Characterization of Two Cell-Wall Polysaccharides from *Fusicoccum amygdali*

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1. The nature of two polysaccharides (s_{20}^0 values 6S and 2S respectively in 1M-sodium hydroxide), comprising a fragment (fraction BB, $[\alpha]_D + 236^\circ$ in 1M-sodium hydroxide), previously isolated from cell walls of *Fusicoccum amygdali*, has been investigated. 2. Both the major (2S) and minor (6S) components were affected by incubation with α -amylase. The 6S polysaccharide was also attacked by exo- β -(1 $\rightarrow$ 3)-glucanase, which is evidence that it contained both α -(1 $\rightarrow$ 4)- and β -(1 $\rightarrow$ 3)-glucopyranose linkages. By fractionation of the products of α -amylase-treated fraction BB it was possible to obtain a water-insoluble polysaccharide, fraction P ($[\alpha]_D + 290^\circ$ in 1M-sodium hydroxide, 67% of fraction BB) and a water-soluble polysaccharide, fraction Q ($[\alpha]_D + 16^\circ$ in 1M-sodium hydroxide, 11% of fraction BB), both of which sedimented as single boundaries with s_{20}^0 values (in 1M-sodium hydroxide) of 1.7S and 4.6S respectively. 3. Evidence from periodate oxidation, methylation analysis, i.r. spectroscopy and partial acid hydrolysis showed that fraction P consisted of linear chains of α -(1 $\rightarrow$ 3)-glucopyranose units with blocks of one or two α -(1 $\rightarrow$ 4)-glucopyranose units interspersed at intervals along the main chain. The 2S polysaccharide, from which fraction P is derived, evidently also contains longer blocks of α -(1 $\rightarrow$ 4)-glucopyranose units, that are susceptible to α -amylase action. 4. Fraction Q consisted of glucose (88%) with small amounts of galactose, mannose and rhamnose. Evidence from digestion with exo- and endo- β -(1 $\rightarrow$ 3)-glucanases, periodate oxidation and methylation analysis suggests that fraction Q consists of a branched galactomannorhamnan core, to which is attached a β -(1 $\rightarrow$ 3)-, β -(1 $\rightarrow$ 6)-glucan. In the cell wall, chains of α -(1 $\rightarrow$ 4)-linked glucopyranose units are linked to fraction Q to form the 6S component of fraction BB.

In the preceding paper (Buck & Obaidah, 1971) a highly dextrorotatory polysaccharide fraction ($[\alpha]_D + 236^\circ$ in 1M-sodium hydroxide) was isolated from cell walls of *Fusicoccum amygdali*. Analytical ultracentrifugation revealed that this fraction contained two components, with s_{20}^0 values (in 1M-sodium hydroxide) of 2S and 6S respectively. The characterization of these two components is described in the present paper.

MATERIALS AND METHODS

Preparation of cell-wall fraction BB. This was done as described in the preceding paper (Buck & Obaidah, 1971).

Paper chromatography. This was done on Whatman no. 1 paper by downward irrigation with the following solvents: A, butan-1-ol-pyridine-water-benzene (5:3:3:1, by vol.); B, propan-1-ol-ethyl acetate-water (7:1:2, by vol.); C, butan-1-ol-pyridine-water (6:4:3, by vol.); D, propan-1-ol-ethyl acetate-water (6:1:3, by vol.); E, pyridine-ethyl acetate-water (2:5:7, by vol.).

Detecting reagents were: (a) AgNO_3 -NaOH (Trevelyan, Procter & Harrison, 1950); (b) aniline-hydrogen phthalate (Wilson, 1959). Oligosaccharides containing β -(1 $\rightarrow$ 3)-glucopyranose linkages were identified by a method based on that of Abdullah & French (1966). Paper chromatography was done in the first dimension with solvent A for 72 h. After drying, the paper was lightly sprayed with a solution containing 10 units of purified basidiomycete QM 806 exo- β -(1 $\rightarrow$ 3)-glucanase/ml in 5 mM-sodium acetate buffer, pH 4.8, and incubated at room temperature for 20 min. After drying the paper, chromatography was done in the second dimension with solvent A for 48 h. Spots on the diagonal are those resistant to the exo- β -(1 $\rightarrow$ 3)-glucanase, and other spots are the products of the exo-enzyme action.

