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Enzymology of Fructan Synthesis in Grasses

PROPERTIES OF SUCROSE-SUCROSE-FRUCTOSYLTRANSFERASE IN BARLEY LEAVES (*HORDEUM VULGARE* L. cv GERBEL)

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ABSTRACT

Fructan synthesis was induced in excised primary leaf blades of *Hordeum vulgare* L. cv Gerbel by illumination in 30 millimolar fructose. This treatment induced a 26-fold increase of sucrose-sucrose-fructosyltransferase (SST, EC 2.4.1.99) activity within 24 hours. Acid invertase (EC 3.2.1.26) activity remained about constant. By preparing protoplasts from induced leaves, approximately 80% of the invertase activity was removed with the cell walls while SST was retained. The protoplast homogenate was used to partially purify and characterize SST. Acid precipitation (pH 4.75) and anion exchange chromatography (fast protein liquid chromatography on Mono 'Q') resulted in a recovery of about 80% of total SST activity. The principal activity (SST 1), accounting for 85% of the activity recovered, was purified about 200-fold. It was essentially free of invertase activity and catalyzed the synthesis of a trisaccharide which co-chromatographed with isokestose (1F-β-fructosylsucrose). The remaining 15% of SST activity (SST 2) was purified about 35-fold. It retained substantial invertase activity and catalyzed the synthesis of only one trisaccharide which co-chromatographed with kestose (6F-β-fructosylsucrose). It is concluded that barley leaves which store mainly fructan of the phlein type (β-2-6 polyfructosylsucrose), nevertheless contain sucrose-sucrose 1F-β-D-fructosyltransferase as the key enzyme of fructan synthesis.

Numerous cereals and other grasses of the Poales adapted to temperate or cool climates contain fructan, a polyfructosylsucrose, as the main carbohydrate reserve in vegetative tissue (8, 12, 13). Enormous quantities of fructan may be accumulated in leaves (up to 75% of dwt[1] of barley leaves [18]), stems and roots depending on the stage of plant development and on environmental conditions (12, 13). Also inflorescences (4) and seeds of wheat (6) were found to contain fructan during certain stages of development. Undoubtedly, fructan plays a prominent role in photosynthate partitioning in grasses and consequently, in determining grain yields of cereals.

Knowledge about the enzymology of fructan metabolism in grasses is surprisingly scarce considering the eminent economic importance of these plants. None of the fructan biosynthetic enzymes has been purified and characterized so far in grasses and most of the current hypotheses about fructan synthesis have been deduced from findings made with other plants.

The enzymology was first elucidated in tubers of Jerusalem artichokes (*Helianthus tuberosus*) storing inulin, a linear β-2-1 linked polyfructosylsucrose (2). Inulin synthesis proceeds by the concerted action of two enzymes. SST (EC 2.4.1.99) catalyzes the exergonic, essentially irreversible reaction of fructosyltransfer from one molecule of sucrose to another forming the trisaccharide isokestose (1F-β-fructosylsucrose) and glucose. Thereafter, FFT (EC 2.4.1.100) is responsible for chain elongation by catalyzing an easily reversible fructosyl transfer from any fructan molecule of DP≥3 to another one of DP≥2.

All the fructosyl residues in the fructan chains of Jerusalem artichoke that are linked to the primary acceptor sucrose are therefore derived from the isokestose produced by SST. SST is therefore the key-enzyme by which photosynthates are diverted from sucrose into fructan.

The enzymology of fructan synthesis in grasses and other monocots might, however, not be so simple, since they contain fructans that are more heterogeneous in size and structure. Two polymer series of fructan differing in their interfructosyl linkages occur in Poales: phlein (or levan), a β-2–6-linked polyfructosylsucrose, predominates but members of the inulin series (β-2–1-linked polyfructosylsucrose) and branched fructans containing both series appear to occur also in the same plant species (7, 12, 16, 22).

In previous studies we found that SST activity changes rapidly and drastically in barley leaves upon induction or repression of fructan synthesis under various conditions such as cold treatment, excision and illumination, and supply of sugars, suggesting that SST also plays a key role in regulation of fructan metabolism in barley (18, 20–22). Since SST in grasses has not been characterized so far we made an attempt to purify and characterize it from barley leaves induced for fructan synthesis. In particular, SST had to be differentiated from abundantly occurring acid invertases (EC 3.2.1.26) also known to have fructosyltransferase activity (17). Probably because of this complication, the very existence of an SST distinct from invertase in grasses has not been demonstrated unequivocally up to now.

