

Unusually Branched Pectin Isolated from a Medicinal Food, *Artemisia indica* Willd. var. *indica*

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Abstract Unusually branched pectin was isolated from leaves of a traditional medicine, *Artemisia indica* Willd. var. *indica* that was grown in Okinawa, Japan. D-Galacturonic acid, L-arabinose, D-galactose, L-galactose, L-rhamnose, D-xylose, acetic acid, methyl ether and methyl ester were identified via chemical, HPAEC and NMR analyses. The molecular mass was 18.5 kDa. The polysaccharide was fractionated on DEAE chromatography and divided into neutral and acidic fraction. Almost D-galacturonic acid residues were moved into acidic fraction and all of the carbon atoms (C1-C6) on ¹³C-NMR spectrum were assigned to double (coupling) signals suggesting that long D-galacturonic acid side-chains (homogalacturonan) were involved. By methylation analysis, (1→4)-linked α-D-GalpA (major), terminal, (1→5)-, (1→2,3)-, (1→2,5)-, and (1→3,5)-linked α-L-Araf were identified. Terminal, (1→6)- and (1→3,6)-linked β-D-Galp, (1→2)- and (1→2,4)-linked α-L-Rhap, (1→4)-linked α-D-Xylp, and (1→4)-linked β-D-GlcpA were also identified. The pectin was consisted of homogalactouronan main-chain, rhamnogalacturonan main-chain, double galacturonan side-chains, arabinogalactan side-chain, and galactoglucuronoxlyose side-chain. This study is the first to report on involving double homogalacturonan side-chains. The pectin molecules seem to cross-linking each other between carboxyl groups of D-GalpA residues through cations, such as B³⁺, Ca²⁺ and Mg²⁺, to hold nutrients, inorganic matters, and water in the cell walls and intercellular areas of leaves of *Artemisia indica* Willd var. *indica*.

Keywords: unusually branched pectin, *Artemisia indica*, medicinal food, NMR analysis, methylation analysis, chemical structure

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1. Introduction

Pectins are most complex polysaccharides present cell walls and intercellular areas in plants. They are consisted of (1→4)-linked α-D-galacturonan (homogalacturonan), rhamnogalacturonan I: (1→4)-linked α-D-galacturonic acid-(1→2)-linked α-L-rhamnose, some of which substitute at C-4 with galactan, arabinan or arabinogalactan, and rhamnogalacturonan II: (1→4)-linked α-D-galacturonic acid residues, some of which substitute at C-2 or C-3 with 5-10 different glycosyl residues including apiose. Pectin produced commercially from some fruits is used as a gelling agent in food industry. We previously isolated pectin from traditional fruit *Citrus depressa* and *C. tankan* grown in Okinawa, Japan [1,2,3]. The former is consisted of homogalacturonan and rhamnogalacturonan I in which (1→3)- or (1→4)-linked β-D-galactan at C-4 of α-L-rhamnose residue was substituted as a side-chain [1,2]. It is industrial useful as drinks in Okinawa, Japan.

The genus *Artemisia* are widespread in India, Thailand, Malaysia, China, Korea and Japan and used as traditional medicine. One of *A. indica* Willd. var. *indica* mainly grows in Okinawa, Japan. The plant is used as herbal medicinal food for long time in Okinawa. Although, a part of structures of pectin from *Artemisia princeps* have been published [4,5], there is no report from *A. indica* Willd. var. *indica*. We present herein structural characteristics of a pectin isolated from leaves of the plant. This paper is the first to report on cross binding pectin.

2. Materials and Methods

2.1. Polysaccharide Preparation

The leaves of *A. indica* Willd. var. *indica* were purchased at public market in Naha City, Okinawa, Japan. Leaves were crushed to a powder with a mixer and kept in refrigerator at 4°C. The powder (20g) was soaked in ethanol overnight and soaked again in acetone to remove lipids, then dried *in vacuo*.

The defatted powder was suspended in water at 90°C for 2 h, and filtered through filter aid (Celite 545, Nakarai, Japan). Ethanol (2 vols) was added to the filtrate and the polysaccharide was dried *in vacuo*. The crude polysaccharide was dissolved in distilled water at room temperature and the solution was passed through the filter aid. Then, the filtrate was precipitated by adding 2 volumes of ethanol and the resulting solid was dried *in vacuo*. The semi-purified polysaccharide was dissolved in distilled water and deionized by passing through a cation exchange column composed of Amberlite 120A H⁺ (Organo, Japan). After neutralization with 0.1 M NaOH, the solution was subsequently lyophilized [6,7].