Enzymes. Hog pancreatic α -amylase was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. and was assayed at 30°C as described by Robyt & Whelan (1968). Sweet-potato β -amylase was obtained from the Worthington Biochemical Corp., and assayed at 30°C by using the conditions described by Hobson, Whelan & Peat (1950). Basidiomycete QM 806 exo- β -(1 $\rightarrow$ 3)-glucanase was prepared and purified as described in the

preceding paper (Buck & Obaidah, 1971). *Aspergillus niger* amyloglucosidase was a gift from Dr B. E. Ryman. Pullulanase was prepared from *Aerobacter aerogenes* by the method of Bender & Wallenfels (1966) and had 5 units of pullulanase activity/mg when assayed as described by Abdullah *et al.* (1966). A freeze-dried preparation of *Helix pomatia* digestive enzymes was obtained from Industrie Biologique Francaise S.A., Gennevilliers, France. An endo- β -(1 $\rightarrow$ 3)-glucanase was isolated from this enzyme complex by the method of Noble & Sturgeon (1968) and had 10 units of β -(1 $\rightarrow$ 3)-glucanase activity/mg when assayed as described by Huotari, Nelson, Smith & Kirkwood (1968).

I.r. spectroscopy. I.r. spectra of samples, dispersed in KBr discs (Barker, Bourne & Whiffen, 1960), were measured with a Unicam SP.200G spectrophotometer.

Optical rotations. These were measured on 1% solutions of samples in 1M-NaOH in a 1 dm cell.

X-ray diffraction patterns. These were measured by Mr P. L. Bird, Analytical Services Laboratory, Imperial College. Samples were placed in Lindeman glass capillaries and diffraction patterns were obtained with a Philips Debye-Scherrer powder diffraction camera, by using nickel filtered Cu K α radiation ($\lambda = 0.15418$ nm) obtained with a Philips PW1310 X-ray generator, operating at 50 kV and 36 mA. The d spacings were calculated from Bragg's (1912) equation.

Analytical ultracentrifugation. This was done with a Beckman model E Analytical Ultracentrifuge by using the schlieren optical system. Centrifugation was done at 160 000g with solutions of samples in 1M-NaOH at 23°C, by using a 12 mm. Kel F 4° single-sector cell. Sedimentation coefficients were determined over a range of concentrations and s_{20}^0 values were obtained by extrapolation to zero concentration.

Analytical methods. These were as described in the preceding paper (Buck & Obaidah, 1971). In addition analysis of products, after periodate oxidation or methylation, was done, after 1M-H₂SO₄ hydrolysis, by g.l.c. of the alditol peracetates (Sawardeker, Sloneker & Jeanes, 1965; Björndal, Lindberg & Svensson, 1967a).

G.l.c. This was done with a Pye series 104 gas chromatograph, equipped with a flame ionization detector. Separations were made at a gas flow rate of 40 ml of N₂/min on a column (160 cm $\times$ 0.4 cm), containing 3% (w/w) of ECNSS-M on Gas Chrom Q (100–120 mesh). For separation of alditol peracetates of partially methylated sugars a constant temperature of 180°C was used. Separations of alditol peracetates derived from the products of periodate oxidation were done by using a temperature programme of 8°C/min from 100°C to 200°C. Quantitation was done by measuring peak areas, after calibration with standards.

Mass spectrometry. This was kindly done by Dr E. S. Waight, Department of Chemistry, Imperial College. Mass spectra of the peracetates of partially methylated alditols were obtained by using a Perkin-Elmer Model 270 combined gas chromatograph-mass spectrometer. Identifications were based on comparison with mass spectra of authentic compounds.

