Biosynthesis of β-2, 6-Fructosan in the Stem of Timothy (*Phleum pratense* L.)

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Abstract

Phlein (β-2, 6-fructosan) synthesis in *Phleum pratense* was examined using $U^{14}C$ fructose and sucrose. Oligosaccharides were actively synthesized from fructose or sucrose in both stem and haplocorm, while phlein from sucrose only in haplocorm. Oligosaccharides were also formed from sucrose by the enzyme from haplocorm, but phlein was not. No evidence was obtained of the involvement of nucleotide sugars in phlein synthesis.

Introduction

Fructosans occurring in higher plants are grouped into inulin (β-2,1) and phlein (β-2,6) in terms of fructose linkage. In general temperate grasses accumulate phlein as principal carbohydrates in stems and leaves. Inulin synthesis has been investigated by many workers[1,5-8,16], but we have only a few studies on phlein synthesis in grasses[14,15,17,18]. POLLOCK[4] has recently suggested the involvement of sucrose in its formation from the time course distribution of sugars in the leaf base of *Dactylis glomerata* fed with [14]CO₂. Up to the present, however, the synthetic mechanism has not been fully elucidated. The aim of this study is to clarify more details of the polymer synthesis in stem and haplocorm of *phleum pratense* using labelled sucrose and fructose.

Materials and Methods

Plant materials and preparation of sugars

  Phleum pratense (cv. Climax) was grown in the Experimental Farm of Obihiro University as described in a previous paper[2]. The upper part of stem (about 15 cm) and root tip were cut off. A radio-active sugar was absorbed into the excised plants from the roots in a test tube which contained 0.2 m*l* of sugar solution (50 μCi). After feeding a labelled compound at 20 C for 2 h, stem and haplocorm were ground in a chilled mortar, separately. To the filtrate through gauze were added successively zinc sulfate and barium hydroxide solutions to remove preteins as previously described[2]. The supernatant obtained by centrifugation at 8,000 *g* for 5 min was treated with Amberlite IR 4B and Dowex 50×8, respectively, followed by freeze drying. The powder was dissolved in 2 m*l* of water for the analysis of sugar.

Chromatography and measurement of radioactivity

  Descending paper chromatography was carried out using *n* propanol : ethyl acetate : water (7 : 1 : 2 V/V) or ethanol : ammonium acetate (pH 7.5, 1M) (7.5 : 3 V/V) as solvent. The sample applied was 0.2 m*l*.

The spots of sugars were developed with 0.2% naphtho resorcinol phosphate reagent[3]. Autoradiogram was prepared by contact of the above paper chromtogram with X-ray film for 3 weeks in the dark. One milliliter of the sample was chromatographed on Sephadex G 75 column (2.2 × 50 cm) using water as eluant at a flow rate of 20 ml/h. The eluate was collected in 4 ml franction. The sugar concentration in each fraction was determined by anthrone reagent[11].

The radioactivities of the spot on paper chromatogram and of the eluate by gel filtration were measured by liquid scintillation counting.

Enzyme preparation and reaction

One hundred grams of haplocorms were homogenized in 100 ml of Tris-HCl buffer (pH 7.2, 50 mM) containing 10 mM ascorbic acid at 4 C for 5 min using a mixer. Filtered through gauze, the homogenate was centrifuged at 10,000 × g at 0 C for 10 min. The supernatant was saturated up to 90% with ammonium sulfate and left for 1 h at room temperature, followed by centrifugation at 10,000 × g at 0 C for 20 min. The precipitate was dissolved in 10 ml of Tris-HCl buffer (pH 7.2, 5 mM) containing 1 mM ascrbic acid over night. The dialysate was used as enzyme preparation. The reaction mixture consisted of 1 ml of enzyme solution and 2 ml of phosphate buffer (1/15 M, pH 5 or 7) including substrates. After incubation at 30 C. 200 μl of the sample, from which proteins were previously removed, were spotted on a filter paper for chromatography. The radioactivity of the spot developed with the reagent was measured as described above.

Chemicals

U[14]C sucrose and U[14]C fructose were purchased from New England Nuclear Chemicals, and adenosine triphosphate (ATP), guanosine triphosphate (GTP) and uridine triphosphate (UTP) from Kyowa Hakko Industry Co. Ltd. Phlein was prepared from haplocorms of *P. pratense* as reported earlier[12]. Infrared absorption spectrum of our compound was the same as that of phlein reported by SUZUKI[19].

