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PATHWAY OF FRUCTOSAN SYNTHESIS IN LEAF BASES OF *DACTYLIS GLOMERATA*

CHRISTOPHER J. POLLOCK
Welsh Plant Breeding Station, Plas Gogerddan, Aberystwyth, SY23 3EB, U.K.

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Key Word Index—*Dactylis glomerata*; Gramineae; cocksfoot leaves; fructosan synthesis; polysaccharide; oligosaccharide.

Abstract—Chemical analysis of leaf base tissue of *Dactylis glomerata* failed to detect any low MW oligosaccharide intermediates during fructosan synthesis. Extracts of tissue harvested at various times after the incorporation of $^{14}CO_2$ showed a decline in radioactivity in sucrose and an equivalent rise in high MW fructosan with no significant accumulation of radioactivity in oligosaccharides. No evidence was obtained for the existence of nucleotide fructose in the tissue, indicating that fructosan synthesis occurs by direct transfer of fructosyl residues from sucrose to the polymer.

INTRODUCTION

Fructosan synthesis in *Dactylis glomerata* is induced by low temperature and is concentrated in the basal portions of the leaves below the ligule [1]. The mechanism of fructosan synthesis has not been studied fully in the Gramineae, but the structure of the polymer [2] and the distribution of MW within this family suggest that the mechanism may differ from that operative in the Compositae [1]. Fructosan synthesis in the Compositae proceeds through the concerted action of two enzymes which transfer fructosyl residues. The kinetic properties of these enzymes are such that during accumulation, a continuous series of oligosaccharides exists between sucrose, with a degree of polymerization (DP) of 2, and inulin with a DP of *ca* 35 [3]. The aim of this study was to examine changes in the oligosaccharide fraction of leaf bases of *D. glomerata* during fructosan accumulation. Gel filtration on small pore acrylamide columns was used to resolve this fraction and to separate it from high MW fructosan. Such a system has been used to resolve fructo-oligosaccharides from onion bulbs up to a DP of 10 [4].

RESULTS AND DISCUSSION

Plants were used for experiments 4 weeks after transfer to 5°, during rapid fructosan synthesis [1]. Leaf base material harvested over the course of the studies had a mean fructosan content of 39 mg/g fr. wt, and there was no significant variation between material harvested at different times during the experiments. Leaf base extracts of material harvested 4 weeks after transfer to 5° were fractionated on Bio-Gel P2 and the eluate assayed for free and combined fructose. The elution positions of marker sugars fructose (DP 1), sucrose (DP 2), raffinose (DP 3) and stachyose (DP 4) were respectively 148, 143, 125 and 120 ml. Only 2 major peaks were detectable, the first, which eluted with the void volume (60 ml), consisted of the unresolved high MW fructosan, and the second co-eluted with marker sucrose (143 ml). This material co-chromatographed with sucrose after PC in a variety of solvents, yielded glucose and fructose on hydrolysis and was presumed to be sucrose.

When extracts of leaf bases were fractionated from plants fed $^{14}CO_2$ (100 μCi per 12 plants) and the eluate assayed for radioactivity, 3 further peaks were observed co-eluting with marker fructose, raffinose and stachyose, respectively. No radioactivity was observed in oligosaccharides of higher DP. The distribution of radioactivity in the major fractions obtained by extracting leaf bases at various times after exposure to $^{14}CO_2$ is shown in Table 1. Changes were monitored for 48 hr after exposure, since significant accumulation of radioactivity in fructosan had been observed in this time [1]. Incorporation peaked 8 hr after feeding and had fallen substantially by 48 hr. The neutral component of the cold-water soluble

Table 1. Distribution of radioactivity in extracts of leaf bases of *D. glomerata* heat-killed after exposure $^{14}CO_2$

Time after feeding (hr)	Radioactivity (μCi/g fr. wt)* Cold-water insoluble fraction	Cold-water soluble fraction	Neutral component of cold-water soluble fraction	Remainder of cold-water soluble fraction
4	0.02	0.56	0.51	0.02
8	0.04	1.67	1.47	0.18
24	0.05	1.26	1.10	0.14
48	0.04	0.85	0.79	0.04

*All values are the mean of 3 determinations.

