THE PECTIC SUBSTANCES OF *Zosteraceae*

PART IV. PECTINASE DIGESTION OF ZOSTERINE

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ABSTRACT

Pectinase digestion of zosterine furnished apiogalacturonan (AGU), an oligosaccharide mixture, and D-galacturonic acid, thereby suggesting a structure that contains, in part, galacturonan chains free of glycosidic bonds with neutral sugars. AGU, which was homogeneous and resistant to pectinase, was composed of D-galacturonic acid and D-apiose residues in a ratio of ~4:5. Partial hydrolysis with acid, periodate oxidation, and methylation studies proved AGU to possess a branched structure with a linear, α-(1→4)-linked D-galacturonan chain substituted in the 2-, 3-, or 2,3-positions by D-apiose residues. Pseudoaldobiouronic acids, consisting of D-xylose or L-arabinose residues linked to D-galacturonic acid, were isolated from the oligosaccharide mixture, thereby demonstrating the presence of glycosidic bonds between the galacturonan chain and neutral fragments of zosterine.

INTRODUCTION

Zosterine isolated[1-3] from various *Zosteraceae* species is a homogeneous, pectic polysaccharide containing D-galacturonic acid (60%), D-galactose, D-xylose, L-arabinose, D-apiose, and trace amounts of L-rhamnose and an *O*-methyl-D-xylose. The pectic acid obtained on partial hydrolysis of zosterine with acid is a linear galacturonan consisting of α-(1→4)[and probably α-(1→5)]-linked residues of D-galacturonic acid[4]. In a previous communication[5], we reported the characterization of zosterine by using enzyme preparations from various invertebrates.

We now describe the application of pectinase digestion in a structural study of zosterine.

EXPERIMENTAL AND RESULTS

General experimental conditions. — Partition chromatography was performed by the ascending and descending techniques on Whatman No. 3 or Filrak FN 12 paper with the following solvent systems (v/v): (*A*) butyl alcohol–acetic acid–water (4:1:1); (*B*) butyl alcohol–benzene–pyridine–water (5:1:3:3, upper layer); (*C*) ethyl acetate–acetic acid–pyridine–water (5:1:5:3); (*D*) butanone saturated with dilute

ammonia; (*E*) butyl alcohol–ethanol–water (40:11:19). Thin-layer chromatography (t.l.c) was performed on silica gel "KSK" (>200 mesh). Sugars were chromatographed as described earlier[6], and their methylated derivatives were developed with chloroform–methanol (9:1). The detection reagents were (**1**) aniline hydrogen phthalate; (**2**) ammoniacal silver nitrate; (**3**) conc. sulphuric acid; (**4**) 0.5% of orcinol in 30% sulphuric acid (200 ml) +1% aqueous ferric chloride (4 ml) for 2–3 min at 180°. Reagents **3** and **4** were employed for t.l.c. only. Gas–liquid chromatography (g.l.c.) was carried out by using a Pye–Argon chromatograph fitted with a β-ionization detector and straight, glass columns (120×0.5 cm); or a "Tsvet-2" chromatograph fitted with a flame-ionization detector and stainless-steel columns (100×0.3 cm). The columns were packed with (*a*) 8% SE-30 on 80–100 mesh Chromosorb W or (*b*) 5% neopentylglycol succinate on 60–80 Chromosorb W, treated with acid and hexamethyldisilazane. Samples (10–15 μg) were analysed with argon or nitrogen as carrier gas at a flow rate of 60 or 30 ml/min. G.l.c. of sugars as trimethylsilyl (TMS) derivatives[7] was performed by using column (*a*) at 180 and 155°; methylated methyl glycosides were chromatographed on column (*b*) at 130 and 150°. I.r. spectra were run on a Zeiss UR-20 spectrometer, n.m.r. spectra (60 MHz) on a Zeiss ZKR-60 spectrometer, and mass spectra on a Sumy MX-1303 spectrometer. Molecular weights were determined as described elsewhere[8]. Gel filtration and chromatography on DEAE-cellulose[9] and electrophoresis[10] on polyacrylamide gel were performed as described previously. Uronic acid was determined by the decarboxylation procedure[11]. Periodate uptake[12] and release of formaldehyde[13] were measured on periodate oxidation. Ash content was determined by heating polysaccharide samples to constant weight at 600°. Solutions were evaporated *in vacuo* at 30–40°.

