Purification and enzymic properties of the fructosyltransferase of *Streptococcus salivarius* ATCC 25975

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The recombinant fructosyltransferase (Ftf) of *Streptococcus salivarius* was expressed in *Escherichia coli* and purified to electrophoretic homogeneity after a combination of adsorption, ion-exchange and gel-filtration chromatography. The N-terminal signal sequence of the Ftf was removed by *E. coli* at the same site as in its natural host. The purified Ftf exhibited maximum activity at pH 6.0 and 37 °C, was activated by Ca^{2+}, but inhibited by the metal ions Cu^{2+}, Zn^{2+}, Hg^{2+} and Fe^{3+}. The enzyme catalysed the transfer of the fructosyl moiety of sucrose to a number of acceptors, including water, glucose and sucrose via a Ping Pong mechanism involving a fructosyl–enzyme intermediate. While this mechanism of catalysis is utilized by the levansucrases of *Bacillus subtilis* and *Acetobacter diazotrophicus* and the values of the kinetic constants for the three enzymes are similar, sucrose was a far more efficient fructosyl-acceptor for the Ftf of *S. salivarius* than for the two other enzymes.

Key words: fructan, kinetic analysis, levansucrase, Ping Pong mechanism, transfructosylase.

INTRODUCTION

We previously reported the cloning and sequencing of the β-D-fructosyltransferase (Ftf) from *Streptococcus salivarius* ATCC 25975 [1]. The Ftf of *S. salivarius*, along with that of *Streptococcus mutans* [2] and the levansucrases (sucrose: 2,6-β-D-fructan 6-β-D-fructosyltransferase; EC 2.4.1.10) of bacilli [3–6], *Erwinia herbicola* [7], *Pseudomonas syringae* [8], *Rhanella aquatilis* [9], *Zymomonas mobilis* [10], *Acetobacter diazotrophicus* [11] and *Aerobacter levanicum* [12,13], form a family of enzymes that synthesize fructans from sucrose with either a levan or inulin structure [14]. In the case of the oral streptococci, Ftfs are considered important virulence proteins, since the fructans they synthesize may enhance the cariogenicity of dental plaque [14,15]. Fructans formed during the ingestion of sucrose are rapidly hydrolysed in the absence of dietary carbohydrate by bacterial fructan hydrolases and subsequently fermented to acid. This process produces prolonged periods of low pH in dental plaque that have the potential to initiate the irreversible demineralization of dental enamel [14,16–19].

Except for the levansucrases of *Bacillus subtilis* [20–23] and *A. diazotrophicus* [11], there have been no systematic kinetic studies of these enzymes. Since the Ftfs and levansucrases can utilize a range of acceptors other than fructan, they can all be described as transfructosylases catalysing the reaction sucrose + acceptor → glucose + acceptor–fructose. In the case of the levansucrase of *B. subtilis*, kinetic analysis of the transfructosylation reactions has led to the conclusion that catalysis proceeds via a Ping Pong mechanism involving the formation of a transient covalent fructosyl–enzyme intermediate [23]. The Ping Pong mechanism has been confirmed by thermodynamic and kinetic studies [20], whereas the existence of a fructosyl–enzyme complex has been verified by the isolation of a fructosyl intermediate covalently linked by an ester bond to the β-carboxyl of an aspartic acid residue [21]. A Ping Pong mechanism of catalysis has also been proposed for the levansucrase of *A. diazotrophicus* [11].

In this study we report the purification and characterization of the recombinant Ftf of *S. salivarius* expressed in *Escherichia coli*, and the analysis of its mechanism of catalysis. The Ftf is shown to produce high M_r fructan at low sucrose concentrations, unlike other similar enzymes that have been studied in detail [11,20–23].

MATERIALS AND METHODS

Chemicals and enzymes

All chemicals, enzymes and reagents were of analytical grade or equivalent and were purchased from Ajax chemicals (Sydney, Australia), BDH/Merck (Sydney, Australia), Genesearch Pty Ltd (Brisbane, Australia) or Sigma Chemical Co. (St. Louis, MO, U.S.A.). The radioactively labelled compounds [U-^{14}C]fructosyl-labelled sucrose, [U-^{14}C]sucrose and [U-^{14}C]glucose were obtained from NEN (Dupont Co., Boston, MA, U.S.A.). The test-combination D-glucose/D-fructose kits were purchased from Boehringer Mannheim GmbH (Mannheim, Germany) and the Coomassie plus protein assay reagent from Pierce (IL, U.S.A.). Broad-range SDS/PAGE and native gel molecular-mass markers were obtained from Bio-Rad (Sydney, Australia).

Phagemids, bacterial strains and growth conditions

The Ftf from *S. salivarius* ATCC 25975 [24] was expressed in *E. coli* strain NM522 harbouring the phagemid pKRK1969 [1]. *E. coli* was grown with shaking (150 rev./min) at 37 °C in 8 litres of Luria–Bertani medium [25] supplemented with ampicillin (100 $\mu g \cdot ml^{-1}$).

