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Purification and Characterization of the Enzymes of Fructan Biosynthesis in Tubers of *Helianthus tuberosus* Colombia

II. Purification of Sucrose:Sucrose 1-Fructosyltransferase and Reconstitution of Fructan Synthesis in Vitro with Purified Sucrose:Sucrose 1-Fructosyltransferase and Fructan:Fructan 1-Fructosyltransferase

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Sucrose:sucrose 1-fructosyltransferase (1-SST), an enzyme involved in fructan biosynthesis, was purified to homogeneity from tubers of *Helianthus tuberosus* that were harvested in the accumulation phase. Gel filtration under native conditions predicted a molecular mass of about 67 kD. Electrophoresis or gel filtration under denaturing conditions yielded a 27- and a 55-kD fragment. 1-SST preferentially catalyzed the conversion of sucrose into the trisaccharide 1-kestose (GF_2). Other reactions catalyzed by 1-SST at a lower rate were self-transfructosylations with GF_2 and 1,1-nystose (GF_3) as substrates yielding GF_3 and 1,1,1-fructosylnystose, respectively, as products. 1-SST also catalyzed the removal of the terminal fructosyl unit from both GF_2 and GF_3, which resulted in the release of sucrose and GF_2, respectively, and free Fru. The purified enzyme did not display β-fructosidase activity. An enzyme mixture of purified 1-SST and fructan:fructan 1-fructosyltransferase, both isolated from tubers, was able to synthesize fructans up to a degree of polymerization of at least 13 with sucrose as a sole substrate.

Fructans consisting of up to 50 β(2→1)-linked fructosyl units are the main storage carbohydrates in tubers of *Helianthus tuberosus*. Enzymes that are proposed to be involved in fructan synthesis in *H. tuberosus* are 1-SST (EC 2.4.1.99) and 1-FFT (EC 2.4.1.100) (Edelman and Jefford, 1968). According to the model of fructan biosynthesis devised by those authors, 1-SST catalyzes the synthesis of the trisaccharide GF_2 from two molecules of Suc, whereas 1-FFT mediates the redistribution of fructosyl units between GF_2 and larger fructans (GF_n, $n > 2$; for full chemical names, see Lewis, 1993). Although it is stated that the current enzymological evidence is not sufficient to sustain the above-described model (Cairns, 1993), this two-step model of fructan biosynthesis is still feasible as a working hypothesis in studies on fructan metabolism.

A fructosyltransferase with a molecular mass of about 70 kD has recently been purified from tubers of *H. tuberosus* (Lüscher et al., 1993; Koops and Jonker, 1994). This enzyme catalyzes polymerization reactions, by which the maximum DP increases, by transferring fructosyl units from oligofructans (GF_n, $n \geq 2$) onto fructan acceptors with a similar or higher DP (Koops and Jonker, 1994). This enzyme also mediates depolymerization reactions, by which the maximum DP decreases, by transferring fructosyl units from higher-molecular-mass fructans onto Suc (Lüscher et al., 1993; Koops and Jonker, 1994). Since the enzyme recognized GF_2 as the smallest fructosyl donor substrate, it was designated as 1-FFT in both studies.

The existence of SST, an enzyme that predominantly or exclusively catalyzes the synthesis of the trisaccharide GF_2 from Suc, has, at least in Asteraceae, not been demonstrated unequivocally. An enzyme purified from chicory roots (Van der Ende and Van Laere, 1993) could accomplish the synthesis of GF_2 from Suc at nonphysiological Suc levels, but preferentially catalyzed the hydrolysis of Suc at concentrations below 400 mol m^{-3}. The chicory enzyme was therefore designated as invertase. 1-SST was claimed to have been purified to homogeneity from tubers of *H. tuberosus* (Praznik et al., 1990). In this study, dormant tubers, which do not actively accumulate fructans but do contain invertase (Venuat et al., 1993), were used as a source for protein extraction. Enzyme activity in the most pure fractions was estimated by hexose release, which does not allow discrimination between invertase and SST activity. SST may have been purified successfully from members of Liliaceae, such as onion (Shiomi et al., 1985; Angenent et al., 1993) and asparagus (Shiomi and Izawa, 1980), although the crude protein extract used in the latter study may have been contaminated with bacteria (Cairns, 1992). A multifunctional fructosyltransferase was very recently purified from barley (Duchateau et al., 1995). This enzyme, because of its ability to synthesize 6-kestose from Suc, was initially designated as Suc:Suc 6-fructosyltransferase

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Abbreviations: Chaps, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DP, degree of polymerization; 1-FFT, fructan:fructan 1-fructosyltransferase; GF_2, 1-kestose; GF_3, 1,1-nystose; GF_4, 1,1,1 fructosylnystose; 6-SFT, Suc:fructan 6-fructosyltransferase; 1-SST, Suc:Suc 1-fructosyltransferase.

(Simmen et al., 1993). A later study (Duchateau et al., 1995) revealed that the enzyme could also catalyze the transfer of fructosyl units onto Glc, oligofructans, and water (invertase activity), which rationalized renaming the enzyme 6-SFT.

The present paper describes the purification of a fructosyltransferase from tubers of *H. tuberosus*, which has an activity profile distinctly different from that of the earlier-purified 1-FFT (Koops and Jonker, 1994) and which has no invertase activity. In contrast to 1-FFT from *H. tuberosus* (Koops and Jonker, 1994), this enzyme can use Suc as the smallest donor substrate in synthetic transfructosylation reactions, of which the synthesis of GF_2 is the predominant reaction. This enzyme is therefore designated 1-SST. The present paper also describes experiments that verify the two-enzyme model of fructan synthesis in *H. tuberosus* (Edelman and Jefford, 1968). Purified 1-SST and 1-FFT were recombined to simulate the fructan-synthesizing potential of the original crude material. The ability of this enzyme mixture to synthesize fructans was evaluated with Suc as the only substrate.

