Carbohydrate Polymers 102 (2014) 376–384

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Extraction of pectin from *Premna microphylla* turcz leaves and its physicochemical properties

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ARTICLE INFO

Article history:
Received 23 August 2013
Received in revised form 15 October 2013
Accepted 28 November 2013
Available online 6 December 2013

Keywords:
Premna microphylla turcz leaves (PMTL)
Alcohol insoluble solids (AIS)
Low-methoxyl pectin
Ammonium oxalate
Morphology

ABSTRACT

Premna microphylla turcz leaves (PMTL) have been used for preparing a "green tofu" by Chinese for a long history. Chemical composition analysis indicated alcohol insoluble solids (AIS) of PMTL contained high amount of pectin. Water, ammonium oxalate, hydrochloric acid and sodium hydroxide were used to extract different pectic fractions sequentially. Ammonium oxalate was found to be the most effective extracting agent, reflecting on a high yield (20.61%) and a significant change of morphology of AIS. The resulted oxalate-soluble pectin (OXSP) showed high galacturonic acid content (76.15%) and average molecular weight (980.67 kDa), low neutral sugar content (6.41%) and degree of methoxylation (14.90%). All of the characteristics have contributed excellent gelling and thickening properties of OXSP. These results may allow an improved use of PMTL as a resource of low-methoxyl pectin, and observation of the morphology of residues can be helpful for evaluating the efficiency of extracting agents.

1. Introduction

Pectins are complex heteropolysaccharides ubiquitously present in the cell wall of land plants. It contains 1,4-linked partially methoxylated α-D-galacturonic acid (GalA) residues as backbone, in which homogalacturonan, rhamnogalacturonan-I and rhamnogalacturonan-II were isolated and characterized as major pectic polysaccharides (Willats, Knox, & Mikkelsen, 2006). Depending on the degree of methoxylation (DM), pectins are commonly categorized as high-methoxyl pectin (HMP, DM > 50%) or low-methoxyl pectin (LMP, DM < 50%). There is considerable interest in manufacturing LMP because LMP can be used as a gelling agent in low-sugar products and the gels obtained from LMP are heat reversible, softer, more spreadable and relatively independent of pH compared to HMP gels (Kumar & Garg, 2009). However, pectin obtained at industrial level is typically HMP, whereas LMP has to be prepared from HMP by reducing the DM through acid demethoxylation, alkali demethoxylation, or demethoxylating reaction of pectin methyl esterase. These demethoxylations must be carried out carefully to avoid the potential depolymerization of pectin. An alternative approach for manufacturing LMP is to 'mine' plant cell walls that are enriched with LMP.

Premna microphylla turcz, which belongs to the species of *Premna*, Verbenaceae, is a deciduous shrub, locally called "dou fu chai". It distributes broadly in the mountainous regions in east, middle and south of China. Currently, the *Premna microphylla* turcz has drawn the attention of Chinese farmers and has been large-scale cultivated to be an important commercial crop due to its various applications, for example, leaves of *P. microphylla* turcz (PMTL) were used to treat skin cuts and infections, dysentery, appendicitis, swelling, headaches, and viper bites (Zhan, Tang, & Shan, 2009). However, the most remarkable characteristic of its fresh leaves is that can be rubbed and squeezed in water to yield a mucilaginous juice. For a long history, this juice has been used to prepare a "green tofu" by local people through addition of materials containing Ca^{2+}. Preliminary experiments indicated that the extract from PMTL is primarily pectic polysaccharides (Wang et al., 2008). However, information about the cell wall composition of PMTL, extraction and physicochemical properties of the pectic substances, as well as their applications are still poorly understood.

In the lab level, new materials for pectin extraction are always explored by a sequential extraction procedure using hot water, aqueous ammonium oxalate solutions, hot dilute hydrochloric acid, and cold dilute sodium hydroxide solutions as extracting agents successively (Christiaens et al., 2012; Eriksson, Andersson, & Aman, 1997). However, most previous studies focused on the yield, properties and structure of different pectic fractions. Little attention has been given to the change of morphology of plant cell wall material after each extraction step. Observation of the morphology of residues may also be very interesting and useful, which may help

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http://dx.doi.org/10.1016/j.carbpol.2013.11.069

scientists to understand the efficiency of extracting agents and the proportion of pectic fractions directly.

The paper is for the first time to explore PMTL as a potential LMP sources by (1) analyzing the cell wall composition of PMTL; (2) observing the morphology of residue after each extraction steps; and (3) presenting proportion and physicochemical properties of different pectic substances.

2. Materials and methods

2.1. Materials

Premna microphylla turcz leaves, bought from the local market, Nanchang, Jiangxi province, China, were cleaned and dried at 60 °C for 24 h. Then the leaf sample was milled using a medicinal herb grinder (Model RT-02, Wuyi Medicine Machine Manufactory, China) and sieved to pass through a 60 mesh size screen, and stored at room temperature in a vacuum packed container before use.

2.2. Preparation of alcohol insoluble solids (AIS) from PMTL

Preparation of an alcohol insoluble solid (AIS) is the most commonly used method for the isolation of cell wall material from plant (Kunzek, Kabbert, & Gloyna, 1999). The alcohol insoluble solids were obtained according to the method of Chau and Huang (2004) with slight modifications. Dried PMLT powder was stirred vigorously in 85% (v/v) alcohol (PMTL:alcohol 1:30 w/v) at room temperature for 1 h. The suspension was further boiled for another 1 h. The supernatant was removed after centrifugation at 4092 × *g* for 10 min (Feige, TDL-5-A, Beijing Feige Instrument Co., Beijing, China), and another portion of 85% alcohol was added. The extraction was continued until absence of sugars in supernatant as shown by negative reaction in the phenol-sulfuric test (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). After filtration, the filtrates were washed with acetone and air-dried.

