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Structural characterization of water-soluble polysaccharides from *Opuntia monacantha* cladodes in relation to their anti-glycated activities

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Abstract

An aqueous extract of polysaccharides from *Opuntia monacantha* cladodes (POMC) was preliminarily purified by 5 kDa molecular weight cut-off ultrafiltration membrane to remove impurities with low molecular weight. Then the retentate was fractionated by ethanol solution and chromatographed on a DEAE Sepharose Fast Flow anion-exchange column to yield a major fraction (POMC IV) which was eluted by 0.5 M NaCl. POMC IV was subjected to further purification on a Sephadex G-50 gel filtration column. Two major fractions, POMC V and VI, were collected. By analyses using gel permeation chromatography (GPC), high-performance liquid chromatography (HPLC) and gas chromatography (GC), POMC V, which had a molecular weight of 28.7 kDa, was comprised mainly of rhamnose, arabinose and glucose in the molar ratio of 9.15:1.00:6.84, with 3.07% (w/w) of glucuronic acid, while POMC VI, which had a molecular weight of 10.8 kDa, was comprised mainly of rhamnose, mannose and glucose in the molar ratio of 8.72:1.00:6.19, with 4.68% (w/w) of glucuronic acid. Six distinct-absorbance peaks, at 1742, 1633 and 1417 cm^{-1} in the infrared (IR) spectra of POMC V, and at 1729, 1596 and 1407 cm^{-1} in the IR spectra of POMC VI, resulted from the presence of uronic acids. The peaks at 1043 and 890 cm^{-1} were characteristic of rhamonse and β-D-glucose, respectively. From the profiles of ^{13}C and ^{1}H nuclear magnetic-resonance (NMR) spectra, the main (1 → 2)-α-L-rhamnopyranose units were obviously characterized by six strong signals at 99.24 (C-1), 77.52 (C-2), 70.19 (C-3), 71.33 (C-4), 69.81 (C-5) and 17.45 ppm (C-6). The signal at 175.92 ppm was due to C-6 of β-D-glucuronic acid units. The ^{1}H spectrum signal at 1.20 ppm was assigned to the CH_3 of α-L-rhamnopyranose units. The evaluation of anti-glycation activity suggested that POMC had good potential for inhibiting the formation of advanced glycation endproducts. Time- and dose-dependent effects were also observed for all POMC samples.

Keywords: *Opuntia monacantha* cladode; Polysaccharide; Nuclear magnetic resonance; Infrared spectrum; Molecular weight

1. Introduction

Water-soluble polysaccharides, long-chain polymers with high molecular weight, are generally presented in cell wall of plants with a variety of structures (Mazumder, Morvan, Thakur, & Ray, 2004). The medical abilities of water-soluble polysaccharides, to prevent the formation and reformation of post-surgical adhesions, to promote antibacterial activity, to stop bleeding during surgery, and to decrease the levels of glucose, total cholesterol and triglyceride in blood, have attracted the interests of researchers in related fields (Park, Khan, & Jung, 2006; Saenz, Sepulveda, & Matsuhiro, 2004). Glycation is a spontaneous non-enzymatic amino-carbonyl reaction between reducing sugars and proteins. Proteins are modified by glucose through the glycation process, which results in the formation of advanced glycation endproducts. The contribution of advanced glycation endproducts to diabetes and aging has received considerable attention in recent years (Thornalley, Langborg, & Minhas, 1999). It has been reported that antioxidants and radical scavengers can

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inhibit this process (Nakagawa, Yokozawa, Terasawa, Shu, & Juneja, 2002).

Opuntia monacantha is a cactus species widely grown in tropical and subtropical regions (Khales & Baaziz, 2005). It is one of the few crops that can be cultivated in areas which offer little growing possibility for common fruits and vegetables (Ennouri et al., 2007). The use of *Opuntia* cladodes is recommended for beneficial therapeutic properties (Galati, Monforte, Tripodo, Aquino, & Mondello, 2001). It has been employed traditionally as a herbal medicine, for treating diabetes, burns, bronchial asthma and indigestion, in many countries over the world (Kim et al., 2006). A significant amount of polysaccharides exists in the tissues of *Opuntia* cladode and they contribute much to these pharmacological properties.

