Properties of Fructan:Fructan 1-Fructosyltransferases from Chicory and Globe Thistle, Two Asteracean Plants Storing Greatly Different Types of Inulin

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Remarkably, within the Asteraceae, a species-specific fructan pattern can be observed. Some species such as artichoke (Cynara scolymus) and globe thistle (Echinops ritro) store fructans with a considerably higher degree of polymerization than the one observed in chicory (Cichorium intybus) and Jerusalem artichoke (Helianthus tuberosus). Fructan:fructan 1-fructosyltransferase (1-FFT) is the enzyme responsible for chain elongation of inulin-type fructans. 1-FFTs were purified from chicory and globe thistle. A comparison revealed that chicory 1-FFT has a high affinity for sucrose (Suc), fructose (Fru), and 1-kestose as acceptor substrate. This makes redistribution of Fru moieties from large to small fructans very likely during the period of active fructan synthesis in the root when import and concentration of Suc can be expected to be high. In globe thistle, this problem is avoided by the very low affinity of 1-FFT for Suc, Fru, and 1-kestose and the higher affinity for inulin as acceptor substrate. Therefore, the 1-kestose formed by Suc:Suc 1-fructosyltransferase is preferentially used for elongation of inulin molecules, explaining why inulins with a much higher degree of polymerization accumulate in roots of globe thistle. Inulin patterns obtained in vitro from 1-kestose and the purified 1-FFTs from both species closely resemble the in vivo inulin patterns. Therefore, we conclude that the species-specific fructan pattern within the Asteraceae can be explained by the different characteristics of their respective 1-FFTs. Although 1-FFT and bacterial levansucrases clearly differ in their ability to use Suc as a donor substrate, a kinetic analysis suggests that 1-FFT also works via a ping-pong mechanism.

Although most plants store insoluble starch as a reserve polysaccharide in the amyloplast, several plant families store soluble polymers of Fru in the vacuole. Inulin, the simplest of these fructans, occurs in plants belonging mainly to the dicotyledonous plant families Asteraceae, Campanulaceae, and Boraginaceae (Hendry, 1993). It consists of one α-Glc and a variable number of β-Fru moieties exclusively linked by 2–1 bounds to each other (Lewis, 1993). The concentration and degree of polymerization (DP) of the inulins stored varies between species. Inulin can reach about 20% of the fresh weight (roughly 80% of the dry weight) in chicory (Cichorium intybus) taproots, making this plant useful for commercial production and extraction of inulin (Schittenhelm, 1996). The inulin stored in chicory has a rather low mean DP of about 10 to 15 (Wilson et al., 1999). Higher DP inulins are found, for example, in artichoke (Cynara scolymus; Hellwege et al., 2000), globe thistle (Echinops ritro), and Viguiera discolor (Itya et al., 1997).

It has been shown by different groups (for review, see Van Laere and Van den Ende, 2002) that inulin can be synthesized in vitro from Suc by the combined action of Suc:Suc 1-fructosyltransferase (1-SST) [G-F + G-F → G + G-F-F] and fructan:fructan 1-fructosyltransferase (1-FFT) [G-Fm+n + G-Fn, ↔ G-F(m+1) + G-F(n+1)] with m > 1 and n ≥ 1. The validity of this model in vivo has been corroborated by simultaneous expression of heterologous 1-SST and 1-FFT transforming potato (Solanum tuberosum) into an inulin-producing crop (Hellwege et al., 2000).

Although the DP of inulin produced in vitro from Suc can be influenced by the ratio of 1-SST to 1-FFT in the reaction mixture (Van den Ende and Van Laere, 1996a,b), it can be expected that also the properties of the 1-FFT enzymes involved are responsible for the variation in DP found in different plants (Hellwege et al., 1998, 2000).

Several plant 1-FFT enzymes have been purified and characterized. A general feature of 1-FFT enzymes is their inability to use Suc as a donor substrate (Edelman and Jefford, 1968; Jeong and Housley, 1992; Koops and Jonker, 1994; Lüscher et al., 1993, 1996; Van den Ende et al., 1996b; St. John et al., 1997), clearly distinguishing them from 1-SST, Suc:fructan 6-fructosyl transferase (6-SFT), invertases, and bacterial levansucrases. A number of 1-FFT cDNAs have been cloned (Hellwege et al., 1998; van der Meer et al., 1998; Goblet JP, Canon L, Van Cutsem PJ, unpublished data; Kawakami et al., 2002).

