FEBS Letters 385 (1996) 39–42

Inulin synthesis by a combination of purified fructosyltransferases from tubers of *Helianthus tuberosus*

Marcel Lüscher, Christian Erdin, Norbert Sprenger, Urs Hochstrasser, Thomas Boller, Andres Wiemken*

Department of Botany, University of Basel, Hebelstrasse 1, CH-4056 Basel, Switzerland

Received 16 March 1996

Abstract Sucrose-sucrose 1-fructosyltransferase (1-SST) was purified 100-fold from tubers of *Helianthus tuberosus* L. The purified enzyme was essentially devoid of invertase activity and could be separated by isoelectric focusing into five isoforms which all were composed of two subunits (59 and 26 kDa). Fructan-fructan 1-fructosyltransferase (1-FFT) was purified from the same source [M. Lüscher et al. (1993) New Phytologist 123, 437–442). When incubated individually with sucrose, 1-FFT was inactive while 1-SST formed isokestose (trimer) and, upon prolonged incubation, some nystose (tetramer). When a combination of the two enzymes was incubated with sucrose, a series of oligofructosides with a degree of polymerization of up to 20 was formed. Amino acid sequences of tryptic peptide fragments from both 1-SST and 1-FFT indicate that these enzymes are highly homologous with plant invertases.

Key words: Fructan; Fructan-fructan 1-fructosyltransferase; *Helianthus tuberosus*; Inulin; Sucrose-sucrose 1-fructosyltransferase

1. Introduction

Inulin belongs to a class of fructose-based, highly soluble polysaccharides collectively called fructans. Fructans are the major non-structural carbohydrates in many plant species, particularly in the prevalent and evolutionarily advanced orders of Asterales, Liliales and Poales (e.g. chicory, onions, wheat) [1]. Fructans are deposited in vacuoles and play an important role as carbohydrate reserves in addition to or as an alternative to starch (e.g. in cereals: [2], for reviews see [3-5]). They are also involved in osmoregulation (e.g. in flowers: [6]) and are believed to function as protectants against drought and cold stress [1,7,8]. Great interest is currently focused on fructans because of their potential value in food technology [9].

Our current view of inulin (β-2-1-polyfructofuranosyl sucrose) biosynthesis in plants is based upon the model proposed for fructan synthesis in tubers of *Helianthus tuberosus* by Edelman and Jefford [10], according to which fructan is synthesized from sucrose via a trisaccharide intermediate by the concerted action of two distinct fructosyltransferases: (i) sucrose-sucrose 1-fructosyltransferase (1-SST, EC 2.4.1.99) which produces the inulin trisaccharide isokestose (*O*-β-D-fructofuranosyl-2-1-sucrose) and glucose from sucrose, and (ii) fructan-fructan 1-fructosyltransferase (1-FFT, EC 2.4.1.100) that transfers fructose moieties between fructan molecules. However, as discussed by Cairns [11], the model has not been validated unequivocally since the two enzymes have not been purified to homogeneity. Meanwhile, 1-FFT has been purified and characterized from dormant and growing tubers [12,13], but the purification of the 1-SST had not been reported.

Here we report the purification of the *H. tuberosus* 1-SST to electrophoretic homogeneity and show that in combination 1-SST and 1-FFT can synthesize long-chain inulins in vitro from sucrose.

2. Materials and methods

2.1. Plant material

Tubers of *Helianthus tuberosus* L. (cultivar Columbia) were collected during the growing stage in September 1995 at the Botanical Garden of Basel. The tubers were immediately washed, frozen in liquid nitrogen and kept at −20°C until further use.

