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Note

Observations on the crystallization of oligogalacturonates

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Abstract

Oligogalacturonates were produced by the limited enzymic hydrolysis of polygalacturonic acid and purified by ion-exchange chromatography. The fractions obtained were of limited polydispersity, determined by analytical ion-exchange chromatography. Oligomers with an average degree of polymerization of 10–15 were readily crystallized from aqueous salt solutions at neutral pH as single crystals. Crystal morphology of the salts examined, Na^+, K^+ and Ca^{2+} were characteristic of the salt. The wide-angle X-ray diffraction patterns obtained for the sodium salt were consistent with published fibre diffraction data of this salt form.

Keywords: Pectin; Oligogalacturonate; Crystallization

1. Introduction

The pectic polysaccharides are major components of the primary cell wall and middle lamella of dicotyledenous food plants. Structurally they consist of a backbone of α-(1→4)-D-galacturonosyl residues interrupted with typically 10% substitution of α-(1→2)-L-rhamnopyranosyl residues. A portion of the rhamnosyl residues are branch points for neutral sugar side-chains. Further structural diversity is obtained from the methyl esterification of a portion of the galacturonosyl residues [1,2].

The oligalacturonate regions of the backbone can adopt a number of conformations in the solid state which, in part, is dependent on the counterion. Initial X-ray fibre diffraction studies concluded that the pectic polysaccharides formed a twofold ribbon-like structure [3], while later studies suggested a threefold helical structure of pitch 1.32 nm [4]. The most detailed study has been made by Walkinshaw and Arnott [5]. In the sodium salt form the pectate adopts a 3_1 helical structure, packed into an orthogonal unit cell of dimensions $a = 0.84$, $b = 1.43$, c (fibre axis) $= 1.34$ nm of space group symmetry $P2_1$. As $b \approx a\sqrt{3}$, the strong reflections can be indexed on an hexagonal lattice of dimensions $a' = b' = 0.84$ and $c = 1.34$ nm. Partial conversion of the sodium form to the acid form retained the

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3_1 helical structure and the space group symmetry of the unit cell, but altered the unit cell dimensions to $a = 0.99$, $b = 1.23$ and $c = 1.33$ nm. The X-ray data for calcium pectate is poor [6] and an alternative model for the conformation of pectin in the junction zones of calcium pectate gels has been proposed [7]. Recently solid state NMR has claimed to provide evidence for the presence of 2_1 and 3_1 helical conformations, and intermediate helical aggregated forms in concentrated polygalacturonate gels [8]. Molecular modelling studies predict that these helical structures are almost equally preferred and therefore polymorphism in the crystalline conformations may be expected [9].

The ability to prepare single-crystals would facilitate X-ray and spectroscopic studies and allow the development of detailed atomic models for the different salt forms of pectin. While it is generally difficult to prepare single crystals of polysaccharides, oligomeric fractions often crystallize more readily and, if the fraction is of limited polydispersity, then crystal perfection can be improved [10]. This article describes the preparation and crystallization of oligogalacturonate fractions.

2. Experimental

Materials.—Polygalacturonic acid was obtained from Fluka.

Preparation of oligogalacturonic acid.—A 1% w/w soln of polygalacturonic acid in 100 mM NaOAc buffer pH 4.5, was treated, at 20 °C, with 0.6 units/100 mL of an *endo*-polygalacturonase (*Aspergillus niger* was obtained from Sigma, and purified as described [11]). A unit is defined as the amount of enzyme required to produce 1 μmol/min of reducing end-group from polygalacturonic acid at 40 °C. After incubation for 24 h the enzyme was inactivated by rapid heating to 90 °C. The product (88% yield) was filtered, treated with Dowex (H^+) X8, to remove Na^+, concentrated tenfold by rotary evaporation and freeze-dried.

Fractionation of oligogalacturonic acid.—The product (500 mg) was fractionated by high-performance preparative ion-exchange chromatography (HPAEC), using a Q Sepharose fast-flow column (47×3.2 cm) at a flow rate of 5 mL/min eluted with 50 mM acetate, pH 5.5 and an acetate salt gradient of 0–500 mM over 9.25 h; 9 mL fractions were collected. Depending on the salt form of the oligogalacturonate required, Na^+ or K^+ were used as the buffer counterions.

