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Extent of ß-glucan Chain Elongation by Ryegrass (*Lolium multiflorum*) Enzymes

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SUMMARY

The chain length and linkage composition of water-soluble β-glucans produced in vitro *from UDP-[^{14}C]glucose by ryegrass membranes was determined by methylation analysis. The methylated β-glucans were acid-hydrolysed and the partially methylated sugar products reduced to the corresponding methylated alditols. Mass spectra were recorded for the permethylated alditols following their separation by reversed-phase h.p.l.c. 64% of the radiolabelled β-glucosyl residues were 3-substituted, 33% were 4-substituted and 3% were non-reducing terminal residues indicating that the average degree of polymerization of the radiolabelled sequences was 33. This result demonstrates that substantial elongation of β-glucan chains was occurring* in vitro *and that chain elongation was from the non-reducing end.*

INTRODUCTION

Saccharide polymerization from nucleotide diphosphate sugars has been clearly demonstrated by the formation of microfibrils of 1,3-β-glucan by cell-free preparations from the fungus *Phytophthora cinnomomi* (Wang & Bartnicki-Garcia, 1976) and the yeast *Saccharomyces cerevisiae* (Larriba *et al.*, 1981). Synthesis of microfibrils of 1,4-β-glucan has also been claimed for cell-free preparations from *Acetobacter xylinum* (Colvin, 1980; Delmer, 1983). In many other *in vitro* systems evidence for polymerization has rested on indirect evidence involving incorporation of [^{14}C] sugars into an insoluble fraction (Fincher &

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Stone, 1981). However, this does not distinguish transfer of one (or a few) residues to insoluble acceptors from concerted glucosyl transfer to produce polysaccharides (Kauss, 1974; Villemez, 1974). Furthermore, determination of the size of the radioactive polysaccharide does not necessarily assist in making this distinction. Thus gel filtration has been used to show the presence of radioactivity in high molecular weight fractions but these results could also be explained (Kauss, 1974; Villemez, 1974; Bowles & Northcote, 1976; Henry & Stone, 1982) by glycosylations of high molecular weight polysaccharides involving transfer of only one (or a few) glycosyl residues to each acceptor. The length of the newly synthesized sequences of glucosyl residues may be determined directly by estimation of the ratio of labelled internal residues to labelled reducing terminal residues (Richards & Whelan, 1973; Tanaka, 1981) in the polysaccharide chains. This method has been used to detect labelled reducing terminal residues in 1,3-β-glucans produced from UDP-[^{14}C]glucose by membrane preparations from *Saccharomyces cerevisiae* (Shematek *et al.*, 1980). However, in polysaccharide biosynthesis, addition of glycosyl residues directly from nucleotide sugars would be expected to occur at the non-reducing end of the growing polymer (Robbins *et al.*, 1967). Thus a method for determining the proportion of radioactivity in non-reducing, rather than reducing terminal residues, is required to estimate the extent of elongation of β-glucan chains growing at the non-reducing end. We have used methylation analysis to identify the non-reducing terminal residues and intrachain residues in β-glucans synthesized *in vitro*.

The water-soluble β-glucans produced from UDP-[^{14}C]glucose by unfractionated membrane preparations from cells of suspension-cultured ryegrass (*Lolium multiflorum*) endosperm were methylated and the partially methylated glucitols produced separated by h.p.l.c. to allow estimation of the radioactivity in terminal and internal chain residues and thus determine the length of newly synthesized sequences of glucosyl residues.

RESULTS AND DISCUSSION

Separation and identification of partially methylated glucitols

The usual method of separating partially methylated sugars is by gas–liquid chromatography (g.l.c.) following reduction and acetylation to

form permethylated peracetylated alditols (Dutton, 1974). The measurement of radioactivity in these compounds requires their quantitative recovery from the column effluent, for scintillation counting or the use of radio–gas chromatography (Baltuskonis *et al.*, 1978). Recovery from the g.l.c. effluent may not always be quantitative (Jausen & Baglan, 1968) and radio–gas chromatography suffers from lack of sensitivity. High performance liquid chromatography (h.p.l.c.) permits the quantitative collection of the permethylated sugars so that small amounts of radioactivity in the terminal glycosyl residues of long polymers may be determined accurately by scintillation counting.