MATERIALS AND METHODS

Plants and Growth Conditions. Seeds of barley (*Hordeum vulgare* L. cv Gerbel) were soaked in slowly running tap water for 24 h and sown in pots of 12 cm diameter on commercially available soil (about 100 seeds per pot). The seedlings were grown in a growth cabinet with a 14 h, 25°C/10 h, 15°C day/night cycle. The RH was 70% and the photon flux density 500 to 600 μmol photons m^{-2} s^{-1}. The seedlings were watered daily with tap water and grown for 8 d.

Induction of SST Activity in Leaf Blades. Fructan synthesis was induced in 8 d-old blades of primary leaves by continuous illumination (500–600 μmol photons m^{-2} s^{-1}) of the excised blades for 24 h (20). During this treatment the lowest parts of the blades were immersed either in distilled water or in a solution of 30 mM fructose.

[1] Abbreviations: dwt, dry weight; SST, sucrose-sucrose-fructosyltransferase; FFT, fructan-fructan-fructosyltransferase; DP, degree of polymerization; fwt, fresh weight.

Isolation of Protoplasts. Mesophyll protoplasts from untreated primary leaf blades were isolated as described earlier (19). The method had to be slightly modified for the isolation of protoplasts from the induced leaves. Since the high fructan content increased the density of the protoplasts, the density of the solutions for isolation and purification of protoplasts had to be adapted. The leaf blades (1 g fwt) were stripped of their abaxial epidermis and floated in a Petri dish on the surface of 8 ml of the following solution: 0.6 M sorbitol, 20 mM citrate (NaOH)-buffer (pH 5.5) (= medium I), with 2% (w/v) cellulase R-10 'Onozuka' (Serva, Heidelberg, GFR), and 0.1% (w/v) Pectolyase Y-23 (Seishin Chem., Noda, Chiba, Japan). After incubation for 2 to 3 h at 25°C in the light, 8 ml of 'medium I' supplemented with 60% (v/v) Percoll (Pharmacia, Uppsala, Sweden) were added and the resulting protoplast suspension was passed through a 200 μm Nylon gauze into a centrifuge tube. Onto this suspension two layers of equal volume (10 ml) of medium I, the first supplemented with 25% (v/v) Percoll, were loaded. This density gradient was centrifuged at 350*g* for 5 min and the protoplasts were collected from the 25/0% Percoll interphase. The protoplast suspension was then diluted with 6 volumes of 0.6 M betaine (Fluka, Buchs, Switzerland), 20 mM citrate (NaOH)-buffer (pH 5.5) (final volume approximately 15 ml), and loaded onto a layer of 10 ml of medium I. The density gradient was centrifuged at 150*g* for 5 min after which the protoplasts were in a loosely packed sediment.

Extraction and Assays of SST, Invertase, and α-Mannosidase. Primary leaf blades (1 g fwt) were homogenized in a mortar with 1 ml of 50 mM citrate-phosphate-buffer (pH 5.7) (McIllvaine) at 0 to 4°C. The leaf homogenate was centrifuged at 2000*g* for 10 min and the supernatant was dialyzed against 5 mM McIllvaine-buffer (pH 5.7) for 16 h at 4°C. Alternatively, a protoplast lysate was prepared by dialysis of the suspension of protoplasts as above, inducing osmotic lysis of the protoplasts. The dialyzed leaf homogenate and protoplast lysate were centrifuged at 2000*g* for 5 min and the supernatants were used as leaf or protoplast extracts, respectively. All the sediments obtained during the preparation of the extracts were resuspended and checked for enzyme activities in order to estimate possible losses. Less than 10% of total SST and less than 15% of invertase activity were found in these fractions. The activities of SST, invertase and α-mannosidase were assayed as described before (18). One μmol of substrate (sucrose/*p*-nitrophenyl-α-mannoside) used per min at pH 5.7 and 30°C was referred to as 1 unit of SST, invertase and α-mannosidase activity.

