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AQUILINAN, AN ACIDIC POLYSACCHARIDE FROM *PTERIDIUM AQUILINUM*

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Abstract—A water-soluble, acidic polysaccharide, aquilinan, was isolated from storage rhizomes and young fronds of bracken and shown to be homogeneous. The polysaccharide contained galactose, xylose, fucose and arabinose residues together with chains of repeating α(1-2')glucuronosylmannose units. A method of correcting the mannose content for degradation during the hydrolysis of the aldobiuronic acid is described.

INTRODUCTION

The parenchymatous tissues of the storage rhizomes and young fronds of bracken (*Pteridium aquilinum* (L.) Kuhn) contain an acidic polysaccharide which can be extracted with water [1]. Indeed, the liquid which exudes freely from these tissues on incision contains substantial amounts of this polysaccharide in solution. In this paper evidence for its homogeneity is presented, and its monosaccharide composition and the structure of the acidic portion are described. The neutral portion is complex in structure and awaits full characterization. The name "aquilinan" is proposed for the polysaccharide.

RESULTS

Homogeneity

The polysaccharide comprised on average 3·4% of the dry wt of the rhizomes, with only minor seasonal changes (±0·4%). Attempts to fractionate the intact polysaccharide by partial precipitation with ethanol, cetyl trimethyl ammonium bromide (CETAB) [1] and Ba(II) and Al(III) ions were unsuccessful. On electrophoresis in borate buffer the polysaccharide ran as a single spot with slight tailing towards the anode. Chromatography on Sepharose 4B revealed a single, somewhat diffuse peak starting at the exclusion volume, whereas the polysaccharide was wholly excluded from all grades of Sephadex up to and including G-200. Its molecular weight may therefore be as much as several million daltons, although in molecular-sieve chromatography the possibility of aggregation cannot be wholly discounted. From these results, the polysaccharide was considered substantially homogeneous.

Monosaccharide composition

The constituent neutral monosaccharides had been established as galactose, xylose, fucose, mannose, and arabinose. The presence of glucuronic acid was confirmed by PC. The rates at which the neutral sugars were released by acid hydrolysis are shown in Figs. 1 and 2. From the slow release of mannose it was suspected that this sugar was combined in an aldobiuronic acid, which in fact was later isolated. In these circumstances it is

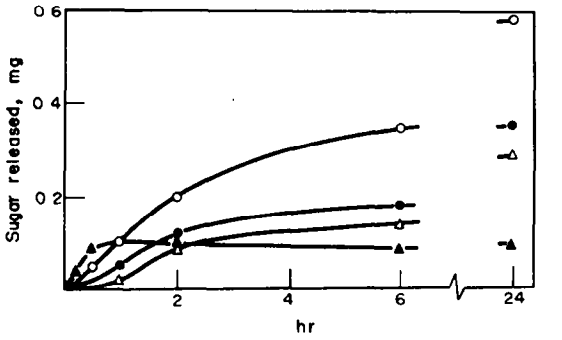

Fig. 1. Release of neutral monosaccharides with 0·5 M H_2SO_4. ○: Galactose, ●: fucose, △: xylose, ▲: arabinose, □: mannose.

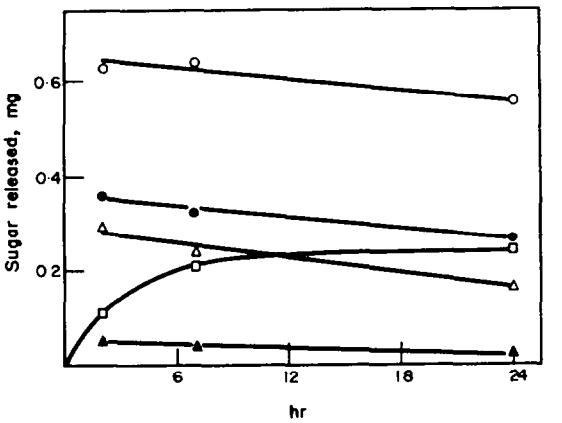

Fig. 2. Release of neutral monosaccharides with 0·005 M H_2SO_4.

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not possible to establish the proportion of the neutral sugar in the unmodified polysaccharide by normal means, as severe acid conditions are necessary for hydrolysis and much degradation takes place simultaneously [2]. The highest mannose content reached in the hydrolysate will therefore be substantially less than the amount of mannose originally present in the polymer. The following treatment of the results makes allowance for this degradation.

