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Fructan Synthesis in Excised Barley Leaves[1]

Identification of Two Sucrose-Sucrose Fructosyltransferases Induced by Light and Their Separation from Constitutive Invertases

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Excised leaves of barley (*Hordeum vulgare* L.) exposed to continuous light accumulate large amounts of soluble carbohydrates. Carbohydrates were analyzed in deionized extracts by high-pressure liquid chromatography on an anion exchange column coupled with pulsed amperometric detection. During the first few hours of illumination, the main sugar to accumulate was sucrose. The levels of glucose and fructans (oligofructosylsucroses) increased later. The trisaccharide 1-kestose (1-kestotriose) predominated initially among the fructans. Later, 6-kestose (6-kestotriose) and tetra- and pentasaccharides accumulated also. Total extracts from barley leaves were chromatographed on a MonoQ column, and each fraction was assayed for enzymes of interest by incubation with 200 mM sucrose for 3 h, followed by carbohydrate analysis. Freshly excised leaves yielded two peaks of invertase, characterized by formation of fructose and glucose, but had almost no trisaccharide-forming activities. In leaves exposed to continuous light, two new enzyme activities appeared that generated fructan-related trisaccharides and glucose from sucrose. One of them was a sucrose-sucrose fructosyl-1-transferase (1-SST), producing 1-kestose exclusively: the peak fractions of this activity contained almost no invertase. The other was a sucrose-sucrose fructosyl-6-transferase (6-SST), producing 6-kestose. It comigrated with one of the constitutive invertases on MonoQ but was separated from it by subsequent chromatography on alkyl Superose. Nevertheless, the preparation retained invertase activity, suggesting that this enzyme may act both as fructosidase and fructosyltransferase. When incubated with 1-kestose in addition to sucrose, this enzyme formed less 6-kestose but instead produced large amounts of the tetrasaccharide bifurcose (1&6-kestotetraose), the main fructan tetrasaccharide accumulating in vivo. These results suggest that two inducible enzymes, 1-SST and 6-SST, act in concert to initiate fructan accumulation in barley leaves.

Fructans (polyfructosyl sucroses) are important carbohydrate reserves in many plant species, including most Asterales (Edelman and Jefford, 1968; Meier and Reid, 1982) and many temperate grasses (Meier and Reid, 1982; Chatterton et al., 1989; Smouter and Simpson, 1989; Pollock and Cairns, 1991). The Asterales contain linear β-2,1-linked fructans called inulins (Edelman and Jefford, 1968; Meier and Reid, 1982). In contrast, the grasses possess fructans with a more complicated, branched structure containing a preponderance of β-2,6 linkages of the levan type in addition to the β-2,1 linkages (Meier and Reid, 1982; Carpita et al., 1989; Chatterton et al., 1990; Pollock and Cairns, 1991).

A general hypothesis for the biosynthesis of fructans was put forward by Edelman and Jefford (1968). On the basis of studies of inulin formation in *Helianthus tuberosus* L., they proposed that fructans are synthesized by two specific fructosyltransferases. The first, SST (EC 2.4.1.99), was suggested to use Suc with its relatively energy-rich glycosidic linkage as a fructosyl donor to form 1-kestose (1^F-fructosyl Suc, 1-kestotriose). The second, fructan-fructan fructosyltransferase (EC 2.4.1.100), was proposed to use 1-kestose or higher β-2,1-linked fructans as fructosyl donors and 1-kestose, or higher β-2,1-linked fructans as fructosyl acceptors to build gradually longer fructans. An SST forming 1-kestose has also been characterized in *Asparagus officinalis* and proposed to act as a key enzyme for fructan synthesis in this species, which contains fructans derived from neokestose (Shiomi and Izawa, 1980). Neokestose (6^G-fructosyl Suc) is thought to be formed here by a 6^G-fructosyltransferase using 1-kestose as fructosyl donor and Suc as acceptor (Shiomi, 1981). However, doubts have recently been cast on the evidence for this pathway (Cairns, 1992).

In the grasses, despite of the preponderance of β-2,6 linkages in their fructans, 1-kestose is generally present in addition to 6-kestose (6^F-fructosyl Suc, 6-kestotriose). Based on this fact and on detailed work with barley leaves (Wagner et al., 1983; Wagner and Wiemken, 1986; Wagner et al., 1986; Wagner and Wiemken, 1987; Obenland et al., 1991), our group previously suggested SST to be the key enzyme for fructan accumulation in the grasses also, and we reported the partial purification of two SST activities from barley protoplasts, one producing 1-kestose and the other 6-kestose (Wagner and Wiemken, 1987). However, these studies were subject to recent criticism (Cairns et al., 1989; Cairns and Ashton, 1991; Pollock and Cairns, 1991; Winters et al., 1992), which focused on two main points. First, as aptly summarized (Pollock and Cairns, 1991), invertases from various sources can catalyze fructosyl transfer to Suc, frequently yielding 6-kestose but also 1-kestose and neokestose under appropriate conditions. Hence, the apparent SST activities measured in

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Abbreviations: DP, degree of polymerization; SST, sucrose-sucrose fructosyltransferase; 6-SST, sucrose-sucrose 6-fructosyltransferase; 1-SST, sucrose 1-fructosyltransferase.

vitro might be due to transferase activities of invertases (Pollock and Cairns, 1991), and variations in the production of the fructan-related trisaccharides in vivo might be explained by modulation of the transferase activities of invertase by the Suc concentration, pH, and temperature (Cairns and Ashton, 1991; Pollock and Cairns, 1991). Second, in the purification of SST reported earlier (Wagner and Wiemken, 1987), barley protoplasts were taken as a source of the enzyme. This approach was taken to exclude interference by cell wall invertases. However, as demonstrated recently, commercial sources of the pectolyase used for protoplast preparation may contain large amounts of fungal SST activities, introducing new possibilities for artifacts (Cairns and Ashton, 1991; Winters et al., 1992).

