Carbohydrate Research, 62 (1978) 127–133

EVIDENCE FOR A SINGLE TYPE OF LINKAGE IN A FRUCTOFURANAN FROM *Lolium perenne*

JELKA TOMAŠIĆ†*, HAROLD J. JENNINGS‡, AND CORNELIS P. J. GLAUDEMANS†

†*Laboratory of Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases, NIH, Bethesda, MD 20014 (U.S.A.)*; ‡*National Research Council, Ottawa K1A OR6 (Canada)*

(Received July 22nd, 1977; accepted for publication, August 8th, 1977)

ABSTRACT

On extraction with water, rye grass (*Lolium perenne*) yielded a mixture of polysaccharides. Fractionation thereof led to the isolation of two fructofuranans, one of which preponderated. Analysis of the main polysaccharide by ^{13}C-n.m.r. spectroscopy indicated that it is composed of fructofuranosyl residues linked only (2 → 6), and terminated by a glucosyl group linked as in sucrose.

INTRODUCTION

In 1928, Haworth and co-workers[1,2] elaborated the structure of inulin as a polysaccharide consisting of (2 → 1)-linked D-fructofuranosyl residues. Two years later, Hibbert and Tipson[3] showed that the polysaccharide (levan) produced from sucrose by *Bacillus mesentericus* is a D-fructofuranan having, at least mainly, intersaccharidic linkages of the (2 → 6) type. Since then, other bacterial levans have been shown to contain mostly (2 → 6)-linked D-fructofuranosyl residues, although branching involving (2 → 1)-linkages is quite common: for instance, in those from *Aerobacter levanicum*[4] and *Streptococcus salivarius*[5].

Our laboratory at the NIH is involved in a study of homogeneous immunoglobulins having anti-(2 → 1) or anti-(2 → 6) specificity[6,7]. It has been found that all homogeneous anti-D-fructofuranans bind levans, but that, within this group of anti-D-fructofuranans, there is a sub-group of immunoglobulins that shows specificity for predominantly (2 → 6)-linked D-fructofuranans only, and that is incapable of binding to inulin.

The inulin from dahlia tubers[1,2] is entirely composed of (2 → 1)-linked D-fructofuranosyl residues, except that it is terminated by a sucrose residue. The bacterial levans, in addition to mostly (2 → 6)-linkages, all contain small proportions of (2 → 1)-linked D-fructose units[4,5]. It is possible that the presence of both linkages in bacterial levans is the reason for the wider, myeloma immunoglobulin-binding capabilities. It thus became important to have available a polysaccharide containing

*Visiting Fellow, from the "Rudjer Bošković" Institute, Zagreb, Yugoslavia, 1975–1976.

only D-fructofuranosyl residues linked (2 → 6). Polysaccharides having a high proportion of this type of linkage have been isolated from plants[8-10]; a convenient source is rye grass (*Lolium perenne*). Lindberg *et al.* [11] found that a levan from this plant contains a D-fructofuranan linked mostly (2 → 6). We have evidence that rye grass contains not one levan, but two levans, one of which preponderates, and we have investigated that fraction.

EXPERIMENTAL

Materials. — Perennial ryegrass (*Lolium perenne*) was obtained from the Department of Agriculture, Beltsville, MD, U.S.A. Genetron 13 (trichlorotrifluoroethane) is a product of Matheson, Coleman and Bell. DEAE-cellulose (DE 52, preswollen, Whatman) was obtained from Angel–Reeves Co., and Sephadex gels from Pharmacia, Sweden. Anti-D-fructofuranans (ABPC-48 and UPC-10) and anti-D-galactans (X-24, J-539, and T-601) were generously provided by Dr. M. Potter, NCI, Bethesda, MD, U.S.A.

Methods. — Paper chromatography was performed on Whatman 3 MM paper in 5:12:4 pyridine–ethyl acetate–water. Monosaccharides were detected with silver nitrate–base[12]. Paper electrophoresis was effected on 3 MM Whatman paper in 0.1M boric acid–sodium tetraborate buffer, pH 8.0, for 90 min at 500 V. Oligosaccharides were detected with aniline–diphenylamine–phosphoric acid spray[13]. The carbohydrate content of fractions was determined by the phenol–sulfuric acid method according to Dubois *et al.*[14]. Total hydrolysis with acid was performed with 44% formic acid for 4 h at 100°. Formic esters possibly formed were saponified with 0.5M LiOH, and neutralization was effected by passage through ion-exchange columns.