2.3. Pectin fractionation

A sequential extraction of pectic polysaccharides was carried out according to the method of Taboada et al. (2010) with some modifications: (1) distilled water at 80 °C for 30 min; (2) 50 mM $(NH_4)_2C_2O_4$ (pH 4.8), acting as chelating agent, at 25 °C for 30 min; (3) 30 mM hydrochloric acid (pH 1.5) at 85 °C for 30 min; and (4) 50 mM NaOH in the presence of 20 mM $NaBH_4$ at 0 °C for 30 min. A solid/liquid ratio of 1:30 (w/v) was employed in all cases. Each extractant was applied three times followed by three times washing with distilled water and adjusting pH to ~4.5. The extracts and the washing water were combined and represented, respectively, water-soluble pectin (WSP), oxalate-soluble pectin (OXSP), acid-soluble pectin (HSP) and alkali-soluble pectin (ASP). Then the extracts were dialyzed with dialysis membranes (molecular weight cut-off 6000–8000 Da), concentrated under reduced pressure at 45 °C and freeze-dried.

2.4. Scanning microscopy analysis

The residues after each extraction step was collected and freeze-dried. Then the AIS and residues were applied to scanning microscopy analysis using an environmental scanning electron microscope (ESEM) (Quanta200F, FEI Deutschland GmbH, Kassel, Germany) at 30 kV and 3.0 spot size. Samples were prepared by sticking them onto double-sided adhesive tape attached to a circular specimen stub. Low vacuum mode was used while operating the ESEM.

2.5. Chemical characterization of AIS and pectin fractions

2.5.1. Proximate analysis of AIS

The moisture content of AIS was measured using a halogen moisture analyzer (Model HR83, Mettler-Toledo, Switzerland), and ash content was determined according to AOAC method (AOAC, 1997). Protein content was determined by Kjeldahl method and multiplied the nitrogen content with a factor of 6.25. Lignin was gravimetrically determined as Klason lignin according to method of Femenia, García-Pascual, Simal, and Rosselló (2003). The amount of soluble (SDF), insoluble (IDF) and total dietary fiber (TDF) was determined according to AACC method 32–45.01 (AACC International, 2010). Contents of K, Ca, Na, Mg, Fe were determined using an inductively coupled plasma optical emission spectrometer (ICP-OES, PerkinElmer Optima 5300DV).

2.5.2. Acid hydrolysis

To determine the uronic acid and neutral sugar contents of AIS and pectins, AIS and pectic fractions were hydrolyzed. AIS were treated with 12 M aqueous sulfuric acid for 60 min at 35 °C followed by complete hydrolysis to monosaccharides with 2 M H_2SO_4 at 100 °C for 60 min (Chau & Huang, 2004). The pectic fractions were hydrolyzed with 2 M TFA at 120 °C for 1.5 h in sealed tubes (Yapo, 2009).

2.5.3. Uronic acid content analysis

The uronic acid content in the AIS and pectins was determined by the m-hydroxybiphenyl method (Blumenkrantz & Asboe-Hansen, 1973) using hydrolysate as described above. Galacturonic acid monohydrate (China Sigma–Aldrich, Shanghai, China) was used as standard.

The type of uronic acid was first qualitative analyzed using paper chromatography (Rao, Beri, & Rao, 1951), then further confirmed according to Taylor and Conrad (1972), reduction of uronic acid to glycosyl units was carried out before hydrolysis by treating with a water-soluble carbodiimide and then with sodium borohydride. After that, the reduced samples were hydrolyzed and taken for neutral sugar analysis as described in Section 2.5.4.

Uronic acid extractability (expressed as the amount of uronic acid extracted from the AIS) was used to quantify the effectiveness of extracting agents according to the following equation (Taboada et al., 2010):

$$\text{Uronic acid extractability}\,(\%) = \frac{m(\text{uronic acid})_{\text{pectin}}}{m(\text{uronic acid})_{\text{AIS}}} \times 100 \qquad (1)$$

Where $m(\text{uronic acid})_{\text{pectin}}$ and $m(\text{uronic acid})_{\text{AIS}}$ is the amount of uronic acid in the pectin and AIS, respectively.

2.5.4. Neutral sugar analysis

The residual H_2SO_4 and TFA after acid hydrolysis was removed by adding with $BaCO_3$ and blowing off with nitrogen, respectively. Then samples were analyzed by gas chromatography (GC) after conversion of hydrolysate to alditol acetates (Liang et al., 2012). The alditol acetates were analyzed using an Agilent 6890 system GC (Agilent Technologies, Palo Alto, CA, USA) fitted with a DB-1701 column (30 m, 0.25 mm I.D., 0.25 μm film thickness; kept at 170 °C for 2 min, 170–250 °C at 10 °C/min, then held at 250 °C for 30 min) with a flame ionization detector (FID).

2.6. FT-IR spectrum and degree of methoxylation (DM)

The FT-IR spectra of the pectins were recorded on a Nicolet 5700 spectrometer (Thermo Co., Madison, USA) and compared with that of citrus pectin P9135 (Sigma–Aldrich, Shanghai, China). The dried samples were ground with KBr powder (spectroscopic grade) and pressed into pellets for spectra measurement in the frequency

range of 4000–400 cm^{-1}. Data was collected and analyzed with Ominic 7.2 software. The DM was calculated from FT-IR spectra according to our previous method (Liang et al., 2012).

2.7. Gel-permeation chromatography

Gel-permeation chromatography was performed at room temperature on a Sepharose CL-6B column. Samples (10 mg) were loaded on the column (60 cm × 1.6 cm) and eluted with degassed 0.1 M NaCl at a flow rate of 20.0 mL/h. Fractions were assayed for carbohydrate content using the phenol-sulfuric test (DuBois et al., 1956). The results are expressed as a function of $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e, V_0, and V_t are the elution volume of the fraction, the void volume, and the total volume of the column, respectively. Dextran standards (T-2000, T-500, T-70, and T-40, Sigma–Aldrich, Shanghai, China) and glucose (Mw 180, Sigma–Aldrich, Shanghai, China) were used to calibrate the column and to establish a standard curve.