To clarify the enzymology of fructan biosynthesis in barley, we reexamined the accumulation of fructans in excised leaves in vivo and compared it with the activities of Suc-metabolizing enzymes in vitro. The data presented here demonstrate that exposure of leaves to continuous light first induces accumulation of 1-kestose in vivo and, concomitantly, the appearance of an SST-forming 1-kestose in vitro. Later, the leaves start to accumulate 6-kestose and large amounts of the tetrasaccharide bifurcose in vivo. At the same time, the appearance of an SST forming 6-kestose is observed in vitro. When supplied with 1-kestose and Suc, this enzyme produces bifurcose in preference of 6-kestose in vitro. Both inducible SST activities can be separated from the constitutive invertases, which are almost completely devoid of SST activity in vitro. Our data strongly indicate that the constitutive invertases are unimportant for fructan biosynthesis in barley, contrary to recent suggestions (Cairns and Ashton, 1991; Pollock and Cairns, 1991), but that two specific inducible SST activities are of central importance. We suggest a new model of the biosynthesis of fructans in grasses.

MATERIALS AND METHODS

Plant Material and Incubation Conditions

Seeds of barley (*Hordeum vulgare* L. cv Express) were germinated on moist paper in a plastic box at 25°C for 24 h. The seedlings were sown in a mixture of two parts of vermiculite and one part of commercial soil (UFA Haus und Garten, Bern, Switzerland) and grown for 8 to 10 d in a growth chamber at a daylength of 14 h and a temperature regimen of 25°C (day) and 15°C (night). The RH was 70%, and the PPFD during the day was 300 μmol m^{-2} s^{-1}. To induce fructan synthesis, primary leaves were excised 4 h after the start of the light period, placed upright in a plastic tray filled with distilled water, and exposed to continuous white light with a PPFD of 300 μmol m^{-2} s^{-1} at 25°C for up to 24 h.

Extraction of Water-Soluble Carbohydrates

Samples of 0.5 g fresh weight of excised primary leaves were boiled twice under reflux for 5 min with 10 mL of 25% ethanol. The two extracts were combined and concentrated by rotary evaporation at 40°C. The samples were resuspended in 2 mL of water and deionized by successive passage through cation (Serdolit CS-2, H^+ form; Serva, Germany) and anion (Serdolit AS-6, formate form, Serva) exchange resins.

Analysis of Carbohydrates

Samples were centrifuged in an Eppendorf microcentrifuge at maximum speed for 5 min. Trehalose was added as an internal standard to a final concentration of 100 μg mL^{-1}. After appropriate dilution, 10 μL of the sample was chromatographed on a CarboPac PA-100 anion exchange column using a Dionex DX-300 gradient chromatography system coupled with pulsed amperometric detection. Before the sample was injected, the column was equilibrated in 100 mM NaOH for at least 10 min. Monosaccharides were eluted in 100 mM NaOH at a flow rate of 1 mL min^{-1} for 5 min. Subsequently, tri- and tetrasaccharides were separated by a sodium acetate gradient of 0 to 200 mM in 100 mM NaOH from 5 to 15 min at the same flow rate. Finally, higher fructans were eluted by washing the column with 600 mM sodium acetate in 100 mM NaOH from 15 to 20 min.

Fructans were identified by cochromatography with the following reference standards: 1-kestose (1-kestotriose), kindly provided by M. Lüscher, Zürich, Switzerland, and Dr. M. Hirayama, Meiji Seika, Kaisha, Japan; 6-kestose (6-kestotriose), bifurcose (1 and 6-kestotetraose), and three isomers of nystose (1,1-kestotetraose, 6,1-kestotetraose, and 6,6-kestotetraose), kindly given by Dr. P. Bancal, Clermont-Ferrand, France; and neokestose, prepared as described previously (Wagner and Wiemken, 1987) from an extract of onion (*Allium cepa*) bulbs by semipreparative TLC on silica gel G1500 glass plates (Schleicher & Schuell, Feldbach, Switzerland), using a Mobil-Rf (Scilab, Therwil, Switzerland) for sequence chromatography (Buncak, 1984). The TLC plates were developed three times with acetone:water (84:16, v/v). Half of each plate was sprayed with anthrone (0.3 g of anthrone dissolved in 10 mL of acetic acid, 20 mL of ethanol, 3 mL of phosphoric acid, and 1 mL of H_2O), and neokestose, and other sugars were made visible by heating at 110°C for 3 min. Neokestose from the nonstained half was scraped off and used as a standard. Standard solutions of Fru, Glc, Suc, and 1-kestose were used for calibration. For quantification of 6-kestose and bifurcose, standards were hydrolyzed in 1 N HCl for 15 min at 100°C. After neutralization with 1 N NaOH, Glc and Fru concentrations were determined by anion exchange HPLC. The Fru to Glc ratios were 1.8 for 6-kestose and 2.8 for bifurcose. Nystose concentration was estimated using the same factor of peak area to weight as for 1-kestose.

Preparation of Enzyme Extracts

Leaf blades were ground at a ratio of 2 mL/g fresh weight in 25 mM methylpiperazine (HCl) buffer (pH 5.25) containing 1 mM DTT, 1 mM benzamidine, 1 mM EDTA, 0.1 mM PMSF, and 0.5% insoluble PVP (Polyclar AT; Serva, Heidelberg, FRG). The homogenate was centrifuged at 100,000*g* for 30 min, and proteins in the supernatant were precipitated by adding ammonium sulfate to 70% saturation and stirring on ice for at least 1 h. The precipitate was collected by centrifugation at 20,000*g* for 15 min and resuspended in 1 mL of 25 mM methylpiperazine (HCl) buffer (pH 5.75). Aliquots of 500

μL were desalted by passing them three times through Bio-Gel P-10 columns (300 × 8 mm) equilibrated with the same buffer, using centrifugation at 350*g* for 5 min. All procedures were conducted at 0 to 4°C.