Conditions for reversed-phase h.p.l.c. of partially methylated sugars were reported by Cheetham & Sirimanne (1980). One complication with this system is that each methylated sugar gives two peaks due to the separation of α and β anomers. To simplify the fractionation we reduced the partially methylated glucoses and separated the methylated glucitol derivatives in the ammonium acetate–ethanol solvent of Cheetham & Sirimanne (1980). The identity of partially methylated glucitols was established by comparison of the retention times with those of permethylated glucitols produced from barley 1,3;1,4-β-glucan and cellobiose. Retention times were 2,3,6-tri-*O*-methyl-D-glucitol, 3·9 min; 2,4,6-tri-*O*-methyl-D-glucitol, 4·5 min; and 2,3,4,6-tetra-*O*-methyl-D-glucitol, 6·5 min.

Mass spectra of partially methylated glucitols

The permethylated glucitols in the h.p.l.c. effluent were also characterized by mass spectrometry. Their identity was established by rationalizing major mass fragments in the mass spectrum with their molecular structure and by comparison with spectra of standards isolated with polysaccharides of known structure. Previously only the mass spectrum of fully methylated glucitols has been recorded (Golovkina *et al.*, 1968).

The mass spectra of the three permethylated glucitols separated by h.p.l.c. are shown in Fig. 1. In addition to the peaks shown a large peak at $m/z = 60$ was observed in all spectra presumably due to acetic acid introduced during h.p.l.c. separation. Molecular ion peaks were not detected. Some peaks may be interpreted as resulting from primary fragmentation with cleavage of C–C bonds followed by elimination of methanol ($m/z = 32$) to produce more intense secondary fragment

peaks. However, other peaks can only be explained by rearrangements involving migration of methoxy groups or hydrogen atoms as suggested by Golovkina *et al.* (1968).

