amount of SOD inhibiting the rate of reaction by 50% at 25 °C. Intracellular reduced GSH was determined by an Assay Kit (Jiancheng BioEngineering) as described previously (*26*). Briefly, the assay was carried out via an enzymatic recycling reaction of GSH in combination with a chromogenic reaction with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) in mixture contained 30 μL of cell supernatant, 200 μL of PBS buffer (150 mM, pH 7.7), and 20 μL of DTNB in a total volume of 250 μL. After incubation at 30 °C for 5 min, this reaction finally led to the formation of 5-thio-2-nitrobenzoate with yellow color, and the absorption maximum at 412 nm was measured at 412 nm against the blank. The GSH content was determined from the standard curve of known concentration of GSH and expressed as nanomole/10^6 cells.

Statistical Analysis. Data were expressed as means ± SD. Statistical differences between the treatments and the control were evaluated by the student's *t*-test. $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Capillary Electrophoresis Separation. For evaluating acid hydrolytic efficiency of pumpkin polysaccharide and improving the accuracy for sugar composition analysis of pumpkin polysaccharide, a new PMP procolumn derivatization HPCE method was developed. In this study, the HPCE separation of eight standard monosaccharides derivatized with PMP was investigated by changing pH value and borate concentration of buffer. The separation was carried out in an uncoated capillary (58.7 cm × 75 μm i.d., effective length 48.5 cm), and the baseline separations among all the investigated monosaccharides were successfully obtained under the optimum conditions of 200 mM sodium tetraborate decahydrate ($Na_2B_4O_7$•$10H_2O$) as buffer at pH 10.8, applied voltage 10 kV, and capillary temperature 25 °C. A typical electropherogram of PMP derivatives for eight standard monosaccharides is shown in **Figure 1A**, and the peaks were identified in the order of xylose, arabinose, glucose, rhamnose, galactose, mannose, glucuronic acid, and galacturonic acid by comparing the retention time of the unknown peaks with that of the standards under the same conditions.

Optimization of Acid Hydrolysis Procedures. The acid degradation of polysaccharides depends on their conformation, and hence the resistance stress to hydrolysis is altered from one polysaccharide to another. Therefore, an optimization of acidic hydrolysis condition is necessary for the accurate analysis of each polysaccharide. In this study, TFA, an effective acid in degrading polysaccharides, was chose to hydrolyze pumpkin polysaccharide into component monosaccharides, and the factors affecting hydrolysis of pumpkin polysaccharide with TFA were investigated by changing the hydrolysis time, acid concentration, and hydrolytic temperature to achieve complete hydrolysis of the polysaccharide polymer.