Purification and characterization of the recombinant Ftf

Recombinant Ftf was expressed and extracted from *E. coli* NM522 harbouring pKRK1969 as previously described [26]. The clear lysate containing Ftf activity was dialysed overnight at 4 °C

Abbreviation used: Ftf, fructosyltransferase.

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against 10 mM potassium phosphate buffer, pH 6.5, before being loaded onto a hydroxyapatite column (1.6 cm × 20 cm; Bio-Gel HTP, Bio-Rad) and eluted with a 10–200 mM gradient of the same buffer. The fractions containing Ftf activity were pooled and dialysed against 20 mM piperazine/HCl buffer, pH 5.2. A sample (50 ml) was loaded onto a 1 ml Resource-Q column (Pharmacia Biotech, Melbourne, Victoria, Australia) connected to an FPLC system (Pharmacia, Biotech) and eluted with a 0–20 mM NaCl gradient in 20 mM piperazine/HCl buffer, pH 5.2. Fractions containing Ftf activity were pooled, dialysed against water and freeze-concentrated. The concentrated proteins were made up to 500 μl in 50 mM potassium phosphate buffer, pH 6.5, containing 150 mM KCl and further fractionated by gel filtration on a Sephacryl-S300 HR 26/60 column (2.6 cm × 60 cm, Pharmacia Biotech) connected to the FPLC system.

At each stage of the purification, the polymer-forming activity of the Ftf was quantified using [U-^{14}C]fructosyl-labelled sucrose as previously described [27]. The degree of purity was also monitored at each stage of purification by SDS/PAGE [28].

The M_r of the denatured and native forms of Ftf were determined by discontinuous SDS/PAGE using 9% (w/v) polyacrylamide gels as well as non-denaturing PAGE using 8%, 10%, 12% and 14% (w/v) polyacrylamide gels as described by Speicher [29].

The purified Ftf (10 μg), separated by SDS/PAGE for 5 h to confirm its purity, was electroblotted onto a PVDF membrane (Bio-Rad) [29] and its N-terminal sequence was determined at the Biomolecular Resource Facility (Australian National University, Canberra, Australia).

Analysis of the reaction products produced by the Ftf

The reaction products produced by the Ftf were analysed by both paper chromatography and TLC. Aliquots (20 μl) of the 300 μl assay mix, containing 100 nM Ftf and 50 mM [U-^{14}C] sucrose (92.5 kBq·ml^{-1}; 2.5 μCi·ml^{-1}), were transferred at 0 s, 20 s, 40 s, 1 min, 2 min, 5 min, 10 min, 20 min, 30 min, 1 h, 2 h and 4 h into 300 μl of ethanol pre-heated at 80 °C. After incubating for a further 15 min at 80 °C, the quenched samples were dried *in vacuo*. Each sample was redissolved in 10 μl of water and a 4 μl aliquot was analysed by ascending paper chromatography on Whatman #3 filter paper (Whatman Paper Ltd., Maidstone, Kent, U.K.) [20]. The reaction products were visualized by autoradiography after exposure to Hyper film™-MP (Amersham Pharmacia, Sydney, New South Wales, Australia) for 24 h. Another 4 μl aliquot from each time point was analysed by TLC on pre-coated 20 cm × 20 cm silica-gel TLC-ready-foils (F1500, Schleicher and Schuell, Dassel, Germany) as described by Cairns and Pollock [30] and the radioactive products were visualized by autoradiography as described above. Fructans with a degree of polymerization of more than 15 did not migrate in either system and remained at the site of application [30].

Transfructosylation from sucrose to sucrose: sucrose hydrolysis and fructan synthesis

The ability of Ftf to hydrolyse and/or polymerize the fructosyl moiety of sucrose was determined at 37 °C by measuring the amount of D-glucose and D-fructose produced by the enzyme. Fructan production was calculated from the difference between the amount of free glucose and free fructose. Each reaction mixture (2 ml) contained 25 nM Ftf and sucrose (0, 1, 2, 3, 5, 10, 20, 40, 50, 75, 100 or 500 mM) in 100 mM potassium phosphate buffer, pH 6.0, supplemented with 1 mM $CaCl_2$ and 0.1 mM histidine [31]. Aliquots (50 or 100 μl) were removed at 0.0, 0.5, 1, 2, 5, 10, 20, 30, 40, 50 and 60 min, quenched in ethanol and dried *in vacuo* as described above before being redissolved in 2 ml of water. The amount of glucose and fructose produced at each time point was quantified at 37 °C using the TC D-glucose/D-fructose enzymic assay kit supplied by Boehringer Mannheim GmbH according to manufacturer's instructions, except that comparisons were made with glucose or fructose standard curves assayed in the same manner at the same time. The initial velocity for the rate of formation of glucose, v_G {d[G]/dt; defined as the molarity of glucose released per minute (M·min^{-1})}, and fructose, v_F (d[F]/dt), at each sucrose concentration was calculated from these data using the 'Sigma-Plot' program (version 3.06, SPSS Inc., Chicago, IL, U.S.A.). In all instances the initial velocity at a given sucrose concentration possessed a linear regression coefficient (r^2) within the range 0.922–0.999.