Methylation of polysaccharides. Both fractions *P* and *Q* were methylated in good yield by the methyl iodide-sodium hydride-dimethyl sulphoxide method of Anderson

& Cree (1966). The absence of absorption at 3500 cm⁻¹ indicated that methylation was complete.

Periodate oxidation and Smith degradation. Fractions *P* and *Q* (50 mg of each) were separately stirred with 25 mM-sodium metaperiodate (20 ml) at 4°C in the dark. At intervals samples were withdrawn for measurement of periodate reduction (Goldstein & Smith, 1958), formic acid production (Barker & Somers, 1966) and formaldehyde liberation (Hay, Lewis, Smith & Unrau, 1965).

After 240 h the mixtures were treated with an excess of ethylene glycol, dialysed exhaustively against water and reduced with sodium borohydride (50 mg) at room temperature for 24 h. Excess of borohydride was destroyed with 1M-acetic acid and the reduced oxopolysaccharide was dialysed exhaustively against water and freeze-dried.

A 4 mg portion of the reduced oxopolysaccharide was stirred with 0.05M-H₂SO₄ at room temperature for 24 h and the suspension was centrifuged (20 000g, 30 min). The residue was washed with water until free from acid and freeze-dried.

Exclusion chromatography. Solutions (0.5%, w/w) of cell-wall fractions or dextrans of known molecular weight in 0.5M-NaOH were passed individually under identical conditions through a column (1.4 cm $\times$ 29 cm) of Sephadex G-200 equilibrated and eluted with 0.5M-NaOH. Fractions (0.05 ml) were collected for total hexose determination. Sephadex and dextrans for calibration were obtained from Pharmacia (G.B.) Ltd., London W.13, U.K. Dextrans were reduced with NaBH₄, before dissolving them in 0.5M-NaOH.

RESULTS

Action of enzymes on cell-wall fraction BB. The two components of fraction *BB* could not be separated by fractional precipitation from a solution in 1M-sodium hydroxide or by chromatography on a column of Sephadex G-200; most of the fraction was excluded from the gel. Separations on agarose gels or silica gels, that allow separation of higher-molecular-weight substances, were not feasible because of the instability of these gels to the alkali required for solution of the polysaccharide fraction. The action of enzymes was therefore investigated to determine if selective action on one component could be obtained with the aim of fractionating the products of enzyme action.

(a) α -Amylase. Cell-wall fraction *BB* (100 mg) was incubated with hog pancreatic α -amylase (150 i.u.) in 5 ml of 0.02M-sodium phosphate buffer, pH 6.9, containing 6.7 mM-sodium chloride and erythritol (1.5 mg), at 37°C. After 3 h the fraction was no longer stained blue by iodine and the reaction was complete after 24 h, when 11.7% of the total hexose had been liberated. Examination of the supernatant by paper chromatography (solvent *A*) revealed sugars with the mobilities of glucose, maltose and maltotriose. The identities of the

oligosaccharides were confirmed after elution from the chromatogram by incubating samples separately with β -amylase and emulsin and rechromatographing them in solvents *A* and *B*. The sugar corresponding to maltose was unaffected by emulsin or β -amylase, whereas that corresponding to maltotriose failed to react with emulsin, but gave maltose and glucose on treatment with β -amylase.

The residue after 24h incubation had $[\alpha]_D + 239^\circ$ and analytical centrifugation revealed mainly one component with a sedimentation rate similar to that of the original 2S component, although with increased band width, and only a small amount of a faster sedimenting component.

(b) β -Amylase. Cell-wall fraction *BB* (20 mg) was digested with a β -amylase–buffer incubation mixture (2 ml) containing human serum albumin (1 mg), glutathione (0.3 mg), sodium citrate buffer, pH 5.0 (25 μ mol), erythritol (5 mmol) and β -amylase (1140 i.u.), at 37°C for 24h. The supernatant contained 2.5% of the cell-wall total hexose and examination by paper chromatography (solvent *A*) indicated that maltose was the only product. The residue was completely resistant to further action of β -amylase and still was stained dark blue by iodine.