MATERIALS AND METHODS

Plant Material

Helianthus tuberosus cv Colombia was grown on the trial fields of the Agricultural Research Department, Center for Plant Breeding and Reproductive Research, location De Haaff. Tubers were harvested in July and August, 1992, and stored at −80°C.

Preparation of Crude Protein Extract

Extraction of proteins from the frozen tubers (−80°C) was according to the method described for 1-FFT (Koops and Jonker, 1994). The protein extract from about 800 g of tubers was adjusted to 45% (w/v) saturation with $(NH_4)_2SO_4$. The insoluble proteins were pelleted by centrifugation (10,000*g*, 30 min) and discarded. The 45% supernatant was brought to 70% (w/v) saturation by further addition of $(NH_4)_2SO_4$. The pellet, obtained after a second centrifugation (10,000*g*, 30 min), was redissolved in 65 mL of a 50 mM phosphate buffer, pH 6.5, with 1 mM DTT and 1 mM PMSF (Sigma-Aldrich, Bornem, Belgium), and desalted by dialysis against 10 mM phosphate buffer, pH 6.5, with 1 mM DTT and 1 mM PMSF for 16 h. After buffer replacement, dialysis was continued for another 3 h. The whole procedure was performed at temperatures between 0 and 4°C. The buffer components were obtained from Acros Chimica (Geel, Belgium) unless indicated otherwise.

Purification of 1-SST by Liquid Chromatography

Anion-Exchange Chromatography

All columns and column packings were obtained from Pharmacia unless indicated otherwise. The centrifuged (30,000*g*, 30 min) dialysate was applied to a 25 × 120 mm Q Sepharose Phast Flow column (4°C), prewashed with 10 mM bis-Tris, pH 6.5, 1 mM DTT, 1 mM PMSF, and 5 mM EDTA in Milli Q water (Millipore). Bound proteins were eluted with a NaCl gradient (0–300 mM) in the same buffer at a flow rate of 5 mL min^{-1}. Fractions of 10 mL were collected and frozen in liquid N_2. For chromatography on Mono Q (final purification step), a 5 × 50 mm column was pre-equilibrated with 10 mM phosphate buffer, pH 6.5, 1 mM DTT, 1 mM EDTA, and 0.1% (w/v) Chaps. Bound proteins were eluted with a NaCl gradient (0–500 mM) at a flow rate of 0.5 mL min^{-1}. Fraction size was 0.5 mL.

Hydrophobic Interaction Chromatography

The Q Sepharose fractions with 1-SST activity were adjusted to 400 mM with solid $(NH_4)_2SO_4$. Fractions of 20 mL were loaded onto a 50 × 15 mm column of Phenyl Sepharose High Performance or Phenyl Sepharose High Substitution, each pre-equilibrated with 10 mM bis-Tris buffer, pH 6.5, containing 500 mM $(NH_4)_2SO_4$, 1 mM DTT, 1 mM PMSF, 2 mM EDTA, and 0.1% (w/v) Chaps (buffer A) at 12°C. Elution of bound proteins was carried out using a linear gradient (0–100%) of pre-equilibration buffer without $(NH_4)_2SO_4$, containing 25% (v/v) 2-ethoxyethanol, at a flow rate of 1 mL min^{-1}. Fraction size was 2 mL.

Con A Chromatography

Phenyl Sepharose fractions up to 10 mL were injected onto a 5 × 50 mm Con A Sepharose column, which had been prewashed in 20 mM bis-Tris, pH 6.5, 250 mM NaCl, 0.5 mM $CaCl_2$, 0.5 mM $MnCl_2$, 1 mM DTT, and 1 mM PMSF. Bound 1-SST was eluted with 500 mM α-CH_3-mannopyranoside in the same buffer. Flow rate was 1 mL min^{-1}, and fraction size was 2 mL.

Hydroxylapatite Chromatography

1-SST containing fractions of one Con A run were pooled and applied to a 5 × 200 mm column packed with spherical (15 μm) hydroxylapatite (Merck Nederland, Amsterdam, The Netherlands). The column was pre-equilibrated in 2 mM $CaCl_2$, 10 mM NaCl, 1 mM DTT, 1 mM PMSF, and 0.1% (w/v) Chaps. Proteins bound to the column were eluted with a stepped gradient (0–100%) of pre-equilibration solution containing 500 mM phosphate buffer, pH 6.5, at a flow rate of 0.5 mL min^{-1}. Fraction volume was 1.5 mL.

Gel-Permeation Chromatography

Mono Q fractions (2 mL) were loaded onto a Hiload 16 × 600 mm Superdex 75 prep grade column that was prewashed in 10 mM phosphate buffer, pH 6.5, and 1 mM DTT. Proteins were eluted in the same buffer at a flow rate of 0.5 mL min^{-1} at 20°C. Fractions of 0.5 mL were collected. To analyze the 27- and 55-kD proteins of SST, either 2% DTT or 8 M urea was added to the phosphate buffer, and gel filtration was performed under the same conditions.

1-SST Assay

1-SST activity of column fractions was routinely assayed at 35°C. Aliquots of 15 μL were mixed with 15 μL of 500 mM Suc in 200 mM citrate/phosphate buffer, pH 5.0. After 3 h of incubation the reaction was stopped by boiling the incubation mixture in a water bath for 5 min. The assay mixtures were analyzed by HPLC. GF_2 synthesis was taken as a measure of 1-SST activity.

Protein concentration was determined by the Bradford protein microassay (Bio-Rad) using BSA as a standard.

Electrophoresis

SDS-PAGE was performed on Excel Gel 8–18 (Pharmacia) as described by Koops and Jonker (1994). Nondenaturing PAGE was performed on a Phastsystem using precast Phastgels (homogenous 20) according to the recommendations of the manufacturer (Pharmacia). For nondenaturing PAGE protein fractions were desalted and concentrated by centrifugation through Microcon-30 ultrafiltration tubes (Grace BV, Amicon division, Capelle, The Netherlands). For sequential analysis of 1-SST by native PAGE and SDS-PAGE, two identical Mono Q fractions were prerun in a nondenaturing Phastgel; the location of the protein band in one lane was determined by silver staining and the corresponding band was excised from the nonstained lane. The 1-SST-containing gel slice was briefly washed in SDS sample buffer and directly applied onto the Excel Gel 8–18 for analysis by SDS-PAGE.

