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Isolation and structural characterisation of pectin from endocarp of *Citrus depressa*

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Abstract

A polysaccharide was extracted with hot HCl from the endocarp of *Citrus depressa* grown in Okinawa, Japan. The yield was 4.1% on a fresh weight basis. Composition of the polysaccharide was 89.3% total carbohydrate, 79.2% uronic acid, 4.1% ash and 8.8% moisture. The degree of methoxylation was estimated to be 66.2%. The polysaccharide was composed of D-GalA, D-Gal, L-Ara and L-Rha in the ratio of 100:10.3:1.53:0.94, respectively. The molecular mass was estimated to be approximately 4.1×10^4. NMR spectra indicated that the polysaccharide was mainly composed of (1→4)-linked α-D-GalA and (1→4)-linked β-D-Gal. Methylation analysis results identified 1→2 and 1→2,4 linked Rha, 1→4, 1→3, 1→6 and 1→3,6 linked Gal. The results indicated that the polysaccharide was a pectin, which was relatively simpler than other pectins. The polysaccharide was classified as high-methoxyl pectin. The pectin turned into a gel by the generalised method.

Keywords: *Citrus depressa*; Pectin; High-methoxyl; Structural characterisation; Endocarp

1. Introduction

Pectic substances are complex plant polysaccharides. They are present in all the higher plants and occur in the intercellular or middle lamellar region, and provide the channels for passage of nutrients and water. In cell walls, pectic substances serve as one of the main agents cementing the cellulose fibrils, and may be linked covalently to other polymers. Pectic substances are classified as protopectins, pectins (high-methoxyl pectins – rapid set pectin and slow set pectin – and low-methoxyl pectins) and pectic acids. Pectins influence growth, development and senescence, and affect textural properties of plant tissues and fruits (Inari, Yamauchi, Kato, & Takeuchi, 2002). Pectins are widely used in the food industry because of their gel-forming properties. They are used as gelling agents and stabilisers in jams, jellies and acid milk products. Citrus peel, apple pomace and sugar beet pulp are reported to be good sources of pectins (Kar & Arslan, 1999).

Pectin is reported to be a complex heteropolysaccharide with homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II as main structures (Willats, McCartney, Mackie, & Knox, 2001). Homogalacturonan is reported to be constituted of (1→4)-linked α-D-galacturonic acid residues. Some carboxyl groups, methyl-esterified and some hydroxyl groups, partially acetylated at O-2 and/or O-3, have a profound effect on the functional properties on pectin. Rhamnogalacturonan I is reportedly composed of (1→4)-linked α-D-galacturonic acid and (1→2)-linked α-L-rhamnose, which are alternatively combined with each other in the backbone; and some of the rhamnose residues contain side chains, such as arabinan, galactan and arabinogalactan at 4-*O*-rhamnose (Vincken et al., 2003). Rhamnogalacturonan II is reported to have a very complex structure. It is composed of a (1→4)-linked α-D-galacturonic acid residue backbone and unique sugars such as D-apiose, 2-keto-3-deoxy-D-manno-2-octulosonic acid (Kdo), 3-deoxy-D-lyxo-2-heptulosaric acid (Dha) and others, which

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are present in side chains attached at O-2 or O-3 of some of the galacturonic acid backbone (Ridley, O'Neill, & Mohnen, 2001; Willats, Knox, & Mikkelsen, 2006).

Pectins have several biological and physiological functions, such as reduction of serum cholesterol (Brown, Rosner, Willett, & Sacks, 1999), stimulation of phagocytes and macrophages (Iacomini et al., 2005; Popov, Popova, Ovodova, Bushneva, & Ovodov, 1999), spleen cells proliferation (Wang, Liu, & Fang, 2005; Zhao et al., 2006), anticomplementary activity (Samuelsen et al., 1995; Yamada, 1994) and inhibition of hyaluronidase and histamine release (Sawabe, Nakagomi, Iwagami, Suzuki, & Nakazawa, 1992). It is also reported that citrus pectin and its derivative (modified citrus pectin) have inhibitory effects on fibroblast growth factor signal transduction (Liu et al., 2001; Liu et al., 2002), suppression of LPS-induced inflammatory responses (Chen et al., 2006) and preventive effect on cancer growth and metastasis (Nangia-Makker et al., 2002; Nangia-Makker, Hogan, Honjo, et al., 2002; Platt and Raz, 1992).

Citrus depressa is a citrus plant grown naturally in Okinawa, Japan. The fruit is eaten, and utilised as a stain remover and cleaner for the local textile industry. The fruit is very sour like lemon, used as a seasoning and processed into snacks and juices. It was reported that the fruit contained appreciable amounts of polymethoxy flavonoids, particularly nobiletin, which have effects on diabetes (Li et al., 2006), inflammation (Tanaka, Sato, Akimoto, Yano, & Ito, 2004; Yoshimizu et al., 2004), cancer (Ito, Ishiwa, Sato, Mimaki, & Sashida, 1999; Minagawa et al., 2001; Sato et al., 2002) and rheumatism (Ishiwa et al., 2000). The fruit is regarded as a significant agricultural product in Okinawa, Japan, and is expected to be utilised effectively in the future. We have isolated and characterised a lot of polysaccharides from algae and plants grown in Okinawa. The objectives of the study are (1), to isolate pectin from *C. depressa* endocarp; (2), to structurally characterise pectin from the endocarp of *C. depressa*; and (3), to assess the gelation properties of isolated pectin, by adding sucrose (60%) and citric acid (0.05 M) to the pectin solution.