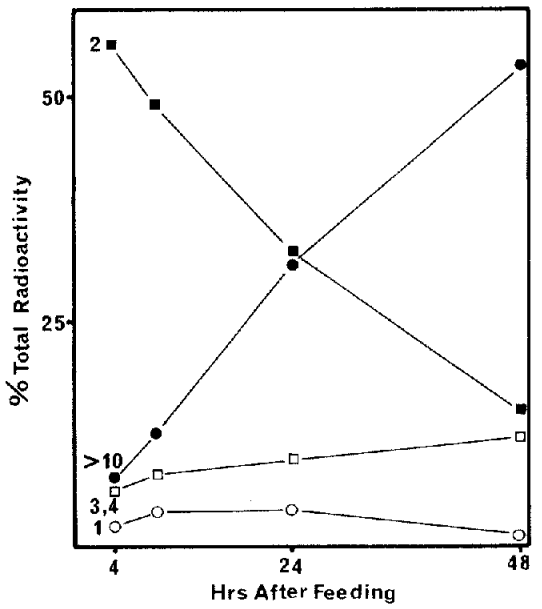

Fig. 1. Changes in the radioactivity of individual sugar components of extracts from leaf bases of *D. glomerata* harvested at different times after exposure to $^{14}CO_2$. ●, Fructosans (DP > 10); □, tri-and tetrasaccharides (DP 3 & 4); ■, disaccharides (DP2) and ○, monosaccharides (DP1). All values are the mean of 3 determinations.

material comprised at least 88% of the label extracted from the plants. The relative amounts of radioactivity found in different fractions of the neutral component after gel filtration are shown in Fig. 1. There was a steady decline in radioactivity in sucrose (DP 2) and an equivalent rise in radioactivity in fructosan of DP > 10. There was a less extensive rise in radioactivity in material of DP 3 and 4, and a small and variable amount in a monosaccharide which was identified as glucose by PC in a variety of solvents.

Material of DP 3 and 4 was completely separated from mono- and disaccharides by PC and was resolved into two components which co-chromatographed with marker raffinose and stachyose. In 3 replicate experiments this material was eluted, hydrolysed under mild conditions and separated by PC. Of the radioactivity present prior to hydrolysis, 72% remained close to the origin, 21% co-chromatographed with fructose and 5% was unresolved. After elution of the material close to the origin, hydrolysis under more vigorous conditions and re-separation by PC, 28% of the label co-chromatographed with galactose, 25% with glucose, 15% remained near the origin and 5% was unresolved. Such results are consistent with this material being composed of raffinose and stachyose. These sugars have been described previously as constituents of the soluble sugar fraction of grasses [5] and are thought to be translocated sugars [6, 7]. Their structure, probable role, and labelling kinetics argue against any role as intermediates of fructosan synthesis in this tissue.

The evidence presented is consistent with the direct transfer of fructose residues from sucrose to the growing polymer chain, without the intermediate series of oligosaccharides observed in inulin biosynthesis. There is no significant accumulation of radioactivity in glucose and all the radioactivity initially present in sucrose is finally isolated in fructosan. This suggests that glucose released during transfructosylation from sucrose is metabolized back to sucrose by an active sucrose synthetic system. Such a pattern is also proposed for inulin biosynthesis [3].

Uridine diphosphofructose has been isolated from plant tissues which synthesize inulin [8, 9], and it has been proposed that it could be an intermediate in the synthetic process. The amounts isolated, however, were small and the extraction methods used did not satisfy the criteria laid down by Bieleski to minimize losses and group transfer reactions due to the action of phosphatases [10]. Leaf bases of *D. glomerata* were examined for nucleotide fructoses as potential intermediates of fructosan synthesis. Extraction procedures were checked for recovery of sugar nucleotides and sugar phosphates [11]. UDP-[U-^{14}C]glucose or [U-^{14}C]fructose-6-P was added to unlabelled leaf base material ground in liquid N_2 prior to killing. 90–95% of the radioactivity was recovered in material eluted from DEAE-cellulose columns by NH_4HCO_3 [12] and this material co-chromatographed with the respective markers on ascending TLC. Elution and hydrolysis of these areas gave 75–80% recovery of added radioactivity in the respective monosaccharides. UDP fructose could not be separated from other sugar nucleotides by conventional partition chromatography [8]. In this study, radioactive extracts of leaf bases were examined for the presence of material which co-chromatographed with UDPG and yielded fructose on hydrolysis. Plants were fed $^{14}CO_2$ (1 mCi per 12 plants) and the distribution of label in the major fractions determined (Table 2). Use of higher activity $^{14}CO_2$ did not lead to *pro rata* increases in tissue radioactivity although some increase in activity of the water soluble extract was observed. In 3 replicate experiments, the acidic component of this extract comprised a constant 6% of the total and further separation of this indicated that *ca* 2.5% of the total radioactivity was in phosphate esters. The majority of this radioactivity was in material