Enzyme preparations. — "Pectinase standardisiert, purum", Fluka (enzyme 1) and "Pectinase" (*ex Aspergillus niger*), Koch–Light (enzyme 2) were used.

Origin of specimens. — *Zosteraceae* plants were collected at the sublittoral of the Sea of Japan in the summer of 1968. Zosterine was obtained from fresh, plant material[3].

Pectinase digestion of zosterine. — (*a*) Enzyme 1 (0.8 g) was added to a solution of zosterine (40 g) in water (4 l), and the mixture was kept for 48 h at 37°, concentrated to 1 litre, and poured into ethanol (3 l). The resulting precipitate was collected by centrifugation to give crude AGU (11 g). The residual solution was evaporated to yield a mixture (*ca.* 25 g) of D-galacturonic acid and higher oligosaccharides which were not further investigated.

The crude AGU was dissolved in water, and reprecipitated with ethanol (3 volumes). The precipitate was dissolved in water and, after dialysis, the solution was freeze-dried to yield purified AGU (6–7 g) as a white powder, $[\alpha]_D^{20}$ +80.5° (water) (Found: mol. wt., 22,000; C, 39.9; H, 6.4; galacturonic acid, 39.6; methoxyl, 1.52; N, 0.8; ash, 4.86%).

(*b*) A 1% aqueous solution of zosterine (10 g) was digested, with simultaneous dialysis, with enzyme 2 (0.2 g) for 48 h at 37° to give crude AGU (non-dialysable fraction A) which was purified as above to yield material (*ca.* 2.0 g) having $[\alpha]_D^{20}$

+80.1° (water) (Found: mol. wt., *ca.* 22,000; C, 39.87; H, 6.4; galacturonic acid, 39.72; methoxyl, 1.52; N, 0.0; ash, 2.9%). The AGU samples from (*a*) and (*b*) were identical.

The material (fraction B) which dialysed was a mixture of D-galacturonic acid and a number of oligosaccharides which, on acid hydrolysis, afforded all the zosterine sugars; only traces of apiose were obtained. The mixture (1 g) was applied to a column containing 200 ml of Dowex-1 x8 (acetate form) resin. Elution with water removed neutral compounds, and a gradient of water containing 0→2M acetic acid gave fractions of galacturonic acid and acidic oligosaccharides which were isolated by using preparative, paper chromatography (solvent *C*). A neutral oligosaccharide **1** and three acidic oligosaccharides[2–4] were obtained in this way.

Oligosaccharide **1**. — The sugar (5 mg), R_{GalA} 1.7 (solvent *C*), gave xylose and arabinose in a molar ratio of *ca.* 1:1 on acid hydrolysis.

Oligosaccharide **2**. — The sugar (15 mg), $[\alpha]_D^{20}$ +50° (water), R_{GalA} 0.5 (solvent *C*), gave xylose and galacturonic acid in a molar ratio of *ca.* 1:1 on acid hydrolysis. The disaccharide gave no colour with triphenyltetrazolium chloride (TTC), indicating[14] the possible presence of a (1→2)-linkage.

The sugar was stable to alkali, suggesting[15] the absence of (1→3)-linkages. Sodium hydroxide (5mM, 2–3 ml) was added to a drop of the disaccharide solution containing 2–3 mg of **2**. The mixture was heated for 10 min at 70°, neutralized with Amberlite IR-120 (H^+) resin, concentrated, and chromatographed on paper in solvent *C*. Unchanged **2** was the only material detected.

Disaccharide **2** (1–2 mg) was reduced with borohydride, and subsequent acid hydrolysis gave xylose as the only unchanged sugar. Thus, galacturonic acid was the reducing end-group of **2**. The i.r. spectrum of **2** indicated[16] the β-D configuration of the xylosidic linkage.

2 (10 mg) was treated with 1% methanolic hydrogen chloride for 18 h at 20°. The acid was neutralized with silver carbonate and the filtered mixture was evaporated. A small portion of the product was oxidised with 30mM sodium metaperiodate in acetate buffer (pH 4.5) in the dark for 24 h at 20°. The resulting polyaldehyde was reduced with borohydride, and the product was hydrolysed with M sulphuric acid in the usual way. Paper chromatography (solvents *A* and *B*) of the hydrolysate revealed neither xylose nor galacturonic acid. A second portion was permethylated with methyl iodide–silver oxide in the usual manner, and the product was methanolysed with 72% perchloric acid–methanol (1:10) in a sealed tube for 3 h at 95°. After removal of acids with Dowex-1 (HCO_3^-) resin, the methyl 2,3,4-tri-*O*-methyl-D-xylosides were detected by t.l.c. The methylated methyl xylosides were hydrolysed with M sulphuric acid for 3 h at 95°. Paper chromatography (solvent *E*) and t.l.c. showed the presence of 2,3,4-tri-*O*-methyl-D-xylose only.