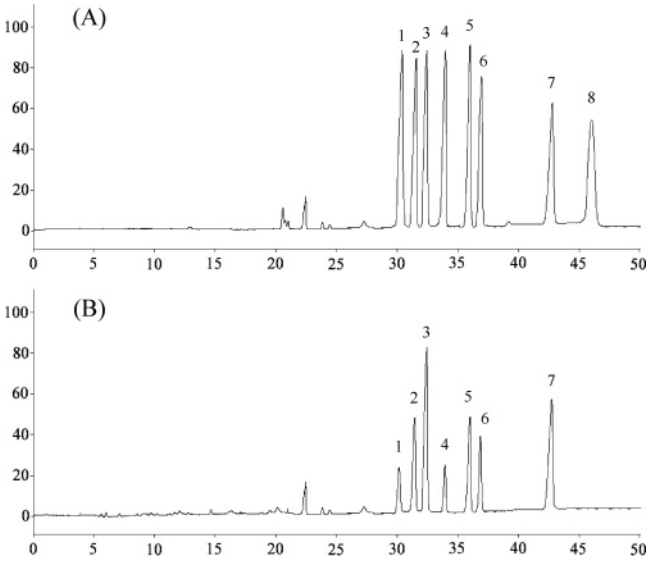

Figure 1. Electropherograms of PMP derivatives of standard monosaccharides (**A**) and acid hydrolysates of pumpkin polysaccharide (**B**) by HPCE. Pumpkin polysaccharide was hydrolyzed with 2.0 M TFA at 100 °C for 6 h. The analysis was carried out on an unmodified fused silica capillary (58.7 cm × 75 μm i.d., effective length 48.5 cm) using 200 mM sodium tetraborate decahydrate as buffer at pH 10.8, applied voltage 10 kV, capillary temperature 25 °C, and detection wavelength at 254 nm. The peaks are identified as follows: 1, xylose; 2, arabinose; 3, glucose; 4, rhamnose; 5, galactose; 6, mannose (internal standard); 7, glucuronic acid; 8, galacturonic acid.

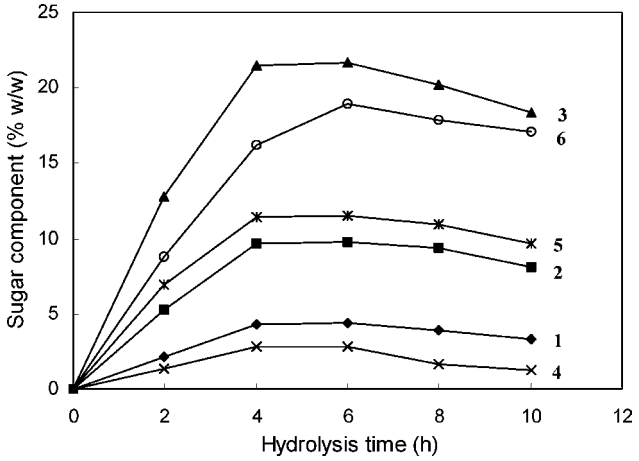

Figure 2. Release of composition monosaccharides (gram/100 g dry sample) from pumpkin polysaccharide subjected to treatment with 2 M TFA at 100 °C versus different hydrolysis times. Hydrolysis procedure and HPCE condition are as described in Materials and Methods. Curves: 1, xylose; 2, arabinose; 3, glucose; 4, rhamnose; 5, galactose; 6, glucuronic acid.

In the experiment, the polysaccharide sample was hydrolyzed with 2.0 M TFA at 100 °C for different times, labeled with PMP for the released component monosaccharides, and then analyzed by HPCE. As shown in **Figure 2**, the release of component monosaccharides in pumpkin polysaccharide was

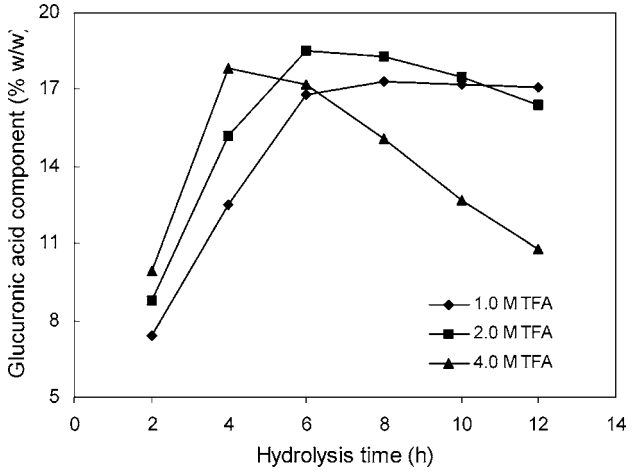

Figure 3. Recovery of glucuronic acid (gram/100 g dry sample) released from pumpkin polysaccharide subjected to treatment with 1.0, 2.0, and 4.0 M TFA at 100 °C versus different hydrolysis times. Hydrolysis procedure and HPCE condition are as described in Materials and Methods.

