Mutation of aspartic acid residues in the fructosyltransferase of *Streptococcus salivarius* ATCC 25975

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The site-directed mutated fructosyltransferases (Ftfs) of *Streptococcus salivarius* ATCC 25975, D312E, D312S, D312N and D312K were all active at 37 °C, indicating that Asp-312 present in the ‘sucrose box’ was not the nucleophilic Asp residue responsible for the formation of a covalent fructosyl–enzyme intermediate required for enzyme activity. Analysis of the kinetic constants of the purified mutated forms of the enzyme showed that Asp-312 was most likely an essential amino acid involved in determining acceptor recognition and/or stabilizing a β -turn in the protein. In contrast, when the Asp-397 of the Ftf present in the conserved triplet RDP motif of all 60 bacterial and plant

family-32 glycosylhydrolases was mutated to a Ser residue, both sucrose hydrolysis and polymerization ceased. Tryptophan emission spectra confirmed that this mutation did not alter protein structure. Comparison of published data from other site-directed mutated enzymes implicated the Asp residue in the RDP motif as the one that may form a transient covalent fructosyl intermediate during the catalysis of sucrose by the Ftf of *S. salivarius*.

Key words: fructan, invertase, levansucrase, neuraminidase, site-directed mutagenesis.

INTRODUCTION

The purified recombinant fructosyltransferase (Ftf) of *Streptococcus salivarius* catalyses the transfructosylation of sucrose by a Ping Pong mechanism involving a fructosyl–enzyme intermediate [1], in a manner similar to that of the levansucrases (sucrose: 2,6- β -D-fructan 6- β -D-fructosyltransferase; EC 2.4.1.10) of *Bacillus subtilis* and *Acetobacter diazotrophicus* [2–6]. In an aqueous environment in which sucrose is the only substrate, two transfructosyl reactions initially occur resulting in the release of glucose. Either the fructose residue is transferred to water (sucrose hydrolysis), or to another sucrose molecule to initiate the formation of a fructan molecule (fructan chain elongation) as shown diagrammatically in Figure 1, where E, S, G, F and A represent the enzyme, sucrose, glucose, fructose and acceptor (i.e. water or a growing fructan chain in a sucrose solution), respectively. Comparison of the kinetic constants of the Ftf of *S. salivarius* with the levansucrases of *B. subtilis* and *A. diazotrophicus* suggests that the molecular basis of the first step of the catalytic pathway, the formation of a fructosyl–enzyme intermediate, EF, from sucrose, may have been conserved in these enzymes [1].

Figure 1 Ping Pong mechanism of catalysis

The acidic amino acids, Asp and Glu, have been found to have a catalytic function in most glycosyltransferases and glycosylhydrolases, either as proton donors or as nucleophiles [7–9]. A stable covalently bound fructosyl–enzyme intermediate has also been isolated from the levansucrase of *B. subtilis* by quenching with a rapid decrease in pH [3]. Protease digestion of the stable fructosyl–enzyme intermediate has revealed that the fructosyl residue is bound by an ester bond to the β -carboxyl group of an Asp residue [3]. However, the position of the Asp residue in the protein remains unknown. Among two possible candidates in the Ftf of *S. salivarius* is an Asp residue, Asp-312, present in the centre of a highly conserved domain designated the ‘sucrose box’ consisting of the 20 amino acid sequence, WSGSATVNSDGS-IQLYYTKN [10,11]. The second potential Asp residue is Asp-397, originally identified in the conserved triplet motif, RDP, of 13 bacterial and plant Ftfs following multiple sequence alignment of these proteins. Conservation of the motif, RDP, has also been reported in several plant invertases and bacterial sucrases [12] and recently the equivalent Asp residue, Asp-309, of the levansucrase of *A. diazotrophicus* has been mutated [13]. The mutated levansucrase of *A. diazotrophicus*, D309N, possesses a similar K_m for sucrose but a 75-fold reduction in the value of k_{cat} , implying that it plays a significant role in catalysis in this enzyme [13]. In light of these observations, we have altered Asp-312 and Asp-397 in the Ftf of *S. salivarius* by site-directed mutagenesis and determined the resultant effects on catalytic function and conformation of the protein.

MATERIALS AND METHODS

Chemicals and enzymes

All chemicals of analytical grade or equivalent were purchased from Ajax chemicals (Sydney, Australia), BDH/Merck (Sydney, Australia) or Sigma Chemical Co. (St. Louis, MO, U.S.A.). Radioactively labelled deoxynucleotides and [14 C]fructosyl-

Abbreviation used: Ftf, fructosyltransferase.

