SOME NON-CELLULOSIC β-D-GLYCANS FROM PLANT SOURCES

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by

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SUMMARY

The structures of some non-cellulosic β-D-Glycans from three plant sources have been investigated and each was found to be characterised by a main chain consisting of β-(1→4)-linked D-pyranosyl sugars. The polysaccharides were, however, different in structural features in a manner apparently related to their respective locations within the organs of the plants concerned.

The polysaccharides were isolated and purified using standard fractionation methods including chromatographic techniques and selective precipitation methods. Structural information was obtained by employing techniques such as methylation analysis (involving use of gas - liquid chromatography - mass spectrometry), optical rotation measurements, mass spectrometry and n.m.r. spectroscopy on the original polysaccharides and on degraded products obtained by methods such as acid- or enzyme-catalysed hydrolysis and Smith degradation.

The polysaccharide from a gum exudate found in Strelitzia reginae flowers has been shown to be a highly substituted β-(1→4)-D-xylan, having side-chains containing predominantly terminal arabinofuranosyl, galactopyranosyl and glucopyranuronosyl groups; in addition there are small

proportions of (1→3)-linked galactopyranosyl, (1→2)-linked mannopyranosyl and 3,4-di-O-substituted glucuronosyl groups.

The hemicellulosic components from Agave sisalana leaves have been shown to be xylan and xyloglucan. The xylan, which forms the major component, is mainly linear with about one-fifth of the xylosyl chain units singly-substituted with 4-O-methylglucuronic acid or xylosyl groups. On the other hand the xyloglucan has more than half of the glucosyl chain residues substituted at O-6 with side-chains containing xylopyranosyl and galactopyranosyl, arabinofuranosyl or fucopyranosyl units.

Finally, the major polysaccharide component from the tubers of Satyrium corrifolium was characterised as a glucommanoglycan consisting of D-mannopyranosyl and D-glucopyranosyl residues in a linear chain of β-(1→4)-linkages.

Part of the work reported in this thesis has been published; viz;

> A complex xylan from corolla ducts of Strelitzia reginae - W.T. Mabusela and A.M. Stephen, S. Afr. J. Chem., 40, 7-11 (1987).

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ABBREVIATIONS

∿	approximately
abs.	absorbance
ca.	approximately
Ara	arabinose
concn	concentration
cm	centimetre
d.p.	degree of polymerisation
eV	electron volt
f	furanose
fuc	fucose
g	gram
Gal	galactose
GalA	galacturonic acid
g.l.c.	gas-liquid chromatography
Glc	glucose
GlcA	glucuronic acid
h	hour(s)
Hz	Hertz
i.r.	infrared
L	litre
M	Molar
Man	mannose
m/e	ratio of mass to electron charge
4-Me-GlcA	4-O-methyl-D-glucuronic acid
mg	milligram
min	minute(s)

mL	millilitre
mm	millimetre
mM	millimolar
m.s.	mass spectrometry
mol	mole
$\bar{M}_w$	weight-average molecular weight
ug	microgram
uL	microlitre
n.d.	not determined
nm	nanometre
NMMNO	N-methylmorpholine-N-oxide
n.m.r.	nuclear magnetic resonance
p	pyranose
p.m.a.a.	partially methylated alditol acetate
Rha	rhamnose
s.e.c.	steric-exclusion chromatography
TFA	trifluoroacetic acid
TMS	tetramethylsilane
u.v.	ultraviolet
v/v	volume per volume
w/w	weight per weight
Xyl	xylose

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CHAPTER 1

1. INTRODUCTION

1.1 Occurrence and nature of non-cellulosic β-D-glycans in plants.

The term β-D-glycans describes a fairly wide range of polysaccharides characterised by a basal chain consisting of D-sugar units that are linked mainly in the β-configuration. An individual polysaccharide species may be composed solely of such a linear chain, or as in a great number of species, the sugar units in the main chain may be further glycosylated to varying degrees to give a branched polysaccharide. β-D-Glycans are widely distributed among plants, and, as in the case of other polysaccharides, one of the aims of studying them is to seek correlations between their physiological roles in the plants and their molecular structures. Another objective is the investigation of their potential use as chemotaxonomic markers,[1] an area where gum exudates have received most attention.[2] Some of these polysaccharides are of industrial interest, and the cell wall polysaccharides of edible plants are important as dietary fibre.[3] β-D-Glycans may be categorised according to the sugar units constituting the basal chain.

β-D-Xylans: These are glycans that are based on a structure comprising β-(1→4)-linked D-Xylp residues. They are found in all land plants,[4] present in higher proportions in annual and hardwood plants and in lesser quantities in softwoods.[5] Furthermore, they are considered to be quantitatively the major components of the secondary cell walls of dicotyledons, whereas in monocotyledons they form the dominant component of the primary cell walls.[6] True D-xylans, composed solely of D-xylose, are rare; the majority carry short side chains, and may be neutral or acidic depending on the type of units constituting these side chains. Neutral side chains are often composed of L-Araf residues, while GlcpA and/or its 4-O-methyl ether are usually the acidic side chains. Partial acetylation of the chain sugar units is also an established feature of certain D-xylans. While they are found as constituents of some plant gums, D-xylans occur mainly among the hemicellulosic components of plants, where they are found in close association with cellulose and lignin.

Xyloglucans: This is a class of polysaccharides consisting of cellulose-like molecules (i.e. a basal chain of β-(1→4)-linked D-Glcp residues) which carry glycosyl substituents at O-6. They are widely distributed among plants,[7] occurring in higher proportions in dicotyledons than in monocotyledons.[8]

They are found as components of plant seeds, as well as in cell walls. Of significant interest with regard to industrial applications is the capacity of these polysaccharides to form gels.

Glucomannoglycans: These are plant tissue glycans that consist of β-(1→4)-linked D-Glcp and D-Manp residues in linear chains. Although the ratio of the constituent sugars varies widely, the proportion of Manp is generally higher than that of Glcp. In certain species these polysaccharides may be partially acetylated. If D-Galp groups are attached to the chain units to an appreciable extent, the polymers are called galactoglucomannans. Such branching results in greater solubility of these polymers. They are known to occur in seeds, tubers and leaves of a limited number of plant families,[9] while they are also found as cell wall components of different families of hardwoods and softwoods.

β-D-glucans: The β-(1→4)- linkage is ubiquitous in plant polysaccharides that are based on a backbone structure consisting of D-Glcp units, as is illustrated by the unlimited occurrence of cellulose in plants. The β-(1→3)-linkage is a rare feature which may entirely characterise a single polysaccharide species (e.g. callose), or may be interspersed with β-(1→4)

linkages as in the so-called mixed-linkage glucans. Callose is believed to be a structural polysaccharide, occurring in small amounts in individual plants but widely distributed throughout the plant kingdom.[10] It is insoluble in water and in dimethyl sulphoxide, while it dissolves with difficulty in alkali. Laricinan, another β-(1→3)-linked D-glucan, is one of the cell-wall components in the compression wood of tamarack.[11] There is evidence for the presence of a range of β-(1→3)-linked D-glucans in cotton fibre.[12] Mixed-linkage glucans are found in the seeds of the Gramineae and in nonendospermic tissue, in the hemicelluloses of grasses and clover,[13-15] and in cell-wall preparations from a number of monocotyledons.[6,16] It has been suggested that their role in the plant is not only structural, but also as energy reserves.[6]

β-D-Mannans: These polysaccharides, which consist essentially of a linear β-(1→4)-linked chain of D-Manp residues, are found as major components in seeds of species from certain genera of the Palmae family. Glycosylation of the mannosyl residues, mostly at O-6 with D-Galp groups, gives rise to a derived group called galactomannans. This class of water-soluble polymers with mucilaginous properties is of great industrial importance, a well-known example in this

respect being guaran, which occurs as an endosperm component of Cyamopsis tetragonoloba.

β-D-Galactans and L-arabino-D-galactans: D-Galactans are composed of β-(1→4)-linked D-galactosyl residues. Relatively few polysaccharide species from this group have been fully characterised, due to problems associated with their separation from other polysaccharides. They occur in seeds, leaves, bark, tubers and as hemicellulosic components of compression wood.[17] The D-galactan from citrus pectin is a commercial product. A certain degree of branching, with GlcA and GalA units as side-groups, has been indicated for a galactan from red spruce.[18]

When the linear β-(1→4)-linked D-galactan is heavily substituted with L-arabinosyl groups, the L-Ara content exceeding 25%, the polysaccharides are referred to as arabinogalactans, classified[19] as Type I. These are found in many kinds of seeds, though not to any significant extent in cereal grains.[19] Another group of arabinogalactans (Type II) differs from the above in that the galactan core is highly branched, consisting usually of β-(1→3) and β-(1→6) linkages. Their complexity is further extended by the incorporation of a range of other neutral monosaccharide units (such as Rha, Man and Xyl), as well as uronic acid groups, and

furthermore, they sometimes occur associated with protein in appreciable amount. Most of these occur as gum exudates from stems, bark and leaves of a large variety of plants. The ubiquity and complexity of this group of β-glycans, some of which are of great industrial importance, is reflected in a number of reviews.[1,5,19-23]

1.2 Isolation procedures for non-cellulosic β-D-glycans

The isolation of a polysaccharide in a state of acceptable homogeneity may be a fairly formidable task. The first step is its removal from the plant material. Primarily, this involves solubilisation of the polysaccharide, which is often dependent on the matrix within which it is located. The total isolation procedure may take one of various pathways, which may be less complicated for water-soluble gum exudates than for hemicelluloses found in highly lignified softwoods. Several problems that are encountered in the isolation of polysaccharides have been discussed by Aspinall.[24] Most methods that can be used for the isolation of β-D-glycans are applicable to polysaccharides in general. In the present context, these methods are discussed with emphasis on their application to xylans, xyloglucans and glucomannoglycans, since the

investigation forming the subject of this thesis has been directed at β-D-glycans of these types.

A high yield, chemical purity and homogeneity are the overall objectives when a polysaccharide is isolated. Minimal degradation is also an aim of any isolation procedure. The simplest means of extracting polysaccharides from plant materials is with water, either in the cold or at high temperatures. While this can be achieved to a certain extent with xyloglucans from seeds and glucomannoglycans from bulbs, tubers, rhizomes, seeds and leaves, its success with xylans appears to be confined to those that occur as gum exudates. The efficiency of water as an extractant for some β-D-glycans is often limited, so that it may become essential to use aqueous alkali for extraction of residual material from associated materials like cellulose. A further limitation sometimes arises from the presence of significant amounts of lignin, (especially in softwoods), which often necessitates a delignification step prior to any form of extraction of the polysaccharide. It is, however, unfortunate that most, if not all known methods will not effect removal of lignin without simultaneously causing significant structural changes in the native hemicelluloses.[25]

Xyloglucans have been reported[8] to show strong non-covalent association (most probably through hydrogen bonding) with cellulose. This form of association can be disrupted almost entirely by the use of aqueous alkaline solutions, under a nitrogen atmosphere, for extraction. While potassium hydroxide solutions have been noted for their efficiency in the extraction of xylans and xyloglucans, sodium hydroxide appears to have a preference for glucomannoglycans. The addition of small amounts of sodium borohydride to such alkaline media is often recommended in order to minimise degradation that proceeds from the reducing ends of the polysaccharides. Although the use of alkaline media for extraction has been found to be the most efficient in terms of high yield, the method suffers from the disadvantage of causing hydrolysis of acetyl ester groupings, in polysaccharides containing such groups in their native state. Thus, dimethyl sulphoxide, alone or containing 10% water, has been found to be one of the best extractants for polysaccharides, if retention of such labile substituents is a consideration. However, the extracting ability of this solvent is often poor. N-methylmorpholine-N-oxide has been shown to be a good solubilising agent for polysaccharides[26] including cellulose;[27] however, there is some evidence that this promotes degradation.[28]

After a β-D-glycan has been removed from plant material by any of the methods described above, it is seldom pure and/or homogeneous, which necessitates fractionation as a further step in the isolation procedure. Fractionation methods may be used to separate polysaccharides from one another or from non-carbohydrate material. General methods applicable in either case have been reviewed in several publications (see, for example, reference 29), and therefore the present discussion is confined to those used in the isolation of the β-D-glycans under investigation. The simplest, and very widely used method of fractionation is precipitation by the addition of a water-miscible organic solvent (e.g. ethanol) to an aqueous solution of the polysaccharide material. While this method is extremely effective in the separation of carbohydrates of low molecular-weight from high molecular-weight components, little fractionation of polysaccharides of different sugar composition and constitution is achieved. Selective precipitation of polysaccharides as salts or complexes is often a successful means of purification. A typical example is afforded by the purification of acidic xylans by precipitation as cetyl trimethylammonium or cetylpyridinium salts, which effects separation from neutral polysaccharides including those that are xylans. The separation of acidic xylans from neutral may also be achieved by

means of ion-exchange chromatography, if conditions are such that the acidic components are retained on the column, while the neutral are eluted completely; the acidic polysaccharides are subsequently eluted under different conditions. These methods are equally useful for the purification of xyloglucans which are often neutral, even though their extraction conditions, especially from holocellulose, are somewhat similar to those of xylans. DEAE-cellulose, DEAE-Sephadex, DEAE-Sephacel and DEAE-Sepharose packings are extensively used in ion-exchange chromatography of this type. Further purification of xyloglucans, xylans and glucomannoglycans may also be achieved by precipitation as alkaline copper (e.g. with Fehling's solution) or barium complexes. Fractionation based on molecular weight differences (steric-exclusion chromatography) is also widely applied.

1.3 Molecular structure variations of non-cellulosic β-D-glycans

The basic approach towards characterisation of polysaccharide structures involves investigation of their composition, constitution, configurational aspects of constituent sugars and their linkages and, where possible, the molecular size of the polysaccharide. Presently, this can be achieved by

application of a number of methods including mainly those of a chemical nature, and physical, spectroscopic and enzymatic methods as appropriate. As in section 1.2, discussion will be confined to the polysaccharides under study, namely, xylans, xyloglucans and glucomannoglycans.

D-xylans may, in the simplest manner, be generally represented as in Fig. 1:

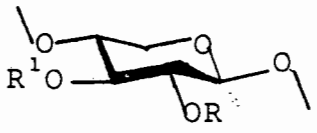


Figure 1 : General structural representation of xylans

where, for example, R = 4-Me-D-GlcA(1→

and/or R^1 = L-Ara-f-(1→

Side chains, R/R^1, may sometimes be more complicated than illustrated above as will be shown below. The most widely occurring type of acidic xylans in hemicelluloses of plants are 4-O-methylglucuronoxylans.

The position of attachment for the 4-Me-D-GlcpA group is usually O-2 of D-Xylp residues. Linkage through O-3 has been found as a variant in xylans from certain plant sources.[30]

In xylans from hardwoods of some dicotyledonous angiosperms, the number of D-Xylp residues per uronic acid group usually varies from 5 to 15. While xylan chain lengths may vary from 45 to 212 residues, several have exhibited some degree of branching.[31] Where the distribution of uronic acid groups along the xylan chain has been determined, for example, in birch wood,[32] it has been found to be irregular. The occurrence of D-GalpA in addition to 4-Me-D-GlcA in Betula verrucosa xylan has been noted by Shimizu and Samuelson.[33] D-GalpA units have also been found, as the sole uronic acid components in side chains, in xylans from the seeds of some species of the genus Ocimum.[34-36] While substitution by acetyl groups may be random, the 2-O-position of xylosyl residues is often the most preferred site. The presence of these ester groupings is known to improve the solubility properties of such xylans. D-Glcp units occur as constituents of acidic xylans isolated from immature sugar maple,[37] while glucoxylans found in common barberry leaves contain in addition terminal D-Galp and L-Araf groups.[38] The occurrence of L-Rha residues in small

amounts in the main chain is another variation of the xylan structure, observed in a polysaccharide fraction from lucerne stems.[39] Fucosyl units are also known to be incorporated, in a highly branched product from cultivated rose cells.[40] Although D-GlcpA as such is found as a constituent of xylans only rarely, compared to its 4-O-methyl ether, a polysaccharide containing the former, linked through O-2 of D-Xylp residues, was isolated from soybean hull cell walls.[41] In contrast to the complex heteroxylans from various dicotyledons, a linear homoxylan consisting of about 80 sugar units was isolated from delignified tobacco stalks.[42]

In softwoods, branching of the xylan core has been observed, and in some instances both single and double branching of D-Xylp residues have been found in the same homogeneous fraction, for example, that from the hemicelluloses of slash pine.[43] Evidence that uronic acid groups are localised rather than randomly positioned along the xylan chain of larch[44] and redwood[45] xylans, was afforded by isolation, after acid hydrolysis, of a substantial proportion of acidic oligosaccharides in which uronic acid groups were located on each of mutually joined D-Xylp units.

Heteroxylans from a large number of species in the Gramineae family have been thoroughly investigated.

This is largely due to the fact that cereals and grasses belong to this family, and their contribution to the bulk of edible plant materials is such that the importance of their hemicelluloses as dietary fibre is generally acknowledged. The heteroxylans from Gramineae bear some resemblance to those from other angiosperms and gymnosperms. Structural studies have indicated that although L-Araf units may occur mostly as single terminal substituents at O-3 of D-Xylp residues, variations where such oligosaccharides as β-D-Galp-(1→4)-β-D-Xylp(1→ and D-Xylp-(1→2)-Araf(1→ may be appended at the 2-O-positions of Araf residues do occur.[6,14,46,47] L-Galactosyl units are occasionally found as end-groups in side-chains of xylans from certain plants.[48-50] D-GlcpA units occur only rarely without being accompanied by its 4-O-methyl ether in monocotyledonous xylans. The usual linkage position for D-GlcpA is through O-2 of D-Xylp residues, but O-3 and even O-4 are also known as alternative positions of attachment.[46] The structural unit 4-Me-D-GlcpA-(1→4)-D-Xylp-(1→4)-D-Gal-(1→ occurs as a rare component in side-chains of hemicellulosic xylans from bamboo leaves[51] and oat stems.[52] When a xylan is highly substituted with L-Araf groups, double substitution at O-2 and O-3 of D-Xylp residues is sometimes observed, although monosubstitution at the latter position is most common.

The covalent association of xylans with lignin is well established,[53] where the modes of linkage include the formation of ferulic esters of arabinoxylans, ferulic acid being one of the potential precursors of lignin. Another variation is the esterification of carboxyl groups in 4-Me-D-GlcpA groups through phenolic groups of lignin.[54,55]

Exudates from angiosperms contain heavily substituted xylans, most of which are characterised by the presence of uronic acid and other sugar units in short side-chains. Most frequently, the uronic acid units are linked directly to the xylan chain through O-2 and occasionally some may carry substituents at O-2. Substitution of D-Xylp residues at O-2, or at both O-2 and O-3, with single units of L-Araf, L-Arap and D-Xylp is quite common. In brea gum[56] there is a possibility of the presence of (1→2)-linkages in the main chains, where uronic acid residues are linked through O-4. Doubly-substituted xylosyl chain units frequently form the major proportion of xylosyl residues in gums from certain Watsonia species,[57, 58] where the trisaccharide units α-D-Galp-(1→3)-α-L-Araf-(1→2)-L-Araf-(1→ and a high proportion of L-Araf end-groups occur as substituents. Less common acidic side-chains such as α-D-GalpA-(1→3)-L-Araf-(1→,

D-GlcpA-(1→3)-L-Araf-(1→, β-D-GlcpA-(1→5)-L-Araf-(1→ and D-GalpA-(1→2)-Rhap-(1→ have been found to occur in mucilaginous xylans from several species of the Plantago genus, where they are linked through O-3 of D-Xylp residues.[59] A mucilaginous xylan from the seed coat of Hyptis suaveolens was found to incorporate as much as 20% of L-fucosyl units.[60] Highly branched arabinoxylans, where all D-Xylp chain residues are substituted at both O-2 and O-3, have been isolated from certain species of the Lauraceae family.[61-63]

A general representation of the structure of xyloglucans may be drawn schematically as in Fig. 2:

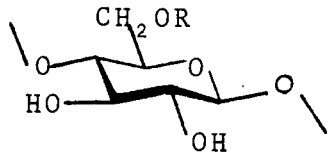


Figure 2 : General structural representation of xyloglucans

where, for example, R = α-D-Xylp-(1→

Structural studies, involving such techniques as methylation analysis, partial acid hydrolysis and use

of cellulase enzymes, carried out on these polysaccharides have indicated a high degree of consistency in their structures.[6,21,64,65] The extended chains of β-(1→4)-linked D-Glcp residues are glycosylated with α-D-Xylp units at approximately half the number of 6-O-positions. To some of the Xylp units, β-D-Galp groups may be appended at O-2. In some instances,[66,67] it has been shown that further β-D-Galp groups may be linked to O-2 of these β-D-Galp units. Where terminal L-Fucp groups are present as additional constituents, for example, in sycamore extracellular xyloglucan, they are linked through O-2 of the β-D-Galp units.[6,68] The presence of L-Araf end-groups has been indicated for some xyloglucans, including one isolated from tobacco stalks,[69] in which they are joined in the α-configuration to O-2 of Xylp units. Other structural variations that have been established are the occurrence of D-Glcp residues in side-chains,[70] and the presence of D-Xylp units joined in ways other than the conventional.[71]

The predominant structural feature of glucomannoglycans is the β-(1→4)-linkage of the constituent D-Glcp and D-Manp residues, as implied by methylation analysis. This can be illustrated in a simple diagram as in Fig. 3:

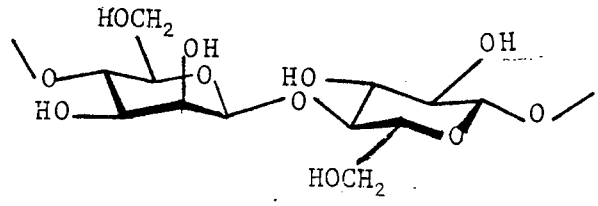


Figure 3 : General structural representation of glucomannoglycans

Although D-Manp residues are often found in higher proportions than D-Glcp residues, it has been demonstrated through acetolysis and partial acid hydrolysis experiments that the latter may be linked in a contiguous manner. α-D-Galp end-groups occur occasionally, most often linked through O-6 of either D-Glcp or D-Manp residues. Branching may occur at O-2,[72] at O-3[73] or at O-2 and O-3 of mannosyl and glucosyl residues. Another common structural variation is that of acetylation, where acetyl groups may be located at either O-2 or O-3 of mannosyl residues, as illustrated in a polysaccharide from Scots pine.[74] Limited branching has been indicated by the presence of appreciable molar proportions of mannosyl and glucosyl units as non-reducing end-groups, as was demonstrated by methylation analysis of the polysaccharide from Norway spruce.[75] From seeds of the leguminous plant, Cassia tora,[76] there has been isolated a highly

branched polysaccharide containing terminal D-Xylp and D-Galp groups. Evidence from methylation analysis suggests that these groups are linked to a glucomannoglycan structure at 6-O-positions. On the other hand, the polysaccharide from Cassia absus seeds appears to have its D-Galp units linked through O-2 of D-Manp chain residues.[77,78] Galactoglucomannoglycans may also contain acetyl groups.[79]

Conformational analysis of polysaccharides linked in different ways has shown that they can be classified into several groups according to their structural shapes. Thus, it appears to be a general rule for homopolysaccharides of the β-(1→4)-glycan type to assume the extended, ribbonlike structure;[80] a typical example being cellulose. β-(1→4)-Xylans also adopt the same shape, and glycosylation of the linear chain units appears not to alter their original arrangements, as has been demonstrated in the heteroxylans from corm-sacs of Watsonia pyramidata[81] and from wheat flour.[82]

CHAPTER 2

2. GENERAL EXPERIMENTAL CONDITIONS

2.1 Paper and thin-layer chromatography

The following solvent systems were used: (all v/v)

A. Ethyl acetate-pyridine-water (8:2:1)
B. Ethyl acetate-pyridine-water (10:4:3)
C. 1-butanol-acetic acid-water (2:1:1)
D. Ethyl acetate-acetic acid-formic acid-water (18:3:1:4)
E. 1-butanol-ethanol-water (4:1:5, upper phase)
F. Chloroform-methanol-water (20:20:7)
G. Chloroform-methanol (97:3)

Analytical p.c. was performed on sheets of Whatman no.1 paper and t.l.c. on Merck aluminium sheets coated with silica gel 60F, thickness 0,2mm.

Preparative p.c. was performed on sheets of Whatman 3 MM paper, pre-washed with deionised water then dried. The sample was dissolved in water and applied in a thin band near the top of the sheet. After elution of the paper sheets and subsequent location of the components, the bands were cut from the sheets. The components were isolated by extraction with deionised water and

concentration of the extract to a small volume followed by freeze-drying.

Reagents and techniques for detecting components separated by p.c. and t.l.c. were as follows:

1. Spraying with a solution of p-anisidinium hydrochloride in aqueous 1-butanol, followed by heating at 110°C for 5-10 minutes

2. Dipping the paper successively through (a) a 0,6% solution of $AgNO_3$ in acetone and, after drying (b) a 2% solution of NaOH in ethanol

3. Carbohydrate was detected on t.l.c. by spraying with p-anisaldehyde-sulphuric acid-ethanol (1:1:18, v/v) followed by heating at 110°C for 5-10 minutes

2.2 Gas-liquid chromatography (g.l.c.)

G.l.c. analysis of alditol acetates or partially methylated alditol acetates (p.m.a.a.'s) was carried out using a Carlo-Erba 4200 gas chromatograph coupled to a Columbia Supergrator 3A integrator for quantitative analysis. It was fitted with one of the following columns:

column A: glass (2m x 3mm i.d.) column packed with 3% OV-225 on Chromosorb W-HP, 80-100 mesh

column B: fused silica capillary DB-225 (30m x 0,32mm i.d.), film thickness 0,25 microns (J & W Scientific, Inc.)

column C: glass (2m x 3mm i.d.) column packed with 3% ECNSS-M on Gas Chrom Q, 100-120 mesh

column D: glass capillary OV-225 SCOT (25m x 0,35mm i.d.)

The carrier gas was helium. Mixtures of alditol acetates were analysed on column A, isothermally at 220°C, or on column B isothermally at 215°C. Mixtures of p.m.a.a.'s were analysed on columns A, C and D, isothermally at 170°C or on column B isothermally at 215°C. Columns B and D were used with a splitter injection system. A flame-ionisation detector was employed. For g.l.c.-m.s., an identical gas chromatograph was coupled, through a jet separator, to a VG Micromass 16F mass spectrometer, and columns A and B were used.

Components in mixtures analysed were identified by comparison of retention times with standards run under

identical conditions, by co-injection of standards in some cases, and by g.l.c.-m.s. Quantitative analyses were achieved by using the molar response factors of Sweet and Albersheim[83] for mixtures of p.m.a.a.'s and empirically determined molar response factors for mixtures of alditol acetates.

2.3 Steric-exclusion chromatography (s.e.c.)

Average molecular weights ($\bar{M}_w$) were estimated by steric-exclusion chromatography[84] on the following columns:

column 1: Sepharose 4B (60 x 0,9cm); eluent M NaCl
column 2: Bio-Gel P-10 (52 x 1,5cm); eluent M NaCl
Other columns used are described in the text.

Samples (1-2mg) were dissolved in eluent (1mL) before being applied to columns and fractions (1-1,6mL) of the column effluent were collected. The emergence of carbohydrate in the fractions was monitored by the phenol-sulphuric acid method.[85]

2.4 Ion-exchange chromatography:

(a) on DEAE-Sephadex A-50

This was carried out on a preparative scale using a column (15 x 2,5cm) of exchanger in the Cl^- form previously equilibrated with potassium phosphate buffer pH 6,4. Samples (∿200mg), dissolved in the same buffer (∿5mL) were applied to the column and eluted initially with this buffer. Subsequently, elution was continued with a gradient of NaCl, 0 → 1M, in the same buffer. Aliquots (20-50μL) of the fractions collected (4-5mL) were assayed for carbohydrate as in s.e.c. Resolved components were recovered by pooling the appropriate fractions and concentration of solution volumes under reduced pressure to 30-50mL. The concentrated solutions were dialysed against water for 3 days and freeze-dried.

(b) on DEAE-cellulose

A column (30 x 2,5 cm) of DEAE-cellulose prepared in the Cl^- form was previously equilibrated with 0,05M Tris-HCl buffer (pH 8,6). Samples (5-10mg), dissolved in this buffer (∿ 3mL), were applied to the column and eluted initially with the same buffer. Subsequent elution was with a gradient of NaCl, 0 →1M, in this

buffer. Emergence of carbohydrate material from the column was monitored as in s.e.c.

In both (a) and (b) the concentration of NaCl was determined by conductivity measurements on a conductivity meter calibrated with solutions of known concentration of NaCl.

2.5 General conditions

Optical rotations were measured from aqueous solutions for underivatised samples, and from chloroform solutions for methylated products, at 20°C (± 3°C) on a Perkin-Elmer Model 141 polarimeter. Infra-red spectra of methylated derivatives in chloroform were recorded on a Perkin-Elmer Model 237 spectrophotometer.

90 MHz ^{1}H n.m.r. spectra were obtained on a Bruker WH-90 spectrometer. Samples were prepared for n.m.r. by dissolving them in 99,7% D_2O after freeze-drying 2-3 times from D_2O solutions. The spectra were recorded at 20°C and 80°c. ^{1}H chemical shifts were measured with reference to internal acetone, δ2,20 downfield from TMS, but were expressed relative to TMS.

Mass spectrometry was performed on a VG Micromass 16F mass spectrometer, operating at 70eV or at 20eV.

2.6 Sugar analyses

Neutral sugars were determined by g.l.c. as alditol acetates, prepared by the method of Albersheim et al.[86] The proportions of neutral sugars constituting polymeric products were determined by two methods. In Method 1, hydrolysis in 2M TFA at 100°C for 18h or 8h, depending on whether the polysaccharide contains uronic acid or not, preceded conversion to alditol acetates and g.l.c. Results were corrected for degradation of proportions of the sugar residues during the hydrolysis.[87]

Method 2 provides for a simultaneous determination of acidic and neutral sugars, following the procedure recommended by Dudman et al.[88] A sample of the glycan (1-2mg) was subjected to methanolysis in 1M HCl in dry methanol (1mL) at 100°C for 18h in a sealed glass tube. After cooling, the solution was neutralised with Ag_2CO_3, the resulting suspension was centrifuged and the supernatant liquor was evaporated to dryness. The residue was dissolved in 96% ethanol and $NaBD_4$ (10mg) added. The solution was sonicated for 10 minutes and then stirred overnight (∿16h) to effect reduction of methyl-esterified carboxyl groups of uronic acids to 6,6-dideuterio-substituted glycosyl

residues. Excess $NaBD_4$ was then decomposed by adding glacial acetic acid dropwise and the solution was treated with Amberlite IR-120 (H^+) resin for 0,5h before filtration and evaporation of the filtrate to dryness. The residue was dissolved in 2M TFA and heated at 120°C for 1h in a sealed glass tube to hydrolyse the methyl glycosides. After cooling, TFA was removed by freeze-drying and the aldoses were converted to alditol acetates as before.

Uronic acid was also determined colorimetrically by the method of Blumenkrantz and Asboe-Hansen[89] (see Appendix).

2.7 Carboxyl-reduction of acidic polysaccharides[90]

An aqueous solution of the polysaccharide was adjusted to pH 4,75 and treated with solid 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. During the ensuing reaction, the pH of the mixture was maintained at 4,75 by titration with 0,05M HCl. After 1h, the reaction mixture was treated with a 3M solution of $NaBD_4$, while the pH was maintained at 7,0 by titration with 4M HCl. After 1h the reaction mixture was rendered slightly acidic to destroy excess borodeuteride, and the solution was dialysed exhaustively against distilled water. The product was isolated by freeze-drying. It

was necessary to repeat the procedure in order to achieve complete reduction. The reduced product was analysed for sugar composition as described in 2.6.

2.8 Methylations and related experiments

Methylations were carried out by the Hakomori method[91] as modified by Phillips and Fraser,[92] using as base potassium methylsulphinylmethanide (2M), prepared by addition of dry DMSO to dry KH at 0°C.[93]

Polysaccharides containing uronic acid were deionised with Amberlite IR-120 (H^+) resin and freeze-dried prior to methylation. In general, samples were vacuum-dried over P_2O_5 for at least 18h before being dissolved in dry DMSO for methylation, conducted under purified N_2. Contact time with the base ranged from 0,5h-3h, the presence of excess base being confirmed by removal of a drop of the reaction mixture for testing with triphenylmethane.[93] Solutions of the alkoxides were frozen in ice before addition of methyl or trideuteriomethyl iodide, after which solutions were stirred at room temperature for at least 0,5h. Polymeric products were recovered by addition of water and $CHCl_3$, followed by dialysis and recovery of the organic phase in the non-dialysable portion. After evaporation of $CHCl_3$, products were purified by passage

through a column of Sephadex LH-20, eluted with ethanol-chloroform (2:1 v/v), the column effluent being monitored for carbohydrate with the anthrone-sulphuric acid reagent.[94] Oligomeric products were recovered from the reaction mixture by addition of chloroform and extraction of salts and DMSO with water. The products were not purified further. Where complete methylation of uronic acid-containing polysaccharides was not achieved, further methylation was carried out by the Purdie method.[95]

Reduction of methyl-esterified carboxyl-groups was achieved by the method of Åman et al.,[96] wherein the reducing agent, lithium aluminium hydride (LAH) or lithium aluminium deuteride (LAD), was heated for 30 minutes at 80°C in dry THF, the suspension was centrifuged and portions of the supernatant were added to a solution of the methylated glycan in dry THF. The resulting solution was heated at 80°C for 18h and the reduced products were recovered by addition of moist ethyl acetate to decompose excess LAH, filtration of the suspension through celite, washing of the residues with $CHCl_3$ and, finally, evaporation of the combined filtrate and washings to dryness.

Completeness of methylation and reduction procedures was assessed by recording i.r. spectra, monitoring the

disappearance of the hydroxyl and carbonyl stretches respectively.

Base-degradation of methylated uronic acid-containing polysaccharides was carried out by the method of Lindberg and Lönngren.[97] The methylated polysaccharide, previously dried over P_2O_5, was dissolved in dry DMSO containing 5% (v/v) of 2,2-dimethoxypropane and a trace of p-toluene sulphonic acid in a serum vial sealed with a rubber cap. The solution was treated with methyl sulphinyl anion (2M) in DMSO. After 18h at room temperature the reaction mixture was poured into 10% acetic acid. The solution was extracted with chloroform (3 times), and the combined organic phase was washed with water (3 times) and concentrated to dryness. The residue was suspended in 10% aqueous acetic acid and the suspension was kept at 100°C for 1h, then cooled and freeze-dried. The product was purified by passing through a Sephadex LH-20 column, using $CHCl_3$-acetone (2:1 v/v) as eluent.