2.8. Gelling capacity

A pectin gels were prepared according to our previous method (Liang et al., 2012) with modifications. Pectin was dissolved in a diluted citric acid/sodium citrate solution (pH 3.0). Solution of pectin was rapidly mixed with $CaCl_2$ solution to give pectin solutions with the desired pectin concentration (1% w/w) and Ca^{2+} concentrations (30 mg Ca^{2+}/g pectin) without heating. After preparation, the gel samples were transferred into a Brookfield sample bottle (60 mm diameter × 60 mm height), sealed with matched rubber stoppers.

Gel strength of pectin gels after resting for 24 h were determined by a Brookfield CT3-100 Texture Analyzer (Middleboro, USA). The gels were deformed by compression at a constant speed of 1.0 mm/s to a distance of 3 mm from the gel surface using a cylindrical probe TA10 (diameter = 12.7 mm). The initial maximum force, recorded when the probe had penetrated 3 mm into the pectin gels, was taken as gel strength.

2.9. Flow behavior of OXSP

The flow behavior of OXSP was determined on an ARES Rheometer (TA, USA). A parallel plate geometry (50 mm diameter, 1.0 mm gap) was used. Steady shear viscosity was determined at various concentrations and temperatures, and compared with four commercial pectin P9135 (GalA 81.23%, DM 60.91%), P9311 (GalA 67.96%, DM 31.65%), P9436 (GalA 77.62%, DM 70.36%) and P9561 (GalA 91.36%, DM 86.35%) (China Sigma–Aldrich, Shanghai, China) at concentration of 2%.

2.10. Statistical analysis

All of the experiments were done in triplicate. Statistical analysis was carried out using SPSS (version 16.0, Chicago, United States). The results were expressed as mean ± standard deviations and compared using the Tukey test at 5% confidence level.

3. Results and discussions

3.1. Cell wall composition of PMLT

During the preparation of alcohol insoluble solid (AIS) as cell wall material, *Premna microphylla* turcz leaves were heated with alcohol, the mixture was stirred vigorously and the residue was washed with acetone. Therefore, enzymes were inactivated and low molecular-weight components (low molecular weight sugars, pigments, and organic acids) were removed. It was found that the dried PMLT were composed of 77.82% of AIS and 22.18% of alcohol soluble materials (Table 1). This AIS content was slight lower than that in yellow passion fruit rinds (82.3%) (Yapo & Koffi, 2006), but higher than that in many other materials such as murta fruit (36.3%) (Taboada et al., 2010), and Japanese quince (35.5–38.4%) (Thomas, Guillemin, Guillon, & Thibault, 2003). All of these materials have been suggested by the authors to be good resources of pectin.

Table 1
Chemical compositions and mineral content of alcohol insoluble solid (AIS) of *Premna microphylla* turcz leaves.[a]

Composition	AIS
Yield[b]	77.82 ± 3.21
Moisture	6.57 ± 0.56
Protein content	13.39 ± 1.11
Ash	7.86 ± 0.37
Total dietary fiber	69.45 ± 2.24
Soluble dietary fiber	13.10 ± 0.41
Insoluble dietary fiber	56.35 ± 1.78
Klason Lignin	16.83 ± 1.15
Total Carbohydrates	64.93 ± 2.15
Galacturonic acids	23.94 ± 1.33
Total neutral sugars[c]	40.99 ± 1.41
Glucose	25.26 ± 1.69
Xylose	7.12 ± 1.08
Galactose	3.63 ± 0.13
Arabinose	2.85 ± 0.33
Rhamnose	1.68 ± 0.07
Mannose	0.45 ± 0.01
Minerals	
K	0.55 ± 0.02
Ca	2.47 ± 0.05
Na	0.23 ± 0.01
Mg	0.33 ± 0.02
Fe	0.03 ± 0.00

[a] Values are means ± standard deviations of triplicate measurements.
[b] g/100 g dried leaves.
[c] Total neutral sugars are sum of rhamnose, arabinose, galactose, glucose, xylose and mannose.

Then, the AIS of PMLT was characterized. It contained, on average, 6.57% moisture, 7.86% ash and 13.39% protein. The total dietary fiber (TDF) content of the AIS was 69.45%. Insoluble dietary fiber (IDF) (56.35 g/100 g AIS) was the predominant fiber fraction (81.14% of TDF), while Klason lignin (16.83 g/100 g AIS) accounted for 29.87% of the IDF. In addition, a relative higher amount of calcium (2.47%) was found in AIS when compared with other minerals such as potassium, sodium, magnesium, and ferrum. The high content of calcium was usually found in the raw materials that rich in low-methoxyl pectin, such as creeping fig seeds (Liang et al., 2012) and leaves of Krueo Ma Noy (Singthong, Ningsanond, Cui, & Douglas Goff, 2005).

Glucose (25.26%) was the main neutral sugar in AIS, followed by xylose (7.12%), galactose (3.63%), and arabinose (2.85%). The amounts of the other neutral sugars, namely, rhamnose, mannose, were relatively low. The total neutral sugars (sum of the individual neutral sugars) and total carbohydrates in AIS of PMTL were 40.99 and 64.93%, respectively, showing the predominance of polysaccharides in PMTL cell wall material.