2. Materials and methods

2.1. Materials

C. depressa fruits were collected in January, 2001 from Ogimi, Okinawa, Japan. The endocarp was separated and air-dried in an oven at 40 °C for 24 h. The dried endocarp was crushed or ground into powder with a blender. A high-methoxyl pectin (Taiyo Kagaku Co., Ltd., Japan) was used as a standard.

2.2. Extraction of polysaccharide

Polysaccharide was extracted by a modified method of the acid extraction procedure of Ralet and Thibault (1994). The powdered endocarp sample (3 g) was suspended in 0.05 M HCl (250 ml) and stirred at 85 °C for 1 h. The suspension was filtered, and the filtrate was adjusted to pH 4.5 with 0.5 M NaOH. Ethanol (EtOH: 2–3 vols) was added to the solution and the precipitate was washed with EtOH twice. The precipitate was dried in a vacuum chamber at 40 °C.

The dried precipitate was dissolved in distilled water and passed through a column (5 × 20 cm) of Amberlite IR-120B (H^+ form). The eluate was adjusted to pH 4.5, dialysed against distilled water, and freeze-dried. The freeze-dried material was dissolved in 0.05 M NaOAc buffer (pH 4.8). The solution (200 mg/50 ml) was loaded on a column (2.6 × 28 cm) of DEAE-sepharose equilibrated with 0.05 M NaOAc buffer at a flow rate of 2 ml/min. The column was washed with 250 ml of the same buffer and the bound material was eluted with 600 ml of 1 M NaOAc buffer (pH 4.8). The eluate was dialysed against distilled water and freeze-dried.

2.3. De-esterification of the polysaccharide

The polysaccharide (250 mg/50 ml) was dissolved in distilled water and then chilled to 4 °C. Cold NaOH (0.1 M) was added slowly to the polysaccharide solution to a final concentration of 0.05 M. The mixture was kept at 4 °C for 1 h with mild stirring at intervals. The pH of the mixture was then adjusted to 4.5 with 1 M HCl, the mixture was dialysed against distilled water, and then freeze-dried.

2.4. Determination of total carbohydrate, uronic acid, moisture, ash and degree of methoxylation (DM) of the polysaccharide

The composition of total carbohydrate and uronic acid were determined by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and the carbazole–sulfuric acid method (Bitter & Muir, 1962), respectively, using D-galacturonic acid as a standard. Moisture content was determined by drying the polysaccharide at 110 °C for 2 h and calculating the amount of evaporated water. The ash content was determined by incinerating the polysaccharide overnight in a muffle furnace at 550 °C and weighing the residue. Degree of methoxylation (DM) was calculated as molar ratio from the contents of methanol and galacturonic acid. The content of methanol was determined by the colorimetric method of Inari and Takeuchi (1997).

2.5. Determination of the polysaccharide components

The polysaccharide (50 mg) was dissolved in distilled water (20 ml) and sulfuric acid was added to a final concentration of 1.5 M. The solution was heated at 100 °C for 3 h. The hydrolysate was neutralised with $BaCO_3$ and filtered. The hydrolysate was analysed by high-performance anion exchange chromatography on a DX 500 liquid chromatograph (Dionex Co., Ltd., Sunnyvale, CA), fitted with a column of CarboPac PA1 (4 × 250 mm) and a pulsed

amperometric detector. The column was eluted at a flow rate of 1 ml/min at 35 °C with 15 mM NaOH for neutral sugars and with 100 mM NaOH/150 mM NaOAc for uronic acids.

2.6. Molecular mass determination of the polysaccharide

The molecular mass of the polysaccharide (1 mg/ml) was determined by high-performance liquid chromatography (HPLC), using an LC-6A chromatograph, (Shimadzu, Tokyo, Japan) on a column of TSK-gel $GMPW_{XL}$ (7.8 × 300 mm, Tosoh Corporation, Tokyo, Japan). HPLC was performed at room temperature, at a flow rate of 0.3 ml/min, with refractive index detection (RID-6A, Shimadzu). The column was conditioned with 0.15 M sodium chloride in 0.05 M sodium phosphate buffer (pH 7.2), and elution was carried out with the same buffer. Standard pullulans (Showa Denko K.K., Tokyo, Japan) including P-400 (molecular mass, 4.04×10^5), P-100 (1.12×10^5), P-20 (2.28×10^4), and P-5 (5.9×10^3) were used as molecular mass markers.

2.7. Specific rotation and infrared spectra of the polysaccharide

Specific rotation was measured at 589 nm on a polarimeter (DIP-180, JASCO International, Tokyo, Japan) with a cell of 5 cm length at 25 °C. The polysaccharide solution (0.2%) was prepared in distilled water. Infrared spectra were recorded with an FTS-3000 spectrophotometer (Bio-Rad Laboratories, Hercules, CA) in transmittance mode from 4000 to 400 cm^{-1} in KBr discs. The KBr discs were prepared by dispersing solid samples in the salt.