Table 2. Distribution of radioactivity within the water-soluble fraction from extracts of *D. glomerata* cold-killed after exposure to $^{14}CO_2$

Time after feeding (hr)	Radioactivity in water-soluble fraction (μCi/g fr. wt)	Percentage water-soluble radioactivity in		
		Acidic fraction	Sugar phosphates	Nucleotide sugars
4	2.67	6.1	1.1	0.3
8	3.83	6.8	1.9	0.3
24	4.77	6.4	0.6	0.3
48	2.01	6.4	0.3	0.4

All values are the mean of 3 determinations.

with the same mobility as hexose phosphates. No change was observed in the proportion of radioactivity in material with the same mobility as UDPG and no evidence was obtained for the presence of fructose after hydrolysis of this material. However, radioactivity was detected in glucose after hydrolysis of this material and in both glucose and fructose after hydrolysis of the hexose phosphate fraction.

The constant small amount of radioactivity isolated from the sugar nucleotide fraction and the absence of detectable nucleotide fructose suggests that fructosan synthesis in *D. glomerata* is not dependent on enzymes which use nucleotide fructose. Although the evidence is consistent with the direct transfer of fructosyl residues from sucrose in a manner analogous to bacterial levansucrase [3], preliminary studies suggest that simple cell-free extracts of leaf bases of *D. glomerata* do not synthesize fructosan either from fructose or from sucrose.

EXPERIMENTAL

The growth of plants of *D. glomerata* ssp. *glomerata* Bc 4095 in controlled environments at 5° and their exposure to $^{14}CO_2$ were as described previously [1]

Extraction and separation of metabolites. For investigation of neutral sugars, the tissue was killed in boiling 80% EtOH and cold-H_2O extracts were made as described previously [1]. Aliquots of these extracts were separated into acidic, basic and neutral components by ion exchange chromatography [13] and the neutral component fractionated on a 150 × 1.5 cm column of Bio-Gel P2 eluted with H_2O. Free and combined fructose was estimated by the ketose specific modification of the anthrone method [14]. Recoveries of fructose and radioactivity after gel filtration were between 95 and 98%. Material eluted after the void vol. was further separated by PC. All PC was descending, on Whatman No. 4 paper. Fractions were pooled, reduced in vol. to 200 μl and separated using EtOAc–*n*BuOH–Py–H_2O (6:6:5:4). Material with mobility less than sucrose was eluted with H_2O, reduced in vol. to 0.5 ml, treated with M TFA for 1 hr at 60° and reduced in vol. to 50 μl. After PC in EtOAc–Py–H_2O (8:2:1), material with mobility less than galactose was eluted with H_2O, reduced in vol. to 0.5 ml, treated with 2 M TFA for 1.5 hr at 105°, reduced again in vol. to 50 μl and separated by PC in EtOAc–Py–H_2O (8:2:1). Identification of mono- and disaccharides was by co-chromatography with known markers in the above solvents; all chromatograms were visualized by $AgNO_3$–NaOH [15].

For investigation of phosphorylated sugars, extraction and separation methods were based on those described in ref. [12]. Tissue was excised, weighed and freeze-clamped between 100 g Al plates cooled with liquid N_2. The tissue was ground in liquid N_2 and 5 ml MeOH–$CHCl_3$–HCO_2H (7 M) (12:5:3) at −20° added. After killing for 18 hr at −20°, material was fractionated into H_2O-soluble, $CHCl_3$-soluble and H_2O-insoluble material, and the H_2O-soluble fraction separated into neutral and acidic components on cellulose ion-exchange media [12]. Aliquots of the acidic components were separated on Whatman TL cellulose by double development in PrOAc–HCO_2H–H_2O (11:5:3) [12]. Compounds coincident with marker UDPG and F-6-P were separately removed from the plate, eluted in H_2O, reduced in vol. to 0.5 ml, hydrolysed in 0.01 M TFA (100°, 15 min), reduced to 50 μl and separated by PC (EtOAc–Py–H_2O, 8:2:1).

Radioactivity in aq. samples, H_2O insoluble material and on sections of PC was estimated as described in ref. [1].

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