2.2. Purification of 1-SST and 1-FFT

Frozen tubers (200 g) were homogenized with a Waring blender in 200 ml of 50 mM MES (NaOH), pH 5.8, containing 10 mM DTT, 5 mM ascorbic acid, 5 mM EDTA, and 5% Polyclar AT (Serva, Heidelberg, Germany). The slurry was filtered through a cotton cloth, centrifuged at 10 000 × *g* for 20 min, and precipitated overnight at 4°C with ammonium sulfate (65% saturation). The precipitated proteins were collected by centrifugation at 10 000 × *g* for 15 min, resuspended in buffer A (50 mM MES (NaOH), pH 5.5, with 1 mM $MnCl_2$, 1 mM $CaCl_2$, 500 mM NaCl, 0.02% Na azide), centrifuged again (15 000 × *g* for 10 min), and loaded onto a column (16 ml volume) of ConA-sepharose (Fluka, Buchs, Switzerland) equilibrated with buffer A. The column was washed with three volumes of buffer A, and glycosylated proteins were eluted with 1.5 volumes of 500 mM α-methyl-mannopyranoside in buffer A. This eluate was loaded onto a 1 ml hydroxylapatite column (Biogel HT, BioRad), equilibrated with buffer B (20 mM Bis-Tris (HCl), pH 6.0). After washing the column with 4 ml of buffer B, the adsorbed proteins were eluted with 3 ml buffer C (0.3 M phosphate (NaOH), pH 5.6). The eluate was supplemented with ammonium sulfate (2 M final concentration) and loaded onto a 1 ml Alkylsepharose column (HR 5/5, Pharmacia) equilibrated with 50 mM citric acid (NaOH) buffer, pH 5.0, containing 2 M ammonium sulfate. Proteins were eluted with a linear gradient of ammonium sulfate (30 ml, from 2 to 0 M). Fractions containing 1-SST activity were desalted over Biogel P4 (BioRad) into buffer B, and loaded onto a 1 ml MonoQ HR 5/5 column (Pharmacia) equilibrated with buffer B. Proteins were eluted with a linear NaCl gradient (30 ml, from 0 to 300 mM) in buffer B. Fractions containing 1-SST activity were pooled and loaded onto a 0.2 ml hydroxylapatite column (Biogel HT, BioRad) equilibrated with buffer B. After washing the column with 1 ml of buffer B, proteins were eluted with 1 ml of buffer C in 50 µl fractions. Fractions containing 1-SST activity were combined and represented the preparation of purified 1-SST. Isoelectric focusing and staining of the gel for 1-SST activity was done as described [14]. Pieces from unstained lanes of the IEF gel corresponding to 1-SST bands were cut out, finely chopped (<0.5 mm) with a razor blade, incubated in sucrose solution and analysed for 1-SST activity by HPLC.

1-FFT was purified to electrophoretic homogeneity essentially as

*Corresponding author. Fax: (41) (61) 267 23 30.

Abbreviations: 1-FFT, fructan-fructan 1-fructosyltransferase; 6-SFT, sucrose-fructan 6-fructosyltransferase; 1-SST, sucrose-sucrose 1-fructosyltransferase; IEF, isoelectric focusing.

PII S0014-5793(96)00343-2

described before [12], and the steps with hydroxylapatite and Alkyl-sepharose described above were also included.

Protein was determined according to Bradford [15] using BSA as a standard.

2.3. SDS-PAGE and sequencing of tryptic peptides from 1-SST and 1-FFT

The purified preparations of 1-SST and 1-FFT were analysed on 12% SDS-PAGE and proteins were stained with silver nitrate [14]. Sequences of tryptic peptides of the 1-SST protein (59 kDa band) and the 1-FFT protein (73 kDa band) were obtained. Proteins were separated on SDS-PAGE gels, electro-transferred to a polyvinylidene difluoride membrane (Immobilon PVDF, Millipore), and visualized with Coomassie Blue R. The band of interest was cut out and digested with trypsin. Tryptic peptides were separated by reversed-phase HPLC and sequenced by automated Edman degradation [16].

2.4. Enzyme assays

1-SST and 1-FFT activities were determined by incubating enzyme solutions or finely chopped pieces from isoelectric focusing gels in a final volume of 50 µl 100 mM phosphate (NaOH) buffer, pH 5.6, 0.02% Na azide and sucrose at the concentration indicated (50–200 mM) for 2 to 24 h at 25°C. To stop the reaction, the mixture was held for 1 min in a boiling water bath, then centrifuged at $10\,000 \times g$ for 10 min, and used for sugar analysis by HPLC.

For the 1-SST/1-FFT combination experiments, purified 1-SST (0.1 nkat) was incubated in combination with 1-FFT (1 nkat) as described above and 10 µl aliquots were taken at the time points indicated and analyzed by HPLC.

2.5. Sugar analysis by HPLC

Sugars were analyzed by anion exchange HPLC and detected by pulsed amperometry [17].

3. Results and discussion

3.1. Purification of the H. tuberosus sucrose-sucrose 1-fructosyltransferase (1-SST)

A 100-fold purification of *H. tuberosus* was achieved (Table 1) by ammonium sulfate precipitation, lectin affinity-, hydroxylapatite-, hydrophobic interaction- and ion exchange chromatography. After the final purification step on the MonoQ column, analysis by SDS-PAGE invariably revealed two bands indicating that 1-SST is composed of two subunits with a size of 59 and 26 kDa (Fig. 1A, lane S). The purified 1-SST preparation from the MonoQ yielded five isoforms separable on gel isoelectric focusing with pIs of 5.09, 5.02, 4.98, 4.94, 4.89 (Fig. 1B). Each isoform was composed of the two subunits of 59 and 26 kDa (Fig. 1A, lanes 1–5).