It is assumed, *inter alia*, that both hydrolysis of the aldobiuronic acid and degradation follow or approximate to first-order kinetics, and that the hydrolysis of the polysaccharide down to the aldobiuronic acid is much faster than the subsequent stages, then it can be shown that the rise and subsequent fall in free mannose in the hydrolysate are described by a function of the form:

$$M_t = \frac{k_H}{k_D - k_H} M_p(e^{-k_H t} - e^{-k_D t}) \qquad (1)$$

where M_t is the amount of free mannose present after time t, k_H and k_D are the rate constants for hydrolysis and degradation respectively, and M_p is the amount of mannose in the original polymer, which we wish to find. When M_t is plotted on a logarithmic scale this function rises to a maximum A and thereafter falls, soon approximating to a straight line with gradient $-k_D$ and intercept B when extrapolated to the M_t axis (see Fig. 3). It can be shown that

$$A = \frac{k_H}{k_H + k_D} M_p \quad \text{and} \quad B = \frac{k_H}{k_H - k_D} M_p$$

thus

$$M_p = \frac{2A}{1 + A/B}$$

The linearity of the later portion of the curve is a satisfactory test of first-order or pseudo first-order kinetics.

The above analysis holds good only if $k_H > k_D$, as appears to be the case here. In the special situation where $k_H = k_D$ equation (1) becomes meaningless. If $k_D > k_H$ a curve of the same shape results, approximating to a straight line of gradient $-k_H$ at large values of t and with:

$$B = \frac{k_H}{k_D + k_H} M_p \quad \text{as before but} \quad A = \frac{k_H}{k_D - k_H} M_p$$

A priori it is not in general possible to distinguish between these two situations when confronted with an experimental curve of this shape, but if there is any doubt an approximate value of k_H may be found from the initial rate of appearance of monosaccharide: this approximation is satisfactory unless k_H is much smaller than k_D.

It should be noted that the hydrolysis of resistant polysaccharides such as alginic acid cannot be handled in this way, since monosaccharides are not released from these polymers at the same rate as bonds are broken [3].

The molar ratios of the different monosaccharides in the polysaccharide were calculated to be as follows: galactose:fucose:xylose:mannose:glucuronic acid:arabinose = 7·5:4·7:4·3:3·1:2·7:1. These figures are averages from rhizomes collected at three different sampling dates. No significant seasonal differences were found.

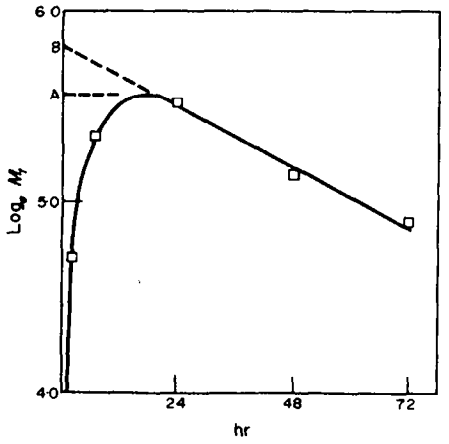

Fig. 3. Release of decomposition of mannose. Natural logarithm of free mannose content of hydrolysate.

Partial hydrolysis and isolation of oligosaccharides

Mild hydrolysis of the polysaccharide followed by preparative PC allowed one acidic and three neutral oligosaccharides to be isolated. Oligosaccharide A contained glucuronic acid and mannose, and co-chromatographed in three different solvents with authentic α-D-glucuronosyl-(1-2')-D-mannose. Oligosaccharide B contained galactose and fucose, oligosaccharide C galactose only, oligosaccharide D galactose and xylose. Slower-running neutral components were of complex composition.

Characterization of the acidic fraction

The polymeric residues from partial acid hydrolysis were also examined. Refluxing for 2 hr in 0·1 M HCl, for example, left *ca* 25% of the polysaccharide in a form precipitable with MeOH (3 vols) and more than half of this was excluded or partially excluded from Sephadex G-50. Fractions eluted in the range $V = 1{\cdot}3$–$1{\cdot}6$ V_t from a Sephadex G-50 column were pooled and hydrolysed. Their monosaccharide composition was found to be similar to that of a CETAB-precipitated fraction from the 2 hr/0·1 M HCl hydrolysis of the polysaccharide. In both cases, mannose and glucuronic acid were accompanied by galactose and xylose. Two cycles of hydrolysis and CETAB precipitation, however, yielded a residue which on further mild hydrolysis released oligosaccharide A, and on total hydrolysis released only mannose and glucuronic acid.