2.8. ^1H NMR and ^{13}C NMR spectra of the polysaccharide

Samples were dissolved in D_2O and then freeze-dried. The dried samples (2%) were redissolved in D_2O, and the solutions were examined in 5 mm o.d. tubes. Chemical shifts were expressed in δ (ppm), relative to the resonance of sodium 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (TSP, 0.00 ppm) as an internal standard. The ^1H and ^{13}C NMR spectra were recorded on an FT-NMR spectrometer at 500.00 and 125.65 MHz (JNM α500, JEOL Ltd., Tokyo, Japan) at 80 °C. One-dimensional ^1H and ^{13}C spectra were recorded using 45° pulse width, 32,768 data points, and 1 s pulse delay for ^1H, and 60° pulse width, 16,384 data points, and 0.5 s pulse delay for ^{13}C, respectively. Two-dimensional spectra of COSY and NOESY were recorded using standard JEOL procedures. COSY and NOESY spectroscopy were performed with 256 (t2) × 128 (t1) × 2 data matrix, using a spectral width of 3.05 × 3.05 kHz, which gave a digital resolution of 11.92 × 11.92 Hz in f2 and f1, respectively. A mixing time of 100 ms was used in the NOESY experiment. The spectral assignments were carried out using the two-dimensional spectra and references.

2.9. Separation of homogalacturonan region

Separation of the homogalacturonan region from the polysaccharide was carried out by the method of Thibault, Renard, Axelos, Roger, and Crépeau (1993). The de-esterified polysaccharide (0.1%) was hydrolysed in 0.1 M HCl at 80 °C for 72 h in a sealed tube. An acid-insoluble fraction (homogalacturonan fraction) was separated by centrifugation of the reaction mixture. The acid-insoluble fraction was resolubilised by neutralisation with 0.5 M NaOH, dialysed against distilled water, and freeze-dried.

The molecular mass of the homogalacturonan (4 mg in 2 ml) was measured by gel filtration chromatography on a column of Sephadex G-150 (2 × 60 cm), eluted with 0.05 M NaOAc buffer (pH 4.1), at a flow rate of 13.2 ml/h. The studied fraction (3 ml) was monitored by the phenol–sulfuric acid method. Standard pullulans, including P-100 (molecular mass, 1.12×10^5), P-50 (4.73×10^4), P-20 (2.28×10^4), and P-5 (5.9×10^3), were used as molecular mass markers.

2.10. Methylation analysis

The polysaccharide was methylated twice, with CH_3I and NaOH–DMSO reagent (Anumula & Taylor, 1992; Needs & Selvendran, 1993). The polysaccharide (5 mg) was dried in a vacuum oven at 40 °C and suspended in DMSO (2 ml) under N_2. NaOH–DMSO reagent (1 ml) was added to the solution, and stirred and sonicated for 90 min. CH_3I (1 ml) was added slowly to the solution, and then stirred and sonicated for 1 h. After dialysis of the solution, methylated polysaccharide was extracted with chloroform. The methylated polysaccharide was then hydrolysed using 2 M TFA (2 ml) for 2 h at 120 °C. Reduction and acetylation were carried out by the method described by Carpita and Shea (1989). The hydrolysate was dissolved in 1 M NH_4OH (0.2 ml). DMSO (1 ml) containing 20 mg of $NaBH_4$ was added and the mixture was incubated at 40 °C for 90 min. After that, glacial acetic acid (0.2 ml) was added to the mixture. Anhydrous 1-methylimidazole (0.2 ml) and acetic anhydride (1 ml) were then added and the reaction mixture was incubated at ambient temperature for 10 min. After extraction with chloroform and washing with water, partially methylated alditol acetates were obtained. The partially methylated alditol acetates were separated by injection onto a capillary column (30 m × 0.25 mm, i.d., DB-1, J&W Scientific) in a gas chromatograph (GC-17A, Shimadzu Co., Ltd.), equipped with a flame ionisation detector. The injector and detector temperatures were 210 and 270 °C, respectively. After injection, oven temperature was maintained at 150 °C for 5 min, then raised at 5 °C/min to 250 °C, and this temperature was kept for 5 min. The identities of the peaks were confirmed with GC–MS (QP-5000, Shimadzu Co., Ltd.).

2.11. Gel formation of the polysaccharide

A gel of the polysaccharide was prepared by the method of Ralet, Axelos, and Thibault (1994). The polysaccharide (1%) was dissolved in distilled water and then sucrose (60%) was added to the solution. The mixture was heated at 100 °C for 30 min with magnetic stirring. Then citric acid (0.5 M) was added slowly to reach 0.05 M in the final concentration with stirring. The slurry was poured into a test tube and capped with a marble, and after that it was left overnight at ambient temperature.

3. Results

Hot acid extraction was used for pectin extraction in the study because these conditions are usually used in the food industry. The yield of the polysaccharide was 4.1%, based on fresh weight basis, and a white fibrous material was obtained. The composition of the polysaccharide was 89.3% total carbohydrate, 79.2% uronic acid, 4.1% ash, and 8.8% moisture. The results indicated that the polysaccharide was high in uronic acid. The degree of methoxylation (DM) of the polysaccharide was 66.2%. Acid hydrolysis and HPLC analysis of the hydrolysate of the polysaccharide identified D-galactose, L-arabinose and L-rhamnose. The HPLC results also indicated that the uronic acid of the polysaccharide was D-galacturonic acid, which is the main component of pectin. The molar ratio of D-galacturonic acid, D-galactose, L-arabinose and L-rhamnose was estimated to be 100:10.3:1.53:0.944, respectively.

The molecular mass of the polysaccharide was measured by gel chromatography, and only one broad peak was observed. The polysaccharide molecular mass was estimated to be approximately 4.1×10^4, according to a standard calibration curve obtained from definite molecular mass pullulans. The specific rotation of the polysaccharide was +188° at 25 °C and this value indicated that the orientation of the polysaccharide was mainly due to α-glycosidic linkages (Misaki, 1973).