Analytical chromatography.—HPAEC chromatography of the oligogalacturonic acid fractions was carried out using a Dionex PA1 column eluted at 1.0 mL/min with 100 mM NaOH, and either a gradient of 0.2–0.85 M NaOAc over 60 min for the crude enzymolysis product, or 0.3–0.6 M over 15 min, followed by 0.6 M isocratic NaOAc for 10 min, to analyse individual column fractions. Carbohydrate in the eluent was monitored by a pulsed electrochemical detector (Dionex) after post column addition of 0.2 M NaOH. The column was primarily calibrated with purified oligogalacturonates with degree of poymerisation 3 (Sigma) and 6 (obtained by preparative ion exchange chromatography and characterisation through electrospray mass spectroscopy, which gave an $[M - H]$ ion at 1073.5 and a $[M - 2\ H]$ ion at 536.2)

Crystallization.—The Na^+ and K^+ forms of the oligogalacturonate were crystallized from 0.5% w/w aq soln containing 0.3 M of KCl or NaCl at 1 °C. The Ca^{2+} form was prepared by addition of $CaCl_2$ (0.05 M) at a rate of 0.015 mol/h to a gently stirred solution of 0.05% w/w sodium oligogalacturonate (47 mL) at 20 °C.

Microscopy.—Scanning electron microscopy (SEM) was carried out using a Leica Stereoscan 360 (Leo, Cambridge, UK). The crystalline precipitate was mounted on an adhesive tab on an aluminium pin stub (Agar Scientific, Stansted, UK) and sputter coated with a layer of gold (~ 25 nm) using a Emitech K550 coater (Emitech, Ashford, UK). The specimen was examined using an accelerating voltage of 20 kV.

X-ray diffraction.—Initial studies of sodium oligogalacturonate were made at the Cu K_α wavelength of 0.154 nm, using a flat plate camera flushed with helium to reduce air scatter, and maintained at a relative humidity of 98%. A suspension of the crystals was packed

into glass capillary tubes using centrifugation. Calcite dusted on the outside of the tube was used for calibration. X-ray diffraction patterns were recorded photographically. Additional powder diffraction patterns were recorded using a Philips Scientific PW 1820 vertical goniometer with an Anton Parr TTK camera. Data were recorded over the angular range 5.0–42.0° 2θ at a scanning speed of 0.01° 2θ/s, with a step size of 0.02° 2θ. Identification of peak positions was accomplished using the Peak Search facility of PC-APD. This automatically locates peaks in a crystalline diffraction pattern by detecting minima in the second derivative of the diffractogram. The standard peak search settings were used, except that the minimum peak width was changed from 0.0 to 0.2° 2θ, causing a slight smoothing of data, but reducing the detection of 'phantom peaks' arising from instrumental noise. Potassium and calcium oligogalacturonate diffraction data was obtained photographically using the flat plate camera with calcite used for calibration.

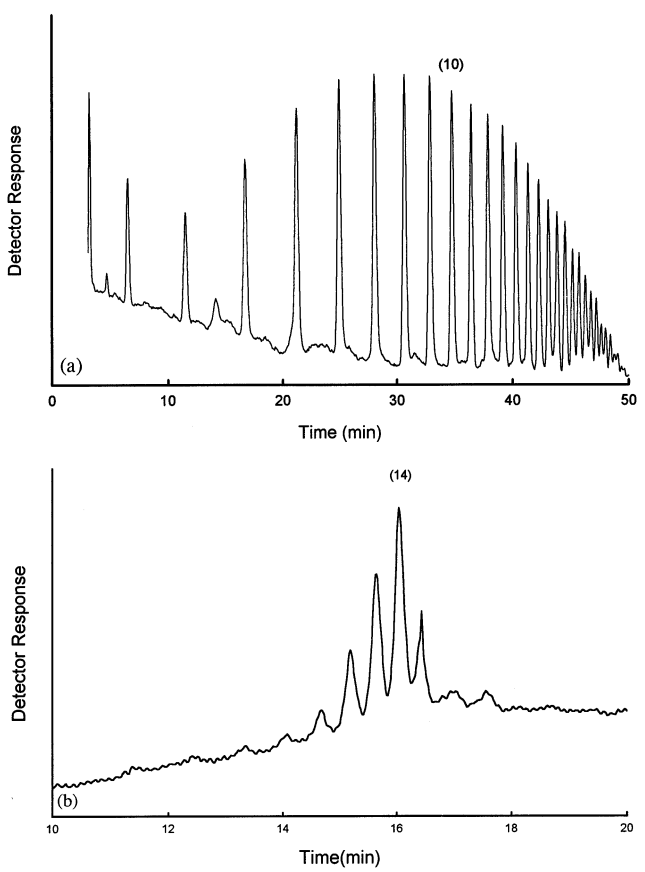

Fig. 1. Analytical ion-exchange chromatograms of oligogalacturonic acid fractions (a) initial product of enzymolysis; (b) fraction obtained from preparative ion-exchange chromatography. Number in brackets above the chromatogram represents the degree of polymerisation of the associated peak.