Methylation analyses were carried out by hydrolysing the methylated products in 2M TFA at 100°C for 8h or 18h, and then removing TFA by freeze-drying. The hydrolysates were examined in some instances by p.c. (solvent E) using the mixture of partially methylated sugars from Virgilia oroboides gum[98] as a standard.

The aldoses were then converted to p.m.a.a.'s for g.l.c. analysis.

CHAPTER 3

3. A XYLAN FROM STRELITZIA REGINAE FLOWERS

3.1 Introduction

The plants of Strelitzia reginae (Musaceae; Zingiberales) are well known for their unusually structured orange and blue flowers. They are indigenous to South Africa and have been found growing in the wild along the east coast. During the flowering season they secrete a gum exudate into the corolla ducts. The major polysaccharide component of this exudate has been the subject of the present investigation, the motivation for which is discussed below.

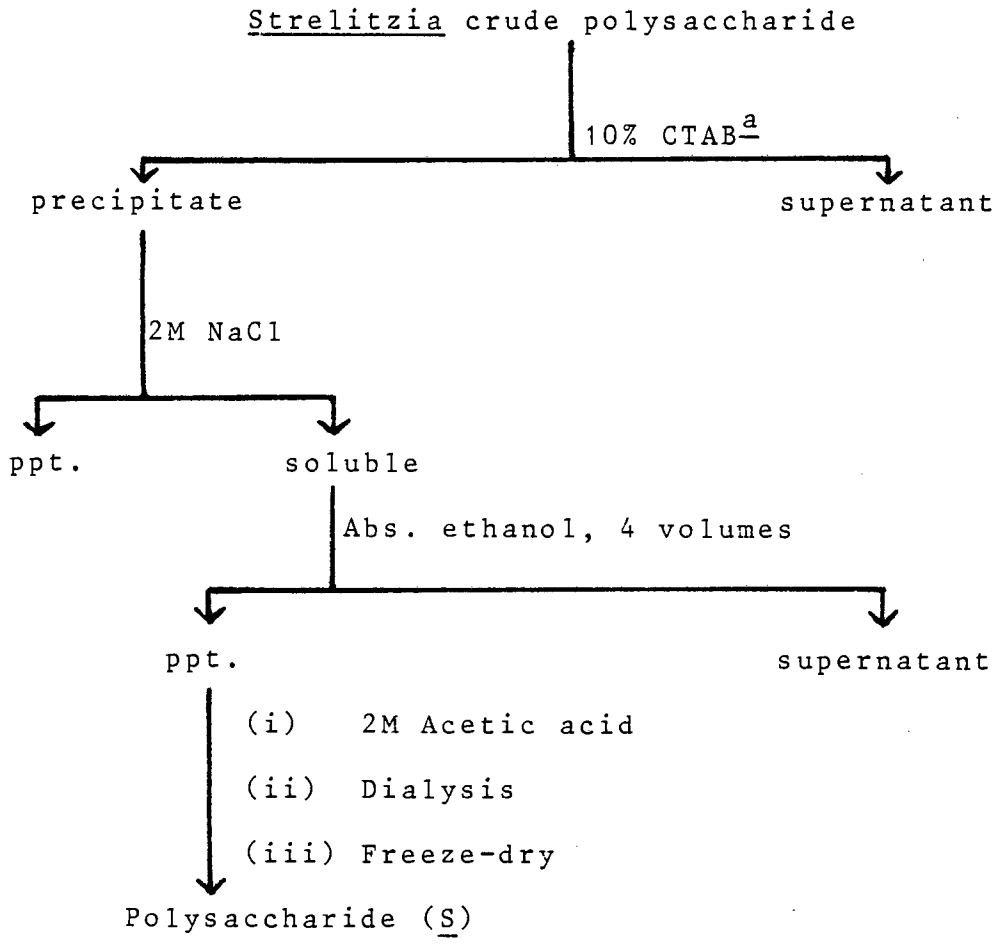
The nature of the polysaccharides on stigmatic surfaces and on pollen may well be involved in mechanisms of species recognition.[19,99] In certain monocotyledones the stigmatic exudates consist of arabinogalactans, often associated with protein,[99-102] but the surface polysaccharide from the stigma of Strelitzia reginae contains significant amounts of xylosyl as well as galactosyl and arabinosyl units. The polysaccharide from the corolla ducts also contains the same neutral sugar components, and, because of its abundance relative to the stigmatic polysaccharide, it has been examined in detail. Another objective of this investigation was to relate the structural features of

this polysaccharide to those of other gums from monocotyledonous plants, particularly the highly-substituted xylans from corm sacs of Watsonia pyramidata (Iridaceae; Iridales)[57] and seed-boxes of W. versveldii.[58]

3.2 Isolation and degree of homogeneity of the sample

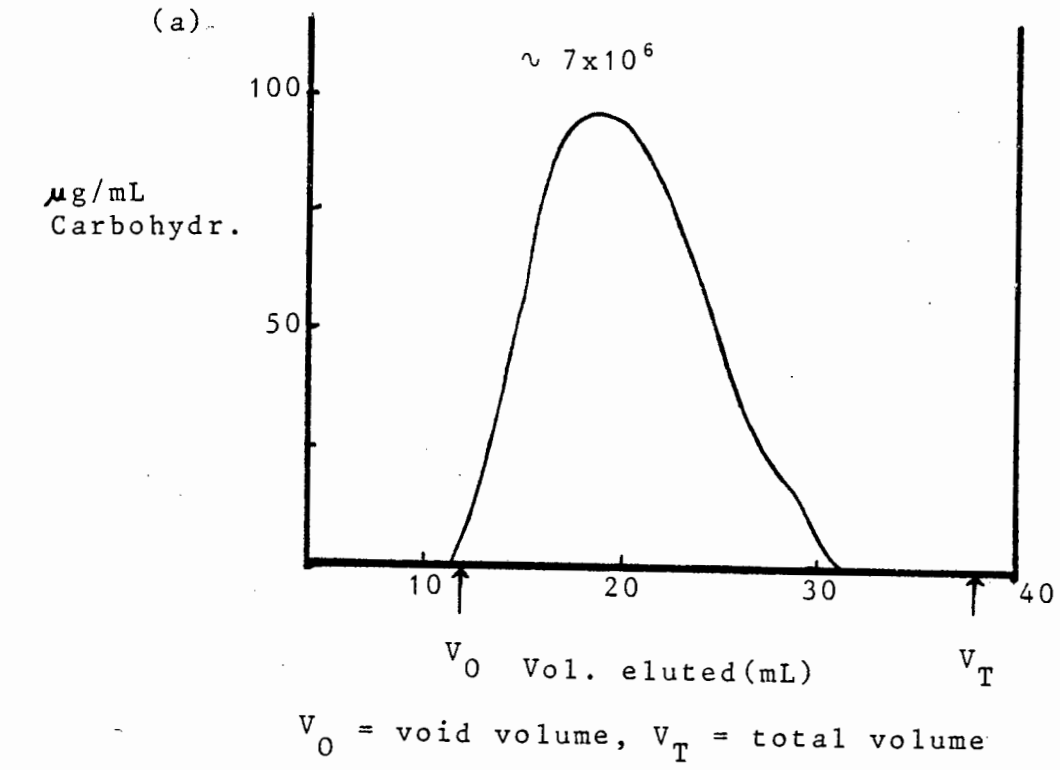
The water-soluble polysaccharide material from the Strelitzia reginae flowers was partly separated from low molecular-weight carbohydrates by fractionation with aqueous ethanol. Repeated ethanol precipitation of the polysaccharide from aqueous solution afforded material which, upon complete hydrolysis, yielded (according to p.c.) xylose, arabinose, galactose, glucose, mannose and uronic acid. The polysaccharide was further purified as its cetyltrimethylammonium complex[103] (see scheme 1 and section 3.9.1 for details). The homogeneity of the sample was investigated firstly by steric-exclusion chromatography (s.e.c.) using Sepharose 4B: it eluted as a single peak whose shape indicated a fair amount of polydispersity (Fig. 4(a)). Ion-exchange chromatography on DEAE-cellulose, eluting with a NaCl gradient also gave a single peak (Fig. 4(b)). The chemical composition and physical properties of the sample are presented in Table I. Although the $[\alpha]_D$ value is

characteristic for a xylan, the occurrence of mannose among the sugar constituents is clearly an unusual feature. The high $\bar{M}_w$ may also be noteworthy; whereas $\bar{M}_w$ value of as high as 31 000 have been reported for hemicellulosic xylans, similar information for xylans in gum exudates appears to be lacking.



SCHEME 1 : Purification of Strelitzia polysaccharide

a CTAB = Cetyltrimethylammonium bromide



V_0 = void volume, V_T = total volume

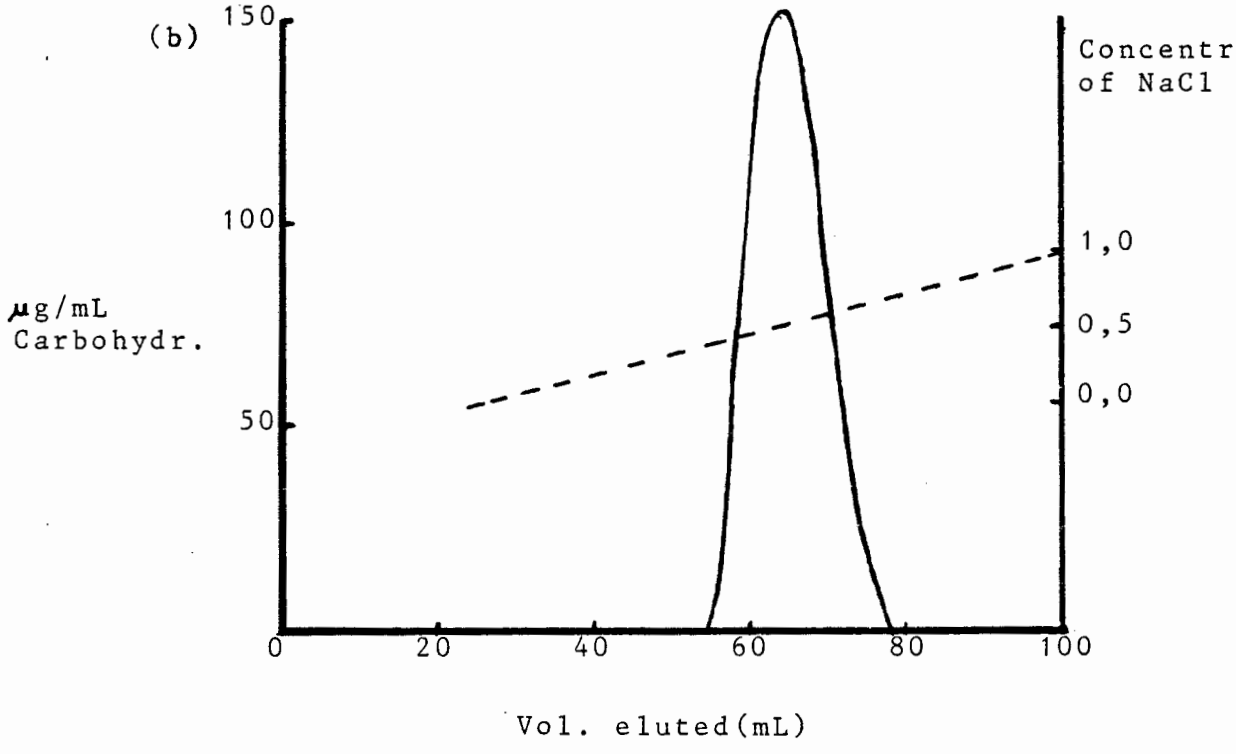


Figure 4 : Column chromatography of Strelitzia polysacchardie S

(a) on Sepharose 4B

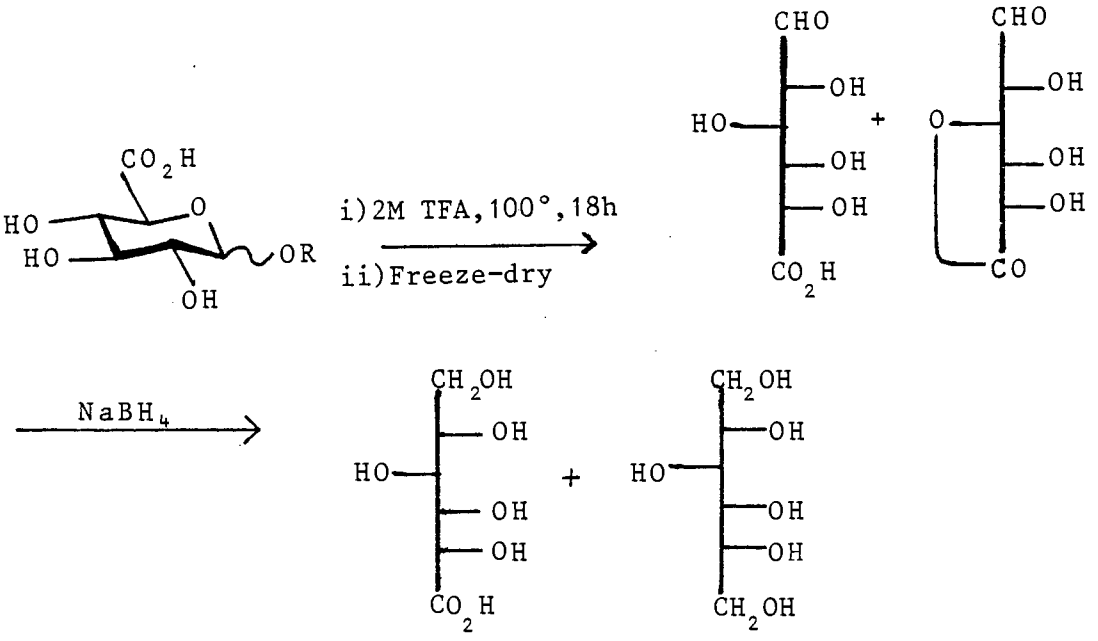
(b) on DEAE-cellulose

TABLE I - CHARACTERISTICS OF PRODUCTS FROM STRELITZIA REGINAE

		S	RS	DS	SDIA	SDIB	SDIC	ORS
$\bar{M}_w$		7.10^6	n.d.[a]	84 000	n.d.	2 000	650	n.d.
$[\alpha]_D$		-56°	n.d.	-28°	n.d.	-87°	-29°	n.d.
mol %	Xyl	37	34	46	100	91	67	77
	Ara	24	20	2	-	-	-	3
	Gal	21	25	28	-	9	22	13
	Man	3	6	4	-	-	-	-
	Glc	-	15	-	-	-	-	7
	GlcA	15	-	19	-	-	11	-

a n.d., not determined

A small proportion of glucitol hexa-acetate (∿ 3%), not shown on the table, was detected upon g.l.c. analysis of alditol acetates derived from the hydrolysate of the polysaccharide. However, this was shown to be an artefact formed by the process illustrated in scheme 2. Thus, when the borohydride reduction step was avoided by the preparation of peracetylated aldononitriles [104] for g.l.c. analysis of the hydrolysate, no glucose derivative could be detected.



SCHEME 2 : Formation of glucitol from glucuronic acid

3.3 Carboxyl reduction

The glycosyluronosidic bond in acidic polysaccharides is well-known for its resistance to acid hydrolysis. This property and the poor volatility of alditol acetates derived from uronic acids often preclude a direct simultaneous determination of acidic and neutral sugars by g.l.c. of their alditol acetates. Sugar analysis using Method 2 (section 2.6) serves to overcome that problem, but the procedure involving reduction of the carboxylic acid groups in the polysaccharide prior to complete hydrolysis may also be applied as an alternative or supplementary method. Furthermore, the latter method has the advantage of generating a reduced derivative of the original polysaccharide, which may serve as a substrate in experiments similar to those performed on the original species. Thus a portion of the polysaccharide under study, S, was carboxyl-reduced by the method of Taylor et al.,[90] using $NaBD_4$ to label the former carboxyl carbon with two deuterium atoms. The sugar composition of the reduced product, RS, is given in Table I. G.l.c.-m.s. of the derived alditol acetates showed that only the hexa-acetate from glucose incorporated deuterium atoms, from which it followed that GlcA was

the only uronic acid present in the polysaccharide structure. This was in agreement with results obtained through sugar analysis by Method 2.

The reduced product was much less water-soluble than the original polysaccharide. The decreased solubility suggests that the carboxylic acid groups may play a significant role in the solubilisation of the polysaccharide, through favouring hydration to a greater extent than the less polar hydroxymethyl groups in the reduced product.

3.4 Methylation studies

The preparation of a fully methylated derivative of the polysaccharide could not be readily achieved by the Hakomori procedure[92] only. Thus, the partially methylated product obtained by this method was completely methylated by subsequent treatments according to the method of Purdie and Irvine,[95] 5 times successively. A portion of the fully methylated product (MS) was submitted to LAD-carboxyl-reduction yielding RMS, part of which was re-methylated to give the product MRMS. All three products were fully hydrolysed and the partially methylated sugars were converted to p.m.a.a.'s for analysis by g.l.c. and g.l.c.-m.s. The results are presented in Table II.

TABLE II - METHYLATION ANALYSIS OF *STRELITZIA REGINAE* POLYSACCHARIDE AND ITS DERIVATIVES

Sugar derivative	MS[a]	RMS	MRMS	MDS	RMDS	BDMS
2,3,5-Ara[b]	21[c]	20	15	7	4	25
2,3,4-Xyl	6	5	5	10	6	8
2,3-Xyl	3	3	5	9	8	8[d]
2-Xyl[e]	8	8	7	13	21	17[d]
3-Xyl[e]	5	4	4	8	10	8
Xyl	14	15	8	1	2	12
2,3,4,6-Gal	17	15	23	23	19	17
2,4,6-Gal	5	5	5	5	4	4
2,6-Gal	3	2	3	-	-	-
3,4,6-Man	4	8	8	7	8	-
2,3,4,6-Glc[f]	-	-	8	-	-	-
2,3,4-Glc	-	7	-	-	12	-
2,6-Glc	-	-	5	-	-	-
2-Glc	-	4	-	-	6	-

a MS, methylated polysaccharide; R, reduced; DS, partially-hydrolysed polysaccharide; BD, base-degraded (including trideuteriomethylation)

b 1,4-Di-O-acetyl-2,3,5-tri-O-methylarabinitol, etc.

c Approximate molar proportions. Allowance made for uronic acid in MS and MDS

d Trace only of OD_3 at C-2 only in 2,3-Xyl, but ~50% in 2-Xyl

e Relative proportions estimated by selected-ion monitoring m.s.

f All Glc derivatives the result of carboxyl-reduction

In the hydrolysate of the methylated polysaccharide (MS), arabinose was present exclusively as its 2,3,5-tri-O-methyl derivative, which indicated that the arabinosyl units in S are in the furanosyl form and occur as non-reducing end-groups. Most of the galactosyl groups are terminal, but about one-quarter occur as (1→3)-linked residues. The presence of the latter residues, though characteristic of a large number of plant polysaccharides,[2] is not a common structural feature in substituted xylans. The detection of small amounts of 2,6-di-O-methylgalactose would suggest further substitution of a few (1→3)-linked galactosyl residues at O-4 if it is not attributable to undermethylation at this position. Approximately one-sixth of the Xylp is terminal and a comparable quantity is (1→4)-linked. While all the branched Xylp units are (1→4)-linked, some appear with substituents at O-2 or O-3 as well and the rest are glycosylated at both O-2 and O-3. The relative ratio of the monosubstituted (1→4)-linked Xylp residues indicates a preference for substitution at O-3 rather than at O-2. Comparing the analyses of MS and RMS with MRMS, suggests that undermethylation could account for some of the Xyl detected in the hydrolysate of MS and RMS. The only derivative of mannose detected was the 3,4,6-tri-O-methyl ether implying that all mannosyl residues are (1→2)-linked, as in many

plant gum exudates.[2] A comparison of the analyses of RMS and MRMS indicates that most of the glucuronic acid is terminal with the rest being doubly-substituted through O-3 and O-4. The use of LAD for the reduction of carboxylate ester groups labelled C-6 with two D atoms, thus identifying unequivocally the GlcA as the source of the Glc derivatives shown by g.l.c.-m.s. The mass spectral data for the p.m.a.a.'s in question, which are from the analysis of MRMS, i.e. (a) 1,6,6-trideuterio-1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol and (b) 1,6,6-trideuterio-1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl-D-glucitol, are presented in Fig. 5. The occurrence of (3,4)-disubstituted GlcA residues within a xylan structure is unusual; in most xylans uronic acid groups occupy terminal positions. On the other hand, Dutton and Unrau[105] have, through periodate oxidation studies, found evidence for GlcA substituted at O-4 in apple- and cherry-wood xylans.

3.5 Partial acid hydrolysis

After a series of pilot experiments designed to establish conditions suitable for the removal of Araf units, with minimal degradation of the rest of the polysaccharide, a sample was subjected to partial acid hydrolysis in 5mM H_2SO_4 for 18h at 100°C. This treatment resulted in the isolation of a degraded

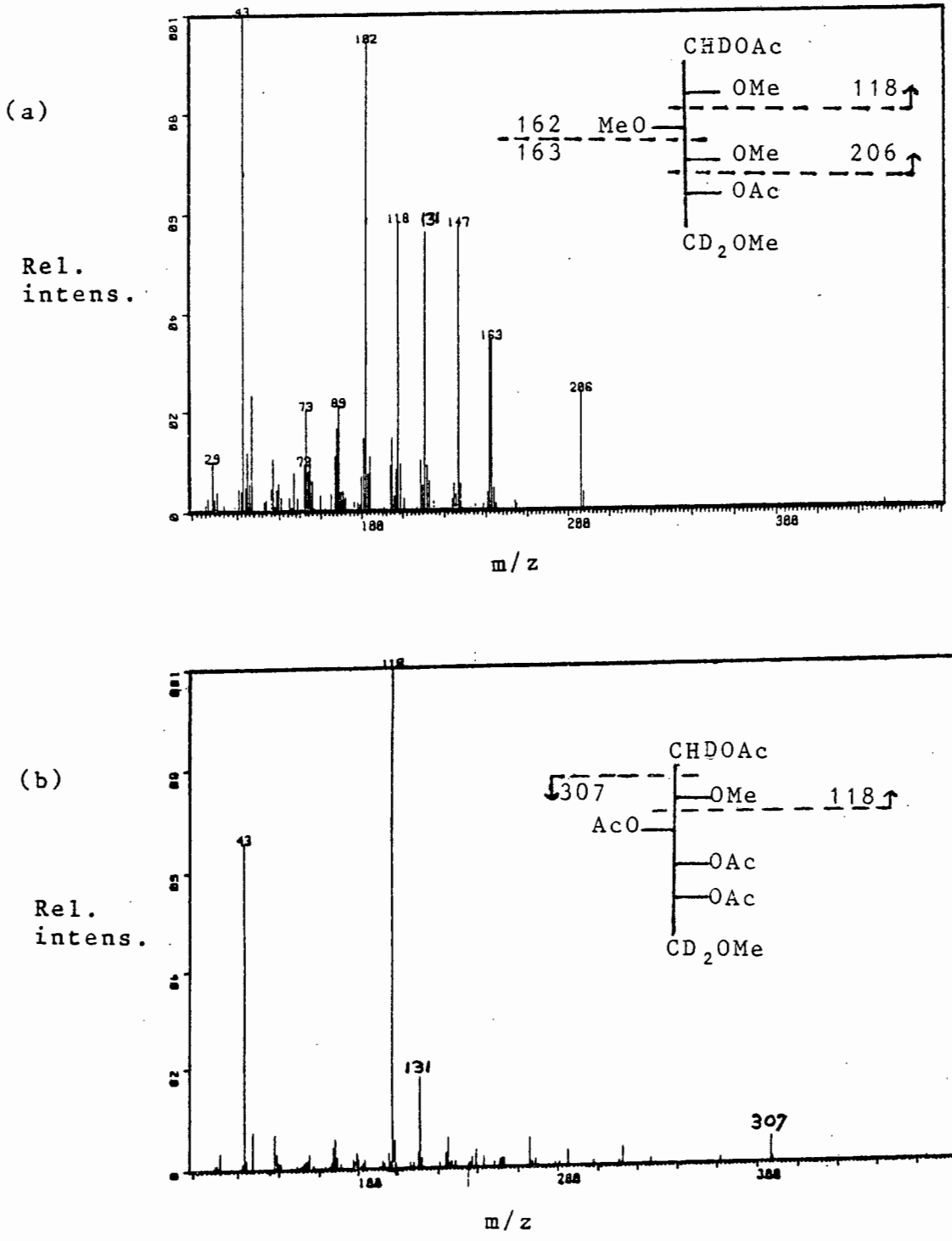


Figure 5 : Mass spectra and some fragmentation pathways of (a) 1,6,6-trideuterio-1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol and (b) 1,6,6-trideuterio-1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl-D-glucitol

polysaccharide (DS), for which the sugar composition and some physical properties are given in Table I. The facile release of arabinose is in agreement with the conclusion reached earlier, that it occurs in the acid-labile furanosyl form. If it is assumed that the Ara groups are, as in most plant polysaccharides, in the L-configuration, the change in $[\alpha]_D$ to a less negative value after partial hydrolysis indicates that the Ara is predominantly α-linked. In addition to the release of Ara, ∿93%, the following percentages of the other constituent sugars were liberated during partial hydrolysis: Gal 12, Xyl 13, Man 10 and GlcA 13. P.c. analysis (solvent A) of the dialysable portion showed that the neutral sugars were present as free monosaccharides, whereas GlcA was detected, as expected, as aldobiouronic acid(s). The relationship between the composition of the degraded polysaccharide found experimentally, and that predicted from loss of Ara only is shown in Table III. From this, it is apparent that, with the exception of Ara, the other sugar constituents are liberated in a proportionally uniform manner.

The degree of homogeneity of DS was determined by chromatography on Sepharose 4B from which it eluted as a single sharp peak (Fig. 6). The estimated $\bar{M}_w$ value of ∿84 000 corresponds to about 1,5% of the expected

TABLE III - COMPARISON OF THE COMPOSITION OF DS WITH THAT EXPECTED FROM LOSS OF ARAf RESIDUES ONLY

		Whole polysaccharide(S)	DS Expected	DS Found
Yield from 101mg			78mg	68mg
wt%	Xyl	34	44	42
	Ara	22	-	2
	Gal	23	29	30
	Man	3	4	4
	GlcA	18	23	22

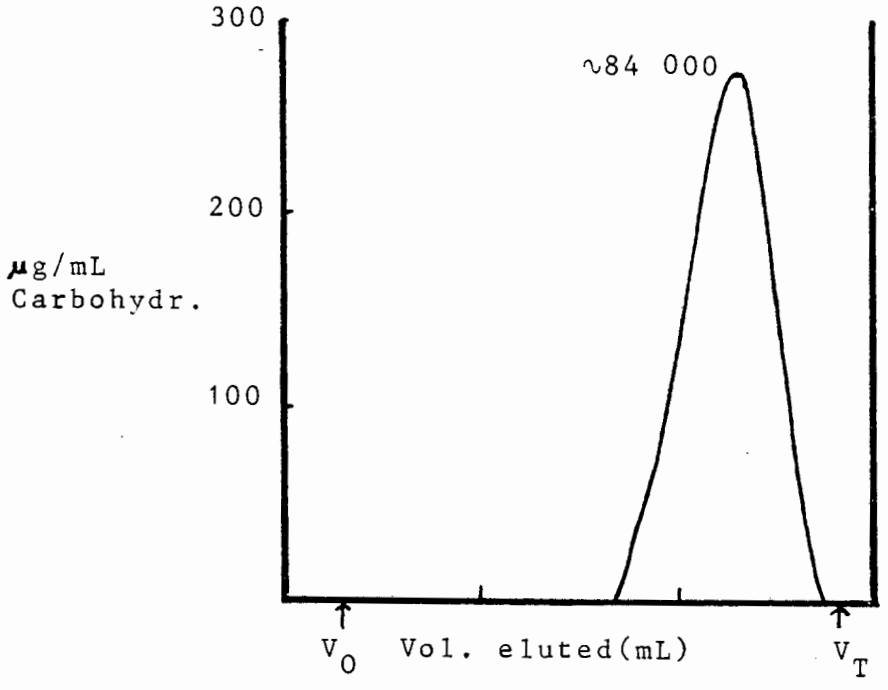


Figure 6 : Chromatography of partially-hydrolysed Strelitzia polysaccharide DS on Sepharose 4B

molecular size based on loss of Ara groups only from a parent polysaccharide of $\bar{M}_w$ ~7 x 10^6. This suggests that scission of the main chain at ~60 points, occurring at approximately equal intervals, accompanied the partial hydrolysis process.

Methylation analysis of DS (see Table II, MDS and RMDS) shows a nearly complete absence of fully-substituted Xylp residues, and a preponderance of monosubstituted (1→4)-linked Xylp. The implication is that much of the Araf occurs as single, terminal groups attached to doubly-branched Xylp in the native polysaccharide. A comparison of the ratio of chain : monosubstituted Xylp residues, particularly in RMS and RMDS, suggests that the doubly-substituted Xylp residues could have Ara in both substituents only if one is a single Araf and the other a different side chain terminated by Araf. From the absence of 3,4-linked Galp in MDS, whereas this was detected in MS, it can be deduced that a small proportion of the Araf groups is joined through O-4 of otherwise (1→3)-linked Galp.

3.6 Base-degradation studies on the methylated polysaccharide (MS)

In order to investigate the location of the uronic acid units, MS was submitted to base-degradation according

to the procedure of Lindberg and Lönngren,[97] which induces β-elimination in the methylated glycosyluronic groups. Removal of the resulting unsaturated uronic acid derivatives by mild hydrolysis was followed by trideuteriomethylation to label exposed hydroxyl groups in the residual polysaccharide structure (BDMS). Hydrolysis and g.l.c.-m.s. analysis of the derived alditol acetates (Table II) showed that CD_3O groups were located predominantly at the 2-O-positions of Xylp residues (Fig. 7). This implies that in the native polysaccharide the GlcA groups are linked to O-2 of Xylp, as is of wide occurrence among plant xylans;[14] furthermore, most of the latter residues also have sugar substituents at both O-3 and O-4, since it was observed that the incorporation of CD_3O groups was greater in the monomethyl than in the dimethyl derivatives of Xylp. There was also evidence for the presence of small amounts of doubly-trideuteriomethylated xylose and xylose trideuteriomethylated at O-3, which may indicate alternative positions of linkage for the uronic acid groups. On the other hand, these derivatives could have resulted artifactually from initial minor undermethylation of the polysaccharide or from a minor partial removal of Araf groups during the preparation of the base-degraded product.