Transfructosylation from sucrose to glucose: exchange reaction of the Ftf

The ability of 250 nM Ftf to exchange the glucose moiety of sucrose was determined at 37 °C in 50 μl reaction mixtures containing 20, 40, 60, 100 or 200 mM sucrose and 50, 100, 120 or 200 mM [^{14}C]glucose (185 kBq·ml^{-1}; 5 μCi·ml^{-1}) in 100 mM potassium phosphate buffer, pH 6.0, supplemented with 1 mM $CaCl_2$ and 0.1 mM histidine [31]. Aliquots (10 μl) were removed at intervals of 30 s or 1 min, quenched in ethanol and dried as described above. After being redissolved in 10 μl of water, an aliquot (5 μl) was analysed by ascending paper chromatography and the radioactive products were identified by autoradiography. The radioactive products were excised from the paper, cut into strips and suspended and shaken at 4 °C for 15 h in Ultima-Gold™ scintillation solution (Packard Instrument Co., Downers Grove, IL, U.S.A.) before being counted in a liquid-scintillation spectrometer (Beckman LS 9000). Under these conditions, the difference in quenching between samples was negligible. The initial rate of exchange, r_G, was defined as the molarity of the fructosyl moiety transferred from sucrose to glucose per minute (M·min^{-1}).

Analysis of data

All experiments were repeated in triplicate and, where appropriate, the results are presented as the means ± S.E.M. The 'Sigma Plot' program (version 3.06) was used to obtain linear regression coefficients (r^2).

RESULTS AND DISCUSSION

Purification and characterization of the Ftf

The purification procedure for the recombinant Ftf expressed in *E. coli* resulted in an electrophoretically homogeneous band (Figure 1) with a specific activity of 58 units per mg of protein. This fraction represented only 0.2% of the total protein and 3% of the original activity for a purification factor of 35-fold (Table 1).

The purified Ftf gave an apparent M_r of 125400 on SDS/PAGE, 180600 by gel filtration and 102000 on native-PAGE (results not shown). N-terminal sequencing revealed that the Ftf expressed in *E. coli*, like that expressed by *S. salivarius*, had been cleaved at TQVKA↓DQVTET to remove its signal sequence [32]. The predicted M_r of the Ftf devoid of its signal sequence is 98450, which was close to the result obtained from the native-PAGE analysis.

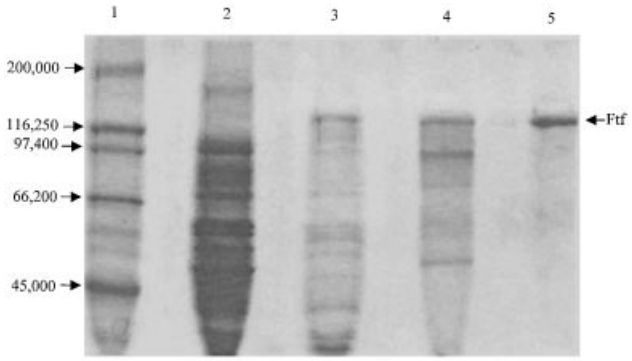


Figure 1 Silver-stained SDS/PAGE gel of Ftf-containing fractions from each purification step

Lanes: 1, M_r markers; 2, *E. coli* cell lysate; 3, hydroxyapatite pool; 4, Resource-Q pool; 5, Sephacryl S-300 purified Ftf. The gel was scanned using a Hewlett-Packard ScanJet 6100C, saved as a TIFF file and printed after importing into MS Word 7.0.

Table 1 Purification of the recombinant Ftf of *S. salivarius* expressed in *E. coli*

Purification step	Specific activity* (units per mg of protein)	Yield of protein (%)	Yield of activity (%)	Purification (fold)
Cell lysate	1.7	100	100	1
HA†	5.5	22	74	3.2
Resource-Q	58	1.1	14.8	34.5
Sephacryl S-300	58	0.2	3	34.5

* Polymer-forming activity was measured using [U-^{14}C]fructosyl-labelled sucrose. One unit of enzyme activity was defined as the amount of Ftf that catalysed the incorporation of 1 μmol of the fructosyl moiety of sucrose into 75% (v/v) ethanol-insoluble polysaccharide per min.

† Hydroxyapatite chromatography.

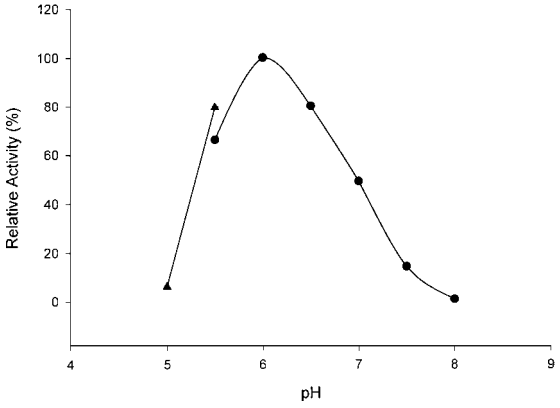


Figure 2 Effect of pH on the activity of Ftf

The effect of pH on enzyme activity was determined at 37 °C in the presence of 10 mM NaF, 100 μM histidine and 1 mM $CaCl_2$ by measuring the amount of glucose released in 60 min from 10 mM sucrose by 25 nM Ftf (means ± S.E.M.; $n = 3$). (▲) 100 mM potassium acetate buffer; (●) 100 mM potassium phosphate buffer.