The two-subunit composition seems to be a common feature of many fructosyltransferases and β-fructosidases of plants, e.g. the fructosyltransferases from tall fescue [14], the sucrose-fructan 6-fructosyltransferase (6-SFT) from barley [16] and the invertases from carrots and *Vigna radiata* [18-20]. They probably arise by posttranslational processing since molecular analysis has shown that cDNAs representing invertases from carrot and *Vigna radiata* and 6-SFT from barley contain a long open reading frame encompassing both subunits.

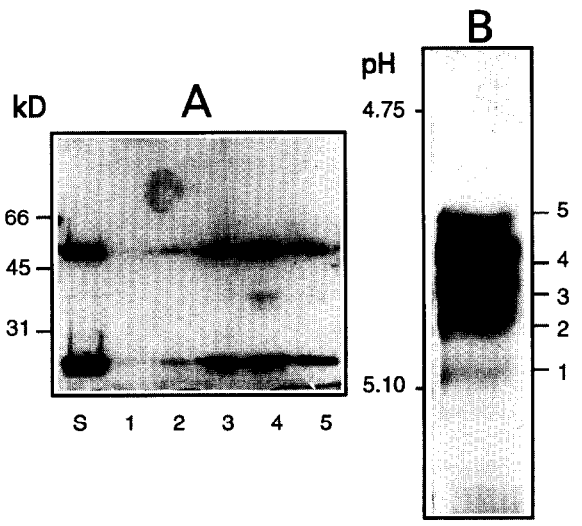

Fig. 1. Electrophoretic analysis of purified sucrose-sucrose 1-fructosyltransferase (1-SST). Purified 1-SST yielded two bands (26 and 59 kDa) on SDS-PAGE (panel A, lane S). When the 1-SST was separated on an isolectric focusing gel, five isoforms were detected in the activity stained gels (panel B, 1–5). All these five isoforms yielded the same two subunits on SDS-PAGE (panel A, lanes 1–5). Numbers on the left of panel A indicate positions of marker proteins.

3.2. Comparison of the amino acid sequence of 1-SST and 1-FFT with other fructosyltransferases and invertases

The purified 1-SST was subjected to SDS-PAGE, and the 59 kD subunit was used to generate tryptic peptides. The amino acid sequences of four of these peptides were compared with the deduced peptide sequences of invertases from *Vigna radiata* [20], *Daucus carota* [18,19] and the barley 6-SFT [16] (Table 2). Although only short peptide sequences of 1-SST are available it is obvious that 1-SST is very similar to these enzymes.

We also determined tryptic peptide sequences from the 1-FFT enzyme, purified to homogeneity as previously reported [12]. The sequences obtained from this 73 kDa enzyme too are highly similar to the deduced peptide sequences of the other fructosyltransferases and β-fructosidases (Table 2).

It has been found earlier that barley 6-SFT is closely homologous with plant invertases as well [16]. Our new finding supports the hypothesis that in evolution, plant invertases might have been recruited repeatedly for generating fructosyltransferases by small mutational changes [1,16].

3.3. Characterization of fructosyltransferase activities

In a short-term experiment (2 h), the purified 1-SST preparation catalyzed almost exclusively transfructosylation from sucrose to sucrose, producing isokestose and glucose (Fig. 2a). Its specific activity was 40 nkat mg^{-1} protein. Invertase activ-

Table 1. Purification of sucrose-sucrose 1-fructosyltransferase (1-SST) from growing tubers of *H. tuberosus*

Purification step	Protein yield (mg)	Specific activity (nkat mg^{-1} protein)	Purification factor
Crude homogenate	906	0.4	1
Ammonium sulphate precipitation	132	1.3	3
ConA sepharose/Hydroxylapatite	2.9	19.7	49
Alkyl sepharose	1.0	23.6	59
MonoQ	0.06	40.2	100

ity (visible in chromatograms by the appearance of fructose) was negligible, accounting for less than 0.1% of the 1-SST activity. All 1-SST isoforms obtained by IEF had similar enzymatic properties.