Ouchterlony double-diffusion, precipitin tests of levan components I and II were conducted against anti-D-fructofuranans and anti-D-galactan ascites fluids. Solutions (0.1%) of levans I and II were used for precipitin tests with anti-levans, and 1% solutions for tests with anti-D-galactans.

Isolation of crude polysaccharides. — Freshly cut grass was immediately dried, and then extracted with ethanol and hexane. Batches (150 g) of dried grass were stirred with water (4 liters) for 24 h at 4°. The plant residue was centrifuged off, and the extracts were combined, and filtered through Whatman No. 1 filter paper by suction. The pH of the filtrate was adjusted to 7.2 with $NaHCO_3$, and the solution concentrated to ~1 liter *in vacuo* (bath temp., not >40°). To remove proteins, portions of the filtrate were mixed with equal volumes of Genetron 13 in a Waring Blendor for 10 min, centrifuged, and the supernatant liquors re-treated with Genetron until the gel interface no longer persisted. The protein-free, supernatant liquors were concentrated to 250 ml, and an equal volume of 3:1 methanol–hexane was added. After the suspension had been stirred for 10 min, the precipitate was centrifuged off, dried in a desiccator, dissolved in water, and the solution lyophilized; 3.3 g of crude polysaccharide was obtained that contained 20.65% of incombustible salts.

Purification on DEAE-cellulose. — Crude polysaccharide (500 mg) was first passed through a short column (2.5 × 5 cm) of DEAE-cellulose, pre-equilibrated in 0.05M Tris-HCl buffer, pH 7.5. The same buffer was used as the eluant, and polysaccharide-containing fractions were detected by the Dubois method[14]. These fractions were pooled, and then directly applied to a second column (2.5 × 95 cm) of DEAE-cellulose. A broad peak was obtained comprising carbohydrate-positive fractions; these were pooled, lyophilized, desalted on Sephadex G-15, and relyophilized. Thus was obtained 230 mg of partially purified levan.

Fractionation on DEAE-Sephadex. — DEAE-Sephadex A-50 was equilibrated in 0.1M borate buffer, pH 8.0. Partially purified levan (100 mg) was applied to a column (2.5 × 95 cm) thereof, and eluted first with 0.1M buffer up to 200 ml; then, a gradient of 0.1–1.0M buffer was applied, the elution being monitored by the phenol–sulfuric acid method. Two maxima were revealed, and these were pooled separately, lyophilized, desalted, and relyophilized, to give levan fraction I (300–400 ml): 60 mg, $[\alpha]_D$ −50° (*c* 0.1, H_2O), and levan fraction II (600–700 ml): 30 mg, $[\alpha]_D$ −60° (*c* 0.1, H_2O). On hydrolysis, both fractions afforded fructose (major) and glucose (minor).

Carbon-13 n.m.r. spectroscopy. — ^{13}C-N.m.r. spectra were recorded for solutions (50–100 mg/ml) of the polysaccharides in deuterium oxide in 10-mm tubes at 37° with a Varian CFT 20 spectrometer operating at 20 MHz in the pulsed, Fourier-transform mode with complete proton-decoupling. Chemical shifts are reported in parts per million (p.p.m.) downfield from external tetramethylsilane contained in a coaxial, inner tube (0.5 mm o.d.), and the ^{2}H resonance of deuterium oxide was used as the field-frequency lock-signal.

RESULTS

Crude levan preparation was obtained after deproteinization of aqueous extracts of perennial ryegrass and subsequent precipitation with methanol–hexane. Acidic components, pigments, and dyes were removed from the crude precipitate by chromatography on DEAE-cellulose at pH 7.5, yielding an only slightly colored material. After acid hydrolysis, this material showed, in paper chromatography, a major spot for fructose, glucose in small amount, and a trace of galactose. Paper electrophoresis of the unhydrolyzed product in borate buffer at pH 8.0 revealed the presence of at least two components, namely, one faster-moving, sharp band, and a slower, diffuse band. Solutions (0.1%) of the material gave precipitin lines with anti-levans UPC-10 and ABPC-48 ascites fluids upon double diffusion in agar, and 1% solutions showed a weak line with anti-D-galactan ascites fluids (X-24, J-539, and T-601) on using the same technique. Polysaccharide solutions absorbed with T-601 (thus removing all galactose-containing polysaccharides) still showed precipitations with anti-levans; this indicated the presence of a small proportion of galactan in the grass extracts.