Chromatography and Analysis of Enzyme Extracts on MonoQ and Alkyl Superose

Desalted enzyme extracts were centrifuged at 25,000*g* for 5 min and injected at a flow rate of 0.5 mL min^{-1} onto a MonoQ HR 5/5 anion exchange column (Pharmacia, Uppsala, Sweden) equilibrated with 25 mM methylpiperazine (HCl) buffer (pH 5.75) in a fast protein liquid chromatography system (Pharmacia). The column was then washed with 3 bed volumes of the same buffer and eluted with a linear gradient of 0 to 200 mM NaCl in a total volume of 40 mL. The flow rate during washing and eluting was 1 mL min^{-1}. Fractions of 0.5 mL were collected. For enzyme assays, aliquots of 25 μL were incubated in a total volume of 50 μL in 200 mM Suc at 27°C for 3 h. The reaction was stopped by boiling for 3 min. After centrifugation and addition of the internal standard, samples were used directly for carbohydrate analysis, as described above.

For further separation by hydrophobic interaction chromatography, fractions 49 to 51 from the MonoQ column were combined, supplemented with ammonium sulfate (2 M final concentration), and injected at a flow rate of 0.5 mL min^{-1} on an alkyl Superose HR 5/5 column (Pharmacia) equilibrated with 50 mM citrate (Na^+) buffer, pH 5.0, containing 2 M ammonium sulfate. The column was washed with the same buffer (3 bed volumes) and eluted with a linear gradient of ammonium sulfate decreasing from 2 to 0 M in 30 mL. Fractions of 0.5 mL were collected, and the enzyme activities were assayed as described above for the fractions from the MonoQ column.

RESULTS

Fructan Synthesis in Vivo

The composition of soluble carbohydrates in excised barley leaves was studied by anion exchange HPLC coupled with pulsed amperometric detection, using an elution gradient well suited to separate Glc, Fru, Suc, and the fructan oligosaccharides of DP 3 and 4 (Fig. 1). Untreated leaves excised 4 h after the start of the light period contained relatively small amounts of Glc, Fru, and Suc. No fructans were present, except for traces of 1-kestose (1-kestotriose) at a retention time of 12.1 min (Fig. 1A). Extracts from leaves exposed to continuous light for 12 h contained much more Suc and Glc and a considerable amount of the trisaccharide 1-kestose; the trisaccharide 6-kestose (6-kestotriose; retention time, 12.7 min) and the tetrasaccharide bifurcose (1 and 6-kestotetraose; retention time, 13.4 min) were present at low levels (Fig. 1B). The identity of bifurcose in the extracts was confirmed by Dr. P. Bancal, using cochromatography with an authentic standard on a C_{18} reversed-phase column as described previously (Bancal et al., 1991). After 24 h in the light (Fig. 1C), leaves accumulated large amounts of the latter oligosaccharides and, in addition, the tetrasaccharide 1,1-nystose (1,1-kestotetraose; retention time, 13.1 min) and one major unidentified compound at a retention time of 14.4 min, probably

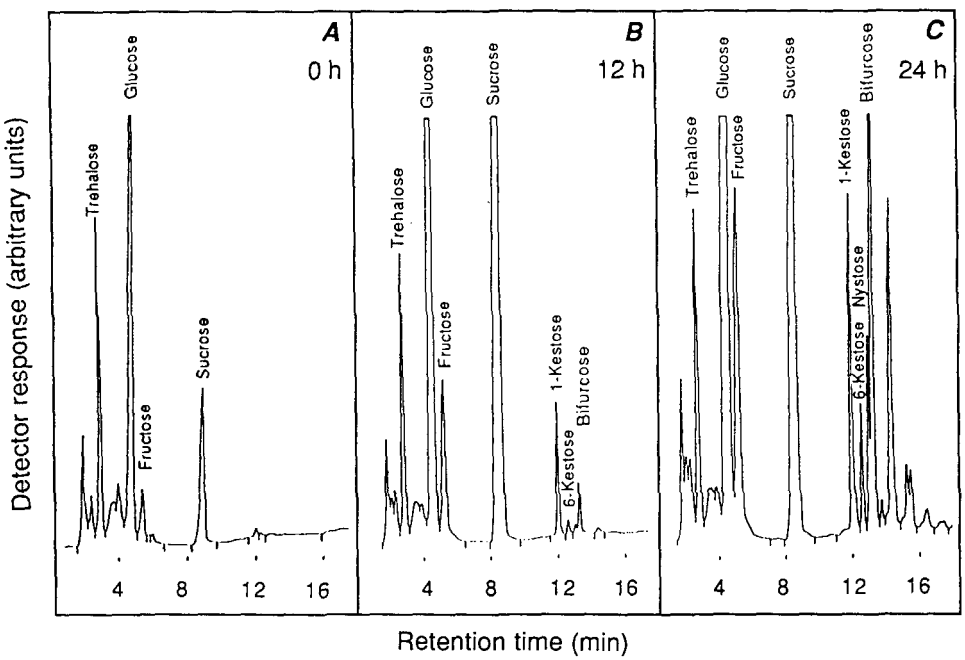

Figure 1. Anion exchange HPLC analysis of neutral carbohydrates in extracts from excised barley leaves obtained after different times of exposure to continuous light. Chromatograms on a CarboPac PA-100 column were recorded by pulsed amperometric detection for extracts obtained after 0 h (A), 12 h (B), or 24 h (C) of exposure to light. Carbohydrates were identified by their retention times. Trehalose was used as an internal standard.

a pentasaccharide of the phlein or branched type (see "Discussion"). There were no major peaks eluting at later retention times, between 15 and 20 min, when fructans with DP > 5 would be expected (Fig. 1C and data not shown). Only trace amounts of oligosaccharides were present at the positions of 6,1-nystose (6,1-kestotetraose) and 6,6-nystose (6,6-kestotetraose) with retention times of 13.7 and 14.0 min, respectively, and neokestose (retention time, 12.9 min) and raffinose (retention time, 11.5 min) were absent (Fig. 1C).

The time course of sugar accumulation in the leaves was studied using carbohydrate analysis (Fig. 2). Suc was the first neutral sugar to be accumulated. After 12 h Suc reached a stable high concentration. Glc increased linearly from 4 to 24 h. Fru also increased, mainly at the beginning of illumination, but remained more or less constant at a comparatively low level afterward. The first oligosaccharide of DP > 2 to be accumulated was 1-kestose. After a lag phase of 8 h, 6-kestose and bifurcose, identified by cochromatography with the respective oligosaccharides, appeared simultaneously. Both accumulated steadily between 12 and 24 h of illumination, but bifurcose reached much higher levels than 6-kestose. After 12 h, 1,1-nystose and an unidentified compound, probably a pentasaccharide, accumulated in considerable amounts.

