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The gum exudate of *Encephalartos friderici-guilielmi*

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ABSTRACT

Studies of the structure of the polysaccharide gum exudate from *Encephalartos friderici-guilielmi* (a Cycad) confirmed its resemblance to those from other species of the same genus. Partial acid hydrolysis gave numerous products which showed a strong correlation with those from *E. longifolius* gum. The course of sequential Smith degradations was monitored by analysis of the molecular weights and composition of the products. The mild acid-hydrolysis step of the Smith degradation proceeded in two stages, the second being very slow but yielding a $(1 \rightarrow 3)$-D-galactan with $< 20\%$ of other residues (D-mannose, and some L-arabinose and L-rhamnose). A tentative formulation of the gum shows L-rhamnose and D-glucuronic acid residues exterior to a branched D-galactan framework; these structures are 3-linked to D-mannose, through D-galactopyranosyl or possibly L-arabinopyranosyl residues, within mannoglucuronoglycan chains. Other units occupy O-4 of D-mannose.

INTRODUCTION

The structure of the gum exudate of *Encephalartos longifolius* has been discussed[1] and comparisons made of the proportions of sugar units found after hydrolysis of the gums of several *Encephalartos*[2,3] species. The exudate from cones of *E. friderici-guilielmi* has been investigated[3] (partial acid hydrolysis, methylation analysis, and sequential Smith degradations) in order to confirm and relate the main molecular features to those of *E. longifolius* gum. A mannose-free fraction, isolated from the exudate of *E. friderici-guilielmi*, consisted[4] of a $(1 \rightarrow 3)$-β-D-galactan with β-D-GlcA 6-linked to 75% of the D-Gal units and terminal α-L-Rha*p* 4-linked to each GlcA. Terminal α-L-Ara*f*, 6-linked to D-Gal*p*, constituted a further mode of substitution.

EXPERIMENTAL

General methods.—Solvent systems used in PC and TLC, and the procedures for partial acid hydrolysis and methylation analysis by GLC–MS, were as de-

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scribed[1]. Smith degradations were performed using the procedures applied to *Grevillea robusta* gum[5].

Isolation and partial hydrolysis of the gum.—The gum exuded from cones of *Encephalartos friderici-guilielmi* was collected as hard, dried nodules from old plants, and as a soft exudate from trees following heavy rain. The gum was dissolved in water, with sonication if necessary, the solution was freeze-dried, and the residue was extracted exhaustively with EtOH. When an aqueous solution of the EtOH-insoluble polysaccharide at pH 2 (H_2SO_4) was heated at 100°C, the rate of initial rise in $[\alpha]_D$ from −31° decreased after 4 h but, after 7 h, there was a further slow, steady increase. After 10 h ($[\alpha]_D$ −5°), the EtOH-insoluble product *A* was collected and analysed for molecular-weight range, and the soluble portion was examined by PC.

Carboxyl reduction.—A sample of gum (790 mg) was carboxyl-reduced (twice)[6]. The product (80%) was fractionated into water-soluble ($[\alpha]_D$ −8°; 19%) and insoluble portions, which were analysed for sugar composition and used for Smith degradation, methylation analysis, and acetolysis.

Sugar analyses.—Proportions of neutral sugars in hydrolysates (2 M trifluoroacetic acid at 100°C, for 8 or 18 h, depending on the presence or absence of uronic acid) were determined by GLC of the derived alditol acetates or aldononitrile acetates. Smith-degradation products were hydrolysed in M CF_3CO_2H at 100°C for 14 h. Uronic acid was determined colorimetrically by the method of Blumenkrantz and Asboe-Hansen[7].

Methylation analyses.—Purified polysaccharides isolated from the native gum were methylated by the Haworth procedure[8] after the addition of a little $NaBH_4$. Portions of aq 50% NaOH and methyl sulphate were added dropwise during periods of 30 min, with vigorous stirring on day 1 under N_2 at 0°C and thereafter at room temperature for 8–10 days. The mixture was then dialysed against tap water and the retentate was freeze-dried.

After decationisation [Amberlite IR-120 (H^+) resin], the degradation products were methylated under Ar, using potassium methylsulphinylmethanide (potassium dimsyl, 2–3 M in Me_2SO) and excess of MeI[9,10]. The product was methylated repeatedly by the Purdie–Irvine procedure[11]. Completeness of methylation was checked by IR spectroscopy of a solution of the product in $CHCl_3$ and by hydrolysis followed by PC (solvent *c*). The product was washed through a column of Merckogel 2000 (Merckogel 20000 if of higher molecular weight) with 1:2 $CHCl_3$–EtOH, and the fractions were monitored using the anthrone reagent[12].