Pectic substances were the main type of cell wall polysaccharides presented in the AIS of PMLT. This was deduced from the presence of large amounts of galacturonic acid (23.94%) while no glucuronic acid were found, and from the occurrence of galactose, arabinose and rhamnose which were also characteristic of pectic polysaccharides. The amount of galacturonic acid found in the AIS of PMTL was higher than that in apple pomace (10–15%) and within the range of that reported for citrus peel (20–30%), sugar beet pulp (15–30%), or sunflower head residues (15–25%) (Yapo & Koffi, 2006), where apple pomace and citrus peels are the two major sources of industrial pectin. Considering the high yield of AIS from

dried PMLT and the content of galacturonic acids in AIS higher than the 10%, which was a threshold for a raw material to be considered as an alternative pectin source proposed by Mohamed and Hasan (1995), this AIS exhibited a great potential to be used for the extraction of pectin. Nevertheless, the sugar composition of AIS also suggested that the AIS contained other different types of polysaccharides including hemicelluloses and cellulose. To understand the proportion and extractability of different pectic polysaccharides and to optimize the conditions for isolation of pectin from raw materials, a sequential extraction procedure was applied in this study.

3.2. Evaluation of extracting agent efficiency

3.2.1. Extraction yields, galacturonic acid content and uronic acid extractability

The yields of the polysaccharide fractions from different extraction steps and their chemical composition data are listed in Table 2, where the extraction yield of WSP, OXSP, HSP and ASP was 1.86, 20.61, 4.22 and 7.96% on a dry matter basis (w extract/w AIS), respectively, which made up to a total extract yield of 34.65%. The yield of OXSP in this study was the highest and accounted for 59.48% of the overall extract yield, while the yield of HSP was low, indicating PMTL contained low amount of acid-soluble pectin but were abundant in calcium-bound pectin. Pectic proportion seemed to vary with sources and sample features. Raynal, Mourgues, and Conte (1991) fractional extracted the plum *prune d'ente* using water, oxalate and HCl. Their main extracts were water-soluble pectin. Happi Emaga, Robert, Ronkart, Wathelet, and Paquot (2008) used a sequential extraction of pectin from peels of banana and plantain, and showed that the banana peel were rich in acid-soluble pectin, whereas plantain peels mainly contained an ammonium oxalate-soluble pectin.

A high yield was not enough to draw a conclusion that $(NH_4)C_2O_4$ was a good agent for pectin extraction from AIS of PMTL if without the verification of the content of galacturonic acid. The OXSP exhibited the highest galacturonic acid content (76.15%), whereas the GalA contents of WSP, HSP and ASP were 43.46, 16.23 and 49.41%, respectively (Table 2). The total galacturonic acid content of OXSP was higher than the 65% limit established by the FCC purity specification for pectin, and was higher than that of pectic polysaccharides isolated from such other sources as *Canna edulis* Ker residue (Zhang, Wang, Yu, & Wu, 2011) or mango and lime peels (Koubala et al., 2008) which also used ammonium oxalate as

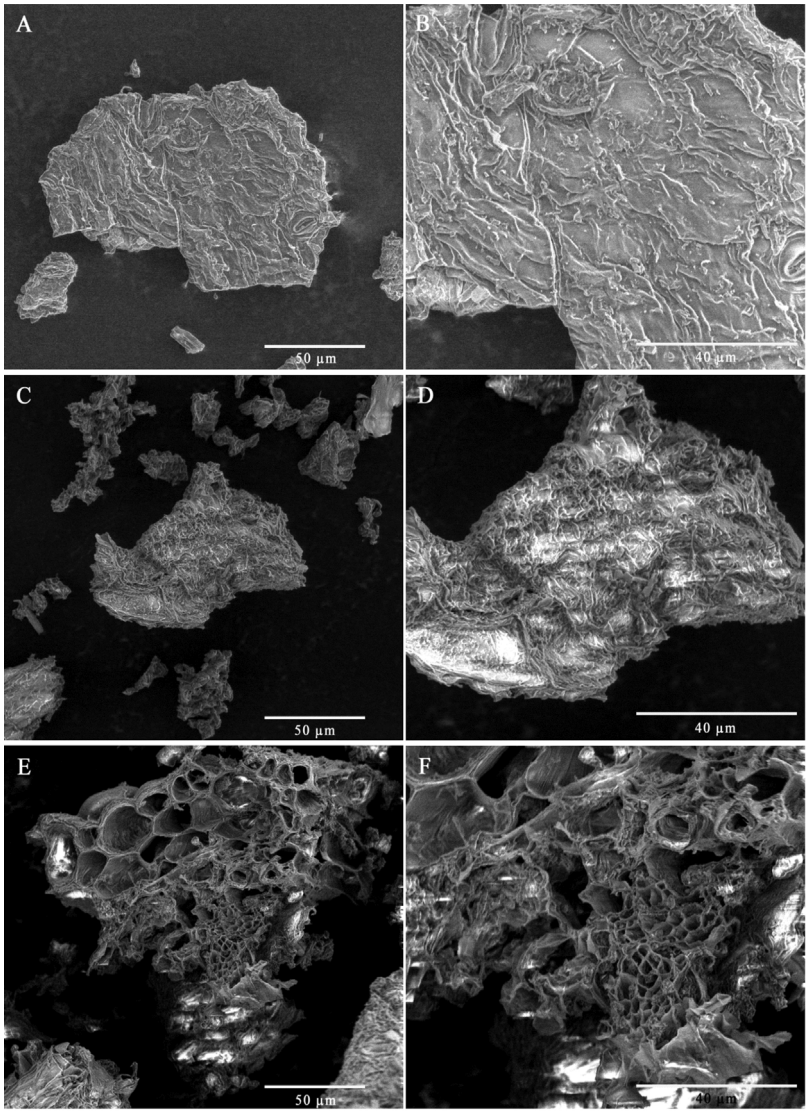

Fig. 1. Environmental scanning electron micrograph of residues after each extraction step. (A and B) AIS; (C and D) residue after water extraction; (E and F) residue after ammonium oxalate extraction; (G and H) residue after acid extraction; (I and J) residue after alkaline extraction.