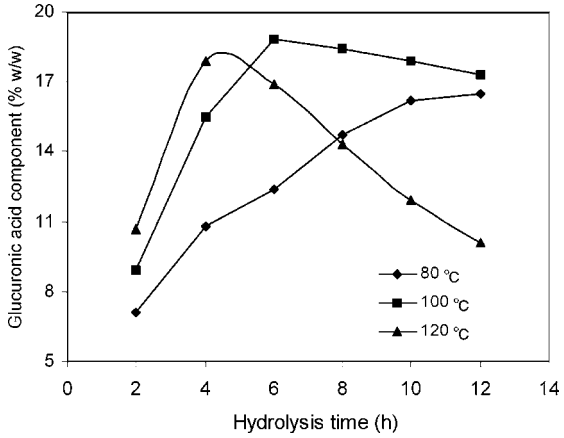

Figure 4. Recovery of glucuronic acid (gram/100 g dry sample) liberated from pumpkin polysaccharide subjected to treatment with 2.0 M TFA at 80, 100, and 120 °C versus different hydrolysis times. Hydrolysis procedure and HPCE condition are as described in Materials and Methods.

significantly dependent on the hydrolysis duration and went through a peak and then decreased. When hydrolysis was carried out after 4 h, the highest recoveries of the neutral sugars such as xylose, arabinose, glucose, rhamnose, and galactose released from pumpkin polysaccharide were achieved, respectively, whereas the depolymerization of component uronic acid (glucuronic acid) was still incomplete (**Figure 2**). When hydrolysis time was prolonged from 4 to 6 h, the total recovery yields of glucuronic acid significantly increased in the range of 16.2−18.9% (w/w %, grams/100 g dry sample) and then decreased slightly, suggesting that uronic acid linkages in the acidic pumpkin polysaccharide were more stable and resistant to TFA hydrolysis than neutral sugar linkages. The incomplete hydrolysis of acidic polysaccharides has been reported to be due to the unusual stability of the glycosyl uronic acid linkage, which hinders quantitative depolymerization under normal acid hydrolysis conditions (*9*). Therefore, the complete hydrolysis of glucuronic acid linkages was very important for the accurate determination of component sugars of uronic acid-rich pumpkin polysaccharide.

To further confirm the influence of acid hydrolysis, the hydrolysis results of pumpkin polysaccharide with different molarity acid (1.0, 2.0, and 4.0 M TFA) at 100 °C for different times were characterized by HPCE analysis for the liberation rate of the glucuronic acid. As depicted in **Figure 3**, although the release of glucuronic acid from pumpkin polysaccharide was considerably accelerated with the increase of TFA concentration, the hydrolysis with 2.0 M TFA for 6 h gave the highest recovery of free glucuronic acid by 18.5%, followed by 17.8% for hydrolysis with 4.0 M TFA for 4 h, and 17.3% for hydrolysis with 1.0 M TFA for 8 h in decreasing order. When hydrolysis time was further prolonged, the destruction rate of glucuronic acid was greater than the release rate from pumpkin polysaccharide, and such destruction was markedly accelerated at higher concentrations of TFA. In addition, similar effects on the liberation of the neutral sugars from pumpkin polysaccharide were observed under the same hydrolysis condition (data not shown). Furthermore, the effect of hydrolysis temperature on glucuronic acid recovery was also investigated. It can be seen in **Figure 4** that, as the duration of hydrolysis increased, there was a corresponding increase in the glucuronic acid recovery and the maximum recoveries were obtained by 18.8% (w/w) and 17.9% (w/w) after hydrolysis at 100 °C for 6 h or 120 °C for 4 h, respectively. For acidic hydrolysis at 80 °C, a lower recovery of glucuronic acid was obtained and an incomplete release of glucuronic acid from pumpkin polysaccharide was observed within tested hydrolysis time (12 h). A further increase in the duration of hydrolysis at 100 °C beyond 6 h and 120 °C beyond 4 h resulted in the decrease of the recovery, reflecting the degradation of the glucuronic acid that had been released. As a result, the hydrolysis with 2.0 M TFA at 100 °C for 6 h was chosen as the optimum condition to achieve the highest hydrolysis yields of pumpkin polysaccharide, basically without affecting the degradation of all the neutral sugars (**Figure 2**).