Isolation of Saccharide Standards and Identification of the Saccharides Formed by SST. Isokestose was extracted from tubers of *Helianthus tuberosus* L., stored at 4°C for 3 months (14). Bulbs of *Allium cepa* were used as the source of neokestose and isokestose (14, 15). Kestose was produced by the action of yeast invertase (Boehringer, Mannheim, GFR) upon incubation with 0.2 M sucrose at pH 4.8 (17). The extraction and purification of the oligosaccharides by TLC was carried out according to the methods reported earlier (18). The TLC method described (silicagel foils developed three times with acetone/water, 87/13, v/v) was found to separate the three trisaccharides isokestose, kestose, and neokestose. In addition, HPLC and GLC were used to separate and identify the saccharides formed in the enzyme assays as described (3). Furthermore, the glucose:fructose ratio of the saccharides formed by SST was determined. The saccharides formed in the enzyme assays were separated from sucrose, glucose, and fructose by TLC and extracted from the chromatograms with water (18). An aliquot of the extract was used for the determination of total fructose (9). Another aliquot (100 μl) was hydrolyzed by adding 1 ml of 1 N HCl and incubating for 15 min at 100°C. Thereafter, 1 ml of 1 N NaOH was added and the glucose content was measured with the glucose-oxidase-method (Boehringer, Mannheim, GFR).

Enzyme Separation by Ion Exchange Chromatography. Protoplasts were isolated from leaf blades after illumination of the blades standing in 30 mM fructose as described above. The protoplast suspension was dialyzed against 2 mM methylpiperazine (HCl)-buffer (pH 4.75) for 16 h at 4°C. The precipitate formed from the lysed protoplasts was centrifuged at 2000*g* for 10 min (acid precipitation) and the supernatant was concentrated to one-fourth of its volume in a dialysis bag embedded in solid PEG 20,000 at 0°C. The sample (0.8 ml) was then incubated with 0.1 g Polyclar AT (Serva, Heidelberg, GFR) for 10 min at 20°C. After another centrifugation (5 min, 12000*g*) the supernatant (purified protoplast extract) was passed through a 0.22 μm membrane filter (GSWP, Millipore, Molsheim, France) and injected onto the ion exchange column ('Mono Q' HR 5/5, prepacked anion exchange column, equilibrated with 20 mM methylpiperazine [HCl]-buffer, pH 4.75) in a fast protein liquid chromatography system (Pharmacia, Uppsala, Sweden). The extract (500 μl containing 500 μg of protein) was applied to the column at a flow rate of 1 ml min^{-1} and the column was eluted with 12.5 ml of the equilibration buffer followed by 15 ml of a linear gradient of 0 to 0.25 M NaCl in the same buffer. Fractions of 1 ml were collected and assayed for SST and invertase activity as well as for protein content.

Protein Determination. Protein was determined by the method of Peterson (10). In the fractions obtained by ion exchange chromatography protein was measured according to Bradford (1). BSA was used as a standard.

RESULTS

Activities of SST and Invertase in Extracts of Leaf Blades and of Mesophyll Protoplasts. Primary leaf blades of barley seedlings grown for 8 d under optimal conditions contain virtually no fructan (18). However, if the leaf blades were excised and allowed to continue photosynthesis by illumination in water, SST activity was readily induced (Fig. 1). An even higher induction was

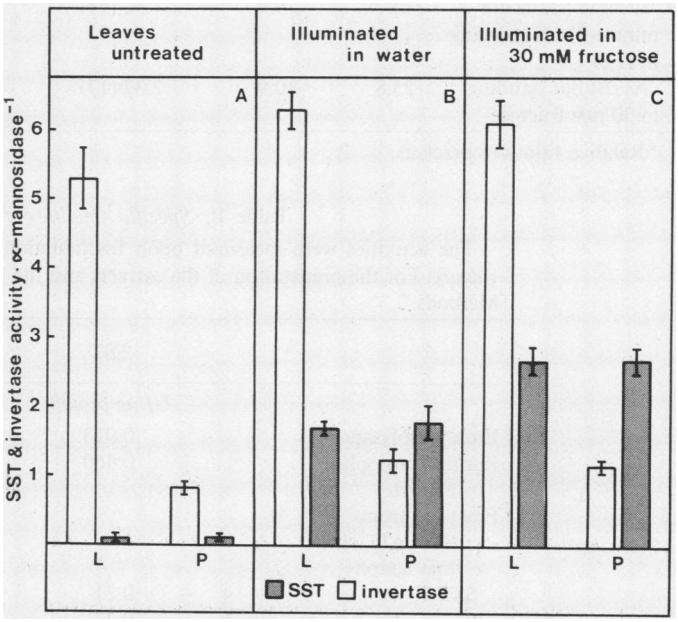

FIG. 1. Activities of SST (dark bars) and acid invertase (white bars) in leaf extracts (L) or protoplast extracts (P) from primary leaf blades of *H. vulgare*. SST activity was induced by illumination of excised leaves for 24 h, standing in water or 30 mM fructose, respectively. To compare leaves and protoplasts, the enzyme activities are expressed per α-mannosidase activity (see text). Standard errors are indicated.