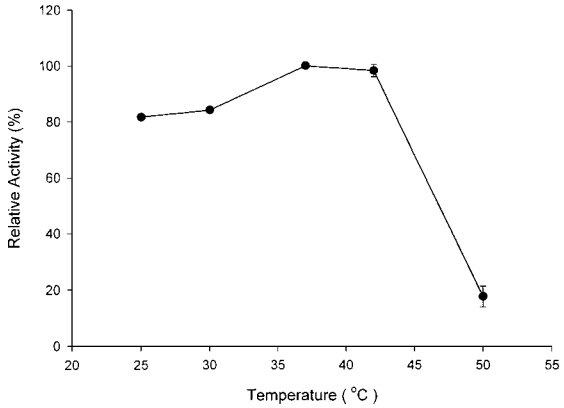


Figure 3 Effect of temperature on the activity of Ftf

Enzyme activity was evaluated as described for Figure 2 (means ± S.E.M.; $n = 3$).

Table 2 Effect of metal ions on the activity of Ftf

Enzyme activities were determined as described in Figure 2 in the absence of added $CaCl_2$. Results are given as means ± S.E.M.; $n = 3$.

Compound (1 mM)	Enzyme activity (%)
None	100.0 ± 0.7
NaCl	116.0 ± 0.1
KCl	115.1 ± 0.1
$MgCl_2$	103.5 ± 3.1
$CaCl_2$	149.4 ± 4.9
$Zn(CH_3COO)_2$	56.4 ± 6.1
$FeSO_4$	62.7 ± 2.8
$FeCl_3$	69.8 ± 1.0
$HgCl_2$	16.3 ± 0.4
$CuSO_4$	23.3 ± 1.0
EDTA (disodium)	17.2 ± 0.2

Effects of pH, temperature and metal ions on Ftf activity

The influence of pH and temperature on Ftf activity was examined in order to define the best conditions for subsequent kinetic studies. For the conditions studied, the catalytic activity reached a maximum at pH 6.0 (Figure 2) and at 37 °C (Figure 3).

The effects of different metal ions on the Ftf activity were also examined (Table 2). Addition of the metal-ion chelator, EDTA, reduced the enzyme activity by 83%, consistent with previous results that had indicated a role for Ca^{2+} in stabilizing enzyme activity [14,31,33]. This was confirmed by the addition of 1 mM Ca^{2+}, which enhanced the activity by 150%. Neither Na^+, K^+ nor Mg^{2+}, the metal ions other than Ca^{2+} used in the preparation of enzyme, affected Ftf activity.

Unlike the situation with the levansucrase of *B. subtilis*, where Fe^{3+} serves to stabilize this enzyme [34,35], the Ftf of *S. salivarius* was partially inhibited by 1 mM Fe^{3+} as well as 1 mM Zn^{2+}. The heavy metals Cu^{2+} and Hg^{2+}, at a concentration of 1 mM, inactivated the enzyme to a greater degree (Table 2). The mechanism by which the heavy metals inhibit the cell-free Ftf is not known. However, the Cu^{2+}-mediated inactivation of the cell-associated enzyme appears to depend on free-radical inactivation of the enzyme rather than any competition with the Ca^{2+} ion [31,36].