The methyl-esterified carboxylate groups were reduced[13] using $LiAlD_4$ in THF at 60°C for 18 h. The excess of $LiAlD_4$ was decomposed using moist EtOAc, aq tartaric acid was used to decompose any aluminium–polysaccharide complex, and the product was extracted into $CHCl_3$. The methylated products were analysed after hydrolysis.

Base-catalysed degradation.—The β-elimination degradations were performed as follows.

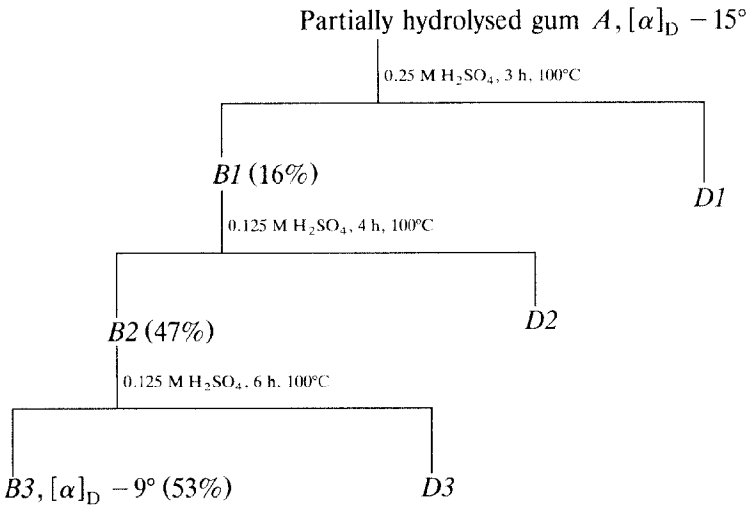
(*a*) *Using potassium dimsyl*[14] *as catalyst.* A solution of the methylated polysaccharide (5–10 mg) containing methyl glucuronate in Me_2SO was stirred with an excess of potassium dimsyl under Ar for 18 h. The product was methylated with an excess of MeI or CD_3I and analysed as for Hakomori methylations.

(*b*) *Using a non-nucleophilic base (Hünig's base or DBU) as catalyst.* A solution of the methylated uronic acid-containing polysaccharide (8–12 mg) in dry toluene (1 mL) was purged with Ar and cooled (ice–water), and Ac_2O (0.7 mL) and either DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) or Hünig's base (*N*,*N*-diisopropylethylamine; 1 mL) were added[15]. Heat was evolved and the solution became green. The tube was sealed and the reaction was allowed to proceed at 100 or 110°C for 24–48 h. The mixture was then washed with M HCl, and the material in the toluene layer was monitored by hydrolysis and PC for completeness of degradation of the uronic ester units. Thereafter, the toluene solution was dried ($MgSO_4$), the residue was saponified (NaOMe) with simultaneous borodeuteride-reduction ($NaBD_4$), and the product was hydrolysed with aqueous 10% HOAc at 100°C for 1 h in order to remove the degraded acid residues preferentially. The mixture was freeze-dried and the residue was deuteriomethylated, prior to complete hydrolysis and conversion of the products into alditol acetates for methylation analysis.

Acetolysis of the carboxyl-reduced gum.—The insoluble portion of reduced gum was acetolysed[16] in 16:19:3:2 HOAc–Ac_2O-concd H_2SO_4–H_2O at 100°C for 15 min. The product was *O*-deacetylated and the resulting mono- and oligo-saccharides were separated on a short column of charcoal–Celite by elution with aq EtOH.

Sequential hydrolyses of partly degraded gum A.—A solution of *A* in 0.25 M H_2SO_4 was heated at 100°C, then as shown in Scheme 1. Each hydrolysate was neutralised and dialysed (SpectraPor tubing; mol-wt cut off, 3500), the diffusates being monitored by PC before proceeding with the hydrolysis. *B1–B3* were analysed for sugar composition. *B2* and *B3* were submitted to methylation analysis (including carboxyl reduction, hydrolysis, and GLC), and to base-catalysed degradation of their methylated derivatives. *B3* was examined by steric-exclusion chromatography, ^1H NMR spectroscopy, and partial hydrolysis with acid.