(c) Pullulanase. To assess whether the α -(1 $\rightarrow$ 4)-glucan contained any α -(1 $\rightarrow$ 6)-D-glucopyranose branch points, as in amylopectin, cell-wall fraction *BB* (before and after separate treatments with α -amylase and β -amylase) (10 mg) was incubated with purified pullulanase (1 unit) in 0.02 M-citrate–phosphate buffer, pH 5.0, (2 ml) at 37°C for 24h. Examination of the supernatant after centrifugation (20000g, 30 min) for total hexose and by paper chromatography (solvent *A*) showed that no soluble sugar was liberated in any of the cases tested.

(d) Exo- β -D-(1 $\rightarrow$ 3)-glucanase. When cell-wall fraction *BB* was incubated with basidiomycete QM 806 exo- β -D-(1 $\rightarrow$ 3)-glucanase (10 units) in 0.05 M-sodium acetate, pH 4.8 (1 ml), at 37°C for 24h, 22.5% of the total hexose was liberated and glucose and gentiobiose were the only sugars detected in the supernatant by paper chromatography (solvent *A*). The residue, which was resistant to further action of the β -(1 $\rightarrow$ 3)-glucanase, had $[\alpha]_D + 279^\circ$ and still stained dark blue with iodine. Its i.r. spectrum had an absorption maximum at 850 cm^{-1} (α -glucan) but none at 890 cm^{-1} (β -glucan). Examination by analytical ultracentrifugation showed the 2S component to be essentially unchanged, but the 6S component was completely absent.

Fractionation of the products of α -amylase action on cell-wall fraction BB. The residue, after treating fraction *BB* with α -amylase, was fractionated as described in Scheme 1. This procedure allowed the

isolation of a water soluble β -D-glucan, fraction *Q*, $[\alpha]_D + 16^\circ$ (11% of fraction *BB*) and an alkali-soluble water-insoluble α -D-glucan, fraction *P*, $[\alpha]_D + 290^\circ$ (67% of fraction *BB*).

Properties of fraction P. Fraction *P* was composed mainly of glucose with 0.2% galactose, a trace of mannose and 0.1% total amino acids. It appeared to contain only one component, with s_{20}^0 value 1.7S, when examined by analytical ultracentrifugation. Its elution profile from Sephadex G-200 (Fig. 1) indicated the main part of the fraction to have a $\bar{M}_w$ of approx. 100 000, with a proportion of material of lower molecular weight, probably not detected by the schlieren optical system of the analytical ultracentrifuge. Its i.r. spectrum, which had absorption maxima at 855 cm^{-1} and 930 cm^{-1} , but no maximum at 890 cm^{-1} , was almost identical with that of an *A. niger* cell-wall α -(1 $\rightarrow$ 3)-glucan (fraction IV R, Johnston, 1965a). However, its X-ray diffraction pattern (*d* spacings: 9.5s; 6.17m; 5.00w; 4.55s; 4.09vs; 3.75vw; 3.08vw; 2.76vw; 2.53vw; 2.34vw) showed some differences from the *A. niger* glucan (*d* spacings: 9.5s; 5.00m; 4.18vs; 3.63vw; 3.22vw; 2.91vw; 2.72w; 2.09vw; 1.99w) (abbreviations: s, strong; vs, very strong; m, medium; w, weak; vw, very weak).

Action of enzymes on fraction P. (a) Amyloglucosidase. Incubation of fraction *P* (10 mg) with *A. niger* amyloglucosidase (1.0 mg) in 0.04 M-sodium acetate buffer, pH 4.8 (1 ml), at 37°C for 24h did not liberate any glucose that could be detected by glucose oxidase or paper chromatography.

(b) *H. pomatia* enzymes. When fraction *P* (10 mg) was incubated with *H. pomatia* digestive enzymes (1 mg) in 0.01 M-citrate–phosphate buffer, pH 5.8, at 37°C for 24h, no sugars were detectable in the supernatant either by paper chromatography or with the Nelson–Somogyi reagent.