De-esterification was carried out to facilitate the analysis of the spectra in IR and NMR. The infrared spectra of the polysaccharide, the de-esterified polysaccharide, the standard pectin and the de-esterified standard pectin are shown in Fig. 1. A major absorption at around 3400 cm^{-1} was attributed to stretching of hydroxyl groups and an absorption at 2900 cm^{-1} was due to C–H stretching of CH_2 groups. An absorption at 1610 cm^{-1} was caused by C=O stretching vibration of ionic carboxyl groups, and an absorption at 1740 cm^{-1} by C=O stretching vibration of methyl-esterified carboxyl groups; this absorption disappeared in the de-esterified polysaccharides (Chatjigakis et al., 1998; Kamnev, Colina, Rodriguez, Ptitchkina, & Ignatov, 1998; Manrique & Lajolo, 2002). The spectra of the polysaccharide and the de-esterified polysaccharide were consistent with the spectra of the standard pectin and the de-esterified standard pectin, respectively, over wide ranges of wave numbers including the fingerprint region.

In the ^1H NMR spectrum of the polysaccharide (Fig. 2), the very large signal at 3.81 ppm was derived from methyl groups binding to carboxyl groups of GalA. Two signals around 2.1 ppm were derived from acetyl groups binding at 2-*O*- and 3-*O*-GalA, the former was at 2.18 ppm and the latter was at 2.10 ppm (Perrone et al., 2002; Renard & Jarvis, 1999). This indicated that acetyl groups were mainly bound at 3-*O*-GalA. Signals at 1.25 and 1.31 ppm were derived from methyl groups of L-rhamnose, and were assigned to the O-2 linked rhamnose and to the O-2,4

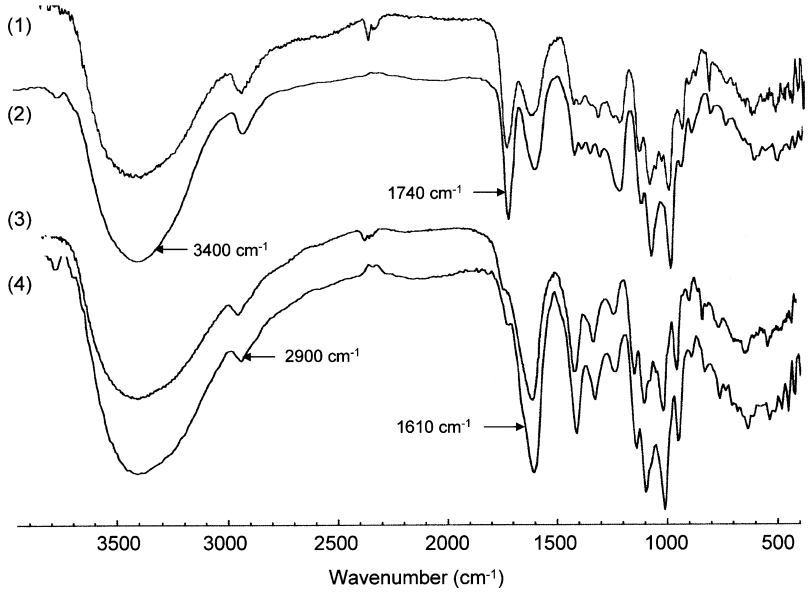

Fig. 1. Infrared spectra of the polysaccharide extracted from *C. depressa*, the de-esterified polysaccharide, the standard pectin and the de-esterified standard pectin: (1) extracted polysaccharide; (2) standard pectin; (3) de-esterified polysaccharide; (4) de-esterified standard pectin.

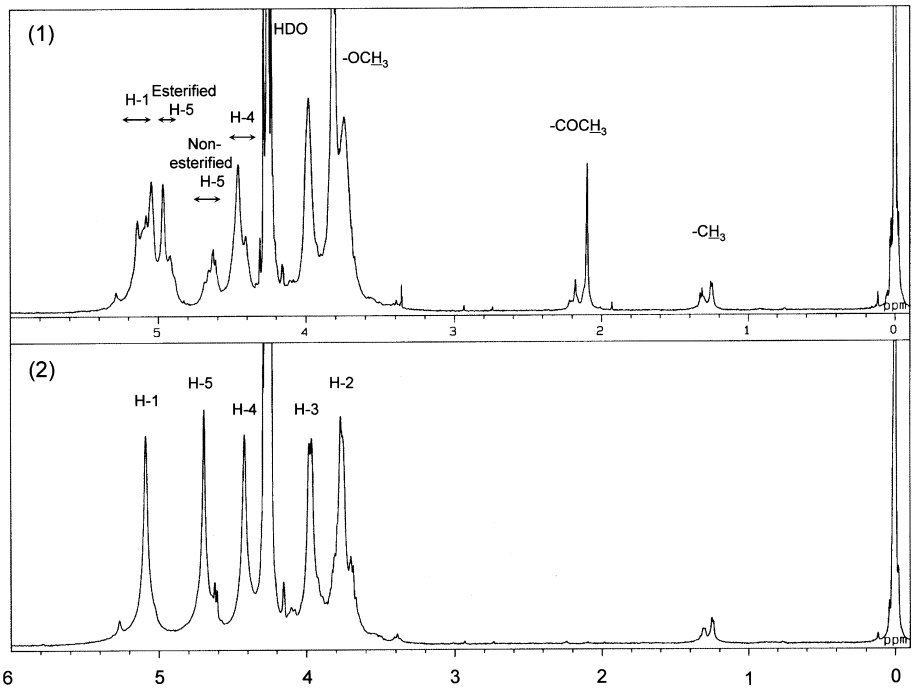

Fig. 2. ^{1}H NMR spectra of the polysaccharide extracted from endocarp and the de-esterified polysaccharide in D_2O at 80 °C: (1) polysaccharide; (2) de-esterified polysaccharide.

