



## Structural characterisation of polysaccharides purified from longan (*Dimocarpus longan* Lour.) fruit pericarp

Bao Yang<sup>a</sup>, Yueming Jiang<sup>a,\*</sup>, Mouming Zhao<sup>b</sup>, Feng Chen<sup>c</sup>, Rui Wang<sup>a</sup>, Yulong Chen<sup>a</sup>, Dandan Zhang<sup>a</sup>

<sup>a</sup>South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, PR China

<sup>b</sup>College of Light Industry and Food Sciences, South China University of Technology, Guangzhou 510640, PR China

<sup>c</sup>Department of Food Science & Human Nutrition, Clemson University, SC 29634, USA

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### ABSTRACT

In this work, crude polysaccharides were extracted from longan fruit pericarp by hot water. After removal of proteins and purification by Sephadex G-100 gel filtration column, polysaccharides of longan fruit pericarp (PLFP) were subjected to structural identification. Gas chromatography analysis indicated PLFP comprised of L-arabinofuranose (32.8%), D-glucopyranose (17.6%), D-galactopyranose (33.7%) and D-galacturonic acid (15.9%). The glycosidic linkages were determined by methylation analysis and gas chromatography/mass spectrometry (GC/MS). The results showed that the backbone consisted of  $\rightarrow$ 5)-L-Araf-(1 $\rightarrow$ ,  $\rightarrow$ 6)-D-Glcp-(1 $\rightarrow$ ,  $\rightarrow$ 3)-D-Galp-(1 $\rightarrow$ ,  $\rightarrow$ 3)-D-GalpA-(1 $\rightarrow$  and  $\rightarrow$ 6)-D-Galp-(1 $\rightarrow$  with a molar proportion of 2:1:1:1. The infrared spectra and nuclear magnetic resonance (NMR) spectra further confirmed that the configuration of L-arabinofuranose was of  $\alpha$ -form, while D-glucopyranose, D-galactopyranose and D-galacturonic acid were of  $\beta$ -form. The molecular weight of PLFP was measured to be 420 kDa by gel permeation chromatography. By determination of the anti-glycated activity, PLFP showed a good potential in inhibiting the glycation reaction *in vitro*.

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### 1. Introduction

It is well known that polysaccharides play an important role in the growth and development of living organisms. In addition, there is much evidence that polysaccharides possess a variety of bioactivities, such as antioxidation, immunomodulation, anti-tumour, and hypoglycemic activity (Kardošová & Machová, 2006; Schepetkin & Quinn, 2006). Therefore, polysaccharides from various sources have recently emerged as an important class of bioactive natural products (Dourado et al., 2004). For example, some botanical polysaccharides have been commercially developed into important components of therapeutic drugs and skin care products (Deters, Dauer, Schnetz, Fartasch, & Hensel, 2001; Wang & Fang, 2002).

Longan (*Dimocarpus longan* Lour.) is an exotic subtropical fruit mainly planted in Southeast Asia, especially in China (Yang, Zhao, Shi, Yang, & Jiang, 2008b; Yang et al., 2008a). It is a desirable fruit well accepted by consumers over the world due to its sweet juicy mouthfeel and good health effect (Rangkadilok et al., 2007). In addition, its flesh, seed and pericarp have often been used as bioactive ingredients in many traditional Chinese medicines for different treatments, such as improving women's health after giving birth to a child and increasing the immunomodulatory capacity. Our previous work indicated that longan fruit pericarp contains a

large quantity of polysaccharides (Yang et al., 2008a; Yang et al., 2008b), but their structural information still remains unknown. Therefore, it is worthwhile to purify the polysaccharides and characterise their structures.

In the present study, crude polysaccharides were extracted from longan fruit pericarp by hot water. After purification by gel filtration chromatography, the molecular weight and monosaccharide composition were determined. Gas chromatography/mass spectrometry, Fourier infrared spectrophotometry and NMR spectrometry were employed to identify the chemical structure. The anti-glycated activity of polysaccharides of longan fruit pericarp (PLFP) was also evaluated.