Smith-degradation experiments.—The native gum (870 mg) was treated at room temperature with 0.1 M $NaIO_4$ (8.4 mmol) for 5 days, then with barium acetate (1.2 g). The mixture was centrifuged, $NaBH_4$ (1.2 g) was added to the supernatant solution, and, after several days, borate was removed conventionally with acidified MeOH. A solution of the product in M CF_3CO_2H was stored for 5 days at room temperature, then one-half was freeze-dried, and the residue was extracted with 1:1 MeOH–Me_2CO. The soluble (SS) and insoluble (IS) fractions were subjected to methylation analysis and assayed for bound glycolaldehyde and uronic acid. The acid-hydrolysis step was continued on the remaining half and the mol-wt distribution of the products was monitored at intervals. After 73 days, the solution was warmed to 45°C and kept thereat until day 88. The carbohydrate product was extracted with 2-propanol, and the soluble and insoluble fractions were analysed.



Scheme 1. Products of partial, acid hydrolysis of *Encephalartos friderici-guilielmi A*; diffusates *D1–D3* and degraded polysaccharides *B1–B3* (yields in parentheses).

In an analogous manner, partially hydrolysed gum *A* was degraded to give first- (after 18 days in M CF_3CO_2H) and second-limit (after 88 days) products. The carboxyl-reduced gum was oxidised, reduced with $NaBH_4$, and hydrolysed in M CF_3CO_2H at room temperature for 7 days. The product was fractionated on a column of Trisacryl GF05 by elution with pyridinium acetate (pH 5.0), to give the mol-wt distributions of the carbohydrate components.

RESULTS AND DISCUSSION

The composition of the exudate from *Encephalartos friderici-guilielmi* (3MeRha, < 1; Rha, 30; Fuc, < 1; Ara, 7; Xyl, 2; Man, 4; Gal, 32; GlcA + 4MeGlcA, 24) differs in the higher Rha content and significantly lower Man content, but otherwise resembles that of *E. longifolius* gum[1]. The $[\alpha]_D$ values were similar (−31° and −28°, respectively). The modes of linkage of the individual units are identical (Table I) with those from *E. longifolius* gum. Base-catalysed β-elimination experiments showed that GlcA was largely 6-linked to Gal with 50% of the Gal being further 3-substituted. Little Man survived, GlcA being both exterior and interior to Man in the original polysaccharide. Much of the Rha exterior to GlcA was destroyed, but terminal L-Ara*f* was detected.

During the "autohydrolysis" process to give *A* (72%), > 50% of the Rha and Ara and minor amounts of Gal and GlcA were lost, the GlcA content rose from 24 to 29%, and the mean molecular weight fell (from $> 2 \times 10^6$ to 60 000). Methylation analysis of *A* indicated that most of the Rha*p* that remained was terminal, Ara was pyranoid, the proportion of T-GlcA had more than doubled, that of 4-linked GlcA had decreased, and that of 6-linked Gal had increased as that of

TABLE I

Molar compositions (by methylation analysis) of *E. friderici-guilielmi* gum and partial-hydrolysis product *A*

Polysaccharide	Gum [a]	*A* [a]
Modes of linkage		
T-Rha*p* [b]	21	10
→ 4)-Rha [c]	5	2
T-Ara*p*	3	2
→ 3)-Ara*p*	3	1.5
T-Gal*p*	–	1.5
→ 3)-Gal*p*	4	4
→ 6)-Gal*p*	8	11
→ 3,6)-Gal*p*	23	15
→ 3,4,6-Gal	2	tr
→ 2,3)-Man*p*	1	2 [d]
→ 2,3,4)-Man	3	4
T-Glc*p*A	5	12
→ 4)-GlcA	20	9

[a] Quantities expressed as mol% for gum and per 72 units for *A*. [b] Includes some T-Ara*f*. [c] → 2)-Rha*p* and → 3)-Rha*p*, trace amounts. [d] Includes → 2)- and → 2,4)-Man.

3,6-linked Gal decreased. Presumably, the acid-labile sugars had been detached from O-4 of GlcA and O-3 of Gal.

PC[1] of the diffusates *D1–D3*, obtained during partial hydrolysis of *A* (Scheme 1), revealed β-Gal-(1 → 3)-Gal and β-Gal-(1 → 3)-Ara in *D1*. The major acidic component was β-GlcA-(1 → 6)-Gal (**1**).

