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Determination of galacturonic acid from pomelo pectin in term of galactose by HPAEC with fluorescence detection

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ABSTRACT

Galacturonic acid (GalA) in pomelo pectin was converted to galactose (Gal) by saponification and reduction reaction of carboxy group. The characterization of the product was investigated by ^{1}H NMR spectroscopy. The reduced pectin was then hydrolyzed with trifluoroacetic acid (TFA) and analyzed by high-performance anion-exchange chromatography (HPAEC) with fluorescence detector. The content of GalA was calculated from an increased Gal after reaction. The GalA content obtained from this method was higher than that obtained from HPAEC-pulsed amperometric detection (PAD), which affords to analyze GalA directly after hydrolysis. The established method was validated and the results showed good linearity, high precision and high sensitivity. This method could be further applied for the analysis of GalA content in pomelo pectin.

1. Introduction

Pectin is one of the categories of complex polysaccharides found in the primary cell wall of most plants. The dominant component is galacturonic acid with neutral sugars primarily galactose, arabinose, rhamnose, and xylose. Pectin has been used as ingredients in various fields, including medicinal, pharmaceutical, cosmetics and food industries, for its gelling, stiffening and stabilizing properties. The two main sources of commercially available pectin are apple pomaces and citrus fruit rinds (Wang, Pagan, & Shi, 2002).

Pomelo (*Citrus maxima* Merr.), family Rutaceae, is a native citrus fruit of Asia especially in China, southern Japan, Vietnam, Malaysia, Indonesia and Thailand. Fruit is always round shape and big size with a very thick skin but soft and easy to peel away. The fruit of pomelo is eaten fresh or as juices, and then the massive amounts of the middle and outer layer (as waste products) are disposed, causing a severe problem in the community. The middle layer (albedo) of fruit peel is reported to contain pectin and can be use as a source of pectin and dietary fiber (Ittimongkol, Srisukh, Nitibhon, & Saraya, 2004; Norziah, Fang, & Karim, 2000; Sriamornsak, 2003).Pectin has been reported to possess a variety of pharmacological activities such as several immunostimulating activity, anti-metastasis activity, anti-ulcer activity, anti-nephrosis activity and cholesterol decreasing effect (Fernandez, Sun, Tosca, & McNamara, 1994; Guo et al., 2000; Nergard et al., 2005; Olano-Martin, Rimbach, Gibson, & Rastall, 2003; Paulsen & Barsett, 2005; Schwartz et al., 1988; Tinker, Davis, & Schneeman, 1994; Willats, Knox, & Mikkelsen, 2006; Yamada, 1994, 1996). Moreover, pectin oligosaccharides were observed to have prebiotic properties (Hotchkiss, Olano-Martin, Grace, Gibson, & Rastall, 2003). The content of galacturonic acid in pectin is very important for biological studies and also pharmaceutical and food industry (Ridley, O'Neill, & Mohnen, 2001).

Several methods have been used for quantitative analysis of galacturonic acid such as colorimetric method (Brummer & Cui, 2005; Chaplin, 1994; Dubois, Gilles, Hamilton, Reber, & Smith, 1965), thin-layer chromatography (Brummer & Cui, 2005; Chaplin, 1994; Yamada et al., 1991), gas chromatography (GC) (Brummer & Cui, 2005; Chaplin, 1994), high-performance liquid chromatography (HPLC) (Brummer & Cui, 2005; Chaplin, 1994; Lv et al., 2009) and capillary electrophoresis (Brummer & Cui, 2005; Chaplin, 1994; Goubet, Ström, Dupree, & Williams, 2005). Pectin must first be hydrolyzed in hot concentrated acid or enzyme to uronic acid and neutral monosaccharides prior to analysis. Acid hydrolysis can cause the degradation of galacturonic acid resulting in the low recovery (Garna, Mabon, Wathelet, & Paquot, 2004). The enzymatic hydrolysis of pectin gives high recovery of galacturonic acid however it requires different types of enzyme activities (Deng, O'Neill, & York, 2006; Kashyap, Vohra, Chopra, & Tewari, 2001; McCleary, 1986). Although pectin hydrolysis by acid gave lower amount of galacturonic acid, almost neutral sugars are still stable.