When the enzyme assay with purified 1-SST preparation was run for a prolonged period of time (24 h), most of the sucrose disappeared and was converted into isokestose; fructose and nystose (the inulin tetramer) as well as traces of the inulin pentamer were also detected (Fig. 2b). Fructose, sucrose, nystose and the inulin pentamer were also formed when purified 1-SST was incubated with 100 mM isokestose (data not shown). Thus, under these conditions, the preparation exhibits some fructan exohydrolase (β-fructosidase) and fructan-fructan 1-fructosyltransferase (1-FFT) activity (ca. 11% and 19% of the 1-SST activity with sucrose, data not shown). Although these activities might be due to contaminating enzymes, we consider it highly likely that they are genuine activities of 1-SST since they were exhibited by all five isoforms, separated by IEF. The β-fructosidase activity of 1-SST was apparent only with isokestose and not with sucrose, its preferred substrate. This is reminiscent of the properties of barley 6-SFT, which exhibits an appreciable β-fructosidase activity only in the absence of its preferred fructosyl acceptor substrates (isokestose or other fructans) [17,21].

As expected [12], purified 1-FFT yielded sucrose and nystose (and subsequently higher oligomers of the inulin series) when incubated with isokestose; it was devoid of activity when incubated with sucrose (data not shown).

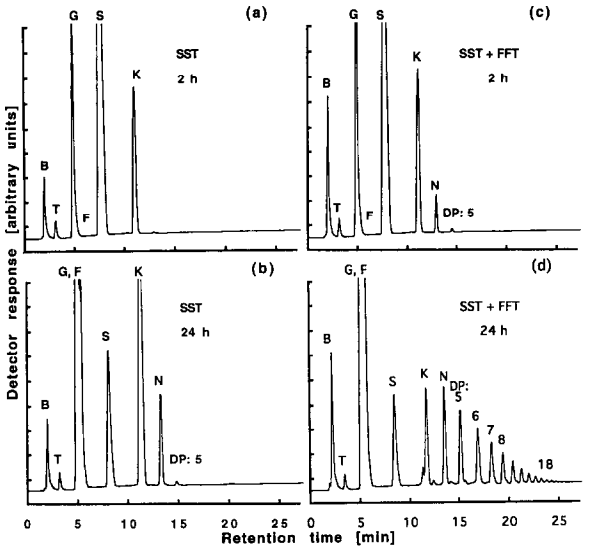

Fig. 2. Products formed from sucrose by purified sucrose-sucrose 1-fructosyltransferase (1-SST) alone or in combination with purified fructan-fructan 1-fructosyltransferase (1-FFT). 1-SST (0.1 nkat) was incubated with 100 mM sucrose alone (a,b) or in combination with 1 nkat 1-FFT (c,d) for 2 h (a,c) and 24 h (b,d) at 25°C. Peaks in the HPLC chromatograms: B, buffer; T, trehalose (internal standard); G, glucose; F, fructose; S, sucrose; K, isokestose; N, nystose; 5–18, inulin oligofructans with degrees of polymerization 5–18.

3.4. *Inulin production by a combination of 1-SST and 1-FFT*

The model of Edelman and Jefford [10] was tested using combinations of purified 1-SST and 1-FFT (Fig. 2c,d). In the presence of sucrose (100 mM), the combination of purified 1-FFT and purified 1-SST rapidly produced substantial amounts of nystose and traces of the inulin pentamer after only 2 h of incubation at 25°C (Fig. 2c). These products were formed when the concentration of isokestose relative to that of sucrose was still low (≤0.1; i.e. isokestose ≤10 mM and sucrose ≥90 mM, see Fig. 2c). After prolonged incubation (24 h), inulin oligomers up to a degree of polymerization of 20 were detected (Fig. 2d). Similar results were obtained when only 50 mM sucrose was supplied, i.e. with a concentration that may occur in vivo (data not shown).

These experiments with combined enzymes clearly demonstrate that the affinity of the 1-FFT for isokestose and higher inulin polymers as fructosyl acceptor substrates is sufficient to allow de novo inulin synthesis from sucrose even in the presence of relatively high concentrations of sucrose, a known, potent fructosyl acceptor substrate for 1-FFT [10]. Taken together the results validate the classic model of Edelman and Jefford [10], and they show that the combination of 1-SST and

Table 2. Comparison of peptide sequences of 1-SST and 1-FFT from *Helianthus tuberosus* (H.t. 1SST and H.t. 1FFT) with deduced amino acid sequences from sucrose-fructan 6-fructosyltransferase of *Hordeum vulgare* (H.v. 6SFT) [16], vacuolar acid β-fructosidase from *Daucus carota* (D.c. aInv2) [18], cell wall β-fructosidase from *Daucus carota* (D.c. cwInv) [19] and vacuolar acid β-fructosidase from *Vigna radiata* (V.r. aInv) [20]