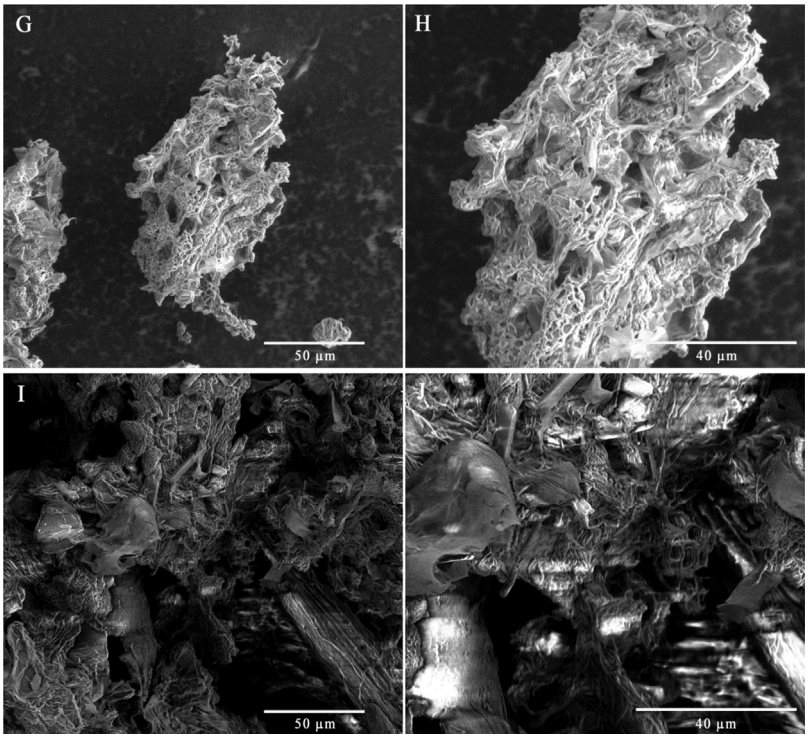

Fig. 1. (*continued*).

extract agent. In this study, uronic acid extractability for WSP, OXSP, HSP and ASP was calculated to be 3.39, 65.48, 2.85 and 16.46%, respectively, which made up to a total of 88.18% recovery. The amount of GalA in OXSP accounted for 74.26% of the total GalA recovered, indicating that under the extraction conditions used, the $(NH_4)C_2O_4$ extraction of pectin from AIS of PMTL was the most effective.

3.2.2. Evaluation of extracting agent efficiency by observing morphology of residues

In order to further verify the efficiency of agents on extraction of pectins, the morphology of residues after each extraction steps were observed using environmental scanning electronic microscopy. As shown in Fig. 1, the cell wall material before extraction was relatively compact, intact, and relatively smooth (Fig. 1A and B). After extracted by distilled water, some loosely bound parts was removed from surface of cell wall material, leading to a bit coarse morphology (Fig. 1C and D). This part of pectins may bind to the cell wall through non-covalent and non-ionic bonds, therefore which was most likely to be extracted by water, giving the WSP-fraction. The feature of cell wall material was significantly changed after using ammonium oxalate as chelating agent, and the apparent morphology of cell wall material became porous (Fig. 1E and F), indicating ammonium oxalate was an effective extracting agent. This results agreed with the yield of OXSP we discussed above. Based on the principle of ammonium oxalate extraction, it can also infer that the main pectic polysaccharides existed in the cell wall of PMTL were mainly low methoxylated, which bound to calcium in the plant cell wall. After using HCl as an extract agent, there were still many pores in the cell wall material, and the structure of cell wall material seemed to be collapsed (Fig. 1G and H). The structure was further collapsed after using sodium hydroxide as an extracting agent (Fig. 1I and J), suggesting ASP played an important role in maintaining the structure of cell wall material. These results may provide some information when search technological conditions for industrial manufacture of pectin from PMTL.

Table 2
Yield, extractability, chemical composition and degree of methoxylation (DM) of pectic fractions extracted from AIS of *premna microphylla* turcz leaves.[a]

	Pectic fractions			
	WSP	OXSP	HSP	ASP
Yield (%)	1.86 ± 0.18a[c]	20.61 ± 1.21b	4.22 ± 0.24c	7.96 ± 0.37d
Galacturonic acids (GalA, %)	43.46 ± 2.42a	76.15 ± 2.18b	16.23 ± 1.97c	49.41 ± 2.82a
Uronic acid extractability (%)	3.39 ± 0.52a	65.48 ± 1.97b	2.85 ± 0.19a	16.46 ± 1.70c
Total neutral sugars (%)[b]	25.69 ± 1.51a	6.41 ± 0.28b	42.12 ± 1.80c	19.81 ± 0.26d
Rhamnose (Rha)	5.82 ± 0.76a	1.91 ± 0.12b	4.12 ± 0.21c	3.47 ± 0.35c
Arabinose (Ara)	8.56 ± 0.15a	2.20 ± 0.15b	6.51 ± 0.16c	5.03 ± 0.27d
Galactose (Gal)	7.14 ± 0.57a	1.71 ± 0.07b	6.41 ± 0.55a	3.34 ± 0.23c
Glucose (Glc)	2.62 ± 0.18ab	0.06 ± 0.01b	24.62 ± 2.26c	4.97 ± 0.22a
Xylose (Xyl)	1.55 ± 0.21a	0.50 ± 0.02b	0.44 ± 0.03b	3.01 ± 0.11c
Mannose (Man)	–	0.03 ± 0.03a	0.02 ± 0.00a	–
Molar ratio of Rha/GalA	0.14 ± 0.03a	0.03 ± 0.00b	0.27 ± 0.02c	0.08 ± 0.01d
DM (%)	25.09 ± 2.37a	14.90 ± 1.53b	5.64 ± 2.12c	–

[a] Data are presented as means ± standard deviations of triplicate measurements.
[b] Total neutral sugars are sum of rhamnose, arabinose, galactose, glucose, xylose and mannose.
[c] Mean values in the same row with different letters are significantly different (Tukey test, $p < 0.05$).

