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OLIGOSACCHARIDE INTERMEDIATES OF FRUCTAN SYNTHESIS IN *LOLIUM TEMULENTUM*

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Abstract—Evidence is presented that fructan accumulation in leaves of *Lolium temulentum* plants grown at 5° proceeded via the synthesis of trisaccharide intermediates. Studies on the oligosaccharide components of this tissue indicated that the major intermediate was probably 1^F-fructosyl sucrose (isokestose) but that two distinct series of oligofructosides could be isolated. One of these had chromatographic properties identical to the 1,2-linked inulin series from *Helianthus tuberosus*. The relationship of this synthetic pattern to the structure of grass fructans and their accumulation in other species is discussed.

INTRODUCTION

It is widely accepted that fructan synthesis in Compositae and Liliaceae proceeds via the synthesis of a trisaccharide, 1^F-fructosyl sucrose (isokestose), from two molecules of sucrose, and the subsequent transfer of fructose residues from this compound to other oligosaccharide acceptors. This results in the occurrence *in vivo* of homologous series of oligofructosides with increasing degrees of polymerization (DP) [1, 2]. Such series cannot be detected during fructan synthesis in the grass *Dactylis glomerata*. Here fructan biosynthesis appears to proceed by the direct transfer of fructose residues from sucrose to a high DP acceptor [3]. There is also evidence that reversible transfer of fructose residues occurs between chains during fructan synthesis in this system [4].

There is little evidence on the mechanism of fructan synthesis in other grass species, but there are reports of the occurrence in *Lolium* species of trisaccharides including 1^F-fructosyl sucrose [5] and that such compounds become labelled when leaf sections are fed $^{14}CO_2$ [6]. Recently a range of oligosaccharide material was detected in neutral extracts of leaves of *Lolium* sp. and *Festuca pratensis* harvested during vegetative growth in the autumn. Similar extracts prepared from leaves harvested during reproductive growth in the spring contained no detectable oligosaccharides, nor could these be detected in extracts of *Phleum pratense* or *D. glomerata* at either growth stage [Pollock, C. J., unpublished observations].

The mean molecular size of the high DP fructan isolated from *Lolium* and *Festuca* sp. (*ca* 10 000 daltons) is substantially lower than that isolated from *D. glomerata* or *P. pratense* (30 000–40 000 daltons) [7, 8], and is closer to that observed in tubers of *Helianthus tuberosus* [1]. These observations could indicate the presence in grasses of alternative mechanisms of fructan biosynthesis, dependent upon both species and growth stage, and associated with the varying molecular size ranges of the final product. The study reported here was undertaken in order to investigate the mechanism of fructan biosynthesis in grass species where fructo-oligosaccharides could be detected during vegetative growth and to compare the results with those obtained from *D. glomerata* where no such material could be detected [3] and from members of the Compositae and Liliaceae [1, 2].

RESULTS AND DISCUSSION

Preliminary experiments showed that fructan biosynthesis in vegetative tissues of *Lolium temulentum* L. was stimulated by transfer to low temperatures. Mean rates of accumulation for whole plants over a 6 week period were 0.2 mg/g fr. wt/week at 20° and 3.2 mg/g fr. wt/week at 5°. These rates were substantially lower than those observed in studies on *D. glomerata* [9], where the plants used showed considerable physiological adaptation to low temperature [10].

Table 1 shows the results obtained after gel filtration of neutral extracts from cold-treated plants, followed by colorimetric estimation of carbohydrate in the separate fractions. Individual components were identified by their elution volumes relative to known markers. High DP fructan was accumulated more extensively in leaf bases (the portion below the ligule), whereas sucrose was more abundant in leaf blades. In both cases low MW sugars made up a substantial proportion of the total water-soluble carbohydrates.

Plants at this stage were exposed to $^{14}CO_2$ at 20° (20 μCi/plant for 1 hr) and then returned to 5°. Plants were harvested daily for 9 days after feeding $^{14}CO_2$. The distribution of radioactivity within the tissues during this period is summarized in Table 2. In both blades and bases there was a steady decline in total

Table 1. Levels of components of neutral extracts of *L. temulentum* leaf blades and bases harvested 2 weeks after transfer to 5°

Component	Level (mg/g fr. wt)*	
	Leaf blades	Leaf bases
Monosaccharides	0.7 ± 0.2	0.6 ± 0.2
Sucrose	4.3 ± 0.6	2.7 ± 0.4
Trisaccharides	1.1 ± 0.3	1.1 ± 0.4
Oligosaccharides (DP 4–10)	0.5 ± 0.2	0.6 ± 0.2
Fructan (DP > 10)	2.5 ± 0.4	6.8 ± 0.8

*All values represent the means of four determinations ± s.e.