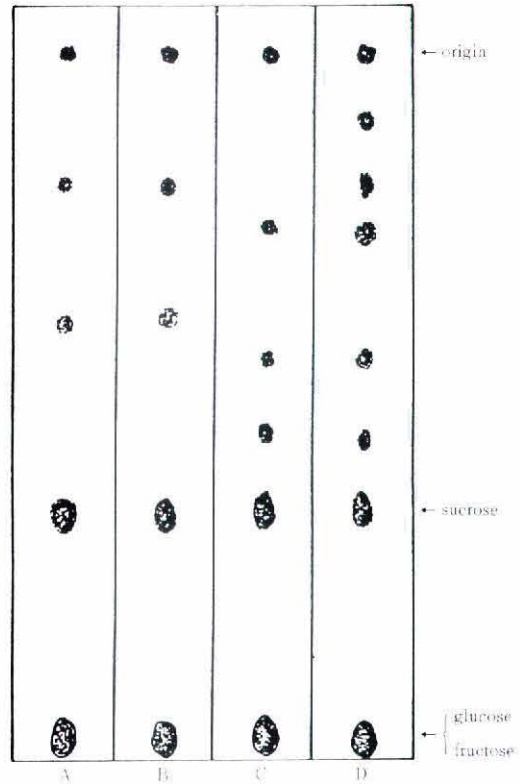## Results

In vivo synthesis of saccharides in stem and haplocorm

Fig. 1. Autoradiogram of sugars.
A : Stem fed with fructose— (14c). B : Haplocorm fed with fructose— (14c).
C : Stem fed with sucrose— (14c).
D : Haplocorm fed with sucrose— (14c).

The autoradiogram (Fig. 1) shows that the radioactivity of fructose or sucrose was incorporated into oligosaccharides and phlein in both stem and haplocorm. As shown in Table 1, sucrose was actively synthesized from fructose irrespective of the tissues tested. The synthesis of oligosaccharides from sucrose was almost the same in stem and haplocorm. The incorporation of radioactivity from sucrose into phlein of high polymerization degree was active in haplocorm, but not in stem.

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Table 1. Percentage of total radioactivity on
each paper chromatogram

Rsucrose value	Relative radioactivity (%)			
	A	B	C	D
0 (origin)	0.2	0.1	0.3	2.0
0.32	0.3	—	0.5	0.3
0.42	2.0	1.8	0.9	1.5
0.66	1.3	3.0	0.7	1.7
1.0 (sucrose)	65.7	30.1	83.6	81.5
1.6 (glucose/fructose)	30.5 100.0	65.0	14.0	13.0
Total %		100.0	100.0	100.0

All values are the mean of triplicate determinations.

A : Stem fed with fructose-(14C).

B : Haplocorm fed with fructose-(14C).

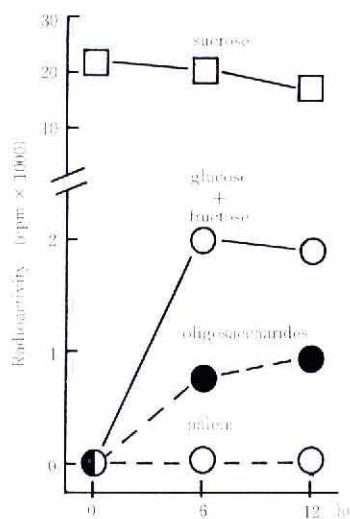C : Stem fed with sucrose-(14C).

D : Haplocorm fed with sucrose-(14C).

Fig. 3. Time course variation
of oligosaccharides.
The reaction mixture
consisted of 50 mg of
sucrose and 10 mg of
phlein, Rsucrose values of
oligosaccharides were 1.
9–2.4.

Fig. 2. Elution profiles of sugars (——) and
distribution of radioactivity (——·——).
A : Stem fed with fructose- (14C).
B : Haplocorm fed with fructose- (14C).
C : Stem fed with sucrose- (14C).
D : Haplocorm fed with sucrose- (14C)

haplocorm than in stem.

*In vitro synthesis of saccharides from sucrose or
fructose*

As shown in Fig. 3, some oligosaccharides were also
formed from sucrose by the enzyme reaction, but the
transfer of fructose residues into phlein was not
recognized even in the presence of the polymer.
Essentially the same results were obtained in the
presence of ATP. UTP or GTP (results are not
detailed here).