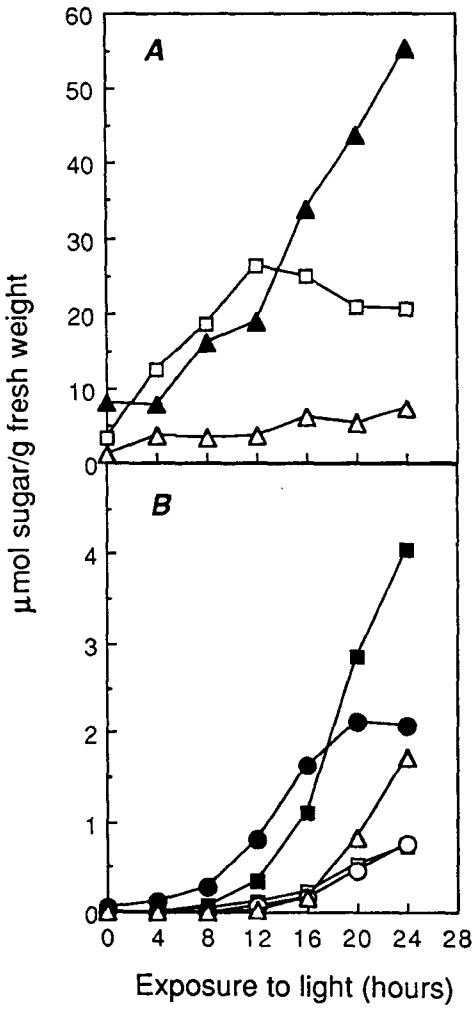

Figure 2. Accumulation of soluble carbohydrates in excised leaves of barley under continuous illumination. In A, Glc (▲), Fru (△), and Suc (□) and, in B, 1-kestose (1-kestotriose, ●), 6-kestose (6-kestotriose, □), bifurcose (1 and 6-kestotetraose, ■), 1,1-nystose (1,1-kestotetraose, ○), and an unidentified compound probably representing a pentasaccharide (kestopentaose, △) were separated and quantified by anion exchange HPLC (see Fig. 1).

Separation and Identification of Invertase and Fructosyltransferase Activities

Extracts from barley leaves were concentrated by ammonium sulfate, dialyzed, and subjected to anion exchange chromatography on a MonoQ column (Fig. 4). Aliquots of the fractions were assayed for enzymes of interest by incubation with 200 mM Suc for 3 h, followed by analysis of the carbohydrates by anion exchange HPLC. Typical chromatograms of the enzyme assays are presented in Figure 3. Invertase was detected by the formation of Fru (Fig. 3A). 6-SST was identified by the formation of 6-kestose; fractions with 6-SST activity typically contained invertase activity also (Fig. 3B). 1-SST was detected by the formation of 1-kestose (Fig. 3C).

Chromatography of total extracts from freshly excised leaves on a MonoQ column yielded two peaks of invertase (Fig. 4A). A minor peak (invertase I) eluted at 80 mM NaCl, and a major peak (invertase II) eluted at 150 mM NaCl. None of the fractions formed any trisaccharide in the enzyme assay. The same two peaks of invertase were present in extracts from the leaves exposed to light for 24 h (Fig. 4B). The activity in the major peak was similar to that in untreated leaves, whereas the activity in the minor peak was clearly increased. In addition, two separate newly induced SST activities were present (Fig. 4B). The 1-SST activity eluted at 110 mM NaCl and contained very little invertase activity. The 6-SST activity comigrated with the minor invertase and eluted at 80 mM NaCl. An analysis of the products formed by fractions 50 and 63, containing the peaks of 1-SST and 6-SST, respectively, demonstrated that the two enzyme activities were well separated and that 1-SST was almost free of invertase, whereas invertase activity was 2.5 times higher than 6-SST activity in the peak fraction of 6-SST (Table I).

It was possible to separate 6-SST activity from a large part of the invertase activity using alkyl Superose (Fig. 5). The 6-SST activity eluted first and contained some residual invertase activity. The main part of invertase activity eluted later, at the same position as minor invertase (invertase I) from uninduced leaves (data not shown), and was free of trisaccharide-forming activities (Fig. 5).

Induction of Invertase and Transferase Activities

Anion exchange chromatography on MonoQ allowed separation of the major constitutive invertase activity (invertase II) from the remaining invertase and fructosyltransferase activities (Fig. 4). To study induction of SST activities more closely, extracts from leaves exposed to continuous light for 0 to 24 h were chromatographed on MonoQ, and fractions 43 to 65 (containing all enzymes of interest except for inver-