Table 1. Recoveries of Six Monosaccharides in Sample Analysis ($n = 5$)

component	added amount (μg/mL)	found amount (μg/mL)	recovery (%)	RSD (%)
xylose	15.0	15.2	101.3	3.4
arabinose	15.0	14.9	99.3	4.2
glucose	15.0	14.4	96.0	4.8
rhamnose	15.0	15.1	101.5	2.7
galabtose	15.0	14.5	96.7	3.0
glucuronic acid	15.0	14.7	98.0	4.1

HPCE Analysis of Pumpkin Polysaccharide Sample. For the accurate quantification of component sugar of pumpkin polysaccharide, the HPCE method was validated in terms of reproducibility, linearity, and sensitivity. In this study, the reproducibility was estimated by making five repetitive injections of a standard mixture solution (10 μg/mL for each analyte) under the selected optimum conditions of 200 mM sodium tetraborate decahydrate at pH 10.8, applied voltage 10 kV, and capillary temperature 25 °C. The results showed that all the values of coefficient of variation were shown as lower than 2.0% for the migration time and 3.7% for the corrected peak areas (A/t), which demonstrated that this method has good reproducibility. In addition, to determine the linearities of eight standard monosaccharides, five calibration solutions from 5.0 to 50 μg/mL were tested. As a consequence, a good linearity with the correlation coefficients (r) in the range of 0.9932−0.9998 was obtained by regression analysis between Y (the corrected peak area, A/t) and X (μg/mL), and the detection limits were lower or near 3.1 μg/mL ($S/N = 3$), which showed that this method was very sensitive. Therefore, the validated results indicated that this method was reliable.

Furthermore, the proposed method was applied to determine the monosaccharide composition in the pumpkin polysaccharide sample under the optimum conditions of hydrolysis with 2.0 M TFA at 100 °C for 6 h. A typical electropherogram of pumpkin polysaccharide sample was shown in **Figure 1B**. As we can see from **Figure 1B**, the PMP derivatives of the component monosaccharides released from the sample by TFA hydrolysis could still be baseline separated and the component monosaccharides in pumpkin polysaccharide could be identified by the migration time of analytes as compared with the electropherogram of the standard mixture solution (**Figure 1A**). The HPCE analytical results showed that pumpkin polysaccharide was composed of xylose, arabinose, glucose, rhamnose, galactose, and glucuronic acid in the percentage of 4.4, 9.8, 21.7, 2.8, 11.5, and 18.9% (grams/100 g dry sample), respectively, and glucose and glucuronic acid were the predominant component monosaccharides in pumpkin polysaccharide. Moreover, recovery experiments were further performed five times by adding the six monosaccharides into the pumpkin polysaccharide sample before hydrolysis and making their analytical concentrations as 15.0 μg/mL, and the assay results are summarized in **Table 1**. The results show that the recoveries of all the six monosaccharides range from 96.0 to 101.3% and the RSD values are lower than 4.8%, which means that this method is precise and practical for the analysis of the pumpkin polysaccharide.