linked rhamnose, respectively (Vignon & Garcia-Jaldon, 1996). Esterification of carboxyl groups GalA may have contributed to the complexity of the NMR spectra, especially signals such as H-1, H-4 and H-5 of galacturonic acid (Andersen, Larsen, & Grasdalen, 1995; Grasdalen, Andersen, & Larsen, 1996; Grasdalen, Bakøy, & Larsen, 1988). After the polysaccharide was de-esterified, the spectrum was simplified and five major signals were found. They were assigned as D-galacturonic acid: H-1, 5.09 ppm; H-2, 3.76 ppm; H-3, 3.97 ppm; H-4, 4.41 ppm; H-5, 4.68 ppm, (Fig. 3) and as reported in the literature (Mukhiddinov, Khalidov, Abdusamiev, & Avloev, 2000). These chemical shifts were in agreement and consistent with those of the de-esterified standard pectin as presented in Table 1, and as reported in the literature (Mukhiddinov et al., 2000). In the NOESY spectrum of the de-esterified polysaccharide, a cross peak between H-1 and H-4 in D-galacturonic acid was observed, which indicated that the polysaccharide contained α-(1→4)-linked D-galacturonic acid residues (Fig. 4).

^{13}C NMR spectra of the polysaccharide and the de-esterified polysaccharide are presented in Fig. 5. In the polysaccharide spectrum, a signal at 55.7 ppm was assigned to methyl groups binding to carboxyl groups of GalA (Keenan, Belton, Matthew, & Howson, 1985) and a signal at 173 ppm was attributed to carboxyl groups bound by methyl groups (Catoire, Goldberg, Pierron, Morvan, & Penhoat, 1998). After de-esterification, the signal at 55.7 ppm disappeared and the signal at 173 ppm was shifted to 177 ppm at which the signal was derived from ionic carboxyl groups. In the spectrum of the de-esterified polysaccharide, six major signals and six minor signals were observed. The six major signals were assigned to D-galacturonic acid: GA-1, 102.0 ppm; GA-2, 71.3 ppm; GA-3, 72.0 ppm; GA-4, 81.1 ppm; GA-5, 74.2 ppm; GA-6, 177.4 ppm. The minor signals were assigned to D-galactose: G-1, 107.2 ppm; G-2, 74.9 ppm; G-3, 76.3 ppm; G-4, 80.5 ppm; G-5, 77.5 ppm; G-6, 63.7 ppm (Catoire et al., 1998; Keenan et al., 1985; Saulnier, Brillouet, & Joseleau, 1988; Westerlund, Åman, Andersson, & Andersson, 1991). These chemical shifts were also in good agreement with those of the de-esterified standard pectin (Table 2).

The polysaccharide was methylated twice and after acid hydrolysis, methylated sugars were converted to partially methylated alditol acetates. As a result of methylation analysis, 1→2 and 1→2,4 linked Rha, 1→4, 1→3, 1→6 and 1→3,6 linked Gal, 1→5 linked Ara were identified (Table 3), which are typical linkage patterns in pectins. The identification of 1→2 and 1→2,4 linked Rha residues suggested the existence of rhamnogalacturonan I in the polysaccharide. The presence of 1→4 and 1→3,4 linked Gal residues, and 1→3, 1→6 and 1→3,6 linked Gal residues indicated that the polysaccharide contained type I arabinogalactan and type II arabinogalactan. About 34.7% of the total Gal residues were 1→4 linked Gal, and with the additional 1→3,4 linked Gal, 41.1% of the total Gal could form type I arabinogalactan, indicating that it was the main side chain. Considering type II arabinogalactan, content of 1→3,6 linked Gal was approximately three times more than that of 1→3 linked Gal, suggesting that type II arabinogalactan could be branched heavily. It was estimated that an average of 3 Gal residues