Partial acid hydrolysis of fraction P. Partial hydrolysis of fraction *P* was done essentially by the method of Johnston (1965b) by heating it with 98% formic acid at 100°C for 10 min, and then with 0.2 M-H₂SO₄ at 100°C for 40 min. After extraction of formic acid with ether and neutralization with Amberlite IR-4B (OH[−] form), paper chromatography (solvents *C* and *D*) indicated a homologous series of nigerodextrins up to nigerohexaose, tentatively identified by their R_{glucose} values and the linear relationship of log R_{glucose} and degree of polymerization. Maltose was also detected.

Methylation analysis of fraction P. Fraction *P* was methylated and the product was hydrolysed as described in the Materials and Methods section. The proportions of methylated sugars are given in Table 1. Only traces of dimethyl sugars were detected.

Periodate oxidation of fraction P. Fraction *P* reduced, per anhydrohexose unit, a limiting

Scheme 1. Fractionation of the products of α -amylase action on cell-wall fraction BB. Conditions for the enzyme incubations are as described in the text.

amount of 0.058mol of periodate with the liberation of 0.025mol of formic acid and 0.006mol of formaldehyde.

Smith degradation of fraction P. Fraction P was oxidized with an excess of sodium metaperiodate for 240h and then reduced with sodium borohydride. The reduced oxopolysaccharide contained glucose (95%), erythritol (2.6%), glycerol (0.7%) and traces of threitol and other unidentified components. This product was hydrolysed with 0.05M-

sulphuric acid for 24h at room temperature. Paper chromatography (solvent *E*) of the soluble product revealed mainly erythritol and glycerol. The insoluble product had $[\alpha]_D + 279^\circ$; examination by analytical ultracentrifugation revealed it to be more polydisperse and to have a lower average sedimentation rate (s_{20}^0 approx. 1.2S) than fraction P. Supporting evidence for the lower average molecular weight and increased polydispersity was provided by chromatography on Sephadex G-200 (Fig. 1).

Properties of fraction Q. Fraction Q was composed of glucose (88.4%), galactose (4.4%), small amounts of mannose and rhamnose, traces of arabinose and xylose and a total of 0.2% amino acids. It had $[\alpha]_D^{16}$ and revealed only one component with an s_{20}^0 of 4.6S (in 1M-sodium hydroxide) when examined by ultracentrifugation; it was almost completely excluded from Sephadex G-200, indicating a mol.wt. above about 200 000. Its i.r. spectrum, with an absorption maximum at 890cm^{-1} , but none at 850cm^{-1} , was almost identical with that of laminarin and indicated a majority of β -(1 $\rightarrow$ 3)-linkages (Michell, 1970). It gave a very diffuse X-ray diffraction pattern, indicating a low degree of crystallinity.

Action of enzymes on fraction Q. (a) Exo- β -D-(1 $\rightarrow$ 3)-glucanase. Fraction Q (5mg) was incubated with Basidiomycete QM 806 exo- β -(1 $\rightarrow$ 3)-glucanase (5 units) in 0.05M-sodium acetate buffer, pH 4.8 (1 ml), containing γ -gluconolactone (0.5 mg), at 37°C for 24 h. Examination of the incubation mixture by

paper chromatography (solvent A) revealed the presence of only glucose and gentiobiose. The free glucose that was liberated accounted for 88% of the glucose in fraction Q.

(b) *H. pomatia* endo- β -(1 $\rightarrow$ 3)-glucanase. Fraction Q (3.5mg) was incubated with purified endo- β -D-(1 $\rightarrow$ 3)-glucanase (0.1 unit) at 37°C for 16 h. After centrifugation the supernatant was deionized with a 1:1 mixture of Amberlite IR-120 (H^+ form) and Amberlite IR-4B (OH^- form) resins. The oligosaccharides formed were tentatively identified by a technique that involved chromatography in one direction, spraying the oligosaccharide zones with QM 806 exo β -(1 $\rightarrow$ 3)-glucanase and then chromatography at right angles to the first direction as described in the Materials and Methods section. The results indicated that the products of action of the *H. pomatia* endo- β -(1 $\rightarrow$ 3)-glucanase on fraction Q include gentiobiose, laminaribiose, laminaritriose, laminaritetraose, laminaripentaose, laminarihexaose and laminariheptaose. In addition an oligomer (R_{glucose} 0.33, solvent A) of unidentified structure, which appeared to be resistant to exo- β -(1 $\rightarrow$ 3)-glucanase action, was formed.