Protective Effect of Pumpkin Polysaccharide against H_2O_2-Induced Cytotoxicity. H_2O_2, a precursor of various ROS, was chosen as an injury model in our experiment. As shown in **Figure 5A**, when exposed to 0.2 mM H_2O_2, cell viability by MTT assay was greatly reduced by 36.2%. Furthermore, the viability of the cells pretreated with a low dose of pumpkin polysaccharide (3 and 10 μg/mL) for 24 h before exposure to 0.2 mM H_2O_2 was not increased significantly, and when the polysaccharide concentration increased to 30, 100, and 300 μg/mL, the pretreatment markedly increased the cell viability ($p < 0.05$). In addition, LDH release into the media was usually used as an index of the integrity of cell membranes or necrosis in response to the oxidant burden (*23*). In this study, the protective effects of pumpkin polysaccharide against H_2O_2-induced toxicity to cultured macrophages were also evaluated on the basis of their effects on LDH leakage into media. As shown in **Figure 5B**, the exposure of H_2O_2 to cultured macrophages caused a 1.6-fold increase of LDH leakage ($p < 0.01$). However, this increase was attenuated in a dose-dependent manner by the preincubation of macrophages with the polysaccharide at the concentrations of 10−300 μg/mL for 24 h ($p < 0.05$). When preincubation with pumpkin polysaccharide at high concentration of 100 and 300 μg/mL, H_2O_2-induced LDH leakage was reduced by 30.7 and 26.5% ($p < 0.01$), respectively. In agreement with the results of the MTT assay, pumpkin polysaccharide significantly suppressed the H_2O_2-induced LDH release in cultured macrophages, indicating that the polysaccharide fractions possessed the anticytotoxic activity. Taken together, pumpkin polysaccharide possessed a significant protective effect against the cytotoxicity caused by H_2O_2 in vitro.

Antioxidant Evaluation of the Polysaccharides. For further insight into the activation mechanism, we examined whether the protective effect was associated with lipid peroxidation. From the results in **Figure 6**, it was found that the intracellular MDA production, a well-known biomarker of overall oxidative damage to cellular constituents such as membrane lipids, was significantly increased by about 2.0-fold after the cells were exposed to 0.2 mM H_2O_2 ($p < 0.01$), and when the cells were pretreated with pumpkin polysaccharide at 10, 30, 100, and 300 μg/mL, the MDA formation was remarkably reduced by 12.8, 27.0, 40.5, and 45.3% ($p < 0.05$), respectively. As a result, there was a significant injury of lipid peroxidation when the macrophages were exposed to H_2O_2 and the pumpkin polysaccharide

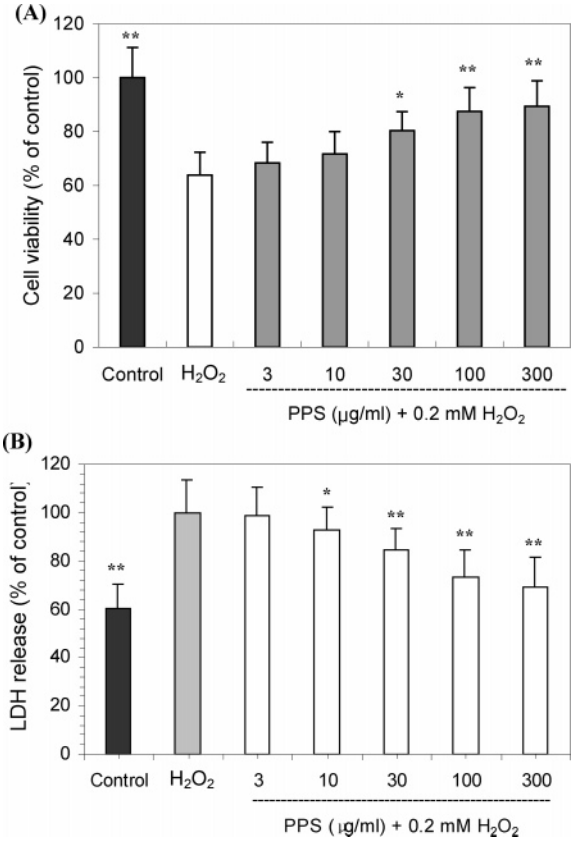

Figure 5. Effects of PPS on H_2O_2-induced cytotoxicity in mouse peritoneal macrophages. Cells were preincubated with indicated concentrations of pumpkin polysaccharide for 24 h and then exposed to 0.2 mM H_2O_2 for another 24 h. Cell viability was assessed by MTT reduction assay, and the cultured medium was collected to assess LDH release. Values represent means ± SD ($n = 5$). *$p < 0.05$, **$p < 0.01$ compared with the positive group treated with H_2O_2 alone.