3. Results and discussion

Analysis [12,13] of the polygalacturonic acid preparation showed that it consisted of > 95% w/w D-galacturonic acid with trace quantities of neutral sugars present, including L-arabinose 0.5, D-galactose 3.5, and L-rhamnose 0.8 mol%. Polygalacturonic acid was subjected to a limited enzymolysis with a fungal *endo*-polygalacturonase to obtain a polydisperse oligogalacturonate preparation. Analytical ion-exchange chromatography of the charged oligomers showed a range of chain lengths with a maximum degree of polymerization (DP) of > 30 (Fig. 1(a)). Attempts to obtain highly crystalline material from these unfractionated oligogalacturonate materials were unsuccessful; all precipitates were amorphous on examination by X-ray diffraction.

Following HPAEC, it was possible to obtain fractions of a limited polydispersity, as shown in Fig. 1(b) for a fraction which has a major component of DP 14, significant amounts of DP 12, 13 and 15 with smaller amounts of other DPs either side of this distribution. In preliminary crystallization experiments it was noted that the tendency of the salt forms to precipitate as a function of counterion concentration was in the order $Ca^{2+} \gg Na^{+} > K^{+}$, while the ease of obtaining a crystalline precipitate was in the order $Na^{+} > K^{+} \gg Ca^{2+}$. Na^{+} and K^{+} oligogalacturonates of DP 14 readily crystallized from aqueous salt solutions at 1 °C. The morphology of the crystalline aggregates is shown in scanning electron micrographs of the Na^{+} and K^{+} forms (Fig. 2(a,b)). Whereas the Na^{+} form has a lobed structure, with the crystallites arranged as a sheaf, the K^{+} and Ca^{2+} forms consist of stacked platelets.

Preliminary X-ray diffraction studies obtained using the flat plate camera, showed that most crystalline material was in the sodium salt form, followed by the potassium salt form and finally the calcium salt form. The calcium