### 2. Experimental

#### 2.1. Plant materials

Fresh longan (*D. longan* Lour. cv. Shixia) fruits at the commercially mature stage were purchased from a local commercial market in Guangzhou, China. Fruits were selected for their uniformity in shape and colour.

#### 2.2. Chemicals

Glucose, phenol and sulphuric acid were obtained from Guangzhou Reagent Co. (Guangzhou, China). Monosaccharide standards

\* Corresponding author. Tel.: +86 20 37252525; fax: +86 20 37252831.

E-mail address: [yjiang@scbg.ac.cn](mailto:yjiang@scbg.ac.cn) (Y. Jiang).

were purchased from Sigma Chemical Co. (St. Louis, MO). All the other chemicals used were of analytical grade.

### 2.3. Extraction and quantification of PLFP

PLFP were extracted by hot water following the method of Zhao, Yang, Yang, Jiang, and Zhang (2007). Briefly, the longan fruit pericarps were air-dried and crushed using a mortar and pestle. After passing through a 60-mesh stainless steel sieve, four grams of the fine pericarp powder was extracted with 100 ml of distilled water in a 150-ml conical flask submerged in a water bath at 55 °C for 120 min. The extract was then filtered through a Whatman No. 1 paper (Whatman plc., Shanghai, China), and concentrated to 25 ml using a vacuum rotary evaporator (BC-R203, Shanghai Biochemical Equipment Co., Shanghai, China) at 65 °C. The proteins in the extract were removed by the Sevag reagent (Navarini et al., 1999). Then, 100 ml of anhydrous ethanol was added into the extract and maintained overnight at 4 °C to precipitate the polysaccharides, which was then obtained by centrifugation at 3,860 g for 15 min.

The content of polysaccharides was determined by the phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), by which the recovery of PLFP was expressed as mg galactose equivalents /g dry pericarp, wherein galactose was prepared in a series of concentrations to make a standard curve for the PLFP calculation. Protein content was estimated using Folin-Ciocalteu reagent and bovine serum albumin was taken as standard (Lowry, Rosebrough, Farr, & Randall, 1951).

### 2.4. Separation and purification of PLFP

One milliliter of 1 mg/ml PLFP was loaded onto a Sephadex G-100 gel column (10 × 500 mm), eluted with 25 ml of distilled water at a flow rate of 0.2 ml/min. Each fraction with 1 ml of eluate was collected. All these fractions were determined by the phenol-sulphuric acid method. The chromatography profile was drawn by Microsoft Excel 2000 (Microsoft, Seattle, WA). The peak with the highest polysaccharide content was collected and then freeze-dried.

### 2.5. Measurement of molecular weight

The molecular weight of the purified PLFP was determined by gel permeation chromatography using the method of Yamamoto, Nunome, Yamauchi, Kato, and Sone (1995). A high-performance liquid chromatography instrument (Waters 5215, Milford, MA) was employed, which was equipped with an Ultrahydrogel column, a model 410 Refractive index detector and operated by the Millennium 32 Workstation. The column was eluted by distilled water at a flow rate of 0.6 ml/min. The PLFP molecular weight was calculated by comparison to a calibration curve made with dextran standards of different molecular weights (4 400, 9 900, 21 400, 43 500, 124 000, 196 000, 277 000 and 845 000 Da).

### 2.6. Infrared spectrum analysis of PLFP

The infrared spectrum of PLFP was determined using a Fourier transform infrared spectrophotometer (Bruker, Ettlingen, Germany) equipped with OPUS 3.1 software. The chromatographically purified PLFP was ground with KBr powder and then pressed into pellets for transformation infrared spectra measurement in a frequency range of 4000–500 cm<sup>-1</sup> (Parikh & Madamwar, 2006).