Methylation analysis of fraction Q. Fraction Q was methylated and the product was hydrolysed as described in the Materials and Methods section. The proportions of partially methylated sugars are given in Table 2.

Periodate oxidation of fraction Q. Fraction Q reduced, per anhydrohexose unit, a limiting amount of 0.18 mol of periodate with the liberation of 0.065 mol of formic acid and 0.018 mol of formaldehyde.

Smith degradation of fraction Q. Fraction Q was oxidized with an excess of sodium metaperiodate for 240 h and then reduced with sodium borohydride. The composition of the water insoluble product is given in Table 3. This product was hydrolysed with 0.05M- H_2SO_4 for 24 h. Paper chromatography of the supernatant (solvent E) revealed glycerol, erythritol and threitol. The composition of the residue is given in Table 3.

Fig. 1. Elution profiles on Sephadex G-200 gel. ●, Fraction P; ■, fraction P after Smith degradation.

Table 1. *Methylation analysis of fraction P*

Products were identified by mass spectrometry (M) and/or the relative retention time (T) on g.l.c. of their alditol peracetate derivatives in comparison with authentic standards as described by Björndal, Lindberg & Svensson (1967a,b).

Product	Molar ratio	Relative retention time (g.l.c.)	Method of identification
Alditol peracetate derivative of			
2,3,4,6-Me ₄ Glc	1.0	1.0	M, T
2,4,6-Me ₃ Glc	224	1.9	M, T
2,3,6-Me ₃ Glc and/or 2,3,4-Me ₃ Glc	6.0	6.1	T

Table 2. *Methylation analysis of fraction Q*

For details see Table 1.

Product Alditol peracetate derivative of	Molar ratio	Relative retention time (g.l.c.)	Method of identification
2,3,4,6-Me ₄ Glc	1.00	1.00	M, T
2,3,5,6-Me ₄ Gal or 2,3,4,6-Me ₄ Gal	0.24	1.12	T
2,4,6-Me ₃ Glc	9.20	1.90	M, T
2,3,6-Me ₃ Glc (and/or Gal) or 2,3,4-Me ₃ Glc (and/or Man)	1.05	2.30	T
Unidentified Me ₂ -hexose	0.27	3.02	T
2,6-Me ₂ Gal	0.22	3.62	T
Unidentified Me ₂ -hexose	0.32	4.70	T
2,4-Me ₂ Glc or 2,3-Me ₂ Glc or 3,4-Me ₂ Glc	0.27	5.30	T

Table 3. *Composition of fractions obtained by Smith degradation of fraction Q*

Relative retention times of the alditol peracetate derivatives in g.l.c. were: glycerol, 1.00; erythritol, 1.37; threitol, 1.45; Ara, 1.78; Xyl, 1.99; Glc, 2.88.

	Molar %					
	Glc	Ara	Xyl	Threitol	Erythritol	Glycerol
Reduced oxopolysaccharide	90	1	Trace	3	2	5
Residue after Smith degradation	95	1	Trace	Trace	Trace	1

DISCUSSION

The major (2S) component of fraction *BB* appeared to be slightly degraded by α -amylase action as evidenced by a lowered sedimentation coefficient and increased band-width on analytical ultracentrifugation. By fractionation of the products of α -amylase action on fraction *BB* it was possible to isolate a glucan, fraction *P*, which comprised 70% of fraction *BB* and sedimented as a single boundary (s_{20}^0 1.7S). The high optical rotation of fraction *P* indicated it to be an α -glucan and the detection of a homologous series of nigerodextrins after partial acid hydrolysis indicated that α -(1 $\rightarrow$ 3)-glucopyranose linkages predominated. Maltose was also found, indicating a small number of α -(1 $\rightarrow$ 4)-glucopyranose linkages. Methylation analysis of fraction *P* indicated it to be a linear glucan, composed of 97.6% (1 $\rightarrow$ 3)-pyranose linkages with 2.4% of (1 $\rightarrow$ 4)- and/or (1 $\rightarrow$ 6)-pyranose linkages, with an average chain length of 230 residues.