Analysis of Sugars and Fructans

Glc, Fru, and Suc were quantified on a 7.8 × 300 Rezex RCM monosaccharide column (Phenomenex, Torrance, CA) run with Milli Q water at 0.75 mL min^{-1} at 85°C. The oligofructans GF_2, GF_3, GF_4, and GF_5 were analyzed by reversed-phase HPLC according to Koops and Jonker (1994). Analyses of oligofructans and fructans with a higher DP were performed by high-pressure anion-exchange chromatography on a Dionex (Breda, The Netherlands) series 4000 ion chromatograph equipped with a 250 × 4 mm CarboPac PA1 anion-exchange column (Dionex) and a 25 × 3 mm CarboPac PA guard column. Fructans were separated with a 60-min linear gradient of 25 to 40 mM NaAc in 100 mM NaOH at a flow rate of 1 mL min^{-1} (Timmermans et al., 1994). Detection was by pulsed amperometry with a gold-working electrode. Fructans were identified by comparison of their retention times with those of fructan standards isolated and purified from *H. tuberosus* (Sigma) according to the method of Heinze and Praznik (1991). The fructan oligomers were quantified according to the method of Timmermans et al. (1994), using rhamnose as an internal standard.

Kinetic Analysis of Purified 1-SST

Mono Q fractions with 1-SST activity were used for kinetic analyses. Buffer and substrate solutions were pipetted into 5-mm-diameter, 0.2-mL tapered glass vial inserts (Chromacol, London, UK) and freeze-dried overnight. The reaction was started by adding 10 μL of 1-SST-containing fractions to the freeze-dried reaction mixtures. The 1-SST assay mixture was incubated for 3 h at 25°C in a water bath, unless indicated otherwise. The time course chosen was a compromise between the incubation time required to obtain sufficient product for analysis and limitation of transfer rate by substrate depletion. Maximum substrate depletion was 30%. The reactions were terminated by boiling the incubation mixtures in a water bath for 5 min. 1-SST activity as a function of temperature was studied according to the procedure described earlier (Koops and Jonker, 1994). Each measurement was performed in duplicate.

Fructan Synthesis with 1-SST and 1-FFT

Purified 1-SST and 1-FFT were used to investigate whether fructans could be synthesized from Suc in vitro. 1-FFT was purified according to Koops and Jonker (1994). The incubations were performed in 1 mL of 100 mM Suc, 2 mM DTT, 20 mM citrate/phosphate buffer, pH 5.5, and 0.01% (w/v) sodium azide at 25°C in a water bath. Reaction time was 80 h. Reaction was stopped by boiling the reaction mixture for 5 min. Reaction samples were deproteinized by centrifugation through Microcon-3 utrafiltration devices (Grace). The filtrate was analyzed by high-pressure anion-exchange chromatography.

RESULTS

Purification of 1-SST

Small tubers harvested in early summer were found to contain the highest 1-SST activity (data not shown) and were used for 1-SST purification. The enrichment of 1-SST during the purification procedure (Table I) was evaluated

Table I. *Purification protocol of 1-SST from tubers of H. tuberosus*

Purification Step	Total Activity	Protein Content	Specific Activity	Purification Factor
	units	*mg*	*mg^{-1} protein*	*fold*
Crude extract	135	1430	0.09	–[a]
45–70% $(NH_4)SO_4$ ppt and dialysis	50.1	469	0.11	1.2
Q Sepharose	39.7	146	0.27	3.0
Phenyl Sepharose	23.3	14	1.7	18.8
Con A	14.8	4.1	3.6	40
Hydroxylapatite	5.0	0.2	25	278
Mono Q	4.7	0.08	59	655

[a] –, No purification.

by incubating fractions with Suc and measuring GF_2 and Glc formation by HPLC. The first fractionation step, i.e. precipitation by $(NH_4)_2SO_4$, was performed in two stages. Approximately one-third of the total amount of proteins, including about 75% of 1-FFT, was removed by the first precipitation with 45% (w/v) $(NH_4)_2SO_4$. 1-SST, together with about 30% of the total proteins, was concentrated by addition of $(NH_4)_2SO_4$ to a final concentration of 70% (w/v) and subsequent centrifugation. The proteins precipitated by 70% $(NH_4)_2SO_4$ were redissolved and dialyzed against phosphate buffer, pH 6.5. This step inevitably resulted in a substantial loss of 1-SST activity (80% at pH 4.5; 30% at pH 6.5). At pH 6.5, 1-SST bound quantitatively to Q Sepharose and was eluted between 200 and 250 mM NaCl. The enzyme bound to Phenyl Sepharose when pre-equilibrated at 500 mM $(NH_4)_2SO_4$. The release of 1-SST activity from Phenyl Sepharose by a negative $(NH_4)_2SO_4$ gradient was marginal. The application of parallel 500 to 0 mM $(NH_4)_2SO_4$ and 0 to 25% (v/v) 2-ethoxyethanol gradients was needed to obtain higher 1-SST recoveries; 1-SST elution was achieved at 250 to 150 mM $(NH_4)_2SO_4$ and 12.5 to 17.5% (w/v) 2-ethoxyethanol. Binding of 1-SST to Con A and the reversibility of this binding by α-CH_3-mannopyranoside was indicative of 1-SST being a glycoprotein. Almost 65% of 1-SST activity was recovered by a batch-wise elution with 500 mM α-CH_3-mannopyranoside. Hydroxylapatite chromatography perfectly discriminated between 1-SST and residual 1-FFT activity, since 1-FFT did not bind to the hydroxylapatite matrix. 1-SST was eluted from the hydroxylapatite by 70 mM phosphate buffer. A second anion-exchange chromatography step, using the high-performance Mono Q packing, was applied as a final step to remove impurities.