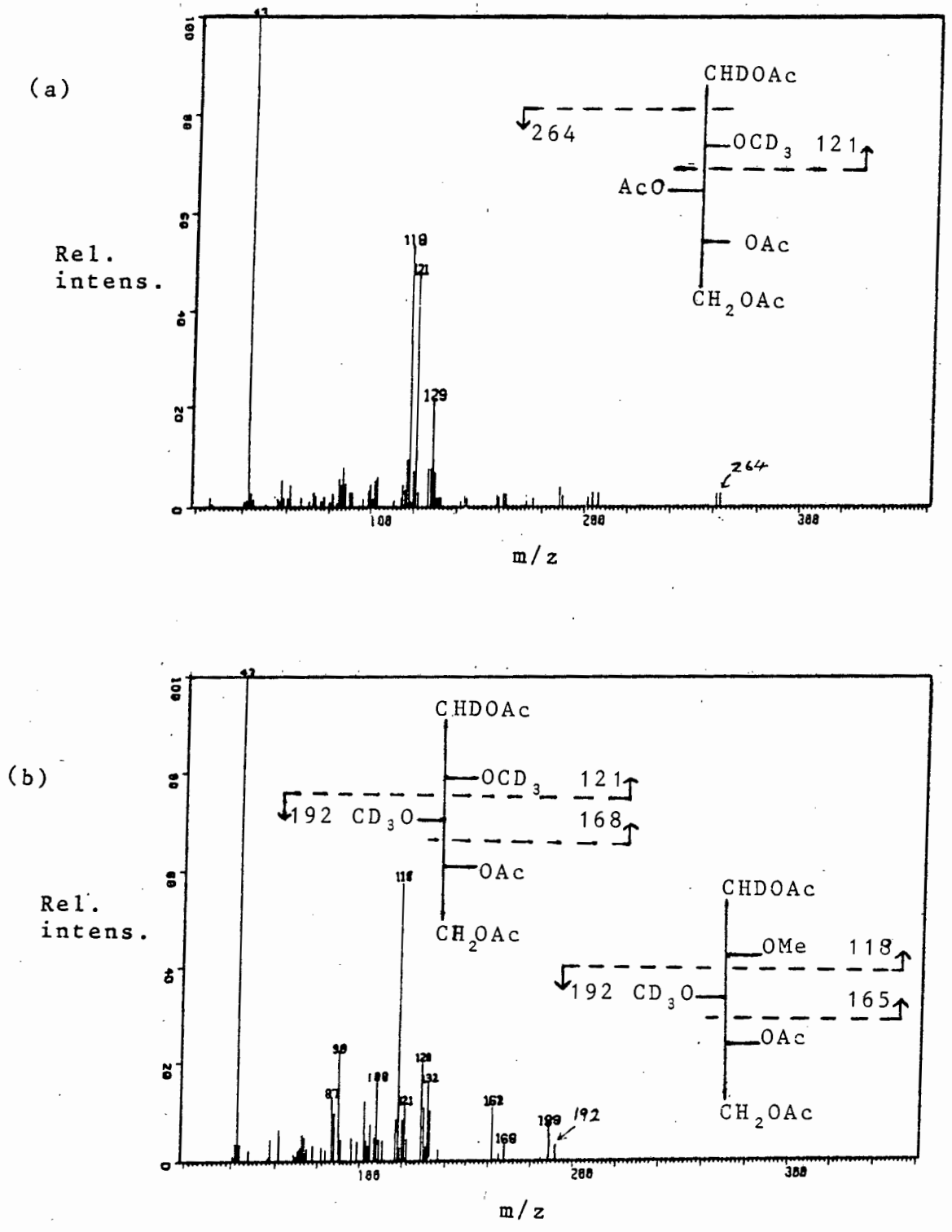


Figure 7 : Mass spectra and expected fragmentations showing the detection of (a) 1,3,4,5-tetra-O-acetyl-2-O-trideuteriomethylxylitol and (b) 1,3,5-tri-O-acetyl-2,3-bis-O-trideuteriomethylxylitol and 1,3,5-tri-O-acetyl-3-O-trideuteriomethyl-2-O-methylxylitol

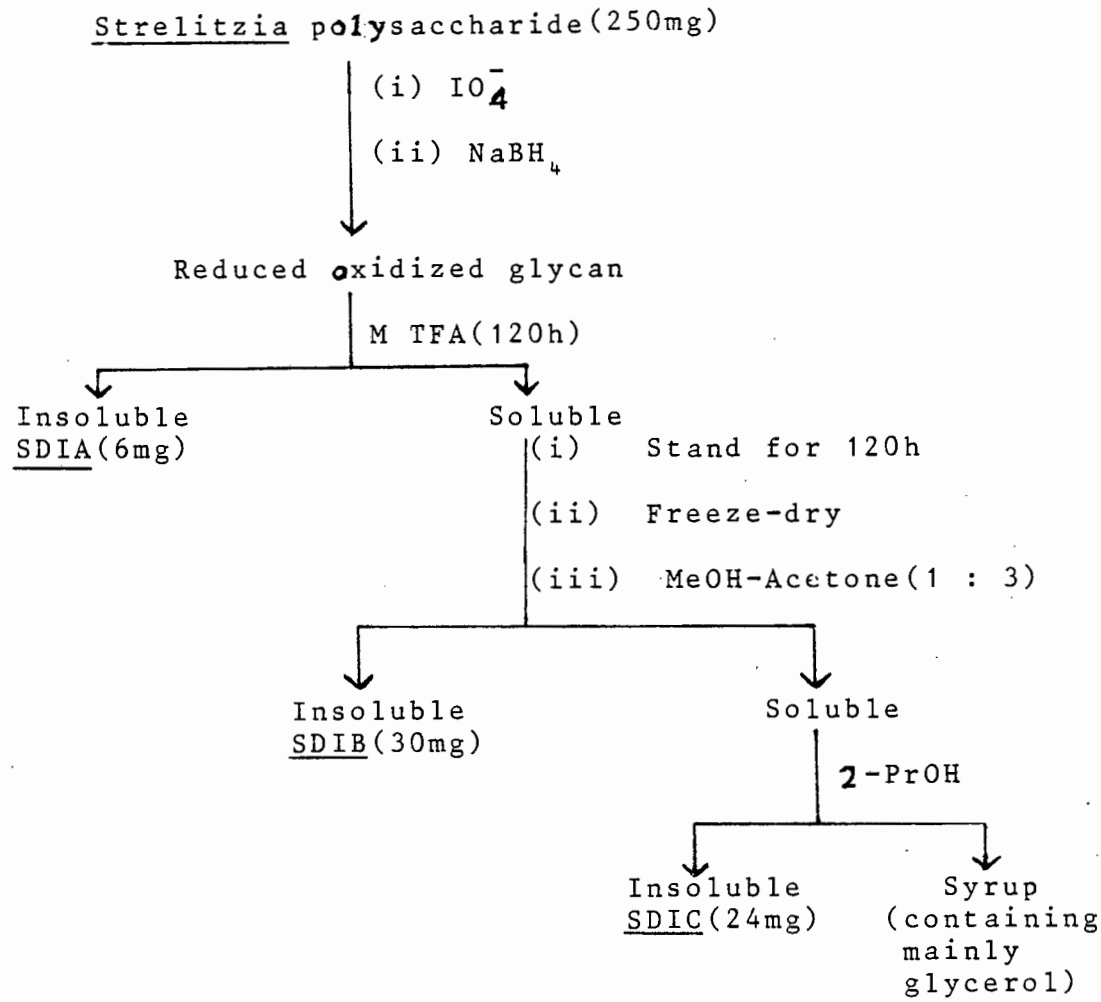
A noteworthy observation in this experiment was the absence of (1→2)-linked Manp residues from BDMS (see Table I), whereas these were detected by methylation analysis of the native and partially-hydrolysed polysaccharide. Since no alternative derivative of Manp was observed, a feasible explanation is that Manp residues were detached along with the modified GlcpA; the structural feature [→2)-Manp-(1→ 4)-GlcpA-(1→] is present within the glucuronomannan portion of many natural polysaccharides,[2] and it is possible that Manp residues (which would largely be decomposed during the β-elimination experiment) are involved in this way, to a limited extent, in the Strelitzia polysaccharide. The aldobiouronic acid group shown above could, of course, be attached to Xylp. The presence of GlcA linked at O-2 of Manp would explain the recovery of Man, upon hydrolysis of the reduced polysaccharide in higher quantities than from the unreduced form.

3.7 Smith degradation studies

The results of methylation analysis of the polysaccharides suggest that about 70% of the Xylp residues are involved in branching. Smith degradation studies were undertaken in order to investigate the extent to which these residues occur contiguously, and

whether there is any regularity in their distribution. Periodate oxidation of the polysaccharide was carried out in duplicate to permit detailed study of the products (X) from one experiment, while those (Y) from the other were submitted to a second Smith degradation. The consumption of periodate by the polysaccharide averaged 0,82 mole per sugar residue, the value calculated from the proportion of oxidisable sugar residues as revealed by methylation analysis being 0,9. The reduced, oxidised products were treated with M TFA at ∿20°C, the hydrolysis process being monitored by observing the change in $\bar{M}_w$ with time. Details for the further processing of X are summarised in scheme 3.

Analysis of X: The Smith-degraded product was fractionated as indicated in scheme 3 into three parts (SDIA, SDIB and SDIC) for which some physical and chemical properties are given in Table I, and methylation analysis data in Table IV. The insolubility of SDIA which precluded its examination by s.e.c., is consistent with its having a fairly linear homoxylan structure, as is shown by methylation analysis; such insolubility can be explained by a favourable strong association of the linear molecules as extended ribbonlike structures through hydrogen bonding. Examination of SDIB and SDIC by s.e.c. on Bio-Gel P-10 showed that each consisted of a range of



SCHEME 3 : First Smith degradation of Strelitzia polysaccharide and fractionation of SDI products

TABLE IV - METHYLATION ANALYSIS OF PRODUCTS OF SMITH DEGRADATION

Sugar derivative	MSDIA[a]	MSDIB	MSDIC	RMSDIC[b]
2,3,4-Xyl[c]	1[d]	1	1	1
2,4-Xyl	-	-	1	1
2,3-Xyl[e]	9	8	2	2
3,4-Xyl[e]	-	-	-	1
2-Xyl[e]	0,35	0,70	0,68	0,62
3-Xyl[e]	0,65	1,30	0,32	0,38
2,3,4,6-Gal	-	1	2	2
2,4-Glc	-	-	-	1

a MSDIA, methylated SDIA

b R, reduced

c 2,3,4-Xyl = 1,5-Di-O-acetyl-2,3,4-tri-O-methylxylitol, etc.

d Numbers represent approximate molar proportions, by g.l.c.

e Relative proportions estimated by selected-ion monitoring m.s.

components differing in molecular-weight (Fig. 8). The results of analysis of SDIA, SDIB and SDIC, indicate that the core structure of the polysaccharide consists of a backbone, composed mainly of (1→4)-linked xylosyl residues, but at least three domains differing slightly in structural features can be recognised. That the Xylp residues (presumed D) are linked in the β-configuration is inferred from the negative sign of the specific rotations measured for SDIB and SDIC. Such domains may be broadly represented as follows, occurring in the approximate relative proportions A:B:C = 1:4:4 in the polysaccharide; the aforementioned relative proportions are based on the individual yields obtained of each SDI product,

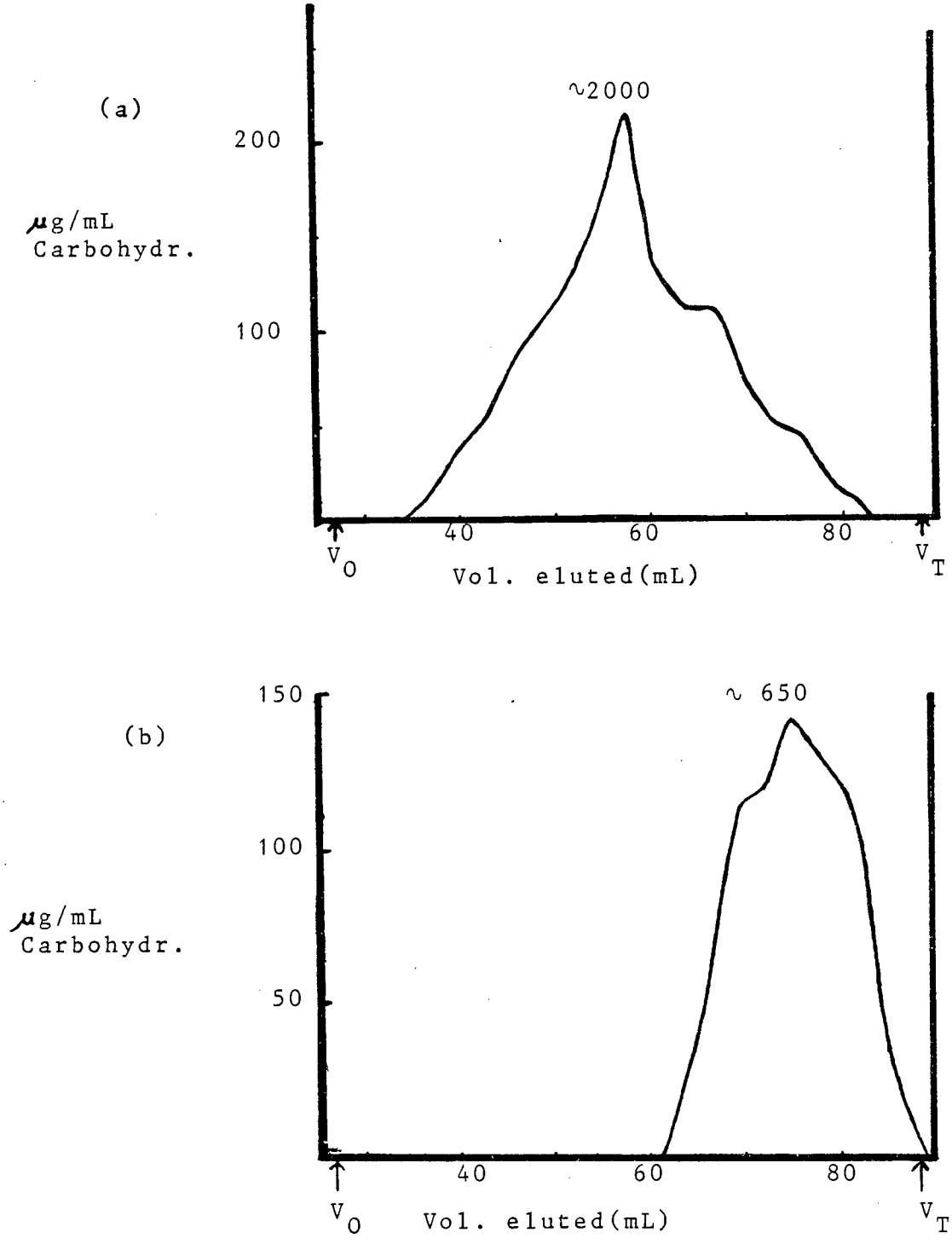


Figure 8 : Chromatography on Bio-Gel P-10 of Smith degradation products (a) SDIB and (b) SDIC

A: $[\text{-4)-Xyl}p\text{-(1-}]_9$ with -4)-Xyl$p$-(1- dispersed among the chain units

2/3
↑
Xyl$p$

B: $[\text{-4)-Xyl}p\text{-(1-}]_8$ with -4)-Xyl$p$-(1- and

2/3
↑
Gal$p$

-4)Xyl$p$-(1- dispersed among the chain units

2/3
↑
Xyl$p$

C: Xyl$p$-(1→4)-Xyl$p$-(1→4)-Xyl$p$-(1-

2↑
Gal$p$-(1→3)-Glc$p$A

and Xyl$p$-(1→4)-Xyl$p$-(1-

3 2/3
↑ ↑
Gal$p$ Xyl$p$

while the sugar-linkage pattern is proposed on the basis of methylation analysis of the SDI products.

The models postulated for domain C are quite arbitrary as a number of different structures for such oligosaccharide units of similar sizes and linkages can be drawn out. That the periodate-immune glucuronic acid residues carry no substituents at O-4 is evidence in support of the proposition that such residues maybe joined to periodate-susceptible (1→2)-linked mannosyl residues through these positions in the native polysaccharide. The characterisation of 3-O-substituted Glc$p$A was achieved by g.l.c.-m.s. analysis

of p.m.a.a.'s derived from reduced, methylated SDIC (RMSDIC) and identification of 1,6,6-trideuterio-1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl-D-glucitol (Fig. 9).

The survival of a large proportion of xylosyl residues after periodate oxidation implies that in the native polysaccharide most of these are substituted at O-2, O-3 or both, by groups such as terminal Araf, Galp or GlcpA. Furthermore, the survival of some galactosyl units indicates that a proportion of them are substituted at the least at O-3, as are the glucuronic

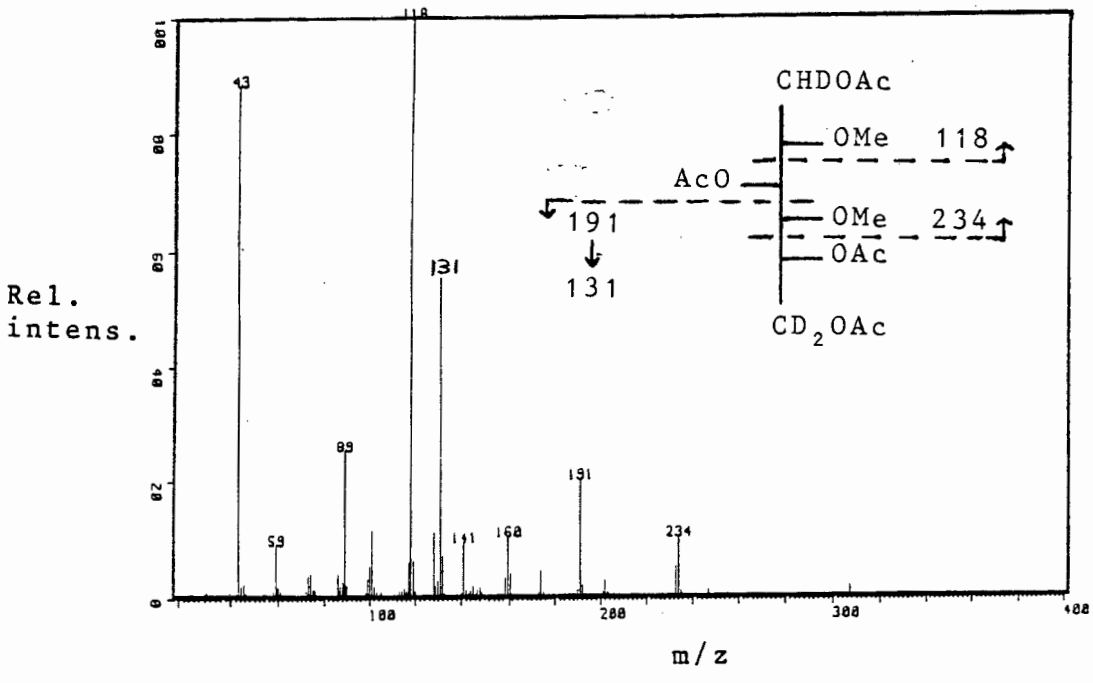


Figure 9 : Mass spectrum and expected fragmentations of 1,6,6-trideuterio-1,3,5,6-tetra-O-acetyl-2,4-di-O-methylglucitol

acid residues. All of these conclusions are in agreement with the results of methylation analysis. The presence of GlcpA in SDIC only and the distribution of Galp in SDIB and SDIC might suggest a certain degree of localisation of these periodate-resistant groups in the polysaccharide structure. As expected, all Araf and Manp units are destroyed by Smith degradation.

Analysis of Y: The Smith-degraded product, after separation from glycerol and other products soluble in 2-PrOH, was subjected to a second Smith degradation. The final total consumption of periodate, 1 mole per sugar residue, agreed with the value calculated from the results of methylation analysis. The Smith-degraded product SDII had $\bar{M}_w$ of 240, estimated from s.e.c. on Bio-Gel P-2 (Fig. 10) which suggested that the SDI products had been reduced to a size between those of a monosaccharide and a disaccharide. Sugar analysis (g.l.c.) showed that the product contained xylose as the only neutral sugar component. The survival of xylose is consistent with the presence in the SDI products of small proportions of xylosyl residues substituted at O-2 or O-3. No galactosyl units survive as all occupy terminal positions. Uronic acid, detected on p.c. analysis of an hydrolysate of SDII is to be expected owing to its presence in the SDI product as a 3-O-substituted unit.

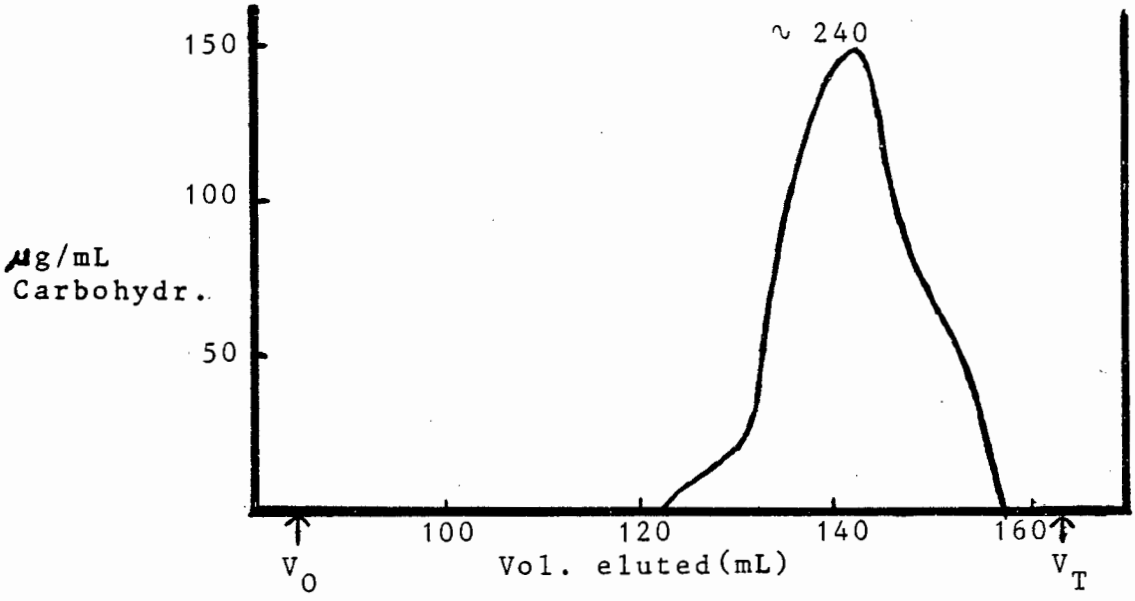


Figure 10 : Chromatography of SDII on Bio-Gel P-2

In polysaccharides of high uronic acid content the acid units have been shown to be somewhat resistant to periodate attack despite the presence of a 1,2-diol system.[106] To obtain further confirmation that the survival of uronic acid during periodate oxidation of the native polysaccharide was not due to this phenomenon but to protection of the uronic acid units by branching, a carboxyl-reduced sample (incorporating D) was periodate-oxidised. Sugar analysis (g.l.c.) of the product (ORS, Table I) showed the presence of a glucose derivative incorporating deuterium atoms, indicating that it was originally derived from

glucuronic acid residues in the native polysaccharide. Thus, both the IO_4^- consumption and the proportions of sugar residues surviving the oxidation remain substantially unchanged for the carboxyl-reduced sample.

3.8 Conclusion

From the results of this investigation certain structural features of the polysaccharide can be postulated. Like most plant xylans, the polysaccharide consists of a backbone structure composed mainly of β-(1→4)-linked Xyl*p* residues. The majority of these residues are substituted at positions *O*-2 and/or *O*-3, those that are singly-substituted at *O*-2 or *O*-3 and those substituted at both positions being present in approximately equal proportions. Most of the substituents consist of single, non-reducing terminal Ara*f*, Gal*p*, or Glc*p*A groups. Direct linkage of Glc*p*A units to the xylan backbone is predominantly through the 2-*O*-position of Xyl*p* chain residues. The polysaccharide also contains a small proportion of side-chains that are unusual in a substituted xylan, in that they consist of such units as (1→3)-linked Gal*p*, (1→2)-linked Man*p* and Glc*p*A substituted at both *O*-3 and *O*-4. Indirect evidence, from base-degradation studies of the methylated polysaccharide and Smith

degradation of the native polysaccharide, suggests that the last two groups occur contiguously, resembling a glucuronomannan-type unit, i.e. [→2)-Manp-(1→4)-GlcpA-(1→], which may be terminated by GlcpA.

Smith degradation studies (section 3.7) have also shown that at least three domains having slightly different structural features can be distinguished in the polysaccharide chain structure. Thus, while all three domains might have a similar distribution of side-chains consisting of one terminal sugar unit, the main difference would be in the distribution and nature of the side-chains consisting of two or more sugar units, and probably the length of the backbone part of the domain. Of the latter type of side-chains, three kinds are recognisable, i.e. those that are linked to the xylan backbone through (a) Xylp, (b) Galp and (c) GlcpA units. Therefore, the domain represented by SDIA has only type (a) side-chains, there being one for every ten chain residues. SDIB represents a domain where for every ten chain residues there is one each of types (a) and (b) side-chains whereas SDIC corresponds to a region in which for every five chain residues there is each of types (a), (b) and (c). Such domains would be separated by unsubstituted periodate-susceptible (1→4)-linked Xylp residues in the native polysaccharide. From the foregoing argument and the

relative proportions in which SDIA, SDIB and SDIC occur, it is apparent that any structural repeating unit postulated for the polysaccharide would have to include a very large number of sugar units.

In its high degree of substitution, the xylan resembles certain others from plant species classified in such orders as Iridales[57,58] and Laurales.[61-63] Unlike the Watsonia polysaccharide[57] and other complex xylans,[51,52] where side-chains such as Galp-(1→3)-Araf-(1→ occur in significant amounts, the Strelitzia xylan has all its Araf occupying terminal positions. Most of the Galp is terminal with a small proportion (1→3)-linked. While GlcA often occurs accompanied by its 4-O-methyl ether in acidic xylans,[14] this has not been found in the present investigation.

3.9 Experimental

3.9.1 Isolation and purification of the polysaccharide

Gum exudate from the corolla ducts of Strelitzia reginae flower heads (collected from Kirstenbosch Botanical Gardens, Cape Town, South Africa, in 1982) was isolated by extrusion, followed by cold water extraction. After homogenisation of the exudate with water, the suspension was centrifuged to remove

insolubles, and the supernatant was freeze-dried. Extraction of the freeze-dried material with 80% ethanol was shown by p.c. (solvent A) to separate the ethanol-insoluble polysaccharide fraction from sucrose, glucose and fructose. The residue was thus redissolved in water and the polysaccharide precipitated by the addition of ethanol up to 80% final concentration, the precipitate being recovered by centrifugation. The process was repeated three times (yield, 2,5g of freeze-dried material).

The crude polysaccharide was purified as follows: (Scheme 1) To a 1% aqueous solution (250mL) of the polysaccharide was added a 10% aqueous solution (125mL) of cetyltrimethylammonium bromide, resulting in immediate formation of a flocculent precipitate. After standing in the refrigerator overnight, the precipitate was recovered by centrifugation. Dissolution of the precipitate in 2M NaCl (250mL) was followed by centrifugation and subsequent addition of ethanol to the supernatant to a final concentration of 80% in order to precipitate the sodium salt of the acidic polysaccharide. The acid form was regenerated by dissolution of the precipitate in 2M acetic acid. Dialysis of the resulting solution for 3 days, followed by freeze-drying, afforded 2,1g of straw-yellow material.

Similar fractionation of material extracted from the flower heads with cold water resulted in very low yields (< 20%) of the polysaccharide, probably due to greater contamination with other water-soluble materials from the flower tissue. The average yield was ∿20mg of purified polysaccharide per flower head.

3.9.2 Partial acid hydrolysis

A dried polysaccharide sample (101mg) in 5mM H_2SO_4 (10mL) was heated in a boiling water bath for 18h, the progress of hydrolysis being monitored polarimetrically (Fig. 11). The optical rotation increased rapidly from

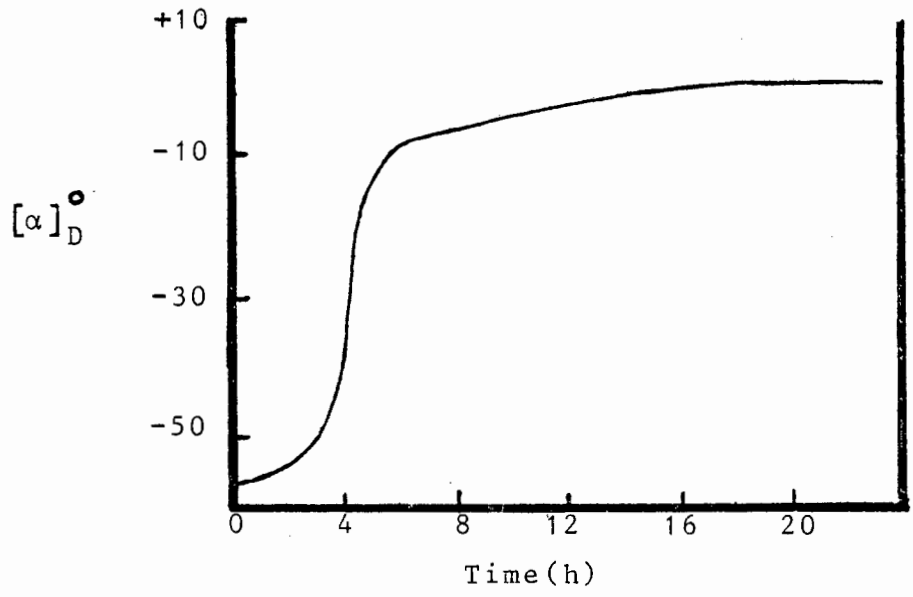


Figure 11 : Partial acid hydrolysis of *Strelitzia* polysaccharide; change in $[\alpha]_D^\circ$ with time on heating at 100°C in 5mM H_2SO_4

-56° to -8° over the first 6h and finally levelled out at +2° after 18h. The solution was cooled, neutralised with $BaCO_3$ and centrifuged. The supernatant was subsequently dialysed against regularly-changed distilled water for 5 days. The dialysable portion was concentrated to a small volume and examined by p.c. The non-dialysable material, recovered by freeze-drying (yield, 68mg) was further examined by s.e.c. on Sepharose 4B and analysed for sugar composition as previously described.

3.9.3 Smith degradation

Two portions of the polysaccharide (250mg each) were separately oxidised, each in 0,22M aqueous $NaIO_4$ solution (50,1mL) in the dark at room temperature. The periodate consumption was monitored by removal of aliquots (0,1mL) at intervals followed by titration with arsenite. After 20h the consumption became constant. Ethane-1,2-diol was added to the reaction mixtures in order to destroy excess IO_4^-, after which they were dialysed against distilled water for 72h. The oxidised polysaccharide samples were reduced with an excess of $NaBH_4$ over 72h. The pH was adjusted to 6 by the addition of acetic acid and the mixtures were freeze-dried. Boric acid was removed by successive treatments with methanol followed by evaporation of

methyl borate at 40°C under reduced pressure. The reduced, oxidised products were isolated via formation of their acetate esters (sodium acetate/acetic anhydride), extraction into chloroform and subsequent deacetylation ($NaOCH_3$, in chloroform-methanol, 1:1 v/v). Hydrolysis of the labile acetal linkages in the deacetylated products was achieved by treatment with M TFA (12,5mL to each) at room temperature. Aliquots (0,5mL) were removed at 48h intervals, neutralised with $NaHCO_3$ and examined by s.e.c. After 5 days, the initially clear solutions had become turbid. From this point the two reaction mixtures (called X and Y) were processed differently.

Examination of X: (See scheme 3)

Centrifugation resulted in separation of a precipitate, which was suspended in water and freeze-dried, affording 6mg of solid material (fraction SDIA). Examination of an aliquot of the supernatant solution by s.e.c. on Bio-Gel P-10 gave peaks, not fully resolved, at positions corresponding to molecular weights of approximately 1100 and 560. Similar behaviour was observed after the solution had been kept in acid for a further 5 days. The solution was then freeze-dried to give a syrupy product which was fractionated with methanol-acetone (1:3 v/v), yielding

30mg of insoluble solid material (fraction SDIB) and a soluble syrup. Further fractionation of the syrup with 2-propanol yielded 24mg of an insoluble solid product (fraction SDIC) and a soluble syrup which was shown by p.c. (solvent E) to contain predominantly glycerol. Each of the above fractions and their respective methylated products were analysed for sugar composition.

Examination of Y:

After 10 days at room temperature the reaction mixture was freeze-dried and the residue was fractionated with 2-propanol. The insoluble fraction (∿60mg) was oxidised in 0,2M aqueous $NaIO_4$ solution (12mL). The periodate consumption became constant after 120h (3,3mmoles per g carbohydrate, expected value 6,7). Excess IO_4^- and IO_3^- ions were removed by addition of a calculated amount of barium acetate, and the precipitated Ba salts were removed by centrifugation. Reduction was carried out by addition of an excess of $NaBH_4$. On the assumption that the low IO_4^- uptake was due to hemiacetal formation [107] the reduced, oxidised product, isolated via the acetate as described above, was submitted to further oxidation followed by reduction. The IO_4^- uptake became constant after 140h (3,5mmoles per g carbohydrate). The $NaBH_4$ -treated

reaction mixture was acidified to pH 6 with acetic acid followed by immediate freeze-drying; boric acid was removed as described above, and residue was dissolved in M TFA (10mL). Aliquots (1mL) were removed at 48h intervals for examination on Bio-Gel P-2. After 96h the solution was freeze-dried. The product was isolated via its acetate ester.

3.9.4 Data obtained from other experiments:

	Starting material	Mass taken(mg)	Yield (mg)	$[\alpha]_D$ (degrees)
	Whole poly-saccharide	98	111	-68
Methyl-ations	DS	20	22	-35
	SDIB	10	11	-122
	SDIC	15	12	-32
Base de-gradation	MS	10	7	n.d.
Carboxyl reduction	S	20	16	n.d.

CHAPTER 4

4. NON-CELLULOSIC β-D-GLYCANS FROM LEAVES OF SISAL (AGAVE SISALANA)

4.1 Introduction

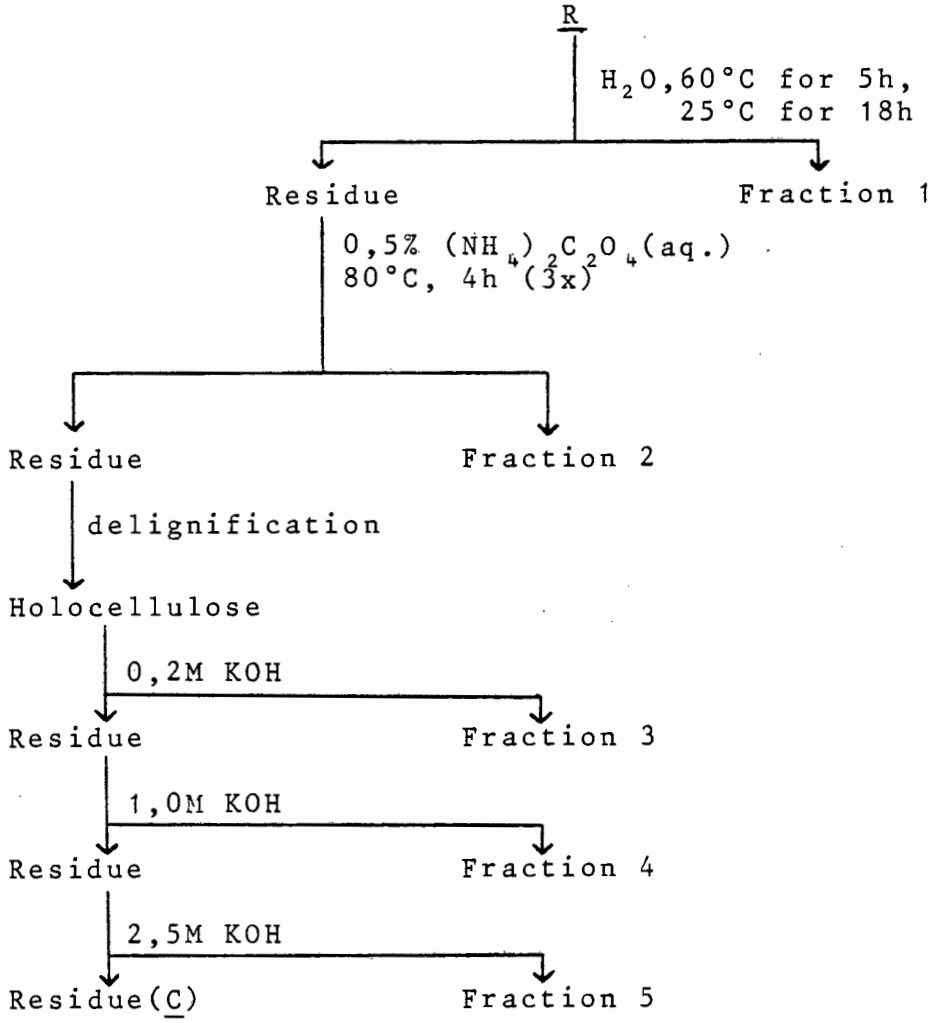
The hemicelluloses and pectins of leaves of sisal (Agave sisalana Amaryllidaceae; Liliales) are of potential significance as components of agricultural waste, one of the main purposes for growing sisal being to recover from the juice a range of saponins. The leaves are also well recognised as a source of commercial fibre, known as cordage fibre and are a source of hemicelluloses and other polysaccharides. In the present study the hemicellulosic composition of sisal has been compared to that of bagasse from sugar cane (Saccharum officinarum, Gramineae; Poales). While both are monocotyledonous angiosperms, sisal and sugar cane belong to the orders Liliales and Poales respectively. According to the classification of angiosperms showing probable interrelationships of the orders,[1] it might be expected that there could be some relationship between the hemicellulosic compositions of sisal and sugar cane bagasse. Hemicelluloses from the latter are of importance in view of the interest in obtaining fermentable carbohydrate materials for the production of

alternative fuels. Previous studies of these hemicelluloses have shown them to be rich in arabinoxylans.[108]

4.2 Extraction and preliminary examination of the polysaccharides

Segments of leaf obtained in the spring of 1981 from a sisal plant grown in North-eastern Natal, South Africa, were extracted with methanol leaving a white residue (7,4% of the original weight of the leaves). This residue could be separated into two physically different portions. One was of the fleshy type, made of ground tissue while the other was mainly fibrous tissue. These were recovered in a mass ratio of 9 : 7 respectively.