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This study was presented the simple and rapid method for the determination of galacturonic acid in pectin samples by chemically de-esterified and reduced pectin to convert galacturonic acid to neutral sugars “galactose” to prevent decomposition of uronate residues during acid hydrolysis. The increased galactose was then analysed by anion-exchange chromatography with the fluorogenic post-column derivatization. The fluorescence of the reaction products was measured upon excitation at a maximum of 331 nm with emission wavelength at 383 nm. The method was validated compared to anion-exchange chromatography with pulsed amperometer detector (HPAEC-PAD).

2. Experimental

2.1. Materials

Pomelo fruit were purchased from Nakhon Pathom province, Thailand. Pomelo peels were cut into small cube size and extracted with hot water, and then filtered and adjusted to pH 4. Ethanol was added to the solution, and washed with ethanol and acetone. The precipitate was dissolved in water, dialyzed and freeze-dried. Pomelo pectin extracted in our laboratory was 80% of degree of methylation (DM).

Commercial citrus pectin (DM = 6%) and apple pectin (DM = 70%) from Wako Pure Chemicals (Osaka, Japan) were used for comparison study. These pectin samples were purified from low-molecular-weight contaminants by dialysis prior to analyses. Monosaccharide standards were purchased from Wako (Japan). All other chemical reagents were of analytical reagent grade.

2.2. Methylation of pectin

Pectin (10 mg) was dissolved in 1 mL water and applied to a Dowex 50X8 cation exchange column (7.5 mm i.d. × 100 mm, H^+ form) to remove cationic contamination, and then, the fraction was freeze-dried. The dried fraction (~5 mg) was suspended in a mixture of 5 mL dry dimethyl sulfoxide and 250 μL methanol, and 150 μL trimethylsilyl diazomethane was added to the sample solution. The sample solution was mixed and kept at room temperature for 1 h after which 75 μL of acetic acid was added to quench the reaction and the sample was freeze-dried three times (75 μL of acetic acid was added after freeze-drying step).

2.3. De-esterification and reduction of pectin

Pectin was de-esterified in 0.1 mol/L NaOH at 4 °C for 9, 16, 24 and 48 h with mild stirring. The pH was acidified to pH 3–4 by 1 mol/L HC1 and the pectin was precipitated by three volumes of 96% ethanol. Precipitates were collected by centrifuged at 2000 × g for 20 min, dialyzed and freeze-dried. The demethylated pectin was dissolved in distilled water and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Dojindo Laboratories, Kumamoto, Japan) was added and kept for 10 min at room temperature. A 40 mmol/L HCl was then added and stirred at 40 °C. After 1 h, 2 mol/L of sodium borodeuteride dissolved in 40 mmol/L of NaOH was added. The reaction was allowed to continue with constant stirring for 16 h at 50 °C. After cooled to room temperature, acetic acid was added and reduced pectin was precipitated by adding 99.5% ethanol and keep for 16 hr at 0 °C. The reduced pectin was separated by centrifuged at 2000 × g for 20 min, dialyzed and freeze-dried. The intact pectin, demethylated pectin and reduced pectin has been investigated the characterization by ^{1}H NMR spectroscopy.

2.4. ^{1}H NMR analysis

^{1}H NMR spectroscopy was used to study the structure of pectin after reaction. ^{1}H NMR spectroscopy was performed by dissolved a sample (approx 4–5 mg) in 0.5 mL of D_2O (99.9%) and freeze-dried repeatedly to remove exchangeable protons. The sample was kept in a desiccator over phosphorus pentoxide in vacuo overnight at room temperature. The thoroughly dried sample was then dissolved in 0.5 mL of D_2O (99.96%) and centrifuged at 2000 × g for 15 min and transferred to an NMR tube (5.0 mm o.d. × 25 cm; Wilmad Glass Co. (Buena, NJ)). 1D NMR experiments were performed on a JNM-400A spectrometer equipped with a 5-mm field-gradient tunable probe with standard JEOL software at 30 °C for all experiments on 500 μL samples. The HOD signal was suppressed by pre-saturation during 3 s for all 1D experiments.