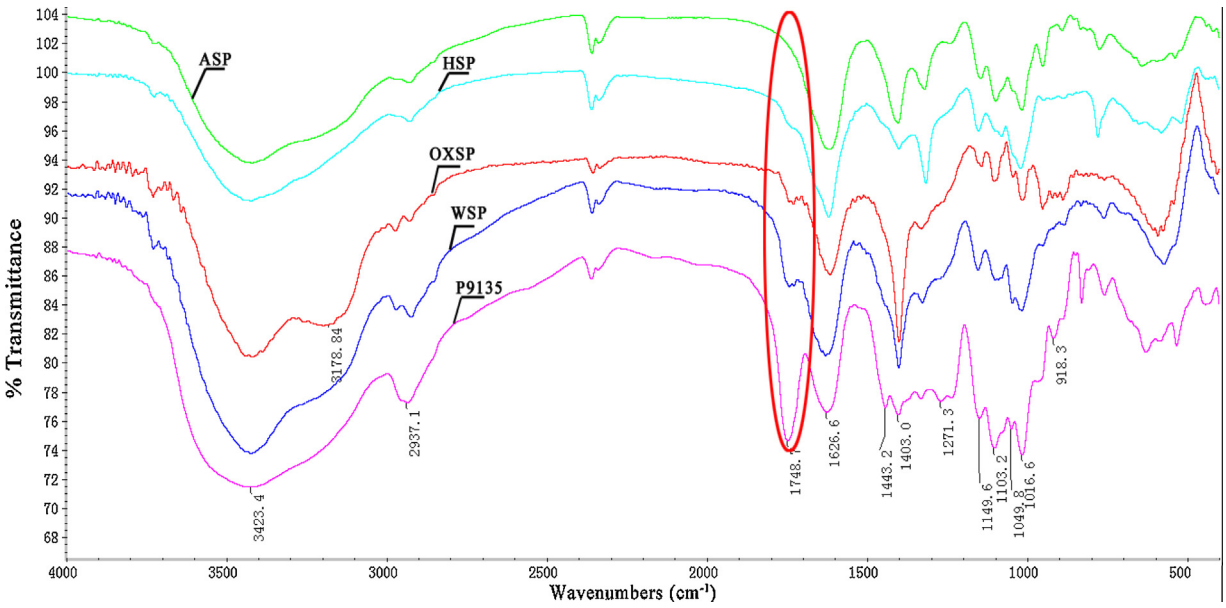

Fig. 2. Fourier transform infrared spectra of pectic fractions from AIS of *Premna microphylla* turcz leaves and commercial pectin standard (P9135).

3.3. Neutral sugars contents and compositions

In this study, the neutral sugar content and composition of pectic polysaccharides varied with the extraction conditions (Table 2). OXSP had lower total neutral sugar content (6.41% (w/w)) than ASP, WSP and HSP (19.81%, 25.69% and 42.12%, respectively). Arabinose, galactose, and rhamnose were the main individual neutral sugars presented in the four fractions, suggesting the presence of arabinan, galactan, and/or arabinogalactan side chains. Xylose mainly existed in ASP but also presented within other pectin fractions in relatively low amounts, while mannose only existed in OXSP and HSP at very low amounts. It is known that rhamnogalacturonan I (RG I) consists of alternating rhamnose and galacturonic acid residues as a backbone, and the ratio of rhamnose:galacturonic acid within a RG I backbone is 1:1. Consequently, the values of the molar ratio of Rha to GalA (0.03) in OXSP suggested that the pectin molecules of the OXSP could consist mainly of polygalacturonic acid-rich "smooth" regions. It is reported that the presence of neutral sugar side chains usually inhibits the formation of junction zones for gelling, because the side chains of pectins may limit the extent of inter-chain association (BeMiller, 1986). Therefore, the high content of galacturonic acid and low proportion of neutral sugars in OXSP suggested that the OXSP might have excellent gelling and thickening capability.

3.4. FT-IR spectrum and the degree of methoxylation

FT-IR spectroscopy was used to support the presented chemical analysis data of the pectic fractions and replenish them with some structure information. The FT-IR spectra of four pectic fractions and one commercial pectin standard (P9135) were examined and compared in Fig. 2. First, the analysis of each band was conducted. The spectrum showed peak at 3423 $cm^{-1}$was due to the stretching of —OH groups. The peaks at 2937 cm^{-1} indicated C—H stretching vibration of CH_2 groups. The band at 1748 cm^{-1} corresponded to methyl esterified or protonated carboxylic groups, whereas the couple of bands at 1626 and 1403 cm^{-1} were attributed to asymmetric and symmetric stretching modes of the COO^- group, respectively. Bands at 1016 and 1103 cm^{-1} indicated vibration of C—C and vibration of backbone respectively. The peak at 1150 cm^{-1} suggested the presence of —CH—OH in aliphatic cyclic secondary alcohol. It was found that all the FT-IR spectra of samples displayed a similar general pattern and exhibited similarities of the absorption patterns to that of commercial pectin standard, confirming that all the extracts were pectic polysaccharides.