Table 2. Distribution of radioactivity in leaf blades and bases of *L. temulentum* at different times after incorporation of $^{14}CO_2$

Days after incorporation	Total incorporation (kBq/g fr. wt)*		Percentage in neutral components*	
	Blades	Bases	Blades	Bases
0	36.6	22.9	45	62
1	43.7	41.8	55	35
3	30.0	36.6	34	26
5	22.9	25.9	34	30
7	20.4	13.7	29	34
9	20.7	14.4	25	36

*All values are the means of triplicate determinations.

radioactivity after day 1, together with a reduction in the proportion of radioactivity associated with neutral compounds. This proportion was always substantially lower than that observed after incorporation of $^{14}CO_2$ by plants of *D. glomerata* under similar conditions [3], reflecting the lower rates of fructan accumulation in these plants.

The neutral material from plants fed $^{14}CO_2$ was fractionated by gel filtration, and the specific activity of the individual components determined at various times after incorporation of $^{14}CO_2$ (Fig. 1). In material from both blades and bases, the specific activity of sucrose fell from day 1 onwards, and this was accompanied by a gradual rise in the specific activity of high DP fructan until both components reached similar values. There were marked differences in the specific activity changes of the trisaccharide components of blades and bases. In base material (Fig. 1a) specific activity peaked at the same time as sucrose and then fell to reach isotopic equilibrium with the high DP fructan, suggesting that both sucrose and trisaccharide were acting as intermediates in the biosynthesis of fructan. In blade extracts, however, (Fig. 1b) the trisaccharide component remained at a higher specific activity.

These differences were studied by characterization of the trisaccharide and oligosaccharide components of blades and bases. Non-radioactive material (50 g each of blades and bases) was harvested 2 weeks after transfer to 5° and low MW sugars separated from high DP fructan by gel filtration of the neutral components of cold-water soluble extracts. Similar preparations were made for comparative purposes from tubers of *Helianthus tuberosus* L. and bulbs of *Allium cepa* L. (cv Stuttgart Giant). The oligosaccharide components of these tissues have been well characterized [1, 5]. Analytical HPLC separations of these preparations indicated the presence of several oligosaccharides with RR_f values greater than sucrose, and preparative gradient HPLC separations were undertaken to obtain individual fractions which were chromatographically homogeneous. The properties of some of these fractions are summarized in Table 3. Extracts of *A. cepa* and *H. tuberosus* contained higher oligosaccharides with RR_f values greater than 4.0, but since these were only present in trace amounts in extracts of *L. temulentum* they were not subjected to further study. The major differences observed between the various preparations was the presence in *L. temulentum* extracts of compound B, an oligosaccharide with a RR_f on HPLC and mobility on PC identical to marker raffinose, and which contained glucose, galactose and fructose in roughly equimolar amounts. On this basis it was provisionally identified as raffinose. The remaining oligosaccharides contained only glucose and fructose in proportions consistent with compounds A, C and D having DP values of 3, 4 and 5 respectively. Their mobilities, together with those of fructose and sucrose, were plotted against apparent DP by a modification of the

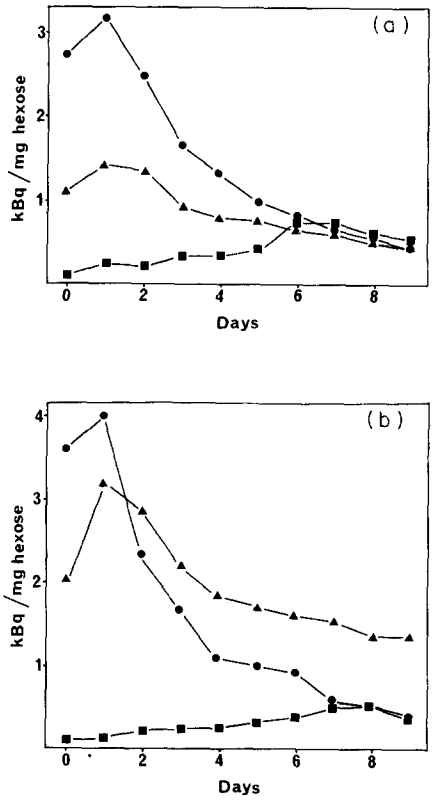

Fig. 1. Changes in specific activity of sucrose (●—●), trisaccharides (▲—▲) and fructan (■—■) in (a) leaf bases and (b) leaf blades of *L. temulentum* following exposure to $^{14}CO_2$. All values are the means of four determinations.