obtained in 30 mM fructose than in water. The activity of acid invertase, in contrast, was less affected by these treatments. The bulk of the invertase activity appears to be localized in the cell walls since it was removed by preparing protoplasts (Fig. 1). SST and invertase activities are expressed per α-mannosidase activity, not per protein, in order to allow a comparison between the leaves and protoplasts. The α-mannosidase activity, localized exclusively within the protoplasts (18), was found to remain constant during induction of SST. Moreover, the total α-mannosidase activities measured in leaf homogenates and protoplast lysates were recovered in the leaf and protoplast extracts (*i.e.* after dialysis and centrifugation). This was not the case with protein. A considerable proportion of protein was lost in the precipitates formed during the preparation of the extracts, particularly in the case of the leaf extracts. Protein was therefore less suitable than α-mannosidase as basis to compare SST and invertase activities in leaves and protoplasts.

The specific activities of SST and invertase in the protoplast extracts (Table I) suggest that protoplasts prepared from SST-induced leaves are an ideal starting material for purification and characterization of SST because of reduced interference by invertase.

Enzyme Separation Procedure. The procedure used for preparing the purified protoplast extract for ion exchange chromatography (acid precipitation) resulted in a nearly 30-fold purification of SST compared with the SST in the protoplast lysate (Table II). About 93% of the protein precipitated during dialysis at pH 4.75 but more than 80% of the total SST activity was recovered in the supernatant.

Table I. *Specific Activities of SST and Acid Invertase in Protoplast Extracts from Primary Leaf Blades of H. vulgare*

The leaves were used directly (A) or after induction of fructan synthesis (B, C) as specified in "Materials and Methods."

Protoplast Extract of	SST	Invertase	SST/Invertase[a]
	mU/mg protein		*ratio*
A. Untreated leaves	1.34	9.6	0.14 (1)
B. Excised leaves illuminated 24 h standing in H_2O	14.7	9.7	1.52 (11)
C. As (B) but standing in 30 mM fructose	25.8	10.9	2.37 (17)

[a] Relative values in brackets.

Almost 80% of the protein loaded onto the 'Mono Q' column was eluted in the first 6 ml of washing buffer (Fig. 2). The remaining protein was eluted with the salt gradient with a recovery of 112%. Most of the SST activity (≥85%) bound to the column and was eluted at a salt concentration of about 120 mM (fractions 17–20). This main peak of SST activity, subsequently called SST 1, was well separated from the bulk of the invertase activity (≥85%) which passed through the column during the washing step (fractions 2–6). A minor amount of SST activity (≤15%), subsequently called SST 2, also passed through the column at the front (fractions 3–6). The degree of purification of SST 1 and SST 2, both on a protein and invertase basis, obtained by the chromatography, is shown in Table II. The recovery of the total SST activity loaded onto the column was 91%, the recovery of invertase activity was 96%.

Product Analysis of the SST 1 and SST 2 Catalyzed Reactions. The products formed upon incubation of SST 1 or SST 2 with sucrose were in both cases trisaccharides. The glucose:fructose ratios measured were 1:2.08 for SST 1 and 1:2.13 for SST 2, respectively.

To evaluate the structures of the trisaccharides formed by the SSTs, they were co-chromatographed with trisaccharide standards obtained from various plant sources ("Material and Methods"). TLC clearly shows a difference between the products of SST 1 and 2 (Fig. 3). The trisaccharide formed by SST 1 co-chromatographed with isokestose. Almost no fructose was formed. Therefore, SST 1 was virtually free of hydrolase activity. Invertase activity measured was less than 2% of SST activity (Table II, fraction 18). In this respect SST 1 resembles SST from tubers of Jerusalem artichokes (14). SST 2, on the other hand, produced exclusively a trisaccharide co-chromatographing with the kestose produced by yeast invertase. Because it forms kestose and shows both transferase and hydrolase activity in equal amounts (Table II, fraction 5), SST 2 resembles yeast invertase and may, therefore, in analogy to yeast, have no biosynthetic function *in vivo*. Obviously, since SST 2 is eluted at the front, it is poorly purified. A definitive answer to the question of its relative hydrolase and transferase activities, as well as a determination that it is not simply invertase or hydrolase, must await further purification.

Product analysis of SST 1 and SST 2 by HPLC or GLC as described by Frehner *et al.* (3) confirmed the data obtained from the TLC.