Since the amount of mannose in the intact polysaccharide was not significantly greater than the amount of glucuronic acid (relative error = 6% in each case), and since no detectable mannose was released by the 0·005 M H_2SO_4 hydrolysis which nevertheless cleaved hexose–hexose bonds in the neutral portion, it is likely that all the mannose and all the glucuronic acid can be accounted for by chains similar to the residue isolated (which presumably was depolymerised to some extent) in being based on the repeating unit α-glucuronosyl-(1-2')-mannose.

DISCUSSION

The structure of the neutral portion of the polysaccharide remains to be elucidated, although galactan chains are probably involved. The acidic portion which is attached to it (or to which neutral side-chains are attached) is similar to the interior chains of Leiocarpan A [4]. It is both taxonomically and biosynthetically interesting that the aldobiuronic acid which forms the repeating unit in these two glucuronomannans is also present in polysaccharides from plants of a number of unrelated genera [4–7], though, in at least one case, only as a disaccharide side-chain attached to the main framework [5]. When the presence of regular repeating units in a plant polysaccharide is demonstrated or suggested, it is often asserted that their polymerisation *in vivo* takes place by the same type of mechanism as is operative in bacteria [4,8,9]. However, we do not believe that the involvement of lipid intermediates can be taken for granted on the basis of polysaccharide structure alone.

One of us (H.J.D.) has characterized the nucleotide sugars extractable from bracken rhizomes, with results which have been reported elsewhere [10,11]: there is a close correspondence between the sugars in the nucleotide fraction and those incorporated in the rhizome polysaccharides, for example GDP-fucose and the large quantity of fucose in the polysaccharide considered here (0·7% of the dry weight of the rhizome). Mannose and glucuronic acid are present as GDP-mannose and UDP-glucuronic acid. It is possible to envisage the synthesis of the glucuronomannan chains as a specific alternate transfer of mannose and glucuronic acid directly onto the non-reducing chain end, the specificity being derived from the physico-chemical properties of the donor and the end of the chain: mannose is neutral and probably donated from GDP, glucuronate negatively charged and donated from UDP. The same basis for specificity could of course operate in a lipid-mediated polymerisation mechanism, which remains entirely possible.

The role of the polysaccharide within the bracken plant is not clear. From the lack of seasonal variation in the amount present it is not likely to be involved in energy storage, and it accounts for just under half of the 8–9% of the 'reserve' carbohydrate fraction, in Williams and Foley's analysis [12], which is unavailable for metabolism. If, as seems likely, the polysaccharide is associated with cell walls bordering the intercellular air spaces which are a conspicuous feature of rhizome parenchyma [13], then its hydrophilic character must assist the plant in resisting drought [14]; and the non-degradable glucuronomannan chains may be involved in disease resistance [15].

EXPERIMENTAL

Plant material. Bracken rhizomes were collected at Drumclog Muir, Dunbartonshire (Grid Ref. NS 552757), and were not normally divided into storage, intermediate and frond-bearing classes before extraction; other studies showed that the polysaccharide could be extracted only in small quantities from the frond-bearing rhizomes. Rhizomes from other sites have been used on occasion as source material, as have the petioles and raches of fronds at an early stage of development (within 3 weeks of emergence).

Extraction. Rhizomes (100 g) were homogenised successively in: (a) MeOH (400 ml) for 4 min at room temp; (b) MeOH (200 ml) for 1 min at room temp; (c) H_2O (200 ml) for 3 min, starting at 32° and finishing at 38°–40°; and (d) H_2O (150 ml) for 1 min at room temp. Homogenates were strained through 2 layers of muslin. Combined MeOH extracts were left for 30 min to settle and the sediment added to the combined aq. extracts. After chilling to 4°, TCA (2 g) was added to precipitate proteins (and with them phenolic substances) [16], and the extracts were centrifuged for 40 min at 13000 *g* and 4°. The polysaccharide was pptd with 3 vols of MeOH containing LiCl (0·2% w/v), redissolved in H_2O and repptd with MeOH. The sticky white ppt was air-dried at 30° or freeze-dried from a little water. Yield: 0·3–0·4 g. This procedure avoided co-extraction of starch from the rhizomes, and was scaled up or down as necessary.

PC. PC was carried out as described previously [17] in the following solvents: A, EtOAc–C_5H_5N–H_2O (12:5:4), running time *ca* 16 hr for monosaccharides and 2 days for oligosaccharides. B, EtOAc–HOAc–HCOOH–H_2O (18:3:1:4) running time *ca* 24 hr. C, EtOAc–HOAc–C_5H_5N–H_2O (50:12:18:10), running time *ca* 36 hr for neutral monosaccharides and 2–3 days for oligosaccharides and uronic acids. (All proportions are by vol.) Detection was by alkaline silver oxide [17], or aniline oxalate used quantitatively in the manner described by Wilson [18]. Whatman No. 1 paper was used throughout, preparative work included. H_2SO_4 hydrolysates were routinely neutralized to pH 5 with $Ba(OH)_2$ prior to chromatography.