The small amount of periodate reduced by fraction *P* and the high glucose content of the reduced oxopolysaccharide is supporting evidence for a majority of (1 $\rightarrow$ 3)-pyranose linkages. The small amount of formaldehyde formed probably arose from the glycol end groups formed when the cell walls were treated with borohydride before

fractionation. The amount of formic acid produced (1mol/45 anhydrohexose units) is higher than expected for the non-reducing end groups and possible (1 $\rightarrow$ 6)-linkages on the basis of methylation analysis and the glycerol content of the reduced oxopolysaccharide; it probably arose from oxidation of the glycol end groups, giving a dialdehyde structure that would be susceptible to over-oxidation (Hough and Perry, 1956).

The reduced oxopolysaccharide contained 2.4% of erythritol, confirming the presence of (1 $\rightarrow$ 4)-pyranose linkages. This value is the same that obtained for the combined (1 $\rightarrow$ 4)- and (1 $\rightarrow$ 6)-linkages by methylation analysis, suggesting the absence of significant amounts of (1 $\rightarrow$ 6)-linkages. Some of the erythritol was released after selective acid hydrolysis, indicating that blocks of at least two (1 $\rightarrow$ 4)-linkages occurred. Long blocks of (1 $\rightarrow$ 4)-linkages are considered unlikely because the glucan was resistant to α -amylase. The lowering of average molecular weight and increase of polydispersity after periodate oxidation indicated that most of the α -(1 $\rightarrow$ 4)-linkages were arranged at internal positions in the chain and not all at the reducing end, as has been suggested for pseudonigeran (Hasegawa, Nordin & Kirkwood, 1969).

A linear α -(1 $\rightarrow$ 3)-glucan containing about 10% α -(1 $\rightarrow$ 4)-linkages, was isolated from cell walls of *A. niger* (Fraction IV R, Johnston 1965a). Dr I. R.

Johnston has kindly provided us with a sample of this glucan and we have found its infrared spectrum to be almost identical with that of the *F. amygdali* α -(1 $\rightarrow$ 3)-glucan. However, marked differences were found in the X-ray diffraction patterns of the two glucans. It is probable that the difference between the two glucans lies in the different proportions and relative positions of the α -(1 $\rightarrow$ 3)- and α -(1 $\rightarrow$ 4)-glucopyranose linkages. Evidently the 2S polysaccharide, from which fraction *P* is derived, additionally contains longer blocks of α -(1 $\rightarrow$ 4)-glucopyranose units, which are susceptible to α -amylase action.

The 6S component of fraction *BB* was attacked by both α -amylase and exo- β -(1 $\rightarrow$ 3)-glucanase, which is evidence that it contained both α -(1 $\rightarrow$ 4)- and β -(1 $\rightarrow$ 3)-glucopyranose linkages. By fractionation of the products of α -amylase action on fraction *BB* it was possible to obtain a water-soluble glucan, fraction *Q*, which appeared homogeneous (s_{20}^0 4.6S) on analytical centrifugation. The low optical rotation and similarity of its infrared spectrum to that of laminarin, suggested that fraction *Q* contained mainly β -linkages.