Molecular sieving on Sephadex G-15, G-25, and G-50 failed to separate the

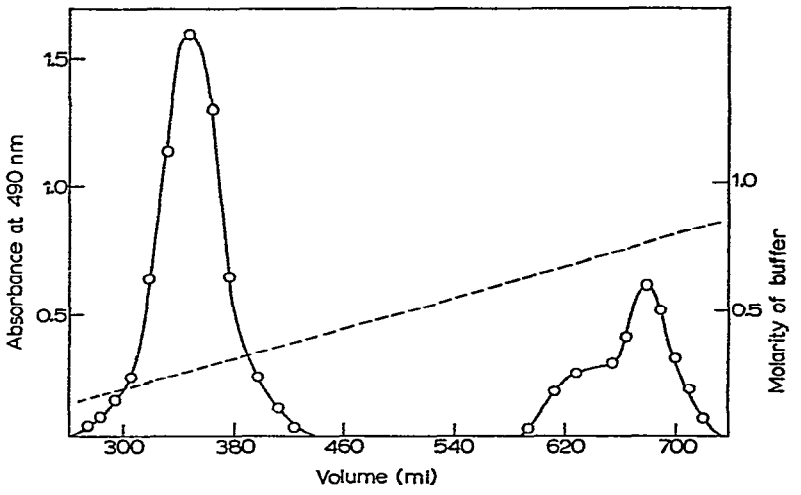

Fig. 1. Chromatography of grass levan on DEAE-Sephadex A-50 in a gradient (0.1–1.0M) borate buffer, pH 8.0.

components in the mixture, as also did chromatography on DEAE-Sephadex A-25 in various borate buffers (0.1M and 0.2M, pH 8.0, and 0.1M, pH 9.0). DEAE-Sephadex A-50 in borate buffer proved to be a convenient medium for a good separation of the two levan components (see Fig. 1). The main levan fraction (I) was eluted, immediately after application of the buffer gradient, as a sharp peak comprising ~60% of the material applied. By paper electrophoresis in borate buffer, only the faster-moving band was detected. The elution of the second fraction started after the buffer molarity exceeded 0.5M, and yielded ~30% of the material applied. Electrophoretically homogeneous fraction II corresponds to the slower-moving band.

Both components yielded fructose and a small amount of glucose on acid hydrolysis. No galactose was detected in hydrolyzates, and precipitin lines no longer appeared with anti-D-galactans upon Ouchterlony double-diffusion in agar. Thus, the galactose-containing component was presumably retained on the DEAE-Sephadex during the separation.

Fraction I precipitates with anti-(2 → 6) immunoglobulins (UPC-10 and ABPC-48), thus indicating the presence of (2 → 6)-linked fructofuranosyl residues. Fraction II also precipitates with immunoglobulins having specificity for (2 → 6)-fructofuranans, but Fig. 1 shows this fraction to consist of at least two components and it was therefore not further investigated.

The ^{13}C-n.m.r. spectrum of the grass levan, shown in Fig. 2, is consistent with its chemically determined structure, namely, that of a linear chain, of low molecular weight, consisting of β-D-(2 → 6)-linked D-fructofuranosyl residues terminating in a (nonreducing) sucrose residue. The chemical shifts of the individual carbon resonances of the levan are listed in Table I, together with those of some relevant, D-fructofuranoside model-compounds.