2.5. Analysis of monosaccharide composition of pectin

Ten milligrams of intact pectin and reduced pectin was hydrolyzed with 2.5 mol/L trifluoroacetic acid (TFA) at 100 °C in a sealed-tube for 6 h. Excess acid was removed by co-distillation with methanol:water (1:1, v/v) after the hydrolysis was completed. One part of the hydrolysate was analyzed by HPAEC-fluorescence detection, and the other was measured by HPAEC-PAD.

2.5.1. HPAEC with fluorescence detector

The chromatographic condition was modified from Toyoda et al. method (Toyoda, Shinomiya, Yamanashi, Koshiishi, & Imanari, 1988). The hydrolysate (1 mg/mL) was determined by anion-exchange chromatography with post-column derivatization. The column used was a TSK gel Sugar AXI (Tosoh Corp., Tokyo, 4.6 mm i.d. × 150 mm,) which was maintained at 70 °C. The mobile phase was comprised of 0.5 mol/L boric acid (pH 8.5 adjusted with 2 mol/L NaOH) delivered at a flow rate of 0.5 mL/min. The derivatizing reagents were 1 mol/L NaOH and 0.5% 2-cyanoacetamide, each of which was delivered at a flow rate of 0.33 mL/min. The reaction bath was controlled at 120 °C. The fluorescence of the reaction product was measured upon excitation at a maximum of 331 nm with an emission wavelength at 383 nm. The polysaccharide levels were calculated based on the peak area response of known amounts of monosaccharide standards.

2.5.2. HPAEC-PAD

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was performed with a Dionex ICS-3000 chromatography system (Sunnyvale, CA, USA) equipped with an ED-3000 electrochemical detector, and a SP gradient pump with an argon degasser. Aliquots of samples (1.0 mg/mL) were filtered (0.20 μm membrane filter) and injected (10 μL) on a CarboPac PA-1 guard column (4 mm i.d. × 25 mm) attached to a CarboPac PA-1 anion-exchange column (4 mm i.d. × 100 mm). A sample containing monosaccharides was eluted isocratically with 10 mmol/L NaOH for 25 min, followed by a linear gradient of 0–150 mmol/L sodium acetate in 100 mmol/L NaOH for 20 min to elute acidic monosaccharides. Before each injection, a column was re-equilibrated by running for 15 min with 100 mmol/L NaOH followed by 10 min with 10 mM NaOH. The eluent flow rate was constantly kept at 1.0 mL/min. Data were collected and analyzed on computers equipped with Dionex Chromeleon 6.80 Sp2 Build 2212 software (Sunnyvale, USA).

3. Result and discussion

^{1}H NMR spectrum of citrus pectin raw material was shown in Fig. 1A. A singlet signal at 3.8 ppm is assigned as a signal of methyl

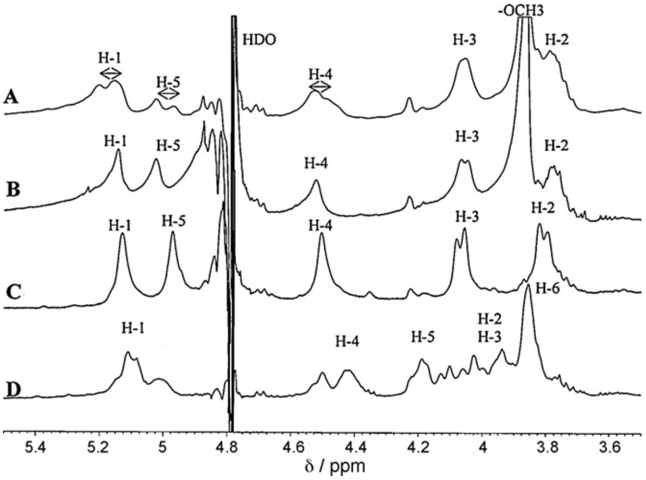

Fig. 1. ^{1}H NMR spectra of intact and chemically modified pectin samples. (A) original pectin, (B) esterified-pectin, (C) de-esterified-pectin and (D) de-esterified and reduced pectin.