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Due to the complicating fact that both products of 1-FFT are also substrates (and vice versa), analyzing the enzymatic mechanism of plant 1-FFTs is a challenging task. Chicory 1-FFT uses 1-kestose and inulin as preferential donors, whereas inulin and Suc are the best acceptors. 1,1 nystose and DP5 seem to be worse donor and acceptor substrates (Van den Ende et al., 1996b). Fru can also be used as an acceptor substrate to produce inulo-\(n\)-oses (Van den Ende et al., 1996a). However, the enzymatic mechanism of the 1-FFT reaction was not studied. The reaction mechanism of bacterial levansucrases was more thoroughly investigated (Dedonder, 1972; Chambert et al., 1974; Song and Jacques, 1999). Essentially, the enzyme interacts with the donor substrate to form a Michaelis-type complex. Subsequently, the terminal Fru is hydrolyzed from the donor substrate, which results in a product that dissociates, whereas the fructosyl unit remains attached to the enzyme. Finally, the acceptor substrate binds to the enzyme and the fructosyl unit is transferred to the acceptor substrate.

The main goal of this manuscript is dual: to show that the difference in DP between a small DP (chicory) or a large DP (globe thistle) species can be explained by the characteristics of their respective 1-FFTs and to verify for the first time whether a plant fructosyltransferase works via a ping-pong mechanism, just like bacterial levansucrases that, in severe contrast to 1-FFT, use Suc as a fructosyl donor.

RESULTS AND DISCUSSION

Fructan Patterns in Chicory and Globe Thistle

Figure 1A shows that an extract from chicory roots shows a regular pattern of increasing DP inulins: 1-kestose shows the highest peak, whereas subsequent inulin peaks gradually decrease and almost disappear (in longer chromatograms) around a DP of 60 (late summer plants harvested before cold). Similar patterns are found in many dicotyledonous species such as Jerusalem artichoke (Helianthus tuberosus; Koops and Jonker, 1994), dandelion (Taraxacum officinale; Van den Ende et al., 2000), Campanula rapunculoides (Vergauwen et al., 2000), and many others (R. Vergauwen, A. Van Laere, and W. Van den Ende, unpublished data). The pattern in roots of globe thistle is completely different (Fig. 1B). All peaks of fructans with a DP > 5 are roughly similar in height, but higher DP fructans only elute during regeneration of the column with 500 mM Na-acetate (Fig. 1B, arrow). The fructan identity of this latter peak was confirmed by mild acid hydrolysis: only Fru and traces of Glc were detected. The mean DP of total soluble sugars (as estimated from the Fru/Glc ratio after subtracting the Glc and Fru already present and originating from Suc), was around 10 and 30 for full-grown roots of chicory and globe thistle, respectively. Moreover, hydrolysis of the total soluble sugars derived from both species revealed roughly equal content of bound Fru. It was estimated that no less than 92% (w/w) of the bound Fru in globe thistle was present in fructans with a DP > 5.

Depending on the developmental stage, sometimes an alternative series of fructans can be detected in chicory (Fig. 1A; Van den Ende et al., 1996a). Chicory 1-FFT can also use Fru as a fructosyl acceptor and can produce, reducing low DP fructans without the terminal Glc. These low DP inulo-\(n\)-oses elute as an alternative series with slightly different periodicity during AEC-PAD (Van den Ende et al., 1996a). Even...
a third series, resulting from the transfer to the 6-OH of free Fru, was demonstrated in chicory under certain circumstances (Timmermans et al., 2001). In globe thistle, these low DP inulono-ose series were less prominent (Fig. 1B).