The molar proportions of Gal, Man, and uronic acid in the retentates *B1–B3* were 42, 22, and 36; 24, 31, and 42; and 7, 45, and 47; respectively. *B3* comprised components of $\overline{M}_w$ 2100 and 3500, and, in ^{1}H NMR spectroscopy, gave H-1 signals[1,17,18] for → 2)-α-Man-(1 → (δ 5.40, 5 H), → 2)-α,β-Man (δ 5.27 and 4.86, ~ 1 H), terminal β-GlcA-(1 → (δ 4.5), and → 4)-β-GlcA-(1 → (δ 4.49). Methylation analysis {methylated *B3* had $[\alpha]_D$ −18° ($CHCl_3$)} showed that 40% of the 2-linked Man residues were also 4-linked, and 50% of the GlcA was terminal. Most of the small proportion of Gal was 6-linked. Partial acid hydrolysis of *B3* yielded neutral and acidic oligosaccharides which, on comparison (PC) with the corresponding products of hydrolysis of *E. longifolius* gum[1], were identified as Man, Ara, β-GlcA-(1 → 2)-Man (**2**), **1**, and the dimer, trimer, and tetramer of **2** (linkages through O-4 of GlcA). The graph of log $[(1/R_{\text{Monomer}}) - 1]$ vs. number of units for the oligomers of **2** was linear. Methylation of *B2* furnished a product {$[\alpha]_D$ −23° ($CHCl_3$)} whose analysis showed modes of linkage for the Gal, Man, and GlcA components comparable to those found for *B3*, though additional → 3,6)-Gal and some → 3)-Gal were also present.

β-Elimination reactions with methylated *B2* and *B3*, using potassium dimsyl, caused the changes → 2)-Man ⟶ T-Man, → 6)-Gal ⟶ T-Gal, and → 3,6)-Gal ⟶ → 3)-Gal. The positions of CD_3 in the products were consis-

tent with GlcA being 2-linked to Man and 6-linked to Gal. The methylated Man residues were largely destroyed, which indicated that they were 4-linked to GlcA. Degradation experiments using DBU were more successful than those using Hünig's base (which brought about little change) but, although the pattern of results observed using potassium dimsyl was confirmed, they were inconclusive as to the proof of a Man to GlcA linkage.

Acetolysis of the carboxyl-reduced gum[6] afforded 5% of β-Gal-(1 → 3)-Gal and β-Gal-(1 → 3)-Ara, identified by PC[1] in three solvent systems, together with (probably) β-Glc-(1 → 6)-Gal (derived ultimately from units of **1**).

Methylation analysis of the gum indicated that Gal and Man in particular, together with 3-linked Ara*p*, would be protected from periodate oxidation. Periodate uptake by the gum was 5.8 g^{-1} (theory, 5.9). Treatment of the periodate-oxidised and borohydride-reduced gum with acid (M CF_3CO_2H, room temperature, 2 days) gave a polysaccharide of mol wt 35 000, which decreased after 4 days with the formation of a major product with $\overline{M}_w$ 7000 and minor components with $\overline{M}_w$ 3000, 4000, and 15 000 (analysed by GPC on Bio-Gel P-10). A first-limit Smith-degradation product, isolated after 5 days, was fractionated with MeOH–Me_2CO to give soluble (*SS*) and insoluble (*IS*) products. Linkage analysis of *IS* {the methylated derivative, isolated from Merckogel 2000, had $[\alpha]_D$ −13° ($CHCl_3$)} proved → 3)-Gal to be the main constituent, which, together with → 3,6)-Gal and → 3)-Ara, made up ~ 80% of the total (see below).

When the mol-wt distribution was monitored during the treatment with M CF_3CO_2H, $\overline{M}_w$ decreased from 14 500 after 6 days to 6700 after 31 and 44 days (Fig. 1). After 66 days, no free sugars were detected in the solution. Bound glycolaldehyde (8 mol%) indicated the presence of degraded residues (probably GlcA) even after 2 weeks at 45°C following 73 days at room temperature in M CF_3CO_2H. The product, when isolated and fractionated (using 2-propanol), showed that, within the polysaccharide fraction {the methylated derivative had $[\alpha]_D$ −18° ($CHCl_3$)}, 80% of the sugar units were Gal, there was 8% of Man (2- and 2,3-linked, and some terminal), 2% of Ara, and a trace of Rha. The Gal was mainly (44%) 3-linked, with ~ 12% present as 3,6-branch points. Terminal Gal comprised 20% and → 6)-Gal 6%. The mol wt of this second-limit block of sugar units was ~ 1000, a minimum value in view of partial glycosidic cleavage (free Gal was detected in solution) during the treatment with acid intended to cleave all of the remaining degraded GlcA units.