According to the literature available about the polysaccharides from *Opuntia* cladodes, most reports are concerned with medical functions (Bwititi, Musabayane, & Nhachi, 2000; Trombetta et al., 2006). Structural information on the polysaccharides is still unknown. In the present work, the water-soluble polysaccharides were extracted from *O. monacantha* cladodes and purified using a 5 kDa molecular weight cut-off ultrafiltration membrane, ethanol solutions with different concentrations, DEAE Sepharose Fast Flow anion-exchange column and Sephadex G-50 gel filtration column, successively. The molecular weight was determined by GPC. The monose compositions were evaluated by GC and HPLC. The structural information was further investigated by IR spectrophotometer and NMR spectrometer, respectively. The anti-glycation activities of POMC after successive isolation and purification procedures were also measured.

2. Materials and methods

2.1. Materials

Fresh *O. monacantha* cladodes specimens were collected from Wenchang county, Hainan province of China, in March, 2006. Cladodes specimens were selected for uniformity of shape and maturity.

2.2. Chemicals

L-arabinose, D-xylose, D-mannose, D-glucose, L-rhamnose, D-glucuronic acid and aminoguanidine were purchased from Sigma Chemical Co. (St Louis, MO, USA). Sephadex G-50 and DEAE Sepharose Fast Flow were purchased from Pharmacia Co. (Stockholm, Sweden). Bovine serum albumin was from Huamei Biological Co. (Shanghai, China). All other chemicals used were of analytical grade.

2.3. Extraction of POMC

POMC extraction was conducted by the method of Yang et al. (2006) with some modifications. *O. monacantha* cladodes specimens were washed and cut into pieces. 200 g samples were weighed and mixed with 2000 ml of distilled water in a mixing grinder. The slurry was incubated in a water bath for 2 h at 60 °C and filtered. The residue was subjected to the above programme twice. The filtrates were combined and forced through an ultrafiltration device (Millipore Pellicon XL, Massachusetts, USA), which was operating at 0.20 MPa of pressure, 40 ml/min of flow rate and 25 °C with a 5 kDa molecular weight cut-off membrane. The retentate was concentrated to 100 ml using a rotary evaporator at 65 °C. The proteins in the retentate were removed using the Sevag reagent (Navarini, Gilli, & Gombac, 1999). After removal of the Sevag reagent, one aliquot was added with anhydrous ethanol to a final concentration of 80% (v/v) and the mixture was kept overnight at 4 °C for precipitating polysaccharides (POMC I). Other aliquots were added, with anhydrous ethanol, to a concentration of 50% (v/v) for precipitating polysaccharides with high molecular weight (POMC II). After centrifugation at 8000 rpm for 15 min, more anhydrous ethanol was added to the supernatant to a final concentration of 80% (v/v) for precipitating the polysaccharides with relatively low molecular weight (POMC III). Because of the high anti-glycation activity of POMC III (Fig. 8), it was subjected to further study.

2.4. Isolation and purification of POMC

DEAE Sepharose Fast Flow column (10 × 100 mm) was used to isolate negative and non-negative polysaccharides from POMC III. The column was eluted with distilled water for 50 min, followed by 0.5 M and 1.0 M NaCl for 50 min, sequentially, at a flow rate of 0.3 ml/min. Fractions (1 ml) were collected by a fraction collector. The major polysaccharide fraction, POMC IV (Fig. 1), was concentrated using a rotary evaporator at 65 °C. This fraction was then loaded onto of Sephadex G-50 gel filtration column (10 × 300 mm), and eluted with 35 ml of distilled water at a flow rate of 0.5 ml/min. Fractions (1 ml) were collected. The major polysaccharide peaks, POMC V and VI (Fig. 2), were collected and then freeze-dried. All of