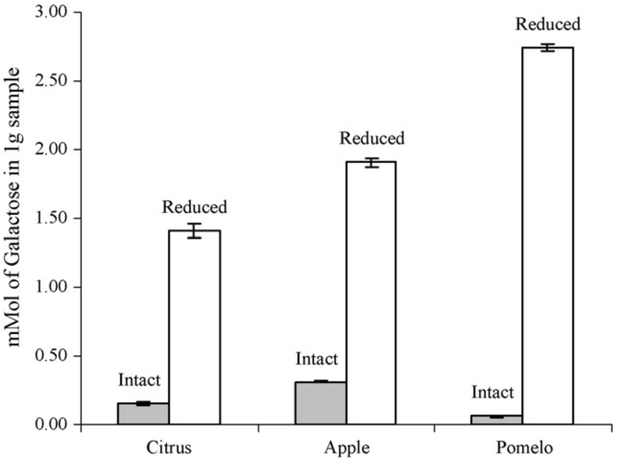

Fig. 2. The diagram of the amount of galactose (mean ± RSD, $n = 6$) in intact and reduced sample analyzed by HPAEC-fluorescent detection.

protons of $-COOCH_3$ and the anomeric signals of galacturonate and methyl galacturonate residues around 5 ppm (4.9–5.1 ppm) were splitted into two peaks. The H-5 protons adjacent to the free carboxyl groups were resonated at 4.6 ppm. In order to show the complete reaction of saponification and reduction, fully methylated pectin was first prepared and determined by ^{1}H NMR spectroscopy (Fig. 1B). The H-5 protons adjacent to the ester groups had slightly shifted to about 5 ppm and H-1 proton signals were found in the lower region (Rosenbohm, Lundt, Christensen, & Young, 2003).

Citrus pectin, apple pectin and pomelo pectin samples were de-esterified in 0.1 mol/L NaOH to convert $-COOCH_3$ group of GalA into carboxyl group. The optimized reaction time was estimated for 16 h. After de-methylation of the ester of galacturonate, the five major signals were found (Fig. 1C) which were assigned as GalA (Tamaki, Konishi, Fukuta, & Tako, 2008). The signal at 3.8 ppm was simply disappeared and signals around 5 ppm became a singlet. After de-esterification, the product was further applied for reduction of carboxyl groups by conditions described above. The completion of the reduction was investigated by ^{1}H NMR spectroscopy. The anomeric protons around 5 ppm were the signals of H-1 of Gal residues and a new signal appeared corresponding to the H-5 at 4.1 ppm (Fig. 1D) which was shown in that of the fully reducted residues (Rosenbohm et al., 2003). Similar results were obtained by investigating the reaction products of apple and pomelo pectin samples by ^{1}H NMR spectroscopy (data not shown).

The composition of monosaccharides in the intact citrus, apple and pomelo pectin samples was analyzed by both HPAEC-fluorometric detection and -PAD methods. After de-esterification and reduction procedures, the increased Gal was analyzed by HPAEC with post-column derivatization system and as the results; the content of Gal in all samples was increased as shown in Fig. 2. The Gal residues increased after reduction are corresponding to the amount of GalA residues in original samples.

The established method was further validated for specificity, linearity, limit of detection (LOD) and limit of quantitation (LOQ), and precision. Each neutral monosaccharide (rhamnose, arabinose, galactose, glucose, xylose and mannose) in pectin could be sep-