2.2. Chemical Component Analysis

The total carbohydrate and uronic acid content were determined by the phenol-sulfuric acid [8] and carbazol-sulfuric acid method [9] using D-galactose and D-galacturonic acid as the standard, respectively.

2.3. Fractionation of the Polysaccharide

Fractionation of the polysaccharide was performed by passage via anion exchange chromatography (DEAE Sepharose: $\phi 25 \times 380$ mm). After applying sample (100 mg in 50 mL in distilled water), the polysaccharide was eluted, and a gradient of sodium chloride solutions was from 0 to 3.0 M.

2.4. High-Performance Anion Exchange Chromatography Coupled with a Pulse Amperometric Detector (HPAEC-PAD)

The polysaccharide was dissolved in purified water and sulfuric acid was added to a final concentration of 1.0 M. The solution was heated at 100°C for 2 h. The hydrolysate was neutralized with BaCO₃ and filtered. The hydrolysate was analyzed by high-performance anion exchange chromatography on DX 500 (Dionex, CA, USA) fitted with a column of CarboPac PAi (4 \times 250 mm) coupled with a pulse amperometric detector (HPAEC-PAD). The column was eluted at a flow rate of 1.0 mL/min at 35°C with 14 mM NaOH.

The hydrolysate was also allied to HPAEC (DX-500, Dionex Co., CA, USA) on a column (A-SC4) equilibrated with 1.7 mM NaHCO₃+1.8 mM NaCO₃ for uronic acid analysis.

2.5. Molecular Mass

The molecular mass of the polysaccharide was determined by high-performance liquid chromatography (HPLC), using LC-6A chromatograph (Shimadzu, Co., Ltd, Japan) on a Superdex 200 with refractive index detection (RID-6A, Shimadzu, Kyoto, Japan). The HPLC operation was performed at room temperature. The column was developed with 50 mM phosphate buffer, and the same buffer supplemented with 150 mM sodium chloride; fractions (3 mL each) were collected at a flow rate of 0.5 mL/min. The column was conditioned with 0.15 M sodium chloride in 0.05 M sodium phosphate buffer (pH 7.2), and elution was conducted with the same

buffer. Standard pullulan (Showa Denko, Tokyo, Japan) with a definite molecular mass were used as molecular mass markers [10].

2.6. ¹³C- and ¹H-Nuclear Magnetic Resonance (NMR) Spectroscopy

The dried sample was dissolved in D₂O (2.0%, W/V). ¹³C- and ¹H-NMR spectra were recorded at 80°C on a α 500 FT-NMR spectrometer (JEOL Co. Ltd, Tokyo, Japan) at 500.00 and 125.65 MHz, respectively. ¹³C- and ¹H-NMR chemical shifts were expressed in parts per million (ppm) relative to the resonance of sodium 3-(trimethylsilyl) propionic-2,2,3,3-d₄ acid (TSP, 0.00 ppm) as an internal standard [1-3].

2.7. Methylation Analysis

Methylation of the polysaccharide was carried out by Ciucanu and Kerek [10]. The methylated polysaccharide was extracted with CHCl₃. The extracted methylated polysaccharide was hydrolyzed with 2 M TFA (2 mL) at 120°C for 2 h. The hydrolysate was dissolved in 1 M NH₄OH (0.2 mL). DMSO (1 mL) containing 20 mg of NaBH₄ was added and the mixture was incubated at 40°C for 90 min. Subsequently acetic anhydride (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 of the polysaccharide were analyzed using a gas chromatograph (GC-14A; Shimadzu Corp., Kyoto, Japan) equipped with a flame ionization detector using a capillary column (DB-1: 40 m \times 0.25 mm, J&W Scientific Inc., CA, U.S.A.). The injector and detector temperatures were 210°C and 270°C, respectively. After injection, the oven temperature was maintained at 150°C for 5 min, and then raised at 5°C/min to 250°C. This temperature was maintained for 5 min. The identities of the peaks were confirmed using GC-MS (GCMS-QP 1000EX; Shimadzu Corp., Kyoto, Japan).

3. Results

3.1. Polysaccharide Preparation from *Artemisia indica* Wild var. *indica*

A. indica Wild var. *indica* is mainly distributed in Okinawa Prefecture, Japan. The plant grows to 20-50 cm with many branched leaves. The leaves (5-15 \times 3-7 cm) are feather shaped, as shown in Figure 1, and used as traditional aromatic herb and medicine. The polysaccharide was prepared and purified as described in the Materials and Methods section. The purified polysaccharide was a colorless, fibrous powder, with a yield of 4.5% (w/w) based on the fresh materials.

The polysaccharide dissolved in distilled water (10 mg/10mL) showed a single peak when applying on Sephadex G50 column chromatography (not shown in Figure).