Pretreatment of both the fibrous (F, 26g) and fleshy (G, 34g) materials involved further extraction with ethanol-benzene (1 : 2, v/v) in order to remove residual pigments and lipid components. The dried insoluble residual material, designated R, in both cases, (23,1 and 29g, respectively) was sequentially extracted with water, aqueous ammonium oxalate, and increasing concentrations of KOH, as depicted in the flow-chart in scheme 4, in order to achieve a primary fractionation of the hemicellulosic polymers. An intervening delignification step was necessary as both



Scheme 4 : Flow-chart for the fractionation of the polysaccharides of sisal leaves after extraction with organic solvents. The fractions are labelled F1-FC in Table V and G1-G5III in Table VI.

materials contained appreciable amounts of lignin (12 and 15% for F and G respectively). The monosaccharide compositions of the polysaccharide fractions recovered from the extracts after freeze-drying are given in Tables V and VI. The richness in xylose of the fractions obtained from F in high yields indicated that the major hemicellulosic components are xylans, as is shown later. The monosaccharide compositions of the fractions from G suggested a certain degree of complexity in terms of hemicellulosic polymer content to the extent that it was essential to carry out some preliminary methylation studies of each fraction in order to acquire more information about their polysaccharide content. Studies undertaken by Aspinall and Cañas-Rodriguez[109] on a fraction corresponding to G2 from another plant of the same species have led to its characterisation as pectic material and therefore, in the present study, investigation of this fraction has been confined to determination of the monosaccharide composition. Since no purification of this pectic material was undertaken, there is an understandable discrepancy between the monosaccharide proportions reported by the aforementioned workers and those presented here.

TABLE V - CHARACTERISTICS OF POLYSACCHARIDE FRACTIONS (F) FROM SISAL FIBRE (see Scheme 4)

Fraction	Yield (g)	Total[a] carbohydr. content (g)	$[\alpha]_D$	Monosaccharide composition[b]						%N
				Rha	Ara	Xyl	Gal	Glc	UA[c]	
F1	0,21	0,18	+38°	11	24	15	9	3	38	1,1
F2	0,75	0,70	n.d.[d]	1	7	19	18	6	50	0,9
F3	1,73	1,67	-50°	-	-	88	0,9	0,9	10	0,2
F4	1,76	1,69	-46°	T[e]	-	90	T	0,7	9	-
F5	0,25	0,22	-45°	T	-	88	T	0,5	11	-
FC[f]	12,73	12,50	n.d.	-	-	4,3	-	95	0,7	-

a Determined by the phenol-sulphuric method[85]

b Determined as mole %, using method 1 (section 2.6) for neutral sugars

c Uronic acid determined by the 3-hydroxydiphenyl method[89]

d Not determined

e Trace

f Sugar composition determined as described in section 4.6.2

TABLE VI - CHARACTERISTICS OF POLYSACCHARIDE FRACTIONS (G) FROM SISAL FLESH (see Scheme 4)

Fraction	Yield (g)	Total[a] carbohydr. content (g)	$[\alpha]_D$/°	Monosaccharide composition[b]								
				Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA[c]	%N
G1	1,71	1,34	-2	9	-	30	5	3	40	11	2	2,6
G2	8,62	5,44	n.d.[d]	7	-	5	3	2	22	4	57	5,1
G3	0,19	0,12	-21	14	-	18	55	-	11	-	2,7	5,7
G4	0,35	0,30	-20	9	5	16	49	3	3	14	2,7	0,7
G5	0,59	0,51	+15	3	5	7	37	3	9	34	3,9	-
GC[e]	10,31	9,87	n.d.	6	T[f]	7	11	T	5	63	8,1	-
GC				10	2	14	27	4	6	27		
	Yield (mg)											
G3I	25		-36	7	-	8	76	-	-	-	8	1,8
G4I	86		+20	-	7	7	20	6	12	48	-	-
G4II	120		-25	11	-	12	61	1	2	5	11	-
G5I	279		+45	-	5	3	36	-	10	46	-	-
G5II	48		-5	-	2	6	60	-	6	23	3	-
G5III	138		+17	20	-	21	26	-	9	-	25	-

a Determined by the phenol-sulphuric acid method[85]

b Determined as mole%, using method 1 (section 2.6) for neutral sugars

c Uronic acid determined by the 3-hydroxydiphenyl method[89]

d Not determined

e Sugar composition determined as described in section 4.6.2

f Trace

4.3 The polysaccharide composition of sisal fibrous material

As indicated in the previous section, the major polysaccharide in the fibrous material has been found to be a xylan, and this was therefore selected for detailed studies. The apparent similarities in monosaccharide compositions and specific rotations of F3, F4 and F5 prompted initial parallel studies of the three polysaccharide fractions. The very low nitrogen content indicated that protein, if present, occurred only in insignificant amounts. When precipitated with ethanol from aqueous solutions, all three polysaccharide fractions gave gelatinous precipitates, but when precipitated from aqueous alkaline solutions, they yielded fibrous, flocculent precipitates. A feasible explanation for this difference in behaviour could be a tendency towards greater solvation of the polysaccharide with ethanol molecules in the aqueous medium (pH ~6) than would be the case in alkaline medium, where the polysaccharide occurs as a salt which would interact more strongly with water molecules.

After each fraction had been purified as its cetyltrimethylammonium complex,[103] xylose was found to be the only neutral sugar present. Furthermore, examination of each purified fraction by s.e.c. showed

similar behaviour as illustrated in Fig. 12(a). The main component had $\bar{M}_w$ ∿150 000, with another peak appearing at the void volume, which was assumed to be due to aggregation favoured by the extended ribbon-like structure of linear xylans, having a low degree of branching,[80] since only a single peak was observed on chromatography of each polysaccharide fraction on DEAE-cellulose column (Fig. 12(b)). The specific rotations of the fractions were more similar (∿-52°) after the purification steps. Thus, further structural studies were pursued using only F3 as substrate.

4.3.1 Methylation studies of F3

Investigation of the monosaccharide linkages in F3 entailed methylation of the purified polysaccharide by the Hakomori procedure[92] and subsequently by the Purdie method[95] (3 times). When no hydroxyl absorption could be observed by i.r. spectroscopy a portion of the methylated product MF3 was hydrolysed for the analysis of p.m.a.a.'s, while the rest was subjected to LAD-reduction, yielding RMF3, part of which was submitted to remethylation to give MRMF3. Sugar analyses of the derived products are presented in Table VII. From the preponderance of 2,3-di-O-methylxylose in the hydrolysate of MF3, it could be deduced that F3 is mainly a linear, (1→4)-linked

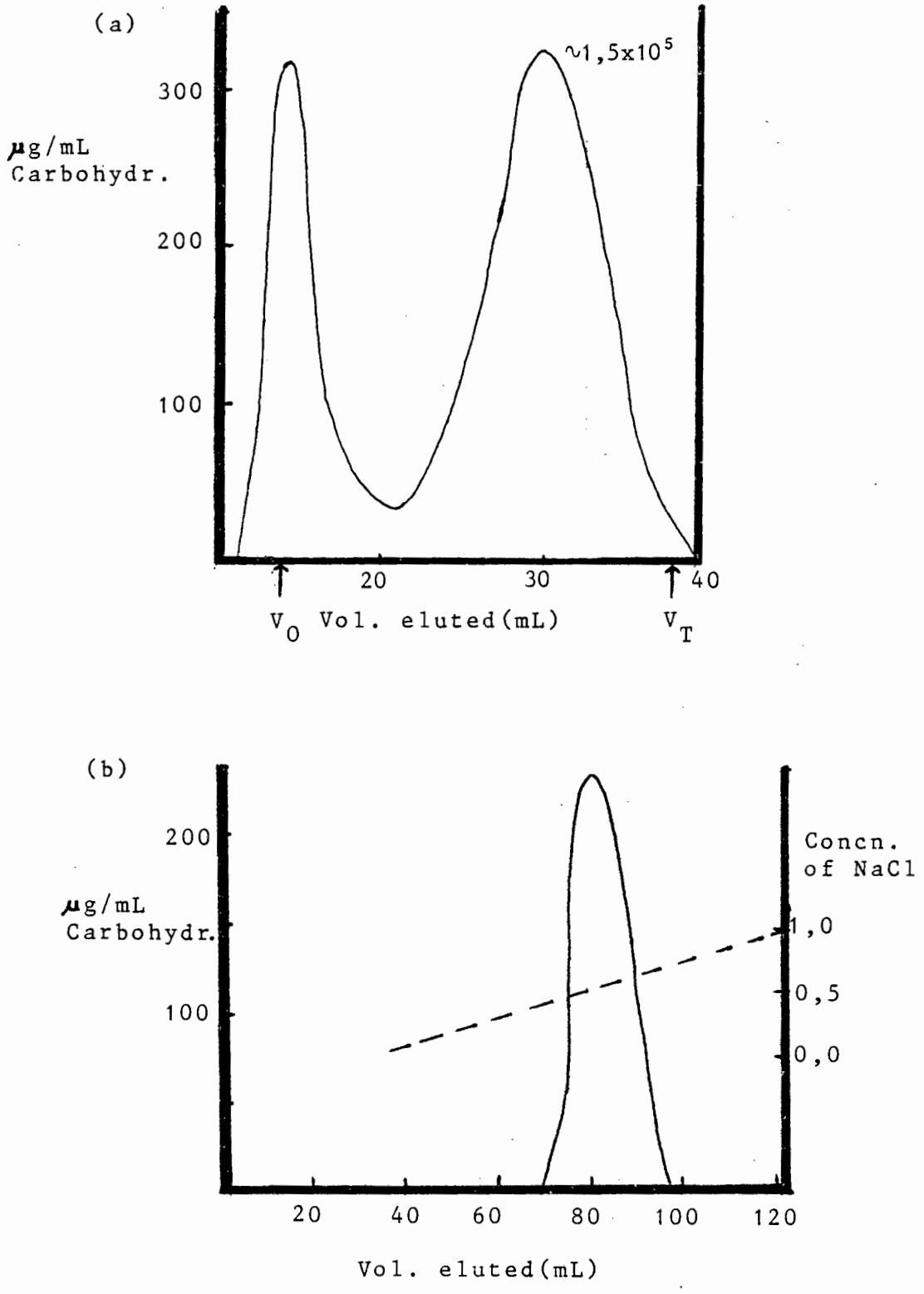


Figure 12 : Column chromatography of sisal xylan F3 (a) on Sepharose 4B and (b) on DEAE-cellulose

TABLE VII - METHYLATION ANALYSIS OF POLYSACCHARIDE FRACTIONS FROM SISAL FIBRE (F) (see Scheme 4)

Sugar derivative	MF3[a]	RMF3	MRMF3	BDMF3	EDO BDMF3	MRMF1
2,3,5-Ara[b]	-	-	-	-	-	6[c]
2,3-Ara	-	-	-	-	-	16
2,3-Me-4-Et-Xyl	-	-	-	-	8	-
2,3,4,-Xyl	10	5	8	10	5	1,6
2,3-Xyl	73	68	66	81[d]	80	14
2-Xyl[e]	6	6	5	5	3	2
3-Xyl[e]	11	12	11	4	4	4
3,4-Rha	-	-	-	-	-	6
3-Rha	-	-	-	-	-	4
2,3,4,6-Gal[f]	-	-	-	-	-	5
2,3,4-Gal	-	-	-	-	-	2
2,3,6-Gal[f]	-	-	-	-	-	21
2,4,6-Gal	-	-	-	-	-	5
2,3-Gal	-	-	-	-	-	0,7
2,4-Gal	-	-	-	-	-	6
2,6-Gal	-	-	-	-	-	0,7
2-Gal	-	-	-	-	-	0,6
2,3,4,6-Glc	-	-	10[g]	-	-	4
2,3,4-Glc	-	9[g]	-	-	-	2
2,4,6-Glc	-	-	-	-	-	2

a MF3, methylated polysaccharide F3; R, reduced; BD, base degraded including trideuteriomethylation, EDO, ethylated, degraded, oxidised

b 1,4-Di-O-acetyl-2,3,5-tri-O-methylarabinitol, etc.

c Approximate molar proportions

d Includes ca 10% of 1,4,5-tri-O-acetyl-2-O-trideuteriomethyl-3-O-methylxylitol

e Relative proportions estimated by selected-ion monitoring m.s.

f Trace incorporation of 2 D-atoms at C-6 of 2,3,4,6-Gal, about 80% incorporation in 2,3,6-Gal

g Glc derivatives are the result of carboxyl-reduction

xylan, the negative specific rotation of the original polysaccharide suggesting β-linkage of the D-xylosyl residues. The methylation results also indicated that in addition to substitution with one xylosyl group for every ten xylosyl residues along the main chain, there is further glycosylation to the same extent with uronic acid groups. The uronic acid groups were shown later to be predominantly 4-O-methylglucuronic acid units including, probably, traces of GlcA groups. Base-degradation of the methylated polysaccharide followed by trideuteriomethylation of the exposed hydroxyl groups (BDMF3), and subsequent analysis of p.m.a.a.'s derived from an hydrolysate by g.l.c.-m.s., indicated that the uronic acid groups are linked through the 2-O-position of Xylp residues (Fig. 13 and Table VII). The similar relative proportions of the monomethylated xylosyl residues in BDMF3 indicated that there was no special preference between O-2 and O-3 for the position of attachment of the xylosyl side-groups.

4.3.2 ^{1}N.m.r. studies of F3

The sisal xylan, F3, was further studied by n.m.r. spectroscopy. The ^{1}H- and ^{13}C-n.m.r. spectra of the polysaccharide are presented in Fig. 14. The presence of GlcA as its 4-O-methyl ether was apparent from the occurrence in the ^{1}H-n.m.r. spectrum of the absorption

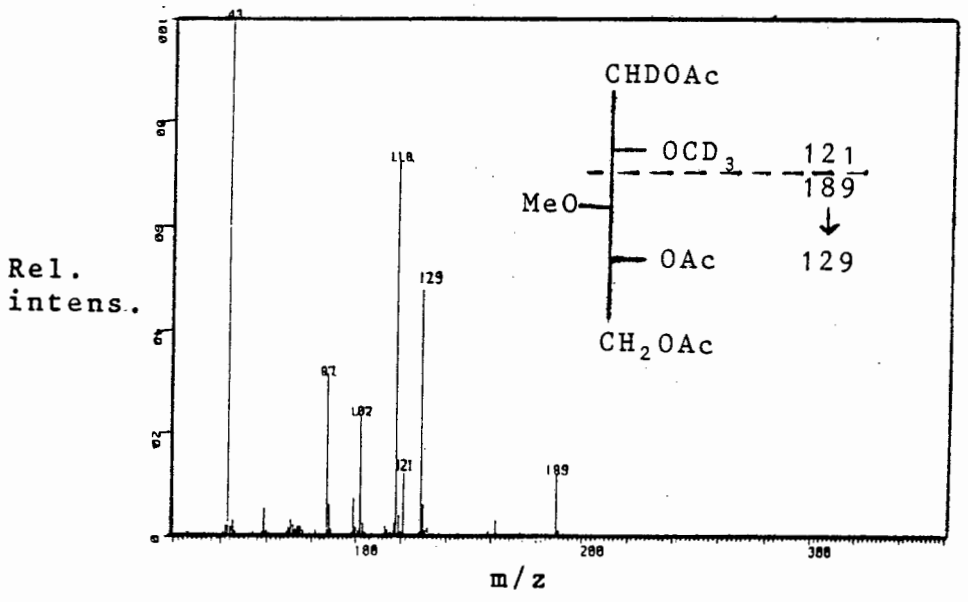


Figure 13 : Mass spectrum and expected fragmentations showing the detection of 1,3,5-tri-O-acetyl-2-O-trideuteriomethyl-3-O-methylxylitol

signal as a singlet at δ3,46, attributable to the methoxyl protons. The observed chemical shift was in agreement with that reported by Utille et al.[110] The anomeric signal due to α-linked 4-Me-D-GlcpA was observed at δ5,22 with the characteristic coupling constant of 3,6Hz. Two anomeric signals were observed for the β-linked D-Xylp residues, both of which had the same coupling constant of 7,2Hz. Glycosylation of some of the xylosyl chain residues with 4-Me-GlcpA and Xylp groups may well account for the presence of the signal at δ4,63, the peak centred at δ4,46 being associated with unglycosylated residues. The relative proportions

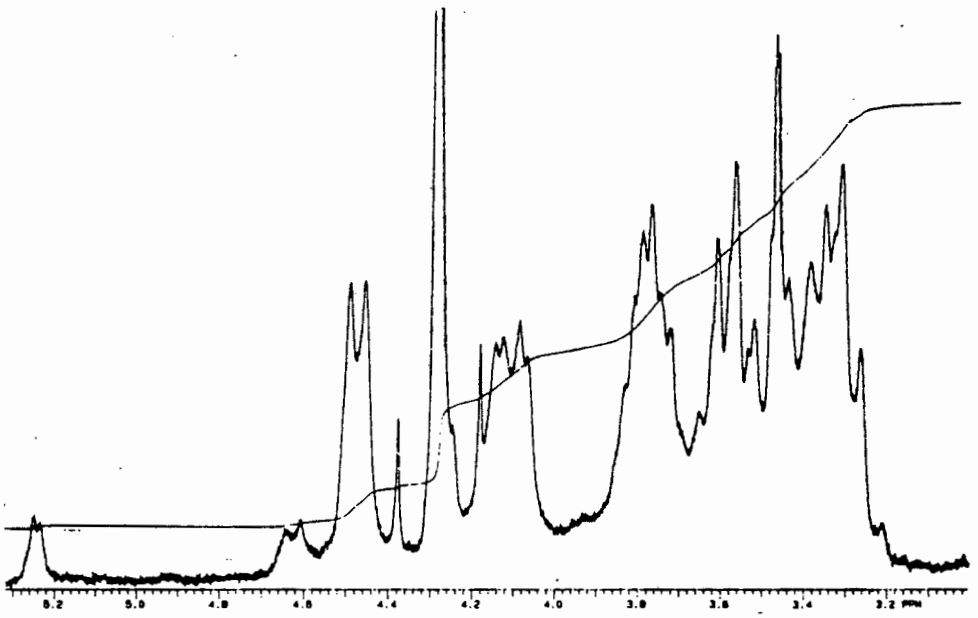


Figure 14(a) : 200MHz ^{1}H-n.m.r. spectrum in D_2O of F3 at 80°C

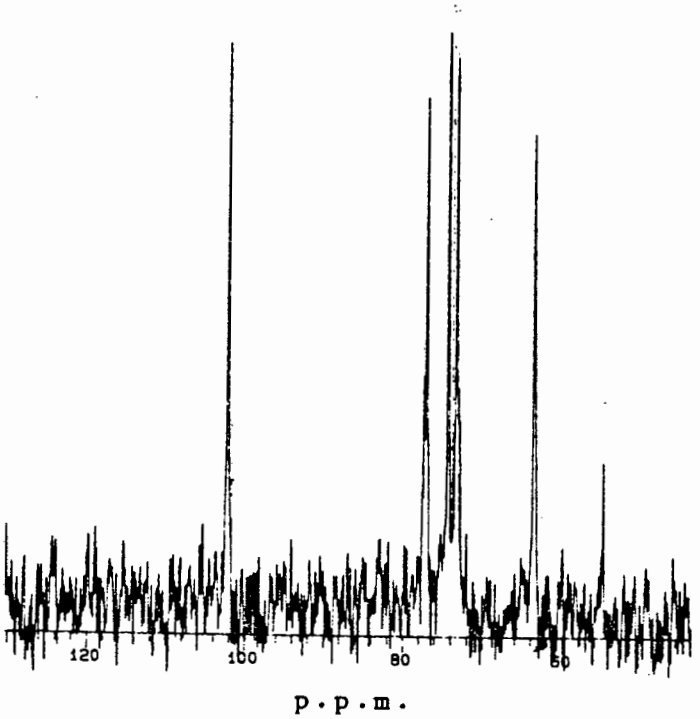


Figure 14(b) : 200MHz ^{13}C-n.m.r. spectrum in D_2O of F3 at 20°C. Spectra in (a) and (b) measured on a Varian XL-200 spectrometer

determined from the integrated peak areas were in agreement with those derived from methylation analysis.

In the ^{13}C-n.m.r. spectrum, the signal occurring at δ102,3 p.p.m. could be assigned to the anomeric carbon atom of β-linked D-xylosyl residues. The anomeric signal due to 4-Me-α-D-GlcpA was probably too small to be observed since no other signal was seen in the anomeric region. Of the other C-atoms (Table VIII) in D-Xylp residues, C-4 absorbed at δ77,15p.p.m., most downfield from the rest, due to the deshielding effect associated with substitution at this position. The assignments made in this discussion are in agreement with reported literature values.[110]

4.3.3. Carboxyl-reduction[90] of F3

The identity of the uronic acid in the polysaccharide was also investigated by reduction of the carboxyl groups with $NaBD_4$ prior to analysis of the monosaccharide composition. Thus, p.c. examination (solvent A) of an hydrolysate of the carboxyl-reduced product showed a spot with R_{Xyl} 1,14 in addition to the presence of xylose as the major component. G.l.c. and g.l.c.-m.s. of derived alditol acetates confirmed that the fast-moving spot observed on p.c. was due to 4-O-methylglucose. The mass spectrum of 6,6-

TABLE VIII - N.M.R. DATA FOR SISAL FIBRE XYLAN (F3), 4-O-METHYL-D-GLUCURONIC ACID, SISAL FLESH XYLOGLUCAN (G5I) AND OLIGOSACCHARIDE ALDITOLS OF B1, B2, B6 AND B7

	^{1}H-n.m.r. data[a]				^{13}C-n.m.r. data[b]		
Compound	δ[c]	$J_{1,2}$ (Hz)	Integral proton	Assignment	p.p.m.[c]	Assignment	
F3	5,22	3,6	1	α-4-Me-GlcA	102,31	β-Xyl	
	4,63	7,2	2	β-Xyl	77,15	C-4	)
					74,48	C-3	)
	4,46	7,2	8	β-Xyl	73,45	C-2	) of Xyl
	3,46	-	i.d.[d]	CH_3 of 4-Me-GlcA	63,75	C-5	)
4-Me-GlcA	5,36	3,6	0,4	α-4-Me-GlcA	95,95	β-4-Me-GlcA	
	4,72	7,2	0,6	β-4-Me-GlcA	92,14	α-4-Me-GlcA	
	3,48	-	3	CH_3 of 4-Me-GlcA	82,49/82,29	C-4	
					60,01/59,92	CH_3 of 4-Me-GlcA	
G5I	5,25	i.d.	0,3	α-Ara			
	5,07	i.d.	0,5	α-Fuc			
	4,90	2,7	3	α- Xyl			
	4,51	7,2	5	β-Glc			
	1,25	6,3[e]	1,4	CH_3 of Fuc			
Oligosaccharide alditols of							
B1	4,90	2,7	1	α-Xyl			
	4,60	7,2	1	β-Glc			
B2	4,90	2,7	2	α-Xyl			
	4,60	7,2	1	β-Glc			
B6	4,90	2,7	1	α-Xyl			
	4,54	7,2	1	β-Glc			
B7	5,08	i.d.	1	α-Fuc			
	4,90	2,7	5	α-Xyl			
	4,51	7,2	8	β-Glc			
	1,24	6,3[e]	3	CH_3 of Fuc			

TABLE VIII (continued)

a From spectra measured at 80°C; assignments for H-1 unless indicated otherwise

b From spectra measured at 20°C; assignments for C-1 unless indicated otherwise

c P.p.m. downfield from TMS, measured with respect to internal acetone

d Ill-defined integration and coupling constants

e $J_{5,6}$

dideuterio-4-O-methylglucitol penta-acetate is given in Fig. 15. The presence of ions having m/z 191 and 261 was characteristic of a hexitol derivative containing a methoxyl group at C-4, while the retention time (Column B) relative to Glc-Ac_6, of 0,86 compared to 0,84 and 0,92 for Man-Ac_6 and Gal-Ac_6 respectively, indicated that the hexitol was a glucose derivative. These results suggested that the uronic acid in the polysaccharide is 4-Me-GlcA, which was in agreement with results obtained by sugar analysis using method 2 (Section 2.6).

4.3.4 Investigation of the uronic acid distribution in F3

Structural studies of glucuronoxylans may sometimes involve investigation of the relative distribution of

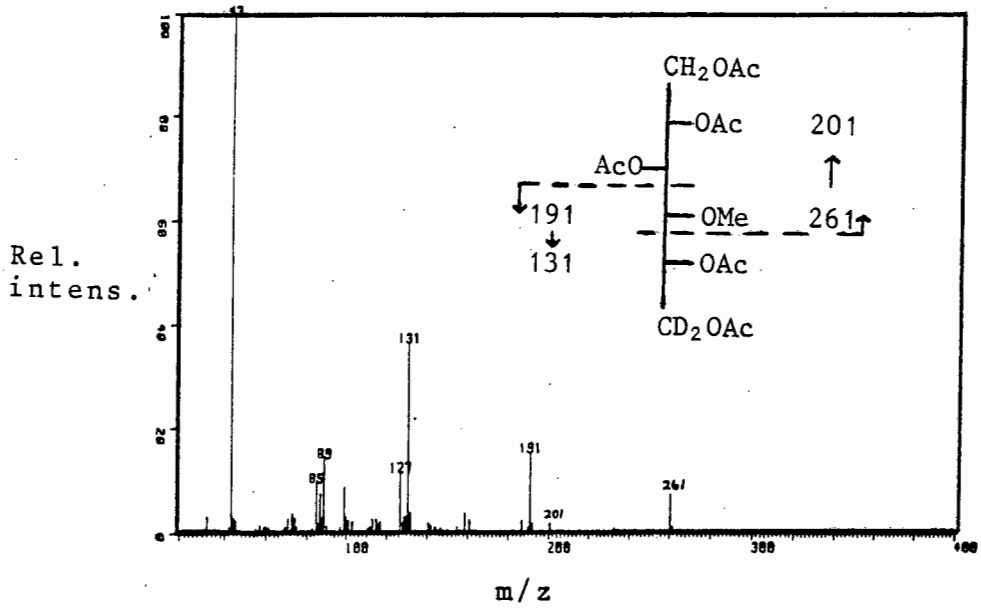
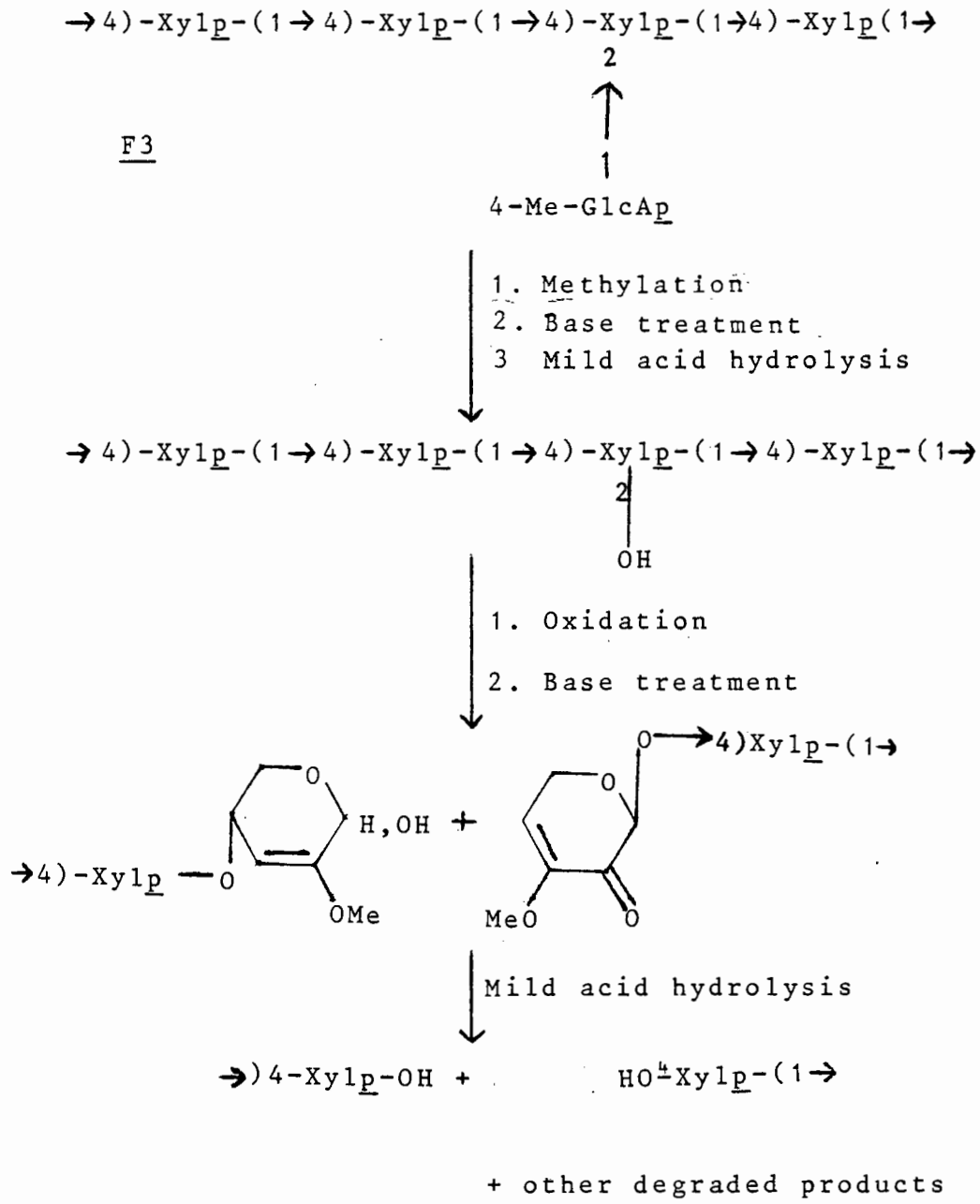


Figure 15 : Mass spectrum and expected fragmentations of 6,6-dideuterio-4-O-methylglucitol penta-acetate

the uronic acid groups along the xylan chain. Such an investigation was undertaken in the present study using the method described by Rosell and Svensson,[82] which involves elimination of the uronic acid residues from the methylated polysaccharide followed by successive oxidation, β-elimination and mild hydrolysis as illustrated in scheme 5.

After elimination of the uronic acid groups by base-degradation, the degraded material showed i.r. absorption in the hydroxyl region (3600-3300cm^{-1}), but



Scheme 5 : Degradation of methylated xylan MF3

none in the carbonyl region (1740-1700cm^{-1}), where the carbonyl group of the uronic acid carboxyl would be expected to absorb. The resulting secondary hydroxyl groups were oxidised to carbonyl groups with the methyl sulphoxide-chlorine complex.[111] The oxidised polysaccharide derivative had i.r. absorption in the carbonyl region (1720cm^{-1}), and subsequent treatment of the material with base resulted in elimination of the substituents at position 4 of each of the oxidised xylosyl residues, i.e. cleavage of the xylan backbone at the points originally holding uronic acid groups. Treatment of the base-degraded material with mild acid resulted in removal of the α,β-unsaturated keto-sugars and appearance of new non-reducing terminal groups, which were subsequently ethylated by the Hakomori procedure.[92] T.l.c. analysis (solvent G) of the generated oligosaccharides using methylated cellobiitol (MCell) as standard indicated the presence of components with R_{MCell} values varying from 0,0 to 1,1. The occurrence of such a wide range of oligosaccharides was indicative of an irregular distribution of uronic acid groups along the xylan chain. A portion of the oligosaccharide products (EDOBDMF3) was hydrolysed for analysis as partially methylated, partially ethylated alditol acetates by g.l.c. and g.l.c.-m.s. The presence of 4-O-ethyl-2,3-di-O-methyl-D-xylitol (Table

VII and Fig. 16) showed that effective cleavage of the xylan chain had been achieved.

4.3.5 Cellulase enzyme degradation studies on F3 xylan

Hemicellulosic xylans are well known for their strong non-covalent association with cellulose in plant tissues, which often necessitates the use of alkaline media for their quantitative extraction from such tissues. However, since this procedure is believed to

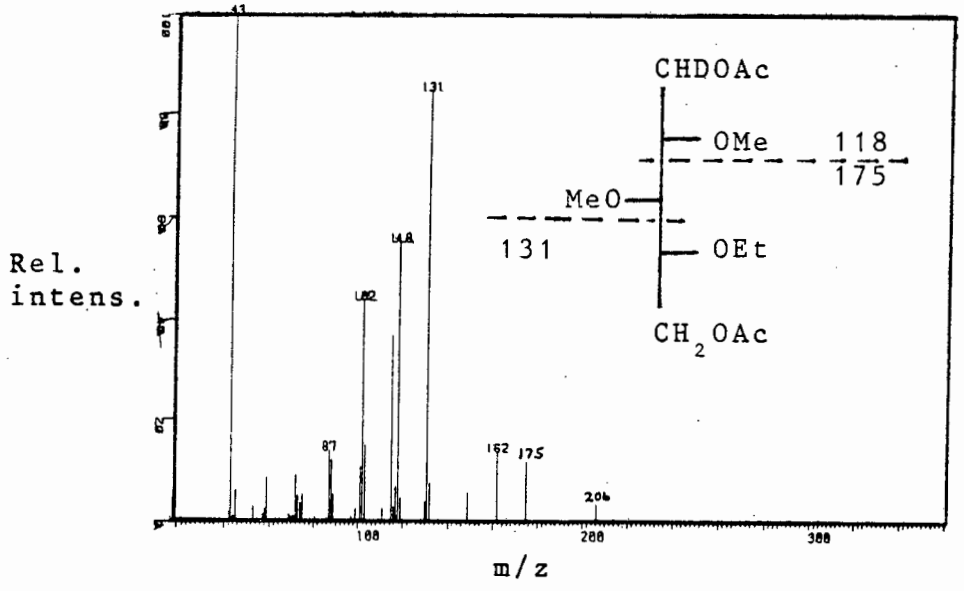


Figure 16 : Mass spectrum and expected fragmentations showing the detection of 1,5-di-O-acetyl-4-O-ethyl-2,3-di-O-methylxylitol

effect some limited degradation of these polymers, the search for alternative methods that would provide a quantitative yield of the native polymers without degradation remains a challenge. In an attempt to find out whether a cellulase preparation from Trichoderma reesei (Rut C-30)[112] could afford specific degradation of cellulose, F was treated with the enzyme. The results obtained showed that in addition to the release of large amounts of glucose, as expected, an appreciable amount of xylose was also liberated, thus indicating that the enzyme has some xylanase activity. Therefore, this enzyme could not be used for the purpose of isolating xylans. However, when it was used to treat purified F3, an interesting result was achieved in that there was evidence for the release of 4-Me-GlcA in addition to xylose. Since no indication of previous observation of this phenomenon could be found in the literature, the reaction was carried out on a semi-preparative scale, with the objective of further substantiating the finding. The 4-Me-GlcA was isolated by preparative p.c. (R_{GlcA} 1,56; solvent D). Further p.c. examination of the isolated product showed the presence of two minor contaminating components, one of which had the same R_f value as GlcA. Thus, it was apparent at this stage that the polysaccharide might contain trace amounts of GlcA in addition to 4-Me-GlcA.

