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Pectic Substances. II.¹⁾ The Location of *O*-Acetyl Groups and the Smith Degradation of Zizyphus-Pectin A

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The *O*-acetyl groups in Zizyphus-pectin A from the fruits of *Zizyphus jujuba* MILLER var. *inermis* REHD. were located at positions 2,3,6 of most of the D-galactopyranosyl residues in galactan side chains. A controlled Smith degradation study supported this conclusion and suggested the presence of continuous branching units in parts of arabinan side chains.

Keywords——*O*-acetyl group location; pectin; Zizyphus-pectin A; fruit; *Zizyphus jujuba* var. *inermis*; 2,3,6-tri-*O*-acetyl D-galactose; side chain structure

The isolation and structural features of the major pectic substance, named Zizyphus-pectin A, from the fruit of *Zizyphus jujuba* MILLER var. *inermis* REHD. (Rhamnaceae) have been reported in the first paper of this series.[1] This material is a well-known oriental crude drug. *O*-Acetyl groups were identified in Zizyphus-pectin A, and the content amounted to 2.3%. The present work was undertaken to elucidate the location of the *O*-acetyl groups. This paper also deals with the results of controlled Smith degradation of the pectin.

The pectin was exhaustively treated with methyl vinyl ether, as a protective reagent for free hydroxyl groups, in the presence of *p*-toluenesulfonic acid in dimethyl sulfoxide.[2] After conversion of the free hydroxyl groups to 1-methoxyethyl ethers, the derivative was deacetylated, then methylated with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide,[3] followed by methylation with methyl iodide and silver oxide in *N,N*-dimethylformamide.[4] The resultant product was subjected to acid hydrolysis, and the final products were analyzed by gas-liquid chromatography-mass spectrometry (GLC-MS) after conversion into alditol acetates.[5] 1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol was detected and identified. This result indicates that 2,3,6-tri-*O*-acetyl D-galactose units are present in the side chains of Zizyphus-pectin A.

The pectin was subjected to periodate oxidation followed by reduction with sodium borohydride.[6] The original polysaccharide was composed of D-galacturonic acid, L-rhamnose, D-galactose, and L-arabinose in the molar ratio of 35 : 1 : 1 : 4.[1] After periodate oxidation, all the D-galacturonic acid residues were decomposed, and the proportions of surviving rhamnose, galactose, and arabinose residues were 70%, 85%, and 28%, respectively. After deacetylation with alkali, the product was subjected to a second periodate oxidation followed by reduction. No change in the contents of L-rhamnose and L-arabinose in the product was found after the second periodate oxidation, while 92% of D-galactose residues were decomposed by this treatment. The reduction product obtained after the second periodate oxidation was treated with 1 N sulfuric acid for 44 h at room temperature. After neutralization and dialysis, the controlled Smith degradation products were isolated by gel chromatography on Sephadex G-15. From the low molecular weight fraction, three products (I, II, III) composed of L-arabinose and glycerol were obtained. The products were methylated with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide. The fully methylated products were hydrolyzed and analyzed by GLC-MS after conversion into alditol acetates.

Methylation analysis indicated the production of 2,3,5-tri-*O*-methyl arabinitol acetate from I, and that of 2,3,5-tri-*O*-methyl arabinitol acetate and 2,3-di-*O*-methyl arabinitol acetate in molar ratios of 1 : 1 from II, and 1 : 2 from III. Based on these results and the structure of the original pectin, I, II, and III were identified as α-L-arabinofuranosyl glycerol, *O*-α-L-arabinofuranosyl-(1→5)-α-L-arabinofuranosyl glycerol, and *O*-α-L-arabinofuranosyl-(1→5)-*O*-α-L-arabinofuranosyl-(1→5)-α-L-arabinofuranosyl glycerol. Rhamnose, galactose, and arabinose were detected in the hydrolysate of the relatively high molecular weight fraction, but no structural analysis of this fraction was done because of its low yield and insufficient purity.

The results of periodate oxidation indicate that the galacturonic acid residues possess no acetyl group. From the results of the second periodate oxidation, it can be concluded that the survival of rhamnose and arabinose is based on the existence of branching residues.

Based on both the acetyl content in the pectin and the result of the first periodate oxidation, we concluded that most of the galactosyl side chain is composed of 2,3,6-tri-*O*-acetyl-D-galactopyranose residues. The incomplete decomposition of galactose units by the second periodate oxidation of the deacetylated product may be attributable to steric hindrance.

Plant pectic polymers generally have backbones consisting of rhamnogalacturonan and side chains, such as a branched arabinan and a linear 1→4-linked galactan.[7] However, little is known about the presence of *O*-acetyl groups in pectic substances. The present report is the first to describe the presence of 2,3,6-tri-*O*-acetylated D-galactopyranosyl units in the side chains of pectic substances.