Figure 1. Photograph of *Artemisia indica* Willd. var. *indica*

3.2. Sugar Components and Fractionation of the Polysaccharide

The polysaccharide contained 67.8, 56.0 and 11.8% (w/w) of carbohydrate, uronic acid and neutral sugar the value of the latter was obtained by subtracting uronic acid from carbohydrate.

Fractionation of the polysaccharide was performed by passage via DEAE anion exchange chromatography

(Figure 2). Three peaks were obtained, but the former two peaks (BI and BII) were not separated completely so they were combined into one (B). The overlapping peaks were due to containing a little amount of uronic acid. The native (before fractionation), neutral and acidic fractions was referred to as A, B and C, respectively.

3.3. Identification of the Polysaccharide Sugar Components

As shown in Figure 3, the anion exchange high-performance liquid chromatogram (HPAEC-PAD) of the acid hydrolysate of the native polysaccharide (A) showed the presence of L-rhamnose, L-arabinose, D-galactose, D-glucose, and D-xylose, the molar ratio of which was 0.8:3.0:1.6:0.4:0.3, respectively. Little amount of L-rhamnose was detected, but D-glucose increased in double in the neutral fraction (B). Almost the same peak pattern compared with that of the native polysaccharide (A) was observed except D-glucose that was disappeared, but unknown sugar: molar ratio, 0.5, was detected in acidic fraction (C). The molar ratios of sugar components were estimated and summarized in Table 1. The unknown sugar will be discussed later on. D-Galacturonic acid (molar ratio, 12.4) and D-glucuronic acid (1.0) was also identified (not shown in Figure).

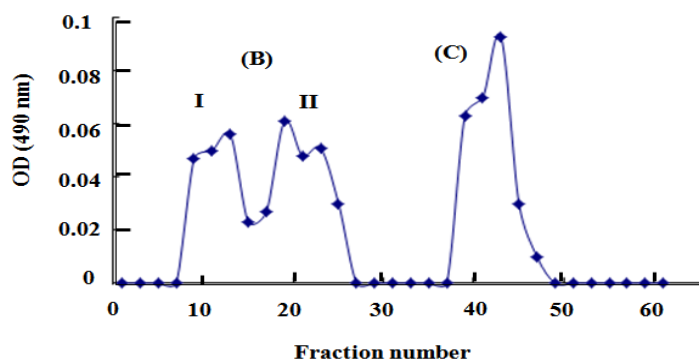


Figure 2. Fractionation of the polysaccharide isolated from *Artemisia indica* on DEAE-Sepharose exchange column. (B), Neutral fraction; (C), Acidic fraction

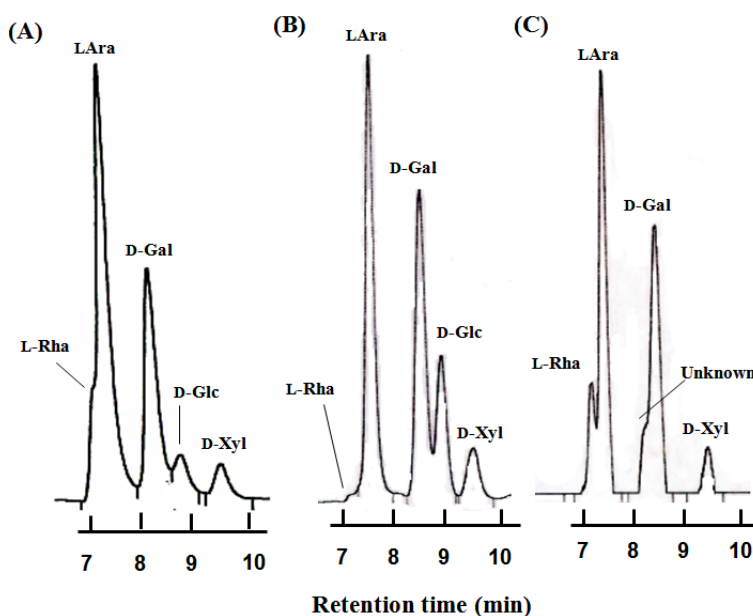


Figure 3. HPAEC-PAD Chromatographs of the hydrolysates of fractionated polysaccharides. (A), Native (B), Neutral fraction (C), Acidic fraction

Table 1. Neutral sugar components of the polysaccharides

Polysaccharides	L-Rhamnose	L-Arabinose	D-Galactose	D-Glucose	D-Xylose	Unknown (*L-Galactose)
Native	0.8	3.0	1.6	0.4	0.3	
Neutral fraction	tr	3.0	2.2	0.9	0.3	
Acidic fraction	0.8	3.0	1.9	0	0.3	0.5

*Suggested from Reference [18] and [19].