Table II. *Specific Activities of SST 1, SST 2, and Acid Invertase*

The activities were measured upon fractionation of a protoplast lysate from primary leaf blades of *H. vulgare*. For the preparation of the extracts and the fractionation procedure see Figure 2 and "Materials and Methods."

		SST	Purification Factor	Invertase	SST/invertase
		U/mg protein	*-fold*	*U/mg protein*	*ratio*
Protoplast lysate		0.013	1	0.006	2.15
Purified protoplast extract		0.360	28	0.150	2.36
Fractions from 'Mono Q' chromatography					
SST 2	4	0.21	16	0.27	0.78
	5	0.47	36	0.81	0.99
	6	0.45	35	0.64	0.71
SST 1	17	3.80	292	0.10	38.0
	18	2.60	200	0.05	55.0
	19	1.61	124	0.05	33.6
	20	2.91	224	0.21	13.9

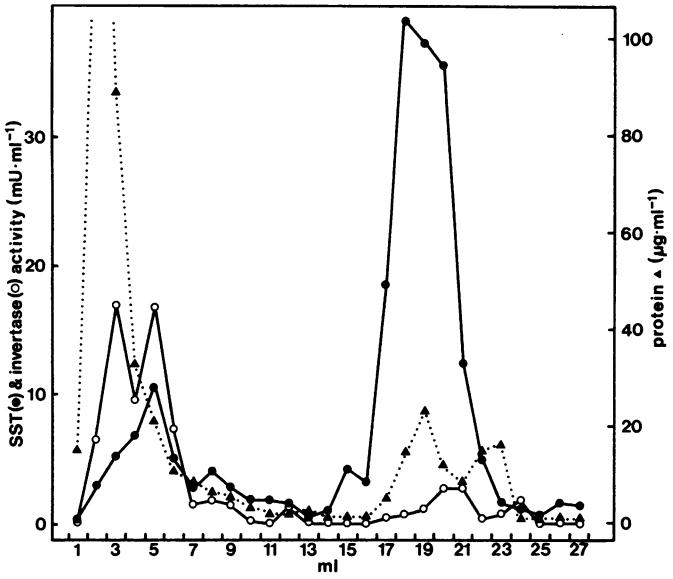

FIG. 2. Elution profile of SST (●), acid invertase (○), and protein (▲) from anion exchange chromatography (fast protein liquid chromatography on 'Mono Q'). A purified protoplast extract (see "Materials and Methods") from SST induced primary leaf blades of *H. vulgare* was loaded. The small peak of SST activity (fraction 3–6) is subsequently called SST 2, the main peak (fraction 17–20) SST 1.

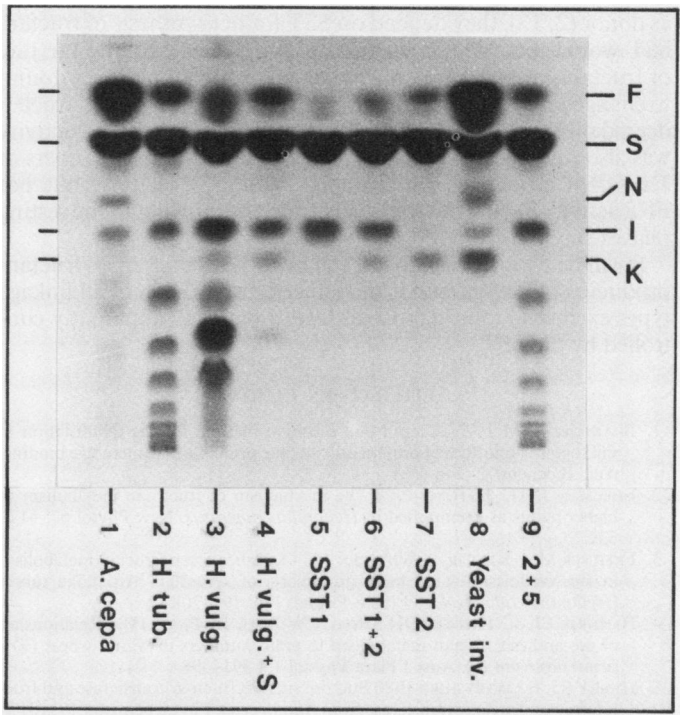

FIG. 3. TLC analysis of the saccharides formed from sucrose by the activity of SST 1 (line 5) and SST 2 (line 7) in comparison with saccharides from various sources (SST 1 and 2 explained in Fig. 2 and Table II): 1, extract of bulbs of *Allium cepa* containing the trisaccharides neokestose and isokestose; 2, extract of dormant tubers of *H. tuberosus* containing isokestose as sole trisaccharide; 3, extract of primary leaf blades of *H. vulgare* in which fructan accumulation had been induced; 4, saccharides formed in the enzyme assay of unfractionated barley leaf extract; 5, products of SST 1; 6, mixture of 5 and 7; 7, products of SST 2; 8, saccharides formed by yeast invertase from sucrose—kestose is the main trisaccharide; 9, mixture of 2 and 5. F, fructose; S, sucrose; N, neokestose; I, isokestose; K, kestose.