Thus, the disaccharide **2** was 2-*O*-β-D-xylopyranosyl-D-galacturonic acid.

Oligosaccharide **3**. — The sugar (20 mg), $[\alpha]_D^{20}$ +77° (water), R_{GalA} 0.25 (solvent *C*), gave xylose and galacturonic acid in a molar ratio of *ca.* 2:1 on acid hydrolysis. The sugar was stable to alkali [absence of (1→3)-links[15]], and the i.r.

spectrum indicated[16] the β-D configuration of the xylosidic linkages. Reduction with borohydride, followed by hydrolysis, showed that the reducing end-group was the D-galacturonic acid residue. The triphenyltetrazolium chloride test indicated the possible presence of a (1→2)-linkage between xylose and galacturonic acid. Thus, trisaccharide **3** is possibly *O*-β-D-xylosyl-(1→2 or 4)-*O*-β-D-xylosyl-(1→2)-D-galacturonic acid.

Oligosaccharide **4**. — The sugar (10 mg), $[\alpha]_D^{20}$ +96° (water), R_{GalA} 0.02 (solvent *C*), gave xylose, arabinose, and galacturonic acid on acid hydrolysis. Reduction with borohydride, followed by hydrolysis, indicated that the reducing end-group was D-galacturonic acid.

Acid hydrolysis of the apiogalacturonan. — (*a*) *Complete hydrolysis*. AGU (0.1 g) was hydrolysed with M sulphuric acid in a sealed tube for 16 h at 95° to yield D-galacturonic acid and D-apiose in a molar ratio of *ca.* 4:5 (paper chromatography in solvents *A*–*C* and t.l.c.). The mixture was treated with Dowex-1 x4 (HCO_3^-) resin to remove galacturonic acid. Elution with water and methanol furnished D-apiose (50 mg), $[\alpha]_D^{20}$ +5° (water), which was identical with an authentic sample, as shown by paper chromatography in solvents *A*–*C*, t.l.c, g.l.c., and by comparison of n.m.r. spectra.

(*b*) *Partial hydrolysis*. AGU (1 g) was hydrolysed with 0.5M sulphuric acid (100 ml) for 5 h at 95°. The precipitate was collected by centrifugation, washed with water, and dried to give the galacturonan (GU) as a white powder (0.3–0.35 g), $[\alpha]_D^{20}$ +258° (dilute ammonia), mol. wt. 10,000. On complete hydrolysis, as above, GU gave galacturonic acid only.

The supernatant solution was neutralized with Dowex-1 x4 (HCO_3^-) resin and evaporated to afford D-apiose (0.5 g).

(*c*) *Autohydrolysis*. AGU (1 g) was treated with Amberlite IR-120 (H^+) resin in water (100 ml) for 10 min at 20°. The filtered solution was refluxed for 4 h and poured into ethanol (500 ml). The precipitate was collected, washed with water, and dried *in vacuo* to give 0.4 g of white material, complete hydrolysis of which afforded galacturonic acid and trace amounts of apiose. The supernatant liquid was treated with Dowex-1 x4 (HCO_3^-) resin and evaporated to yield D-apiose (*ca.* 0.5 g).

Periodate oxidation of AGU. — AGU (0.1 g) was oxidised with 30mM sodium metaperiodate (500 ml) in acetate buffer (pH 4.5) for 48 h at 20° in the dark; 1.16 moles of periodate were consumed and 0.49 mole of formaldehyde was released per "anhydro-sugar" moiety. The excess of metaperiodate was destroyed with ethylene glycol, the mixture was dialysed, and the polyaldehyde was reduced with sodium borohydride (1 g) for 24 h at 20°. The polyalcohol (50 mg) obtained was hydrolysed with M sulphuric acid for 4 h at 95°, and paper chromatography (solvents *B* and *E*) revealed the presence of galacturonic acid and threonic acid (trace). Apiose, threose or erythrose, and glycerol were absent, thus confirming the terminal location of the D-apiose residues in AGU.