3.4. Molecular Mass

The molecular mass of the native polysaccharide was determined by HPLC using a chromatograph on a column of Superdex 200 (not shown in Figure). According to the standard calibration curve obtained from the definite molecular mass pullulan, the molecular mass of the polysaccharide was calculated to be approximately 18.5 kDa.

3.5. ^{13}C - and ^1H -NMR Spectra Analysis

Chemical shifts in the ^{13}C - and ^1H -NMR spectra of the native (A), neutral (B) and acidic (C) fraction were shown in Figure 4a and 4b. Complicated ^{13}C -NMR signals were observed in native polysaccharide, as shown in Figure 4a. From published research [1-3,11-15], the anomeric signals at 102.86, 110.53, 106.16, 102.54 and 102.02 ppm were assigned to 1,4-linked α -D-galacturonopyranose

(GalAp), 1,5-linked α -L-arabinofuranose (Araf), β -D-galactopyranose (Galp), α -L-rhamnopyranose (Rhap) and α -D-xylopyranose (Xylp), respectively. C-6 Methyl groups from L-Rhap residue were assigned to 14.39 and 14.51 ppm. Methyl ether at 55.91ppm and methyl acetic acid at 23.03ppm were also assigned. Two methylester on α -D-GalAp carboxyl groups (C-6) at 173.46 and 173.80 ppm suggested that there are two types of 1,4-linked D-GalpA residues were involved. The results indicate that the polysaccharide is identified to be pectin. In the neutral fraction (B), a major signal from 1,4-linked α -D-GalAp around 102ppm disappeared, but that of D-glucopyranose (D-Glcp) was assigned to 106.90 ppm. The methyl signal (small) from L-Rhap still assigned to 15.61 ppm. The signals from the methyl ether disappeared, but acetyl groups assigned to very small signal. Although, the methyl ester remained at 33.88 ppm, carboxyl group (C6) of α -D-GalAp was not detected.