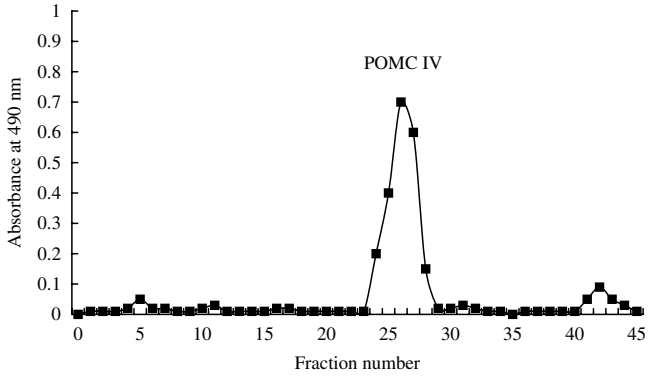

Fig. 1. DEAE Sepharose Fast Flow anion-exchange chromatogram of POMC III.

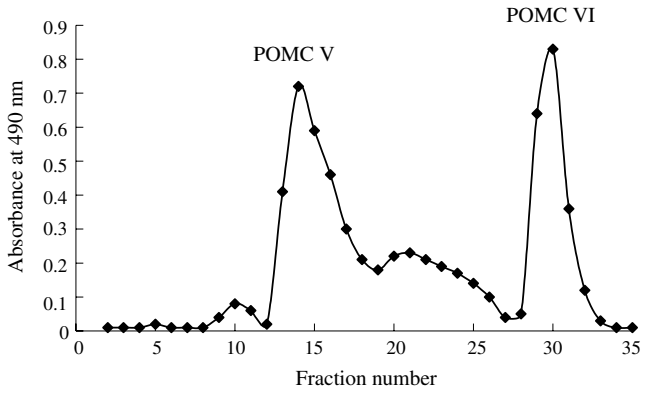

Fig. 2. Sephadex G-50 gel filtration chromatogram of POMC IV.

these fractions were assayed for sugar content by the phenol-sulfuric-acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.5. UV–visible spectrophotometric analysis

One milligram of POMC V (or VI) was dissolved in 10 ml of distilled water. The sample solution was scanned from 200 to 400 nm, using a UV-2102 PC UV–visible spectrophotometer (Unico, Shanghai, China), while the spectra were recorded.

2.6. Analysis of monose compositions

The uronic acid was determined colorimetrically by the 3,5-dimethylphenol method, using glucuronic acid as standard (Scott, 1979).

Twenty milligram of POMC V (or VI) were hydrolysed with 20 ml of 2 M trifluoroacetic acid at 120 °C for 6 h (Erbing, Jansson, Widmalm, & Nimmich, 1995). The resulting solution was concentrated *in vacuo* and the excess of acid was removed by repeated co-distillations with distilled water. The residue was dissolved in distilled water and chromatographed on a DEAE Sepharose Fast Flow column (10 × 100 mm). Elution was carried out with distilled water for neutral sugars, and then with 10% (v/v) aqueous formic acid. The fraction eluted with distilled water was concentrated *in vacuo*, reduced by sodium borohydride and treated with trimethylsilylated reagents according to the method of Guentas et al. (2001). The trimethylsilylated derivatives were loaded onto a HP-5 capillary column of GC, using inositol as internal standard. The operation was performed using the following conditions: H_2: 16 ml/min; air: 150 ml/min; N_2: 20 ml/min; injection temperature: 230 °C; detector temperature: 230 °C; column temperature programming from 130 to 180 °C at 5 °C/min, holding for 2 min at 180 °C, then elevating to 220 °C at 5 °C/min and finally holding for 3 min at 220 °C. The acidic fraction was concentrated *in vacuo* and the residue was dissolved in distilled water (1 ml) and triethylamine (0.02 ml) was added. After 10 min, the product was analysed by HPLC, which was carried out on Waters 2695 Separation Module (Waters, Massachusetts, USA) equipped with a Waters 2487 Dual λ Absorbance Detector (Waters, Massachusetts, USA) and a Whatman Partisil Sax column (250 × 4.6 mm).