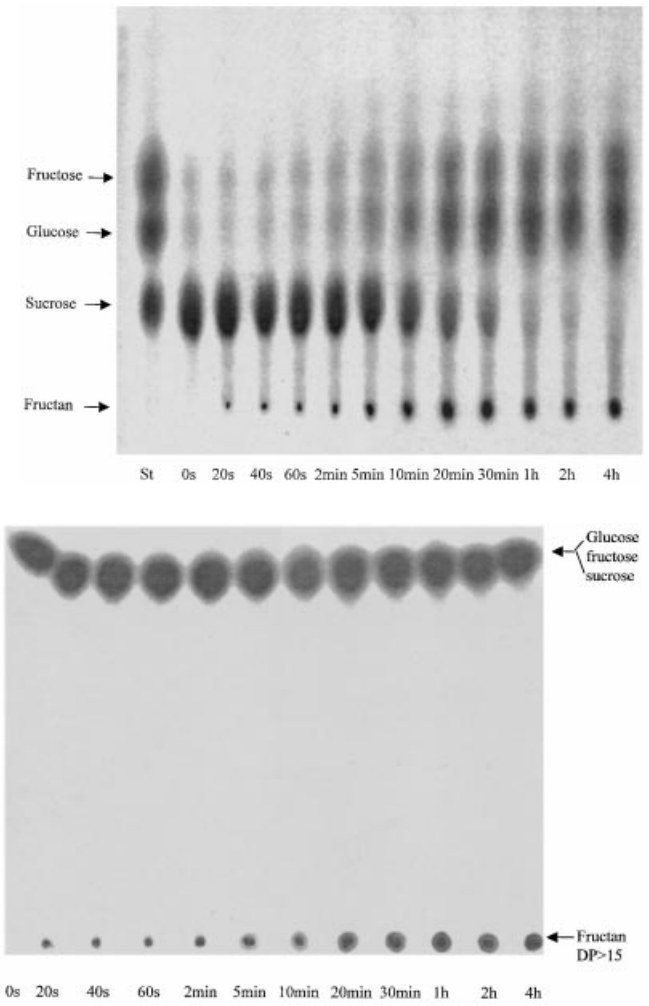


Figure 4 Analysis of transfructosylation of sucrose by (top) paper chromatography and (bottom) TLC

The reaction mixture contained 50 mM [U-^{14}C]sucrose (92.5 kBq · ml^{-1}) and 100 nM Ftf. Samples (20 μl) of the reaction mixture were removed at the end of 0, 20, 40, 60 s, 2, 5, 10, 20, 30, 60 min and 2, 4 h. St, standards. DP, degree of polymerization. Autoradiograms were scanned as described for Figure 1.

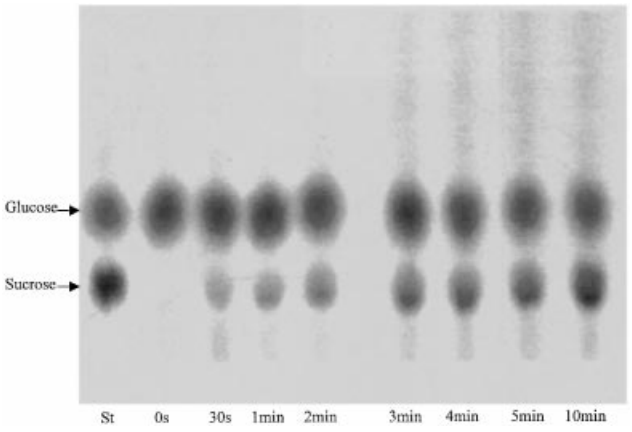
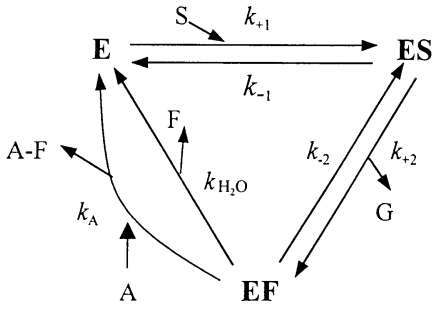


Figure 5 Analysis of the Ftf exchange reaction by paper chromatography

The reaction mixture contained 50 mM [U-^{14}C]glucose (185 kBq · ml^{-1}), 200 mM sucrose and 250 nM Ftf. Samples (10 μl) of the reaction mixture were removed at the end of 0, 0.5, 1, 2, 3, 4, 5 and 10 min. St, standards. The autoradiogram was scanned as described for Figure 1.



Scheme 1 Transfructosylation reaction of the Ftf of *S. salivarius* in which E, S, G, F and A represent enzyme, sucrose, glucose, fructose and fructosyl acceptor respectively and k_{H_2O} is considered to be a pseudo-first-order rate constant which contains the water concentration factor

Mechanism of transfructosylation by Ftf

In the presence of sucrose alone, the Ftf of *S. salivarius* catalysed both sucrose hydrolysis and fructan synthesis. Glucose, fructose and polyfructose (fructan) were produced (Figure 4a). TLC analysis of the fructans showed no evidence for the accumulation of short-chain oligofructans, since only fructans with a degree of polymerization > 15 could be observed at the application site [30]. The appearance of fructan with degree of polymerization $>$ 15 was detected 20 s after initiating the reaction by the addition of the enzyme (Figure 4b). This result is distinctly different to that observed with the levansucrases of bacilli, where high concentrations of sucrose ($\geqslant 0.8$ M) and longer reaction times ($\geqslant 30$ min) are required to synthesize fructan in the absence of added primer [11]. Furthermore, the levansucrase of *A. diazotrophicus* produces large amounts of tri- and tetrasaccharides during the early stages of polymerization [11].

In the presence of glucose, the enzyme also catalysed an exchange reaction. The exchange reaction could be distinguished from the hydrolysis reaction by using [U-^{14}C]glucose and unlabelled sucrose. As a result of the exchange reaction, the fructosyl moiety in the unlabelled sucrose was transferred to the [U-^{14}C]glucose, forming labelled sucrose (Figure 5), implying that the transfer of a fructosyl unit occurred with retention of an α-configuration at the C-1 site of the exchanged glucose molecule. The presence of the exchange reaction in which glucose was acting as an acceptor strongly supported the contention that the Ftf of *S. salivarius*, like the levansucrase of *B. subtilis* and that of *A. diazotrophicus*, utilized a Ping Pong mechanism involving the formation of a transient fructosyl–enzyme complex [11,20–23]. The transfructosylation reactions could thus be described by a sequence of elementary steps as in Scheme 1 from which the kinetic equations of the initial rate of each transfructosylation reaction could be derived by the King and Altman procedure [37].