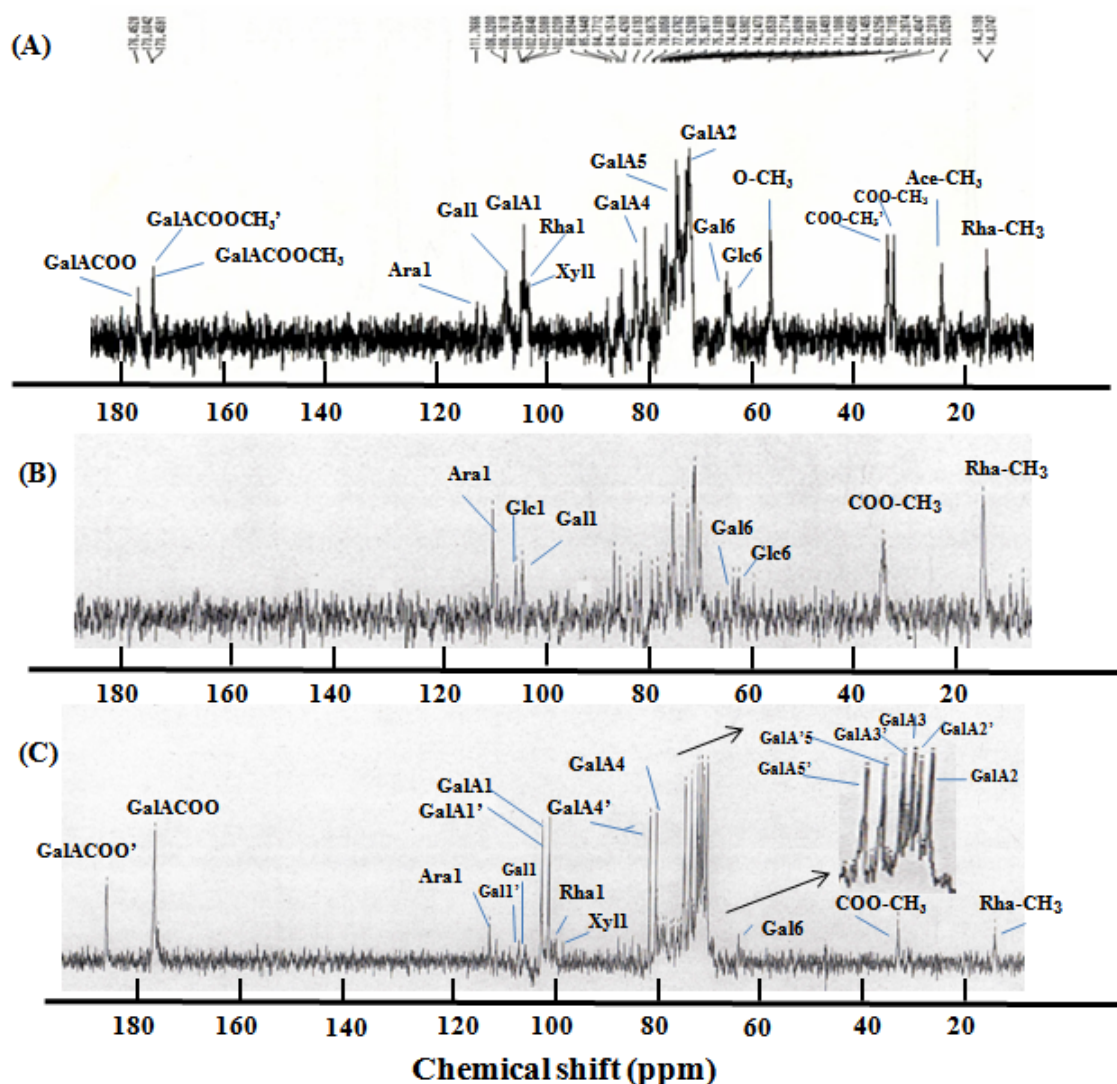


Figure 4a. ^{13}C -NMR Spectra of the polysaccharides at 80°C. (A), Native; (B), Neutral fraction; (C), Acidic fraction

Well-resolved ^{13}C -NMR signals were observed in the acidic fraction (C). Signals from all carbons of 1,4-linked α -D-GalAp residues assigned as double (coupling) peaks: C-1, 102.79 ppm and C-1', 103.01; C-2, 71.15 and C-2', 71.46; C-3, 71.32 and C-3', 71.87; C-4, 81.89 and C-4', 82.08; C-5, 75.41 and C-5', 76.53; C-6, 178.46 and C-6', 187.68. Anomeric ^{13}C of α -L-Araf, β -D-Galp, α -L-Rhap and α -D-Xylp were also assigned to 111.70, 105.87, 102.53, 102.27 ppm, respectively [1-3,11-15]. The carboxymethyl ester was assigned to 33.90 ppm. The results were summarized in Table 2. The double (coupling) signals of α -D-GalAp residues suggested that 1,4-linked α -D-Galacturonan substituted as side-chains in pectin molecules.

Although the chemical shift of anomeric signals of ^1H -NMR (Figure 4b) of the native pectin (A) were overlapped due to a little high viscosity, they were consistent with the presence of α -L-Araf (5.25 ppm), α -D-GalAp (5.16), α -L-Rhap (5.06), and α -D-Xylp (4.98) from published papers [1-3,12-15]. The methyl protons of L-Rhap (1.28), acetyl (2.18), methyl ether (3.36) and carboxyl methyl ester (2.12 and 2.18) groups were also assigned [1,2,3]. The ring protons (H-2 to H-6) of sugar components of the pectin were overlapped, but some signals were assigned. In neutral fraction (B), α -L-Araf (5.32), α -L-Rhap (5.09), α -D-Xylp (5.02), β -D-Galp (4.52) and β -D-Glcp (4.48) were assigned. Small signals of methyl protons from L-Rhap (1.29), methyl ester (2.14) and acetyl group (1.92) were also assigned.

In acidic fraction (C), α -L-Araf (5.32 ppm), α -D-GalAp (5.15), L-Rhap (5.06), and α -D-Xylp (4.98) were assigned [1,2,3,12,13]. Double methyl protons from L-Rhap (1.32 and 1.37 ppm) were assigned. However, there was no

signal from D-Glcp residue. A little broad signal from H-1to H-6 of 1,4-linked α -D-GalAp were observed as compared with those of previous papers [1,2,3] that might be due to presence of two kind of α -D-GalAp residues. The proton from methyl esters were assigned as double signals (2.17 and 2.22). Small acetyl proton was also assigned (1.94). The results were summarized in Table 2.