### 2.7. Analysis of monosaccharide compositions

PLFP (10 mg) was hydrolysed by 10 ml of 2 M trifluoroacetic acid at 100 °C for 4 h (Erbing, Jansson, Widmalm, & Nimmich,

1995). Derivatisation of the released monosaccharides was then carried out using the trimethylsilylation reagent according to the method of Guentas et al. (2001). The trimethylsilylated derivatives were loaded onto a HP-5 capillary column and determined by a flame ionisation detector. The following program was adopted for gas chromatography analysis: injection temperature: 230 °C; detector temperature: 230 °C; column temperature programmed from 130 to 180 °C at 5 °C/min, holding for 5 min at 180 °C, then increasing to 220 °C at 5 °C/min and finally holding for 3 min at 220 °C. Nitrogen was used as the carrier gas and maintained at 1.0 ml/min. Inositol was used as the internal standard.

### 2.8. Methylation analysis

Methylation of PLFP was carried out using the method of Needs and Sevendran (1993) with minor modifications. Two milligrams of dry PLFP was weighed precisely and dissolved in 2.0 ml of DMSO before 200 mg of NaOH was added. The mixture was then treated with an ultrasonic wave attached to an ultrasonic cleaner (KQ-300DE, Kunshan Ultrasonic Equipment Co., Kunshan, China, 40 kHz) for 10 min. After incubation for 1 h at room temperature (25 °C), methyl iodide (1.5 ml) was added for PLFP methylation. The sample was kept in darkness for 1 h before 2.0 ml of distilled water was used to decompose the remained methyl iodide. The methylated polysaccharides were extracted with 3 × 2 ml of chloroform and dried at low pressure on a rotary evaporator (RE52AA, Yarong Instrument Co., Shanghai, China). After hydrolysis with 2 M trifluoroacetic acid, the PLFP hydrolysates were dissolved in 2 ml of 0.2% (m/m) NaOH. Twenty milligrams of NaBH<sub>4</sub> was added to reduce the uronic acid. After incubation at 40 °C for 30 min, 100 µl of glacial acetic acid was used to terminate the reduction. The sample was dried under low pressure, and then acetylated by addition of 2 ml of acetic anhydride and 2 ml of pyridine. The reaction was kept at 100 °C for 1 h. Two milliliters of distilled water was used to decompose the remained acetic anhydride. The acetylated derivatives were extracted with 4 ml of methylene chloride. A gas chromatography / mass spectrometer (GCMS-QP 2010, Shimadzu, Kyoto, Japan) was used to analyse the glycosidic linkage. The acetylated derivatives were loaded into a HP-1 capillary column. The temperature program was set as follows: the initial temperature of column was 150 °C, increased to 180 °C at 10 °C/min, then to 260 °C at 15 °C/min, held for 5 min at 260 °C; injection temperature: 180 °C. The ion source of mass spectrometer was set at 200 °C.

### 2.9. NMR spectroscopy analysis

Two milligrams of PLFP was dissolved in 0.5 ml of dimethyl sulfoxide. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded by a Bruker DRX-400 spectrometer (Bruker, Rheinstetten, Germany) at 25 °C. Chemical shift was expressed in ppm. Tetramethylsilane was used as an internal standard.

### 2.10. Determination of anti-glycated activity

The anti-glycated activity was determined by the method of Tang et al. (2004). A stock solution of 5% bovin serum albumin, 1 M glucose and 0.1% sodium azide in pH 7.4 phosphate buffer was prepared. PLFP or aminoguanidine was dissolved in the stock solution to a concentration of 0.1 or 0.5 mg/ml, respectively. The control group was free of PLFP or aminoguanidine. Bacteria were removed by membrane filtration with 0.2 µm pore size. The solution was held in a 1.5 ml centrifuging tube and incubated in the dark at 37 °C for one, two or four weeks. The content of advanced glycated end product was determined at an excitation wavelength of 370 nm and emission wavelength of 440 nm by a fluorospectro-

photometric method. The percentage of anti-glycated activity was calculated as  $(A_{\text{con}} - A_{\text{sample}}) / A_{\text{con}}$ .  $A_{\text{con}}$  represents the fluorescent determination of the control group.  $A_{\text{sample}}$  represents the fluorescent determination of the sample group.

### 2.11. Statistical analyses

Data were expressed as means standard deviations (SD) of three replicated determinations. Statistical calculation by OriginPro Version 7.5 software (OriginLab Corporation, Northampton, USA) was carried out. One way of variance analysis was applied for determining significant difference at  $P < 0.05$ .