The Mono Q-purified 1-SST was analyzed by nondenaturing PAGE, SDS-PAGE, and gel-permeation chromatography. Electrophoresis of Mono Q fractions revealed one protein band of about 90 kD under nondenaturing conditions (Fig. 1A), but after SDS-PAGE two major bands with molecular masses of 27 and 55 kD (Fig. 1B, lane 2) were obtained. Excision of the protein band from the native gel (Fig. 1A) and reanalysis by SDS-PAGE again revealed the presence of 27- and 55-kD bands (Fig. 1B, lane 3).

We investigated whether the 27- and 55-kD polypeptides represented either subunits, linked by disulfide bonds, or dissociation products of the full-size enzyme. To establish whether the 55-kD polypeptide represented a homodimer of two 27-kD subunits or whether 1-SST represented a heterodimer comprising 55- and 27-kD subunits linked by disulfide bonds, Mono Q fractions were boiled in SDS buffer with or without 5 mM DTT. Immediately after boiling, iodoacetamide was added to the sample containing DTT to a final concentration of 50 mM to preclude artifactual reassociation of the monomers during electrophoresis. The DDT/iodoacetamide treatment did not cause the disappearance of the 55-kD band, whereas omission of DTT did not lead to the appearance of an 82-kD protein. The conclusion is that sulfide bridges are not involved in the linkage of the 27- and 55-kD proteins.

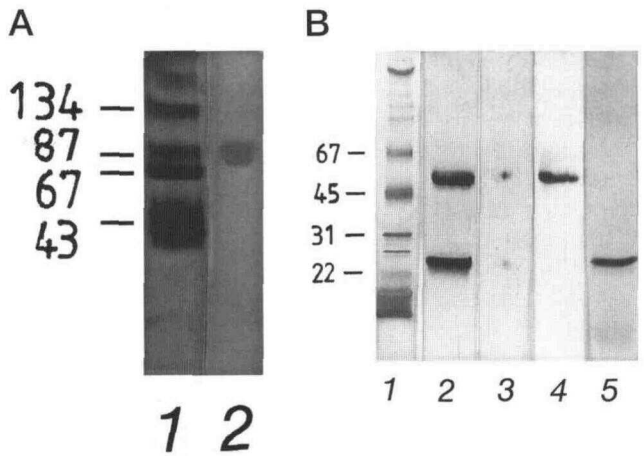

Figure 1. Analysis of 1-SST fractions from tubers of *H. tuberosus* by native PAGE (A) and SDS-PAGE (B). A, Lane 1, Molecular mass marker; lane 2, Mono Q fraction with 1-SST activity. B, Lane 1, Molecular mass marker; lane 2, Mono Q fraction with 1-SST activity; lane 3, Mono Q-purified 1-SST after native gel electrophoresis, excision, and reanalysis by SDS-PAGE; lanes 4 and 5, 1-SST fragments eluting successively during denaturing gel-permeation chromatography in 8 M urea of Mono Q-purified 1-SST.

Following gel-permeation chromatography in 10 mM phosphate buffer, pH 6.5, 1-SST eluted as one single peak with the same retention time as the standard BSA. This is consistent with a molecular mass of about 67 kD for 1-SST. Gel filtration in 2% DTT did not cause dissociation of 1-SST, although enzyme activity was completely lost. When Mono Q-purified 1-SST was subjected to denaturing gel-permeation chromatography in 8 M urea, two separate peaks were obtained. SDS-PAGE confirmed that these peaks represented the 55- and 27-kD polypeptides (Fig. 1B, lanes 4 and 5).

We finally investigated whether both the 27- and the 55-kD polypeptides were necessary for activity. Urea was added to Mono Q-purified 1-SST, and the 27- and 55-kD proteins were separated by gel-permeation chromatography. After removal of the urea by dialysis, 1-SST activity was measured in a control fraction (Mono Q fraction + 8 M urea + dialysis), in fractions containing either the 55- or the 27-kD protein, and in 55- + 27-kD fractions. However, 1-SST activity was completely lost in all fractions after addition and removal of urea. Apparently, the original conformation of the full-size protein could not be restored by recombination of the 27- and 55-kD fragments under nondenaturing conditions.

Enzyme Properties of 1-SST

Mono Q-purified 1-SST was used to study enzyme properties. 1-SST activity was estimated from the rate of GF_2 production from Suc at pH values ranging from 2.5 to 8.0, employing Gly-HCl, citrate-phosphate, and phosphate buffers, and at temperatures ranging from 4 to 50°C. 1-SST activity at 50 mM Suc was maximal at pH values between 3.5 and 5.0. The pH values for half-maximum activity were 2.7 and 6.5. The temperature optimum for GF_2 synthesis at

50 mM Suc was 20 to 25°C. 1-SST activity at 5°C was 50% of that at the temperature optimum, which is equal to a Q_{10} of 1.3 in the range of 20 to 5°C. The other value for half-maximum activity was 37°C.

1-SST activity at pH 6.5 was not significantly affected by 5 mM Ca^{2+} or Mg^{2+}; however, 5 mM Mn^{2+} reduced the activity about 20 ± 4%. Enzyme activity was inhibited by 1 mM of either $CuSO_4$ (to 53 ± 4% of control rate) or $AgNO_3$ (85 ± 8%). The SH-reagents *N*-ethylmaleimide and iodoacetamide (each 1 mM) reduced 1-SST activity by about 10%. 1-SST activity was stimulated by 20 mM pyridoxine (120 ± 3% of control rate) or pyridoxal-HCl (147 ± 5%). Stimulation of polymerizing fructosyltransferase activity by these compounds may be attributed to inhibition of invertase (Cairns, 1989) or to fructan exohydrolase activity (Wagner and Wiemken, 1986). However, no detectable amount of Fru was present when either pyridoxine or pyridoxal-HCl was omitted, suggesting that the increase in the GF_2 synthesis was due to a direct stimulatory effect on synthetic activity.