The 4-Me-GlcA was also examined by n.m.r. spectroscopy, and the assignment of peaks was made by comparing the spectra with those obtained for GlcA under the same conditions. Although both ^{1}H- and ^{13}C-n.m.r. spectra of 4-Me-GlcA also showed evidence for the presence of contaminating material, the characteristic anomeric signals were clearly discernible. From the ^{1}H-n.m.r. data (Table VIII) the anomeric proton of 4-Me-D-GlcpA absorbed at δ4,72 and 5,36 due to the presence in the equilibrium mixture of the β- and α-configurational forms of the uronic acid, respectively. Both signals occurred as doublets with the characteristic splitting constants of 3,6Hz (α) and 7,2Hz (β). The signal due to the methoxyl group was observed as a singlet at δ3,48. In the ^{13}C-n.m.r. spectrum (Fig. 17) the signals due to the α and β anomeric carbon atoms appeared at δ92,14 and 95,95 p.p.m. respectively; the α : β ratio of 2 : 3 determined from the relative intensities of the peaks was in agreement with that found by integrating the corresponding signal areas in the ^{1}H-spectrum. The methoxyl carbon absorbed at δ59,92 and 60,01 p.p.m., this fine twinning being due to the presence of two anomeric forms. The C-4 signal appeared at δ82,29/82,49 p.p.m., slightly downfield from the rest of the ring carbons, implying that the presence of the methoxyl group has a deshielding

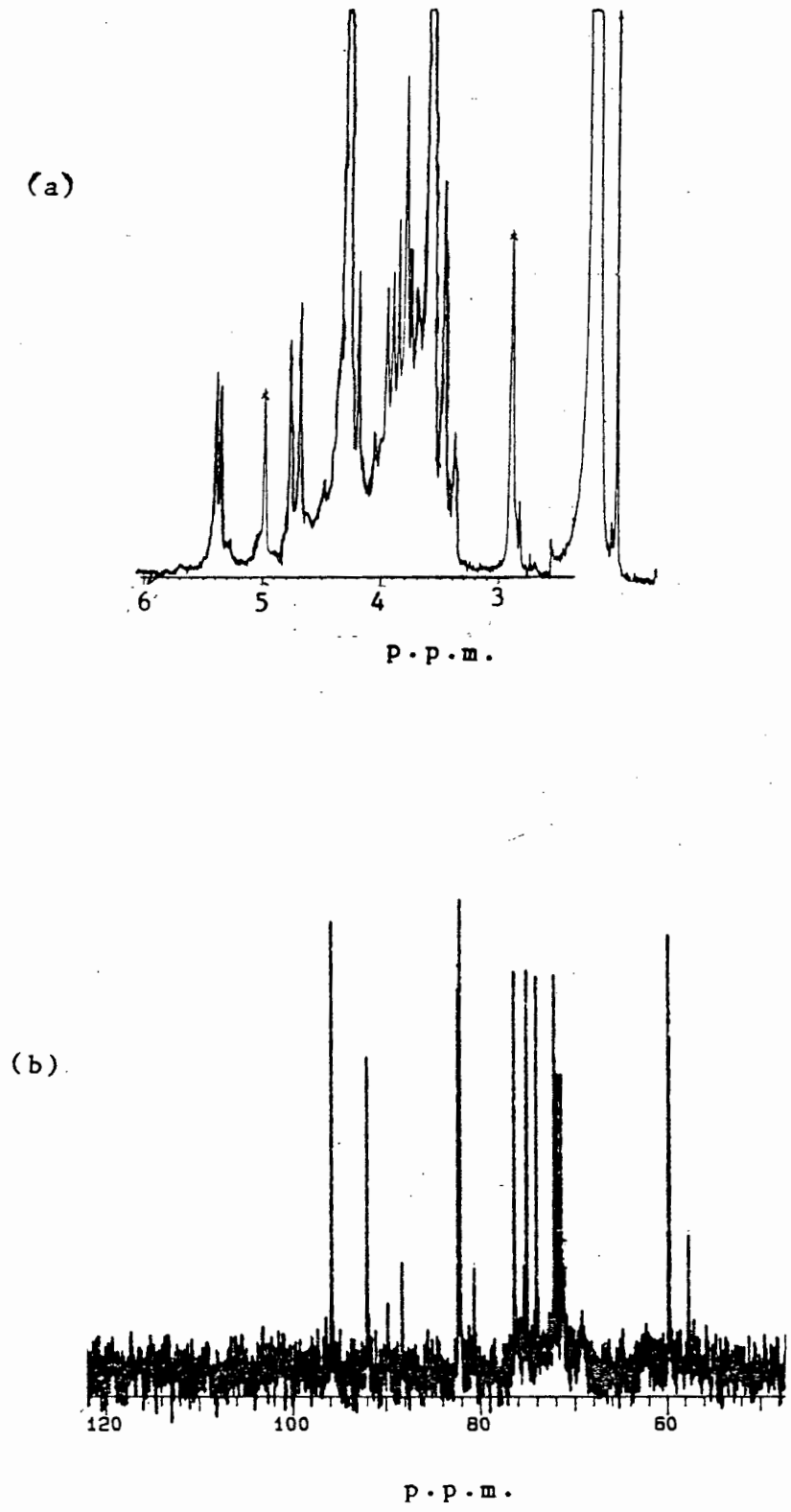


Figure 17 : (a) 90MHz ^{1}H- and (b) 200MHz ^{13}C-n.m.r. spectra in D_2O of 4-O-methyl-D-glucuronic acid at 80°C and 20°C respectively. The ^{13}C-n.m.r. spectrum was measured on a Varian XL-200 spectrometer

effect. The other signals appearing in the spectrum could not be assigned unambiguously.

To further confirm the structure of 4-Me-GlcA its methyl glycoside methyl ester was prepared by refluxing with methanolic HCl, and the methyl ester group was subsequently reduced with LAD. A portion of the reduced product was acetylated to give methyl 2,3,6-tri-O-acetyl-4-O-methyl-D-glucopyranoside (a). Another portion was hydrolysed, reduced with $NaBD_4$ and acetylated, yielding 1,6,6,-trideuterio-1,2,3,5,6-penta-O-acetyl-4-O-methyl-glucitol (b). The products (a) and (b) were examined by mass spectroscopy (Fig. 18). Although the presence of the fragment ions m/z 305 $[M-31]^+$ and 245$[(M-31)-60]^+$ in the mass spectrum of (a) was consistent with a dideuterated, monomethylated hexose derivative structure, the exact location of these substituents was provided by the mass spectrum of (b) thus: the fragment ions m/z 262 and 202 were due to a four-carbon skeleton incorporating one deuterium which could only have been introduced by $NaBD_4$ reduction at C-1 of the original aldose, whereas the ions at m/z 191, 131[= 191-(CH_3COOH)], 89[= 131-(CH_2=C=O)] were derived from a three-carbon skeleton labelled with two deuterium atoms which could only have arisen from LAD-reduction of the carboxylic acid group.

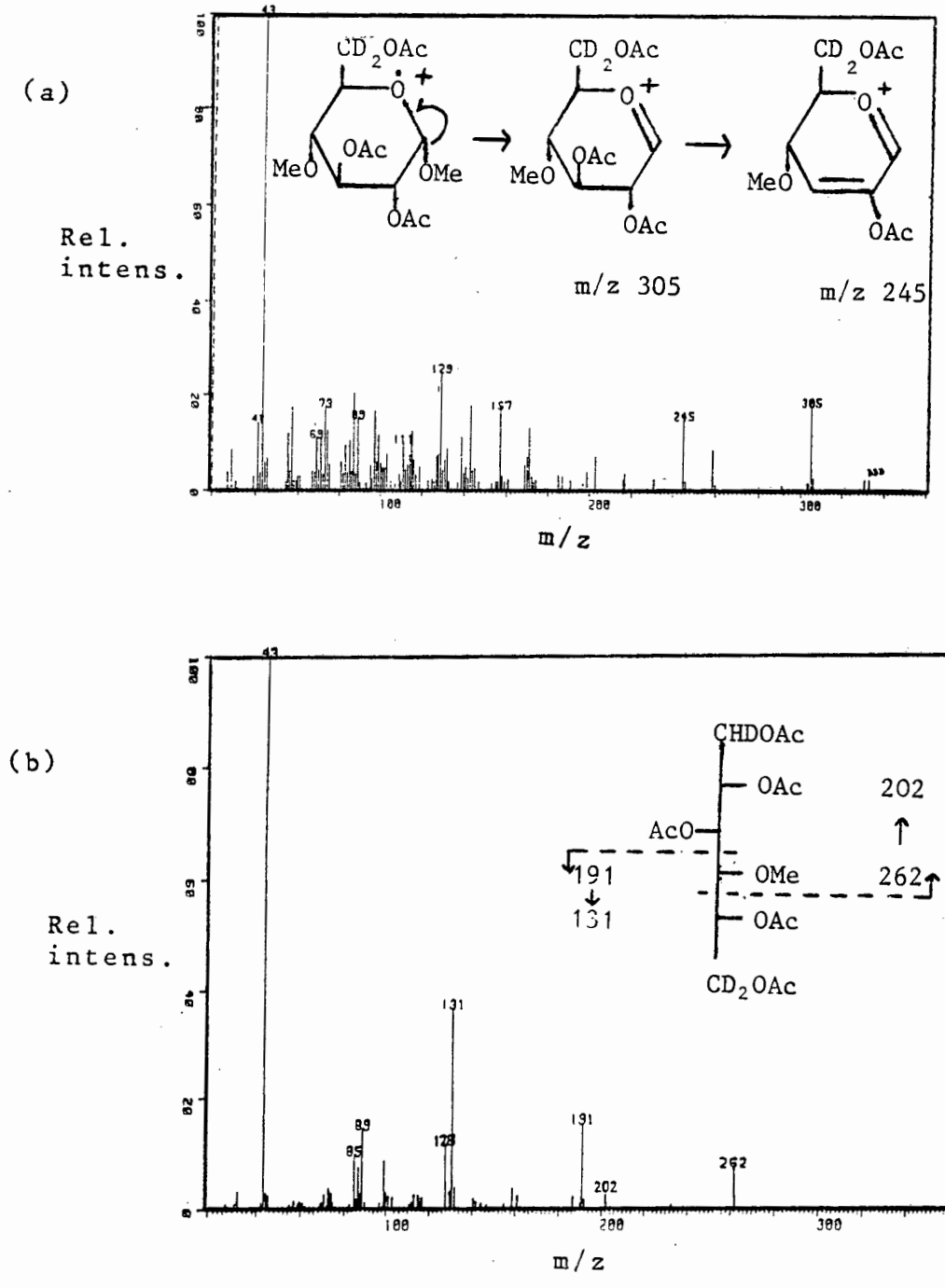


Figure 18 : Mass spectra and some expected fragmentations of (a) methyl 6,6-dideuterio-2,3,6-tri-O-acetyl-4-O-methyl-D-glucopyranoside and (b) 1,6,6-trideuterio-4-O-methyl-D-glucitol penta-acetate

The characterisation of 4-Me-GlcA in this way confirmed the cleavage activity of the cellulase preparation towards the glycosidic bonds involving these units as found in the sisal xylan.

4.3.6 General discussion

From the preceding investigation it has been demonstrated that the major hemicellulosic polysaccharide in the fibrous portion of the sisal plant leaf material is a 4-O-methylglucuronoxylan. As would be expected, most of it was extracted under alkaline conditions, albeit with different concentrations of KOH. The similarity in behaviour observed during the preliminary examination of the fractions extracted with different concentrations of KOH suggests that a saturation factor could have accounted for the less than complete extraction of the polysaccharide at each concentration of alkali. The xylan bears some resemblance to the one isolated from birch wood[32] in having a 4-Me-GlcA group linked to the 2-O-position of one in ten xylosyl chain residues. However, a major difference between the two is the occurrence in the sisal xylan of xylosyl units as side groups in almost the same frequency as the 4-Me-GlcA groups. The absence of arabinose as a sugar

constituent is a major difference between this xylan and those isolated from sugar cane bagasse.[108]

The high uronic acid content of the polysaccharides extracted with water (F1) and ammonium oxalate (F2) (Table V), and the fairly high positive specific rotation of the former suggested that they consisted mainly of pectic material. While F2 was not examined further, F1 was methylated, LAD-reduced and remethylated, and the results for the analysis of p.m.a.a.'s derived from an hydrolysate of the methylated, reduced, methylated product (MRMF1) are presented in Table VII. The presence of 2,3,6-tri-O-methylgalactose incorporating two deuterium atoms at C-6 as the major galactosyl component indicated that the original polysaccharide contained (1→4)-linked galacturonosyl residues, which is characteristic of pectins. The identification of 2,4-di- and 2-monosubstituted rhamnosyl residues suggested that the pectic material could be of the rhamnogalacturonan type. The presence of (1→4)- or (1→5)-linked Ara and (1→4)-linked Xyl indicated that F1 contained arabinan and xylan chains either as independent entities or as side-chains to the rhamnogalacturonan polymer.

The preponderance of Glc as a sugar constituent in the final alkaline-insoluble residue (FC) (Table V) is

indicative of the presence of cellulose almost as the sole carbohydrate polymer in this material. The detection of small proportions of Xyl and uronic acid may be attributed to the presence of residual 4-O-methylglucuronoxylan, which is so strongly associated with the cellulosic polymers that it was not extractable under the conditions employed.

4.4 The polysaccharide composition of sisal fleshy material (G)

In contrast to the results obtained in the analysis of the fibrous material, the major non-cellulosic polysaccharides of the fleshy material appeared to be pectic polymers similar to those studied by Aspinall and Cañas-Rodriguez.[109] Although the next highest yield was obtained from the water extract, the polysaccharide fractions extracted by alkali were selected for more detailed study in order to compare them with those obtained under similar conditions from the fibrous material. To gain some insight into the kind of carbohydrate polymers present in fractions G3, G4 and G5, each was separately methylated by the Hakomori[92] procedure. From analyses of p.m.a.a.'s derived from their hydrolysates by g.l.c. (Table IX) it was apparent that each fraction consisted of more than one polysaccharide species. Thus, while the

TABLE IX - METHYLATION ANALYSIS OF POLYSACCHARIDE FRACTIONS FROM SISAL FLESH (G) (see Scheme 4)

Sugar derivatives	MG3[a]	MG4	MG5	MRMG3I	MG5I	MG5II	MRMG5III	MG1
3,4-Rha[b]	9[c]	-	2	5	-	-	8	6
3-Rha	3	-	1	-	-	-	5	-
2,3,4-Fuc	-	-	-	-	5	2,5	-	-
2,3,5-Ara	13	2	3	5	3	3	4	25
2,3,4-Ara	6	2	-	-	-	-	-	6
2,3-Ara	10	2	3	4	-	1,3	13	9
2,3,4-Xyl	4	12	23	6	27	15	3	-
2,3-Xyl[d]	26	40	12	62	-	31	23	-
3,4-Xyl[d]	-	8	6	-	9	5	-	-
2-Xyl[d]	3	-	-	5	-	-	-	-
3-Xyl[d]	7	5	-	7	-	6	2	-
Xyl	-	-	-	-	-	2,5	-	-
2,3,6-Man	-	2	2	-	-	-	-	-
2,3,4,6-Gal	5	2	5	-	5	1,3	6	4
2,3,6-Gal	-	-	-	4[e]	-	-	18[e]	-
2,3,4-Gal	-	-	-	-	-	-	0,9	5
2,4,6-Gal	8	-	-	-	-	-	4	12
3,4,6-Gal	-	3	6	-	6	4	2[e]	-
2,3-Gal	-	-	-	-	-	-	4	-
2,4-Gal	5	-	-	-	-	-	0,5	28
2,6-Gal	-	-	-	-	-	-	0,8	-
3,6-Gal	-	-	-	-	-	-	0,5[e]	-
2-Gal	-	-	-	-	-	-	-	5
3-Gal	-	-	-	-	-	-	0,5	-
2,3,4,6-Glc	-	-	-	2[e]	-	-	-	-
2,3,6-Glc	-	8	13	-	15	9	-	-
2,3-Glc	-	12	25	-	35	17	-	-
3-Glc	-	-	-	-	-	2	-	-

a M, methylated; R, reduced

b 1,2,5-Tri-O-acetyl-3,4-di-O-methylrhamnitol, etc.

c Approximate molar proportions

d Relative proportions estimated by selected-ion monitoring m.s.

e All derivatives incorporate 2D-atoms at C-6

methylation analysis of G3 demonstrated the presence of features of xylan, galactan and pectic material, G4 appeared to consist of xylan and xyloglucan. The analysis of G5 showed it to be composed mainly of xyloglucan. The apparent diversity in the polysaccharide content of these fractions necessitated their further fractionation.

4.4.1 Studies on fraction G3

As is evident from Table VI a striking feature in the composition of G3 is its high nitrogen content. If all of this nitrogen were due to the presence of protein, the corresponding protein content (%N x 6,25) would be approximately 36%. It was therefore considered necessary to employ fractionation methods that would remove protein not covalently associated with the carbohydrate material. This was first attempted by the Sevag procedure,[113] which involves shaking up an aqueous solution of the protein-containing material with chloroform, resulting in denaturing and precipitation of the protein component. However, subsequent examination of the material remaining in solution and of the precipitate showed that neither differed much in composition from the original material. In a second attempt at fractionation ion-exchange chromatography on a DEAE-Sephadex column was

employed, the eluate being monitored for both carbohydrate and protein. The resulting chromatogram (Fig. 19(a)) showed co-elution of carbohydrate and protein although more fractionation was apparent. While the first component to elute, which was rich in carbohydrate, also contained some protein, most of the protein-rich material with less carbohydrate eluted in later fractions. These results suggest that some protein may be covalently linked to polysaccharide in G3. Paucity of material precluded further fractionation studies. The carbohydrate-rich component G3I was recovered for further examination, but the yield was too low to permit a detailed study of this material.

A comparison of the monosaccharide compositions of G3 and G3I (TAble VI) and their respective methylation analyses (Table IX) shows that G3I was somewhat enriched in xylan, whereas it was devoid of any galactan features. The methylation analysis of G3I was carried out after LAD-reduction and subsequent re-methylation of the methylated product. Therefore the detection of terminal glucosyl and (1→4)-linked galactosyl derivatives each incorporating two deuterium atoms at C-6 implies the presence of both Glcp A and/or its 4-O-methyl ether and Galp A in the original polysaccharide. The occurrence of (1→2)-linked Rhap

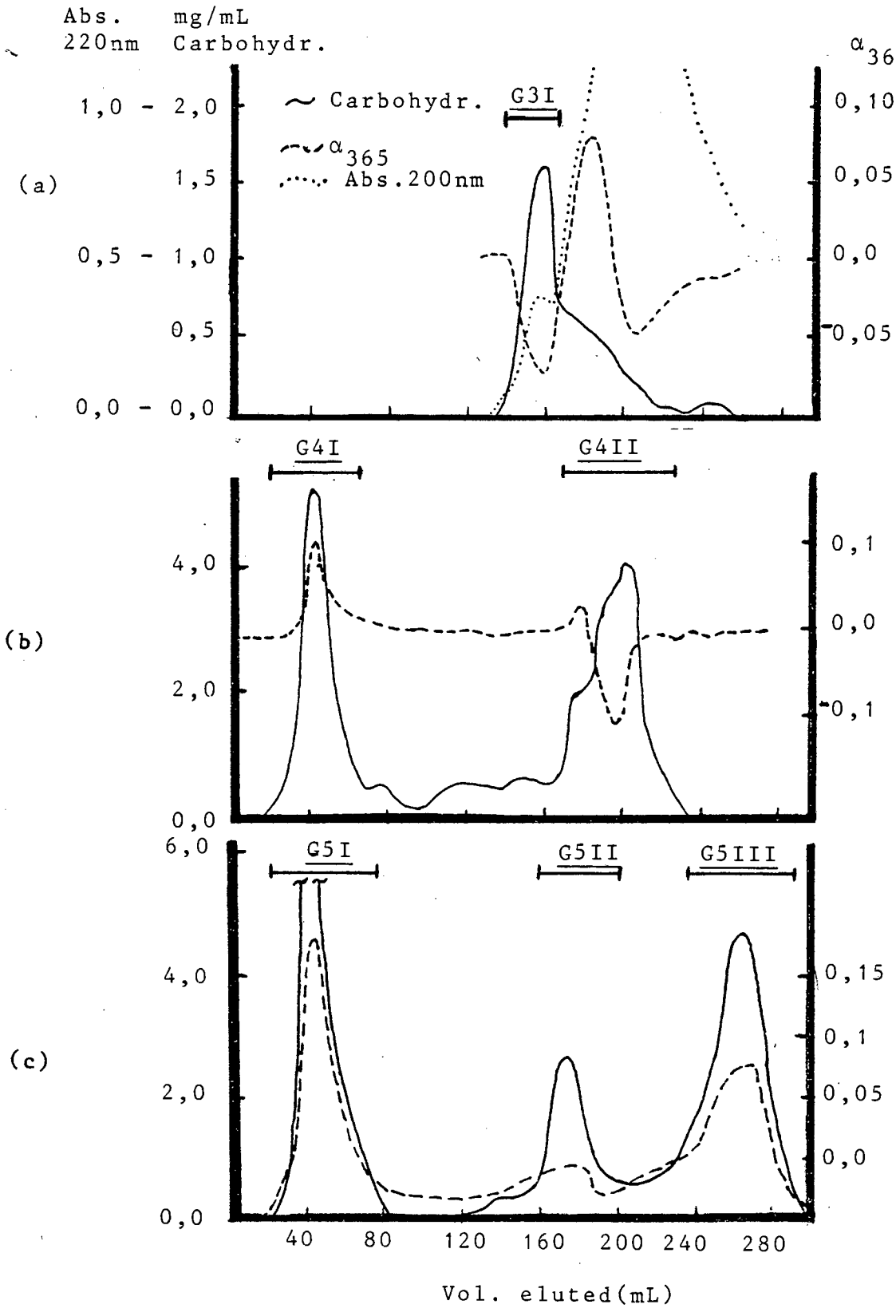


Figure 19 : Fractionation of alkaline extracts (a) G3, (b) G4 and (c) G5 on DEAE-Sephadex A-50; elution was isocratic with buffer for the first 100mL, thereafter a linear gradient of NaCl was employed

in addition to GalpA suggests that G3I retained some of the pectic characteristics observed in G3. Whether the xylan, pectic polysaccharide and protein are all covalently associated remains to be investigated.

4.4.2 Studies on fractions G4 and G5

The fractionation and examination of both G4 and G5 will be discussed in this section because these fractions showed more parallels in their behaviour than the others. The fractionations were achieved by DEAE-Sephadex column chromatography. Whereas G4 could be fractionated into only two components, G5 was resolved into three (see Fig. 19). After recovery of the different components, each was analysed for monosaccharide composition (Table VI). The emergence of G4I and G5I from the column in the early fractions, eluted isocratically with 10mM potassium phosphate buffer, suggested that these were neutral polysaccharides. Furthermore, the positive specific rotation values and the presence of high proportions of Glc accompanied by appreciable amounts of xylose among their sugar constituents indicated structures of the xyloglucan-type. Since G5I was recovered in higher yield than any of the other fractions from G4 and G5, this component was submitted to more detailed structural studies as detailed below (section 4.4.3).

The shape of the corresponding peak in the DEAE-Sephadex chromatogram of G4 (Fig. 19(b)) suggests that G4II may consist of more than one component. However, no attempt was made to resolve these components further. Thus G4II was analysed only for sugar composition (Table VI). The high proportion of Xyl would suggest the presence of xylan polymers, whereas the detection of Rha together with uronic acid in appreciable quantities may be due to associated pectic polymers. The presence of such polymers in G5 was apparent when G5II and G5III were each subjected to methylation studies as well as analysis for sugar composition; the results are discussed later (see section 4.4.4).

4.4.3 Structural studies of xyloglucan G5I

Further purification of G5I was achieved by fractionation via copper-complex formation as described by Jones and Stoodley.[114] The polysaccharide was recovered almost quantitatively as the insoluble copper complex. The purified product was devoid of traces of mannose that were detectable prior to the fractionation process, but the relative proportions of the other sugar constituents remained essentially unchanged. Repetition of the procedure did not bring about any change in the monosaccharide composition and thus the

purified polysaccharide was used as such in subsequent structural studies. When passed through Sepharose 4B, G5I emerged at a point corresponding to the total volume of the column suggesting that it is a polymer of relatively low $\bar{M}_w$ (i.e. < ca. 50 000). Therefore its homogeneity was demonstrated by s.e.c. on Bio-Gel P-10, from which it emerged as a single sharp peak (Fig. 20) corresponding to $\bar{M}_w$ $\sim 1 \times 10^4$. The monosaccharide composition of G5I is given in Table VI.

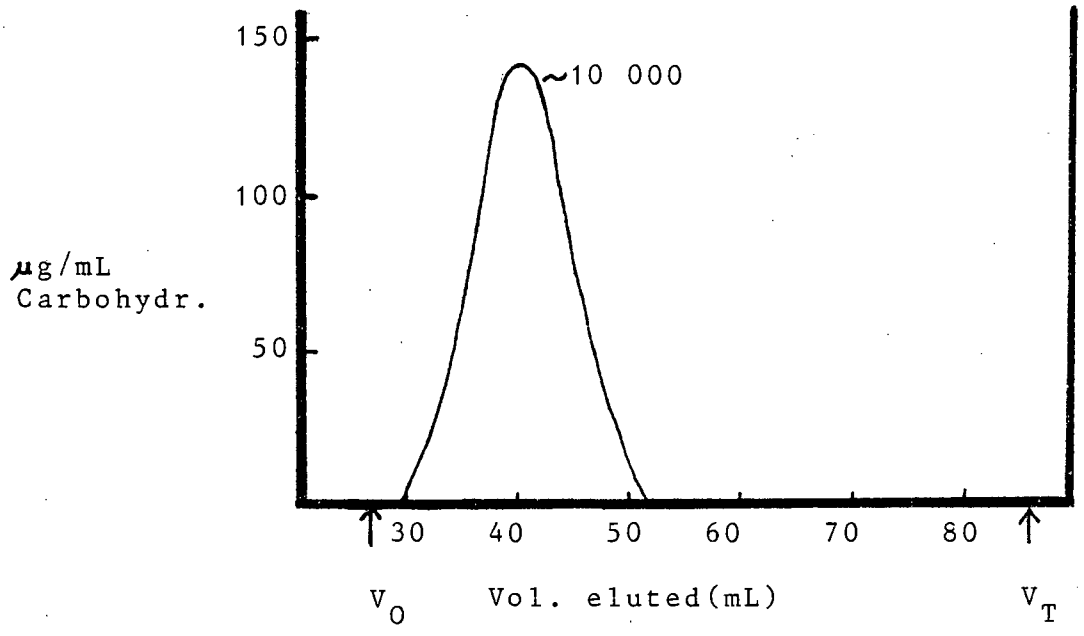


Figure 20 : Chromatography of G5I on Bio-Gel P-10

4.4.3.1 Methylation studies

The determination of the glycosidic linkage composition was carried out by methylation of G5I following the Hakomori[92] procedure. The results of g.l.c. and g.l.c.-m.s. analyses of the derived p.m.a.a.'s are presented in Table IX. The detection of fucose and arabinose solely as their 2,3,4- and 2,3,5-tri-O-methyl derivatives respectively, implies that each occurs as non-reducing terminal groups, the former in the pyranosyl ring form and the latter as a furanosyl end-group. About half of the galactosyl units occur as non-reducing end-groups while the rest are glycosylated at O-2. A major proportion of the Xylp units are terminal, whereas about a quarter are substituted at O-2. All the Glc residues are linked at O-4, with about two-thirds of them also carrying substituents at O-6. The foregoing results conform to a pattern observed for xyloglucans containing L-Fucp, L-Araf and D-Galp as additional sugar constituents: the backbone structure comprises (1→4)-linked Glcp residues to some of which are attached xylosyl units at O-6; some of the latter units being further substituted with Ara, Gal or Fuc. Although this type of xyloglucan has been found as a polysaccharide constituent in a number of dicotyledonous plants,[6] its occurrence in a mono-cotyledonous plant is unusual.

4.4.3.2 ^{1}H-n.m.r. studies of the xyloglucan

Structural studies of xyloglucans in general have shown that the configuration of glycosidic linkages is always β for D-Glc and D-Gal units, whereas D-Xyl, L-Ara and L-Fuc are often α-linked. From the ^{1}H-n.m.r. data of G5I (Table VIII) it was apparent that this trend is upheld. Thus, the signal centred at δ4,51, appearing as a doublet (J 7,2 Hz), was characteristic of H-1 in a β-linked D-Glcp residue. The peaks observed at δ4,90 (doublet, J 2,7 Hz), and 5,07 and 5,25 (both broad singlets) were assigned to H-1's in α-linked D-Xylp, L-Fucp and L-Araf respectively. It is believed that the anomeric proton signal for β-D-Gal was obscured by that of β-Glc, since their chemical shifts are very close and the polysaccharide contains about 5 times more Glc than Gal. The methyl group protons of Fuc absorbed at δ1,25 (doublet, J 6,3 Hz).

4.4.3.3 Cellulase enzyme degradation studies of G5I

The presence in xyloglucans of a cellulose-like backbone consisting of β-(1→4)-linked Glc residues makes cellulase enzymes very potent tools for degradative studies of these polysaccharides. While the xylose-containing side-chains would be expected to inhibit enzyme cleavage activity to some extent, the

fact that often at least one third of the Glc chain residues are unsubstituted, makes possible the generation of a range of oligosaccharides, the subsequent characterisation of which provides a better understanding of the xyloglucan structure. With a similar objective, the xyloglucan under study was treated with the cellulase preparation from Trichoderma reesei (Rut C-30).[112] The generated oligosaccharides were partially fractionated on a Trisacryl GF 05 column giving the elution profile shown in Fig. 21. Fractions I and II were fractionated further by preparative paper chromatography resulting in the isolation of 7 components, designated B1-B7. Some physical and chemical properties of these are summarised in Table X. Fraction III was shown by both p.c. and g.l.c. to consist mainly of free sugars in addition to small amounts of the disaccharide Xyl-(1→6)-Glc. The presence of the latter was demonstrated as follows. A portion of fraction III was reduced with $NaBD_4$, followed by methylation of the resulting alditols. Examination of the methylated alditols by g.l.c. on column B showed the presence of an isolated component (retention time 0,85 relative to nonamethylated cellobiitol) in addition to a cluster of peaks due to methylated hexitols and pentitols. The mass spectrum and fragmentation pathways of this component, leading to some pertinent ions are shown in Fig. 22. The

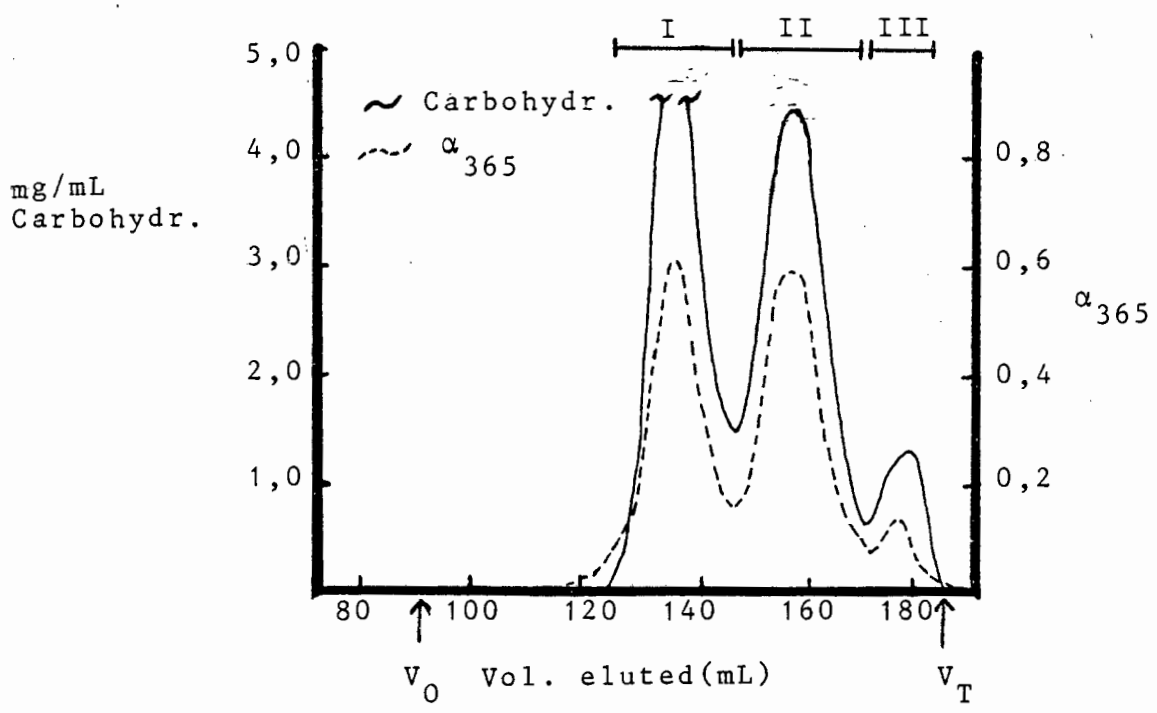


Figure 21 : Fractionation of products of cellulase degradation of G5I on Trisacryl GF 05 column

TABLE X - SOME CHARACTERISTICS OF OLIGOSACCHARIDES, B1-B7, DERIVED BY CELLULASE DEGRADATION OF SISAL FLESH POLYSACCHARIDE FRACTION G5I

		B1	B2	B3	B4	B5	B6	B7
Mass recovered (mg)		20	25	4,8	5,2	5	29	35
wt% of xyloglucan		10	12,5	2,4	2,6	2,5	14,5	17,5
R_{Glc} [a]		0,74	0,58	0,40	0,40	0,29	0,18	0,11
$[\alpha]_D$/°		+34	+60	n.d.	n.d.	n.d.	+46	+38
mole % of sugars [b]	Fuc	-	-	2	-	-	-	8
	Ara	-	-	-	-	-	3	-
	Xyl	36	55	42	48	41	27	37
	Gal	-	-	12	-	-	-	13
	Glc	64	45	44	52	58	70	42

a P.c. (solvent B)

b Sugar composition determined by method 1 (section 2.6)

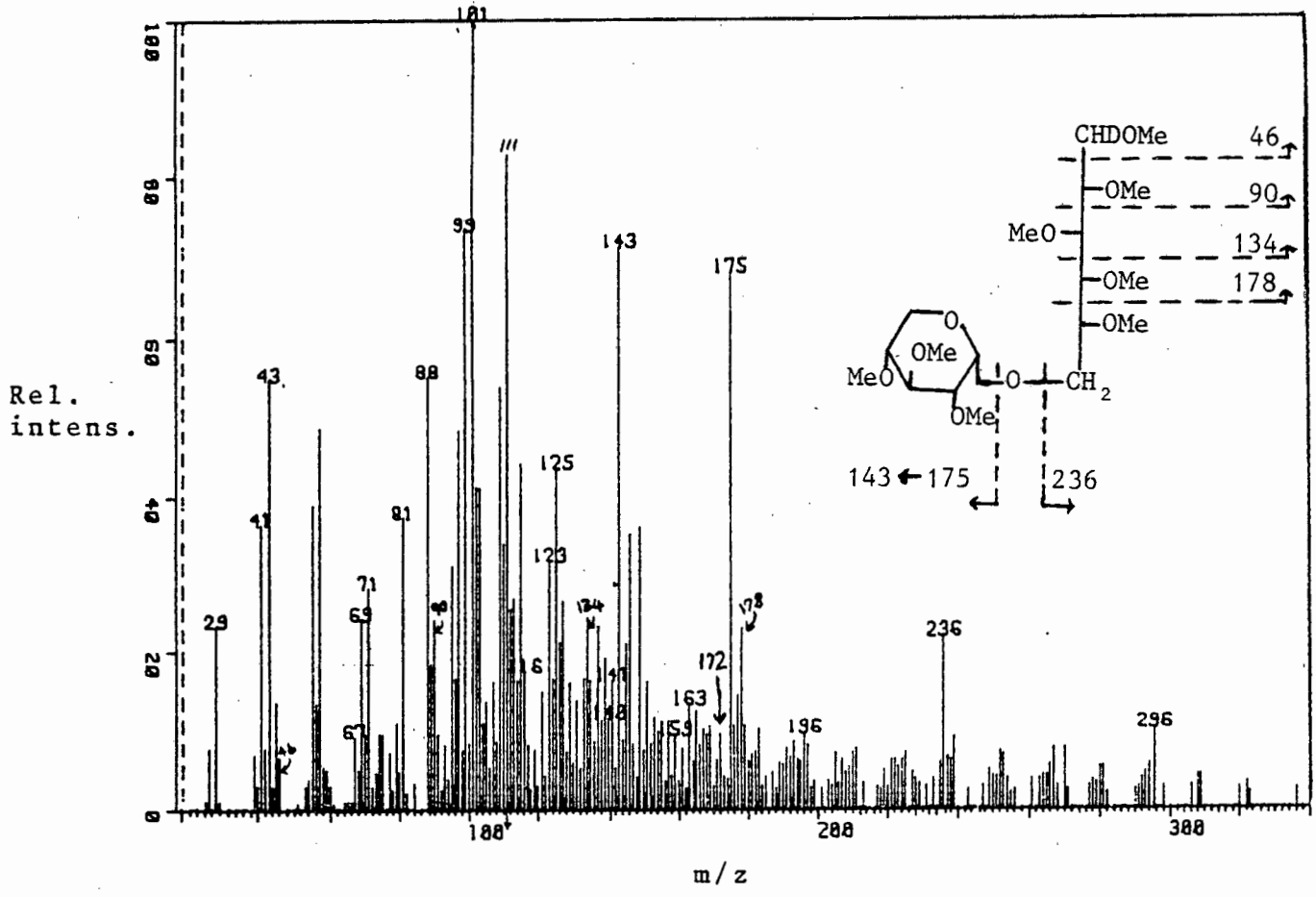


Figure 22 : Mass spectrum and expected fragmentations of the permethylated alditol of xylosyl-(1→6)-glucose

intense ions of the aA series (m/z 175, 143, 111) showed the presence of non-reducing, terminal pentosyl groups, whereas the ions at m/z 134, 178, 236 and 296(abJ_1) demonstrated a hexitol derivative substituted through position 6. Hydrolysis of the material followed by g.l.c. analysis of the derived p.m.a.a.'s revealed the presence of alditol acetates originating from 2,3,4-tri-O-methyl Xyl and 1,2,3,4,5-penta-O-methyl Glc, occurring in approximately equal quantities. The proposed structure of the disaccharide was thus confirmed.