Smith degradation of the partially hydrolysed gum (*A*, 9.6 mmol of periodate consumed per g) yielded a first-limit product (39% after 18 days in cold M CF_3CO_2H) tending to a mol wt of 2400. The second-limit product from *A* (34%, mol wt 1250 after 12.5 weeks in cold M CF_3CO_2H) contained Gal (8) and Man (1) residues, and degraded acid (isolated as erythritol). Methylation analysis showed that, in the product of Smith degradation of *A*, → 3)-Gal [with less T-Gal and → 3,6)-Gal] preponderated and → 3)-Ara, T-Man, and → 2,3)-Man were present. Thus, the periodate-resistant block of sugars comprised an average of seven Gal

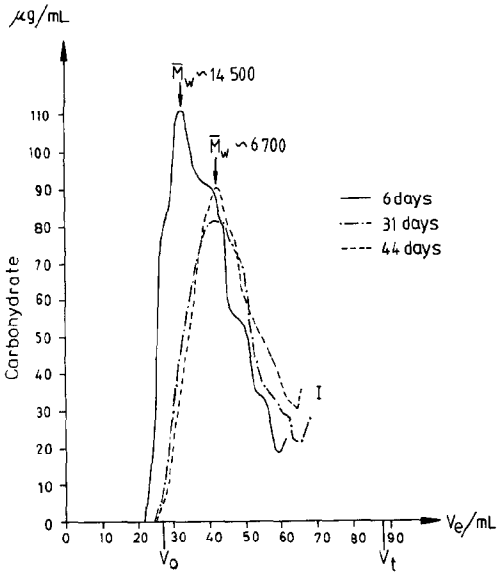


Fig. 1. Monitoring by steric-exclusion chromatography (Bio-Gel P-10, with dextrans of known, narrow ranges of molecular weight as calibrants) of the progress of Smith degradation (hydrolysis step) of *E. friderici-guilielmi* gum polysaccharide. V_o, void volume; V_t, total volume; V_e, volume eluted; I represents interference from iodine.

residues, one branched, two terminal, and the rest 3-linked. The block may have been joined directly to Man (at O-3) or through 3-linked Ara.

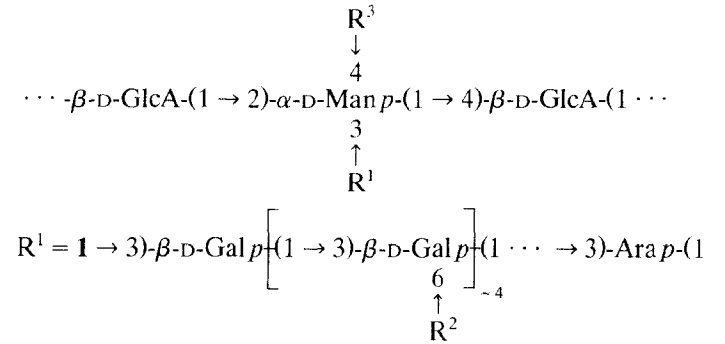
Smith degradation of the carboxyl-reduced gum (6.0 mmol of periodate consumed per g) yielded products which, after treatment for 6 days with cold M CF_3CO_2H, underwent little degradation compared with the product isolated after 3 days. Fractionation on Trisacryl GF05 gave two major polysaccharides with mol wts of >3000 and 3000–800 in the ratio 3:1. In addition to Gal, the chief constituent, Man and Ara in the ratio 2:1 were present, supporting the conclusions on structure reached on Smith degradation of the gum and of *A*. A third fraction contained erythritol and glycerol in the molar ratio 12:7, together with small proportions of bound glycolaldehyde and sugars which included Man and Ara in the ratio 2:1.

On the basis of the three Smith-degradation experiments, the following conclusions were made. (*a*) For *A*, the high molecular weight regions in which unattacked sugars remained linked together consisted of a (1 → 3)-galactan with $\bar{M}_w$ 1250, consistent with a structure made up of seven sugar residues and one polyol. Associated with this unattacked "block" were some Man and Ara (<10 and <5 mol%, respectively) for both the gum and *A*. (*b*) 3-*O*-Methyl-L-rhamnose (3MeRha) was found only in the Smith-degradation products from the reduced gum, and, together with Ara and Man, in all the fractions obtained from the reduced gum, and with the three sugars in the same approximate proportions. This finding could

imply that these sugars were associated with one another in some way. No such oligosaccharide was isolated, however, nor was an oligosaccharide comprising only Ara and Man found. (*c*) Whereas Man was generally highly substituted, the size of the substituent chains varied. This inference was deduced from the fact that Man appeared in all fractions, soluble and insoluble, after Smith degradation.