	84 91	187 201	220 234
H.v. 6SFT	GYHFQTAK	ATPADPNDPLLRRWT	FRDPMTAWYDESDET
D.c. aInv2	SFHFQPNQ	AYPADPSDPLLIEWV	FRDPTTAWRTPEGKW
D.c. cwInv	GYHFQPKQ	AIPANLSDPYLRKWI	FRDPTTAWLDKSGYW
V.r. aInv	SFHFQPEK	AYPADPSDPLLLDWI	FRDPTTAWLTSEGKW
H.t. 1SST	TYHFQPDD		FRDPSTLWLGPDGEY
H.t. 1FFT		AVPVxLxxPLFIxWV	

	327 340	347 358	397 413
H.v. 6SFT	WTPIDPELDLGIGL	FYASTSFYDPAK	TRTNLLLWPVEEIETLR
D.c. aInv2	WVPDNPELDVGIGL	YYASKTFYDSNK	TGSNLLQWPVEEVNKLR
D.c. cwInv	YIPDNTSVDGWAGL	FYASKTFFDPSK	SGKQLMQWPIEELETLR
V.R. aInv	FTPDDVKNDVGVGL	FYASKTFYDQNK	TGSNLLQWPVDEVESLR
H.t. 1SST		FYASKTFYDQHK	TNLIQWPIEETEHLR
H.t. 1FFT	WTPDNPELDVGIGL		xGTHLLHWPVEEVxx

Numbers refer to the position within the deduced barley 6-SFT protein sequence. Bold letters: identity of one of the *H. tuberosus* peptide sequences with at least one other enzyme sequence. Bold, underlined letters: identity between the *H. tuberosus* sequences and all the other enzymes. x: unidentified amino acid.

1-FFT is both necessary and sufficient to allow de novo synthesis of inulin from sucrose in *H. tuberosus*.

Acknowledgements: We wish to thank Dr. M.A. Collinge (Friedrich Miescher-Institute, Basel) for critical reading of the manuscript, Dr. P. Jenö (Biozentrum, University of Basel) for peptide sequencing and Ms. E. Roth for typing the manuscript. This work was supported by the Swiss National Science Foundation, Grant 31-42535.94.

References

[1] Hendry, G.A.F. (1993) New Phytol. 123, 3–14.
[2] Schnyder, H. (1993) New Phytol. 123, 233–245.
[3] Lewis, D.H. (1993) New Phytol. 124, 583–594.
[4] Housley, T.L. and Pollock, C.J. (1993) in: Science and Technology of Fructans, pp. 191–225 (Suzuki, M. and Chatterton, N.J., Eds.) CRC Press, Boca Raton, FL.
[5] Wiemken, A., Sprenger, N. and Boller, T. (1995) in: International Symposium on Sucrose Metabolism, pp. 179–189 (Pontis, H.G., Salerno, G.L. and Echeverria, E.J., Eds.) ASPP Press, Rockville.
[6] Bieleski, R.L. (1993) Plant Physiol. 103, 213–219.
[7] Pontis, H.G. (1989) Plant Physiol 134, 148–150.
[8] Pilon-Smits, E.A.H., Ebskamp, M.J.M., Paul, M.J., Jeuken, M.J.W., Weisbeek, P.J. and Smeekens, S.C.M. (1995) Plant Physiol. 107, 125–130.
[9] Fuchs, A. (1991) Biochem. Soc. Trans. 19, 555–560.
[10] Edelman, J. and Jefford, T.G. (1968) New Phytol. 67, 517–531.
[11] Cairns, A.J. (1993) New Phytol. 123, 15–24.
[12] Lüscher, M., Frehner, M. and Nösberger, J. (1993) New Phytol. 123, 437–442.
[13] Koops, A.J. and Jonker, H.H. (1994) J. Exp. Bot. 45, 1623–1631.
[14] Lüscher, M. and Nelson, C.J. (1995) Plant Physiol. 107, 1419–1425.
[15] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[16] Sprenger, N., Bortlik, K., Brandt, A., Boller, T. and Wiemken, A. (1995) Proc. Natl. Acad. Sci. USA 92, 11652–11656.
[17] Simmen, U., Obenland, D., Boller, T. and Wiemken, A. (1993) Plant Physiol. 101, 459–468.
[18] Unger, C., Hardegger, M., Lienhard, S. and Sturm, A. (1994) Plant Physiol. 104, 1351–1357.
[19] Sturm, A. and Chrispeels, M.J. (1990) Plant Cell 2, 1107–1119.
[20] Arai, M., Mori, H. and Imaseki, H. (1992) Plant Cell Physiol. 33, 245–252.
[21] Duchateau, N., Bortlik, K., Simmen, U., Wiemken, A. and Bancal, P. (1995) Plant Physiol. 107, 1249–1255.