## 3. Results and discussion

### 3.1. Extraction and purification of PLFP

Hot water was used to extract the crude water-soluble polysaccharides, which was determined to be  $10.6 \pm 0.7$  mg galactose equivalents/g dry pericarp, and accounted for  $93.3 \pm 2.7\%$  of the dry extract. In contrast, the protein content in the aqueous extract only accounted for  $1.1 \pm 0.1\%$ , which was due to the removal by the Sevag reagents at the first step of purification. Fig. 1 shows the gel filtration chromatogram of crude PLFP eluted by distilled water. Two major peaks were detected in the profile. The first peak which accounted for 65.4% of the total PLFP was collected. Through comparing with the dextran standard, which molecular weight was 10 kDa, the second peak represented a polysaccharide fraction which molecular weight was lower than this size.

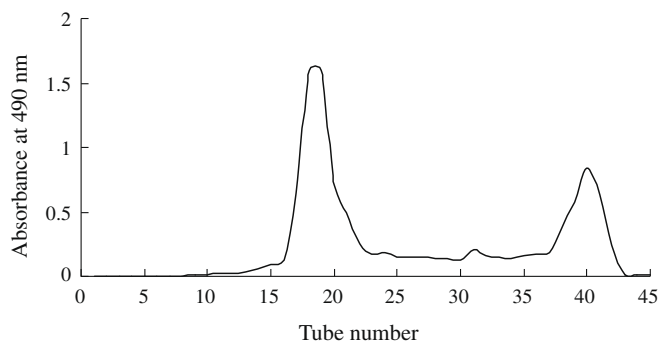


Fig. 1. Gel filtration chromatogram of PLFP using a Sephadex G-100 gel column.

### 3.2. Identification of structure and molecular weight of PLFP

High performance gel permeation chromatography is often employed to determine the molecular weight of polysaccharide (Geresh, Adin, Yarmolinsky, & Karpasas, 2002). Based on the equation of the standard curve made by different dextran standards and the retention time of PLFP, the molecular weight of the purified PLFP was estimated to be 420 kDa.

The purified PLFP was hydrolysed by TFA into individual monosaccharides that were further trimethylsilylated for gas chromatography analysis. The results are shown in Fig. 2. Four monosaccharides, including L-arabinofuranose, D-glucopyranose, D-galactopyranose and D-galacturonic acid, were identified after comparison with the monosaccharide standards. Their molar percentages were 32.8%, 17.6%, 33.7% and 15.9%, respectively. Methylation by methyl iodide is a classical method to form a methoxyl group on the polysaccharide. The methylated PLFP was subjected to acid hydrolysis by TFA and then acetylation. A GC/MS instrument was employed to analyse the derivatives. Table 1 lists the glycosidic linkages of these monosaccharide residues. D-galacturonic acid residue could not be observed in the GC/MS results, due to the reduction into D-galactose by  $\text{NaBH}_4$  before acetylation. Only two types of glycosidic linkage were found for D-Gal from Table 1. However, D-galacturonic acid can not exist as  $\rightarrow 6$ -D-GalpA-(1 $\rightarrow$ ) due to the uronic acid group, it was deduced that all the D-GalpA residues were linked as  $\rightarrow 3$ -D-GalpA-(1 $\rightarrow$ ). By calculation of the molar percentage, the proportion of  $\rightarrow 5$ -L-Araf-(1 $\rightarrow$ ),  $\rightarrow 6$ -D-Glcp-(1 $\rightarrow$ ),  $\rightarrow 3$ -D-Galp-(1 $\rightarrow$ ),  $\rightarrow 3$ -D-GalpA-(1 $\rightarrow$ ) and  $\rightarrow 6$ -D-Galp-(1 $\rightarrow$ ) was approximately 2:1:1:1:1. Moreover, D-Glcp-(1 $\rightarrow$ ) was also found in trace amounts. It indicated that glucose was the first monosaccharide of the backbone.

