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Note

Structural analysis of fructose polymers by gas–liquid chromatography and gel filtration

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Polymers of fructose form important reserves of carbohydrate in a number of families of flowering plants[1]. Two major types have been described, inulins and levans, which differ in the position of the major linkage between fructose residues[2,3]. Structural analysis of grass levans by methylation, hydrolysis and paper chromatographic (PC) separation of the methylated glycosides[4] indicated the presence of small amounts of dimethyl fructose. These could have originated either from incomplete methylation, or from branch points in the polymer. The development of more effective methods for methylation, together with the separation and identification of partially methylated sugar derivatives by gas chromatography (GC) and mass spectrometry (MS) has increased the value of this type of analysis[5], and we report the modification of these methods to permit improved structural analysis of fructosans. This technique also permits the estimation of the degree of polymerisation (DP) by measurement of the ratio of terminal to non-terminal groups. Colorimetric and group assays have been employed with grass levans[6] but the accurate determination of the ratio becomes more difficult with larger polymers. We describe the calibration of an acrylamide gel filtration column with linear dextrans which allows the rapid determination of the mean DP, and also the range of size within the sample.

MATERIALS AND METHODS

Tillers of a Norwegian isolate of cocksfoot *(Dactylis glomerata)* were grown at 5° and fructosans extracted from leaf base material with or without prior exposure to $^{14}CO_2$ as described previously[7]. The water soluble material from 10 g of plant material was purified by passage though a 10 × 1 cm column of ion-exchange resin composed of equal parts of Amberlite IR 120 (Na^+) and IR 400 (Cl^-). The eluate was dialysed for 12 h against flowing tap water and freeze-dried. Inulin from *Helianthus tuberosus* was obtained from BDH (Poole, Great Britain) and recrystallised from ethanol–water. Solid samples were stored over P_2O_5 at room temperature.

Weighed samples of 1–10 mg were methylated by the method of Hakomori[8] as described by Sandford and Conrad[9]. The permethylated samples were hydrolysed

at 60° for 30 min in 2 *M* trifluoroacetic acid, and the neutralised sample reduced with 10 mg sodium borohydride or sodium borodeuteride for 48 h at room temperature in the dark. The sample, together with 5 mg recrystallised *myo*-inositol as an internal standard, was acetylated and the products extracted into 1 cm^3 dichloromethane as described by Talmadge *et al.*[10]. The mixture of partially methylated alditol acetates was separated by GC and the individual components subjected to MS as described previously[11].

Samples (1–3 mg) were separated by gel filtration on a 1.5 × 40 cm column of Bio-Gel P-150 eluted with 50 m*M* $NaPO_4$ buffer at pH 7.0 and a flow-rate of 5.0 cm^3 h^{-1}. Suitable aliquots of the eluate were assayed by the ketose specific modification of the anthrone method[12] using recrystallised inulin as a standard. The column was calibrated with a series of linear dextrans obtained from Sigma (St. Louis, Mo., U.S.A.) and the relationship between molecular weight and elution volume determined by the procedure of Andrews[13].

Levan samples (2 mg) were hydrolysed at 60° for 30 min in 2 *M* trifluoroacetic acid, blown dry in an air stream and separated by descending PC on Whatman No. 4 paper in ethyl acetate–pyridine–water (8:2:1), butan-1-ol–ethanol–water (52:33:15), or butan-1-ol–pyridine–water (1:1:1).

RESULTS AND DISCUSSION

Extraction of 10 g of tissue yielded 520 mg of material after freeze-drying. Chemical analysis of this material by the anthrone method showed that it gave 98.5% of the optical density of the same weight of recrystallised inulin. Acid hydrolysis and PC in a number of solvents yielded spots corresponding in position to fructose and glucose only. Material extracted from plants fed $^{14}CO_2$ had 98% of the radioactivity associated with fructose and glucose after hydrolysis and separation. We consider that our preparations were sufficiently pure to permit further study.

Initial attempts to obtain methylated alditol acetates from this material and from recrystallised inulin were unsuccessful. Using ^{14}C-labelled levan we obtained complete recovery of added label in the final solution after acetylation, but no detectable peaks after GC separation. This suggested degradation of the polymer during derivatisation, rather than loss of the material. Hydrolysis of the unmethylated levan as described by Talmadge *et al.*[10] gave recoveries of less than 20% of the original fructose. Recoveries of 95–98% were obtained by reducing the temperature to 60° and the time of hydrolysis to 30 min.