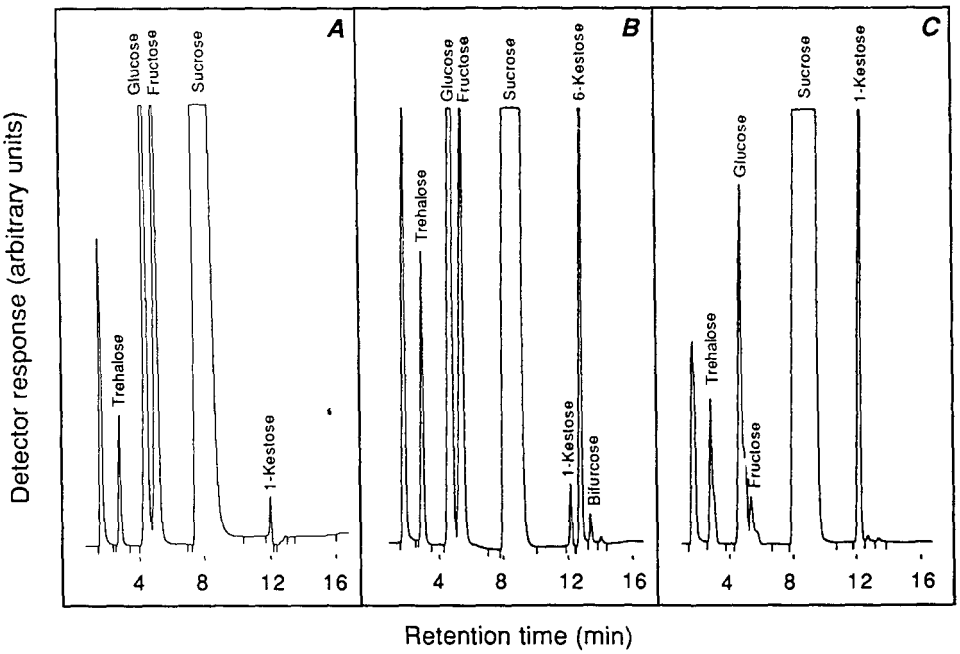

Figure 3. Anion exchange HPLC analysis of carbohydrates present after incubation of different enzyme preparations with 200 mM Suc. A, Fraction 80 containing invertase II. B, Fraction 50 containing 6-SST and invertase. C, Fraction 63 containing 1-SST. Enzyme preparations were obtained from extracts of leaves exposed to continuous light for 24 h by MonoQ chromatography (see Fig. 4) and were incubated in 200 mM Suc at 27°C. Incubation times were 3 h (fractions 50 and 63) and 30 min (fraction 80).

tase II, see Fig. 4) were pooled and analyzed (Fig. 6). The two SST activities were very low in freshly excised leaves; the sensitivity of the detector had to be increased 10-fold to quantify 1-kestose and 6-kestose in the enzyme assays. Activities of 1-SST and 6-SST were induced at least 30- and 100-fold, respectively, during 24 h of exposure to light (Fig. 6). As already shown in Figure 4, activity of invertase I also increased about 10-fold. The enzyme preparations from leaves exposed to light for more than 12 h also produced bifurcose, the tetrasaccharide accumulating in vivo.

Characterization of Partially Purified 1-SST, 6-SST, and Invertases

Extracts from leaves exposed to continuous light for 24 h were chromatographed on MonoQ, and the activities of fractions 50, 63, and 80, containing peaks of 6-SST/invertase I, 1-SST, and invertase II, respectively, were characterized.

Dependence on Substrate Concentrations

Aliquots of each fraction were incubated with different Suc concentrations (Fig. 7). In fraction 80, invertase II, the major invertase of barley leaves, displayed a K_m of about 5 mM Suc and was fully saturated at 25 mM Suc; this fraction formed practically no 1-kestose or 6-kestose at any Suc concentration (Fig. 7A). In fraction 50, invertase (representing invertase I, the minor constitutive invertase, as well as the invertase comigrating with 6-SST on alkyl Sepharose) had a K_m of about 50 mM, whereas 6-SST activity increased linearly up to at least 600 mM Suc (Fig. 7B). This fraction also formed low amounts of 1-kestose, probably because it was contaminated by some 1-SST. In fraction 63, 1-SST activity displayed a K_m of approximately 200 mM Suc (Fig. 7C). This fraction showed no 6-kestose formation at any concentration of Suc and contained only a trace of invertase activity that was saturated at 25 mM Suc, probably a contamination of invertase II.

Temperature Dependence

The temperature optimum of invertase II was at 45°C, and the temperature optima of 1-SST, 6-SST, and invertase I were at about 30°C (Fig. 8). The invertase and 6-SST activities present in fraction 50 differed with respect to their activities at 50°C: invertase was still half-maximally active, whereas 6-SST was fully inactivated (Fig. 8).

Effect of Pyridoxal

The effects of pyridoxal were also examined (Wagner and Wiemken, 1986). Pyridoxal (10 mM) inhibited invertase II by 75%; 1-SST in fraction 63 was not affected by this compound (Table II). The preparation of 6-SST, purified by alkyl Superose chromatography after the MonoQ column, still contained both 6-SST and invertase activities (see Fig. 5). Both of these activities were little affected by pyridoxal (Table II). On the other hand, invertase I, separated from 6-SST on the alkyl Superose column (see Fig. 5), was inhibited more than 50% by pyridoxal (Table II).

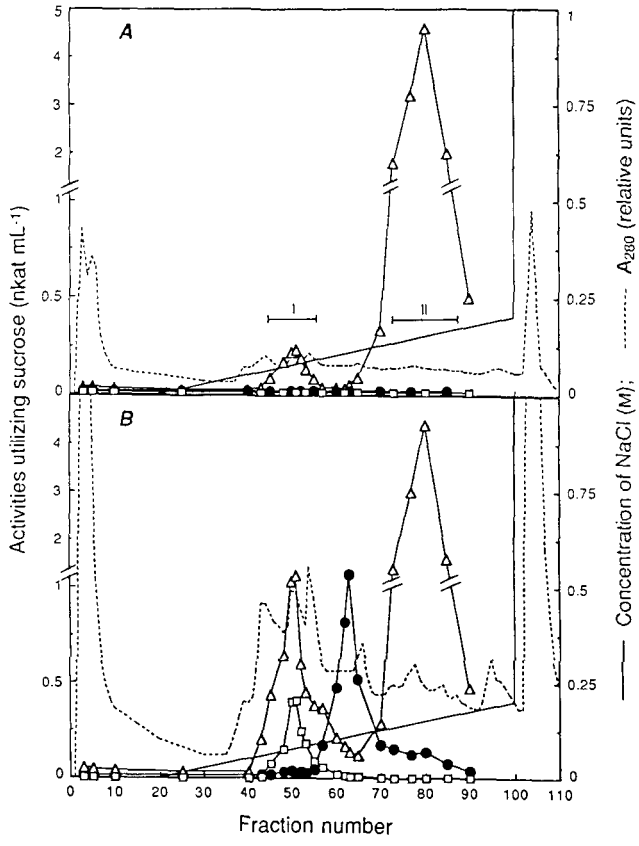

Figure 4. *Separation of SSTs and invertases by anion exchange chromatography on MonoQ.* Extracts (concentrated by ammonium sulfate precipitation) of freshly excised leaves (A) or excised leaves exposed to continuous light for 24 h (B) were eluted with a linear gradient from 0 to 0.2 M NaCl in 40 mL. Aliquots of the fractions were incubated in 200 mM Suc at 27°C for 3 h and subjected to carbohydrate analysis. Fru (△), 1-kestose (●), and 6-kestose (□) formed in the assays were separated and quantified by anion exchange HPLC. The A_{280} was continuously registered.