Fig. 3 shows the infrared spectra of the purified PLFP. A broad stretching intense characteristic peak was shown at around  $3396 \text{ cm}^{-1}$  for the hydroxyl groups, and a weak C–H stretching band was observed at  $2929 \text{ cm}^{-1}$  (Santhiya, Subramanian, &

Table 1

Molar percentages of the glycosidic linkages of PLFP deduced from methylation analysis.

Glycosidic linkage	Molar percentage
$\rightarrow 5$ -L-Ara-(1 $\rightarrow$ )	34.3%
$\rightarrow 6$ -D-Glc-(1 $\rightarrow$ )	16.9%
$\rightarrow 3$ -D-Gal-(1 $\rightarrow$ )	32.0%
$\rightarrow 6$ -D-Gal-(1 $\rightarrow$ )	16.8%

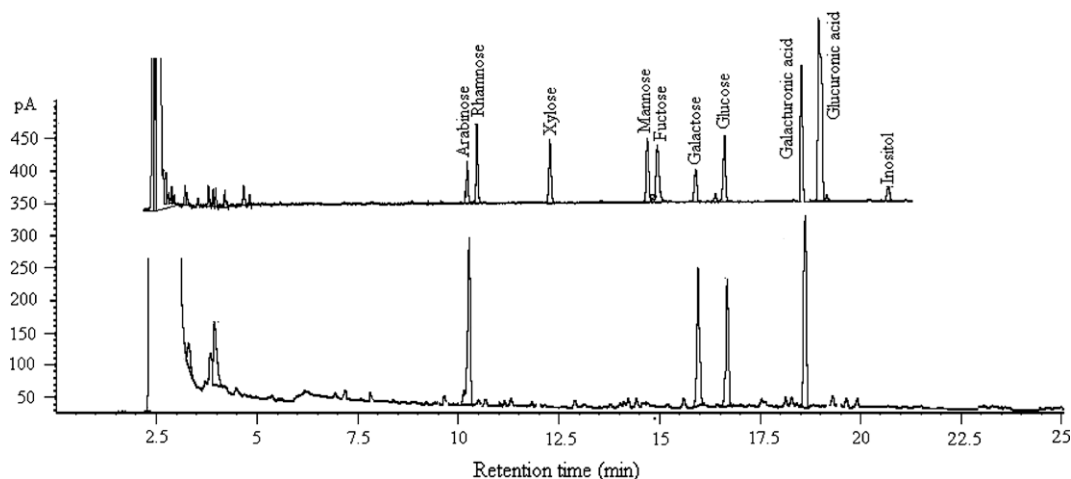


Fig. 2. Gas chromatogram of monosaccharide composition of PLFP. The upper profile represents the sugar standards. The bottom profile represents the PLFP tested.

Natarajan, 2002). The peak around  $2169\text{ cm}^{-1}$  in the infrared spectra also indicated the aliphatic C–H bonds. Uronic acids were characterised by the carboxylic group which could produce three absorbance peaks. The band towards  $1750\text{ cm}^{-1}$  was attributed

to stretching vibration of C=O in the protonated carboxylic acid. Two peaks towards  $1620\text{ cm}^{-1}$  were attributed to the absorbance of the  $\text{COO}^-$  deprotonated carboxylic group (Manrique & Lajolo, 2002). Two distinct absorbance peaks at  $1732$  and  $1618\text{ cm}^{-1}$  in