A general approach towards characterisation of the oligosaccharides B1-B7 involved analysing each for sugar composition. The samples were then reduced with $NaBD_4$, and methylated. The methylated products were analysed both by g.l.c. analysis of the derived p.m.a.a.'s and by direct-insertion m.s. using the e.i. mode; the principles of sequencing oligosaccharides[115,116] were applied and the nomenclature used is that of Kochetkov and Chizhov.[117] For the higher oligosaccharides the results from direct-insertion m.s. were of limited value because the instrument used could not be calibrated to higher than 800 a.m.u. Where sample amounts were sufficient the oligosaccharide alditols were examined by ^1H-n.m.r.

The structures of B1 and B2:

Hydrolysis of B1 and B2 in 2M TFA revealed the presence of Xyl and Glc as the only sugars, though the relative ratios were different for each sample (Table X). After reduction each alditol was examined by ^{1}H-n.m.r. spectroscopy. Both spectra showed the presence of the characteristic signals for H-1's of β-D-Glcp (δ4,6 doublet, J 7,2Hz) and α-D-Xylp (δ 4,9 doublet, J 2,7 Hz); the relative ratios of the integrated areas were Glc : Xyl , 1 : 1 for B1 and 1 : 2 for B2. The methylated derivatives of B1 and B2 appeared as single components when examined by g.l.c. on an SE 30 packed column; their retention times relative to nonamethylated cellobiitol were 2,8 and 6,0 respectively. The results of methylation analysis as p.m.a.a.'s are presented in Table XI. Because the g.l.c. column used for analysis of the methylated derivatives of B1 and B2 was at the time not compatible with the g.l.c.-m.s. instrumentation, they were analysed by direct-insertion m.s. The mass spectra and some fragmentation pathways of each are depicted in Figs. 23 and 24 respectively.

The relative retention time of 2,8 for the methylated derivative of B1 was close to that of methylated raffinose alditol (i.e. 3,0 on the same scale) which

TABLE XI - METHYLATION ANALYSIS OF B1-B7, PRODUCTS OF CELLULASE DEGRADATION OF POLYSACCHARIDE FRACTION G5I

	B1	B2	B3	B4	B5	B6	B7
1,2,3,5,6 -Glc[a]	29[b]	-	7	16	3	4	2
1,2,3,5-Glc	-	31	-	-	12	-	-
2,3,4,6-Glc	-	-	-	-	2	12	-
2,3,4-Glc	40	23	17	20	16	12	10
2,3,6-Glc	-	-	4	5	5	6	7
2,3-Glc	-	-	13	19	18	21	23
2,3,4,6-Gal	-	-	6	-	-	-	6
3,4,6-Gal	-	-	5	-	-	-	9
2,3,4-Fuc	-	-	3	-	-	-	8
2,3,4-Xyl	31	47	32	36	35	29	24
2,4-Xyl	-	-	-	-	3	5	-
3,4-Xyl	-	-	13	4	5	8	11
2,3,5-Ara	-	-	-	-	-	3	-

a 4-O-Acetyl-1,2,3,5,6-penta-O-methylglucitol, etc.
b Approximate molar proportions

indicated that B1 could be a trisaccharide. The hydrolysis data (Table X) suggest a sugar composition of 1 Xyl and 2 Glc units. The sugar ratio is equally supported by ^{1}H-n.m.r. data (Table VIII) on the presumption that the reduced end of the molecule is a glucitol which, of course, would not show any anomeric signal in the spectrum.

The mass spectrum of methylated B1 alditol (Fig. 23) gave ions of the aA (m/z 175, 143 and 111) and cA (236,

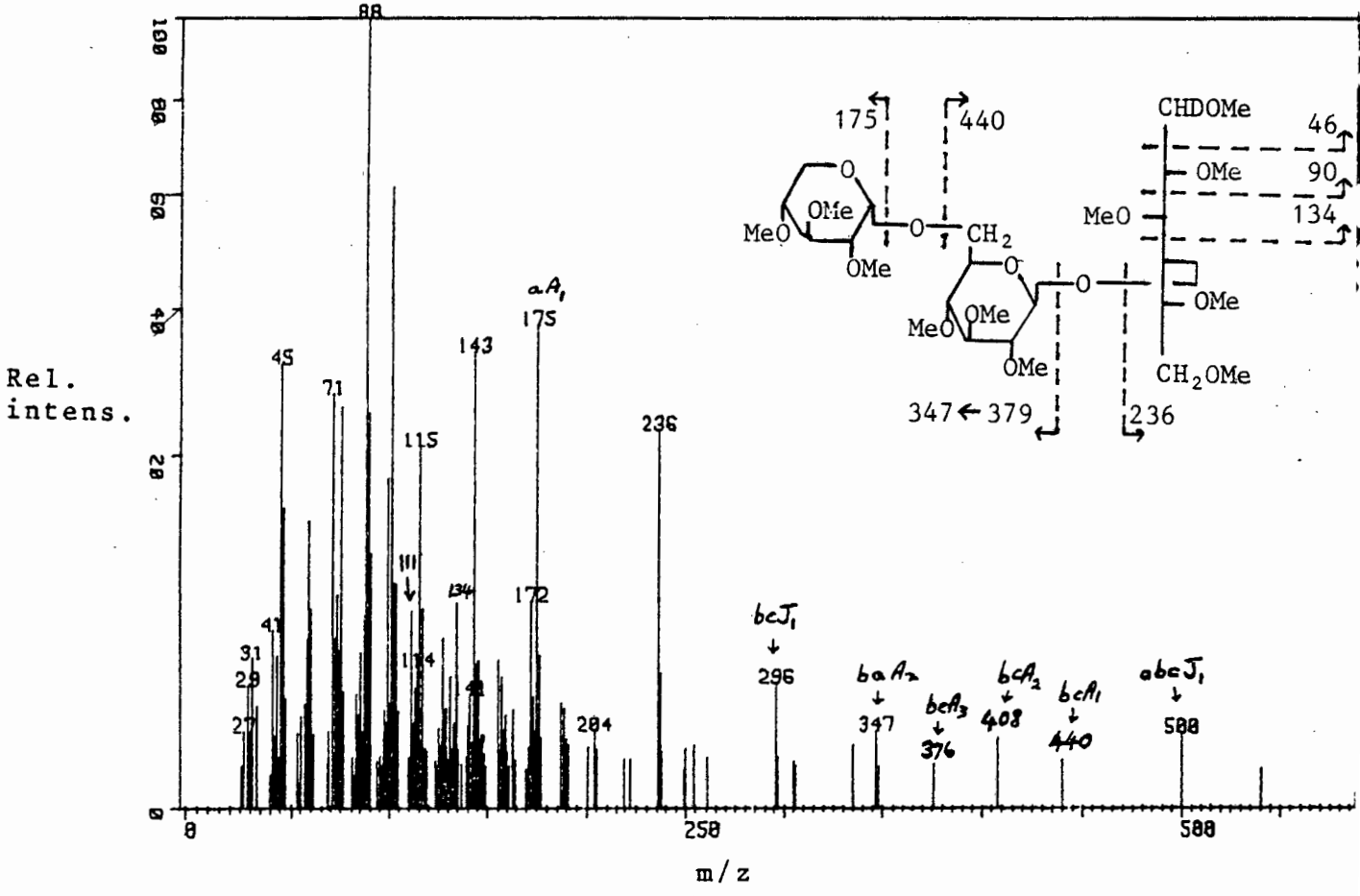


Figure 23 : Mass spectrum and some expected fragmentations of the permethylated alditol of B1

204 and 172) series suggesting the presence of a trisaccharide derivative containing non-reducing, terminal pentose and hexitol units respectively. The presence of ions at m/z 134 and 90 and the absence of an ion at 178 indicated that the hexitol is linked through C-4. That the internal residue is a hexose could be inferred from the presence of the ions at m/z 440(bcA_1), 408(bcA_2), 376(bcA_3), 500($abcJ_1$) and 347(baA_2). The results of methylation analysis by g.l.c. of p.m.a.a.'s (Table XI) showed that xylose is non-reducing and terminal, and glucose is present both as a (1→6)-linked residue and as its 4-linked reduced derivative, glucitol. The combined data were consistent with the structure Xylp-(1→6)-Glcp-(1→4)-Glcp for B1.

From the relative proportions (determined as for B1) of Xyl and Glc in B2, found to be 1 : 1, the relative behaviour of B2 and B1 on p.c. and t.l.c. and that of their methylated derivatives on g.l.c., it appeared reasonable to assume that B2 is a tetrasaccharide with a xylosyl unit additional to the sugar constituents of B1. The presence, in the mass spectrum of methylated B2 alditol (Fig. 24), of ions of the aA (m/z 175, 143, 111) and baA (379, 347, 315) series identified the sequence pentosyl→ hexosyl→ . (The glycosyl units referred to here and in other similarly identified

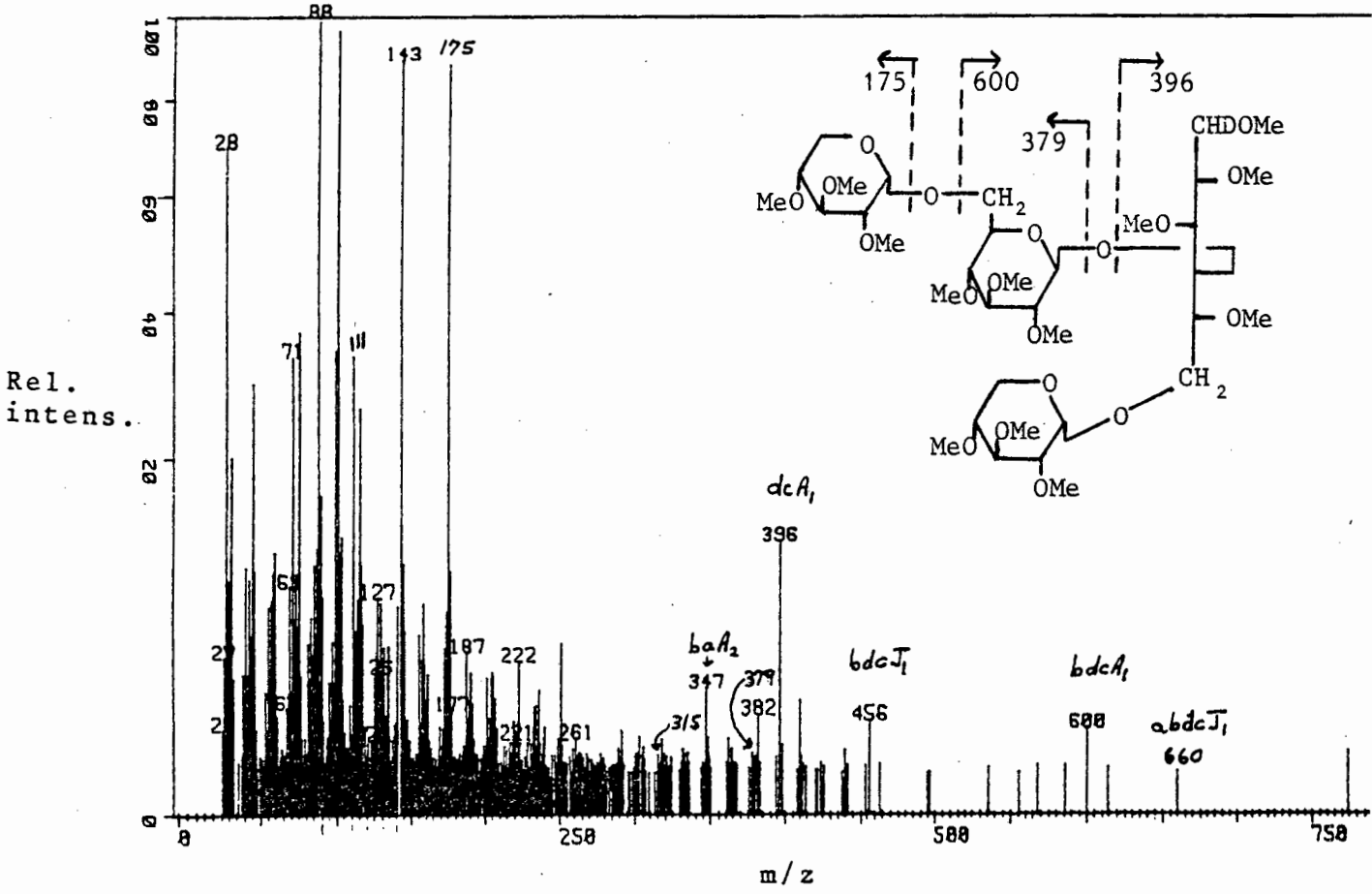


Figure 24 : Mass spectrum and some expected fragmentations of the permethylated alditol of B2

sequences in the following discussion are all linked through C-1 to the adjacent sugar unit). That the alditol moiety was substituted with both pentosyl and pentosyl hexosyl groups, was suggested by the presence of ions at m/z 396(daA_1), 456($bdcJ_1$) 600($bdcA_1$) and 660($abdcJ_1$). Methylation analysis (Table XI) showed the presence of non-reducing terminal xylose, (1→6)-linked glucose and (4,6)-linked glucitol. From these data the structure of B2 was proposed as:

Xylp-(1→6)-Glcp-(1→4)-Glcp
6
↑
1
Xylp

Structural investigations of oligosaccharides B3, B4, B5, B6 and B7:

These oligosaccharides are considered together because of similar problems encountered in their investigation. Firstly, the appearance of mass spectra obtained for them ranged from fair to poor, probably because of their higher molecular weights. Secondly, with the exception of B6 and B7, their ^{1}H-n.m.r. spectra could not be recorded due to the very small amounts of samples available. Thirdly, evidence from t.l.c.

showed varying degrees of heterogeneity. Thus, in addition to the limited data obtained, their structural compositions are discussed in the light of positively identified oligosaccharides derived by similar methods from other xyloglucans, as described in the literature. Furthermore, an assumption is made that the decrease in R_{Glc} observed can be associated with an increase in d.p. by the addition of one or two sugar units. In a literature survey of xyloglucan oligosaccharides, it was observed that there is a trend, whereby higher oligomers appear to be composites of the lower ones, a fact immediately apparent from the relationship between B2 and Xyl-(1→6)-Glc characterised above.

While the mass spectrum of methylated reduced B3 is given in Fig. 25, the intensities of ions which provide confirmation of terminal units present in B4, B5, B6 and B7 are listed in Table XII.

Examination of B3 by t.l.c. (solvent F) revealed that it contained at least two components, differing only slightly in mobility, the minor one being slightly faster. The fragmentation pathways (Fig. 26) implied by the appearance of the mass spectrum of methylated reduced B3 are discussed under the assumption that the components present are pentasaccharides. The presence of ions of the aA series (m/z 189, 157, 125), ions

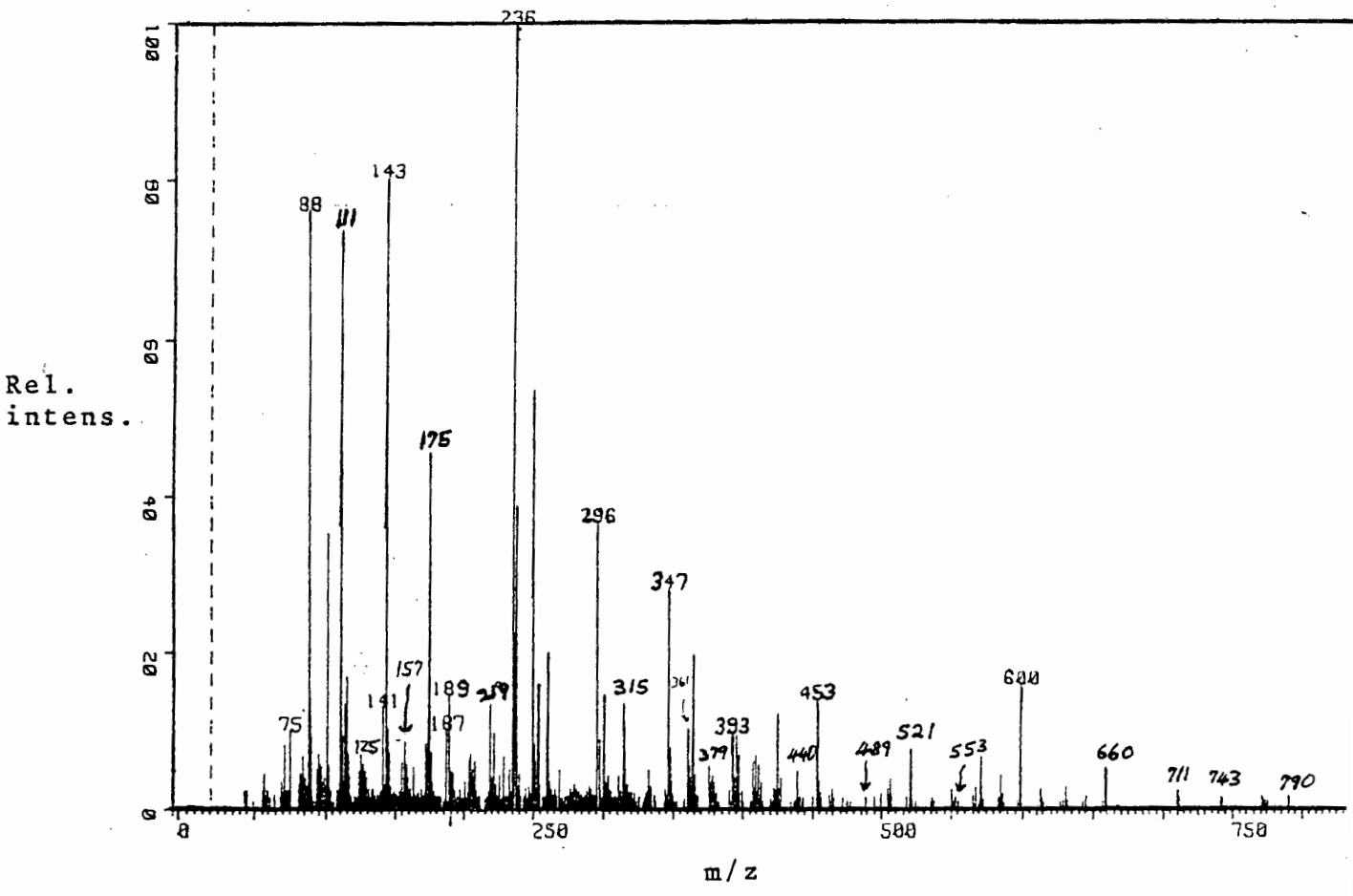
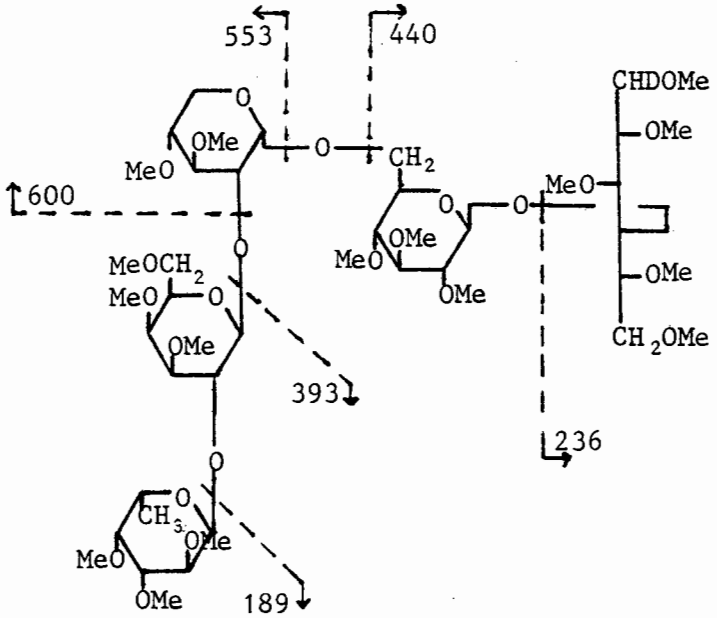
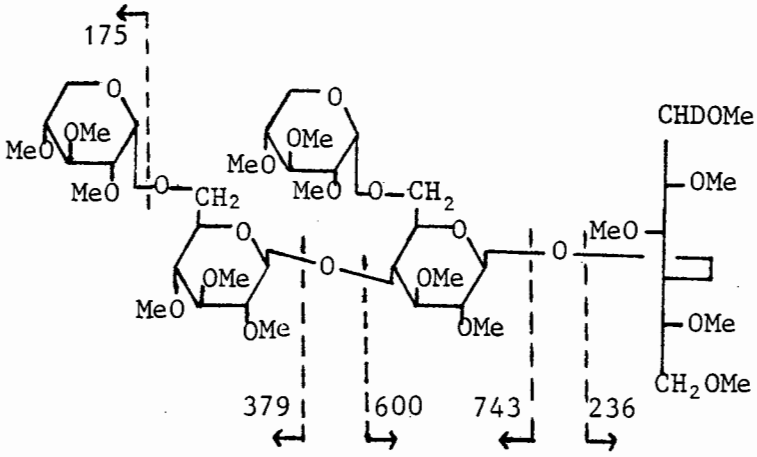


Figure 25 : Mass spectrum of the permethylated alditols of B3

(1)



(2)



(3)

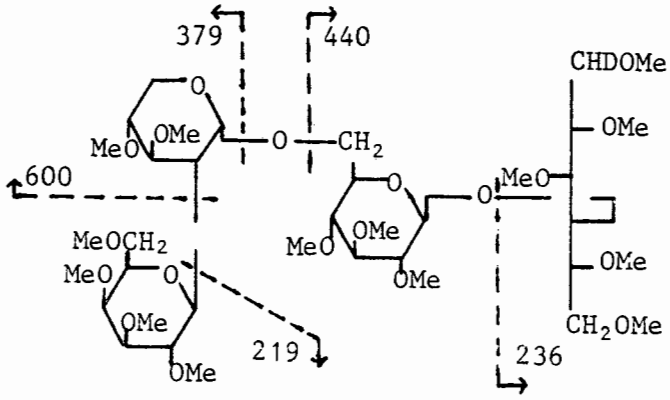


Figure 26 : Some expected fragmentations of the permethylated alditols of B3-(1), (2) and (3)

TABLE XII - RELATIVE INTENSITIES (m/e 88 = 100) OF SOME IONS IN MASS SPECTRA OF METHYLATED DERIVATIVES OF OLIGOSACCHARIDES B4-B7

m/e	B4	B5	B6	B7	Fragment ion[a]
88	100	100	100	100	
90	29,3	23,3	20,0	12,7	
111	44,0	17,3	46,7	22,7	aA_3 (p)
125	-	-	-	22,0	aA_3 (dh)
134	31,3	19,3	-	-	
143	93,3	86,0	55,3	65,3	aA_2 (p)
155	-	-	30,7	12,7	aA_3 (h)
157	-	-	-	32,7	aA_2 (dh)
172	39,3	18,7	-	-	
175	82,7	80,0	49,3	56,0	aA_1 (p)
187	-	-	48,0	36,0	aA_2 (h)
189	-	-	-	34,7	aA_1 (dh)
204	17,3	15,3	-	-	
219	-	-	25,3	26,7	aA_1 (h)
236	65,3	20,0	23,3	20,0	Hexitol
296	10,7	-	7,3	5,3	
303	-	-	9,3	-	
315	14,7	11,3	8,0	16,0	
347	12,7	16,7	12,0	20,7	
361	-	-	-	18,7	
379	-	5,3	9,3	15,3	
393	-	-	-	11,3	
396	-	30,0	-	-	
489	-	-	-	6,0	
519	-	-	-	6,0	
521	-	-	-	8,0	
551	-	-	-	6,7	
600	7,3	-	-	-	

a Symbols aA correspond to those used in ref. 117; (p), pentose; (dh), deoxyhexose; (h), hexose

of m/z 393(baA_1), 361(baA_2) and icns of the cbaA series (m/z 553, 521, 489) identified the sequence 6-deoxyhexosyl → hexosyl → pentosyl →. The ions at m/z 236, 296(deJ_1), 600($cdeA_1$) and 660($bcdeJ_1$) could be associated with the sequence pentosyl → hexosyl→ . hexitol, where the pentosyl (a) and the hexosyl (b) units are both potential positions for attachment of the missing group in the original molecule. Possible linkage through (a) is supported by the presence of the ion at m/z 440(deA_1) which could not be produced if linkage were solely through (b). Since methylation analysis (Table XI) indicated the presence of non-reducing, terminal Fucp, (1→2)-linked Xyl, (1→6)-linked Glc and 4-linked glucitol, then, by a combination of the former sequence and the latter, through (a), it was reasonable to propose the structure Fucp-(1→2)-Galp-(1→2)-Xylp-(1→6)-Glcp-(1→4)-Glcp, B3(1), for one of the components present in B3. However, the proportion of Fuc present in B3 suggests that it is a minor component. This pentasaccharide has previously been characterised by two different research groups.[118,119]

The major component could be associated with a structure involving linkage through (b) of the second sequence identified above. The mass spectrum (Fig. 25) showed the presence of ions of the aA (m/z 175, 143,

111) and baA (379, 347, 315) series thus identifying the sequence pentosyl → hexosyl→. Also present were the ions m/z 743 ($cdbaA_1$) and 711 ($cdbaA_2$). These data combined with methylation analysis (Table XI) were consistent with the structure Xylp-(1→6)-Glcp-(1→4)-(Glcp)-(1→4)-Glcp

6

↑

Xylp

B3(2)

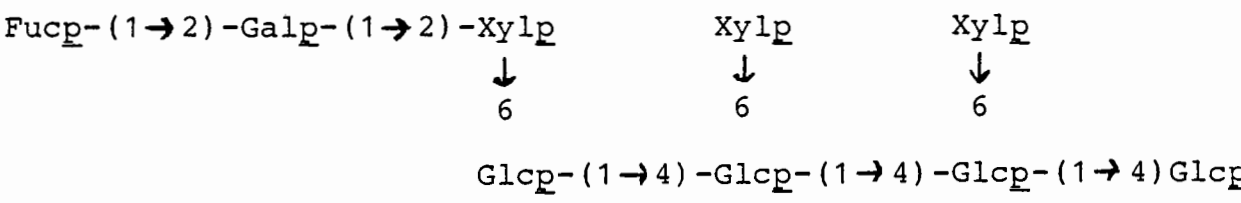
A similar oligosaccharide has been isolated by various groups.[118,120]

Finally, the possibility of a third component was suggested by the presence of the ions of the aA series (m/z 219, 187, 155), which in conjunction with the baA series (m/z 379, 347, 315) would identify the sequence hexosyl → pentosyl. A combination of this, with the pentosyl → hexosyl→hexitol sequence identified above, and a consideration of the methylation analysis (Table XI) would support the proposal of the structure Galp-(1→2)-Xylp-(1→6)-Glcp-(1→4)Glcp, B3(3), for the component in question. Further support is provided by the presence in the mass spectrum of the ion at m/z 790 $[M-45]^+$. Cellulase degradation of xyloglucans from potatoes[121] and runner beans[119] generated a similar oligosaccharide, among others.

The oligosaccharide B4 migrated in a manner similar to B3 both on p.c. and t.l.c. Its sugar composition reflected the presence of xylose and glucose. Examination of its methylation analysis (Table XI) and mass spectral data (Table XII) indicated that it had the same structure as component B3(2), as identified above.

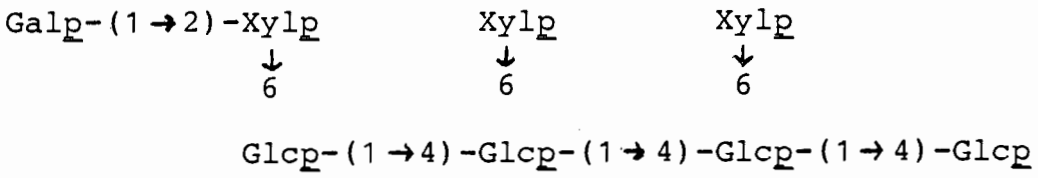
B7 is considered next because it had some structural relationship with the Fuc-containing component in B3. Examination of B7 by t.l.c. (solvent F) showed that it contained two components which could not be completely resolved from each other. The ^{1}H-n.m.r. spectrum of B7 showed the anomeric proton signals expected for α-linked D-Xylp and and β-linked D-Glcp in addition to the H-1 absorption peak of α-linked L-Fucp at δ5,08, as an ill-defined doublet, and the methyl protons' signal at δ1,24 (doublet, J 6,3 Hz) (see Table VIII). Also observed was a signal at δ5,25 (broad singlet) which is where the anomeric proton of α-linked L-Ara would be expected to absorb; however, the presence of Ara could not be confirmed when B7 was analysed for sugar composition. From the mass spectrum of methylated reduced B7 (Table XII) the sugar sequence 6-deoxyhexosyl → hexosyl → pentosyl could be identified with the presence of the following ions, m/z 189, 157, 125, 393, 361, 521 and 489. While these are ions

associated with 3 different A series, the small letters which are used to label the sugar units involved are deliberately omitted, since no evidence was available to indicate the unit to which the side-chain was attached. The presence of ions m/z 236 and 296 showed that the reduced end was a singly substituted hexitol. Considering the chromatographic mobility of B7 relative to B3 (Table X) and taking into account the methylation analysis of B7, the structure B7(1), which has also been assigned to one of the oligosaccharides derived from soybean xyloglucan,[118] was proposed for this component.



The order of side chains is purely arbitrary.

The second component was associated with the presence, in the mass spectrum (Table XII), of ions of the aA (m/z 219, 187, 155) and baA (379, 347, 315) series and the ions m/z 551 ($cbaA_2$) and 519 ($cbaA_3$), which identified the sequence hexosyl→ pentosyl→hexosyl→. These data in conjunction with methylation analysis suggest the structure B7(2) for the component.



B7(2)

A similar oligosaccharide was isolated from a cellulase digest of rice endosperm xyloglucan.[122]

T.l.c. examination of B5 revealed the presence of one major component together with two minor ones. The sequence pentosyl → hexosyl→was identified from the presence, in the mass spectral data (Table XII) of ions of the aA (m/z 175, 143, 111) and baA (m/z 379, 347, 315) series. That most of the hexitol units were doubly-substituted, with at least one substituent being a pentosyl group, was suggested by the higher intensity of the ion m/z 396 relative to ion m/z 236, the latter being due to monosubstitution. Assuming that the major component in B5 is a hexasaccharide, as its relative mobility on p.c. might imply, then a structure consistent with the above data and methylation analysis (Table XI) would be one of cellotriose that is substituted at each 6-O-position with Xylp groups.