Transfructosylation from sucrose to sucrose: sucrose hydrolysis and fructan synthesis by Ftf

In the presence of sucrose alone, Ftf catalyses both sucrose hydrolysis and fructan synthesis. However, fructosyltransferases (levansucrases) are not common two-substrate systems in which

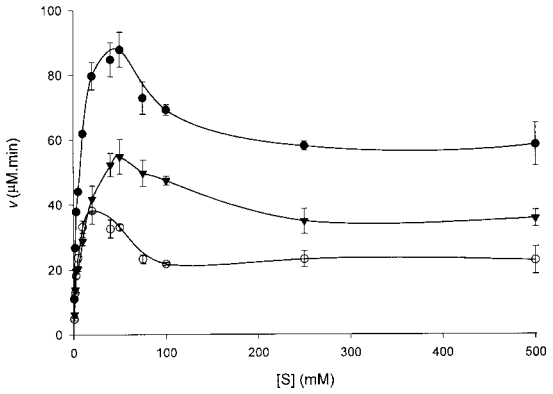


Figure 6 Effect of [S] on Ftf activity

Ftf (25 nM) was incubated at 37 °C in the presence of various [S] (means ± S.E.M.; $n = 3$). (●) v_G, (○) v_F, and (▼) $v_G - v_F$.

sucrose and oligofructan are simultaneously the substrate and product of the forward and reverse reactions. The nature of the fructosyl acceptor (except water) changes as the reaction proceeds. Chambert et al. [23] have proposed a multiple-chain-elongation mechanism in which the fructosyl residues are added randomly to all molecules of the fructan acceptors. Kinetic and chemical studies of the levansucrase of *B. subtilis* [38] further suggest that each fructosyl unit is added one at a time onto an acceptor molecule. While the initial acceptor may be sucrose, the growing fructan chain subsequently acts as an acceptor. Under these circumstances, A in Scheme 1 can be represented by S-$(F)_n$ where n is the number of fructosyl groups transferred to a growing fructan chain for which sucrose, S, is the initial acceptor (i.e. when n is zero). Under the initial reaction conditions it can be assumed that the Ftf does not distinguish between fructan molecules with a degree of polymerization of either n or $n+1$. Thus the molarity of the acceptor $[S\text{-}(F)_n] = [S\text{-}(F)_{n+1}]$ and is therefore constant. The same hypothesis has been used by Chao et al. [39] in their kinetic study of maltodextrin phosphorylase and by Chambert et al. [23] in the kinetic analysis of levansucrase of *B. subtilis*. Consequently, if $k_A[S\text{-}(F)_n]$ is set as k_{AS}, E_o as the total enzyme concentration and $k_{-1} \gg k_{+2}$ (as assumed by others [20,23] on the basis that the formation of the intermediate from the enzyme and sucrose, ES, is controlled by rapid diffusion), then by applying the King and Altman procedure [37], the initial rates of glucose and fructose formation (v_G and v_F) for a Ping Pong mechanism can be calculated from:

$$\frac{E_0}{v_G} = \frac{k_{-1}}{k_{+1}k_{+2}[S]} + \frac{k_{+2}+k_{H_2O}+k_{AS}}{k_{+2}(k_{H_2O}+k_{AS})} \quad (1)$$

and

$$\frac{E_0}{v_F} = \frac{k_{-1}(k_{H_2O}+k_{AS})}{k_{+1}k_{+2}k_{H_2O}[S]} + \frac{k_{+2}+k_{H_2O}+k_{AS}}{k_{+2}k_{H_2O}} \quad (2)$$

from which one can obtain:

$$k_{cat}{}^G = \frac{k_{+2}(k_{H_2O}+k_{AS})}{k_{+2}+k_{H_2O}+k_{AS}} \text{ and } K_m{}^G = \frac{k_{-1}(k_{H_2O}+k_{AS})}{k_{+1}(k_{+2}+k_{H_2O}+k_{AS})}$$

and

$$k_{cat}{}^F = \frac{k_{+2}k_{H_2O}}{k_{+2}+k_{H_2O}+k_{AS}} \text{ and } K_m{}^F = \frac{k_{-1}(k_{H_2O}+k_{AS})}{k_{+1}(k_{+2}+k_{H_2O}+k_{AS})}$$

Table 3 Comparison of the values of apparent kinetic constants for bacterial Ftfs