3.6. Methylation Analysis

The gas chromatograms of native (A), neutral fraction (B) and acidic fraction (C) of methylated pectin are shown in Figure 5. Many peaks in the native pectin were observed and corresponding mass spectra were shown in Figure 4A. From publishing papers [2,16,17], peak number (1) was 2,3,5-tri-*O*-methyl-L-Araf (terminal; relative molar ratio, 1.0), (2) 2,3,4-tri-*O*-methyl-D-Xylp (terminal; 0.1), (3) 2,3-di-*O*-methyl-L-Araf (1 \rightarrow 5-linked; 0.8), (4) 2,3-di-*O*-methyl-D-Xylp (1 \rightarrow 4-linked; 1.0), (5) 3,4-di-*O*-methyl-L-Rhap (1 \rightarrow 2-linked; 1.1), (6) 2,3,4,6-tetra-*O*-methyl-D-Galp (terminal; 0.7), (7) 2,3,6-tri-*O*-methyl-D-Glcp (1 \rightarrow 4-linked; 0.7), (8) 3-mono-*O*-methyl-L-Rhap (1 \rightarrow 2,4-linked; 0.1), (9) 2-mono-*O*-methyl-L-Araf (1 \rightarrow 3,5-linked), (10) 2,4,6-tri-*O*-methyl-D-Galp (1 \rightarrow 3-linked; 0.7), (11) 5-mono-*O*-methyl-L-Araf (1 \rightarrow 2,3-linked; 0.7), (12) 3-mono-*O*-methyl-L-Araf (1 \rightarrow 2,5-linked; 1.1), (13) 2,3-di-*O*-methyl-D-Glcp (1 \rightarrow 4,6-linked; 1.8), (14) 2,3-di-*O*-methyl-D-GalAp (1 \rightarrow 4-linked; 2.3). (15) 2,4-di-*O*-methyl-D-Galp (1 \rightarrow 3,6-linked; 2.3). Peak 14 was identified to be 2,3-di-*O*-methyl-D-GalAp (1 \rightarrow 4-linked; 2.3) because it disappeared in neutral fraction (Figure 5 B), but presented in acidic one (Figure 5C).

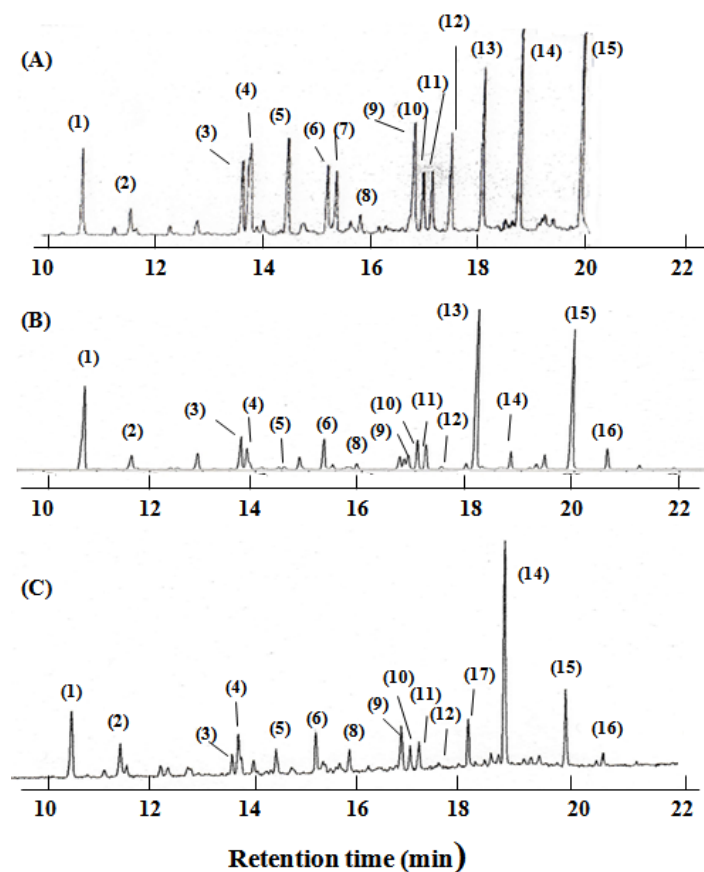


Figure 5. Gas chromatograms of the partially methylated alditol acetates of the pectin: (A), Native pectin; (B), Neutral fraction; (C), Acidic fraction