Alditol acetates could not be obtained from pure fructose using the procedure of Talmadge *et al.*[10] which gave complete derivatisation of aldoses. Derivatisation of ketose sugars was found to require higher concentrations of reducing agent to achieve satisfactory reduction of carbon-2. The final procedure used 10 mg sodium borohydride incubated for 48 h. Overall the method produced total recoveries of derivatised levan of 85% when compared with *myo*-inositol.

The profiles obtained after derivatisation and chromatography of levan and inulin are shown in Fig. 1. In both samples only three partially methylated alditol acetates could be identified, two of which were derived from terminal glucose linked through carbon-1 and terminal fructose linked through carbon-2. These were found in both samples. Fructose residues linked 2,1- as proposed for inulin, would yield

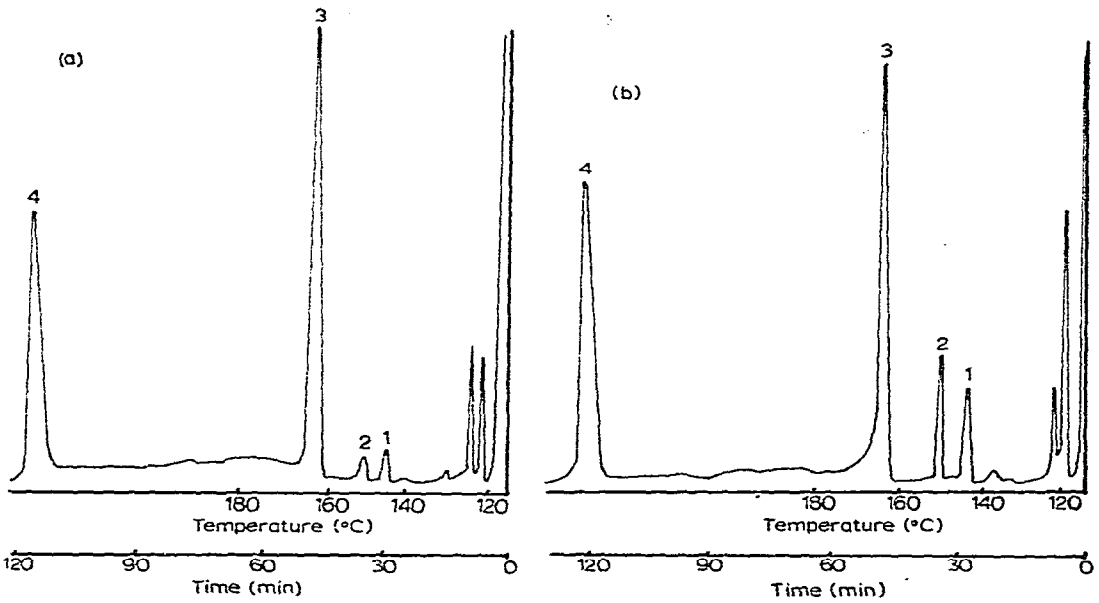

Fig. 1. Gas–liquid chromatogram of derivatised samples of (a) levan from *D. glomerata* and (b) inulin from *H. tuberosus*. Identified peaks are numbered and are derived from (1) terminal fructose, (2) terminal glucose, (3) linkage fructose and (4) *myo*-inositol internal standard. Injections were 1 μl of a 1-cm^3 solution containing 0.5 mg *myo*-inositol and 1.0 mg fructosan. GC conditions: temperature, 120° held for 5 min, then raised to 180° at 1° min^{-1}; carrier gas (nitrogen) flow-rate 50 cm^3 min^{-1}; recorder range 10 mV; chart speed 120 mm h^{-1}.

3,4,6-methyl fructose after methylation and hydrolysis; giving on reduction and acetylation 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl glucitol. The principal peak in the inulin sample was shown to consist of this derivative by means of its retention time and mass spectrum (primary fragments *m/e* 45, 161 and 189).

The principal peak in the levan fraction had the same retention time and mass spectrum as that in the inulin fraction. The putative major linkage in levan, fructose residues linked 2,6- would give 1,3,4-methyl fructose after methylation and hydrolysis, but this would also yield 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl glucitol on reduction and acetylation. By reduction of the permethylated samples with sodium boro-deuteride after hydrolysis it was possible, by MS, to distinguish between 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-glucitol produced from 1,3,4-methyl-fructose and that derived from 3,4,6-methyl fructose. This method has been used previously for cell wall polysaccharide analysis[14]. The principal peak from deuterated samples of permethylated inulin yielded primary fragments of *m/e* 45, 161 and 190, whereas those from levan yielded primary fragments of *m/e* 45, 162 and 189. This is consistent with a major linkage which is 2,1- in inulin and 2,6- in levan, confirming the earlier observations[2,3].