**Purification of 1-FFT from Chicory and Globe Thistle**

Chicory 1-FFT was purified as described (Van den Ende et al., 1996b). A similar purification scheme using ammonium sulfate precipitation, lectin affinity chromatography (concanavalin A [Con A]), and AEC was followed to purify 1-FFT from globe thistle (Table I). Activity losses occurred after each purification step, especially after Mono Q. A rather poor purification of 14-fold and a specific activity of 130 mU mg⁻¹ protein, which is much lower than the one observed for chicory 1-FFT (Van den Ende et al., 1996b) was obtained. However, in the presence of inulin as a substrate, the 1-FFT from globe thistle was stable for at least 3 weeks at 0°C (not shown). Both 1-FFT’s are shown on SDS-PAGE (Fig. 2). Both enzymes were nearly pure and tested negative for 1-SST and invertase activities. Like chicory 1-FFT, globe thistle 1-FFT appears as a heterodimer with subunits of about 52 and 17 kD (Fig. 2).

**Kinetic Analysis of Chicory 1-FFT**

As mentioned in the introduction, kinetic analysis of 1-FFT reactions is a complicated matter. One of the simplest reactions that can be catalyzed by the enzyme is the transfer of Fru from inulin (donor substrate) to Suc (acceptor substrate). Suc can only act as an acceptor in this system (Van den Ende et al., 1996b). Nevertheless, even then theoretically the following reactions can occur:

\[
\text{Donor} + \text{Acceptor} \rightarrow \text{Product 1} + \text{Product 2}
\]

Only reaction 1 can be measured adequately because the addition or subtraction of a Fru moiety from polydisperse inulin molecules hardly produces a different chromatographic pattern. The relative importance of reaction 2 can roughly be estimated from the putative inhibition of reaction 1 at higher inulin concentrations.

For all reactions, different time points were analyzed and only data from the linear range were used. Incubation times were kept short to avoid that the 1-kestose produced affected the reaction not only as a product, but also as an alternative donor and acceptor substrate. With Suc concentrations varying from 0.1 to 300 mm and inulin concentrations between 0.1 and 10 mm, Suc (Fig. 3A) but not inulin (Fig. 3B) did inhibit reaction 1. Therefore, it can be concluded that reaction 2 is negligible in vitro over the range of inulin concentrations tested. Because inulin already starts to precipitate at 10 mm in vitro, inhibition of reaction 1 at the higher inulin concentrations found in vivo cannot be excluded. Apparently Suc, which is only an acceptor in the reaction (Van den Ende et al., 1996b), can also compete with the donor substrate (Fig. 3A).

Only in the region were Suc was not clearly inhibiting the reaction did plots of 1/reaction velocity (v) against 1/[Suc] at different inulin concentrations yield straight and parallel lines (Fig. 4A). Similarly, parallel lines were found when plotting 1/v against 1/[inulin] at different Suc concentrations (Fig. 4B). In the case where donor and acceptor substrates are clearly different, and the donor substrate is not competitive with the acceptor substrate or vice versa, the v can be described by the following equation:

\[
v = \frac{V_{\text{max}} \times D \times A}{K_d \times A + K_a \times D + D \times A}
\]

with D as the concentration of donor substrate (i.e. inulin), A is the concentration of acceptor substrate (i.e. Suc), K_d is the K_m of the donor substrate, and K_a is the K_m of the acceptor substrate.

**Table 1. A typical purification of 1-FFT from 0.6 kg of 5-month-old globe thistle roots**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Activity (mU)</th>
<th>Recovery (%)</th>
<th>Specific Activity (mU mg⁻¹ protein)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>208</td>
<td>1,936</td>
<td>100</td>
<td>9.3</td>
<td>1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ 30–75%</td>
<td>17</td>
<td>905</td>
<td>47</td>
<td>53</td>
<td>5.7</td>
</tr>
<tr>
<td>ConA</td>
<td>6.7</td>
<td>650</td>
<td>34</td>
<td>97</td>
<td>10</td>
</tr>
<tr>
<td>Mono Q pH 6.0</td>
<td>0.54</td>
<td>70</td>
<td>4</td>
<td>130</td>
<td>14</td>
</tr>
</tbody>
</table>