Such an oligosaccharide was also detected among those derived from runner bean xyloglucan.[119]

On t.l.c. B6 gave a streak rather than discrete spots, so that there was no indication of how many components it contained. The complex composition was further reflected by the appearance of its ^{1}H-n.m.r. spectrum. While the anomeric proton signals due to α-linked D-Xylp and β-linked D-Glcp were observed at the expected values, they appeared as complex doublets. This could suggest the presence of oligomers with significantly different molecular structures for which conformational fluctuations cannot readily be averaged out in the spectrometer. The presence of appreciable amounts of terminal non-reducing Glc was also unexpected, since oligosaccharides derived from xyloglucans usually have the Glc unit at the reducing end as the unsubstituted one, rather than at the non-reducing end, a phenomenon that could probably be associated with the specific mode of action of cellulase enzymes. However, the formation of reversion products during cellulase enzyme degradation studies has been reported,[123] and therefore it was considered possible that a similar phenomenon could explain the observed anomalies. Such a process would lead to a consumption of the glucose released by degradation, through a biosynthetic reaction resulting

in glycosylation of some sugar units in the oligosaccharides with glucosyl groups.

The mass spectral data of the methylated alditols of B6 showed the presence of some features indicative of terminal groups found in the other oligomers investigated. Since B6 is the only component that had Ara as one of its sugar constituents, the presence of the ion m/z 303 in addition to the aA series (m/z 175, 143, 111), which identifies the sequence pentosyl pentosyl , could be regarded as suggestive of an Araf-(1→2)-Xylp linkage; the methylation analysis (Table XI) shows the Araf to be terminal. The relative mobility of B6 on p.c. suggests that the average molecular size is that of an octasaccharide, even though such an Ara-containing oligosaccharide of the same d.p. has, apparently, not been reported in the literature.

The detection of small amounts of 2,3,6-tri-O-methyl glucose in the methylation analysis of B3, B4, B5, B6 and B7 would suggest the presence of (1→4)-linked Glc residues that are unsubstituted at O-6 in some of the oligosaccharides contained in these components. Whether such residues are part of oligosaccharides derived by enzymatic degradation or by the biosynthetic reaction discussed above is not certain.

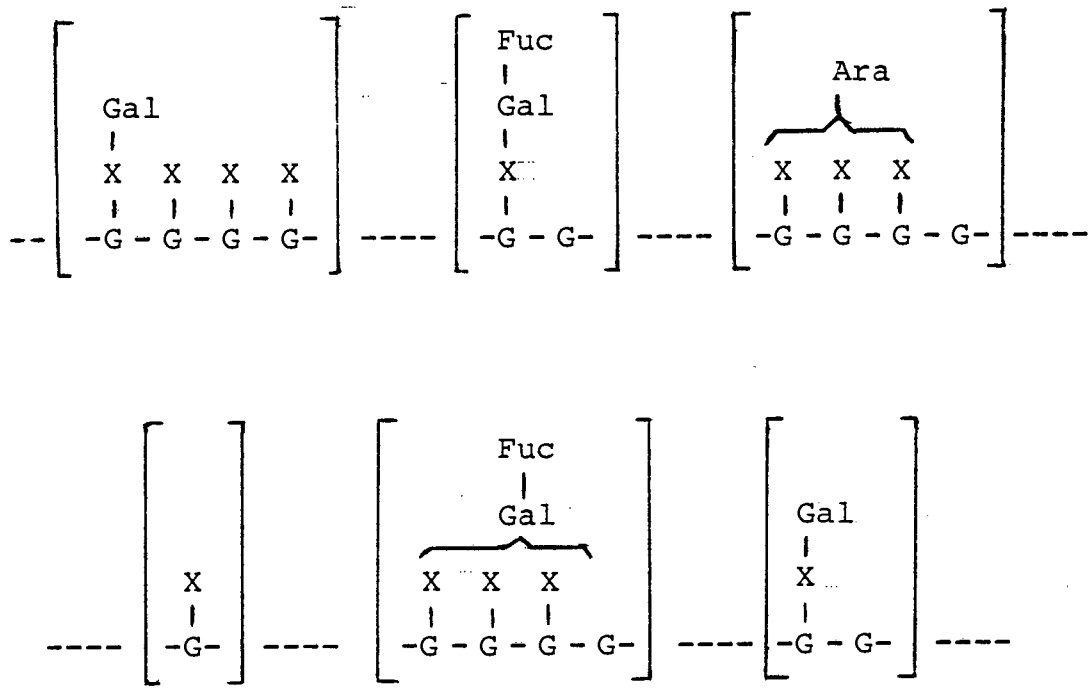
Conclusions

These studies have demonstrated the susceptibility of the xyloglucan to cellulase degradation, generating oligomers of d.p. less than 10. This result is in agreement with the proposed presence of a backbone structure of β-(1→4)-linked glucosyl residues in the polysaccharide. The release of Gal, Fuc and Ara by the enzyme demonstrated that it has other glycosidase activities as well.

The oligosaccharides discussed above give an indication of the structural features of the parent polysaccharide from which they were generated. Taking into account the proportions of the sugars and their linkages in the polysaccharide, the postulated main structural features of the polysaccharide can be visualised in "blocks" as in Fig. 27. The assignments are based mostly on the structures of the higher oligosaccharides, from which it is assumed the lower oligomers may be generated.

4.4.4 Methylation studies of G5II and G5III

G5II and G5III, recovered from the same alkaline extract as the xyloglucan G5I were examined for sugar-linkage composition by methylation analysis. G5II contained an insignificant amount (3%) of uronic acid



G = Glc, X = Xyl

FIGURE 27 : Postulated main structural features of the sisal xyloglucan

whereas for G5III the uronic acid content was 25%. Thus G5II was completely methylated by the Hakomori procedure,[92] whereas full methylation of G5III was achieved only after the Hakomori treatment was followed by 2 successive Purdie[95] methylations. Methylated G5III was LAD-reduced and remethylated to give MRMG5III. Methylated G5II (MG5II)and MRMG5II were then hydrolysed and the derived p.m.a.a.'s were analysed by g.l.c. and g.l.c.-m.s. The results are presented in Table IX.

The methylation results for G5II indicate the presence of a fair amount of (1→4)-linked xylan polymers, with occasional branching at O-2 and both at O-2 and O-3, as implied by the detection of 3-O-methyl Xyl and xylose. In addition to the xylan, there also appears to be present about an equal amount of xyloglucan as indicated by the detection of (1→4,6)- and (1→4)-linked Glcp and an appreciable amount of terminal Xylp. Thus it appears as if G5II is either a mixture or a complex of the two polysaccharide species.

The methylation results for G5III showed that deuterium incorporation had occurred at C-6 of (1→4)-, (1→2)- and (1→2,4)-linked Galp residues. The preponderance of the first form of linkage suggests the presence of a mainly (1→4)-linked chain of GalpA residues in G5III, which when interspersed with the (1→2)- and (1→2,4)-linked Rhap would form a rhamnogalacturonan-type of polymer. This structure is well-known to be fundamental for most pectic polysaccharides, where the chain units may be substituted with arabinan, galactan and occasionally xylan chains of limited length. Therefore the occurrence of (1→5)- or (1→4)-linked Ara and some (1→3)- and (1→4,6)-linked Galp could be associated with such chains. However, the proportion of (1→4)-linked Xylp seems too high for all of it to be incorporated in such side-chains, and it is more

likely that these residues occur in xylan polymers present in G5III.

4.4.5 Methylation and other studies of G1

G1 was also subjected to the Hakomori methylation procedure[92] in order to investigate its sugar-linkage composition and hence deduce the nature of the carbohydrate polymers present. Due to the insignificant uronic acid content, LAD-reduction was deemed unnecessary. The methylation results appear in Table IX. The preponderance of (1→3,6)-linked Galp and terminal Araf, and the presence of fair proportions of (1→3)-linked Galp and (1→5)- or (1→4)-linked Ara, indicate that G1 consists mainly of a Type II arabinogalactan. The detection of small proportions of (1→2)-linked Rhap suggests minimal amount of associated pectic polymers.

Examination of G1 by ^{1}H-n.m.r. spectroscopy (Fig. 28) showed the presence of a pair of doublets centred at δ7,0 in addition to the anomeric proton signal of α-L-Ara at 5,25 (the β-D-Gal signal was obscured by the water peak). Of more interest here was the signal at δ7,0, because the symmetrical appearance of these doublets was characteristic of a p-substituted aromatic system. A phenolic compound with such characteristics

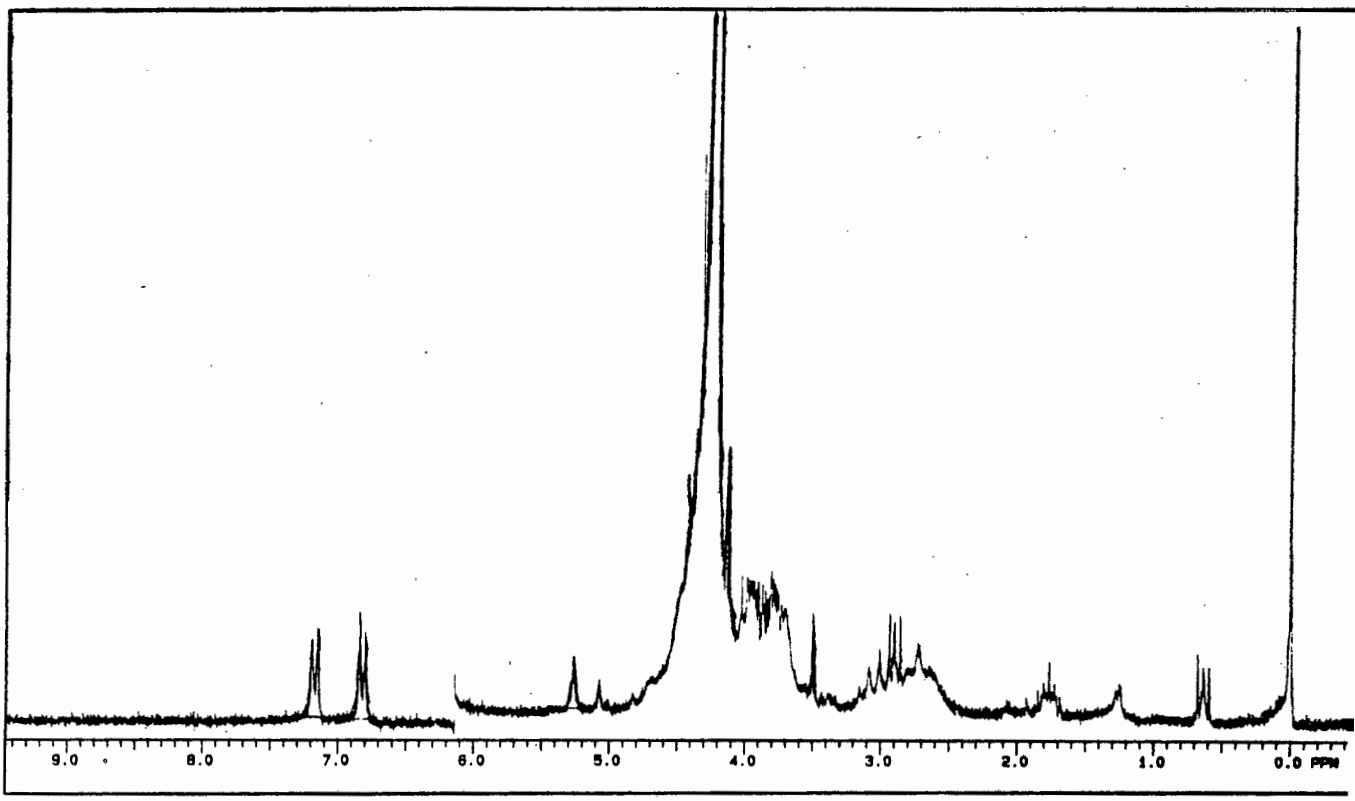


Figure 28 : 200MHz ^{1}H-n.m.r. spectrum in D_2O of G1 at 80°C

which usually occurs in association with plant polysaccharides is p-coumaric acid[124,125] in its ester glycoside form; however, the absence of signals corresponding to the olefinic protons indicated that the phenolic species detected was different from p-coumaric acid. Presuming that the nitrogen content (2,6%) of the non-dialysable material Gl could be due to associated protein, it could be that the phenolic species might be the amino acid tyrosine, since this would give a similar pattern of signals in the aromatic region. Complete hydrolysis of Gl gave a range of amino acids with tyrosine appearing as a component on p.c.. The phenolic content as estimated by u.v. spectroscopy (λ_{max} 273nm) was 10% (w/w of Gl). Tyrosine is a precursor towards the formation of p-coumaric acid, which in turn is one of the starting materials utilized during the biosynthesis of lignin.[126] Its dimer, isodityrosine, has been implicated in the cross-linking of cell-wall glycoproteins.[127] However, quantitative analysis gave the found tyrosine content as less than 1%, and it is therefore suggested that p-hydroxybenzoic and/or p-hydroxyphenylacetic acid might be the phenolic species responsible.[128]

4.5 Conclusion

In the study of the fleshy material (G) from sisal plant leaf just discussed, it has been demonstrated that its polysaccharide composition is fairly diverse, compared to that of the fibrous material (F). Thus, while the water extract of G consisted predominantly of an arabinogalactan, associated with protein, the alkaline extracts revealed the presence of xylan polymers (which appeared to have a strong association with protein and/or with pectic polymers) in addition to xyloglucans which formed the dominant species in these extracts. In contrast, the water extract of F consisted mainly of what appeared to be rhamnogalacturonan-type pectic polymers, while all the alkaline extracts contained, almost exclusively, a 4-O-methylglucuronoxylan. This xylan was found to be of the linear type, with β-(1→4)-linked D-xylosyl residues in a chain, two in ten being substituted with single xylosyl and 4-O-methyl-α-D-glucuronosyl groups. The latter groups are distributed in an irregular manner along the xylan chain. Those xylan polymers in G which were studied by methylation analysis were also found to be mainly linear, suggesting that they may bear some structural relationship to the F xylans.

The xyloglucan isolated from G was subjected to detailed structural studies which included cellulase enzyme degradation experiments. Like all xyloglucans it consisted of a basal chain of β-(1→4)-linked D-glucosyl residues, with about two-thirds of these substituted at O-6 with xylosyl units. About two-fifths of the latter residues are further substituted with Gal-, Ara- and Fuc-containing side-groups. From the enzyme degradation studies it was possible to postulate the main structural features of the polysaccharide in terms of "blocks" (Section 4.4.3).

The major non-cellulosic polysaccharide in G is pectin, most of which could be extracted with ammonium oxalate, but it was noted that it occurred in every extract including the final residue (GC). Its presence in the final residue could be associated with the appreciable amount of uronic acid detected in this material. Pectin was only a minor component in F, the portion extracted with ammonium oxalate being only about 20% (w/w) of the total of 4-O-methylglucuronoxylan isolated. That there could be some residual xyloglucan present in GC was demonstrated by the release of appreciable amounts of Glc and Xyl under hydrolysis conditions as detailed in method 1, Section 2.6. Under these conditions cellulose is not hydrolysed or it is hydrolysed only to an insignificant extent. The

released neutral sugars accounted for about 1% (w/w) of dry GC.

To complete this discussion, an overall picture of the distribution of monosaccharide units in the various extracts of the whole leaf material is presented in Tables XIII and XIV. The preponderance of xylose among the alkaline extracts is consistent with the xylans being the overall major hemicellulosic β-glycans in the sisal plant leaf, as is apparently common in monocotyledonous plants.[6] Although the major hemicellulosic β-glycans from sugar cane bagasse were also xylans consisting of a backbone structure of β-(1→4)-linked Xyl-p residues, these occurred as a range of polymers substituted at O-3 to varying degrees with terminal Ara-f groups.[108] For the purpose of comparison some characteristics of hemicellulose from bagasse are summarised in Table XV.

4.6 Experimental

4.6.1 Extraction and isolation of the polysaccharide fractions

Sisal plant-leaf material in the form of a white residue, after exhaustive extraction with methanol, was supplied by courtesy of Dr J F Elsworth, University of

TABLE XIII - RELATIVE WEIGHT DISTRIBUTION OF MONOSACCHARIDES IN COMBINED FRACTIONS OF SISAL LEAF

Extraction medium	Mass of combined fractions(g)	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA[b]
H_2O	1,52	9[a]	-	27	6	3	40	9	7
Aq. $(NH_4)_2C_2O_4$	6,14	6	-	4	4	2	21	4	59
0,2M KOH	1,78	1	-	1	83	-	2	1	12
1,0M KOH	1,99	1	1	2	81	0,5	0,5	3	10
2,5M KOH	0,74	2	3	4	48	2	7	25	7
Final insoluble residue	22,38	3	-	3	6	-	2	85	5

a expressed as g/100g of the combined fraction from each extract

b UA = uronic acid

TABLE XIV - ABSOLUTE WEIGHT DISTRIBUTION OF MONOSACCHARIDES IN COMBINED FRACTIONS OF SISAL LEAF

Extraction medium	Mass of fraction (g)	% yield[a]	Monosaccharide composition[b]							
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA[c]
H_2O	1,52	2,9	4,0	-	11,7	2,5	1,3	17,4	4,1	3,1
Aq.$(NH_4)_2C_2O_4$	6,14	11,8	1,0	-	7,6	7,06	3,1	37,5	7,4	105,3
0,2M KOH	1,78	3,4	0,5	-	0,6	43,0	-	0,9	0,5	6,2
1,0M KOH	1,99	3,8	0,8	0,4	1,3	47,0	0,3	0,3	1,8	5,8
2,5M KOH	0,74	1,4	0,4	0,7	0,9	10,2	0,5	1,4	5,3	1,5
Final residue	22,38	43,0	16,2	-	17,2	40,0	-	14,7	531,5	28,5

a Expressed as w/w of dry EtOH/benzene-insoluble whole leaf material

b Expressed as µg/mg of total carbohydrate in combined fractions from dry EtOH/benzene-insoluble leaf material

c UA = uronic acid

TABLE XV - SOME CHARACTERISTICS OF A XYLAN-RICH FRACTION FROM SUGAR CANE BAGASSE [108]

SUB-FRACTION[a]	A1	A2	B
% weight ratio	18	45	37
$[\alpha]_D$, degrees	-50	-72	-65
Proportions of neutral sugars (mol%)[b]			
Ara	39	22	14
Xyl	61	63	86
Gal	-	7	-
Glc	-	8	-
Methylated product			
$[\alpha]_D$, degrees	-70	-91	-80
Molar ratio of methylated sugars[c]			
2,3,5-Ara	1,0	1	1
2,3-Xyl	2,3	4	8
2-Xyl	1,3	1	0,5
		[d]	

a A1 and A2 were obtained on Cetavlon fractionation of the water-soluble material A (A1 from precipitate, A2 from supernatant). B was material dissolving in water only after sonication

b By g.l.c. as alditol acetates on 3% OV-225 column

c By g.l.c. and g.l.c.-m.s. of p.m.a.a.'s

d No methyl ethers of Gal or Glc were detected

Cape Town. The material was fractionated by sifting into a fibrous portion, designated F (26g) and a fleshy portion, called G (34g). Each portion was ground to a particle size passing through 40 mesh wire net. The lignin content of F and G, determined by the sulphuric

acid method as described by Adams,[129] was 12% and 15% (w/w) respectively. The ground samples were each extracted for 24h with ethanol-benzene (1 : 2, v/v) in a Soxhlet apparatus and the resulting insoluble residue, designated R in both cases, was air-dried at ca 25°C (yield, 23g and 29g from F and G respectively).

The procedure followed for the extraction of the polysaccharide fractions (Scheme 4) was similar for both samples, the only difference being in the quantities of materials used. Since these quantities were determined proportionally according to the mass of R and that of the holocellulose recovered after delignification, details of the extraction procedure are described with reference to F only. Thus the residue R (23g) was suspended in water (1L) and stirred at 80°C for 5h followed by 18h at 25°C. The slurry was filtered through cloth on a Buchner funnel. The filtrate was centrifuged and the supernatant, after concentration under reduced pressure (at ∿40°C) to about 200mL was dialysed against running tap water for 3 days and against distilled water for 24h. The final solution was freeze-dried to give F1. The insoluble residue was further extracted (3 times) for 4h at 80°C with 0,5% aqueous $(NH_4)_2C_2O_4$ (1L each time). After filtration the combined filtrate was centrifuged and the supernatant was concentrated as above.[a] The pH

[a] The solution containing G2 could not be concentrated to less than 600mL due to its very high viscosity.

was adjusted to 4 with acetic acid and the turbid solution was dialysed and freeze-dried, yielding F2. The recovered insoluble residue was delignified by the method described by Adams,[130] which involved suspension of the material in water (750mL) at 75°C, followed by 3 additions of $NaClO_2$ (12g) and acetic acid (2,9mL) at 1h intervals; the mixture was stirred vigorously during the delignification process. After 3h the mixture was cooled to ca.25°C and filtered through cloth; the insoluble residue was washed with water until free of acid, washed with ethanol and finally air-dried; yield 17,3g of holocellulose (lignin content 2,4%). The yield of holocellulose from G was 13,4g; lignin content 1,8%.

F3, F4 and F5 were obtained by extracting the holocellulose successively with the following concentrations of KOH : 0,2M (1L), 1,0M (500mL) and 2,5 M (500mL) respectively. Each extraction was carried out at ca.25°C, under a nitrogen atmosphere, for 24h, all the KOH solutions being made 10mM with respect to $NaBH_4$ prior to extraction. After filtration, the opalescent filtrates were clarified by centrifugation at ca.5°C and the clarified solutions were neutralised, with cooling, to pH 7 by the addition of acetic acid. The solutions were concentrated and further processed as described above to give F3, F4 and F5. The

alkaline-insoluble residue was washed with water until washings were neutral to litmus paper, then successively with ethanol and ether, and finally air-dried to give FC.

4.6.2 Hydrolysis of FC and GC

The proportions of neutral sugar constituents in these cellulose-enriched polysaccharide fractions were determined by g.l.c. of derived alditol acetates after hydrolysis, as follows: A sample (10mg) was slurried with 72% (w/w) H_2SO_4 (0,7mL) and left at 20°C for 3h. The slurry was diluted with distilled water to give a H_2SO_4 concentration of 1M and heated at 100°C for 5h. The cooled hydrolysate was diluted with distilled H_2O (25mL) and treated with 0,25M $Ba(OH)_2$ to pH 5-6. The precipitated $BaSO_4$ was removed by centrifugation and the precipitate was washed with distilled H_2O. The combined supernatant and washings were evaporated to dryness under reduced pressure at ∿40°C. The aldoses in the residue were converted to alditol acetates as detailed in Section 2.6.

4.6.3 Oxidation and degradation of base-degraded methylated F3 (Scheme 5)

An anhydrous M solution of chlorine in dichloromethane (10mL) was placed under nitrogen in a flask sealed with a rubber cap and cooled to -55°C (dry ice-acetone). With continuous stirring, anhydrous methyl sulphoxide (5mL) was added dropwise to the solution, giving a white complex. Base-degraded methylated F3 (50mg), (prepared as described in Section 2.7) in dichloromethane (3mL) was added dropwise with a syringe while the reaction mixture was kept at -55°C. After 5h of continuous stirring, triethylamine (5mL) was added, and the reaction mixture was allowed to attain room temperature, then dialysed against water overnight, and concentrated to dryness. The product was purified as for other methylated polysaccharides (see Section 2.7); yield, 35mg.

The oxidised material (30mg) in dichloromethane (2mL) was treated with ethanolic M sodium ethoxide (1mL) and the mixture was stirred at ~25°C. After 1h it was neutralised with acetic acid and evaporated to dryness. The residue was dissolved in methanol and Amberlite IR-120(H^+) resin was added to bring the pH to 4. The solution was filtered and concentrated to dryness. The residue was treated with 50% aqueous acetic acid for 2h

at 100°C and the resulting solution evaporated to dryness, yielding 17mg of degraded product. Part of this material (10mg) was ethylated by the Hakomori procedure[92] and the partially methylated, partially ethylated oligosaccharides were examined by t.l.c. (solvent G). A portion of these oligosaccharides was hydrolysed and analysed by g.l.c.-m.s.

4.6.4 Degradation of sisal xylan (F3) with cellulase

The polysaccharide (1g) was dissolved in 0,1M sodium acetate buffer (100mL, pH 4,8) and incubated with the cellulase preparation from *T. reesei* (Rut C-30),[112] (100mg, activity 390 IU/g) at 37°C for 3 days, in the presence of toluene to prevent microbial growth. At the end of this period polymeric material was precipitated by the addition of ethanol to 80% final concentration. The precipitate was removed by centrifugation and the supernatant was evaporated to dryness. The residue was taken up in H_2O (10mL), the solution was treated with Amberlite IR-120(H^+) resin to remove sodium ions and then freeze-dried. The product was shown by p.c. (solvent D) to contain 4-O-methyl-glucuronic acid in addition to large amounts of xylose. The two components were separated by preparative paper

chromatography and the uronic acid was isolated (yield, 66 mg) and examined as detailed in Section 4.3.5.

4.6.5 Purification of sisal xyloglucan (G5I)

The polysaccharide (279mg) was dissolved in H_2O (20mL) and Fehling's solution (3mL) was added dropwise with continuous stirring. When the addition was complete, the suspension was allowed to stand at ∿5°C for 5h and the precipitate was recovered by centrifugation. The gelatinous precipitate was dispersed in H_2O (25mL) and acidified by the dropwise addition of cold 0,5M HCl until all the precipitate was dissolved. To the resulting solution was added 4 volumes of ethanol. The precipitated material was washed several times with ethanol, then dissolved in H_2O and finally freeze-dried. The above procedure was repeated twice; yield 266mg.

4.6.6 Degradation of sisal xyloglucan (G5I) with cellulase

A solution of purified G5I (200mg) in 0,1M sodium acetate buffer (20mL, pH 4,8) was incubated with the cellulase preparation (45mg) under the conditions given in Section 4.6.4. The reaction mixture was then decationised by passing through a column (7 x 1cm)

of Amberlite IR-120(H^+) resin. The eluate was concentrated to 5mL and centrifuged, and the supernatant was applied to a Trisacryl GF 05 column (70 x 2,5cm) previously equilibrated with pyridinium acetate buffer (0,1M, pH 5,0). The column was eluted with the same buffer. Fractions (2,2mL) were collected and were monitored for carbohydrate polarimetrically and by removing aliquots (40µL) for the phenol-sulphuric acid assay. Appropriate fractions (see Fig. 21) were combined, reduced in volume to ∿10 mL and freeze-dried to give I, II and III, each of which was examined by t.l.c. (solvent F) and p.c. (solvent B). I could be resolved into 4 components whereas II revealed the presence of 3 components. I and II were further fractionated by preparative p.c. using solvent B.

G.l.c. examination of methylated oligosaccharide derivatives of B1 and B2 was performed on a glass column (2m x 3mm i.d.) packed with 2,2% SE 30 on Gas-Chrom S (100-120 mesh). The column temperature was maintained isothermally at 300°C.

CHAPTER 5

5. A GLUCOMANNOGLYCAN FROM SATYRIUM CORIIFOLIUM TUBERS

5.1 Introduction

The Satyrium coriifolium plant (Orchidaceae; Orchidales), a native of South Africa, grows wild in the winter-rainfall region of the South-Western Cape and its distribution is also extended to the Eastern Cape.[131] It is a perennial plant which becomes dormant during the non-vegetative season of the year and for its continued survival over that period it is provided with subterranean tubers which serve as storage reservoirs for essential nutrients. Studies of the chief polysaccharide nutrients found in tubers of some Orchidales species have shown them to be glucomannoglycans[9,132] (usually referred to as glucomannans). An investigation into the molecular structure of the major polysaccharide component in the tubers of S. coriifolium has been undertaken in order to relate it to those isolated from other species. In particular the polysaccharide has been compared with that isolated from the tubers of a closely related species,[133] namely, Satyrium carneum.

5.2 Isolation and preliminary examination of the polysaccharide

Successive extraction of ground tuber with ethanol, water and 24% KOH gave, inter alia, alkaline-insoluble fractions D and E, which contained Man and Glc residues in the relative proportions 1 : 1 and 3,3 : 1 respectively, the latter, for the major fraction E, being typical of glucomannan. The polysaccharide was isolated, in 72% yield, as small hard pellets (0,2-0,5mm diameter) fairly similar in shape, which were remarkably insoluble in H_2O, 24% KOH, dimethylsulphoxide or N-methylmorpholine-N-oxide (NMMNO), the last being a well-known solubilising agent for polysaccharides.[26] However, the polysaccharide pellets did dissolve slowly in 24% NaOH at ∿25°C, forming a thick gel of partially dissolved particles at 1,0% concentration. Upon dilution with the NaOH solution to 0,2%, nearly complete dissolution was achieved over 18h, giving a highly viscous solution. After removal of the insolubles (accounting for about 5% w/w of the pellets) the polysaccharide was purified as its copper complex, resulting in quantitative recovery of the glucomannan as an insoluble precipitate (sugar composition, Man:Glc 3,6 : 1; $[\alpha]_D$ -46° in 24% NaOH). The purified material was still insoluble in water, DMSO, NMMNO and 6M aqueous urea. The solubility

of the polysaccharide in NaOH but not in KOH solutions (even at 30% concentration of the latter) might suggest that a specific significant role is played by the cations during the solubilisation process, although it is not clear how this may occur. A test for solubility in LiOH solutions was negative and thus no apparent trend could be deduced with respect to the hydrated ionic radii of the different cations.

Low concentrations (< 5%) of KOH and NaOH are known to cause an increase in viscosity and subsequent gelation in aqueous solutions of otherwise water-soluble glucomannans.[132] This phenomenon was explained as being due to the saponification of acetate ester groups on the glucomannan chains, resulting in increased interchain hydrogen bonding. That the glucomannan under investigation is insoluble in water but not in concentrated NaOH solution, might suggest the absence of acetyl groups.

Neutralisation of alkaline solutions of the polysaccharide gave a gelatinous precipitate whereas addition of ethanol (to 80% final concentration) resulted in a fibrous precipitate which subsequently formed a gel upon dispersion in water. In its tendency towards gel formation, the glucomannan resembles those

isolated from *Amorphophallus konjak*[134] and *Narcissus tazetta*.[135]

Water-soluble material, mainly for n.m.r. studies, was obtained by partial hydrolysis of the insoluble polysaccharide in 72% H_2SO_4 and separation of products by dialysis. The higher molecular weight, non-dialysable material, E1 was isolated by freeze-drying; E2 consisted of the dialysable portion after extraction with 96% ethanol to remove monosaccharides and some lower oligosaccharides, the ethanol extract being designated E3. E1 and E2 contained Man and Glc residues in the same proportions as E; s.e.c. on Bio-Gel P-10 showed them to be highly polymolecular, with $\bar{M}_w$ 10 000 and 600 respectively (Fig.29). E1 had $[\alpha]_D$ -15° in water.