Kinetic parameter	Bacterium		
	S. salivarius	*B. subtilis**	*A. diazotrophicus**
K_m (mM)	5.0 ± 0.3	4.0 ± 0.4	11.8 ± 1.4
$k_{cat}{}^F$ (min^{-1})	1733 ± 70	2100 ± 120	3600 ± 400
$k_{cat}{}^F/K_m$ ($M^{-1} \cdot min^{-1}$)	3.9×10^5	5.3×10^5	3.1×10^5
$k_{cat}{}^G$ (min^{-1})	3809 ± 217	–†	–
$k_{cat}{}^G/K_m$ ($M^{-1} \cdot min^{-1}$)	7.6×10^5	–	–
k_{+1}/k_1 (M^{-1})	13.3 ± 0.3	18 ± 2	12 ± 1
k_{+2} (s^{-1})	1042 ± 50	260 ± 20	550 ± 60
k_{-2} ($M^{-1} \cdot s^{-1}$)	4648 ± 163	4600 ± 300	3300 ± 200
k_{H_2O} (s^{-1})	29.2 ± 0.4	35 ± 4	51 ± 5
k_{AS} (s^{-1})	44 ± 1.1	–	–
k_s ($M^{-1} \cdot s^{-1}$)‡	–	–	25 ± 5
k_{inu} ($M^{-1} \cdot s^{-1}$)§	–	$(7.0 \pm 0.7) \times 10^3$	0.0
k_{lev} ($M^{-1} \cdot s^{-1}$)‖	–	$(3.9 \pm 0.4) \times 10^4$	$(9.7 \pm 1) \times 10^3$

* Data from [11] and [22].
† No data available.
‡ k_s is the kinetic constant for the transfructosylation from sucrose to sucrose for the formation of kestose [11].
§ k_{inu} is the kinetic constant for the transfructosylation from sucrose to inulin [22].
‖ k_{lev} is the kinetic constant for the transfructosylation from sucrose to levan [22].

It is clear from these equations that $K_m{}^F = K_m{}^G$.
From the ratio of the reaction rates, eqn. (3) can be derived:

$$\frac{v_G}{v_F} = 1 + \frac{k_{AS}}{k_{H_2O}} \quad (3)$$

Both k_{H_2O} and k_{AS} are considered as pseudo-first-order rate constants which contain the water concentration factor and the fructan concentration factor respectively.

The initial rates of the reaction, v_G, v_F and $v_G - v_F$ (representing the rate of fructosyl residues being incorporated into fructan) were measured at 37 °C in the presence of various concentrations of sucrose (Figure 6). At approx. 50 mM sucrose, all three parameters reached a maximum, with substrate inhibition being observed at higher concentrations of sucrose (Figure 6).

The apparent values of K_m, k_{cat} and k_{cat}/K_m for both v_G and v_F were determined by linear regression of double-reciprocal plots of v_G and v_F against sucrose concentration ($r^2 = 0.994$ and 0.982 respectively) (Table 3). The K_m^G value of 5.0 ± 0.3 mM for sucrose that was determined from v_G was experimentally the same as the K_m^F value of 4.9 ± 0.2 mM determined from v_F, which was in agreement with the equations derived from the Ping Pong mechanism. This value of the K_m was less than that of 12 ± 0.1 mM previously reported [31]. This latter value was determined in the presence of host cells, under the misapprehension that it was the *in situ* cell-bound form that was being evaluated. This study [31] was done before the discovery that the cell-bound Ftf was released from the surface of *S. salivarius* in the presence of its substrate sucrose [33].

According to eqns. (1) and (2), the values for the relationship between the various kinetic constants could also be calculated using these results. Hence:

$$k_{-1}/(k_{+1} \cdot k_{+2}) = K_m{}^G/k_{cat}{}^G = (1.3 \pm 0.1) \times 10^{-6} \text{ M} \cdot \text{min} (n = 3)$$

and

$$\frac{k_{+1}(k_{+2}+k_{H_2O}+k_{AS})}{k_{-1}(k_{H_2O}+k_{AS})} = \frac{1}{K_m} = (201 \pm 13) \text{ M}^{-1} (n = 3)$$

The slope of the regression line for the relationship between the ratio of the reaction rates v_G/v_F and sucrose concentration was very small (3.5×10^{-4} mM^{-1}; results not shown), suggesting that the ratio remained pseudo-constant in the presence of various concentrations of sucrose. Again this was consistent with eqn (3) derived from the Ping Pong mechanism. Thus by extrapolating the value of the ratio of the reaction rates as the concentration of sucrose [S] approached zero, k_{AS}/k_{H_2O} could be estimated as 1.47 ± 0.08 ($n = 3$). The ratio k_{AS}/k_{H_2O} reflects the competition between sucrose (or oligofructan) and water as fructosyl acceptor. Thus the transfer of fructose from the fructosyl–enzyme intermediate to sucrose (or oligofructan) is a more rapid reaction than that to water and nearly 60% of the fructosyl residues transferred from sucrose are incorporated into fructan (yield of fructan, $Y = [v_G - v_F]/v_G$). This is a similar result to that reported for *A. levanicum* levansucrase [12,13].