Table I. *Products formed from Suc by different enzyme preparations*

Activity	Enzyme Preparation[a]	
	1-SST	6-SST
	nkat mL⁻¹	
Product formed[b]		
Glu	1.16	1.44
Fru	0.13	1.03
1-Kestose	1.07	0.04
6-Kestose	0.01	0.40
	factor	
Ratio of activities		
Fructosyltransferase/invertase	8	0.4
1-SST/6-SST	100	0.01
6-SST/1-SST	0.1	10

[a] The enzyme preparations used were fraction 50 (6-SST) and fraction 63 (1-SST) of an extract from leaves exposed to continuous light for 24 h, chromatographed on MonoQ (Fig. 4B). [b] Products formed with 200 mM Suc in vitro at pH 5.7 and 27°C during 3 h of incubation.

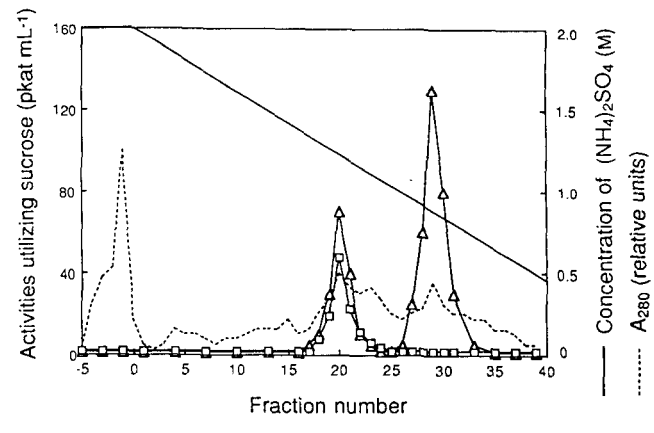

Figure 5. Separation of invertase and 6-SST by hydrophobic interaction chromatography on alkyl Superose. MonoQ column fractions 49 to 51 from leaves exposed to continuous light for 24 h (see Fig. 4B) were pooled and loaded on the column. Symbols are the same as in Figure 4.

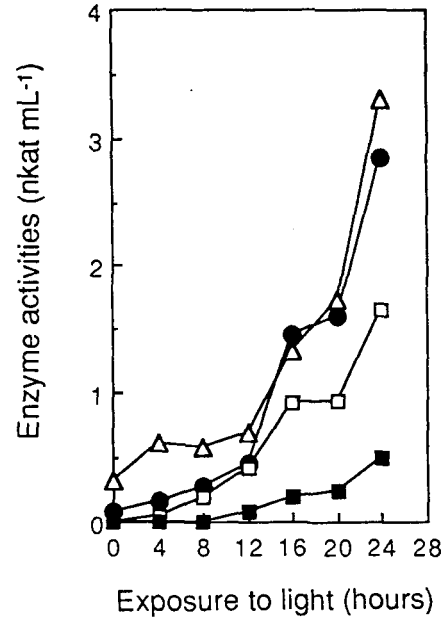

Figure 6. Induction of enzyme activities in excised leaf blades of barley during exposure to continuous light. MonoQ fractions 43 to 65 were pooled (see Fig. 4) and incubated in 200 mM Suc for 3 h at 27°C. Products formed in the assays were separated and quantified by anion exchange HPLC. △, Invertase (Fru-forming activity). ●, 1-SST (1-kestose-forming activity). □, 6-SST (6-kestose-forming activity). ■, Activity producing bifurcose.

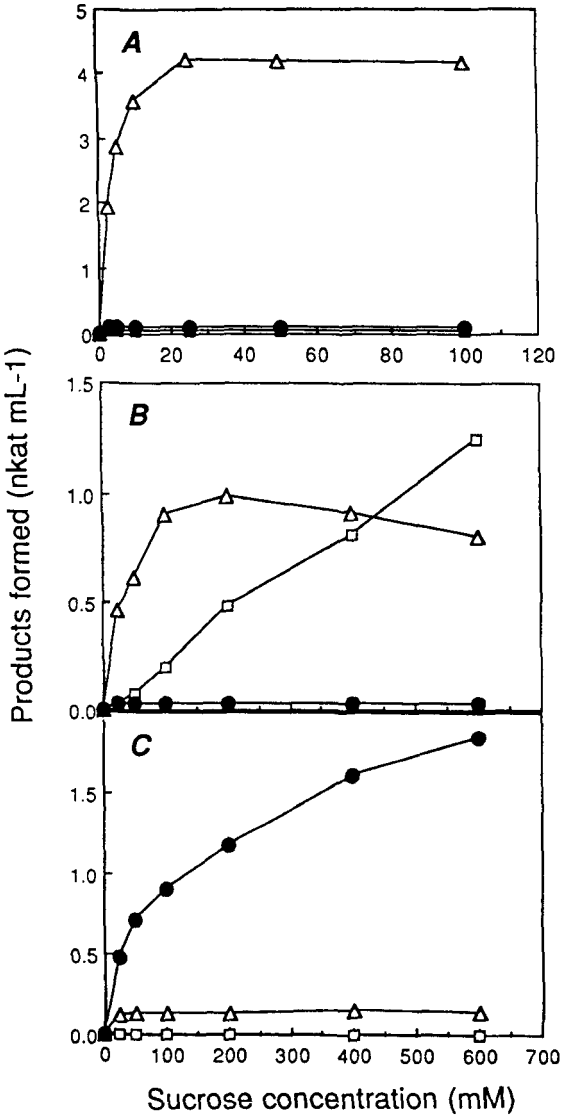

Figure 7. Dependence of the enzyme activities from leaves exposed to light for 24 h on Suc concentration. The leaf extracts were fractionated by Mono Q chromatography (see Fig. 4). A, Fraction 80 containing invertase II. B, Fraction 50 containing 6-SST and invertase. C, Fraction 63 containing 1-SST. The enzyme assays were incubated at 27°C for 12 min (fraction 80) or 3 h (fractions 50 and 63). Products formed in the assays were separated and quantified by anion exchange HPLC. Symbols are the same as in Figure 4.