Electrophoresis. Electrophoresis on glass-fibre paper, in 0·05 M sodium tetraborate, pH 9·2 containing EDTA (0·005 M) was used [19].

Molecular-sieve chromatography. The polysaccharide was dyed with Procion MX3G (I.C.I. Ltd.) prior to chromatography [20], and was chromatographed within 2 weeks [21]. The column effluent was monitored continuously at 254 nm (the extinction coefficient of the free or bound dye at this wavelength is *ca* 2× its maximum in the visible) using a commercial detection system adapted by the inclusion of a purpose-built ×10 amplifier. The stability, sensitivity and low dead volume of this made it ideal for polysaccharide work. The eluting solvent was normally H_2O, at 0·2 to 0·8 ml/min. NaCl (1 M) was sometimes used with identical results.

Attempted fractionation with metal ions. The polysaccharide was not pptd from H_2O by $Ba(OH)_2$ (0·05 or 0·15 M) [22]. It pptd in the presence of $AlCl_3$ (0·2 M) as the pH was raised progressively above 3·5, but fractional precipitation in this way revealed no significant difference in monosaccharide composition between the fraction precipitated and that in solution. EtOH- and CETAB-fractionation studies were carried out earlier in this laboratory by Morton [1].

Fragmentation analysis. The polysaccharide (*ca* 2 mg/ml in H_2O) was hydrolysed with 0·005 M and 0·05 M H_2SO_4 at 100° and the release of monosaccharides followed over up to 72 hr. A second series of hydrolyses, with 0·01 M HCl at 100°, was used to isolate oligosaccharides. A complex mixture of oligomeric components was obtained after 1 hr hydrolysis by neutralizing to pH 5 with KOH and adsorption on a 1 cm thick pad of activated charcoal (BDH Ltd.)-celite (1:1 w/w) sandwiched betwen layers of celite on a 8 cm sintered glass filter. Residual polymeric material, salt, and monosaccharides (with the exception of fucose) were eluted with water and the oligosaccharides desorbed with 40% EtOH. The eluate was brought to pH 7 before evaporation to *ca* 2 ml vol at < 30°. The acidic fraction was separated from the neutral by retention on a small column of anion-exchange resin (AG1-X4) 200–400 mesh, acetate form, 4 × 10 mm) in a Pasteur pipette. Neutral oligosaccharides were eluted with H_2O and the aldobiuronic acid with 10% HOAc. Monosaccharide composition of oligomeric and polymeric fragments was established by micro-scale hydrolysis of *ca* 100 μl of 1 to 2 mg/ml soln with HCl (0·025 M) in a sealed capillary tube for 16 hr at 100°. Hydrolysates were neutralized with a drop of C_5H_5N prior to PC. *Anogeissus leiocarpus* gum, repptd in EtOH, was the starting material for isolation of authentic α-D-glucuronosyl(1-2′)-D-mannose, using the same methods as described for the isolation of aldobiuronic acid from the bracken polysaccharide.

The components of the gums acidic fraction (which also contained a galactose-containing aldobiuronic acid assumed to be β-D-glucuronosyl-(1-6')-D-galactose [4]) were separated by preparative PC. Hydrolysis with 0·1 M HCl was used for the examination of the polymeric residues. 50 ml of 5 mg/ml polysaccharide soln was hydrolysed for 2 hr under these conditions, and neutralized with $AgCO_3$. The polymeric fraction was pptd with EtOH (3 vols) and redissolved in H_2O (5 ml). An aliquot (1 ml) was chromatographed on a 30 × 1·5 cm column of Sephadex G-50, eluting with H_2O and collecting fractions (3·0 ml) which were assayed by $PhOH-H_2SO_4$ [23]. To the remainder was added 1 ml of 5% CETAB. The resulting complex was dissolved in 0·1 M HCl and reprecipitated with EtOH (4 vols). A further batch of polysaccharide (100 mg) was hydrolysed with 0·1 M HCl at 100° for 6 hr, neutralized, pptd with EtOH, redissolved and treated with CETAB in the same way. The pptd acidic portion was redissolved in 0·1 M HCl and subjected to a further cycle of hydrolysis, neutralization, EtOH and CETAB precipitation, and the purified glucuronomannan redissolved in 0·1 M HCl and pptd with EtOH (3 vols).

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