The most significant differences between pectic fractions were in the region where featured the state of carboxylic groups (peaks at 1748 cm^{-1} and 1626 cm^{-1}). The intensity of the band at 1748 cm^{-1} decreased in the order of WSP, OXSP, HSP and ASP, where the peak of 1748 cm^{-1} disappeared for ASP, probably due to the removal of methyl esters during the dilute alkaline condition. Košťálová, Hromádková, and Ebringerová (2010) also reported that alkaline fractions from the seeded fruit of oil pumpkin were only in the carboxylate form. The peak areas of 1748 cm^{-1} and 1626 cm^{-1} are usually used to determine the degree of methoxylation. A linear calibration curve were established between ratio (R) of the peak area at 1748 cm^{-1} over the sum of the peak areas at 1748 and 1626 cm^{-1} and DM of pectins ($DM = 125.1\,R + 2.1023$, $R^2 = 0.9915$), and used in our previous study (Liang et al., 2012). The DM of WSP, OXSP and HSP was calculated to be 25.09, 14.90, and 5.64%, respectively. This result indicated that pectic fractions from AIS of PMLT were low-methoxyl pectins.

It should be noticed that some $(NH_4)C_2O_4$ may still exist in the OXSP sample even extensive dialysis was carried out in the process of purification, because a broad absorption was observed in 3178 cm^{-1} (assigned to NH_4^+ salt) in the sample of OXSP. Mort, Moerschbacher, Pierce, and Maness (1991) also found the great difficulty in dialyzing chelating agents away from pectins, this problem may need scientist to deal with in the future work.

3.5. Molecular weight

The molecular weight distribution of each pectic substance separated by Sepharose CL-6B column is shown in Fig. 3. The molecular weight profiles of WSP contained two peaks with average molecular weight of approximately 958.87 kDa and 20.63 kDa. In contrast, the OXSP showed a large amount of a high molecular-mass fraction eluted in one major fraction of 980.67 kDa. Fraeye et al. (2009) also reported that chelator-soluble pectin from AIS of strawberries contained pectins with the highest molecular weight, and the water soluble pectin showed a peak with rather high Mw and a lower tail originating from shorter chains. HSP in this study showed only one main peak but with lower molecular mass, about 208.08 kDa. ASP

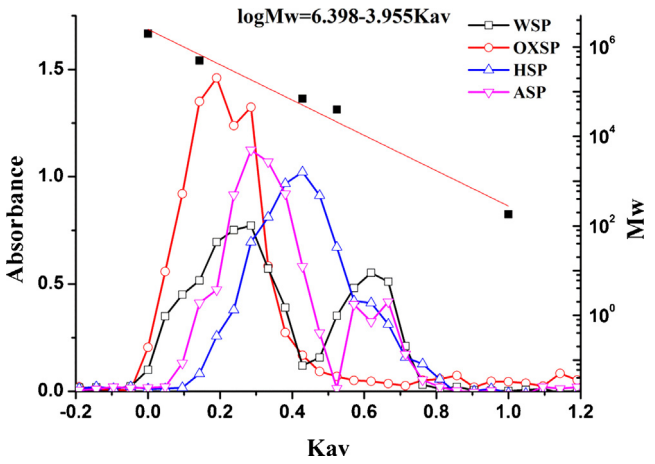

Fig. 3. (A) Molecular weight profiles of pectic fractions. Symbols: (□) for WSP; (○) for OXSP; (△) for HSP; (▽) for ASP. (■) for molecular weight.

behaved similarly with WSP on this Sepharose CL-6B column, displayed more than one peak in the chromatogram (about 387.99 kDa and 10.18 kDa, respectively). The lower molecular weight of HSP and ASP might due to an acid hydrolysis at acidic condition and a β-elimination at alkaline condition, respectively.

3.6. Gelling capability

Until now, only a few source materials have been used for commercial production of pectin as food additive. One of the reasons for this is that most of the pectic materials present in nature do not have any functional properties; in particular, the ability to form gel systems, and this property has been the main requirement of commercial pectin (Pinheiro et al., 2008). Therefore, it is necessary to evaluate the gelling capability of the pectic fractions.

No gelation was observed for HSP and its value of gel strength (hardness) also lower than 1.70 g, which was a critical value we established to distinguish whether gelation has taken place (Liang et al., 2012). For the other pectin fractions, gels had formed after standing for 24 h. The OXSP gel showed the highest gel strength (21.96 g) and gumminess (19.39 g), followed by WSP gel (4.22 and 3.83 g, respectively) and ASP gel (2.99 and 2.58 g, respectively) (Table 3). The parameter gumminess was positive correlation with gel strength (hardness), which was in accordance with the report of Kim, Yoo, Yam, Park, and Yoo (2008). It is also notable that this gel strength of OXSP at 1% pectin concentration were considerably greater than those of pectin from 'Golden delicious' apples at 2% (8.2 g) and 3% (15.3 g) (Rascón-Chu et al., 2009), indicating the excellent gelling ability of OXSP. This results essentially reinforced the pectin molecular structure (sugar composition, DM and molecular weight) results obtained earlier and indicated that structural parameters and gel strength were inter-related. OXSP showed a high content of galacturonic acid (76.15%) coupled with low content of neutral sugars (6.41%) and low DM (14.90%), all these factors being known to have a beneficial effect on the gelation process of LMP. On the other hand, gel strength depends on length of molecule.