Table 3. Methylation analysis of the pectin

No.*	Methylated sugars	Molar ratio of fractions			Mode of linkage
		Native	Neutral	Acidic	
1	2,3,5-tri- <i>O</i> -methyl-L-Arabinofuranose	1.0	1.0	1.0	L-Araf- α -(1 \rightarrow
2	2,3,4-tri- <i>O</i> -methyl-D-Xylopyranose	0.1	0.1	0.3	D-Xylp- α -(1 \rightarrow
3	2,3-di- <i>O</i> -methyl-L-Arabinofuranose	0.8	0.3	0.1	\rightarrow 5)-L-Araf- α -(1 \rightarrow
4	2,3-di- <i>O</i> -methyl-D-Xylopyranose	1.0	0.2	0.5	\rightarrow 4)-D-Xylp- α -(1 \rightarrow
5	3,4-di- <i>O</i> -methyl-L-Rhamnopyranose	1.1	tr	0.3	\rightarrow 2)-L-Rhap- α -(1 \rightarrow
6	2,3,4,6-tetra- <i>O</i> -methyl-D-Galactopyranose	0.7	0.6	0.6	D-Galp- β -(1 \rightarrow
7	2,3,6-tri- <i>O</i> -methyl-D-Glucopyranose	0.6	0.2	0	\rightarrow 4)-D-Glcp- β -(1 \rightarrow
8	3-mono- <i>O</i> -methyl-L-Rhamnopyranose	0.1	tr	0.3	\rightarrow 2,4)-L-Rhap- α -(1 \rightarrow
9	2-mono- <i>O</i> -methyl-L-Arabinofuranose	1.2	0.1	0.4	\rightarrow 3,5)-L-Araf- α -(1 \rightarrow
10	2,4,6-tri- <i>O</i> -methyl-D-Galactopyranose	0.7	0.2	0.2	\rightarrow 3)-D-Galp- β -(1 \rightarrow
11	5-mono- <i>O</i> -methyl-L-Arabinofuranose	0.7	0.2	0.2	\rightarrow 2,3)-L-Araf- α -(1 \rightarrow
12	3-mono- <i>O</i> -methyl-L-Arabinofuranose	1.1	tr	tr	\rightarrow 2,5)-L-Araf- α -(1 \rightarrow
13	2,3-di- <i>O</i> -methyl-D-Glucopyranose	1.8	1.9	0	\rightarrow 4,6)-D-Glcp- β -(1 \rightarrow
14	2,3-di- <i>O</i> -methyl-D-Galacturonopyranose	2.3	0.1	3.8	\rightarrow 4)-D-GalpA- α -(1 \rightarrow
15	2,4-di- <i>O</i> -methyl-D-Galactopyranose	2.3	1.7	1.2	\rightarrow 3,6)-D-Galp- β -(1 \rightarrow
16	2-mono- <i>O</i> -methyl-D-Galacturonopyranose		0.2	0.1	\rightarrow 3,4)-D-GalpA- α -(1 \rightarrow
17	2,3-di- <i>O</i> -methyl-D-Glucuronopyranose	ol	ol	0.5	\rightarrow 4)-D-GlcpA- β -(1 \rightarrow

*, Peak number in Figure 5. ol, Overlapped in peak 13.

In neutral fraction (Figure 5B), terminal L-Araf (peak No. 1), terminal D-Xylp (2), 1 \rightarrow 5-linked L-Araf (3), 1 \rightarrow 4-linked D-Xylp (4), 1 \rightarrow 2-linked L-Rha (5), terminal D-Gal (6), 1 \rightarrow 4-linked D-Glcp (7), 1 \rightarrow 2,4-linked L-Rha (8), 1 \rightarrow 2,5-linked L-Araf (9), 1 \rightarrow 3-linked D-Galp (10), 1 \rightarrow 2,3-linked L-Araf (11), 1 \rightarrow 4,6-linked D-Glcp (13), 1 \rightarrow 4-linked D-GalpA (14), 1 \rightarrow 3,6-linked D-Galp (15) and 1 \rightarrow 3,4-linked D-GalpA (16) were identified. The presence of a little amount of 1 \rightarrow 4-linked D-Galp A (0.1 mol.), 1 \rightarrow 2-linked L-Rhap (trace), and 1 \rightarrow 2,4-linked L-Rhap (trace) indicate that a part of branching segment (rhamnogalacturonan) is also involved in neutral fraction. The results correspond to the profile of DEAE anion exchange chromatogram in Figure 2 where small amount of D-Galp A and L-Rha might be involved in the peak BII.

Although all peaks decreased, the same patterns were observed in acidic fraction (C) as compared with those of native pectin (A). The results indicated that non-reducing terminal L-Araf (peak No. 1; molar ratio, 1.0), terminal D-Xylp (2; 0.3), 1 \rightarrow 5-linked L-Araf (3; 0.1), 1 \rightarrow 4-linked D-Xylp (4; 0.5), 1 \rightarrow 2-linked L-Rhap (5; 0.3), terminal D-Galp (6; 0.6), 1 \rightarrow 2,4-linked L-Rhap (8; 0.3), 1 \rightarrow 3,5-linked L-Araf (9; 0.3), 1 \rightarrow 3-linked D-Galp (10; 0.2), 1 \rightarrow 2,3 linked L-Araf (11; 0.2), 1 \rightarrow 4-linked D-GalpA (14; 3.8), 1 \rightarrow 3,6-linked D-Galp (15; 1.2), 1 \rightarrow 3,4-linked D-GalpA (16; 0.1) were involved in the acidic fraction. Since 2,3,6-tri-*O*-methyl-D-Glcp (peak 7) and 3/4 of 2,3-di-*O*-methyl-D-Glcp (peak 13) disappeared in this fraction, they might be originated from different polysaccharides such as β -D-glucan and/or hemicellulose. Remained small peak at the same retention time of 2,3-di-*O*-methyl-D-Glcp (13) was originated from 1 \rightarrow 4-linked D-glucuronic acid residue which referred as peak number 17 in Figure 4C. The results were summarized in Table 3.