Transfructosylation from sucrose to glucose: the exchange reaction of Ftf

According to the King and Altman procedure [37] for a Ping Pong mechanism,

$$\frac{E_O}{r_G} = \frac{k_{-1}}{k_{+1}k_{+2}[S]} + \frac{1}{k_{-2}[G]} + \frac{1}{k_{+2}} \quad (4)$$

where r_G is the rate of exchange of the fructosyl moiety of sucrose with free glucose. At 37 °C, double-reciprocal plots of r_G against glucose concentration [G], at fixed sucrose concentrations [S], were linear and parallel with each other (r^2 in the range 0.992–0.999; results not shown), consistent with that predicted from eqn. (4) for a Ping Pong mechanism. The secondary plot of the intercepts with the ordinate axis of these lines, $1/r_G = f(1/[G])$, against the reciprocal of the sucrose concentration (1/[S]) was also linear ($r^2 = 0.945$; results not shown). From these plots the values for $1/k_{-2}$, $1/k_{+2}$ and $k_{-1}/(k_{+1}.k_{+2})$ were determined to be $3.6 \pm 0.1 \times 10^{-6}$ M·min, $1.6 \pm 0.1 \times 10^{-5}$ min and $1.2 \pm 0.1 \times 10^{-6}$ M·min respectively following linear-regression analysis. There was no significant difference in the value of $k_{-1}/(k_{+1}.k_{+2})$ determined by the exchange reaction and that of $1.3 \pm 0.1 \times 10^{-6}$ M·min determined from the analysis of sucrose hydrolysis and fructan synthesis.

The rate constants for the Ftf of *S. salivarius* (Table 3) reveal that the transfer of the fructosyl moiety from the fructosyl–enzyme intermediate is the rate-limiting step for the transfructosylation to water and sucrose. However, transfructosylation to glucose is much faster than that to water or to sucrose at the concentrations of added glucose used in this study (> 50 mM). Thus in the presence of glucose the exchange reaction inhibits the formation of fructose and fructan by reducing the steady-state concentration of the fructosyl–enzyme intermediate.

Comparison of the properties of the Ftf of *S. salivarius* with the levansucrases of *B. subtilis* and *A. diazotrophicus*

Table 3 compares the kinetic constants of *S. salivarius* Ftf with those published for the levansucrases of *B. subtilis* [21] and *A. diazotrophicus* [11]. The affinity of the enzyme for sucrose (K_m) and the rate constants for the formation of the fructosyl–enzyme intermediate (k_{+1}/k_{-1}, k_{+2}, k_{-2}) are, for the most part, very similar. In the levansucrase of *B. subtilis*, an aspartic acid has been detected as the fructosyl-binding residue, although the exact position of this aspartic acid in the amino acid sequence is not known [21]. The so-called 'sucrose box', which appears to play a role in sucrose binding (D. D. Song and N. A. Jacques, unpublished work), is also highly conserved in the streptococcal Ftfs and the levansucrases of bacilli [1]. It is perhaps not surprising therefore that the molecular basis of the first step of the catalytic process, the formation of a fructosyl–enzyme covalent intermediate, appears to have been highly conserved between these different enzymes; a fact that is evident from the similar values of the kinetic constants. Despite this observation, however, the efficiencies of the transfer of fructosyl residue from the fructosyl–enzyme intermediate to various fructosyl acceptors differ for the three enzymes for which data are available. At low concentrations of sucrose (< 50 mM), when sucrose and water are the only two possible acceptors, the levansucrases of both *B. subtilis* and *A. diazotrophicus* exhibit only hydrolytic activity [11,20,22,23]. In contrast, the Ftf of *S. salivarius* uses sucrose as an efficient fructosyl acceptor. It does not require the presence of a preformed fructan to prime the polymerization reaction. The Ftf of *S. salivarius* also rapidly synthesizes fructans of high M_r ($\leqslant$ 20 s) without the accumulation of transient small oligofructans (Figure 4b).

The production of high M_r fructans by the Ftf of *S. salivarius* is consistent with a number of physicochemical and linkage studies of the polymers which suggest that they are monodisperse spherical arborescent structures possessing multi-branched chains and M_rs of the order of $(20–100) \times 10^6$ [40–44]. The spherical shape allows the fructan to be retained in dental plaque by virtue of its low diffusive properties, while being effectively hydrolysed from its outermost regions by exo-hydrolases when carbohydrate is in short supply [14]. The low diffusive properties are clearly important in the oral environment, as fructans with a low degree of polymerization would be readily removed from dental plaque biofilms by salivary flow.

In contrast, the kestose accumulated by the levansucrase of *A. diazotrophicus*, a Gram-negative bacterium associated with sugar cane, may be used by the plant host itself [11], since kestose is known to play a key role in plant fructan biosynthesis [45]. In fact the fructans of plants usually exhibit a low degree of polymerization [46,47]. Plant fructans are important storage carbohydrates and are believed to be involved in osmoregulation and in enhancing drought and cold resistance [46,47]. The low degree of polymerization may be important in allowing the fructans to disperse readily within the cells. The results obtained from this study therefore further support the hypothesis of Hernandez et al. [11] that the fructosyltransferases of various origins may have a common ancestor, but that the catalytic specificity of these enzymes could have diverged to meet the needs of different species, dependent upon their ecological niche.

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