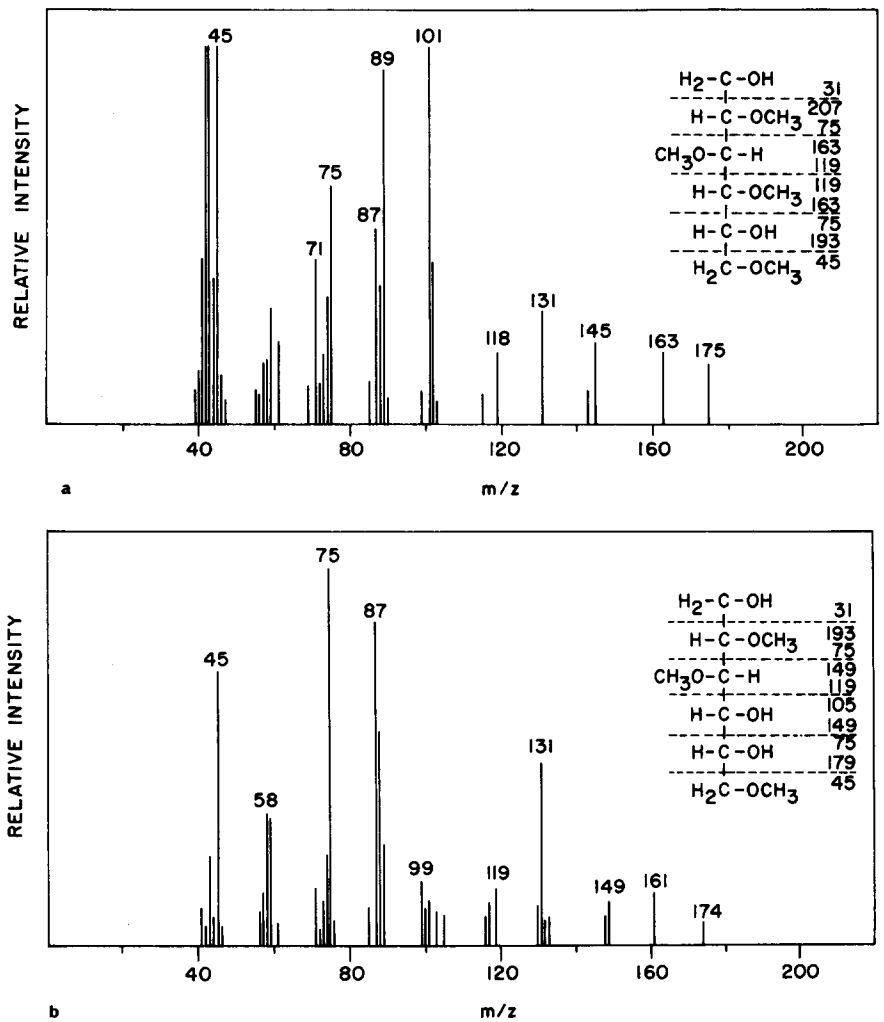

Fig. 1. Mass spectra of permethylated glucitols separated by h.p.l.c. (a) 2,3,4,6-tetra-*O*-methyl-D-glucitol, relative to $m/z = 101$ (42, 43, 45 off-scale); (b) 2,3,6-tri-*O*-methyl-D-glucitol, relative to $m/z = 75$; (c) 2,4,6-tri-*O*-methyl-D-glucitol, relative to $m/z = 75$.

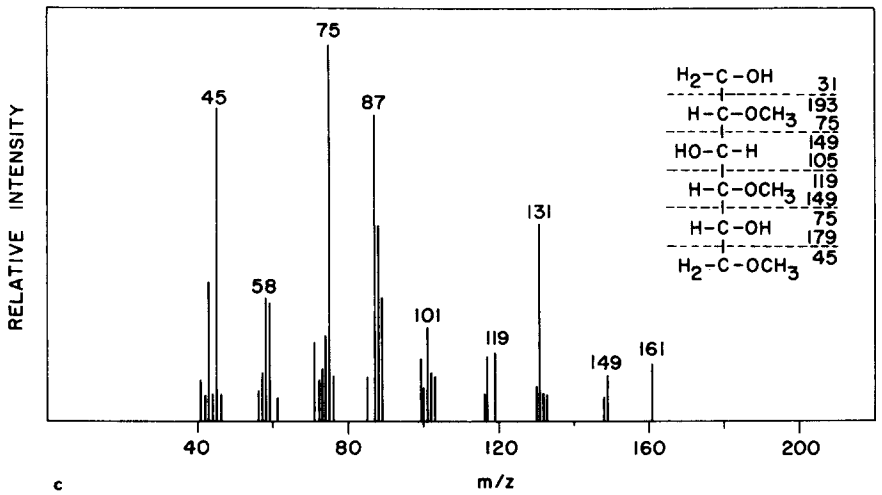

Fig. 1. – *contd.*

Mass spectrum of 2,3,4,6-tetra-*O*-methyl-D-glucitol

Peaks in the mass spectra at m/z = 163, 119, 75 and 45 could be due to primary fragments resulting from cleavage of C-C bonds. Elimination of methoxy groups (m/z = 32) from these primary fragments would explain the peaks at m/z = 131, 87 and 43. The peak at m/z = 175 may be produced by a similar elimination from a primary fragment at m/z = 207 (not detected). The major peaks were m/z = 101 and 45. These are the predominant peaks in the mass spectrum of hexamethyl-glucitol (Golovkina *et al.*, 1968).

A peak at m/z = 101 is found in each of the spectra but was most intense in the spectrum of 2,3,4,6-tetra-*O*-methyl-D-glucitol. In the spectrum of the hexamethyl glucitol the fragment m/z = 101 is the base peak and arises by elimination of a methoxy group from m/z = 133, a primary fragment with three consecutive methylated carbons. The abundance of m/z = 101 in the 2,3,4,6-tetra-*O*-methyl-D-glucitol spectrum may be similarly explained by the presence of three consecutive methyl groups in the parent molecule.

The appearance of m/z = 71 probably involves the elimination of formaldehyde from m/z = 101 and the migration of a hydrogen atom

(Golovkina *et al.*, 1968). The peak at $m/z = 88$ found in each spectrum corresponds to a two-carbon fragment with methoxy groups on both carbons.