The rate of GF_2 production by 1-SST as a function of the [Suc] is presented in Figure 2. Maximum activity was attained only at [Suc] higher than 1 M. The ability of 1-SST to metabolize Suc was studied in a long-term incubation (80 h at 25°C). To prevent microbial growth during incubation, NaN_3 was included in the medium. To ensure that the 1-SST fractions used in this experiment did not contain 1-FFT impurities, the hydroxylapatite chromatography step was performed twice. The 80-h incubation with 1-SST confirmed that 1-SST converted Suc into GF_2 efficiently (Fig. 3), since about 90% of the Suc supplied was consumed by 1-SST. 1-SST was also

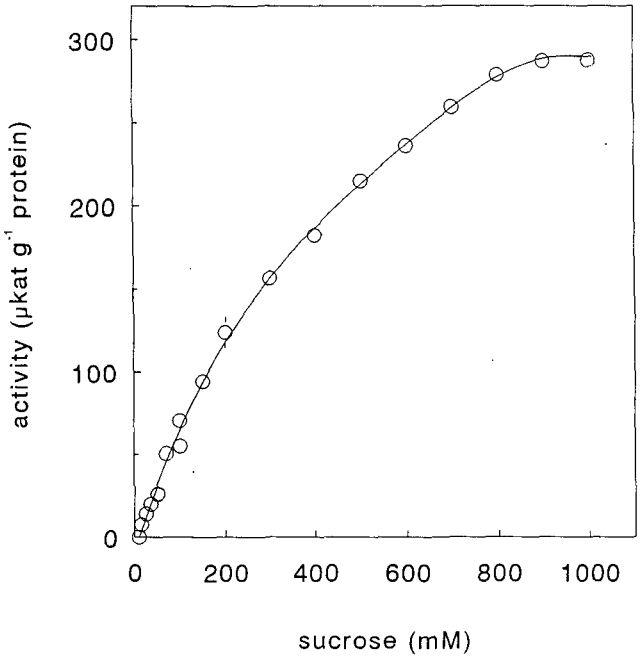

Figure 2. Suc-concentration-dependent rate of GF_2 formation by 1-SST from tubers of *H. tuberosus*. Transfer rates are the means of duplicates ± SD; error bars are omitted when they are smaller than the symbols.

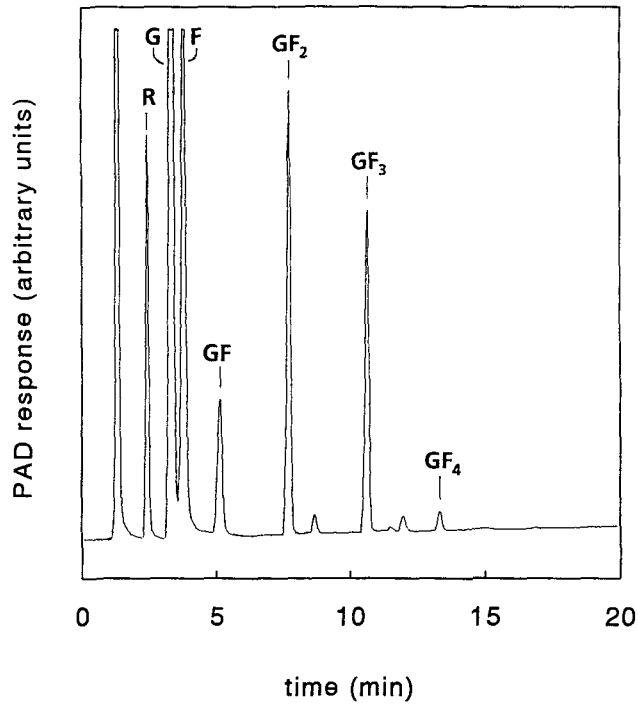

Figure 3. High-performance anion-exchange separations of Glc (G; 9.9 mg at 80 h after incubation), Fru (F; 6.3 mg), GF_2 (9.0 mg) GF_3 (7.2 mg), and GF_5 (0.9 mg) synthesized from Suc (GF; 34 mg at time 0, 3.6 mg at 80 h) by 1-SST purified from tubers of *H. tuberosus*, after 80 h of incubation at 25°C. Rhamnose (R) was used as an internal standard. The labeled oligofructans were identified by comparison with the β(2→1)-linked fructan standards isolated and purified from *H. tuberosus*. PAD, Pulsed amperometry.

able to mediate the synthesis of GF_3 and, although to a much lesser extent, the formation of GF_4 (Fig. 3). Besides these oligofructans, substantial amounts of Glc and Fru were present at the end of the 80-h incubation period. Glc release was stoichiometrically related to GF_2 and GF_3 synthesis. The presence of Fru, however, indicates that the 1-SST preparation has hydrolytic activity as well. In short-term incubations with 1-SST (e.g. Fig. 2) using Suc as the only substrate, we did not find detectable amounts of Fru. This may indicate that 1-SST has no hydrolytic activity against Suc but can possibly remove fructosyl units from GF_2 and/or GF_3 only. To investigate in which reaction free Fru is released, equal amounts of 1-SST were mixed with 10 μmol of either Suc, GF_2, or GF_3. Samples were taken after 2, 4, and 8 h, and the sugar compositions were evaluated by HPLC (Fig. 4). Of the three substrates, Suc (Fig. 4A) was most efficiently consumed by 1-SST. In the first 2 h the synthesis of equimolar amounts of Glc and GF_2 was the predominant reaction. Fru increased only after a substantial amount of GF_2 had accumulated. The 1-to-1 stoichiometry between Glc and GF_2 was lost after 2 h, partly because of some GF_3 synthesis at the cost of GF_2 and probably also because of the release of a terminal Fru from GF_2 (Fig. 4A). The results in Figure 4B confirm that 1-SST can use GF_2