Bifurcose Formation by Partially Purified 6-SST

The products formed by partially purified 6-SST from induced leaves (fraction 50 from the MonoQ column) incubated simultaneously in 200 mM Suc and different amounts of 1-kestose were analyzed. As shown (Fig. 4B), this enzyme formed mainly Fru, Glc, and 6-kestose and, in addition, traces of 1-kestose and bifurcose when incubated with Suc alone. When increasing amounts of 1-kestose were included in the enzyme assay in addition to 200 mM Suc, bifurcose was

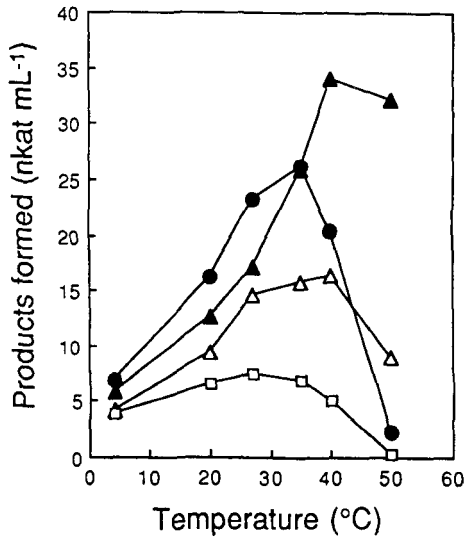

Figure 8. Temperature dependence of the enzyme activities from leaves exposed to continuous light for 24 hr. The leaf extracts were fractionated by Mono Q chromatography. ▲, Fru-forming activity of fraction 80 containing invertase II. ●, 1-Kestose-forming activity of fraction 63 containing 1-SST. □, 6-Kestose-forming activity and △, Fru-forming activity of fraction 50 containing 6-SST and invertase. The enzymes were incubated for 30 min (fraction 80) or 3 h (fractions 50 and 63). Products formed in the assays were separated and quantified by anion exchange HPLC.

produced in increasing amounts, and the release of Glc increased in parallel; at the same time, formation of 6-kestose decreased (Fig. 9). When the enzyme was incubated with 1-kestose in the absence of Suc, neither 6-kestose nor bifurcose was formed (data not shown).

DISCUSSION

Separation and Quantification of Carbohydrates by HPLC

Anion exchange chromatography coupled with pulsed amperometric detection of oligosaccharides has been used to

Table II. *Effect of pyridoxal (10 mM) on the enzyme activities in different fractions*

Fraction	Enzyme	Activity
		% of untreated control
63[a]	1-SST	103
80[a]	Invertase II	25
20[b]	6-SST	93
20[b]	Invertase	87
29[b]	Invertase I	44

[a] Fractions of an enzyme extract from leaves exposed to continuous light for 24 h, chromatographed on MonoQ (Fig. 4B). [b] Fractions of an enzyme preparation (MonoQ pool 49–51) from leaves exposed to continuous light for 24 h, chromatographed on alkyl Superose (Fig. 5).

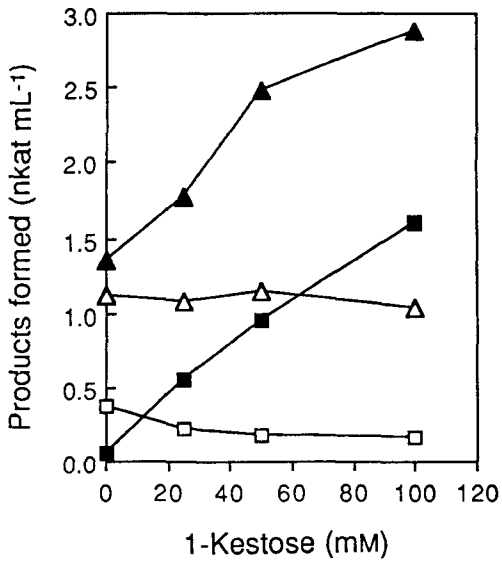

Figure 9. Reaction products formed by a preparation of 6-SST from Suc and 1-kestose. Fraction 50 (obtained by MonoQ chromatography of extracts from leaves exposed to continuous light for 24 h) was incubated in 200 mM Suc and different amounts of 1-kestose at 27°C for 3 h. Fru (△), Glc (▲), 6-kestose (□), and bifurcose (■) formed in the assays were separated and quantified by anion exchange HPLC.

separate and analyze various series of oligosaccharides in grasses (Chatterton et al., 1990). To investigate fructan metabolism and enzymology, it was important to separate not only oligosaccharides but also monosaccharides. A program combining isocratic elution followed by a sodium acetate gradient served this purpose. The main oligosaccharides identified in excised barley leaves exposed to light for 24 h were the trisaccharides 1-kestose and 6-kestose and the tetrasaccharides 1,1-nystose and bifurcose.

Fructan Synthesis in Excised Leaves

The time course of carbohydrate accumulation in the light was similar to that found in our previous studies (Wagner et al., 1983; Wagner et al., 1986): Suc accumulated first, followed by trisaccharides and subsequently by fructans of DP > 3 (Fig. 2). As found earlier (Wagner and Wiemken, 1987), 1-kestose and to a lesser degree 6-kestose were the only trisaccharides accumulated. The most abundant tetrasaccharide was identified as bifurcose; 1,1-nystose was also present. In addition, an additional oligosaccharide was found that migrated slightly more slowly than the pentasaccharide of the inulin type. Because oligosaccharides of the inulin type eluted earlier on this column than comparable oligosaccharides of the phlein or branched type, it is probably a pentasaccharide of one of the latter groups. Our results are in contrast to those of a preliminary report by Smeekens et al. (1991), who found 6-kestose to be the only trisaccharide accumulated in excised barley leaves, although a constitutive high 1-kestose-forming activity was present in vitro. We cannot explain this discrepancy at present.