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this does not necessarily follow from the kinetic mechanism, which only indicates that a product must dissociate before the acceptor substrate forms a Michaelis complex with the enzyme. Although an intermediate was reported to be purified once (Dedonder, 1972), no covalent enzyme-fructosyl intermediate has been fully characterized for any fructosyltransferase. A ping-pong mechanism would imply the following reactions for 1-FFT (e.g. self transfer from 1-kestose to 1-kestose):

\[
\begin{align*}
\text{Enzyme} + \text{G-F-F} & \rightarrow \text{Enzyme} \\
& \approx \text{G-F-F (1-kestose binds as a donor)} \\
\text{Enzyme} \approx \text{G-F-F} & \rightarrow \text{Enzyme-F} + \text{G-F} \\
& \text{(Suc dissociates; intermediate is formed)} \\
\text{Enzyme-F} + \text{G-F-F} & \rightarrow \text{Enzyme-F} \approx \text{G-F-F} \\
& \text{(1-kestose binds as an acceptor)} \\
\text{Enzyme-F} \approx \text{G-F-F} & \rightarrow \text{Enzyme} + \text{G-F-F-F} \\
& \text{(fructosyl transfer; 1,1-nystose formation)}
\end{align*}
\]

The ping-pong mechanism was also proposed by Song and Jacques (1999) for a levansucrase from Streptococcus salivarius American Type Culture Collection 25975 and by Chambert et al. (1974) for Bacillus subtilis levansucrase. However, in contrast to the results presented in Figure 4, no parallel lines were found by Chambert et al. (1974) when plotting 1/v against 1/[levan] at different Suc concentrations and plotting 1/v against 1/[Suc] at different levan concentrations. This discrepancy can be explained by the levan concentration range used (7–28 mM), which was high enough to partially inhibit the Fru transfer from Suc to levan (Fig. 20 in Chambert et al., 1974).

In this way, parallel lines in double reciprocal plots were lost by competitive inhibition of levan as a donor substrate (Cleland, 1979).

For chicory 1-FFT, \( K_d/V_{\text{max}} \) and \( K_a/V_{\text{max}} \) can be estimated from the slopes in Figure 4. Robust average values of 0.004028 ± 0.0007 for \( K_a/V_{\text{max}} \) and 0.01534 ± 0.00075 for \( K_d/V_{\text{max}} \) were obtained. The intercepts obtained on the 1/v axis can further be plotted against 1/inulin (Fig. 5A) or 1/Suc (Fig. 5B) producing an independent concurring estimate of \( K_d/V_{\text{max}} \) and \( K_d/V_{\text{max}} \) of 0.0035 and 0.0143, respectively. Estimates of \( V_{\text{max}} \) from these data would be based on one intercept with very small 1/v value yielding huge uncertainties. It was not possible to make more robust estimates of \( V_{\text{max}} \) using higher inulin and/or Suc concentrations because inulin already starts to precipitate at 10 mM, and high Suc concentrations inhibit the reaction (Fig. 3A).

The inhibition by high Suc concentrations was also estimated by adapting the above equation according to Cleland (1979):

\[
v = \frac{V_{\text{max}} \times D \times A}{K_d \times (1 + A/K_d) \times A + K_a \times D + D \times A}
\]

with \( K^i_d \) as the competitive inhibition constant of Suc. A theoretical curve with a \( V_{\text{max}} \) of 6,600 mU 1-kestose mg\(^{-1}\) protein yielded a good visual fit with the experimental data when \( K^i_d \) was chosen eight times higher than \( K_a \) (Fig. 3). In combination with the experimentally determined \( K/V_{\text{max}} \) values, this yields the following Michaelis constants: \( K_a \approx 25 \) mM, \( K_d \approx 100 \) mM, and \( K^i_d \approx 200 \) mM.

From these reaction kinetics, we can conclude that chicory 1-FFT has three to four times higher affinity for Suc (at the acceptor site) than for inulin (at the donor site). Although it is not a donor substrate, Suc can compete with inulin at the donor site with about 10 times less affinity. We were unable to estimate \( K_m \) correctly because \( V_{\text{max}} \) cannot be measured accurately.