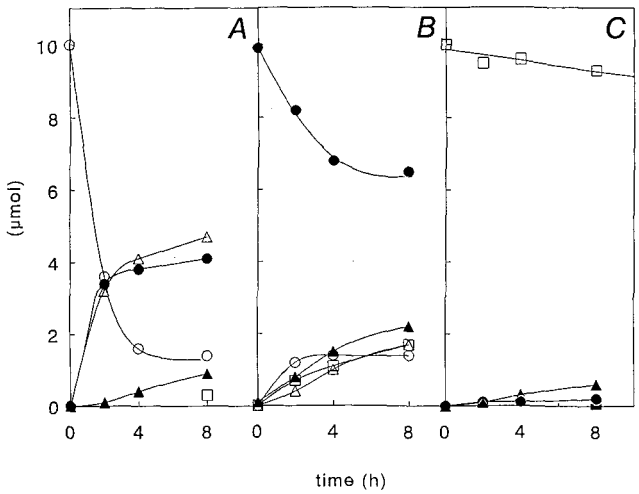

Figure 4. Time course of substrate consumption and product formation by 1-SST from tubers of *H. tuberosus*. Substrates at time 0 were Suc (A), GF_2 (B), and GF_3 (C). Substrate concentration at time 0 was 100 mM. △, G; ▲, F; ○, GF; ●, GF_2; □, GF_3; ■, GF_4.

as a substrate for GF_3 synthesis. A substantial release of Fru, together with a higher Suc level (1.25 μmol present after 2 h) than was expected on the basis of GF_3 synthesis only (0.63 μmol present after 2 h) indicates that GF_3 was also substrate for hydrolytic reactions and that removal of a terminal fructosyl unit from GF_3 was another cause of Suc accumulation. The Suc level after 2 h is slightly lower than can be expected on the basis of the combined effect of GF_3 synthesis and hydrolysis of GF_2. However, in view of the high affinity of 1-SST for Suc (Fig. 4A), some of the Suc formed during incubation will inevitably be channeled into GF_2 synthesis again. GF_3 is a better substrate for hydrolytic reactions (0.08 μmol of Fru present after 2 h) than for synthetic reactions (0.02 μmol of GF_4; Fig. 4C), although both reactions are of minor importance compared to GF_2 synthesis (Fig. 4A) and GF_2 hydrolysis (Fig. 4B).

The substrate and end product levels after 2 h of incubation (Fig. 4, A–C) were used to calculate the relative rate of each possible transfructosylation reaction catalyzed by 1-SST (Fig. 5, A–C). To allow this calculation, it was assumed that only monofructosyl units are transferred per single synthetic or hydrolytic transfructosylation reaction. The HPLC-pulsed amperometry chromatogram (Fig. 3) revealed the presence of some minor unidentified compounds, which may represent F_2 or F_3; however, in view of the small amounts present, the contribution of di- or trifructosyltransferase reactions to the total carbon flow was considered to be negligible. Because of the low affinity of 1-SST for GF_4, the rate of the reactions in Figure 5C are calculated over a 20-h period and normalized to allow a comparison with Figure 5, A and B. The overall transfructosylation scheme (Fig. 5) shows that 1-SST preferentially converts Suc into GF_2. The rate of this self-transfer reaction (Fig. 5A) outstrips that of the reactions with GF_2 and GF_3 as substrates by a factor 3.6 and 80, respectively. Only four out of five possible reactions are presented in Figure 5B, since it was not possible to match the end product concentrations with a reaction scheme that includes the transfer of a fructosyl unit from GF_3 onto Suc, which would result in GF_2 synthesis, as one of the possible transfructosylation reactions. GF_2 is the second preferable substrate for 1-SST. This substrate is rather efficiently used for GF_3 synthesis and is also the most important source of Fru (Fig. 5B). GF_3 is 5 times less susceptible than GF_2 to hydrolytic activity of 1-SST (Fig. 5C).

Fructan Synthesis in Vitro with Purified 1-SST and 1-FFT

From experiments described in the present paper and performed earlier (Koops and Jonker, 1994), it can be concluded that tubers of *H. tuberosus* contain at least two different enzymes that are able to catalyze fructosyltransferase reactions that lead to an increase in the maximum DP. 1-SST can synthesize GF_2 and GF_3 from Suc, whereas 1-FFT uses GF_2 but not Suc, as the smallest fructosyl donor for the synthesis of fructans with a higher DP (Koops and Jonker, 1994). Because of the complementary activity of 1-SST and 1-FFT, it should be possible to synthesize fructans with Suc as the only substrate by recombining purified 1-SST and 1-FFT. Although this possibility was predicted on the early model of fructan synthesis (Edelman and Jefford, 1968), this hypothesis has never been tested in vitro. The model of fructan synthesis was tested with three incubation mixtures (each 1 mL): (a) 100 μmol of Suc + buffer (control); (b) 100 μmol of Suc + buffer + 17 μg of 1-FFT; and (c) 100 μmol of Suc + buffer + 31 μg of 1-SST + 17 μg of 1-FFT. NaN_3 was added to each mixture to prevent microbial growth. The purity of the 1-SST or 1-FFT