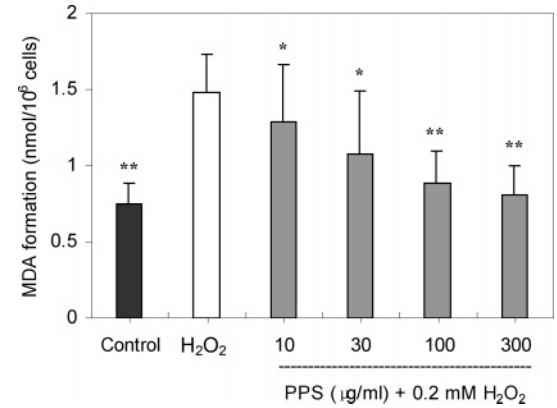

Figure 6. Effects of PPS on H_2O_2-induced MDA formation in macrophages. Macrophages were treated as described in **Figure 5**. The level of lipid peroxidation was evaluated by measuring intracellular MDA content and calculated as nanomole/1 × 10^6 cells. Data are presented as means ± SD of five replicates. *$p < 0.05$, **$p < 0.01$ compared with the positive group treated with H_2O_2 alone.

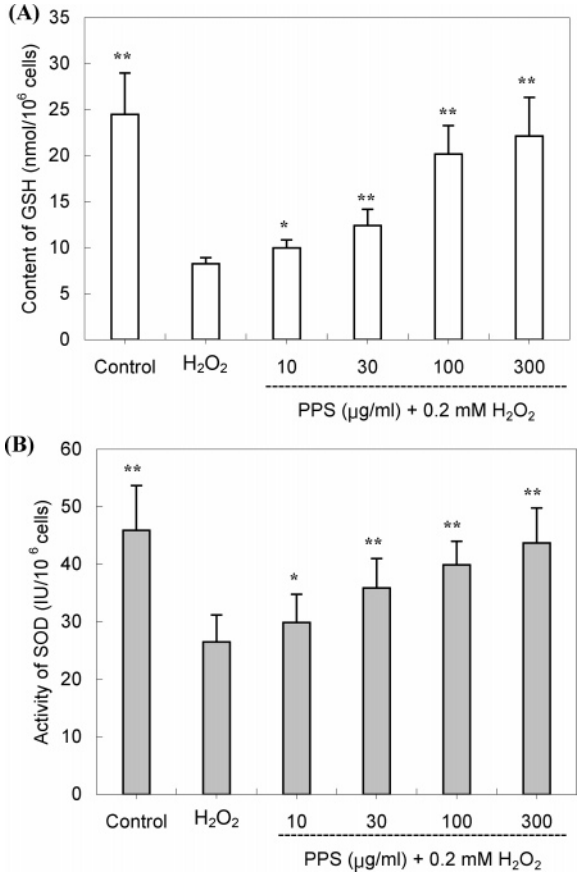

Figure 7. Effects of PPS against H_2O_2-induced oxidative damage in macrophages. Treatment of cells was the same as that in **Figure 5**. GSH level (**A**) and SOD activity (**B**) were assayed as described in Materials and Methods. Values are displayed as the means ± SD ($n = 5$). *$p < 0.05$, **$p < 0.01$ compared with only H_2O_2-treated group.

could exhibit antioxidative effect against the oxidative injury. To further confirm the consequence, the effects of pumpkin polysaccharide on the H_2O_2-induced changes of SOD activity and GSH content in cultured macrophages were further investigated. As shown in **Figure 7A**,**B**, after the cells were exposed to 0.2 mM H_2O_2 for 24 h, intracellular GSH was significantly depleted by about 66.4% ($p < 0.01$) and intracellular SOD activity was decreased by about 42.3% ($p < 0.01$). However, after being preincubated with pumpkin polysaccharide at 10, 30, 100, and 300 μg/mL, GSH levels were recovered to 40.8, 50.6, 82.4, and 90.5% of the control group ($p < 0.05$), respectively, and the pretreatment also rescued the H_2O_2-decreased SOD activity to 65.1, 78.2, 86.9, and 95.2% of the control group ($p < 0.05$), respectively. It was further conformed that pumpkin polysaccharide possessed significantly antioxidative activity.