In Vitro Inulin Biosynthesis by Purified Chicory and Globe Thistle 1-FFTs

Figure 6 shows chromatograms of reactions mixtures of 1-kestose and purified globe thistle (Fig. 6A) or chicory 1-FFT (Fig. 6B) as a function of time. The globe thistle 1-FFT produces high peaks of 1,1 nystose and DP5 as well as much lower peaks of higher
DP inulins. Some fructan with a DP exceeding 18 (termed high DP inulin, see arrow in Fig. 6A) eluted with 500 mM Na-Ac. The chicory 1-FFT only produces smaller inulin with a maximal DP of 14 under these conditions. With both enzymes, at the end of the incubation a similar amount of Suc was formed, suggesting an equal total amount of Fru transfers. Overall, the patterns obtained in vitro with the purified 1-FFT enzymes correlate well with the in vivo inulin patterns in their respective plants (Fig. 1), although the maximal DP reached in vivo might be higher. However, our preliminary experiments demonstrate that prolonged incubations and addition of new 1-kestose substrate allow for the production of higher amounts of high DP fructans, until the saturation point is reached and fructans start to precipitate in vitro. It can be concluded that the different inulin patterns in Asteraceae at the end of the period of active biosynthesis can be explained by the characteristics of their 1-FFTs. A substantial role for 1-FEH in determining the DP and final pattern at the end of the period of active biosynthesis is very unlikely because 1-FEH activities are generally very low compared with the activities of fructan biosynthetic enzymes during the period of active biosynthesis in Asteraceae (Van Laere and Van den Ende, 2002).

By using higher concentrations of the much cheaper commercially available substrate neosugar (Fig. 7A), the globe thistle 1-FFT produced higher peaks of high DP inulin, allowing for manual collection and subsequent mild acid hydrolysis (Fig. 7B). This resulted in an estimated mean DP of 32 and 40, after 93 and 170 h of incubation, respectively. The addition of α-glucosidase to the reaction prevented the accumulation of huge amounts of Suc and was beneficial for faster high DP fructan accumulation (not shown). Despite the fact that Suc is a relatively bad acceptor substrate for the globe thistle 1-FFT (see below), very high Suc concentrations act as a competitive inhibitor for the enzyme. Like all other 1-FFTs (see Introduction), it was verified that Suc cannot be used as a donor substrate by the globe thistle 1-FFT (not shown).

**Figure 3.** Activity of chicory 1-FFT, as measured by the 1-kestose formation with Suc as acceptor and inulin as donor (incubation time 30 min). A, As a function of Suc concentration at 0.1 mM (□), 0.34 mM (▲), 1 mM (●), 3.3 mM (□), or 10.2 mM (△) inulin; B, as a function of inulin concentration at 0.1 mM (●), 0.3 mM (■), 1 mM (▲), 3 mM (●), 10 mM (□), 30 mM (△), 100 mM (●), or 300 mM (▲) Suc. Curves (dashed at 100 and 300 mM Suc for clarity in B) are not an interpolation based on experimental data but are the result of the theoretical model using the following parameters: 6,600 mU 1-kestose (mg protein⁻¹) as $V_{\text{max}}$ and 25, 100, and 200 mM as $K_a$, $K_d$, and $K_{ic}$, respectively. The $V_{\text{max}}$ was arbitrarily chosen to yield an optimal visual fit to the experimental data.
Comparison of the Kinetic Properties of Chicory and Globe Thistle 1-FFT

The purified isoform of globe thistle 1-FFT has a lower affinity for Suc as acceptor (Fig. 8A) and a higher affinity for inulin as donor (Fig. 8B) than chicory 1-FFT. Also Fru is a much less efficient acceptor for globe thistle than for chicory 1-FFT (Fig. 8C), probably explaining why low DP inulo-\(\alpha\)-oses accumulate to a higher extent in chicory (Fig. 1). The kinetic behavior of globe thistle 1-FFT was further analyzed with 1-kestose and inulin as substrates and compared with chicory 1-FFT. Four reactions can occur under these conditions.

Because monodisperse inulin is not available, we can only quantitatively measure the formation of Suc (product of reactions 1 and 2) and 1,1-nystose (product of reactions 2 and 3), and it is impossible to determine the exact kinetic parameters of the reactions. Reaction 4 cannot be measured at all. The only information we can derive, is the fructosyl transfer from 1-kestose (is equal to the Suc production) and the fructosyl transfer to 1-kestose (equals the 1,1-nystose production).