method of French and Wild [11] using values of $1/RR_f$ instead of R_f. The plot generated a straight line ($m = -0.132$, $c = 0.228$, $r = 0.998$). The mobilities of marker raffinose, stachyose and maltotriose did not lie on the curve. Such a relationship indicates that compounds A, C and D were structurally related, and comprised a homologous series in *L. temulentum* similar to those observed in *H. tuberosus* and *A. cepa* [1, 2] with A, C and D representing mono-, di- and trifructosyl sucrose respectively.

These observations indicate that the trisaccharide component of *L. temulentum* extracts isolated by gel filtration comprised a mixture of raffinose and mono-fructosyl sucrose. The distribution of radioactivity between these two compounds in extracts of blades and bases, harvested at different times after incorporation of $^{14}CO_2$, was assessed by HPLC. In leaf blade extracts 82–88% of the recovered radioactivity co-eluted with marker raffinose, whereas in base extracts this value was 35–40%. These proportions did not vary appreciably with time after incorporation. The remaining radioactivity co-eluted with compound A (mono-fructosyl sucrose). The high proportion of radioactivity associated with raffinose in leaf blade extracts would account for the failure of the trisaccharide component to reach isotopic equilibrium with sucrose and high DP fructan. Thus it is suggested that fructan synthesis in *L. temulentum* under these conditions proceeds via sucrose and mono-fructosyl sucrose in a manner similar to that observed in Compositae and Liliaceae [1, 2] but apparently distinct from some other Gramineae [3].

Three distinct forms of mono-fructosyl sucrose have been characterized, differing in the position of linkage of the fructosyl moiety [12]. 1^F-fructosyl sucrose is the only form present in *H. tuberosus* [13], whereas *A. cepa* also contains 6^G-fructosyl sucrose (neokestose) [5]. *Lolium perenne* is reported to contain these compounds together with traces of 6^F-fructosyl sucrose (kestose) [5]. The different forms can be separated by charcoal–Celite chromatography or TLC [14]. Results obtained after TLC separation of HPLC-purified oligosaccharides are summarized in Table 4. Each purified oligosaccharide from extracts of *H. tuberosus* gave only a single spot, whereas those from *A. cepa* and *L. temulentum* were resolved into two components, the less mobile of which had the same $R_{fructose}$ value as the corresponding oligosaccharide from *H. tuberosus*. This suggests that two distinct fructan series exist in *L. temulentum* and *A. cepa*, confirming previous observations on *A. cepa* extracts separated on charcoal–Celite columns [5]. One of the series appears to be the same as the

Table 3. Chromatographic and chemical properties of sugars purified from neutral extracts of *L. temulentum*, *A. cepa* and *H. tuberosus*

Compound	$RR_f(H_2O)$	Ratio (fru:glc)	Monosaccharides present in hydrolysates
Fructose	1.82	—	Fru
Sucrose	2.04	0.97:1	Fru,Glc
A	2.50	1.89:1	Fru,Glc
B*	2.77	1.05:1	Fru,Glc,Gal
C	3.03	3.07:1	Fru,Glc
D	3.70	4.10:1	Fru,Glc

*Compound B was only isolated from tissues of *L. temulentum*. The remaining compounds were present in tissue extracts from all species. There were no significant differences between the properties of these compounds extracted from different species.

Table 4. TLC mobilities of oligofructosides from *H. tuberosus*, *A. cepa* and *H. temulentum*

Compound*	Mobility (as $R_{fructose}$)	
	H. tuberosus	*A. cepa*/*L. temulentum*
Sucrose	0.95	0.95
A	0.74	0.92,0.73
C	0.67	0.75,0.65
D	0.54	0.70,0.53

*Compound B, isolated only from *L. temulentum* extracts, gave a single spot ($R_{fructose}$ 0.37) coincident with marker raffinose.

1,2-linked fructans of *H. tuberosus*, but the identity of the other is unknown.