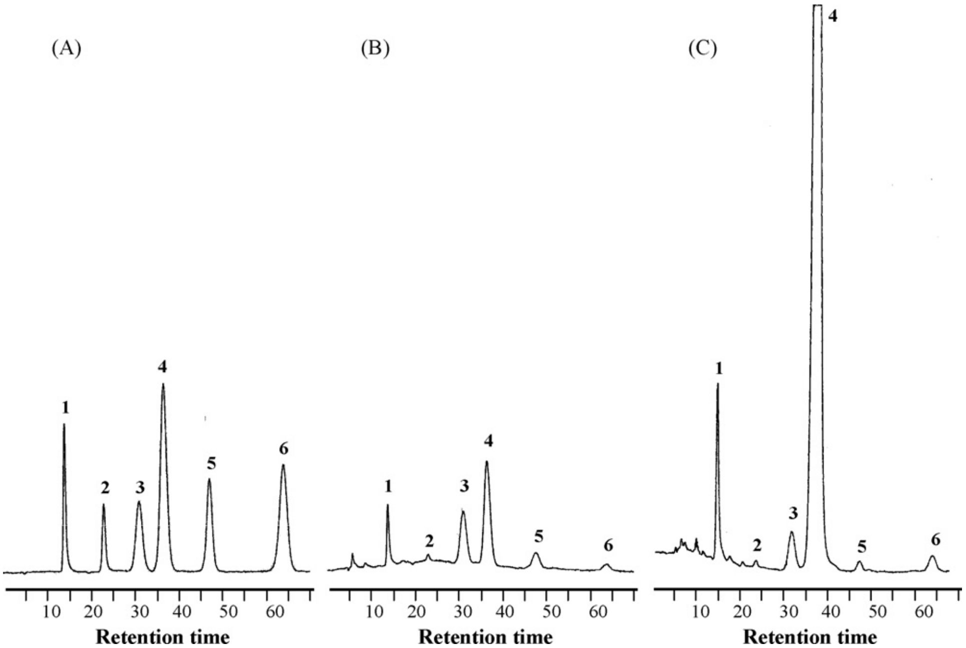

Fig. 3. The HPLC chromatograms of hydrolysated citrus pectin analyzed by HPAEC-fluorescent detection (A) mixture of six monosaccharide standard solution, (B) intact citrus pectin and (C) reduced citrus pectin. 1 = rhamnose, 2 = mannose, 3 = arabinose, 4 = galactose, 5 = xylose and 6 = glucose.

Table 1
Linearity, limit of detection (LOD) and limit of quantitation (LOQ) and repeatability of monosaccharides analyzed by HPAEC-Fluorescence detector and HPAEC-PAD.

	Range (ppm)	r^2	Slope ± SD[a] (×10⁴)	Intercept ± SD[a] (×10⁴)	LOD (ppm)	LOQ (ppm)	Repeatability (%RSD)[c]
Anion-exchange chromatography with fluorescence detector							
Gal	5–100	0.999	1.6 ± 0.001	2.2 ± 2.29	1.0	3.5	2.0
Ara	5–50	0.991	3.0 ± 0.003	2.2 ± 0.64	0.5	1.3	2.4
Rha	5–50	0.993	2.4 ± 0.003	2.3 ± 0.72	0.2	0.5	2.2
Glc	5–50	0.998	1.2 ± 0.002	3.3 ± 1.36	0.7	2.5	2.4
Xyl	5–50	0.995	3.3 ± 0.006	0 ± 0	0.5	1.5	1.5
	Range (ppm)	r^2	Slope ± SD[a]	Intercept ± SD[a]	LOD (ppm)	LOQ (ppm)	Repeatability (%RSD)[b]
Anion-exchange chromatography with PAD							
GalA	50–300	0.983	0.29 ± 0.15	7.13 ± 3.93	3.3	11.0	8.2
Gal	5–50	0.989	0.74 ± 0.25	2.74 ± 1.92	2.9	9.5	4.8
Ara	5–50	0.951	0.32 ± 0.13	7.80 ± 3.14	14.0	47.0	4.1
Rha	5–50	0.983	0.36 ± 0.28	1.70 ± 0.87	2.9	8.3	7.7
Glc	5–50	0.990	0.76 ± 0.40	2.51 ± 2.00	3.0	10.0	5.3
Xyl	5–50	0.997	0.95 ± 0.30	0.71 ± 0.30	1.8	5.8	11.0

[a] Standard deviation ($n = 3$).
[b] Relative standard deviation of the peak area response of six standard injection ($n = 6$).