In conclusion, the present study demonstrated that the developed HPCE method is accurate and useful with high sensitivity and reproducibility for the quantification of pumpkin polysaccharide. Glucose and glucuronic acid were the predominant component monosaccharides in pumpkin polysaccharide. Furthermore, pumpkin polysaccharide exerted a significant cytoprotective effect and antioxidant activity against H_2O_2-induced injury in mouse peritoneal macrophages in vitro. These findings are of significance in the discovery of new functional foods and development of agricultural products.

LITERATURE CITED

(1) Fu, C. L.; Shi, H.; Li, Q. H. A review on pharmacological activities and utilization technologies of pumpkin. *Plant Foods Hum. Nutr.* **2006**, *61*, 70−77.

(2) Zhemerichkin, D. A.; Ptitchkina, N. M. The composition and properties of pumpkin and sugar beet pectins. *Food Hydrocolloids* **1995**, *9*, 147−149.

(3) Fu, C. L.; Tian, H. J.; Cai, T. Y.; Liu, Y.; Li, Q. H. Some properties of an acidic protein-bound polysaccharide from the fruit of pumpkin. *Food Chem.* **2007**, *100*, 944−947.

(4) Li, Q. H.; Tian, Z.; Cai, T. Y. Study on the hypoglycemic action of pumpkin extract in diabetic rat. *Acta Nutr. Sin.* **2001**, *25*, 34−36.

(5) Zhang, Y.; Yao, H. Study on effect of hypoglycemia of different type pumpkin. *J. Chin. Food Sci.* **2002**, *23*, 118−120.

(6) Li, Q. H.; Fu, C. L.; Rui, Y. K.; Hu, G. H.; Cai, T. Y. Effects of protein-bound polysaccharide isolated from pumpkin on insulin in diabetic rats. *Plant Foods Hum. Nutr.* **2005**, *60*, 13−16.

(7) Jun, H. I.; Lee, C. H.; Song, G. S.; Kim, Y. S. Characterization of the pectic polysaccharides from pumpkin peel. *LWT−Food Sci. Technol.* **2006**, *39*, 554−561.

(8) De Ruiter, G. A.; Schols, H. A.; Voragen, A. G. J.; Rombouts, F. M. Carbohydrate analysis of water-soluble uronic acid-containing polysaccharides with high-performance anion-exchange chromatography using methanolysis combined with TFA hydrolysis is superior to four other methods. *Anal. Biochem.* **1992**, *207*, 176−185.

(9) Selvendran, R. R.; March, J. F.; Rings, S. G. Determination of aldoses and uronic acid content of vegetable fiber. *Anal. Biochem.* **1979**, *96*, 282−292.

(10) Benhura, M. A. N.; Chidewe, C. Some properties of a polysaccharide preparation that is isolated from the fruit of *Cordia abyssinica*. *Food Chem.* **2002**, *76*, 343−347.

(11) Garna, H.; Mabon, N.; Nott, K.; Wathelet, B.; Paquot, M. Kinetic of the hydrolysis of pectin galacturonic acid chains and quantification by ionic chromatography. *Food Chem.* **2006**, *96*, 477−484.

(12) Wang, Q. J.; Fang, Y. Z. Analysis of sugars in traditional Chinese drugs. *J. Chromatogr., B* **2004**, *812*, 309−324.