Mass spectra of 2,3,6-tri-*O*-methyl-D-glucitol and 2,4,6-tri-*O*-methyl-D-glucitol

The two trimethylglucitols gave primary fragments at $m/z = 149$, 119, 75 and 45. The base peak was $m/z = 75$ for both trimethylglucitols. This fragment could arise by fission between carbon atoms 2 and 3 or 4 and 5. The primary fragments could lead to $m/z = 117$, 87 and 43 by elimination of methoxy groups and $m/z = 161$ may be produced in the same way from the primary fragment $m/z = 193$ (not detected) formed by fission between carbon atoms 1 and 2.

Golovkina *et al.* (1968) showed that three possible $m/z = 101$ ions may be formed by elimination of a methoxy group from any of the three carbons in the fragment $m/z = 133$. The most abundant of these $m/z = 101$ fragments is the one with methoxy groups remaining on carbons 1 and 3. Thus the absence of possible three-carbon fragments with methoxy groups on carbons 1 and 3 from 2,3,6-tri-*O*-methyl-D-glucitol may explain the lower intensity of $m/z = 101$ in the mass spectrum of 2,3,6-tri-*O*-methyl-D-glucitol compared with that of 2,4,6-tri-*O*-methyl-D-glucitol. The fragment at $m/z = 88$ in the spectrum of 2,4,6-tri-*O*-methyl-D-glucitol could arise by the migration of a methoxy group.

Linkage analysis of [^{14}C] β-glucans produced *in vitro*

The water-soluble β-glucans produced from 1 mM UDP-glucose by ryegrass membranes were methylated, acid-hydrolysed and reduced to produce partially methylated glucitols. Fractionation of the resultant mixture by h.p.l.c. and measurement of the radioactivity in fractions by scintillation counting gave the radioactivity profile shown in Fig. 2. Glucose was the only labelled sugar detected in an acid hydrolysate of the ^{14}C-labelled products. Peak 1 is 2,3,6-tri-*O*-methyl-D-glucitol arising from 4-substituted glucose residues in the β-glucan and peak 2 is 2,4,6-tri-*O*-methyl-D-glucitol arising from 3-substituted residues in the β-glucan. Peak 3 has the same retention time as 2,3,4,6-tetra-*O*-methyl-D-glucitol and represents the non-reducing terminal residues of the β-glucan chains.

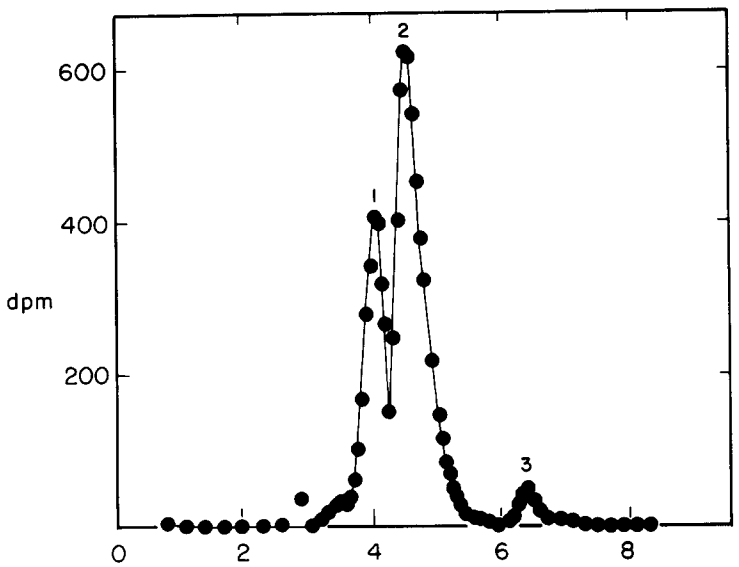

Fig. 2. h.p.l.c. of partially methylated [^{14}C] glucitols released by acid hydrolysis of methylated β-glucans produced from UDP-[^{14}C] glucose by ryegrass membranes. (1) 2,3,6-tri-*O*-methyl-D-glucitol; (2) 2,4,6-tri-*O*-methyl-D-glucitol; (3) 2,3,4,6-tetra-*O*-methyl-D-glucitol.