Periodate oxidation of the galacturonan (*GU*). — GU (50 mg) was oxidized with 30mM sodium metaperiodate (100 ml) as described above, and the polyaldehyde was

reduced with borohydride to give a polyalcohol which was then hydrolysed. Paper chromatography revealed the absence of D-galacturonic acid in the hydrolysate.

Pectinase digestion of the galacturonan. — GU (50 mg) was dissolved in dilute ammonia and freeze-dried. The ammonium salt was digested with pectinase (enzyme 1 or 2) in water (5 ml) for 48 h at 37°. When the mixture was poured into ethanol (5 volumes), no precipitate was formed, and the solution was evaporated to small volume and analysed by paper chromatography which revealed the presence of D-galacturonic acid.

Methylation of carboxyl-reduced AGU. — A solution of AGU (2 g) in water (100 ml) was treated with Amberlite IR-120 (H^+) resin to remove cations, and the solution was freeze-dried. The polysaccharide was dispersed in methanol (500 ml), treated with an excess of diazomethane at 0°, and stored for 24 h at 20°. The procedure was repeated until the yellow colour persisted. The methyl ester of AGU (i.r. band at 1735 cm^{-1}) was dissolved in water (100 ml), sodium borohydride (2 g) was added, and the mixture was stirred for 24 h at 20°, dialysed, and freeze-dried to yield partially methylated apiogalactan (2.2 g) which had i.r. bands at 2930 and 3600 cm^{-1}, but not at 1735 cm^{-1}. A portion of the product was methylated by Hakomori's procedure[17], followed by two successive Purdie methylations[18], to afford permethylated, reduced apiogalactan (MAG, 600 mg), $[\alpha]_D^{20}$ +6.3° (chloroform), which showed no i.r. band for hydroxyl (Found: OMe, 41.0%).

A second portion was treated with diazomethane at 37° until (4 days) complete dissolution in methanol was achieved. The resulting product was permethylated by two successive treatments with the Purdie reagents[18] to afford MAG (0.9 g), $[\alpha]_D^{20}$ +6.1° (chloroform) (Found: OMe, 40.8%). A sample of MAG (0.5 g) was methanolyzed with a mixture of 72% perchloric acid and methanol (1:10) for 6 h in a sealed tube at 100°. Examination of the methanolysate by t.l.c. and g.l.c. indicated the presence of methyl glycosides of 2,3,4-tri-*O*-methyl-D-apiose (main component), and 2,3,6-tri-(trace), 2,6- and 3,6-di-, and 6-*O*-methyl-D-galactose. A portion of the mixture of methyl glycosides was hydrolysed with M sulphuric acid in a sealed tube for 5 h at 95°. Paper chromatography and t.l.c. revealed the above methylated sugars.

Elution of the mixture of methyl glycosides (0.5 g) from silica gel "KSK" (<200 mesh), with a gradient of increasing concentrations of ethanol in chloroform, afforded the following fractions.

Fraction 1 (0.21 g), $[\alpha]_D^{20}$ +3.5° (chloroform), gave a single spot on t.l.c. and peaks on g.l.c. corresponding to those of methyl 2,3,4-tri-*O*-methyl-α,β-D-apiofuranoside. The i.r. and mass spectra were identical with those of an authentic sample.

A portion (10 mg) gave apiose (paper chromatography, solvents *A* and *B*) on demethylation[19] with boron tribromide.

The second portion (0.1 g), when hydrolysed with M sulphuric acid, as above, gave 2,3,4-tri-*O*-methyl-D-apiose (50 mg) indistinguishable from authentic material by paper chromatography, t.l.c., and mass spectrometry.

Fraction 2 (0.15 g) was characterized as a mixture of methyl 2,3,6-tri, 2,6- and 3,6-di-*O*-methyl-D-galactosides by t.l.c. and g.l.c. Acid hydrolysis of the methyl glycosides, followed by paper chromatography and t.l.c. of the free sugar derivatives, confirmed this inference. Demethylation[19] gave galactose.

Fraction 3 (0.05 g) was characterized as methyl glycosides of 6-*O*-methyl-D-galactose by t.l.c. and g.l.c. of the TMS derivatives. Acid hydrolysis, followed by paper chromatography and t.l.c. of the free sugar, confirmed this inference. Demethylation[19] gave galactose.