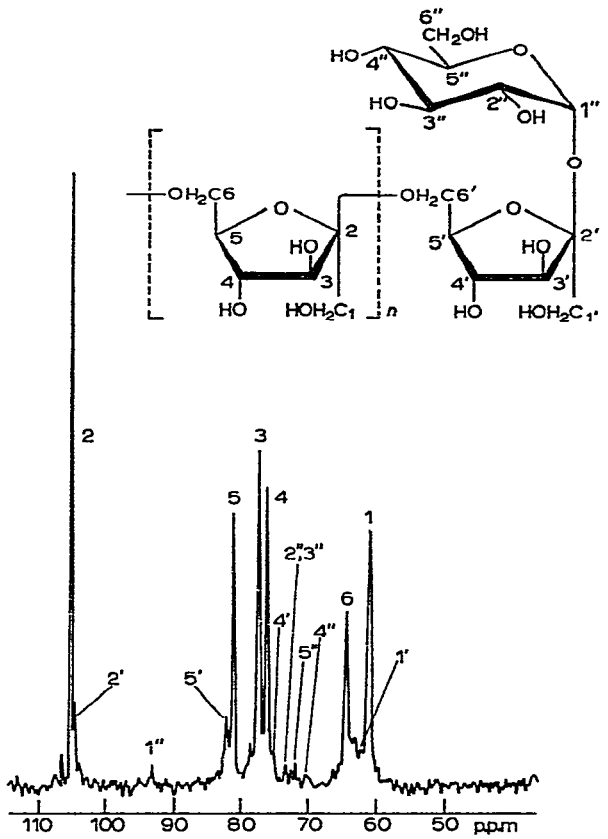

Fig. 2. Fourier-transformed, ^{13}C-n.m.r. spectra of the grass levan in D_2O (100 mg/ml) at 37° and pH 7. (Recorded with an acquisition time of 0.5 s, a pulse angle of 90°, and a spectral width of 4 kHz. The number of accumulated, free-induction decays was 37,284).

TABLE I

CARBON-13 CHEMICAL SHIFTS OF TWO D-FRUCTANS AND THREE MONO-D-FRUCTOFURANOSES[a]

Compound	*Carbon atom*					
	1	*2*	*3*	*4*	*5*	*6*
Sucrose (α-D-glucopyranosyl moiety)	93.3[b]	73.8[b]	73 6	70.4[b]	72.2[b]	61.7
Sucrose (β-D-fructofuranosyl moiety)	62.6[b]	104 9[b]	77.7	75.3	82.5[b]	63.5
β-D-Fructofuranose[c]	63.6	102.6	76.4	75.4	81.6	63.2
Inulin						
[β-D-(2 → 1)-linked D-fructofuranose][d]	62.2	104.5	78.5	76.6	82.4	63.4
Grass levan						
[β-D-(2 → 6)-linked D-fructofuranose][e]	61.3	105.4	77.6	76.5	81.5	64.6
Methyl β-D-fructofuranoside[c]	60.0	104.7	77.7	75.9	82.1	63.6

[a]In p.p.m. from external tetramethylsilane. [b]These resonances are identifiable as signals of low intensity in the ^{13}C-n.m.r. spectrum of the grass levan. [c]Chemical shifts, obtained from ref. 15. [d]At pH 13.0. [e]When recorded at pH 13.0, only the C-3 resonance changes by more than 0.1 p.p.m. (to 77.9 p.p.m.).

The principal feature of the spectrum consists in the six intense, narrow signals, which, because of their narrowness, are consistent with the carbon signals of the fructofuranosyl residues of a homogeneously linked, linear chain thereof. The β-D configuration of the linkages was confirmed by comparing the chemical shifts of these signals with those of some β-D-fructofuranose derivatives (see Table I). The chemical shifts of the C-3, C-4, and C-5 resonances of the levan were more consistent with those of β-D-fructofuranose and methyl β-D-fructofuranoside than with those of the equivalent resonances for the α-D configuration[15]. The position of linkage was not so readily determined by comparison with the mono-D-fructofuranose compounds studied, due to the fact that these all have two hydroxymethyl groups (on C-2 and C-5), one of which (that on C-2) is very sensitive to changes in the aglycon (see Table I). Although this ambiguity presents no problem with the mono-D-fructofuranose derivatives, as the C-1 and C-6 resonances had previously been unambiguously assigned by deuterium-labeling experiments[15], the problem of definitive assignment for the levan remains. However, experience would indicate that any linkage to C-6 of a fructofuranose unit should normally cause a downfield displacement[16] of the signal for C-6. On this basis, only the resonance at 64.6 p.p.m. can be assigned to C-6, leaving the remaining resonance (at 61.3 p.p.m.) to be assigned to C-1. By using the chemical shift of C-6 of methyl β-D-fructofuranoside as a standard reference, this indicated that linkage on C-6 of a fructofuranose unit produces a downfield displacement of 1.0 p.p.m. This value is the smallest recorded for an intersaccharide linkage, its nearest contender being the 2.0-p.p.m. downfield displacement caused by linkage to the hydroxymethyl group on C-8 of sialic acid[16].