Most of the label was in 3-linked glucosyl residues (Table 1). Since the fraction analysed was water-soluble and was prepared using a high UDP-glucose concentration, 1,3-β-glucans and 1,3;1,4-β-glucans would be expected in this fraction but 1,4-β-glucans are unlikely to be present (Smith & Stone, 1973). If it is assumed that the ratio of 1,3-linkages to 1,4-linkages in any 1,3;1,4-β-glucan product is 28:72 (Anderson & Stone, 1978), it can be calculated from the methylation data that 52% of the newly-synthesized water-soluble glucan is 1,3-linked and 48% is 1,3;1,4-linked.

Chain length of [^{14}C] β-glucans produced *in vitro* and the direction of their synthesis

The ratio of radioactivity in internal chain to reducing terminal residues indicates that the average degree of polymerization (DP) of the newly

TABLE 1

Characterization of Water-Soluble β-Glucans Produced from UDP-[^{14}C]Glucose by Ryegrass Enzymes (The Mixed-membrane Preparation from Ryegrass was Incubated with ^{14}C-Labelled 1 mM UDP-Glucose and the Water-soluble Fraction of the Reaction Mixture Characterized by Methylation Analysis)

Glucosyl residues	*% of ^{14}C in each type of glucosyl residue*[a]	*% of total glucosyl residues*[b]	*Relative specific radioactivity*
4-Linked	33	71	0·47
3-Linked	64	26	2·4
Non-reducing terminal	3	3	1·0

[a] The percent of ^{14}C in each type of glucosyl residue was determined by h.p.l.c. (Fig. 1).

[b] The percent of each type of glucosyl residue was estimated by capillary g.l.c. of the peracetylated permethylated alditols (corrected as described by Sweet *et al.*, 1975). Most of this glucan originated in the enzyme preparation and was not produced *in vitro*.

synthesized sequences of glucosyl residues in the 1,3- and 1,3;1,4-β-glucans, produced from UDP-glucose by the ryegrass membranes, was 33.

Estimates of the length of the newly synthesized chains of the individual water-soluble β-glucans cannot be made from these data, but hydrolysis with the β-glucan exo-hydrolase from *Euglena gracilis* suggested (Henry & Stone, 1982) that the DP of the water-soluble 1,3-β-glucans produced is probably also close to the 33 average.

Thus we conclude that these *in vitro* reactions represent true elongation of polysaccharide chains and not simply limited glycosyl transfer to high molecular weight acceptors. The results also indicate that the β-glucans are synthesized by addition of glucosyl residues to the non-reducing end of the growing chains.

Unlabelled polysaccharides in the membrane preparation

The ryegrass membrane preparations contain substantial amounts of unlabelled β-glucans as well as other polysaccharides and glycoconjugates (Mascara & Fincher, 1982). These may be located in compartments of the endomembrane system or be contaminating cell wall

polysaccharides. The unlabelled polysaccharides in the water-soluble fraction of the reaction mixture were characterized by methylation analysis. In addition to permethylated glucitols other permethylated alcohols arising from arabinose and galactose were present (Mascara & Fincher, 1982). The relative amounts of the unlabelled methylated glucitol derivatives were very different to the proportions of radioactive residues of each type (Table 1). The glucosyl residues were 71% 1,4-linked, 26% 1,3-linked and 3% were non-reducing terminal residues. Thus the unlabelled β-glucans contained much more 1,4-linked glucose than the labelled products and the ratio of 1,3- to 1,4-linkages was characteristic of 1,3;1,4-β-glucan found in ryegrass cell walls (Anderson & Stone, 1978).

Newly formed ^{14}C-labelled β-glucan differed from the endogenous β-glucan in containing significantly more 1,3-linked glucose. This suggests the 1,3-β-glucan is not normally produced in large quantities *in vivo*. A conclusion consistent with earlier observations (Henry & Stone, 1982) which showed that the 1,3-β-glucan formed under similar reaction conditions had a specific radioactivity almost seven times that of the other β-glucans. The predominance of 1,3-linkages in β-glucans formed *in vitro* from UDP-glucose is characteristic of preparations from many sources (Brett, 1981).