2.7. Molecular weight measurement

The molecular weights of POMC V and VI were determined by GPC according to the method of Yamamoto, Nunome, Yamauchi, Kato, and Sone (1995) in combination with a Waters HPLC (Waters, Massachusetts, USA) equipped with a Ultrahydrogel column, a model 410 refractive index detector and a Millennium 32 Workstation. The column was eluted with distilled water at a flow rate of 0.6 ml/min. Dextran standards with different molecular weights (4400, 9900, 21,400, 43,500, 124,000, 196,000, 277,000 and 845,000 Da) were used for calibration curve.

2.8. IR spectral analysis

The structural characteristics of the POMC V and VI fractions were determined using a Fourier transform IR spectrophotometer (Bruker, Karlsruhe, Germany) equipped with OPUS 3.1 software. The purified polysaccharides were ground with KBr powder and then pressed into pellets for transform IR spectral measurement in the frequency range of 4000–500 cm^{-1} (Kumar, Joo, Choi, Koo, & Chang, 2004).

2.9. NMR spectroscopy

^1H NMR experiments were recorded on a Bruker Avance 400 spectrometer (operating frequency of 400.13 MHz). ^{13}C NMR experiments were obtained on the same spectrometer (operating frequency of 100.57 MHz). Samples were recorded as solutions in D_2O at 333 K in 5 mm internal-diameter tubes. The chemical shifts were expressed in ppm relative to the resonance of the internal standard Me_4Si. Spectra were calibrated with internal acetone (δ_H 2.1 ppm, δ_C 31.5 ppm).

2.10. Evaluation of anti-glycation activity

Experiments were performed as described by Wu and Yen (1995) with a minor modification. Bovine serum albumin (50 mg/ml) in phosphate buffer (20 mM, pH 7.4) containing 0.02% (w/w) sodium azide was preincubated with POMC or aminoguanidine at concentrations of 0.1, 0.5 and 1.0 mg/ml for 10 min at room temperature (20 °C). 1.0 M glucose solution was added to the reaction mixture and the whole incubated at 37 °C for 4 weeks. The blank group was prepared using distilled water instead of POMC. Fluorescence was determined every week using a fluorescence spectrophotometer (Perkin–Elmer, Wellesley, USA) with an excitation wavelength of 350 nm and an emission wavelength of 450 nm. The anti-glycation activity was calculated by the following equation:

Anti-glycation activity(%)
= (1 − fluorescence of the sample group
/fluorescence of the blank group) × 100

3. Results and discussion

3.1. Isolation and purification of POMC

POMC III was purified using a DEAE Sepharose Fast Flow anion-exchange column to yield one major peak (POMC IV) and several minor peaks (Fig. 1). The negative polysaccharides, POMC IV, eluted by 0.5 M NaCl accounted for 84.6% (w/w) of POMC III. Thus, POMC IV was subjected to gel filtration on a Sephadex G-50 column and yielded two major fractions (POMC V and VI), with fraction numbers of 14 and 31, respectively (Fig. 2). POMC V and VI were collected for further identification of structure and monose compositions. As shown in Fig. 3, no significant absorbance at or near 280 nm was shown in the UV–visible spectral profiles of POMC V and VI. This indicated that their protein contents were very low.

3.2. Monose compositions and molecular weight of purified POMC fractions

Monose compositions of POMC V and VI were determined by the trifluoroacetic acid hydrolysis method. GC analysis of the trimethylsilyl derivatives showed the presence of rhamnose, arabinose and glucose in the molar ratio of 9.15:1.00:6.84 for the POMC V fraction (Fig. 4), while it also showed the presence of rhamnose, mannose and glucose in the molar ratio of 8.72:1.00:6.19 for the POMC VI fraction. The results indicated that rhamnose and glucose were the major monoses constructing the backbones of POMC V and VI. The uronic-acid contents of POMC V and VI, determined spectrophotometrically by the 3,5-dimethylphenol method, were 3.07% and 4.68% (w/w), respectively. Analysis by HPLC of the hydrolysate of the acidic fraction suggested the uronic acid to be glucuronic acid.