Assuming that the transfer to Suc is minimal (very short incubation times), the transfer from 1-kestose to inulin (Suc formed minus 1,1-nystose formed) in-
creases with increasing inulin concentration at a constant 1-kestose concentration of 5 mM (Fig. 9A). The globe thistle 1-FFT has a higher affinity for inulin than the chicory 1-FFT. The transfer from 1-kestose, as measured by the Suc formation, is inhibited by higher inulin concentrations (Fig. 9B), indicating that inulin is starting to act as a donor substrate too (reaction 4). Apparently, the higher the affinity is for inulin as acceptor substrate, the higher the affinity for inulin is as donor substrate. Furthermore, for globe thistle 1-FFT, the addition of only small amounts of inulin to 5 mM 1-kestose leads to a greater increase of the Fru transfer from 1-kestose (Suc production) as compared with chicory 1-FFT. This indicates that 1-kestose is a rather poor acceptor substrate for globe thistle 1-FFT (but not so for chicory 1-FFT). A small hydrolytic activity (Fru production) is detected at low substrate concentration (Fig. 9B).

At a constant inulin concentration of 3 mM, the percentage of fructosyl transfer from 1-kestose to inulin decreases with increasing 1-kestose concentrations. This decrease is more pronounced with chicory 1-FFT (Fig. 9C) than with the globe thistle 1-FFT (Fig. 9B). Although the transfer to inulin decreased to 25% for chicory 1-FFT at 100 mM 1-kestose, it only decreased to 75% for globe thistle 1-FFT. This further indicates that compared with 1-kestose, inulin is a much better acceptor substrate for globe thistle 1-FFT than it is for chicory 1-FFT.

Conclusion and Perspectives

For the first time, it was demonstrated that a plant fructosyltransferase works via a ping-pong mechanism, just like bacterial levansucrases, although 1-FFT and levansucrases use a different fructosyl donor substrate (fructan versus Suc).

Research with transgenic plants harboring different 1-FFTs showed that the properties of the 1-FFT enzymes involved are probably responsible for the
Figure 6. In vitro inulin synthesis with purified globe thistle 1-FFT (A, 100 μg mL⁻¹) and chicory 1-FFT (B, 100 μg mL⁻¹) starting from 10 mM 1-kestose. The different reaction times are indicated. G, Glc; F, Fru; S, Suc; K, 1-kestose; N, 1,1-nystose. The arrow indicates high DP inulin formation by globe thistle 1-FFT in vitro.

Figure 7. A, In vitro inulin synthesis with purified globe thistle 1-FFT (100 μg mL⁻¹) starting from 160 mg mL⁻¹ neosugar. Yeast α-glucosidase was also added (120 mU mL⁻¹). The different reaction times are indicated. G, Glc; F, Fru; S, Suc; K, 1-kestose; N, 1,1-nystose. The identity of the compound eluting after Suc is unknown. The arrow indicates high DP inulin formation by globe thistle 1-FFT in vitro. B, Mild acid hydrolysis of the manually collected high DP inulin peaks from A. G, Glc; F, Fru.
DP variation found (Hellwege et al., 2000). The results presented here further confirm this hypothesis by an in-depth kinetic analysis of chicory and globe thistle 1-FFT. As observed in chicory and other Asteracean dicots (Van Laere and Van den Ende, 2002), no role for 1-FEH determining the DP during the period of active inulin biosynthesis is expected. However, the situation seems different in monocots where FEHs and fructan biosynthetic enzymes are coexpressed during the period of active fructan synthesis (Van den Ende et al., 2003).

The high affinity of chicory 1-FFT for Suc, Fru and 1-kestose as acceptor makes redistribution of Fru moieties from large to small fructans very likely during the period of active fructan synthesis when import and concentration of Suc can be expected to be high. High 1-SST activity might help in keeping Suc concentration low and higher DP-inulin production high. In globe thistle, this problem is avoided by the very low affinity of 1-FFT for Suc and the high affinity for inulin resulting in an almost complete use of the 1-kestose formed by 1-SST for elongation of already relatively long inulin molecules. It will be interesting to clone the globe thistle 1-FFT and compare the amino acid sequence and the three-dimensional structure of the chicory and globe thistle 1-FFT enzymes to understand the different affinities and behavior of these and perhaps other fructosyltransferases.