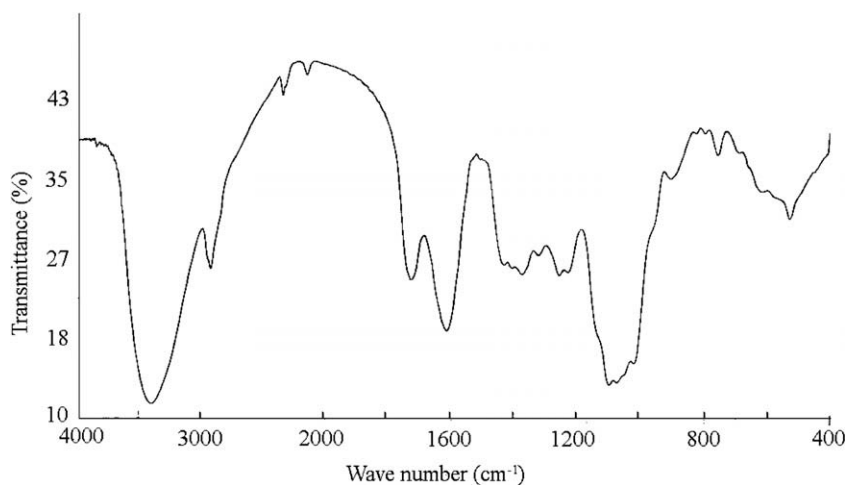


Fig. 3. Infrared spectra of PLFP.

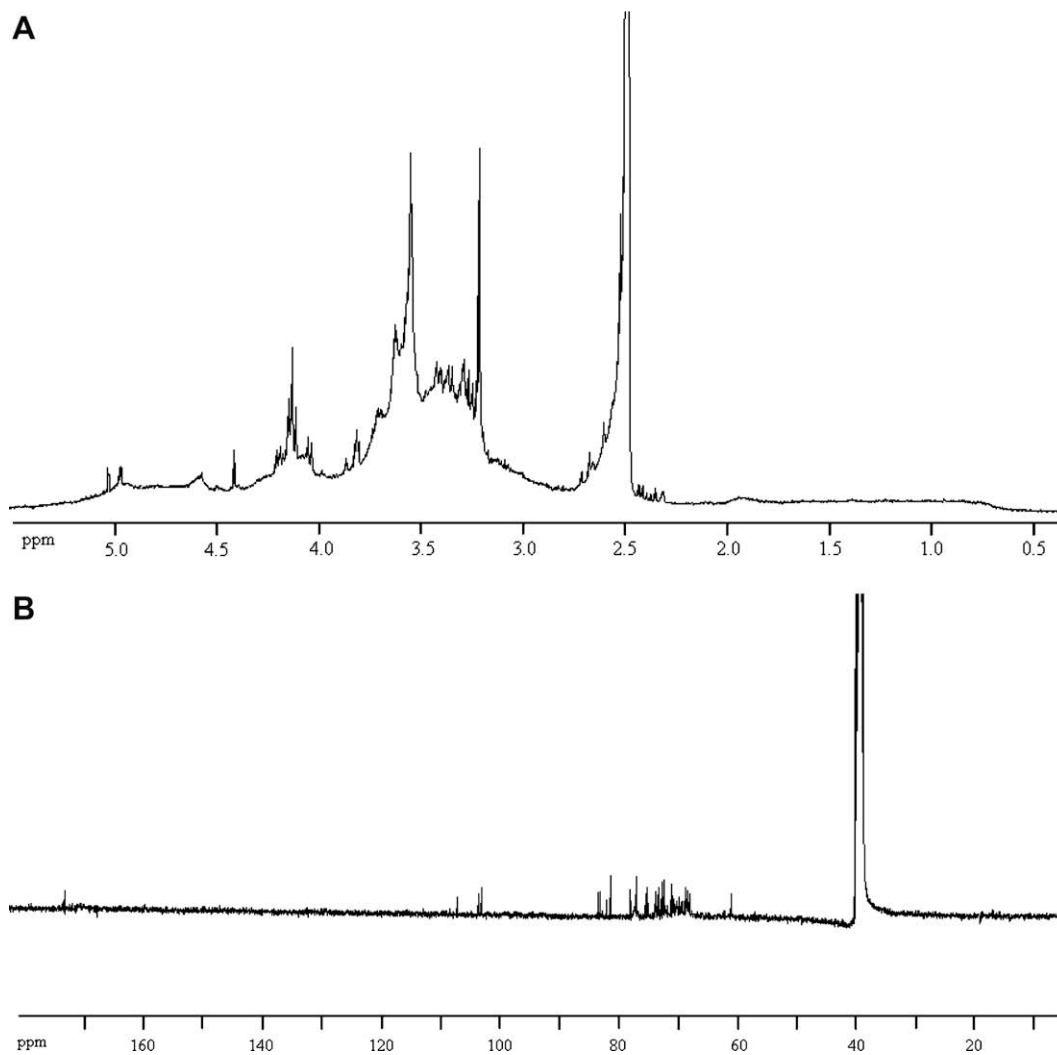


Fig. 4. NMR spectra of PLFP. (A)  $^1\text{H}$  NMR spectra; (B)  $^{13}\text{C}$  NMR spectra.