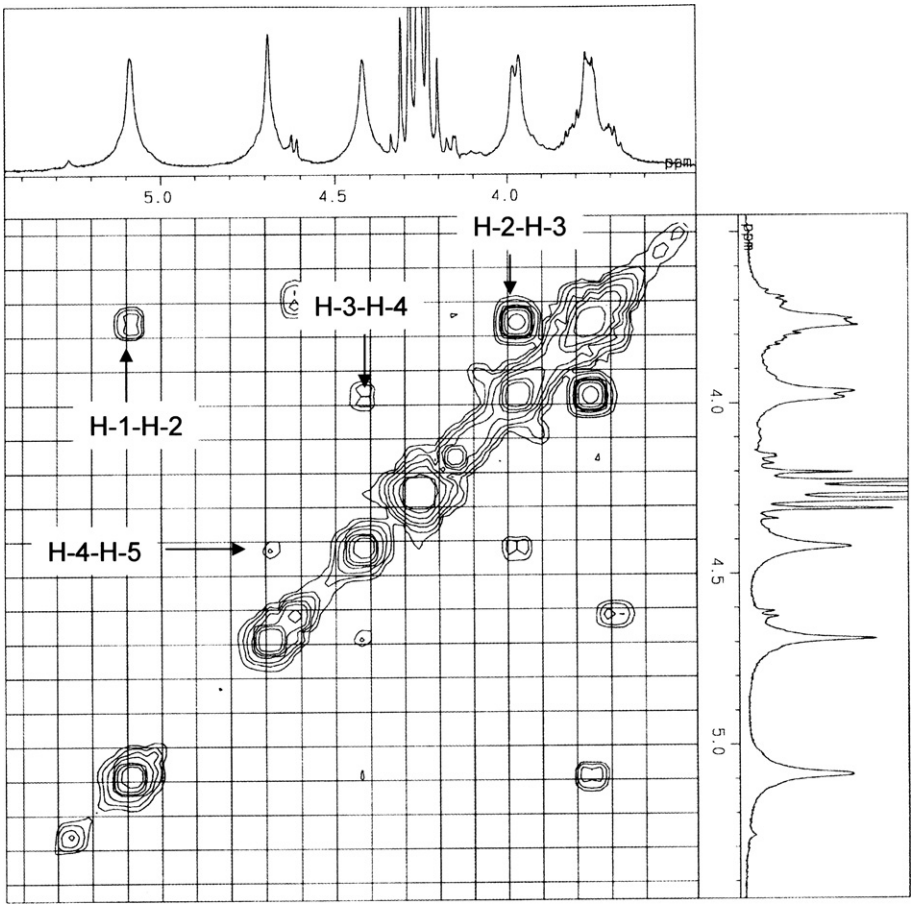

Fig. 3. COSY spectrum of the de-esterified polysaccharide from *C. depressa*.

Table 1
Chemical shifts of the ^{1}H NMR spectra of the de-esterified polysaccharide extracted from endocarp and the de-esterified standard pectin

		^{1}H chemical shifts				
		H-1	H-2	H-3	H-4	H-5
De-esterified polysaccharide	Galacturonic acid	5.09	3.76	3.97	4.41	4.68
De-esterified polysaccharide	Galacturonic acid	5.08	3.76	3.96	4.42	4.71

as side chain of the arabinogalactan were attached at the O-6 branching point of the 1→3 linked Gal backbone.

In order to inspect the length of the homogalacturonan of the polysaccharide, the homogalacturonan was separated from the polysaccharide. The yield was 68.2% and the composition of total carbohydrate and uronic acid were 89.7% and 90.8%, respectively. A ^{1}H NMR spectrum of the homogalacturonan indicated that it was very simple. Only five signals were observed, indicating that the homogalacturonan was composed of only galacturonic acid. The molecular mass of the homogalacturonan was estimated to be approximately 1.8×10^4 by gel chromatography, as a single peak. The gel chromatography results indicated that about 100 galacturonic acid residues were at least sequentially linked. The polysaccharide was hydrolysed for 96 h, to check whether further degradation occurred, but this hydrolysis did not significantly change the polysaccharide molecular mass.

It is known that high-methoxyl pectins (methoxyl content $\geqslant$7%, degree of methoxylation (DM) $\geqslant$43%) can form gels at pH 2.2–3.5 if a co-solute, such as sucrose, is present at a concentration of $\geqslant$55% by weight (Ralet et al., 1994) and low-methoxyl pectins (methoxyl content <7%, DM <43%) can form gels in the presence of an adequate concentration of a divalent cation, such as calcium, across a wide range of soluble-solids contents (Pilgrim, Walter, & Oakenfull, 1991). The solution of the polysaccharide turned into a gel in presence of sucrose (60%) and citric acid (0.05 M), like jams and jellies with viscosity and adhesion. Standard high-methoxyl pectin also turned into a gel under the same conditions.

4. Discussion

The physicochemical and structural characterisation results indicated that the polysaccharide extracted from the endocarp of *C. depressa* was a pectin. The pectin was obtained with a yield of 4.1%, which was slightly higher than recorded yields of lemon (3.0–4.0%) and grapefruit