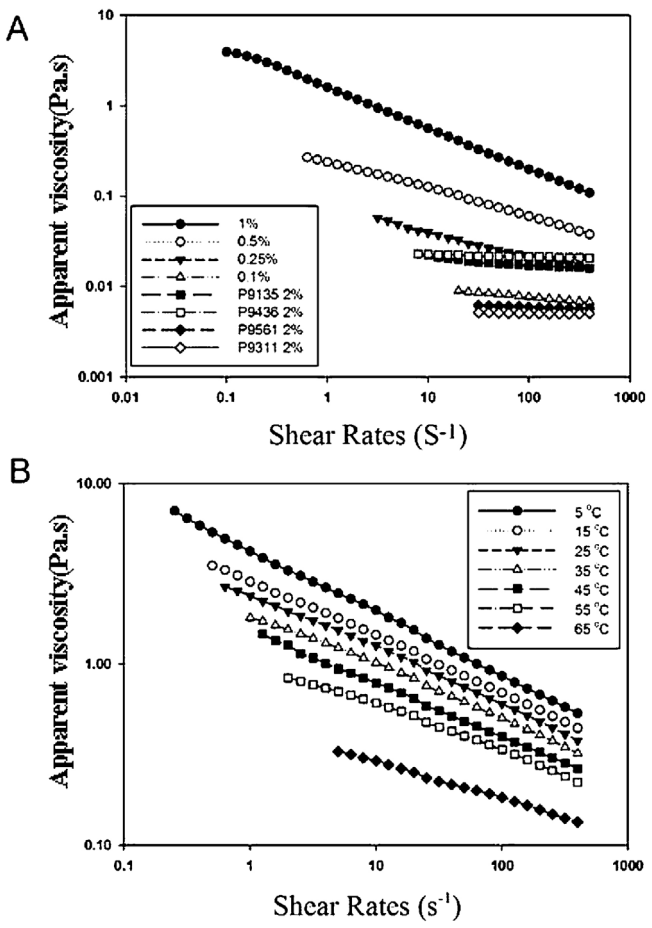

Fig. 4. (A) Steady shear flow curves of OXSP from *Premna microphylla* turcz leaves at different concentrations and commercial pectins at 2%. (B) Steady shear flow curves of 0.5% OXSP at different temperatures.

High molecular weight of OXSP promotes the interactions between pectin chains and increases the length of junction zones created by the associations of pectin chains, rendering pectin gels stronger. Therefore, the highest gel strength of OXSP could also be explained by its high molecular weight. In addition, other parameters, such as springiness, resilience and cohesiveness, were not appreciably different amongst gels of OXSP, WSP and ASP. The gel capability of OXSP suggested that low-methoxyl pectin extracted from PMLT by ammonium oxalate might be a very promising additive for food and non-food applications.

3.7. Flow behavior of OXSP

To explore the potential of OXSP used as thickener, its steady shear viscosity at different concentrations and temperatures were determined. It was found that the higher concentration, the higher apparent viscosity (Fig. 4A). At all concentration investigated, OXSP exhibited shear thinning, and more pronounced at higher

Table 3
Texture profile analysis parameters of pectin gels.[a]

Pectic fractions	Hardness (g)	Adhesiveness (mJ)	Springiness (–)	Cohesiveness (–)	Gumminess (g)	Resilience (–)
WSP	4.22 ± 0.75a[b]	0.026 ± 0.001a	0.96 ± 0.01a	0.91 ± 0.02a	3.83 ± 0.66a	0.73 ± 0.02a
OXSP	21.96 ± 1.64b	0.019 ± 0.000b	0.93 ± 0.02a	0.88 ± 0.02a	19.39 ± 1.36b	0.75 ± 0.05a
HSP	0.99 ± 0.16c	0.000 ± 0.000c	1.06 ± 0.02b	1.01 ± 0.03b	1.00 ± 0.18c	0.77 ± 0.05a
ASP	2.99 ± 0.53ac	0.028 ± 0.001a	0.86 ± 0.05a	0.86 ± 0.02a	2.58 ± 0.45ac	0.73 ± 0.03a

[a] Data are presented as means ± standard deviations of triplicate measurements.
[b] Mean values in the same column with different letters are significantly different (Tukey test, $p < 0.05$).

concentration. This effect could be explained by that the increase of concentration increased the amount of molecule chains, leading to the increase of interactions or chain–chain entanglements of pectin chains and restrictions of the motion of individual chains. The application of a shear to the system will cause the disruptions of ordered structures, which would lead to a decrease viscosity as the rate of deformation increases (Singthong et al., 2005). At dilute polymer systems, the formation of the entanglement is minimum, the entanglement to be disrupted by shear force is limited, so the decrease of viscosity and shear-thinning behavior was not so significant. When the flow behavior of pectin dispersions was compared to that of commercial pectin, it is notable that viscosity value of OXSP at 0.25% was slight higher or similar with that of P9436 and P9135 at 2%, while OXSP at 0.1% showed higher apparent viscosity than P9561 and P9311 at 2% (Fig. 4A).

Regarding the influence of temperature on the viscous properties, data shown in Fig. 4B suggest that these solutions were temperature dependent, as the temperature increased, viscosity decreased. This might be due to the elevated temperature increased the energy dissipation of the molecule, or decreased the intermolecular interactions, which in turn decreased the interference of the hydrodynamic domain (Chen & Chen, 2001). The results about the effect of concentration and temperature on apparent viscosity indicated that the OXSP may be used to thicken aqueous samples, and permits stabilization of emulsions, foams and particulate suspensions.

4. Conclusions

Cell wall composition analysis revealed that AIS of PMLT contained high amounts of pectin. The sequential extraction of cell wall polysaccharides from AIS demonstrated the main pectic substance in PMLT was calcium-bound pectin. Extraction of this pectin resulted in a significant change of morphology of cell wall material, indicating the observation of morphology of extraction residues can be used to evaluate the efficiency of extracting agents and proportion of pectic substances.

In addition, different pectic fractions exhibited different proportions, sugar compositions, molecular weights as well as gelling capabilities. OXSP showed the best gelling capability and a high apparent viscosity due to its high galacturonic acid but low neutral sugars content, high average molecular weight and low DM. The results may allow an improved use of PMTL as a resource of LMP. The resulted LMP has a great potential as a commercial gelling agent or thickener in the food industry.

Acknowledgements

The authors would like to thank the Centre of Analysis and Testing of Nanchang University and State Key Laboratory of Food Science and Technology for their expert technical assistance. This study was supported financially by the National Natural Science Foundation of China (Nr 31260386), and the Research Program of State Key Laboratory of Food Science and Technology, Nanchang University (SKLF-MB-201004).

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