the infrared spectra resulted from the presence of uronic acids. The absorbance of PLFP in the range of 950–1200  $\text{cm}^{-1}$  was where the C–O–C and C–O–H linkages were found (Kacuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000). A stretching peak at 1001  $\text{cm}^{-1}$  in the infrared spectra suggested the presence of a C–O bond. A band of absorption at 763  $\text{cm}^{-1}$  represented symmetrical ring vibration.

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of purified PLFP are shown in Fig. 4. From the profile of  $^{13}\text{C}$  spectra, the main  $\rightarrow 5$ - $\alpha$ -L-Araf-(1 $\rightarrow$ ) linkage was obviously characterised by five strong signals at 108.2, 82.1, 78.4, 83.0 and 68.1 ppm, which originated from C-1, C-2, C-3, C-4 and C-5 of the L-arabinofuranosyl unit (Navarro, Cerezo, & Stortz, 2002). The chemical shift at 5.02 ppm of anomeric H-1 indicated the  $\alpha$  form of L-arabinofuranosyl unit. The signals identified at 104.1, 73.9, 76.4, 70.4, 75.5 and 69.1 ppm could be assigned to the C-1, C-2, C-3, C-4, C-5 and C-6 of  $\rightarrow 6$ - $\beta$ -D-Glcp-(1 $\rightarrow$ ) unit. The chemical shift of H-1 at 4.61 ppm suggested the  $\beta$  form of D-glucopyranosyl residue (Roslund, Tähtinen, Niemitz, & Sjöholm, 2008). The signals at 103.2, 70.3, 81.5, 71.1, 76.2 and 174.6 ppm in the  $^{13}\text{C}$  spectra of PLFP were related to the C-1, C-2, C-3, C-4, C-5 and C-6 of  $\rightarrow 3$ - $\beta$ -D-GalA-(1 $\rightarrow$ ). The chemical shift at 174.6 ppm showed the existence of uronic acid. The signals identified at 83.5 and 61.1 ppm in the  $^{13}\text{C}$  spectra could be assigned to the C-3 and C-6 of  $\rightarrow 3$ - $\beta$ -D-Gal-(1 $\rightarrow$ ), while the signals at 73.2 and 69.1 ppm could be for the C-3 and C-6 of  $\rightarrow 6$ - $\beta$ -D-Gal-(1 $\rightarrow$ ) (Errea & Matulewicz, 2003). The H-1 signals at 4.52 and 4.36 ppm confirmed that the configuration of D-galactopyranosyl residue was the  $\beta$  form.

### 3.3. Anti-glycated activity of PLFP

The anti-glycated activities of PLFP and aminoguanidine are present in Fig. 5. Two concentrations (0.1 and 0.5 mg/ml) were used to evaluate the inhibition effect of PLFP on the formation of advanced glycated end products. The results indicated that at the first week, very low anti-glycated activities were observed for both PLFP and aminoguanidine. 0.5 mg/ml aminoguanidine exhibited an anti-glycated activity of  $80.6 \pm 3.7\%$  at the second week, significantly ( $P < 0.05$ ) higher than PLFP at the same concentration. However, PLFP had a higher anti-glycated activity than aminoguanidine at the fourth week. When a concentration of 0.1 mg/ml was used, an anti-glycated activity of  $58.1 \pm 2.4\%$  was obtained for aminoguanidine at the second week, which decreased to  $10.6 \pm 2.1\%$  after the fourth week.