No evidence was obtained for the presence of other minor linkages in either polymer, implying that the previous report of dimethyl fructoses from levan samples was the result of incomplete methylation[4]. Estimates of DP were made from the areas of the various peaks and were in close agreement with the values obtained by gel filtration (Table I). The gel filtration profiles obtained for levan and inulin are shown

TABLE I

MEAN DP ESTIMATES FOR *HELIANTHUS TUBEROSUS* INULIN AND *DACTYLIS GLOMERATA* LEVAN

Method of assessment	*Degree of polymerisation*	
	Inulin	*Levan*
Peak height analysis from GC of methylated alditol acetates	33	110
Determination of elution volume of profile peak after gel filtration	34	108

in Fig. 2. The levan fractions of highest fructose content were re-fractionated as a check on resolution. Both samples contained material of a range of apparent molecular weight distributed around a peak, the apparent molecular weight of which was the same as that obtained from measurement of mean DP by GC. Within the sample of levan, apparent molecular weights ranged from 6 to 40 kD.

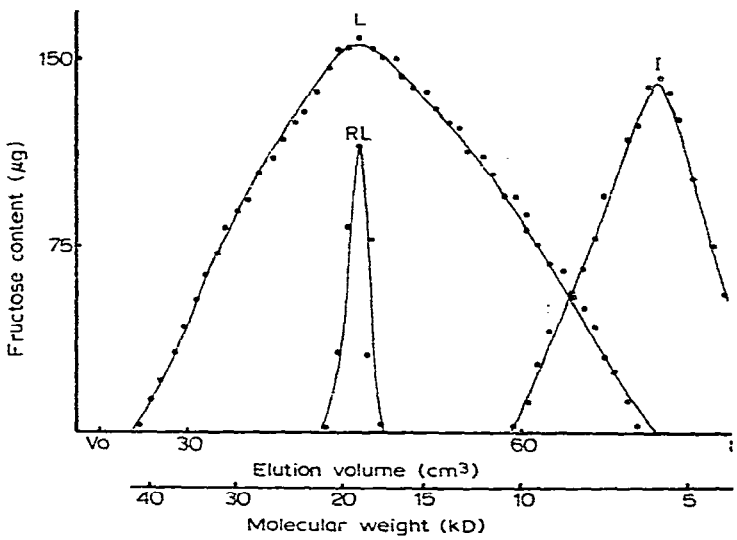

Fig. 2. Elution profiles of levan from *D. glomerata* (L) and inulin from *H. tuberosus* (I) obtained after gel filtration on Bio-Gel P-150. The four levan fractions of highest fructose content were pooled and refractionated (RL) as a check on column resolution.

In conclusion we propose that the combination GC–MS and gel filtration provides an accurate method for structural analysis of frutose polymers, particularly those of higher DP, and that the method is well suited for investigating the changes in molecular weight which have been reported to occur as a result of environmental changes[1].

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REFERENCES

1 D. Smith, in G. W. Butler and R. W. Bailey (Editors), *Biochemistry of Herbage* Vol. 2, Academic Press, New York, 1973, Ch. 3, p. 105.
2 W. N. Haworth and A. Learner, *J. Chem. Soc.*, (1928) 619.
3 S. W. Challinor, W. N. Haworth and E. L. Hirst, *J. Chem. Soc.*, (1934) 676.
4 D. J. Bell and A. Palmer, *J Chem. Soc.*, (1952) 3763.
5 P. Albersheim, D. J. Nevins, P. D. English and A. Karr, *Carbohyd. Res.*, 5 (1967) 340.
6 R. D. Grotelueschen and D. Smith, *Crop. Sci*, 8 (1968) 210.
7 C. J. Pollock and P. A. Ruggles, *Phytochemistry*, 15 (1976) 1643.
8 S. Hakomori, *J. Biochem.*, 55 (1964) 205.
9 P. A. Sandford and H. E Conrad, *Biochemistry*, 5 (1966) 1508.
10 K. W. Talmadge, K. Keegstra ,W. D. Bauer and P. Albersheim, *Plant Physiol.*, 51 (1973) 158.
11 A. G. Darvill, D. P. Roberts and M. A. Hall, *J. Chromatogr.*, 115 (1975) 319.
12 M. A. Jermyn, *Nature (London)*, 177 (1956) 38.
13 P. Andrews, *Biochem. J.*, 96 (1965) 595.
14 H. Björndal, C. G. Hellerqvist, B. Lindberg and S. Svensson, *Angew. Chem., Int. Ed. Engl.*, 9 (1970) 610.