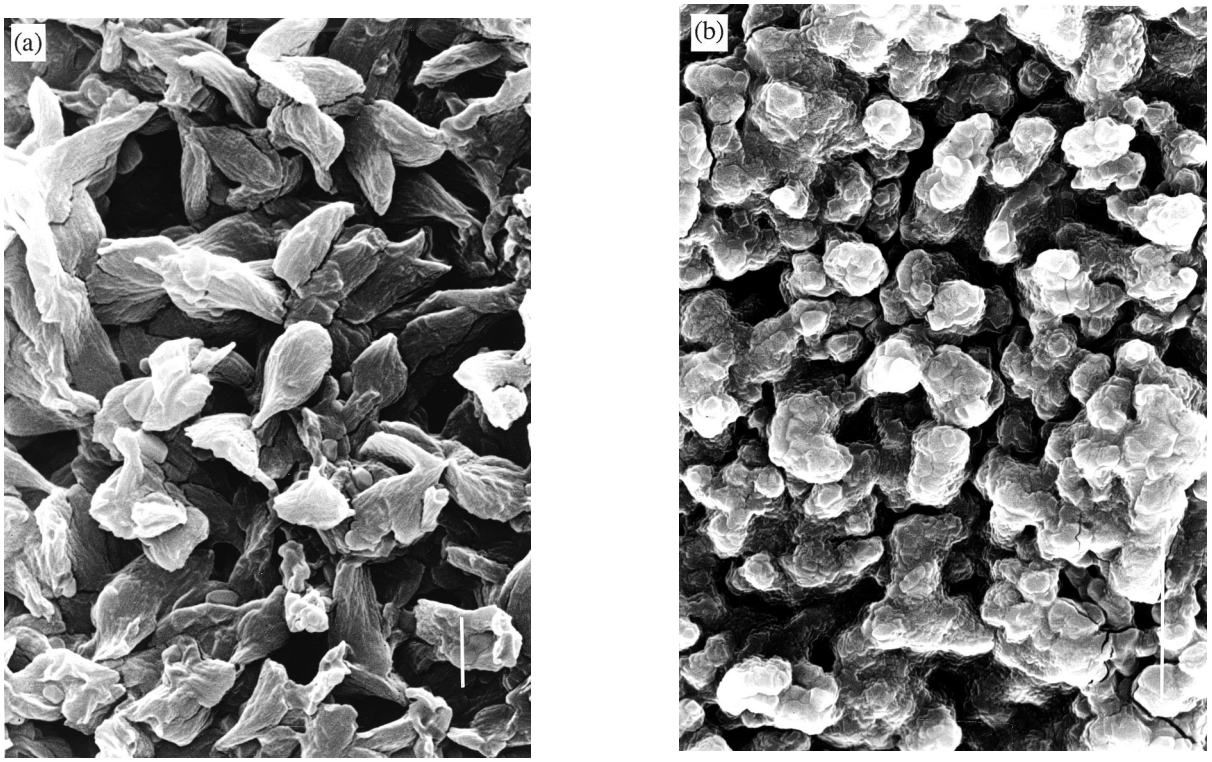

Fig. 2. Scanning electron micrographs of crystallized oligogalacturonates (a) Na^+, scale bar 1 μm; (b) K^+, scale bar 5 μm.

salt showed only two broad, weak reflections, which are recorded in Table 1. Data on the potassium salt varied in quality from sample to sample. Data on the sample showing the most reflections, is also recorded in Table 1. The X-ray powder diffraction pattern for the sodium salt, after fitting and subtraction of a background, is shown in Fig. 3. The data for the pattern is recorded in Table 1. All the observed reflections are consistent with the simplified hexagonal lattice described for sodium pectate fibres [5]. There are no detectable reflections corresponding to 001 or 002 but detectable reflections corresponding to 003 and 006. The data suggest that the sodium salt adopts the same helical structure and packing as that observed in the oriented fibre patterns. All the salt-forms show a reflection at approximately 0.44 nm, which may correspond to the axial rise per repeat unit. However, insufficient information is available to determine whether the helix is threefold for the potassium and calcium salt forms.

4. Conclusions

Oligalacturanates of limited polydispersity are useful materials for the investigation of the solid state conformations of pectin. The

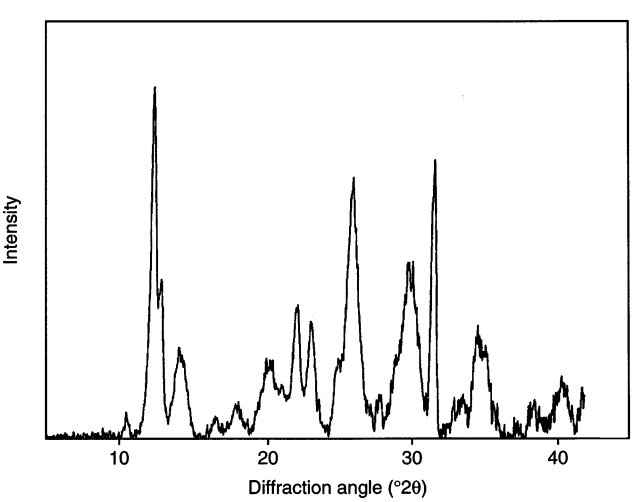

Fig. 3. Wide angle X-ray diffractogram of sodium oligogalacturonate.

Table 1
Crystallographic parameters from X-ray powder diffraction of oligogalacturonate salts

Salt form	Angle (° 2θ)	*d*-spacing (nm)	Intensity [a]
Sodium [b]	10.53	0.84	vw
	12.37	0.72	s
	12.92	0.68	m
	14.43	0.61	m^-
	17.97	0.49	w
	20.05	0.44	w
	22.24	0.40	w^+
	23.12	0.38	w^+
	25.98	0.34	m
	29.75	0.30	w and b
	34.55	0.26	w and b
	40.45	0.22	w and b
Potassium [c]	9.72	0.91	w and b
	11.64	0.76	m^-
	14.06	0.63	w^+
	19.73	0.45	w^-
	23.41	0.38	w^-
Calcium [c]	10.92	0.81	m^-
	20.65	0.43	w

[a] s, strong; m, medium; w, weak; vw, very weak; w and b, weak and broad.
[b] Powder diffractometer data.
[c] Flat plate camera data.

single crystals obtained, provide a basis for refining X-ray diffraction data on oriented fibres, and could provide a means of assigning and interpreting X-ray, or solid-state NMR data obtainable on pectin gels or for the pectin component of plant cell walls.

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