Experimental

Solutions were concentrated at or below 40 °C with rotary evaporators under reduced pressure. Infrared (IR) spectra were recorded on a JASCO IRA-2 infrared spectrophotometer. GLC was carried out on a Shimadzu GC-7AG gas chromatograph equipped with a hydrogen flame ionization detector. GLC-MS was performed with a JEOL JGC-20K gas chromatograph and a JEOL JMS-D100 mass spectrometer. Optical rotations were measured with a JASCO DIP-140 automatic polarimeter.

Preparation of Zizyphus-Pectin A——This was carried out as described in the preceding report[1] of this series.

Determination of Component Sugars——This was carried out by GLC of alditol acetates derived from acid hydrolysates, as described in a previous report.[8] A programmed temperature increase of 5 °C per min from 190 to 220 °C was used.

Treatment with Methyl Vinyl Ether——The dried sample (97 mg) was suspended in dimethyl sulfoxide (10 ml) and then *p*-toluenesulfonic acid (20 mg) was added. The mixture was stirred at 15 °C, then methyl vinyl ether (5 ml), condensed at −10 °C, was added in portions under stirring. The reaction mixture was stirred at 15 °C for 6 h, then dialyzed against distilled water. The non-dialyzable fraction was concentrated to dryness, and the reaction procedure was repeated twice more. The final solution was applied to a column (2 × 44 cm) of Sephadex LH-20. The column was eluted with acetone, and fractions of 5 ml were collected. The eluates obtained from tubes 9 to 12 were combined and concentrated. The reaction procedure of acetalation followed by purification on a Sephadex LH-20 column was similarly repeated eight times. The final product showed no hydroxyl absorption in its IR spectrum.

Deacetylation of the *O*-Acetyl-*O*-(1-methoxyethyl) Derivative——One-third of the product was dissolved in methanol (4 ml), then 0.2 M methanolic sodium methoxide (4 ml) was added under stirring. The mixture was refluxed at 70 °C for 2 h, then concentrated and applied to a column (3 × 38 cm) of Sephadex LH-20; the column was eluted with methanol. Fractions of 10 ml were collected, and the eluates obtained from tubes 9 to 19 were combined and concentrated. The absence of ester absorption bands in the IR spectrum of the residue confirmed that deacetylation was complete.

Methylation of the *O*-(1-Methoxyethyl) Derivative——Half of the product was dissolved in dimethyl sulfoxide (2 ml). Sodium hydride (150 mg) was mixed with dimethyl sulfoxide (10 ml) in an ultrasonic bath for 30 min, followed by stirring at 70 °C for 1 h, then the mixture was added to the sample solution. The reaction mixture was stirred at room temperature for 4 h, then methyl iodide (10 ml) was added and the whole was stirred overnight at room temperature. All procedures were carried out under nitrogen. After addition of water (40 ml), the reaction mixture was extracted five times with chloroform (40 ml each). The combined extract was washed five times with water (200 ml each), then dried over sodium sulfate, and the filtrate was concentrated to dryness. The residue was methylated four

Table I. Relative Retention Times on GLC and Main Fragments in MS of Partially Methylated Alditol Acetates

	Relative retention times[a]	Main fragments (m/z)
1,4-Ac-2,3,5-Me-L-Arabinitol	0.48	43, 45, 71, 87, 101, 117, 129, 161
1,4,5-Ac-2,3-Me-L-Arabinitol	1.08	43, 87, 101, 117, 129, 189
1,4,5-Ac-2,3,6-Me-D-Galactitol	2.03	43, 45, 87, 99, 101, 113, 117, 233

a) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Abbreviations: Ac = acetyl; Me = methyl (*e.g.*, 1,4-Ac-2,3,5-Me- = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-).

times under the same conditions. The final residue was dissolved in chloroform–methanol mixture (2 : 1), then applied to a column (2 × 46 cm) of Sephadex LH-20. The column was eluted with the same solvent, and fractions of 5 ml were collected. The eluates obtained from tubes 9 to 11 were combined and concentrated to dryness. The residue was dissolved in *N*,*N*-dimethylformamide (2 ml), then methyl iodide (3 ml) and silver oxide (0.2 g) were added successively under stirring. The reaction mixture was stirred at room temperature for 20 h in the dark, then filtered. The filtrate was washed with chloroform, then the filtrate and washing were combined and concentrated. The residue was methylated four times more under the same conditions. The final reaction mixture was filtered and washed with chloroform. The filtrate and washing were combined, then water (25 ml) and 10% potassium cyanide (10 ml) were added to the solution. The mixture was extracted with chloroform (35 ml each) five times. The extracts were combined and washed with water (170 ml each) five times, then dried over sodium sulfate, and the filtrate was concentrated. The final solution was applied to a column (2 × 45 cm) of Sephadex LH-20. The column was eluted with chloroform–methanol mixture (2 : 1), and fractions of 5 ml were collected. The eluates in tubes 9 and 10 were combined and concentrated to dryness. The final residue (12.4 mg) was a reddish-yellow syrup, and showed no hydroxyl absorption in its IR spectrum.