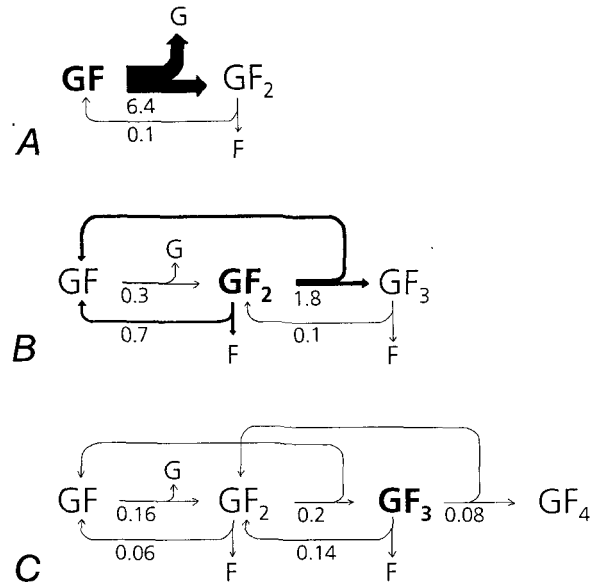

Figure 5. Relative rates of all possible transfructosylation reactions catalyzed by 1-SST from tubers of *H. tuberosus*. The transfructosylation scheme was based on data derived from Figure 4. Figures below the arrows represent the total substrate consumption (μmol) in each individual reaction during the first 2 h of incubation in one of the three starting substrates Suc (A), GF_2 (B), and GF_3 (C).

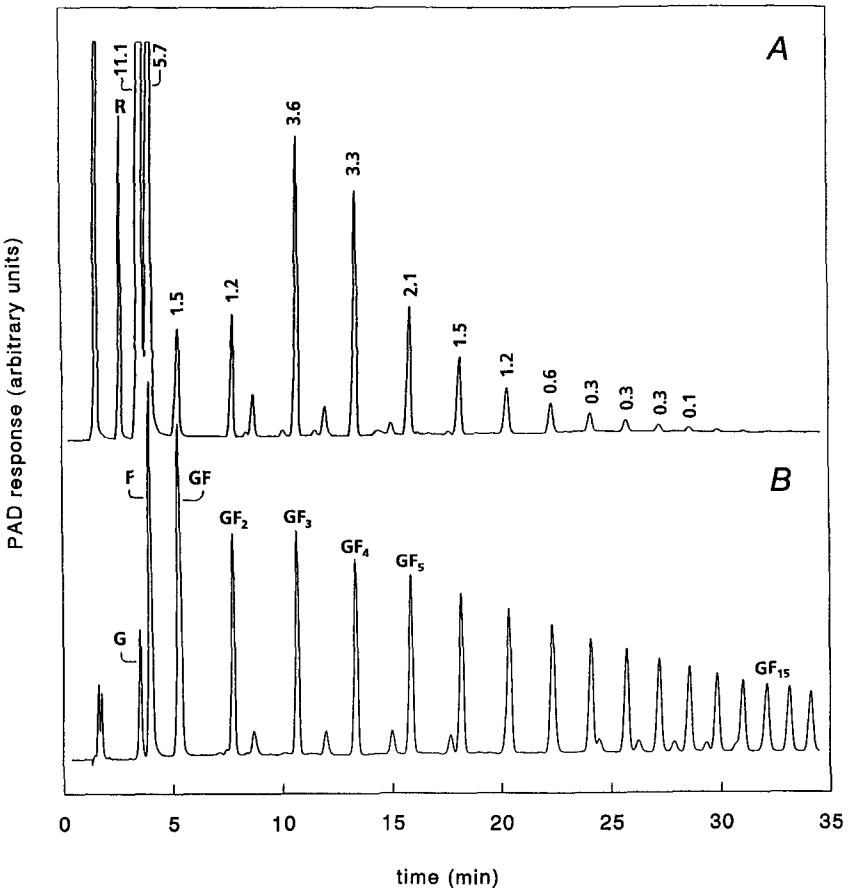

Figure 6. A, High-performance anion-exchange separations of Glc (G), Fru (F), and fructans (GF_n) synthesized from Suc (GF; 34 mg at time 0) by a mixture of 1-SST and 1-FFT, both purified from tubers of *H. tuberosus*, after 80 h of incubation at 25°C. Rhamnose (R) was used as an internal standard. Numerals above peaks represent total amount of substrate or product at the end of incubation. B, Anion-exchange separation of a linear series of fructans (inuline) from *H. tuberosus*. PAD, Pulsed amperometry.

fractions used for this experiment was verified by SDS-PAGE and silver staining.

(a) The absence of free hexoses at the end of the incubation in the control mixture shows that Suc was not susceptible to spontaneous hydrolysis and that microbial growth was prevented (data not shown). (b) As was already established in short-term experiments (Koops and Jonker, 1994), 1-FFT has no activity against Suc; there were no detectable amounts of hexoses or GF_2 present after 80 h of incubation (data not shown). (c) Figure 6 conclusively shows that fructans, at least up to a DP of 13, can be synthesized from Suc by the concerted action of purified 1-SST and 1-FFT. Within the margins tested, the 1-SST:1-FFT ratio did not significantly affect the final DP. When 10 μg of 1-SST was mixed with 24 μg of 1-FFT, the fructan profile did not significantly deviate from that shown in Figure 6. Substantial amounts of free Glc and Fru were present at the end of the incubation period. Glc was a by-product of the 1-SST-mediated self-transfer reaction with Suc. Fru most probably resulted from hydrolytic activity of 1-SST.

DISCUSSION

Research on the biosynthesis of fructans in *H. tuberosus* tubers (Edelman and Jefford, 1968) provided a theoretical foundation for the enzymology of fructan synthesis in plants. The early model of fructan synthesis was further elaborated by the finding that fructosyltransferase activities were localized in the vacuole (Wagner et al., 1983; Darwen and John, 1989). More recently, however, the model of fructan synthesis has been the subject of serious criticism. According to Cairns (1993), the SST/FFT model was not sufficiently sustained because the enzymological evidence was obtained with crude extracts or only partially purified preparations. Moreover, in many cases these extracts were contaminated with invertase. This enzyme, because of its artifactual fructosyltransferase activity, could be equally responsible for fructosyltransferase activity in many of the experiments published up to this time. The present work, which is part of a study to investigate the enzymic properties of fructosyltransferases in fructan-accumulating tubers of *H. tuberosus*, focused on the purification and characterization of 1-SST and provided evidence for the validity of the two-enzyme model of fructan synthesis.