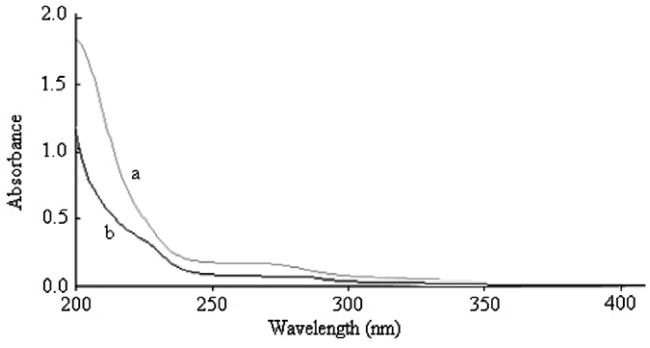

Fig. 3. UV–visible spectra of POMC V and VI from 200 to 400 nm. (a) POMC V and (b) POMC VI.

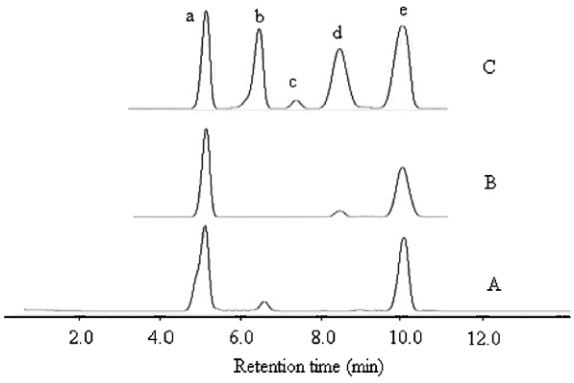

Fig. 4. Gas chromatogram of monose compositions of POMC V (A) and VI (B) Standard monoses (C): a, rhamnose; b, arabinose; c, xylose; d, mannose; e, glucose.

The molecular weights of POMC V and VI were determined by GPC. The equation of the standard curve was drawn as $\log \text{ wt} = 121.9 - 9.17t + 0.242t^2 - 0.00217t^3$ (where wt represents molecular weight, while t represents elution time) using the Millennium 32 software. The molecular weights of POMC V and VI were estimated to be 28.7 and 10.8 kDa, respectively.

3.3. IR spectra of purified POMC fractions

As shown in Fig. 5, the IR spectra of the POMC V and VI fractions displayed a broad stretching intense characteristic peak at around 3435 and 3428 cm^{-1} for the hydroxyl group, and two weak C–H stretching bands at 2931 and 2872 cm^{-1} as well as at 2926 and 2855 cm^{-1}, respectively (Santhiya, Subramanian, & Natarajan, 2002). The peak around 2064 cm^{-1} in the IR spectra of POMC V also indicated aliphatic C–H bonds. Uronic acids were characterized by the carboxylic group which could lead to three absorbance peaks. The band towards 1750 cm^{-1} was attributed to the stretching vibration of C=O in protonated carboxylic acid. Two peaks towards 1620 and 1420 cm^{-1} were attributed to the absorbance of the COO^- deprotonated carboxylic group (Manrique & Lajolo, 2002). Six distinct-absorbance peaks, at 1742, 1633 and 1417 cm^{-1} in the IR spectra of POMC V, and at 1729, 1596 and 1407 cm^{-1} in the IR spectra of POMC VI resulted from the presence of uronic acids. The absorbance of polysaccharides in the range 950–1200 cm^{-1} were where the C–O–C and C–O–H link band positions were found (Kacuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000). Two stretching peaks, at 1116 and 1043 cm^{-1} in the IR spectra of POMC V and at 1122 and 1043 cm^{-1} in the IR spectra of POMC VI, suggested the presence of C–O bonds. Meanwhile, The peak at 1043 cm^{-1} was characteristic of rhamnose. Both POMC fractions showed IR absorption at 890 cm^{-1}, which was characteristic of β-D-glucose. The peak at 600 cm^{-1} was due to O–H out-of-plane vibration (Chiovitti et al., 1997).