Protein glycation plays an important role in the development of diabetic vascular complications *in vivo*. Schiff alkali and Amadori

products are formed in the initial stage of non-enzymatical reaction between protein and glucose, which are transformed into irreversible advanced glycation end products after oxidation, rearrangement and crosslinking (Metz, Alderson, & Thorpe, 2003). Therefore, inhibiting the formation of advanced glycation end products will be beneficial to human health. Aminoguanidine has been used clinically as a glycation inhibitor (Lunceford & Gugliucci, 2005). The inhibition mechanism of aminoguanidine is that it can react with the carbonyl group of a glycated protein to develop inactive substances, which prevent crosslinking of the glycated protein from developing advanced glycation end products. Otherwise, aminoguanidine can combine with free glucose, which lowers the glucose concentration and decreases the development of glycated protein (Thornalley, 2003). In this work, PLFP showed a good and stable anti-glycated activity at the second and fourth week. However, aminoguanidine only produced a strong anti-glycated activity at the second week. A sharp decrease was observed when the incubation period was extended to four weeks. This difference between PLFP and aminoguanidine should be due to their inhibition mechanisms. The antioxidant activity and special structure of PLFP might be responsible for its anti-glycated activity.

### 4. Conclusion

Hot water was used to prepare crude polysaccharides from longan fruit pericarp in this work. The purified PLFP was obtained by Sephadex G-100 gel filtration column. The molecular weight of PLFP was measured to be 420 kDa by gel permeation chromatography. Gas chromatography analysis suggested that PLFP comprised of L-arabinofuranose, D-glucopyranose, D-galactopyranose and D-galacturonic acid with molar percentages of 32.8%, 17.6%, 33.7% and 15.9%, respectively. The assay of glycosidic linkage showed that the backbone of PLFP consisted of  $\rightarrow 5$ -L-Araf-(1 $\rightarrow$ ),  $\rightarrow 6$ -D-Glcp-(1 $\rightarrow$ ),  $\rightarrow 3$ -D-Galp-(1 $\rightarrow$ ),  $\rightarrow 3$ -D-GalpA-(1 $\rightarrow$ ) and  $\rightarrow 6$ -D-Galp-(1 $\rightarrow$ ) with a molar proportion of 2:1:1:1:1. No branching was found in the PLFP chain. The infrared and nuclear magnetic resonance spectra further confirmed that the configuration of L-arabinofuranose was of the  $\alpha$  form, while D-glucopyranose, D-galactopyranose and D-galacturonic acid were of the  $\beta$  form. By determination of anti-glycated activity, PLFP showed a good and stable anti-glycated activity when 0.5 mg/ml was used. However, the anti-glycation mechanism of PLFP is still not clear. It will be interesting to investigate the relationship between PLFP structure and anti-glycated activity in future work.

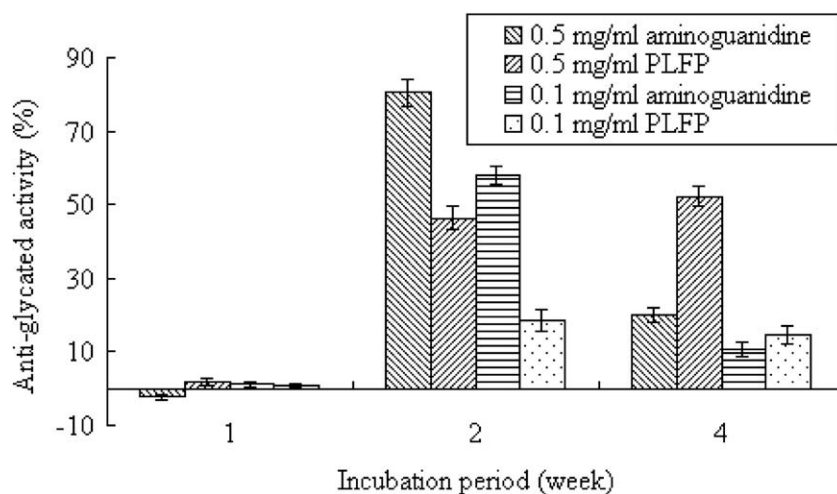


Fig. 5. Anti-glycated activities of PLFP and aminoguanidine during different incubation periods.



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