It is clear that high DP fructans will contribute much less to the osmotic potential of a tissue. Because we hardly understand the physiological and ecological significance of fructans versus Suc or starch as a carbohydrate reserve, the difference between high- and low-DP fructans in this respect remains even more elusive.

MATERIALS AND METHODS

Plant Material and Sampling

Chicory (Cichorium intybus var sativum cv Orches) and globe thistle (Echinops ritro) were sown in a local field with sandy, loamy soil in April during the growing seasons of 2001 and 2002. In analogy with chicory roots, roots were harvested in September before 1-FEH could be induced by cold. Samples were used for carbohydrate analyses and for enzyme purification purposes.

Purification of 1-FFTs

1-FFT from chicory roots (harvested in September) was purified as described (Van den Ende et al., 1996b). 1-FFT from globe thistle was purified using a similar procedure. The roots (0.6 kg) were harvested in September, at the stage of maximal DP, when 1-SST activity was low and 1-FEH activity was not yet induced. Roots were washed, cut in small pieces, and homogenized in 600 mL of 50 mM Na-acetate buffer, pH 5, containing 1 mM Na-EDTA, 10 mM NaF, 1 mM mercaptoethanol, and 0.1% (w/v) Polyclar AT (Serva, Heidelberg). The homogenate was squeezed through cheese-cloth. Ammonium sulfate was added to the supernatant to a saturation of 30% (w/v) and was gently stirred on ice for 30 min. After centrifugation for 20 min, precipitated protein was discarded. Again ammonium sulfate was added to the supernatant to a saturation of 80% (w/v). After a second centrifugation (20 min at 40,000g and 4°C), the precipitate was collected and redissolved in 150 mL of 50 mM Na-acetate buffer, pH 5.0. Undissolved material was spun down for 15 min at 40,000g and 4°C. The supernatant was applied to a Sepharose column (25×100 mm) equilibrated with 50 mM Na-acetate, pH 5.0, containing 1 mM CaCl₂, MnCl₂, and MgCl₂. The column was washed with the same buffer until the absorbance at 280 nm was less than 0.1. Bound proteins were eluted with 50 mL of 1 mM methyl α-D-mannopyranoside in 50 mM Na-acetate buffer, pH 5.0. Fractions were adjusted to pH 6.0 with concentrated HCl and were applied on a Mono Q anion-exchange column (Pharmacia HR 5/5; Pharmacia, Uppsala), which was equilibrated with 20 mM His-HCl buffer, pH 6.0. Proteins were eluted using a linear gradient from 0 to 0.3 M NaCl in 30 min.
and Glc liberation. Subsequently, molar inulin concentration was estimated based on this mean DP ratio after mild acid hydrolysis in 60 mM HCl at 70°C for 75 min. Subsequently, molar inulin concentration was estimated based on this mean DP and Glc liberation.

**Carbohydrate Analyses and Enzyme Activity Determinations**

Carbohydrates, as well as products of enzymatic reactions, were analyzed by AEC-PAD (Dionex, Sunnyvale, CA) as described previously (Van den Ende et al., 1998). Overall, incubation conditions for 1-FFT activity were in 60 mM Na-MES buffer, pH 6.25, and 0°C. When incubated at higher temperatures, chicory and globe thistle 1-FFT showed some 1-FEH activity (for details of the chicory 1-FFT, see Van den Ende et al., 1996b). The reaction was started by adding a suitable amount (5 μg mL⁻¹) for kinetic analyses and 100 μg mL⁻¹ for kinetic synthesis of purified enzyme to the reaction mixture. We refer to the figure legends for details on incubation times used. The reaction was stopped, in general after different periods of incubation, by keeping an aliquot for 5 min in a boiling water bath. Different concentrations and/or binary combinations of Fru, Suc, 1-kestose, inulin, or neosugar were used as substrates. Fructosyl transfer between inulin and Suc was measured as a function of Suc concentration (0.1, 0.3, 1, 3, 10, 30, 100, and 300 mM) at 0.1, 0.34, 1, 3.3, and 10.2 mM inulin. Overall, purified enzymes remained stable in the reaction mixtures for many days, as was seen from the linearity of Suc production from 1-kestose as a function of time. The experiments were repeated at least three times with consistent results.
Fructan 1-Fructosyltransferases from Two Plants Storing Different Inulin