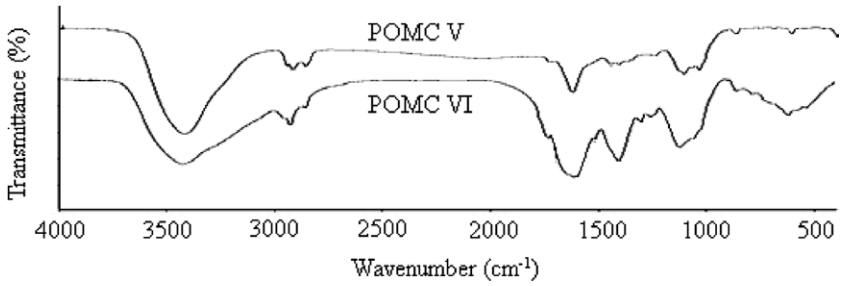

Fig. 5. IR spectra of POMC V and VI in the frequency range 4000–500 cm^{-1}.

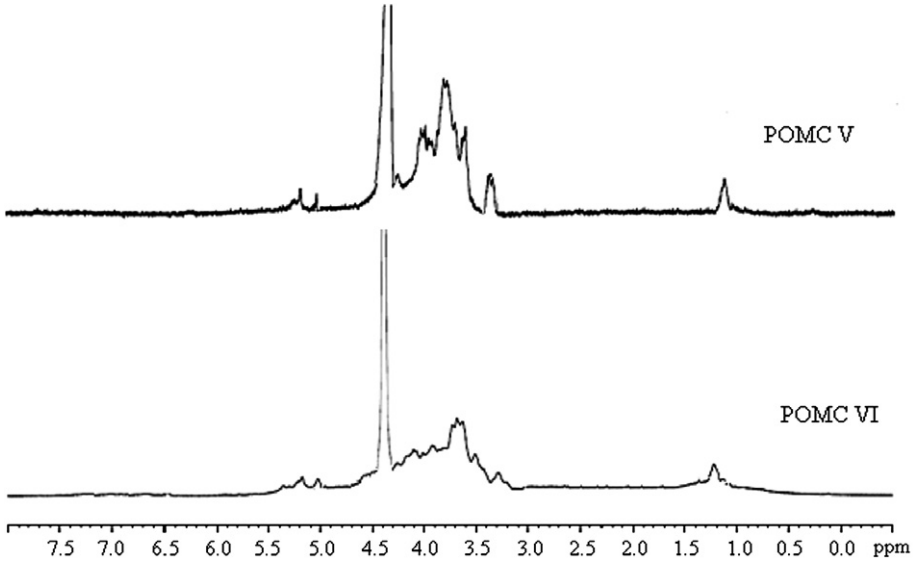

Fig. 6. ^{1}H NMR spectra of POMC V and VI (333 K, 400.13 MHz).

3.4. NMR analysis of purified POMC fractions

The ^{1}H and ^{13}C NMR spectra of POMC V and VI are shown in Figs. 6 and 7. From the profile of ^{13}C spectra, the main (1 → 2)-α-L-rhamnopyranose units were obviously characterized by six strong signals at 99.24, 77.52, 70.19, 71.33, 69.81 and 17.45 ppm, which originated from C-1, C-2, C-3, C-4, C-5 and C-6 of the α-L-rhamnopyranose units (Habibi, Mahrouz, & Vignon, 2005). The signals identified at 103.73, 75.11, 76.79, 77.53, 76.13 and 62.26 ppm could be assigned to C-1, C-2, C-3, C-4, C-5 and C-6 of β-D-glucose (Watt, O'Neill, Percy, & Brasch, 2002). These observations indicated the existence of rhamnose and glucose as the major monose components of POMC V and VI. The signals at 108.57, 81.92, 83.14 and 68.29 ppm in the ^{13}C spectra of POMC V were related to C-1, C-2, C-4 and C-5 of α-L-arabinose. The signals identified at 102.16, 66.91 and 66.15 ppm in the ^{13}C spectra of POMC VI could be assigned to C-1, C-4 and C-6 of α-D-mannose (Silipo et al., 2005). The signal at 175.92 ppm was due to C-6 of β-D-glucuronic acid units. From the profiles of ^{1}H spectra, the signal at 1.20 ppm was assigned to the CH_3 of α-L-rhamnopyranose units, and a broad singlet at 5.26 ppm was assigned to their anomeric H_1 (Habibi et al., 2005). The signal at 4.79 ppm might originate from the anomeric H_1 of β-D-glucuronic acid units (Watt et al., 2002).