Table 2
Content of monosaccharides (% molar ratio) in citrus, apple and pomelo pectin samples determined by HPAEC-PAD and HPAEC-fluorescence detector.

	Citrus				Apple				Pomelo			
	HPAEC-PAD		HPAEC-fluorescence		HPAEC-PAD		HPAEC-fluorescence		HPAEC-PAD		HPAEC-fluorescence	
	Mean	±%RSD	Mean	±%RSD	Mean	±%RSD	Mean	±%RSD	Mean	±%RSD	Mean	±%RSD
GalA	77.0	3.2	81.2	0.9	63.8	5.3	72.6	0.8	63.6	9.4	76.9	0.5
Gal	10.6	8.1	11.1	5.3	14.4	23.0	16.3	2.5	3.8	18.7	2.1	2.1
Ara	5.4	13.6	4.4	3.5	7.0	36.9	2.8	3.5	27.3	17.1	17.3	2.1
Rha	5.9	13.3	1.8	3.8	8.0	16.9	1.2	1.0	3.2	13.2	0.9	2.2
Glc	0.6	25.2	1.3	3.4	3.2	34.7	3.2	3.0	1.0	58.5	2.2	2.2
Xly	0.4	14.7	0.2	6.2	3.6	20.2	3.8	4.6	1.2	22.5	0.5	4.0

Mean ± %RSD ($n = 6$).

arated chromatographically as shown in Fig. 3. The advantage of fluorescence detection is not only its low detection limits but also its higher specificity. The mechanism of reaction of galactose with 2-cyanoacetamide was followed the previous report (Honda, 1996; Honda, Kakehi, Fujikawa, Oka, & Takahashi, 1988). The galactose in the aldehyde form was first reacted with methylene group in 2-cyanoacetamide, and then subsequently dehydrated and cyclized which produced the strong fluorescence heterocyclic derivatives; a 2-pyrrolidone derivatives and 2-pyridone derivatives. Linearity of system was determined by an analysis of three replicates of five concentrations of standard solutions and the plots of area under the curve (AUC) of the peak responses of each sugar against their corresponding concentrations. The linear regression analysis data for the calibration plots showed good linear relationship with $R^2 > 0.99$ over the concentration range for all neutral sugars. The standard deviation (SD) of the slope and the intercept of the three linear regressions prepared on three different days were listed in Table 1. The LOD and LOQ of each neutral sugar were estimated as 3.3 and 10 times of the standard deviation intercept/slope ratio of the calibration curve, respectively. The LOD and LOQ concentrations were found to be 0.2–1.0 and 0.5–3.5 μg/mL for 10 μL injection volume. Six replicates ($n = 6$) of standard solutions were analyzed in the same day to determine method precision and the peak area obtained were used to calculate the relative standard deviation (RSD)% values. The LOD and LOQ suggested high sensitivity of the method and the system precision (repeatability) of the method was less than 2.5%. The results obtained from the method were also compared with those obtained from HPAEC-PAD (Table 1), and these results strongly suggested that the present method might be useful and more reliable.

The content of monosaccharides in citrus and apple pectin samples was summarized in Table 2. The satisfactory recovery of GalA was obtained from HPAEC-fluorescence analysis after converting of GalA to Gal. Moreover, the precision of the method for other neutral sugars showed good reproducibility compared to those obtained by HPAEC-PAD.

The method was further applied for GalA analysis in pectin extracted from pomelo. The results showed good recovery of GalA. GalA determined by the present method was 78% which is higher than that determined by HPAEC-PAD (64%). The relative standard deviation (RSD, %) was less than 2.5% for all neutral sugars except xylose (4%) (Table 2).

4. Conclusion

A method for the determination of GalA in pectin has been modified for high recovery. The analytical approach was based on converting GalA in polysaccharide chain to be the stable neutral sugar Gal following by hydrolysis to monosaccharide before analysis by HPAEC-fluorescence detector. The method was validated and allowed precise and sensitive with good linearity and successfully applied to analyze GalA in pectin extracted from pomelo sample.

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