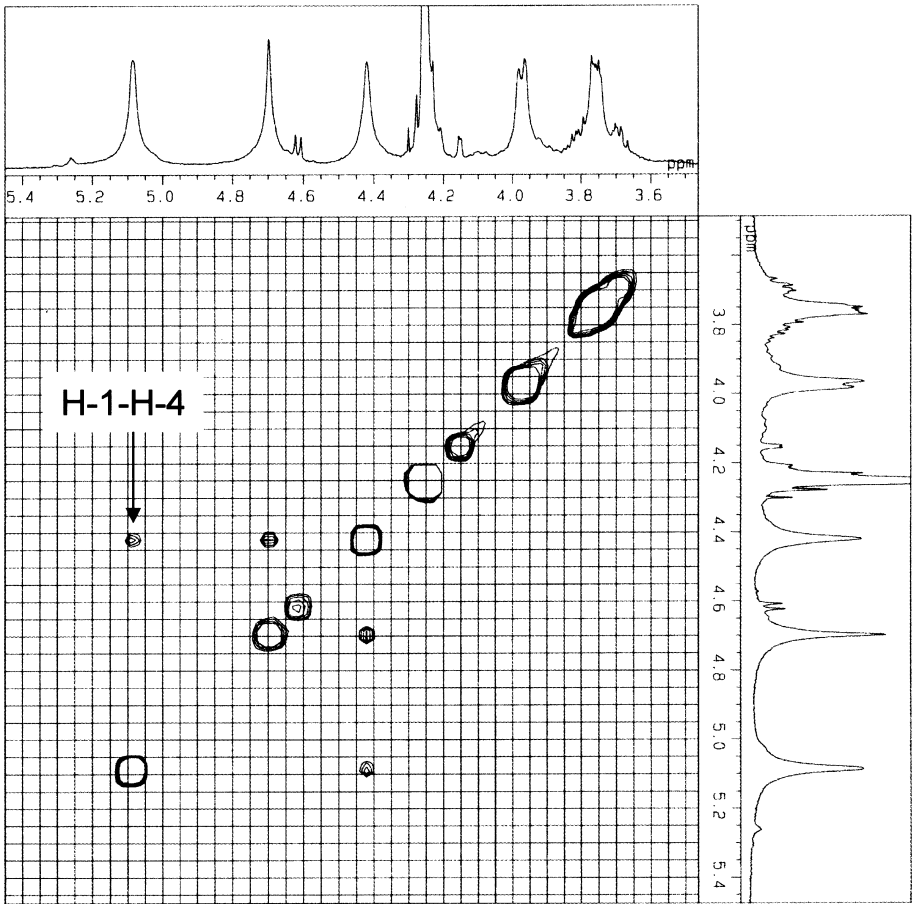

Fig. 4. NOESY spectrum of the de-esterified polysaccharide extracted from *C. depressa*.

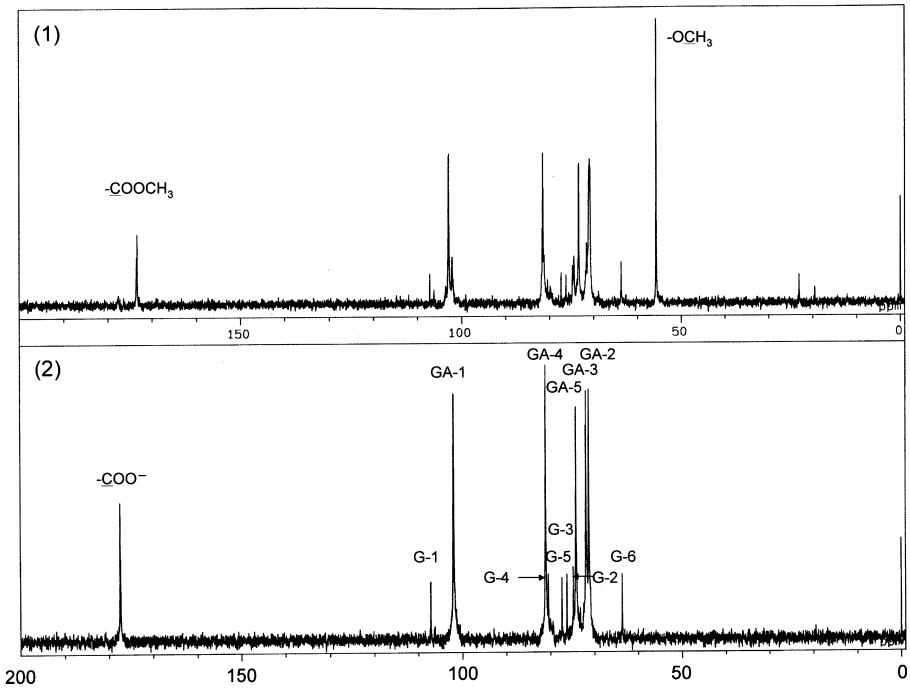

Fig. 5. ^{13}C NMR spectra of the polysaccharide extracted from *C. depressa* and the de-esterified polysaccharide in D_2O at 80 °C: (1) extracted polysaccharide; (2) de-esterified polysaccharide. Abbreviation: GA, galacturonic acid; G, galactose.

Table 2
Chemical shifts of the ^{13}C NMR spectra of the de-esterified polysaccharide extracted from *C. depressa* and the de-esterified standard pectin

		^{13}C chemical shifts					
		C-1	C-2	C-3	C-4	C-5	C-6
De-esterified polysaccharide	Galacturonic acid (GA)	102.0	71.3	72.0	81.1	74.2	177.4
	Galactose (G)	107.2	74.9	76.3	80.5	77.5	63.7
De-esterified standard pectin	Galacturonic acid (GA)	101.9	71.3	71.9	81.1	74.3	177.6
	Galactose (G)	107.2	74.9	76.3	80.5	77.4	63.7

Table 3
Methylation analysis of the polysaccharide extracted from endocarp

		mol.%
Ara	Terminal*f*	4.39
	Terminal*p*	2.61
	1→3 link	3.30
	1→5 link	8.48
Rha	1→2 link	5.15
	1→2,4 link	4.79
Gal	Terminal	14.25
	1→4 link	24.76
	1→3 link	2.32
	1→6 link	11.64
	1→3,4 link	4.93
	1→4,6 link	4.24
	1→3,6 link	6.38
	1→3,4,6 link	2.76

(3.3–4.5%), as reported in the literature (Manabe, 2001). The main sugar was galacturonic acid, and it accounted for 88.7% of the total sugars. The main neutral sugar was galactose, which was comprised of approximately 80% of the neutral sugars. Arabinose and rhamnose were present in small quantities. Other sugars such as xylose and glucose, which are often present in citrus pectins, were not detected. The degree of methoxylation (DM) was 66.2%, and degree of acetylation (DA) was estimated to be 2.1%, by comparing the area of acetyl groups with that of methyl groups of rhamnose in the ^{1}H NMR spectrum. The pectin turned into a gel by a generalised method of adding sucrose (60%) and then citric acid (final concentration, 0.05 M) to a pectin solution. This pectin therefore was classified as a high-methoxyl pectin (medium rapid set), the type of which is utilised in acid jams and jellies, and confectionery uses (Pilgrim et al., 1991; Thibault & Ralet, 2003). The molecular mass of the pectin was estimated to be approximately 4.1×10^4. The results were consistent with the physicochemical properties of commercial pectins (galacturonic acid >65%, degree of methoxylation 30–75%, degree of acetylation <5%, neutral sugars <15%, and molecular mass 1.0×10^5–2.0×10^5), as reported in the literature (Thibault & Ralet, 2003). Except for molecular mass, other physicochemical characteristics of the extracted pectin were within the acceptable ranges of commercial pectins.