Because of the ambiguity caused by the presence of two hydroxymethyl groups in the mono-D-fructofuranose derivatives, we compared the chemical shifts of the β-D-(2 → 6)-linked grass levan with those of inulin [a known β-D-(2 → 1)-linked D-fructofuranose polysaccharide], to ascertain whether we could differentiate between these two kinds of linkages to the two different hydroxymethyl groups (see Table I). The resonance at 63.6 p.p.m. is consistent with that of C-6 having O-6 unsubstituted, as shown by all the mono-D-fructofuranose compounds; we could therefore assign the resonance at 62.2 p.p.m. to C-1. Quantitation of the displacement caused by linkage to C-1 was difficult, because of the great variation of the chemical shift of this resonance for the mono-D-fructofuranose models due to aglycon sensitivity. However, in all cases, it was a small displacement (only 0.9 p.p.m.) compared with the signal of C-1 bearing unsubstituted O-1 that was given by the grass levan.

A close examination of the ^{13}C-n.m.r. spectrum of the grass levan (see Fig. 2) reveals a number of less-intense signals that are associated with the sucrose end-group. In all, seven of the twelve signals can be readily identified, and their identification is sufficient to confirm the structure of the terminal, D-glucose-containing disaccharide residue as that of α-D-glucopyranosyl β-D-fructofuranoside. In addition, integration of the resolved C-2 and C-2′ signals of the respective, linear-chain, fructofuranoside units at 105.4 p.p.m. and of the sucrose moiety at 104.9 p.p.m. gives some measure of the relative size of the grass levan. The ratio of C-2 to C-2′ is ~10:1,

indicating that an average of ten D-fructofuranose units are linked to the terminal sucrose residue.

ACKNOWLEDGMENTS

The authors thank Mrs. A. Martin and Dr. I. C. P. Smith for recording the ^{13}C-n.m.r. spectra, and are grateful to Dr. Gerald Powell (USDA, Beltsville, MD, U.S.A.) for supplying us with *Lolium perenne*.

REFERENCES

1 W. N. HAWORTH AND A LEARNER, *J. Chem. Soc.*, (1928) 619–625.
2 H. D. K. DREW AND W. N. HAWORTH, *J. Chem. Soc*, (1928) 2690–2697.
3 H. HIBBERT AND R. S. TIPSON, *J. Am. Chem. Soc*, 52 (1930) 2582; see also, H. HIBBERT, R. S TIPSON, AND F. BRAUNS, *Can. J. Res.*, 4 (1931) 221–239.
4 D. S. FEINGOLD AND M. GEHATIA, *J. Polym. Sci.*, 23 (1957) 783–790
5 R. H. HANCOCK, K. MARSHALL, AND H. WEIGEL, *Carbohydr. Res.*, 49 (1976) 351–360.
6 M. VRANA, J. TOMAŠIĆ, AND C. P. J. GLAUDEMANS, *J. Immunol.*, 116 (1976) 1662–1663.
7 D. G. STREEFKERK AND C. P J GLAUDEMANS, *Biochemistry*, 16 (1977) 3760–3765.
8 S. W. CHALLINOR, W. N. HAWORTH, AND E. L. HIRST, *J. Chem. Soc.*, (1934) 676–679.
9 W. N. HAWORTH, E. L. HIRST, AND R. R. LYNE, *Biochem J.*, 31 (1937) 786–788.
10 R. A. LAIDLAW AND S. G REID, *J. Chem. Soc.*, (1951) 1830–1834.
11 B. LINDBERG, J. LÖNNGREN, AND J. THOMPSON, *Acta Chem. Scand.*, 27 (1973) 1819–1821.
12 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature (London)*, 166 (1950) 444–445.
13 J. L. BUCHAN AND R. J. SAVAGE, *Analyst*, 77 (1952) 401–406.
14 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
15 S. J. ANGYAL AND G. S BETHELL, *Aust J. Chem.*, 29 (1976) 1249–1265.
16 A. K BHATTACHARJEE, H. J. JENNINGS, C. P. KENNY, A. MARTIN, AND I. C. P. SMITH, *J. Biol. Chem.*, 250 (1975) 1926–1932