Discussion

The incorporation of label into sugars shows that

As shown in Fig. 2 the elution profiles and distribu-
tion of radioactive sugars supports again that the
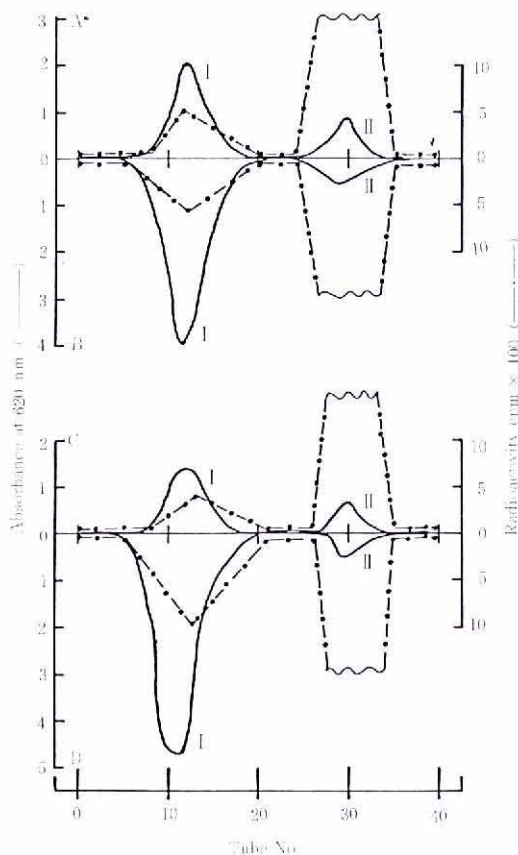polymer synthesis from sucrose was more active in

oligosaccharides are actively synthesized from fructose or sucrose in both stem and haplocorm, while active phlein synthesis occurs only in haplocorm fed with sucrose (Figs. 1 and 2). This suggests that fructose is not a direct precursor for the polymer synthesis, but indirectly involved through its conversion to sucrose.

POLLOCK[15] has recently proposed that phlein seems to be synthesized by the direct transfer of fructose residues from sucrose to the growing polymer chain on the basis of steady decline in sucrose and an equivalent rise in phlein without a significant accumulation of oligosaccharides in the leaf base tissue of *Dactylis glomerata* after exposure to $^{14}CO_2$. On the other hand, ROCHER[17] stated that 6F-fructosylsucrose plays an important role in phlein synthsis as an intermediate from the time variations in various saccharides in the leaf fragments of *Lolium italicum* fed with radioactive sucrose. In our experiments the higher radioactivity was found rather in oligosaccharides than in phlein irrespective of the tissues tested (Table 1). This suggests that the mechanism of phlein synthesis in higher plants is different from that of bacterial levan[3, 4, 9].

DEDONDER[5] reported that a trisaccharide of inulin type is formed through transfructosylation from sucrose by the enzyme from *Helianthus tuberosus*. Later EDELMAN and DICKERSON[7] demonstrated that the transfructosylase highly specific for terminal β-2, 1-linked fructosyl residues occurs in the plant and 1F fructosylsucrose is a key intermediate for the synthesis of long chain inulin. The formation of oligosaccharides by the enzyme was also observed in our system, but phlein synthesis was not recognized. This supports again that phlein synthesis may not be exerted by the direct transfer of fructose residues from sucrose. Production of oligosaccharides is assumed to be due simply to the transferring action of invertase as found in many plants. There is however a possibility that failure to detect radioactivity in phlein is attributed to the presence of phlein hydrolase by which the polymer once formed is degraded again to low molecular saccharides. It has been

proposed that uridine diphosphofructose in plant tissues may be involved in fructosan synthesis[10,13,20]. The present evidence showed that such a nucleotide is not involved in phlein synthesis in *P. pratense*. Considering all the results in this study, it would appear that the polymer synthesis from sucrose must be more complicated than a straight forward transfructosylation. The detailed mechanism should be furhter investigated using more purified enzyme preparation.

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チモシー茎部における β-2,6-フラクトサンの生合成について

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摘　　　　要

U¹¹C シュクロースと U¹¹C-フラクトースを用いて、チモシー(*Phleum pratense* L.)における β-2,6-フラクトサンの合成機構を調べた。茎及び球茎の双方において、フラクトースまたはシュクロースからの少糖類の活発な合成が認められたが、特に球茎においてはシュクロースからのフレイン合成も認められた。チモシー球茎から得た可溶性酵素は、シュクロースから少糖類を合成したが、フレインは合成しなかった。糖ヌクレオチドのフレイン合成への関与は認められなかった。