3.5. Anti-glycation activity

In the present study, bovine serum albumin was chosen as the model protein and glucose was used as the glycation agent. As shown in Fig. 8, all the samples showed low anti-glycation activities (less than 1%) at the first week. During the first three weeks, higher anti-glycation activities of aminoguanidine at 0.1, 0.5 and 1.0 mg/ml were found than of POMC samples at the same concentration. While, at the fourth week, most of POMC samples showed stronger anti-glycation activity than aminoguanidine, except for 0.1 mg/ml of POMC I and II. This indicated that POMC had a good continuity in inhibiting the glycation process. Time- and dose-dependent effects were also observed for all POMC samples. The anti-glycation activity of POMC was gradually improved after successive isolation and purification procedures. POMC V was the fraction obtained by gel filtration chromatography. It exhibited the highest anti-glycation activity (61.2%) among all POMC samples when a 4 week incubation period was taken and a 1.0 mg/ml concentration was used.

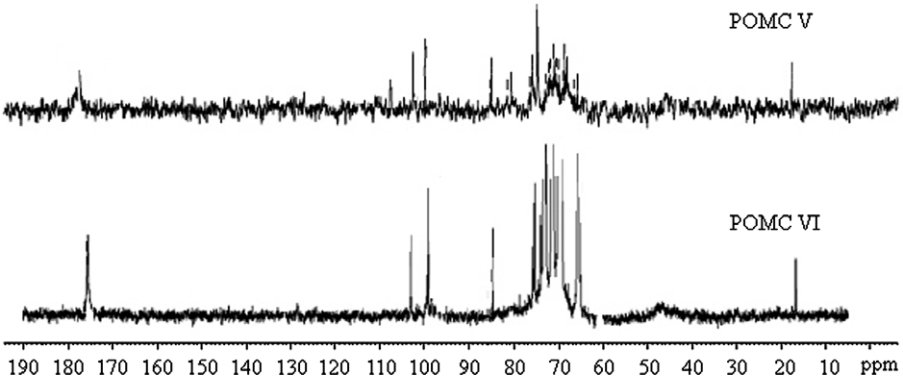

Fig. 7. ^{13}C NMR sepctra of POMC V and VI (333 K, 100.57 MHz).