4. Discussion

Artemisia indica Willd. var. *indica* is used as herbal tradition medicine in treatment of rheumatism, neuralgia, antipyretic and gastrointestinal disease in Okinawa, Japan. This study presents structural investigation of pectin isolated from leaves of the plant.

The pectin is consisted of uronic acid to neutral sugars ratio was 56: 11.8 and molar mass was 18.5 kDa. Based on the NMR and methylation analysis, it substituted with 1,4-linked α -D-GalpA (major), 1 \rightarrow 2- and 1 \rightarrow 2,4-linked α -L-Rhap, terminal (non-reducing), 1 \rightarrow 5-, 1 \rightarrow 2,3-, 1 \rightarrow 2,5-, and 1 \rightarrow 3,5-linked α -L-Araf, terminal, 1 \rightarrow 6- and 1 \rightarrow 3,6-linked β -D-Galp, 1 \rightarrow 4-linked α -D-Xylp, and 1 \rightarrow 4-linked β -D-GlcpA residues. The double (coupling) signals were observed in all carbons (C1 to C6) of D-GalpA residue in acidic fraction, suggesting that the pectin molecule substituted with long homogalacturonan side-chains.

Yamada et al. reported some pectin oligosaccharides isolated from *Artemisia princeps*: D-GlcpA-(1 \rightarrow 2)-L-Rhap, D-GalpA-(1 \rightarrow 4)-L-Rhap, D-Galp-(1 \rightarrow 6)-D-Galp-(1 \rightarrow 6)-D-Galp, L-Araf-(1 \rightarrow 3)-Galp and D-GlcpA-(1 \rightarrow 4)-D-Xylp-D-GalpA [4,5]. The results suggest that C-4 of L-Rhap residue is the branching sugar and arabinogalactan seems to be involved in the *A. indica* pectin. D-Xylp residue was detected in the neutral fraction in addition to acidic one suggesting glucuronoxyloligosaccharide substitutes with D-GalpA at rhamnogalacturonan main-chain. Furthermore, the other literatures [18,19] reported that terminal α -L-Galp substituted with C-4 of D-GlcpA. This suggests that unknown sugar (Figure 2C) seems to be α -L-Galp. Consequently, L-galactoglucuronoxyloligosaccharide substitutes at C-3 of D-GalpA in rhamnogalacturonan segments, because 1 \rightarrow 2,4-linked α -L-Rhap has been identified.

From results and discussion, we propose a chemical structure of the pectin isolated from *Artemisia indica* Willd. var. *indica* as shown in Figure 6. The pectin is consisted of homogalacturonan main-chain, rhamnogalacturonan main-chain, arabinogalactan side-chain, galactoglucuronoxyloligosaccharide side-chain, and double homogalacturonan side-chains. The galactoglucuronoxyloligosaccharide side-chain may substitute with C-3 of D-GalpA on rhamnogalacturonan which correspond to DEAE chromatogram in which continued second small peak (BII in Figure 2) was observed that may contained a small amount of D-GalpA. Since there was no peak of 2,3,4-tri-*O*-methyl-D-Glcp which originated from terminal D-GlcpA by methylation analysis, terminal L-Galp residue might substitute at C-4 of D-GlcpA residue. The arabinogalactan and galactoglucuronoxyloligosaccharides may be involved in the same rhamnogalacturonan segments

5. Conclusions

Thus, we conclude that the pectin isolated from *Artemisia indica* Wild var. *indica* is a novel unusually branched polysaccharide. This study is the first to report on the pectin involving double D-galacturonan side-chains in addition to α -L-arabinogalactan and galactoglucuronoxyl side-chains in the rhamnagalacturonan main-chain. The cross-binding pectin molecules are able to associate between carboxyl groups of D-galacturonic acid residues through cations such as B^{3+} , Ca^{2+} and Mg^{2+} on different molecules to hold nutrients, inorganic matters, and water in the cell walls and intercellular areas of leaves of *Artemisia indica* Willd. var. *indica* [20,21,22,23,24].

Statement of Competing Interests

The authors declare no competing interests.

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