DISCUSSION

Pectinase digestion is a convenient method of studying pectic substances[20]. Zosterine, which is a pectic substance of *Zosteraceae* plants[1–3], is degraded by pectinase, and optimal conditions of digestion were sought in order to obtain the fragments in highest yield. Commercial preparations of pectinase (enzymes 1 and 2) were used at constant temperature (37°), with variation of (*a*) pectinase quantity, (*b*) zosterine concentration (0.1–1.5%), and (*c*) duration of digestion (2–96 h). The most satisfactory results were achieved by using 20 mg of pectinase per each 100 ml of 1% aqueous zosterine for 48 h. The digestion furnished AGU, oligosaccharide fragments, and galacturonic acid. The isolation of the last substance indicated the presence of portions of galacturonan chain free of glycosidic bonds with neutral sugars. The oligosaccharide fragments contained all the zosterine sugars, excepting D-apiose. Digestion with enzyme 1 gave a mixture of higher oligosaccharides, whereas enzyme 2 furnished oligosaccharides of low molecular weight together with monosaccharides. In order to increase the yield of oligosaccharide fragments, pectinase digestion of zosterine with enzyme 2 was carried out with simultaneous dialysis. The lower oligosaccharides were partly separated to furnish a neutral disaccharide **1** (composed of xylose and arabinose residues) and three acidic oligosaccharides **2–4**.

Disaccharide **2** appeared to be 2-*O*-*β*-D-xylopyranosyl-D-galacturonic acid, and trisaccharide **3** might be *O*-*β*-D-xylosyl-(1→2 or 4)-*O*-*β*-D-xylosyl-(1→2)-D-galacturonic acid. Oligosaccharide **4** consisted of xylose, arabinose, and galacturonic acid residues. The last represented a reducing end-group only. Thus, oligosaccharides **2–4** are pseudoaldobiouronic acids. This evidence demonstrates the presence of glycosidic bonds between the galacturonan chain and the neutral fragments of zosterine. AGU, which was obtained as the main product on pectinase digestion, was examined in detail. The polysaccharide was resistant to further digestion with both enzyme preparations. After purification, AGU was examined by gel filtration on Biogel P, chromatography on DEAE-cellulose, and electrophoresis on polyacrylamide gel. A single peak or band was observed in all cases, thereby confirming the homogeneity of AGU. Complete hydrolysis of the polysaccharide with acid afforded D-galacturonic acid and D-apiose in a ratio of *ca* 4:5. The molecular weight of AGU was 22,000, suggesting that it represented *ca.* 25% of the zosterine molecule. Partial hydrolysis with acid and autohydrolysis of AGU gave a galacturonan (GU) and

apiose (as the only monosaccharide); the latter was liberated early in the hydrolysis, demonstrating the lability of its attachment. Galacturonic acid and oligosaccharides were almost completely absent from the products of partial hydrolysis or autohydrolysis. D-Apiose was isolated and identified by comparison with an authentic sample.

The galacturonan was readily oxidised by periodate, with almost complete cleavage of the galacturonic acid residues. In addition, GU was completely digested by pectinase to yield D-galacturonic acid. This evidence demonstrates that GU represented a part of the pectic acid molecule and consisted of α-(1→4)-linked D-galacturonic acid residues.

Periodate oxidation of AGU, followed by reduction with borohydride, afforded a polyalcohol which yielded galacturonic acid as the only monosaccharide on acid hydrolysis. The apiose residues were completely destroyed. These data suggest a branched nature for the AGU molecule, with terminal apiose residues. Further information on the structure of AGU was furnished by methylation studies. Because permethylation of polysaccharides containing a high proportion of uronic acid residues may be difficult to achieve, AGU was esterified with diazomethane, followed by reduction with borohydride, prior to successive methylations. Methanolysis of the product afforded 2,3,4-tri-*O*-methyl-D-apiose (main component), and 2,3,6-tri- (trace only), 2,6- and 3,6-di-, and 6-*O*-methyl-D-galactose. Other methylated D-apiose derivatives were not detected. This evidence accords with the periodate-oxidation data and suggests that AGU possesses a branched structure involving a linear α-(1→4)-linked D-galacturonan chain substituted in the 2-, 3-, and 2,3-positions by terminal D-apiose residues.

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