EXPERIMENTAL

β-Glucan synthesis by cell-free preparations

Particulate β-glucan synthases were isolated from cells of suspension-cultured ryegrass *Lolium multiflorum* endosperm as previously described (Smith & Stone, 1973; Henry & Stone, 1982). The β-glucan synthases were incubated at 25°C for 15 min in 50 mM morpholinopropane sulphonic acid, pH 7·5 containing 30 kBq of UDP-[^{14}C]glucose in a total volume of 4 ml. The reaction was stopped by boiling for 10 min.

Isolation of β-glucan produced

Unincorporated UDP-[^{14}C]glucose was removed by washing the freeze-dried reaction products with 5 ml each of 66% ethanol containing

0·85 ml EDTA, 66% ethanol, 70% ethanol, ethanol and acetone. This procedure was shown by Smith & Stone (1973) to remove UDP-glucose from the incubation mixtures. The water-soluble products were extracted from the residue three times with 3 ml of water in a boiling water bath. The water-soluble products from six replicate reaction mixtures were combined and dried by evaporation under reduced pressure. This procedure yielded 2·5 kBq of ^{14}C-labelled product for analysis.

Determination of monosaccharide composition

The identity of the ^{14}C-labelled glycosyl residues in the isolated fraction was determined by acid hydrolysis and paper chromatography. Total acid hydrolysis was achieved using sulphuric acid as described by Saeman *et al.* (1963). The acid hydrolysate was neutralized with barium carbonate and filtered. The filtrate was concentrated by evaporation at 40°C and chromatographed for 16 h on Whatman No. 3MM paper with ethyl acetate:pyridine:water, 10:4:1 (v/v), as solvent. The chromatogram was cut into 1 cm strips and counted in a scintillation fluid containing 75 g of PPO and 0·8 g of POPOP in 2·5 litres of xylene and 625 ml of Triton X-114.

Methylation hydrolysis and reduction of β-glucans

β-Glucans of known structure (cellobiose, Sigma Chemical Co., St Louis, Missouri) and 1,3;1,4-β-glucan from barley (Clarke & Stone, 1966) and those produced by ryegrass synthases were methylated by the method of Hakomori (1964). The methylated glucans were hydrolysed first with 90% (w/w) formic acid for 1 h at 100°C and then with 0·2 M sulphuric acid for 14 h at 100°C as described by Bouveng & Lindberg (1965). The methylated sugars released were reduced with 1% (w/v) $NaBH_4$ in 1 M NH_4OH. After 4 h at room temperature and then 10 min at 40°C, excess borohydride was destroyed by the dropwise addition of 4 M acetic acid until evolution of gas ceased. The solution was evaporated to dryness and the borate was removed by repeated addition and evaporation from the sample at room temperature of methanol as described by Valent *et al.* (1980).

Gas chromatography

The methylated glucitols were analysed by gas–liquid chromatography following acetylation with 1 ml of acetic anhydride at 100°C for 1 h. The acetic anhydride was removed under a stream of nitrogen and replaced with chloroform before injection into the chromatograph. A Silar 10C SCOT column (0·5 cm i.d. × 25 m) was used with a temperature program of 150°C rising to 230°C at 2°C min^{-1} (Shibuya, 1981). Injection port and detector temperatures were maintained at 250 and 300°C respectively.

Liquid chromatography

Methylated glucitols were separated on a Spherisorb ODS column (4·55 mm i.d. × 25 cm) using 1% ammonium acetate : ethanol, 9 : 1 (v/v) as solvent at a flow rate of 1 ml min^{-1} (Cheetham & Sirimanne, 1980). A Multiref 902 (Optilab, Vallingby, Sweden) differential refactometer was used as detector.

Electron-impact mass spectrometry

The mass spectra were recorded by direct probe insertion of h.p.l.c. fractions that had been dried at room temperature in a vacuum desiccator over P_2O_5. A Joel JMS-D100 double focusing mass spectrometer was used with probe temperatures of 90–150°C.

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