Fraction *Q* was attacked by a purified *H. pomatia* endo- β -(1 $\rightarrow$ 3)-glucanase, giving, among other materials, gentiobiose and a series of oligomers that were tentatively identified as laminaridextrins from laminaribiose to laminariheptaose, suggesting that the glucan consisted of chains of β -(1 $\rightarrow$ 3)-linked glucopyranose residues with small numbers of β -(1 $\rightarrow$ 6)-linkages. Further structural information on fraction *Q* was obtained from the action of basidiomycete QM 806 exo- β -(1 $\rightarrow$ 3)-glucanase. Nelson, Johnson, Jantzen & Kirkwood (1969) found that this enzyme will remove a glucosyl residue that has a free C-3 hydroxyl group and that is attached to another glucosyl residue by a β -(1 $\rightarrow$ 3)-linkage. The exo-glucanase released most of the glucose from fraction *Q* as a mixture of glucose and gentiobiose; these were the only mono- and oligo-saccharides detectable. This confirmed the presence of β -(1 $\rightarrow$ 3)-linkages and indicated that the β -(1 $\rightarrow$ 6)-linkages occur singly and not in blocks of two or more units.

The small amount of periodate reduced by fraction *Q* and the large amount of glucose in the reduced oxopolysaccharide is consistent with the presence of (1 $\rightarrow$ 3)-pyranose linkages. The amount of formic acid liberated indicates one non-reducing end group or (1 $\rightarrow$ 6)-pyranose linkage in every 15 residues. The formaldehyde liberated is in excess of that expected from the reducing end group of a polymer of mol.wt. greater than 200 000 and may have arisen from cleavage of furanose residues. Cleavage of (1 $\rightarrow$ 2)- or (1 $\rightarrow$ 3)-linked galactofuranose residues would give rise to arabinose; evidence for this structural feature was provided by the detection

of 1.3 mol of arabinose/100 mol in the reduced oxopolysaccharide (only a trace of arabinose was present originally in fraction *Q*).

The isolation of 2,4,6-tri-*O*-methylglucose as a major component after methylation and hydrolysis confirmed the importance of (1 $\rightarrow$ 3)-glucopyranose links in the structure of fraction *Q*. The minor trimethylhexose formed was probably a mixture of 2,3,6-tri-*O*-methylgalactose, 2,3,6-tri-*O*-methylglucose and 2,3,4-tri-*O*-methylglucose, since Smith degradation of fraction *Q* gave rise to small amounts of threitol and erythritol [from (1 $\rightarrow$ 4)-linked galactopyranose and glucopyranose or mannopyranose residues]; the residue after Smith degradation contained a small amount of glycerol suggesting the existence of unbranched (1 $\rightarrow$ 6)-pyranose linkages. The high proportion of tetra-*O*-methylgalactose showed that about 40% of the galactose in fraction *Q* was present as non-reducing end groups. Barker, Basarab & Cruickshank (1967) found that in a galactomannan peptide isolated from *Trichophyton mentagrophytes*, all of the galactose was present as terminal galactofuranose residues attached to mannose residues in a highly branched structure.

The formation of four different dimethylhexoses indicated that fraction *Q* has a branched structure. As most of the glucose in fraction *Q* was released as glucose and gentiobiose by exo- β -(1 $\rightarrow$ 3)-glucanase the only branch involving significant amounts of glucose would be 1,3,6; however, the amount of 2,4-di-*O*-methyl-D-glucose formed was not more than a quarter of the amount of tetra-*O*-methyl-D-glucose. This suggests that the galactomannorhamnan moiety is branched with some chains terminated by glucopyranose residues; these non-reducing glucopyranose residues may well be 'stubs' of α -(1 $\rightarrow$ 4)-linked units remaining after α -amylase action on the 6S polysaccharide.

The above evidence suggests that the 6S polysaccharide consists of a branched galactomannorhamnan core to which are attached chains of α -(1 $\rightarrow$ 4)-linked glucopyranose residues and a branched β -(1 $\rightarrow$ 3)-, β -(1 $\rightarrow$ 6)-glucan. The nature of the attachment regions is not known and further study is required to establish if the phosphodiester and peptide linkages, shown in the preceding paper (Buck & Obaidah, 1971) to be associated with the heteroglycan fraction of the cell walls, are involved. The limited action of β -amylase on the α -(1 $\rightarrow$ 4)-glucopyranose chains suggests that these are terminated by other types of linkage.

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