1-SST was purified 655-fold from fructan-accumulating tubers of *H. tuberosus*. SDS dissociates 1-SST into 27- and 55-kD polypeptides. The reason is unknown, although dissociation by SDS has also been reported for other fructosyltransferring enzymes. SDS dissociates the acid invertase from carrot into 43- and 25-kD polypeptides. These polypeptides were found to represent the N- and C-terminal fragments, respectively, of a 68-kD protein (Unger et al., 1992). The 6-SFT (69 kD under native conditions) puri-

fied from barley is unstable under denaturing conditions. SDS-PAGE of 6-SFT yielded 20- and 50-kD fragments (Duchateau et al., 1995). For 1-SST from *H. tuberosus*, recent data obtained by amino acid sequencing of the purified 1-SST and DNA sequencing of the corresponding cDNA from a cDNA library of *H. tuberosus* tubers reveals that the 27- and 55-kD proteins represent the C- and N-terminal parts, respectively, of 1-SST (with an estimated molecular mass of 75 kD for the unprocessed translation product; I.M. van der Meer and A.J. Koops, unpublished observations). These data conclusively show that the 27- and 55-kD fragments are derived from one protein.

The most recent study on the purification of 1-SST from Asteraceae describes a procedure to obtain the enzyme from dormant tubers of *H. tuberosus* in two chromatographic steps (Praznik et al., 1990). Gel filtration predicted a molecular mass of 69 kD for this protein, which corresponds well with 1-SST from fructan-accumulating tubers (gel filtration data in this report). However, the temperature optimum (34°C), the pH optimum (5.4), and the activity profile as a function of the [Suc] (K_m of 42 mM) of the enzyme from dormant tubers (Praznik et al., 1990) differ from those of the enzyme described in the present paper. The enzyme purified from dormant tubers may therefore represent an SST isoform that is not active or present in fructan-accumulating tubers.

As shown for many other SST-containing preparations (for an overview, see Pollock, 1986), the rate of GF_2 formation from Suc is barely saturable (Fig. 2). Often, the rate versus concentration curves of this reaction are interpreted as first-order Michaelis-Menten equations with K_m values usually higher than 100 mM. However, a full description of the kinetics of a self-transfer or any bisubstrate reaction needs four kinetic parameters. Determination of the kinetic parameters requires a set of experiments in which the substrate concentrations at the donor and acceptor site are varied independently (Mahler and Cordes, 1971). As this requirement is impossible to meet for any self-transfer reaction, the K_m values reported in the literature for SST (and FFT) do not have much significance. In many studies with partially purified SST or FFT preparations, Fru release is interpreted as an indicator of contamination by invertase and is used for assessment of residual invertase activity. The present investigations, however, show that Fru release may also result from SST-mediated hydrolysis of oligofructans and that synthetic and hydrolytic activity may reside on the same protein. The hydrolytic activity of SST resembles that of fructan exohydrolases, which hydrolyzes fructans by stepwise removal of terminal nonreducing fructosyl residues. In Asteraceae, fructan exohydrolases also have negligible activity against Suc (Simpson and Bonnet, 1993).

The present work shows that two enzymes are needed to synthesize fructans from Suc and that these enzymes are essentially different proteins. 1-SST and 1-FFT differ in their chromatographic, electrophoretic, and enzymic properties. For example, 1-FFT is not able to catalyze the initial step of fructan synthesis, whereas 1-SST is not able to catalyze the formation of fructan polymers with a DP higher than 5 [GF_n, $n > 4$]. 1-SST is able to transfer fructosyl units between Suc as efficiently as 1-FFT between GF_2, GF_3, or GF_4 molecules. The net number of fructosyl units transferred by 1-SST during GF_2 formation from Suc, expressed per unit of time and protein (Fig. 2), compares to the number of fructosyl units transferred by 1-FFT during GF_3, GF_4, or GF_5 synthesis (Koops and Jonker, 1994). Although 1-SST and 1-FFT have some overlapping activity (both enzymes can catalyze the formation of GF_3 and GF_4), GF_3 and GF_4 synthesis is much more efficiently catalyzed by 1-FFT (Koops and Jonker, 1994).

GF_2 formation from two molecules of Suc is the reaction most favorably catalyzed by 1-SST (Fig. 5). However, some futile cycling of carbon may occur, since 1-SST can also mediate the removal of a terminal fructosyl unit from GF_2, which results in one molecule of Suc and, for fructan enzymes, nonutilizable Fru (Fig. 5). 1-SST activity is poorly coordinated with that of FFT. The 1-SST-mediated GF_2 synthesis from Suc is favored by high [Suc] (Fig. 2), whereas the second phase of fructan synthesis, the 1-FFT-mediated conversion of GF_2 into fructans with a higher DP, is competitively inhibited by Suc (Koops and Jonker, 1994). The experiment described in Figure 6 shows that despite the poorly balanced enzymic properties of 1-SST and 1-FFT, it is possible to synthesize fructans from Suc after recombining purified 1-SST and 1-FFT. Although we were able to demonstrate the synthesis of fructans with a DP of up to 15 after 80 h of incubation, the molecular mass distribution of the fructans obtained still does not resemble the molecular mass profile in a tuber homogenate. However, fructans appeared to be synthesized in a cascade-like manner. Fructosyl units are probably transferred between fructans with the same DP, as can be concluded from the finding that GF_{n+1} is synthesized only after some GF_n has accumulated (Koops and Jonker, 1994). Therefore, the DP of fructans in a tuber homogenate might be a function of tuber age and possibly of GF_2 supply, which in turn, depends on the Suc supply at the phase of tuber filling and 1-SST activity.

On the basis of present investigations it can be concluded that the basic concept of the early model of fructan synthesis in *H. tuberosus*, one enzyme for the trisaccharide synthesis and one enzyme for the synthesis of the higher fructans, is still supported by experimental evidence.

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