Analysis of the *O*-Methyl Derivative——A part of the product was hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated in the manner described in a previous report.[9] GLC-MS was carried out under the same conditions as in a previous report.[10] The relative retention time of the product with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in GLC and its main fragments in the mass spectrum are listed in Table I.

Periodate Oxidation——The pectin (493 mg) was oxidized with 0.05 M sodium metaperiodate (200 ml) at 7 °C in the dark. The periodate consumption was measured by a spectrophotometric method.[11] The oxidation was completed after 3 d, and the maximal value of the consumption was 0.57 mol per mol of anhydrosugar unit. The reaction mixture was successively treated with ethylene glycol (1 ml) at 7 °C for 3 h and sodium borohydride (1 g) at 22 °C for 14 h, then adjusted to pH 5 by addition of acetic acid. The solution was dialyzed against running water for 2 d. The non-dialyzable fraction was concentrated and applied to a column (5 × 86 cm) of Sephadex G-15. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 29 to 36 were combined and lyophilized. Yield, 470 mg.

Deacetylation and the Second Periodate Oxidation——The product obtained by the first periodate oxidation still showed ester absorptions (1230 and 1720 cm^{-1}) in its IR spectrum. The product (420 mg) was dissolved in 0.01 N sodium hydroxide (80 ml) and left at room temperature for 10 min. The solution was neutralized with 1 N acetic acid and diluted up to 85 ml with water, then 0.1 M sodium metaperiodate (85 ml) was added. The oxidation was performed at 6 °C in the dark, and was completed after 3 d. The maximal value of periodate consumption was 0.24 mol per mol of anhydrosugar unit. The reaction mixture was successively treated with ethylene glycol (1 ml) at 6 °C for 1 h and sodium borohydride at 6 °C for 16 h. After neutralization, dialysis, and gel chromatography as described above, the product (392 mg) was obtained.

Controlled Smith Degradation——The product (264 mg) was dissolved in 1 N sulfuric acid (28 ml), and after standing at 18 °C for 44 h, the solution was neutralized with barium carbonate and filtered. The filtrate was concentrated and applied to a column (5 × 81 cm) of Sephadex G-15. The column was eluted with water, and fractions of 10 ml were collected. The eluates obtained from the column were divided into five groups: Fr. 1, tubes 54 to 61; Fr. 2, tubes 62 to 72; Fr. 3, tubes 82 to 86; Fr. 4, tubes 87 to 94; Fr. 5, tubes 95 to 102. The yields were 5 mg for Fr. 1, 10 mg for Fr. 2, 8 mg for Fr. 3, 17 mg for Fr. 4, and 44 mg for Fr. 5. Product III was obtained from Fr. 3. Fr. 4 was dissolved in water, and applied to a column (2.6 × 93 cm) of Sephadex G-15. The column was eluted with water and fractions of 5 ml were collected. The eluates obtained from tubes 57 to 68 were combined and lyophilized. Product II (3 mg) was obtained from this fraction. Fr. 5 was dissolved in water, and applied to a column (5 × 81 cm) of Sephadex G-15. The column was eluted with water and fractions of 10 ml were collected. The eluates obtained from tubes 94 to 97 were combined and lyophilized. Product I (6 mg) was obtained from this fraction. Acid hydrolysis followed by analysis of component sugars indicated that the presence of rhamnose, galactose, and arabinose in Fr. 1, and rhamnose and arabinose in Fr. 2.

Analysis of the Products, I, II, and III——Thin-layer chromatography (TLC) was performed on Merck precoated Kieselgel 60 plates. As a developing solvent, the following mixture was used: *n*-butanol–acetic acid–water (2 : 1 : 1, v/v). The detection of spots on TLC plates was done by spraying 0.2% orcinol in 20% sulfuric acid followed by heating of the plates at 110 °C for 5 min. The *Rf* values of I, II, and III were 0.63, 0.57, and 0.51, respectively. On the other hand, samples were trimethylsilylated in the usual way,[12] then subjected to GLC. GLC was carried out under condition C, using a column (0.3 cm × 2 m long spiral glass) packed with 2% OV 101 on Uniport HP (80 to 100 mesh) and with a programmed temperature increase of 4 °C per min from 180 to 300 °C at a helium flow rate of 50 ml per min. The retention times (min) of I, II, and III were 13.0, 26.9, and 42.5, respectively, as their trimethylsilyl ethers.

Methylation Analysis of I, II, and III——Methylation was carried out with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide as described above. The reaction was repeated twice. The final product was hydrolyzed with dilute sulfuric acid in acetic acid, then reduced, acetylated, and analyzed by GLC-MS as described above. The relative retention times of the products with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in GLC and their main fragments in the mass spectra are also listed in Table I.

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