Enzymology of Fructan Synthesis

In recent analyses, doubts were expressed concerning the existence of specific SST activities in monocots (Cairns et al., 1989; Cairns and Ashton, 1991; Pollock and Cairns, 1991; Cairns, 1992; Winters et al., 1992. There were three main concerns. First, the long-term dialysis and lengthy purification protocols used in the analysis of *Asparagus* SST (Shiomi, 1980; Shiomi, 1981) may lead to problems with bacterial contamination (Cairns, 1992). Second, some of the SST activities reported may actually be side reactions of invertases; invertases from various sources, including the well-characterized yeast invertase, can catalyze fructosyl transfer to Suc, yielding various trisaccharides in addition to their main products, Fru and Glc (Straathof et al., 1986; Wagner and Wiemken, 1987; Cairns et al., 1989; Cairns and Ashton, 1991; Pollock and Cairns, 1991). Third, the SST partially purified and characterized from barley protoplasts (Wagner and Wiemken, 1987) may have been contaminated by SST from the fungal enzymes used to prepare protoplasts (Cairns and Ashton, 1991; Winters et al., 1992).

In the present study, we avoided lengthy steps in purification, using size exclusion chromatography instead of dialysis for desalting, and we dispensed with preparation of protoplasts. We also developed a novel assay technique for the enzymes of interest, separating and quantitating all products generated from Suc by anion exchange HPLC and thereby allowing simultaneous detection of invertase and SST activities in each assay. Chromatography of the total protein extracts from barley leaves on a MonoQ anion exchange column (Fig. 4) revealed the presence of several different enzymes that use Suc as a substrate.

Freshly excised leaves do not accumulate fructans in vivo. These leaves are almost completely devoid of SST activity, but they contain high constitutive invertase activities. These invertases have practically no fructosyltransferase activities when incubated with 200 mM Suc. In contrast, excised leaves exposed to continuous light for 24 h have two new, additional enzyme activities. One can be defined as specific 1-SST, because it forms practically only 1-kestose and Glc from Suc. This enzyme is in all likelihood the same as the one previously characterized from barley protoplasts (Wagner and Wiemken, 1987), and it probably corresponds to the SST activities found in *Lolium* (Housley and Pollock, 1985; Cairns and Pollock, 1988) and in *Agropyron* (Chatterton et al., 1988). The other is a 6-SST that produces 6-kestose as the only trisaccharide but has some invertase activity. A large part of this invertase activity, corresponding to an invertase also present constitutively in uninduced leaves, can be separated from 6-SST by further chromatography on alkyl Superose. However, some invertase remains associated with 6-SST on this column also. This invertase activity, like 6-SST itself, was insensitive to pyridoxal, in contrast to the other invertases, indicating that the invertase activity may be a genuine side reaction of the newly induced 6-SST, as proposed in earlier studies (Bancal and Gaudillère, 1989). It is clear, however, that the newly induced 6-SST is different from the invertases present in uninduced leaves, which are devoid of 6-SST activities. The appearance of the new enzyme activities in vitro correlates

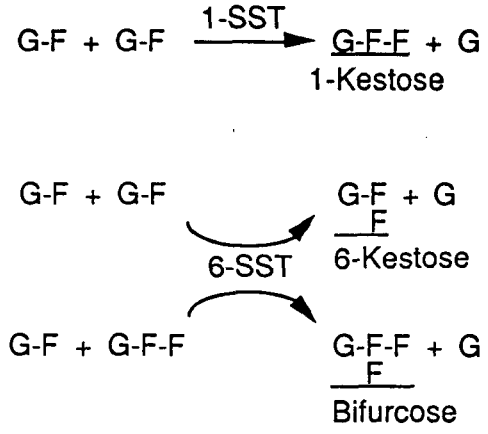

Figure 10. Scheme of the proposed activities of 1-SST and 6-SST during fructan biosynthesis in excised barley leaves exposed to continuous light. G, Glc; F, Fru.

well with the accumulation of fructans in vivo. Leaves exposed to light start to accumulate 1-kestose after 4 h and, in addition, 6-kestose and bifurcose after 8 h in vivo. Increased levels of 1-SST are seen in extracts from leaves after 4 h of exposure to light and increased levels of 6-SST after 8 h.

The main invertase has a surprisingly low K_m of 3 to 5 mM Suc. We have recently found that this enzyme is localized in the epidermis (D. Obenland, U. Simmen, K.H. Bortlik, T. Boller, and A. Wiemken, unpublished data), and its high affinity for Suc may be connected to its function in Suc mobilization in a heterotrophic sink tissue. This invertase also differs from the other enzymes investigated by its elevated temperature optimum and by its sensitivity to pyridoxal.

We found that the peak fraction of 6-SST from the MonoQ column produces not only 6-kestose when incubated with Suc but it can also form bifurcose, the most abundant tetrasaccharide accumulating in vivo, when incubated with Suc and 1-kestose (Fig. 9). The observation that 6-kestose formation is decreasing as bifurcose formation is increasing suggests that the two products result from competing reactions at the same enzyme. Because the enzyme does not form bifurcose with 1-kestose alone, and because Glc is released in parallel to bifurcose formation, we propose that the enzyme characterized as 6-SST can also act as Suc-fructan ^{6}F-fructosyltransferase, transferring the fructosyl residue from Suc to the 1-kestose to form bifurcose (Fig. 10). This enzymic reaction would explain a key step in the elongation-trimming pathway suggested for fructan synthesis in wheat (Bancal et al., 1991, 1992). Although our results are suggestive, further work is needed to conclusively demonstrate that 6-SST is a bifunctional and perhaps multifunctional enzyme in fructan biosynthesis of barley.

In conclusion, our results dispel doubts that barley possesses specific SST activities. Furthermore, they confirm that an inducible 1-SST is the key enzyme diverting Suc into the biosynthetic pathways of the fructans by formation of 1-kestose (Wagner et al., 1986; Wagner and Wiemken, 1987; Obenland et al., 1991). In addition, the novel inducible 6-SST may be equally important, producing not only 6-kestose from Suc but also the main tetrasaccharide accumulating in barley, bifurcose, by transfer of a fructosyl residue from Suc to the 1-kestose generated by 1-SST.

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