Thus, the (1 → 3)-galactan chain was associated with Man and Ara, but some may have been cleaved from these sugars during degradation. This conclusion is borne out by the fact that the first-limit insoluble fraction from the whole gum, on methylation and sub-fractionation using Merckogel 2000, yielded some methylated galactan, $[\alpha]_D$ +26° ($CHCl_3$). The methylated sub-fraction of higher molecular weight (eluted first) had $[\alpha]_D$ −13° ($CHCl_3$); and the sugar units were T-Gal, 7; → 3)-Gal, 53; → 3,6)-Gal, 11; → 3,4,6)-Gal, 4; → 3)-Ara, 15; → 2,3)-Man, 2; → 4)-Rha, 2; and T-GlcA, 4. A third component, obtained from the soluble fraction by preparative PC (R_{Gal} ~ 0.1, solvent *a*), consisted of T-Gal, → 3)-Gal, → 4)-Gal, T-Man, and T-Rha in similar proportions, with T-Ara, → 3)-Ara, → 2,3)-Man, and → 2,3,4)-Man making up the remaining 20%. This finding showed that much of the highly substituted Man [observed as 6-*O*-methylmannose (6MeMan) on methylation analysis] was substituted by short periodate-resistant chains or single periodate-resistant sugar residues. Therefore, it was essentially the size of the galactan chain that determined the size of the unattacked block. Furthermore, this galactan chain was not likely to be 4-linked to Man, as no 6MeMan was found on methylation analysis of the fractions of higher molecular weight. Since Man was 2-substituted by GlcA, the galactan chain must have been on position 3, attached either directly or indirectly, possibly through → 3)-Ara*p*.

The conclusions are expressed (Fig. 2) in the form of partial structures deduced from the Smith-degradation experiments, together with information on the gum



R^2 = → 4)-β-D-GlcA-(1, → 4)-β-D-GlcA-(1 → 6)-β-D-Gal*p*-(1, or no substituent. L-Rha*p*-(1 residues (0, 1, or 2) are attached to otherwise terminal GlcA. R^3 = 3-*O*-Me-L-Rha*p*-(1, or **2**, or no substituent.

Fig. 2. Partial structures for *E. friderici-guilielmi* gum deduced from methylation analysis, partial hydrolysis, and Smith-degradation experiments; **1** and **2** are defined in the text. L-Ara*f* residues are attached, probably to Gal, in the gum.

and its Smith-degradation products obtained by methylation analysis and partial hydrolysis.

Past research has shown the general similarity in sugar composition of the gums from different *Encephalartos* species[2,3], and two (the whole gum from *E. longifolius*[1] and a mannose-free fraction obtained on GPC from *E. friderici-guilielmi* gum[4]) have been examined in detail. The acidic galactan moiety present in the latter fraction resembles closely the core structure reported for *Cussonia spicata* gum[19]. Application of partial hydrolysis and sequential Smith degradation to *E. friderici-guilielmi* gum and to the products of autohydrolysis and carboxyl reduction established that the structures of the two gums are based upon identical patterns. A mannoglucuronoglycan core is common to both (the structure of *B3* characterises this feature), although less prominent in *E. friderici-guilielmi* gum, and, whereas there may be some variation in the pattern of sugars and glucuronic acid in the substituted acidic arabinogalactan moieties 3-linked to mannose and the distribution of rhamnose units in the periphery, there are no other major differences. As is well known for the Smith degradation of polysaccharides in which uronic acids form a substantial proportion of the units in the core, degraded acid units, the acetal linkages of which are difficult to hydrolyse, provide the key as to the extent of molecular weight diminution[5]. A first limit is reached in the normal way after glycolaldehyde-acetal fission, and the second limit much later as the acetal cleavage is completed. Thus, the first- and second-limit values for $\overline{M}_w$ of autohydrolysed *E. friderici-guilielmi* gum (*A*) were 2400 (insoluble portion) and 1250.

There is uncertainty as to whether → 3)-L-Ara*p* constitutes a link between the galactan and the core, and, indeed, whether all the substituted acidic galactan regions are attached to the mannoglucuronoglycan chain, although Smith-degradation experiments indicated these features. The general conclusion is that most of the Man, of which there is considerably less than in *E. longifolius* gum, is 4-substituted, but the positioning of 3MeRha, possible uronic acid-containing units, and L-Ara*f* in the model is arbitrary.

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