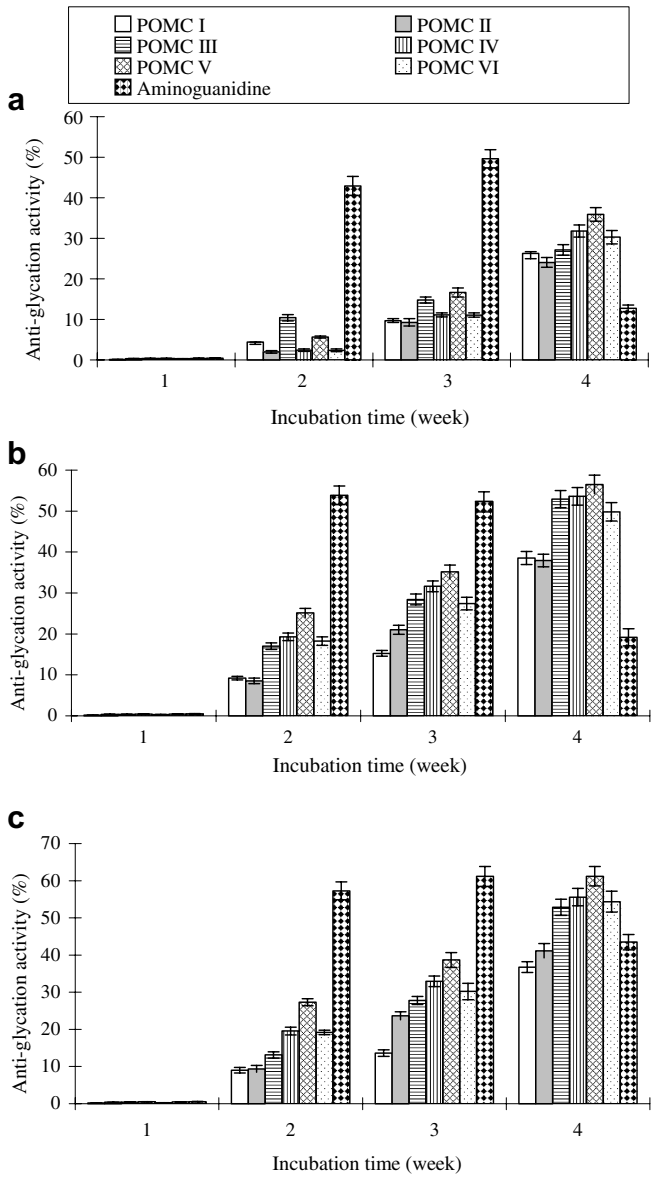

Fig. 8. Anti-glycation activities of POMC I–VI and aminoguanidine at various concentrations for different incubation times. Values are represented as mean ± standard deviation of three replications. (a) 0.1 mg/ml of POMC I–VI and aminoguanidine; (b) 0.5 mg/ml of POMC I–VI and aminoguanidine and (c) 1.0 mg/ml of POMC I–VI and aminoguanidine.

Protein cross-linking and fluorescence formation are the major end results of the glycation reaction. The inhibition of free radical generation derived from the glycation process (and subsequent inhibition of the protein modification) is considered to be one of the mechanisms of the anti-glycation effect (Wu & Yen, 1995). Many dietary antioxidants, such as vitamin C, phenolic acids and flavonoids, have shown ability to reduce *in vitro* and *in vivo* protein glycation (Rahbar & Figarola, 2003). However, the literature about anti-glycation activities of polysaccharides is limited. In this study, POMC showed a stronger anti-glycation activity at the 4th week than did aminoguanidine, which was the first compound to be extensively studied as a powerful inhibitor of advanced glycation endproduct formation (Rahbar & Figarola, 2003). This suggested that POMC might have good potential in treating diabetes complications.

4. Conclusion

POMC V and VI were obtained by isolation and purification with a 5 kDa molecular weight cut-off ultrafiltration membrane, ethanol solutions with different concentrations, DEAE Sepharose Fast Flow anion-exchange column and Sephadex G-50 gel filtration column, successively. By analyses using GPC, HPLC and GC, POMC V, which had a molecular weight of 28.7 kDa, was comprised mainly of rhamnose, arabinose and glucose in the molar ratio of 9.15:1.00:6.84, with 3.07% (w/w) of glucuronic acid, while POMC VI, which had a molecular weight of 10.8 kDa, was comprised mainly of rhamnose, mannose and glucose in the molar ratio of 8.72:1.00:6.19, with 4.68% (w/w) of glucuronic acid. Six distinct-absorbance peaks at 1742, 1633 and 1417 cm^{-1} in the IR spectra of POMC V and at 1729, 1596 and 1407 cm^{-1} in the IR spectra of POMC VI, resulted from the presence of uronic acids. The peaks at 1043 and 890 cm^{-1}were characteristic of rhamonse and β-D-glucose, respectively. From the profiles of ^{13}C and ^{1}H NMR spectra, the main (1 → 2)-α-L-rhamnopyranose units were obviously characterized by six strong signals at 99.24 (C-1), 77.52 (C-2), 70.19 (C-3), 71.33 (C-4), 69.81 (C-5) and 17.45 ppm (C-6). The signal at 175.92 ppm was due to C-6

of β-D-glucuronic acid units. The ^1H spectrum signal at 1.20 ppm was assigned to the CH_3 of α-L-rhamnopyranose units. The evaluation of anti-glycation activity suggested that POMC showed good activity in inhibiting the formation of advanced glycation endproducts. Further chemical analysis should be carried out for better understanding the structure information and anti-glycation methanism of POMC. Harvesting time might affect the structure information on POMC, and it would be interesting to further investigate the effect of this factor.

Acknowledgements

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