TLC separations of the mono-fructosyl sucrose of *L. temulentum* leaf bases extracted 5 days after exposure to $^{14}CO_2$ showed that 85% of the recovered radioactivity cochromatographed with marker fructosyl sucrose from *H. tuberosus*, and the remainder with the more mobile component visualized after TLC separation of the corresponding purified fractions from *A. cepa*. This indicates that 1^F-fructosyl sucrose is the major intermediate form even though the high DP product in Gramineae is generally accepted to have a different major linkage [15]. It has been suggested, however, that 1^F-fructosyl sucrose may be formed during the synthesis of the predominantly 2,6-linked bacterial levan [16]. What is not understood is the synthetic inter-relationships between the contrasting series of oligofructosides and the final product, nor the significance of the two apparently distinct synthetic mechanisms present in Gramineae. Such an understanding will require a more detailed structural analysis of individual components of the fructan pool, together with characterization of the enzymes involved in the various processes.

EXPERIMENTAL

Materials. Plants of *L. temulentum* (Ba 3081) were grown from seed in controlled environments (20°, 8 hr light for 4 weeks followed by 5°, 8 hr light for 2 weeks) using a liquid medium [4]. After incorporation of $^{14}CO_2$ plants were held at 5° (8 hr light) until harvested. Bulbs of *A. cepa* cv Stuttgart Giant and tubers of *H. tuberosus* were obtained locally.

Methods. The techniques used on *L. temulentum* for incorporation of $^{14}CO_2$ by whole plants, followed by separation, killing and extraction of tissues, and the initial fractionation of the extracts obtained have been described previously; as have the methods used for the estimation of radioactivity [3]. Only the neutral components of cold-water soluble material were fractionated further.

Bulbs and tubers (200 g) were mechanically homogenized in hot 80% EtOH. Filtered extracts were concd under red. pres. fractionated by ion exchange chromatography [9] and the neutral material lyophilized.

Fractionation of neutral extracts. Neutral extracts from all sources were fractionated by gel filtration on Bio-gel P2 [3] and the carbohydrate contents of the fractions estimated as described previously [7], together with radioactivity where appropriate. Specific portions of the eluates were pooled, lyophilized and redissolved in H_2O to give a final concn of 5–10 mg/ml (fructose). These fractions were studied further by HPLC using LDC Constametric pumps in constant flow mode; columns packed with Spherisorb 5-NH_2; and where appropriate an LDC Gradient Master solvent programmer or LDC Refractomonitor II refractive index detector. Two solvent systems were used, both MeCN–H_2O, acidified to pH 5.0 with HOAc: solvent I (85:15); solvent II (65:35). Analytical runs for determination of RR_f (H_2O) and relative abundance of oligosaccharides up to DP 10 were on 4.0 × 250 mm columns. Samples (20 μl) were separated isocratically at 2 ml/min (*ca* 100 kg/cm^2) using solvent II. Mass determinations were by refractive index measurements using sucrose as an external standard. Radioactivity was determined after collection of fractions directly into scintillation vials.

Prep. HPLC for purification of individual oligosaccharides was on 15 × 250 mm columns. Samples (400 μl) were separated at 5.0 ml/min (*ca* 80 kg/cm^2) using a convex exponential gradient ($M = 0.2$) between 100% solvent I and 100% solvent II. The run time was 30 min. Fractions were collected and assayed for free and combined fructose [4]. Fractions comprising individual peaks were pooled, lyophilized and redissolved in 0.5 ml H_2O. Repurification by the same method was performed where necessary until samples were obtained where >95% of the mass eluted as a single peak after separation by analytical HPLC as above. Individual peaks eluting after sucrose were designated alphabetically in order of their elution.

Characterization of oligosaccharides. Oligosaccharides (aliquots 1–2 μl) were separated by TLC on Si gel plates pre-run in 1.0 M NaH_2PO_4 and dried. The plates were developed with *iso*-PrOH–Me_2CO–H_2O (2:2:1) [14] and then stained with urea–HCl [17].

Further aliquots (50 μl) were hydrolysed in 0.02 M TFA (100°, 30 min), dried under red. pres. and the residue redissolved in 0.1 ml H_2O. Component sugars were identified after descending PC (Whatman No. 4 paper). Solvents used were PrOH–EtOAc–H_2O (7:1:2 and 6:1:3) and EtOAc–C_5H_5N–H_2O (6:1:3). Papers were stained in alkaline $AgNO_3$ [18]. The monosaccharide ratios of individual hydrolysates were estimated after analytical HPLC of 20 μl aliquots in solvent I.

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