5.3 Methylation studies

The glycosyl linkage composition of the glucomannan was investigated by methylation using the Hakomori procedure.[92] The methylation procedure performed on purified polysaccharide material was repeated twice before isolation of the fully methylated product. Hydrolysis of the product followed by g.l.c. analysis of the derived p.m.a.a.'s (Table XVI) showed that the preponderant sugars in the hydrolysate were the

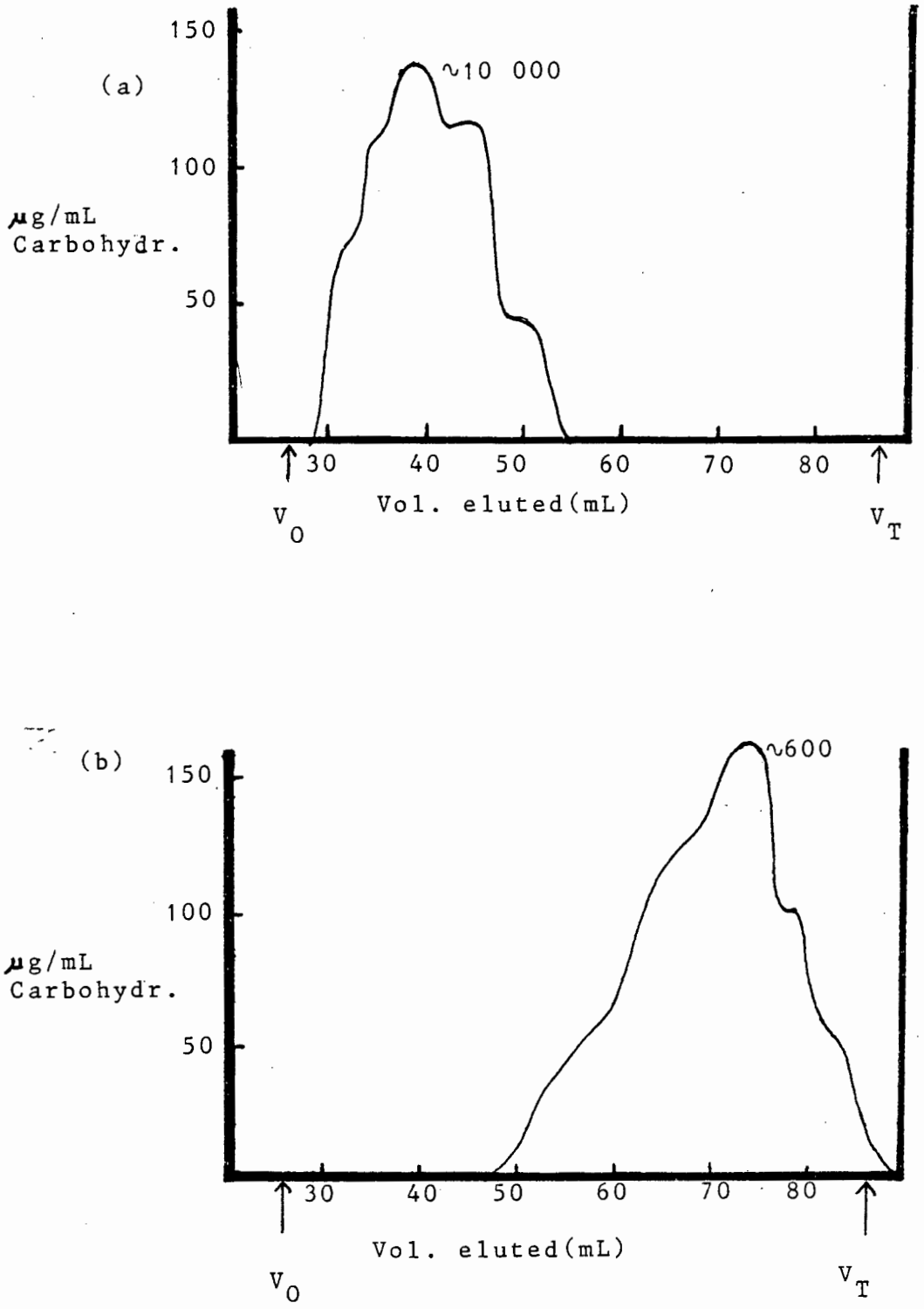


Figure 29 : Chromatography of (a) E1 and (b) E2 on Bio-Gel P-10

TABLE XVI - METHYLATION ANALYSIS OF GLUCOMANNAN, E, AND DEGRADATION PRODUCTS E1 AND E2

Sugar derivative	ME[a]	ME1	ME2
1,2,3,5,6-Hex[b]	-	-	16[c]
2,3,4,6-Man	0,8	3	13
2,3,4,6-Glc	0,2	1	4
2,3,6-Man	78	76	50
2,3,6-Glc	21	20	17

a ME, methylated E

b 4-O-Acetyl-1,2,3,5,6-penta-O-methylhexitol, etc.

c Approximate molar proportions

2,3,6-tri-O-methyl derivatives of Manp and Glcp which occurred in relative proportions of 3,8 : 1 respectively. Only trace amounts of the 2,3,4,6-tetra-O-methyl derivatives were detected. Methylation analysis of E1 and E2 (the latter after reduction with $NaBH_4$) gave similar results, except that in E2 the reducing and non-reducing end-groups were detected in appreciable proportions, because of the low $\bar{M}_w$ of this fraction. These results indicate that the glucomannan consists of (1→4)-linked Manp and Glcp residues in a linear chain. There was no indication of branching of the chain, whereas the occurrence of minimal branching has been observed in other glucomannans.[9,135]

The presence of contiguously-linked Glc residues in certain glucomannans has been demonstrated by the detection of cellobiose among partial hydrolysis products of the corresponding polysaccharide. The occurrence of such a structural feature was investigated as follows: the ethanol-soluble material, E3, (which was shown by p.c. to contain mainly mannose, glucose and a range of disaccharides with only traces of trisaccharides) was reduced with $NaBH_4$ and the resulting alditols were methylated. G.l.c. analysis (column B) of the methylated alditols showed the presence of four components in the disaccharide region (Fig. 30) probably due to the four disaccharides possible from a statistical combination of Man and Glc in β-(1→4)-linkage. That one of these components was derived from cellobiose was indicated by comparing its retention time with that of standard methylated cellobiitol. The increase in intensity of the corresponding peak upon co-injection with the standard further confirmed the assignment. From this result it could be deduced that in the glucomannan chain there are at least two Glc residues that are mutually linked, as has been observed in other glucomannans.[9,135]

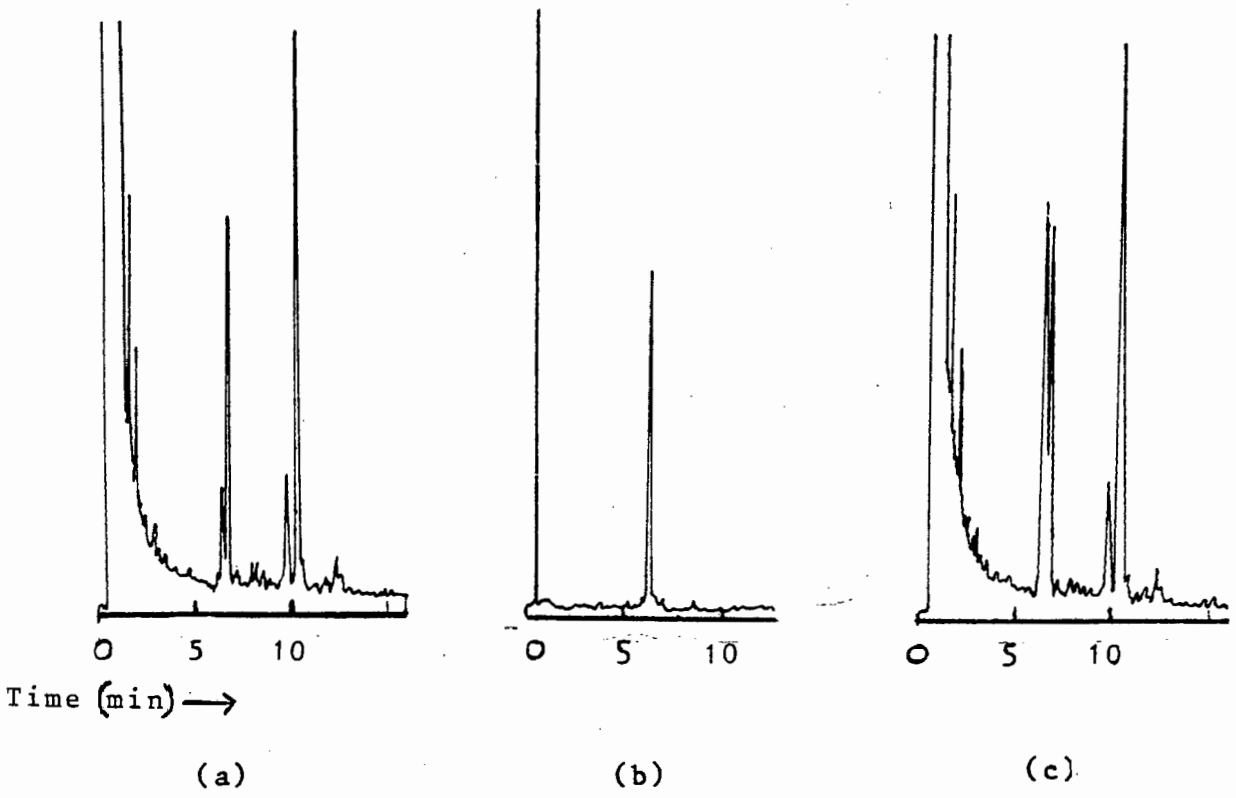


Figure 30 : G.l.c. traces of (a) methylated alditols of E3 (b) methylated cellobiitol and (c)=(a)+(b)

5.4 ^{1}H-n.m.r. studies

The configurations of the glycosidic linkages were determined by measuring the ^{1}H-n.m.r. spectra of E1 and E2; the spectrum of E2 is given (Fig. 31). For both samples the spectra showed the presence of a broad singlet (ill-defined doublet) at δ4,75 due to the anomeric proton of β-D-Manp, whereas the doublet centred at 4,50 (J 8,0 Hz) was characteristic of H-1 in β-D-Glcp In the spectrum of E2, the additional signals

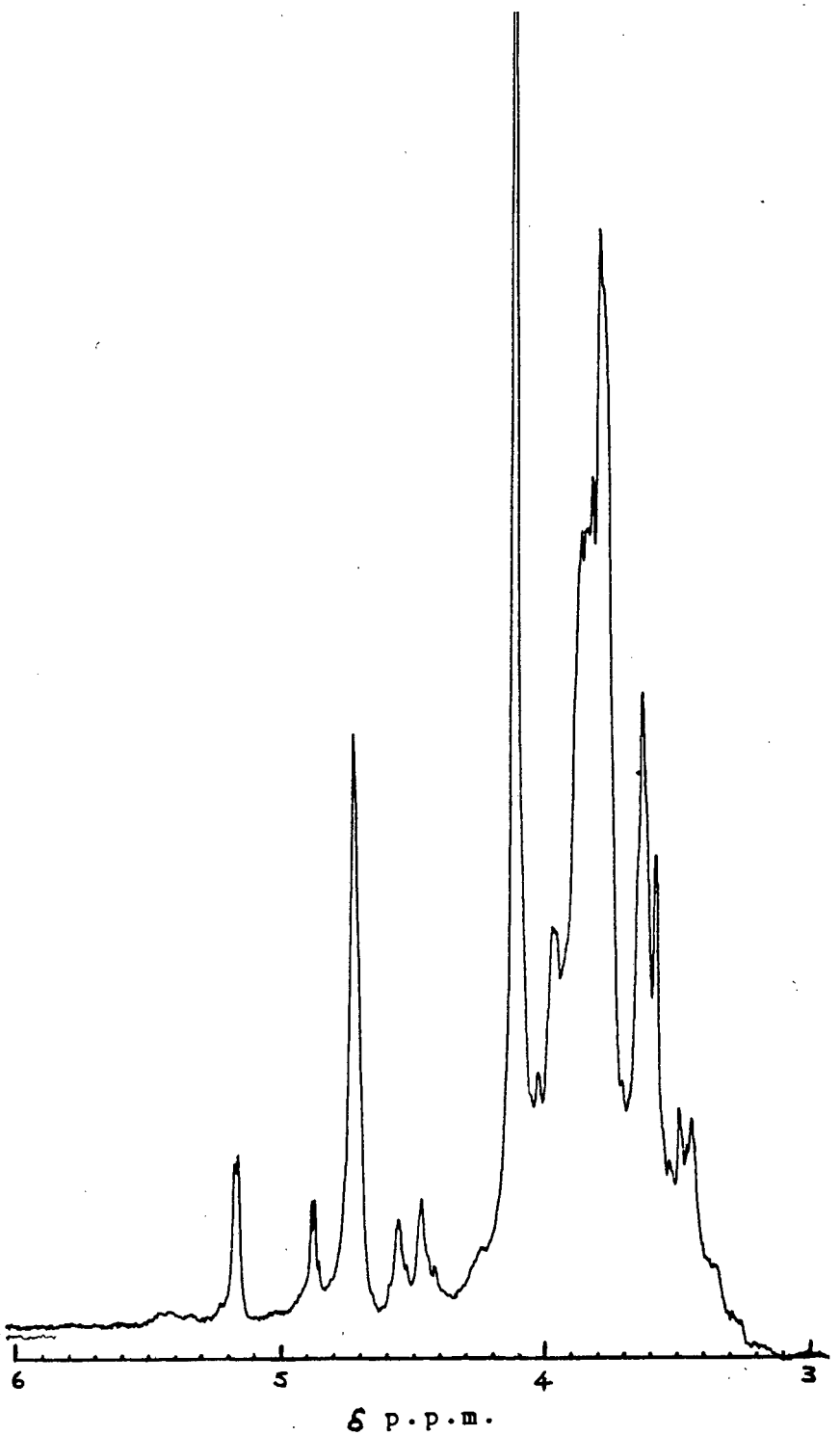


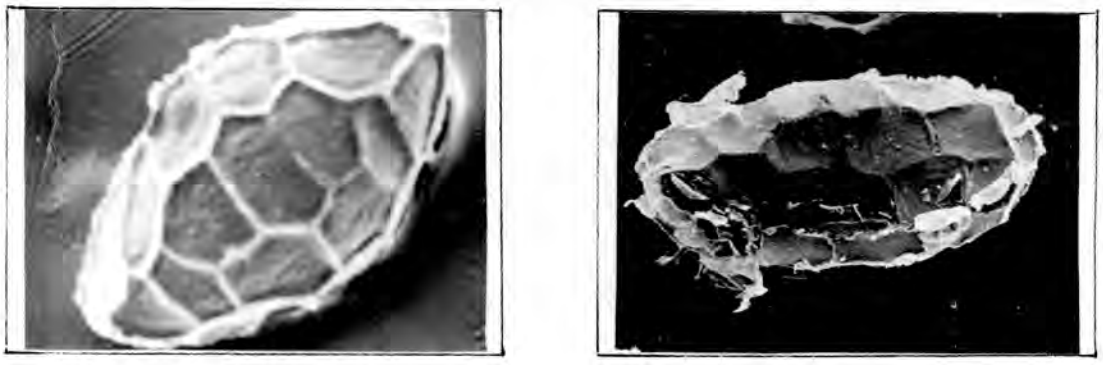
Figure 31 : 90MHz ^{1}H-n.m.r. spectrum in D_2O of E2 at 80°

at δ5,18 and δ4,88 (doublets, J 1,5 Hz for both) could be associated with H-1's of α-and β-D-Manp respectively, located at the reducing ends of the oligosaccharides. The signals for H-1's of reducing Glc were probably too small to be observed; that of β-D-Glc could well be obscured by those of β-linked chain residues.

5.5 Electron microscopy and X-ray diffraction studies

In view of their apparently highly organised macrostructure, the glucomannan pellets were examined by electron microscopy and X-ray diffraction methods. The electron micrograph of one pellet under magnification is shown in Fig.32, from which the well-defined shape of the pellets is clearly apparent. The presence of calcium ions in the pellets was indicated by the energy dispersive X-ray analyser system stated in the experimental section. The Ca^{2+} content was estimated as 0,073% by atomic absorption spectroscopy. The significance of the presence of the Ca^{2+} ions, if any, was not clear. The identity of the counter-ions could not be ascertained; in particular, the absence of phytate was evident from the fact that no phosphorus was detected. In addition to being associated with counter-ions, the Ca^{2+} ions may also be involved in

complexation with hydroxyl groups of the polysaccharide.



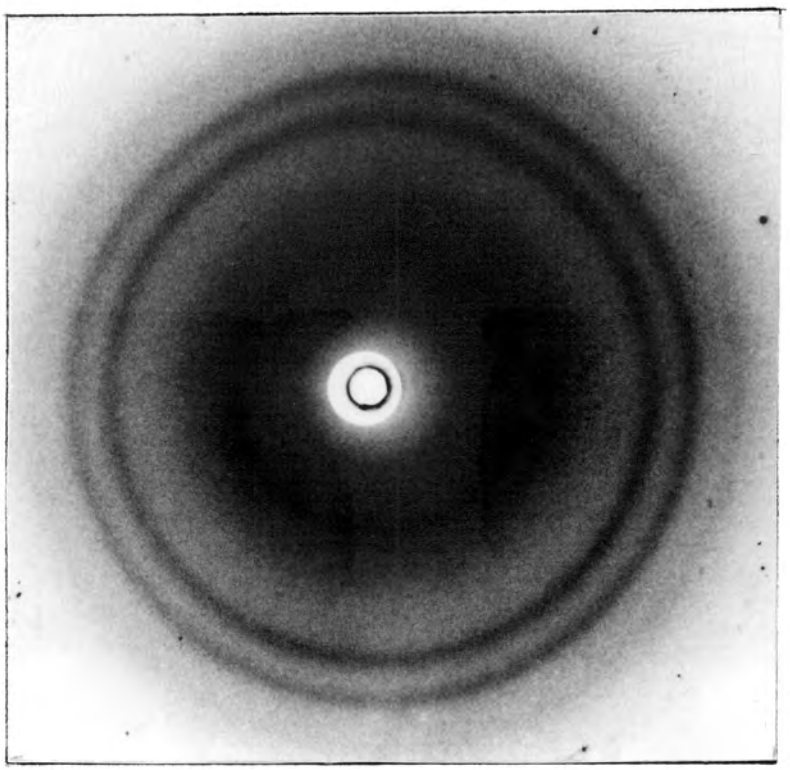
(a) (b)

dimensions: 0.75 x 0.40mm 0.29 x 0.23mm

Figure 32 : Electron micrographs of glucomannan pellets
(a) Satyrium coriifolium
(b) Satyrium carneum

The X-ray diffraction photograph (Fig. 33) shows a series of relatively sharp diffraction rings suggesting a high degree of regularity in the polysaccharide structure. The derived interplanar spacings are presented in Table XVII. Although these spacings do not correlate well with other known crystalline forms of plant polysaccharides, there was some resemblance to the diffraction spacings of mannan II, an alkaline-treated mannan preparation from algal cell

(a)



(b)

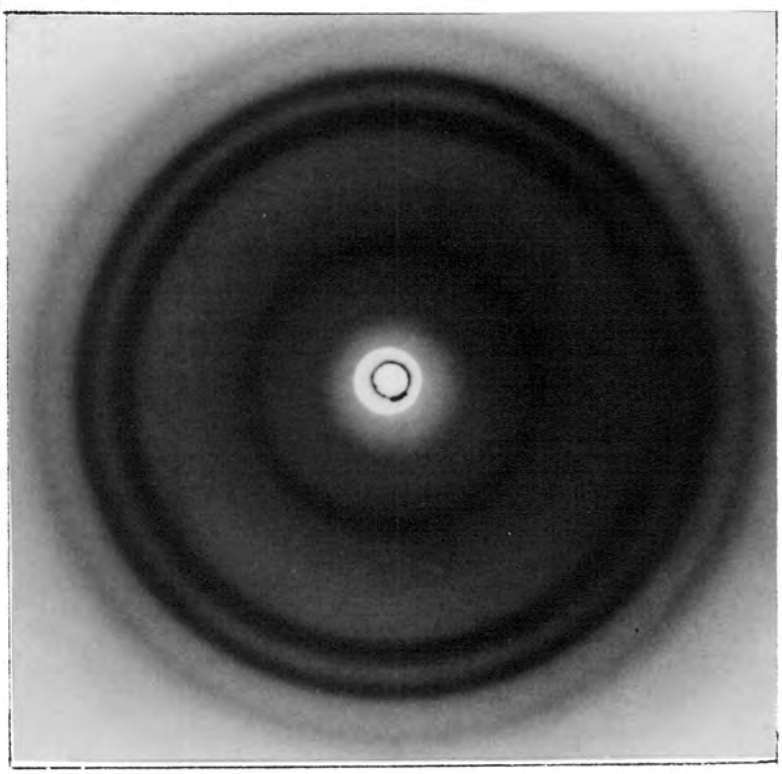


Figure 33 : X-ray diffraction photographs of glucomannan pellets
(a) Satyrium coriifolium
(b) Satyrium carneum

TABLE XVII - X-RAY INTERPLANAR SPACINGS OF (a) SATYRIUM CORIIFOLIUM AND (b) SATYRIUM CARNEUM GLUCOMANNAN PELLETS

(a)		(b)	
Intensity*	Spacing in nm	Intensity*	Spacing in nm
S	0,815	S	0,815
S	0,448	VS	0,448
S	0,397	S	0,397
M	0,348	M	0,348
VW	0,301	VW	0,301
VW	0,269	VW	0,269
W	0,255	W	0,255

* S, strong; M, medium; W, weak; V, very

walls,[136] which gives strong diffraction rings at 0,826 and 0,450nm.

5.6 Conclusion

The major polysaccharide component found in the tubers of Satyrium coriifolium has been characterised as a glucomannan consisting mainly of a linear chain of β-(1→4)-linked D-Manp and D-Glcp residues. In this respect it bears some resemblance to glucomannans isolated from other Orchidales species,[9] but is somewhat different in being unbranched. A major

difference is in its macrostructural appearance as unusually water-insoluble pellets, a feature which it seems to share only with a glucomannan from tubers of Satyrium carneum.[131] The methods used in the structural studies of the latter glucomannan were essentially similar to those used in the present study and both glucomannans were isolated as hard pellets insoluble in either water or 24% KOH but soluble in 24% NaOH. Therefore, a detailed comparison of the two polysaccharides is appropriate. They resemble each other in many respects, but the pellets have certain contrasting features which are summarised below:

	S. coriifolium	S. carneum
Colour	brown	near white
Solubility in:		
(i) NMMNO	-	+
(ii) 6M aqueous urea	-	+
Metal ions detected	Ca(0,073%)	K(0,68%)
Yield (from a single tuber)	72%	63%

The difference in solubility behaviour in NMMNO and 6M urea can not readily be explained. The respective diffraction patterns (Fig.33 and Table XVII) of the two samples are virtually identical (except for the relative intensity of the diffraction ring at spacing 0,448nm) thus suggesting the presence of very similar

"crystalline" forms. Furthermore, the polysaccharides have similar structural features in that they both consist of a linear chain of β-(1→4)-linked D-Manp and D-Glcp residues, occurring in the relative proportions of 3,6 : 1. The presence of mutually-linked Glc residues was shown to occur to about the same extent in both, and the specific rotation values of -46° (S.coriifolium) and -48° (S. carneum) provide further evidence for the similarity of the two polysaccharides. Therefore, it is unlikely that the insolubility of the polysaccharide from S. coriifolium in NMMNO and 6M urea is due to the structural features described above. The solubilising action of these solvents towards polysaccharides is believed to involve disruption of interchain hydrogen bonding; insolubility of a polysaccharide may, therefore, suggest that such a disruptive process is somehow hindered. That the S.coriifolium polysaccharide remained insoluble in the two solvents even after it had been previously dissolved in 24% NaOH indicates that whatever hindered dissolution of the original pellets was not eliminated by solubilisation in the alkaline medium. Therefore it is apparent that an explanation for this unusual behaviour would entail more detailed studies of these pellets; non-carbohydrate material possibly associated with the polysaccharide, which may also account for the

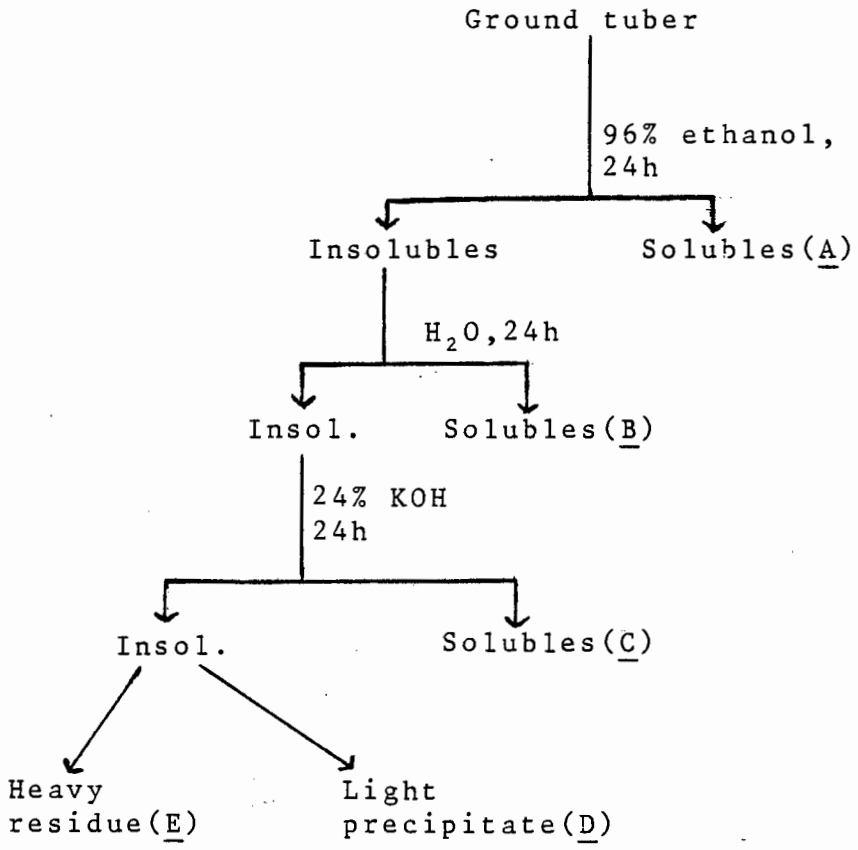
different colours of the pellets, could be involved, as may the different metal ions detected.

Further similarity of the polysaccharide pellets from the two species was demonstrated by their insolubility in DMSO and LiOH solutions, and the tendency to form gelatinous precipitates upon neutralisation of their solutions in alkaline media. Considering that these glucomannans occur as reserve nutrients, it is likely that in the living plant they exist in more soluble forms or as gels resembling the mucilaginous glucomannan globules found in Orchis morio.[9] Aggregation into insoluble pellets was probably promoted by storage of the tubers under ethanol and at sub-zero temperatures.

5.7 Experimental

5.7.1 Isolation of the glucomannan (Scheme 6)

Satyrium coriifolium tubers, collected in February 1985, were donated by courtesy of the late Professor E.A.C.L.E. Schelpe (Bolus Herbarium, U.C.T.). The freshly collected tubers were brushed free of soil and immediately immersed and stored in 96% ethanol to inactivate any enzymes that might promote degradation of polysaccharides present. One tuber was ground up in



SCHEME 6 : Isolation of glucomannan from Satyrium coriifolium tuber

ethanol (50mL) and the suspension was stirred for 18h at ∿25°C. After centrifugation the supernatant was evaporated to dryness yielding A 0,28g; p.c. examination showed the presence of free glucose. The residue was further extracted with distilled H_2O (50mL) for 24h. After centrifugation the supernatant was dialysed against water for 48h and freeze-dried; yield 0,33g (B). Examination of an hydrolysate of B by

p.c. revealed the presence of Glc as the only sugar constituent. Extraction of the H_2O-insoluble residue with a 24% KOH solution (50mL), made 10mM with respect to $NaBH_4$, was carried out under N_2 for 24h. The supernatant obtained after centrifugation was neutralised with acetic acid, dialysed against H_2O for 72h and freeze-dried; yield 0,24g (C). Hydrolysis of C released Glc as the preponderant sugar together with a small proportion of Man. The final, insoluble residue which was washed repeatedly with H_2O until washings were neutral to litmus paper, consisted of two phases. The less dense material, D, was separated from the dense particles E by decantation of an aqueous suspension in which the latter had settled. More water was added and the process was repeated until E was free of D; yield D 0,47g, E 3,38g.

5.7.2 Partial acid hydrolysis of the glucomannan

The glucomannan E (500mg) was dissolved in 72% (w/w) H_2SO_4 (5mL); complete dissolution was achieved after shaking at ∿25°C for 2h. The solution was diluted to ∿8%, cooled in ice-water and neutralised with $NaHCO_3$. The neutral solution was dialysed against regularly-changed distilled H_2O (a total of 5L) for 48h. Freeze-drying of the non-dialysable material gave E1 (340mg). The dialysate was evaporated to dryness under reduced

pressure at ~40°C. The oligosaccharides were freed of salts by acetylation in pyridine-acetic anhydride (2 : 1, 5mL) at 100°C for 6h; after addition of water to the reaction mixture the acetates were extracted into chloroform and deacetylated with sodium methoxide in $CHCl_3$-MeOH (1 : 1, 6mL). A solution of the deacetylated products in H_2O was neutralized with Amberlite IR-120(H^+) and then filtered. The filtrate was freeze-dried. Extraction of the freeze-dried material with 96% ethanol gave E2 as the ethanol-insoluble product (yield, 80mg). Evaporation of the ethanol-extract to dryness afforded E3 (46mg).

5.7.3 Scanning Electron Microscopy (by courtesy of Dr A.L. Rodgers, U.C.T.)

The glucomannan pellets were examined using a Cambridge S180 Scanning Electron Microscope (SEM), operating in the secondary electron collection mode at a nominal beam potential of 15kV and beam current 100μA. Images were recorded on an Ilford F4 roll film at 60 second frame period and 800 lines per frame on a carefully focussed camera. The SEM was equipped with an energy dispersive X-ray analyser system used for routine elemental analysis.

5.7.4 X-ray diffraction analysis (by courtesy of Professor E.D.T. Atkins, University of Bristol, U.K.)

A single pellet was mounted on a thin walled glass capillary tubing. X-ray diffraction photographs were obtained using point collimation from an Elliott Rotating-target generator using nickel-filtered CuKα radiation. The camera was evacuated during exposure to reduce air scatter. Exposure times of 5h were required with specimen to film distances of 40mm.

5.7.5 Atomic absorption spectroscopy

Atomic absorption spectroscopic measurements were performed by courtesy of Miss J. Wyrley-Birch (U.C.T.) on a Varian Techtron Model AA6 instrument. Calcium and potassium contents were determined using a nitrous oxide/acetylene and an air/acetylene flames respectively for atomisation of samples. Absorption measurements were taken at 422,7nm for calcium and at 766,5nm for potassium.

CHAPTER 6

6. CONCLUSION

Some non-cellulosic β-D-glycans from plant sources have been studied, and their main structural features, together with their origin and expected physiological functions, are summarised in Table XVIII. From this table it is clear that the only structural feature shared by all is the presence of a main chain consisting of β-(1→4)-linked D-pyranosyl sugars. The polysaccharides differ widely with respect to sugar composition and in the extent to which the main chains are substituted. Thus, while the glucomannoglycan from Satyrium coriifolium is unsubstituted, the xylans from Strelitzia reginae and Agave sisalana represent the highest and lowest degrees of substitution respectively, with that of the xyloglucan from A. sisalana being intermediate between the two extremes. Further differences are illustrated in their possible physiological functions in relation to the plant organs from which they were isolated.

The xylan from S. reginae, isolated from a gum exudate secreted into the corolla ducts of the flowers, resembled in its sugar composition a polysaccharide found on the stigmatic surfaces of these flowers and hence physiological function is probably similar. Although it consists of the essential structural

TABLE XVIII - SOME β-(1→4)-D-GLYCANS OF PLANT ORIGIN

Polysaccharide		Plant source	Structure	Physiological functions
Xylan		Strelitzia reginae flowers	β-(1→4)-D-Xylan, heavily substituted; side-chains contain Araf, GlcpA, Manp and Xylp	Possibly related to species recognition for reproductive purposes
Hemicelluloses	Xylan	Agave sisalana defatted leaves	β-(1→4)-D-Xylan, low degree of substitution; single 4-Me-GlcpA or Xylp substituents	Structural, modifying and binding cellulose fibres
	Xyloglucan		β-(1→4)-D-Glucan, substituted with Xylp, to some of which are appended Araf-, Fucp- or Galp-containing groups	
Glucomannoglycan		Satyrium coriifolium tubers	β-(1→4)-linked linear chain of D-Glcp and D-Manp residues	Storage reserve

features expected for a xylan, some unusual features, notably the presence of 3-O-substituted Galp, GlcpA substituted at both O-3 and O-4, and (1→2)-linked Manp, are also found in this polysaccharide. Furthermore, the trend whereby a xylan containing high proportions of both Araf and Galp may have Gal→Ara as one of its side-chains is not observed, since methylation analysis of the polysaccharide has shown all Araf units to be terminal, non-reducing groups. Evidence for the high degree of substitution of this polysaccharide was provided mainly by Smith degradation studies, which showed that about 70% of xylosyl units in the original polymer survived the first Smith degradation; the majority of these occurred as (1→4)-linked residues in the degraded product. Of the substituted xylosyl residues in the original polysaccharide singly- and doubly-substituted residues were present in approximately equal proportions.

The hemicelluloses from A. sisalana leaves are presumed to serve a structural function by virtue of their strong association with cellulose, the universal structural polysaccharide of plants. The xylan, present largely in the fibrous tissue of the leaves, formed the major component of the two hemicelluloses. It is more simple in structure compared to that from S. reginae; for every ten sugar residues in the main

chain two are singly-substituted, one with 4-Me-Glc*p*A and the other with Xyl*p*. The xyloglucan, found only in the ground tissue of the leaves, has more than half of the 6-*O*-positions in the glucan chain substituted with Xyl*p* units. Some of the latter are further substituted with either Gal*p*, Ara*f* or Fuc*p*-(1→2)-Gal*p* groups, structural features which are unusual for xyloglucans isolated from monocotyledonous plants.

The glucomannoglycan which appears as the major polysaccharide reserve in *S. coriifolium* tubers has been found to have the most simple structure among the β-D-glycans investigated in this study, in that the only sugar constituents, D-Man*p* and D-Glc*p* (3,6 : 1 ratio) occur along a linear chain in β-(1→4)-linkage. However, this polysaccharide had some unusual solubility properties; it was insoluble in water and a number of other aqueous and non-aqueous solvents, but dissolved slowly in 24% NaOH, although not in other alkaline solutions. The polysaccharide also showed remarkable gelling properties, a well-known feature of some polymers of this class. X-ray methods showed an ordered arrangement of sugar residues in the small insoluble pellets formed by this polysaccharide, and a scatter of Ca^{2+} ions upon the surface; the Ca^{2+} ions may contribute to the unusual physical behaviour of this glucomannoglycan.

APPENDIX

APPENDIX:

URONIC ACID DETERMINATION

The quantitative determination of uronic acid in acidic polysaccharides is of fundamental importance in elucidation of their structures. Earlier methods include that involving decarboxylation followed by quantitation of the CO_2 released from the uronic acids,[137] and colorimetric methods requiring reagents such as orcinol[138] or carbazole[139] for the generation of chromophores. The decarboxylation method was noted for its superiority in terms of accuracy,[140] although it is less rapid compared to the colorimetric methods. The colorimetric method utilizing the 3-hydroxydiphenyl reagent was developed by Blumenkrantz and Asboe-Hansen[89] who found it to be simpler, quicker, more sensitive and more specific than other methods. Later Scott[141] introduced the 3,5-dimethylphenol reagent which, although comparable in sensitivity was shown to be more specific than 3-hydroxydiphenyl and therefore particularly useful at uronic acid levels less than 4%. However, the latter method involved fairly elaborate procedures and this did not appear as suitable for routine analysis with the aim of pursuing further structural studies. Furthermore, most analyses were carried out on samples with uronic acid levels higher than 4%, and therefore the 3-hydroxydiphenyl method was adopted. The method is based on the formation of a chromogen, 5-formyl-2-furancarboxylic acid,[141] from uronic

acids heated at 100°C in concentrated $H_2SO_4/Na_2B_4O_7$, which when further reacted with the reagent gives a chromophore absorbing at 520nm. Glucuronic acid was used as the calibration standard; galacturonic acid, (another common constituent of plant polysaccharides) gives a somewhat lower response.

Generally, samples were dried under reduced pressure over P_2O_5 for at least 18h prior to analysis. Aqueous solutions of accurately weighed portions (∿5mg) of the samples were then prepared. Water-insoluble samples were initially solubilised in a minimum amount of 72% (w/w) H_2SO_4 at ∿20°C (2-3h) and then diluted to 8% with respect to H_2SO_4. Any undissolved particles were removed by centrifugation. To 0,4mL of the uronic acid-containing solution, 2,4mL of a solution of $Na_2B_4O_7$(0,0125M) in concentrated H_2SO_4 was added, followed by cooling in ice. After thorough mixing of the contents in a Whirli mixer, the tubes were heated at 100°C for 5 min. After cooling to ∿25°C, 40µL of 3-hydroxy-diphenyl (0,15%) in 0,5% aqueous NaOH was added followed by mixing as before. Absorbance was measured at 520nm within 5 minutes after mixing. In order to minimize interference, particularly by neutral sugars, blank samples were run in each case by replacing the 3-hydroxydiphenyl reagent with 40µL of 0,5% NaOH.

The uronic acid content of a number of samples was determined by this method. A comparison of some results with values obtained titrimetrically is presented below:

Source of polysaccharide	% uronic acid (w/w)	
Gum exudate of:	Colorimetric	Titrimetric
Strelitzia reginae	17,6	18,9
Hakea sericea, H_2O-soluble	14,4	12,9
Acacia karroo	17,9	15,2
Acacia tortilis*	13,2	9,3

* protein content: 12%

The results appear to be in fair agreement in view of the fact that experimental errors in the two methods would mostly be of a different nature; for example, the titrimetric procedure requires all acid groups to be in the carboxylic form. Furthermore, the presence of protein in the A. tortilis sample could be responsible for the relatively low titrimetric value.

Correlation of the results obtained on quantitative determination of 4-O-methylglucuronic acid in polysaccharides by the Blumenkrantz method with those given by other methods indicated that the response was comparable to that of GlcA. The response of glucuronolactone, determined using a commercial sample was also found to be comparable.

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