It was noticed that the cell wall lytic enzymes used for the preparation of protoplasts had significant SST activity. This activity was found to bind tightly to a 'Mono Q' column and eluted at much higher salt concentrations than the SST 1 of barley, namely into the fractions 27–30 (*cf.* Fig. 2). Since in these fractions no SST activity was detected upon fractionation of protoplast extracts it was concluded that the protoplasts were free of the SST activity contained in the cell wall lytic enzymes.

Interestingly, SST 1 and 2 clearly differ in their pH optima. SST 1 has a pH optimum at 5.7, as found earlier for total SST activity in cell-free extracts of leaves (18). In comparison, SST 2 has a pH optimum near 5.2, similar to the optimum of acid invertase activity (19).

DISCUSSION

In earlier work we tried to verify the existence of SST distinct from invertase in cell-free extracts of barley leaves (18–20). The two activities differed markedly with respect to several characteristics measured, such as pH optima, temperature dependency and stability, inhibitors, and the patterns of induction or repression (18–20). However, those results are not conclusive. The bulk of invertase of the leaves appears to be cell wall invertase since it is lost during preparation of protoplasts (Fig. 1). It has been shown that only a small part of total invertase activity is associated with the vacuoles, where SST activity is localized (18). Hence, only the characteristics of the vacuolar invertase activity and the SST activity should be compared.

In this work we have chosen to use a more direct approach, namely to separate SST from invertase. We took advantage of two procedures to increase SST activity over invertase activity in barley leaves. First, SST activity was strongly induced in excised leaf blades by a combination of illumination and sugar supply. Second, the bulk of invertase was eliminated by preparing protoplasts from the leaves. Thereafter, using a simple scheme for purification of SST from the protoplast lysate, the existence of an SST distinct from invertase in a grass was established.

The simple scheme of purification, involving only acid precipitation followed by ion exchange chromatography, gave a good yield of total SST activity (about 80%). This was essential to estimate the relative importance of the different SST activities detected. Interestingly, the principal SST activity (about 85%) showed no invertase activity and produced isokestose (1F-β-fructosylsucrose), thereby demonstrating activity similar to the SST from Jerusalem artichokes (2). A small part of SST activity (about 15%), separated from the principal SST but not from invertase, produced kestose (6F-β-fructosylsucrose).

Since barley appears to store mainly fructan of the phlein series (β-2–6-inter-fructosyl linkages) as the other grasses (7, 8), it is remarkable that the principal, if not the only real SST found, produced isokestose, the first member of the inulin series of fructan (β-2–1-inter-fructosyl linkages).

Careful analysis of the extracts of grasses as well as of other monocots containing various types of fructans revealed that isokestose is widely if not universally distributed in fructan-producing plants (11, 12, 16). Moreover, all the cell-free systems of plants containing various types of fructans studied in more detail up to now appear to catalyze the formation of isokestose from sucrose (5, 12, 15). This may indicate that the presence of an SST producing isokestose is a prerequisite for all fructan-producing plants and that this enzyme may therefore play a key role diverting photosynthates from sucrose into fructan metabolism (12, 20, 22). The differences in size and structure of fructans, characteristic for different plants, would then be the result of specific transferase activities of the FFT-type. Since these enzymes can use sucrose only as fructosyl acceptor and not

as donor (2, 13), they depend on SST for net synthesis of fructan. SST would then be the pacemaker enzyme controlling the rate of fructan synthesis. Indeed, the activity of SST has been found to change rapidly upon induction of fructan synthesis and fructan degradation in barley leaves (20). A rapid change of SST activity was also found in other plants (13), in particular in the tubers of Jerusalem artichokes which rapidly lose SST activity but not FFT activity during the transition from the growing to the resting phase (2).

It will be a future task to test the hypothesis that, in all fructan-producing plants, carbon is introduced into fructan of all linkage types exclusively through isokestose, a pathway apparently controlled by the SST.

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