The infrared spectra, and ^{1}H NMR and ^{13}C NMR spectra of the polysaccharide were consistent with the spectra of standard commercial pectins. NMR spectra identified the presence of α 1→4 linked GalA and β 1→4 linked Gal residues. Other sugars could not be assigned because they were present in small quantities. In methylation analysis, the main partially methylated alditol acetate was from 1→4 linked Gal residues, which was in agreement or consistent with the NMR results. The 1→2 Rha and 1→2,4 Rha residues indicated that the polysaccharide contained rhamnogalacturonan I moiety. The results indicated that about half of the Rha residues were linked at O-4 with side chains, such as type I arabinogalactan and type II arabinogalactan. In type I arabinogalactan, it was shown that the backbone was composed of β 1→4 linked Gal residues and it had one O-3 branched Gal residue per 6 Gal. The Gal residues and/or Ara residues might be linked at non-reducing ends. In type II arabinogalactan, the results indicated that the backbone was 1→3 linked Gal residues, and there were three O-6 branched Gal residues per 4 Gal. The type II arabinogalactan was short and branched widely.

From the results, the schematic structural model of the polysaccharide from the endocarp of *C. depressa* is tentatively proposed as in Fig. 6. The polysaccharide was a very

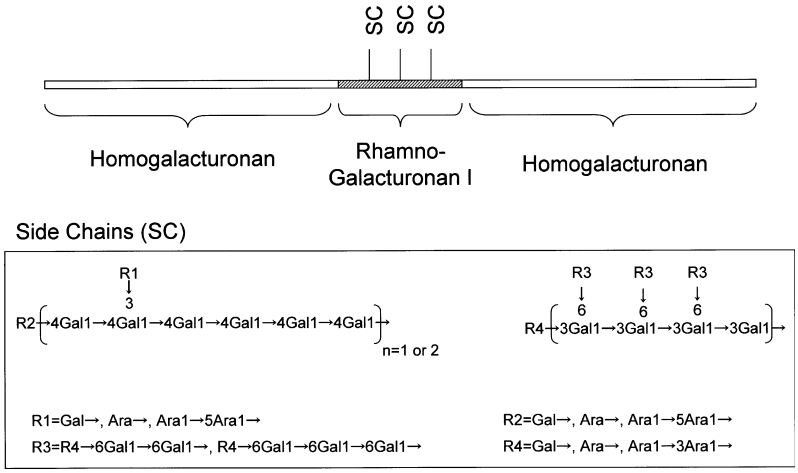

Fig. 6. Schematic structure model of the pectin of endocarp from *C. depressa*. Galacturonic acid residues were methoxylated (65%) and acetylated (2%).

simple pectin that was composed of only GalA, Gal, Ara and Rha residues. Almost all sugar components of the polysaccharide (pectin) were GalA, and the polysaccharide consisted mostly of homogalacturonan moiety. It was considered that the rhamnogalacturonan I moiety was small, and the side chains were also short. Considering the sizes of the polysaccharide and the homogalacturonan, the polysaccharide perhaps could be made up of 2 homogalacturonan moieties and 1 rhamnogalacturonan moiety. In addition, about 65% of galacturonic acid residues had methyl groups and a few of them were acetylated. The main components of the side chains were relatively long type I arabinogalactan and heavily branched type II arabinogalactan. From this experiment, it was difficult to consider that the rhamnogalacturonan I of this polysaccharide contained arabinan as a side chain, because the content of arabinose was much lower than galactose. The polysaccharide was also extracted under severe conditions, indicating that linkages of arabinose may be cut, and 1→2,4 rhamnose as a branching point was low, as indicated by methylation analysis. Based on our estimate, it was assumed that only 2 or 3 side chains may exist in the rhamnogalacturonan region. It may be difficult to assume that the site for arabinan binding was prepared. It might be considered that arabinose residues may be in branches of arabinogalactans. As a matter of fact, other schematic models may be possible.

C. depressa is regarded as a healthy food product, and good source of polymethoxyflavonoids. Pectin was extracted from the edible part or endocarp of *C. depressa* with high yield. It was classified as high-methoxyl type (medium rapid set) and it gelled under commonly used conditions (sucrose 60% and citric acid 0.05 M), by forming a high-methoxyl pectin characteristic junction zone (Oakenfull, 1991). The extracted pectin has the potential for utilisation in the food industry as a binder and stabiliser in jams, jellies, beverages and fruit juices.

In the case of physiological effects, inhibitory effects of FGF signal transduction were reported to be related to a high degree of methoxylation, a low total neutral sugar content and a high molecular mass of pectin (Liu et al., 2001; Liu et al., 2002). On suppression of LPS-induced inflammation, higher DM pectins had a higher suppression effect than lower DM (Chen et al., 2006). The pectin from *C. depressa* endocarp is expected to have similar physiological effects. The pectin extracted from the endocarp had high galactose content in neutral sugars, and may be a good source of modified citrus pectins, if the molecular weight could be reduced. More research is needed on extracted pectin from *C. depressa* for effective utilisation in the food, physiological and pharmaceutical industries.

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