(13) Honda, S.; Akao, E.; Suzuki, S.; Ckuda, M.; Kakehi, K.; Wakamura, J. High-performance liquid chromatography of reducing carbohydrates as strongly ultraviolet-absorbing and electrochemically sensitive 1-phenyl-3-methyl-5-pyrazolone derivatives. *Anal. Biochem.* **1989**, *180*, 351−357.

(14) Honda, S.; Togashi, K.; Taga, A. Unusual separation of 1-phenyl-3-methyl-5-pyrazolone derivatives of aldoses by capillary zone electrophoresis. *J. Chromatogr., A* **1997**, *791*, 307−311.

(15) Honda, S.; Suzuki, S.; Taga, A. Analysis of carbohydrates as 1-phenyl- 3-methyl-5-pyrazolone derivatives by capillary/microchip electrophoresis and capillary electrochromatography. *J. Pharm. Biomed. Anal.* **2003**, *30*, 1689−1714.

(16) Daotian F.; Roger, A. Monosaccharide composition analysis of oligosaccharide and glycoproteins by high-performance liquid chromatography. *Anal. Biochem.* **1995**, *227*, 377−384.

(17) Honda, S.; Yamamoto, K.; Suzuki, S.; Ueda, M.; Kakehi, K. High-performance capillary zone electrophoresis of carbohydrates in the presence of alkaline earth metal ions. *J. Chromatogr., A* **1991**, *588*, 327−333.

(18) Kuo, Y. C.; Sun, C. M.; Tsai, W. J.; Ou, J. C.; Chen, W. P.; Lin, C. Y. Chinese herbs as modulators of human mesangial cell proliferation: Preliminary studies. *J. Lab. Clin. Med.* **1998**, *132*, 76−85.

(19) Yang, X. B.; Zhao, Y.; Wang, Q. W.; Wang, H. F.; Mei, Q. B. Analysis of the monosaccharide components of *Angelica* polysaccharides by high performance liquid chromatography. *Anal. Sci.* **2005**, *21*, 1177−1180.

(20) Zhao, Y.; Yang, X. B.; Sun, X. L.; Jiang, R.; Zhang, S. Y. Enantiomeric separation of synthetic 2,3-dihydroxy-3-phenylpropionate compounds by β-cyclodextrin-modified capillary electrophoresis. *J. Chromatogr., A* **2006**, *1108*, 258−262.

(21) Kim, G. Y.; Choi, G. S.; Lee, S. H.; Park, Y. M. Acidic polysaccharide isolated from *Phellinus linteus* enhances through the up-regulation of nitric oxide and tumor necrosis factor-a from peritoneal macrophages. *J. Ethnopharmacol.* **2004**, *95*, 69−76.

(22) Carmichael, J.; DeGraff, W. G.; Gazdar, A. F.; Minna, J. D.; Mitchell, J. B. Evaluation of a tetrazolium-based semiautomatic colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.* **1987**, *47*, 936−942.

(23) Mi, Y. L.; Zhang, C. Q. Protective effect of quercetin on aroclor 1254-induced oxidative damage in cultured chicken sermatogonial cells. *Toxicol. Sci.* **2005**, *88*, 545−550.

(24) Uchiyama, M.; Mihara, M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal. Biochem.* **1978**, *86*, 271−278.

(25) Sun, Y.; Oberley, L. W.; Ying, L. A simple method for clinical assay of superoxide dismutase. *Clin. Chem.* **1988**, *34*, 497−500.

(26) Van den Berg, J. J.; Op den Kamp, J. A.; Lubin, B. H.; Roelofsen, B.; Kuypers, F. A. Kinetics and site specificity of hydroperoxide-induced oxidative damage in red blood cells. *Free Radical Biol. Med.* **1992**, *12*, 487−498.

Received for review January 27, 2007. Revised manuscript received April 3, 2007. Accepted April 13, 2007. This work was supported by funds from Shaanxi Normal University and the science foundation of Shaanxi Province in China (No. SF200224 and No. 2002K10-G2).

JF070241R