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Table 1 Bacterial strains and phagemids

Bacterial or phagemid	Description*	Source/reference
Bacteria		
<i>Streptococcus salivarius</i> ATCC 25975	(No description)	ATCC [14]
<i>Escherichia coli</i> NM522	F' <i>lacI</i> ^q Δ(<i>lacZ</i>) <i>M15 proA</i> ⁺ <i>B</i> ⁺ / <i>supE thi</i> Δ(<i>lac-proAB</i>)Δ(<i>hsdMS-mcrB</i>)5 (<i>r</i> _K [−] <i>m</i> _K [−] <i>McrBC</i> [−])	[15]
<i>Escherichia coli</i> BMH71-18 <i>mutS</i>	<i>thi supE</i> Δ(<i>lac-proAB</i>) [<i>mutS</i> ::Tn10][F' <i>proAB lacI</i> ^q Δ <i>M15</i>]	Clontech
Phagemids		
pIB131	Ap ^r , fl origin replication, β-galactosidase, T3 and T7 polymerase promoters	1B1 Corp.
pKRK1969	Ap ^r ; Ftf ⁺ ; 3.29 kbp of insert of <i>S. salivarius</i> ATCC 25975 chromosomal DNA in pIB131	[11]
pKRK1801	pKRK1969; <i>MluI</i> site → <i>BsrI</i> ; new <i>DpnI</i> site; <i>ftf</i> coding for D312N	This study
pKRK1802	pKRK1969; <i>MluI</i> site → <i>BsrI</i> ; new <i>DpnI</i> site; <i>ftf</i> coding for D312E	This study
pKRK1803	pKRK1969; <i>MluI</i> site → <i>BsrI</i> ; new <i>AvaI</i> site; <i>ftf</i> coding for D312S	This study
pKRK1804	pKRK1969; <i>MluI</i> site → <i>BsrI</i> ; new <i>StyI</i> site; <i>ftf</i> coding for D312K	This study
pKRK1805	pKRK1969; <i>MluI</i> site → <i>BsrI</i> ; new <i>DpnI</i> site; <i>ftf</i> coding for D312I	This study
pKRK1806	pKRK1969; <i>MluI</i> site → <i>BsrI</i> ; new <i>BglI</i> site; <i>ftf</i> coding for D397S	This study

* Apr, ampicillin resistance.

labelled sucrose were obtained from NEN (DuPont Co., Boston, MA, U.S.A.). Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, T4 polynucleotide kinase, DNase I, lysozyme and DNA molecular mass markers were obtained from Genesearch Pty Ltd (Brisbane, Australia), Promega (Sydney, Australia) or Boehringer–Mannheim GmbH (Mannheim, Germany). The T7 DNA polymerase and T7 DNA sequencing kit were purchased from Amrad Biotech (Melbourne, Australia), the test-combination D-glucose/D-fructose kits from Boehringer–Mannheim GmbH, and the Coomassie Plus Protein Assay Reagent from Pierce (Rockford, IL, U.S.A.). Broad range SDS/PAGE molecular mass markers were obtained from Bio-Rad (Sydney, Australia) and Gradipore electrophoresis gel stain from Gradipore Ltd (Sydney, Australia).

Phagemids, bacterial strains and growth conditions

Escherichia coli strains NM522 and BMH71-18 *mutS* (defective in mismatch repair) were used throughout this study (Table 1). All phagemids were derivatives of pKRK1969 containing the gene coding for the Ftf of *Streptococcus salivarius* ATCC 25975 [11] (Table 1). *E. coli* cells harbouring pKRK1969 or one of its derivatives were grown with shaking (150 rev./min) at 37 °C in Luria–Bertani media [16] supplemented with ampicillin (100 µg/ml).

Site-directed mutagenesis

Phagemid pKRK1969 was purified by CsCl gradient centrifugation using standard protocols [17]. Site-directed mutagenesis made use of the Transformer[™] Site-Directed Mutagenesis Kit supplied by Clontech Laboratories (Palo Alto, CA, U.S.A.) [18]. Oligonucleotides used for mutagenesis (Table 2) were either synthesized using a Pharmacia Gene Assembler Plus or purchased from Beckman Instruments (Ryde, Australia). The oligonucleotides were desalted using NAP[™]-10 Columns (Pharmacia Biotech, Melbourne, Australia) according to the instructions supplied by the manufacturer. *E. coli* was electroporated in 1 mm cuvettes (Bio-Rad Gene Pulser; 1.6 kV, 200 W, 25 µF).

The mutated phagemids were screened by determining whether they could be cut at the newly introduced restriction sites. Those phagemids giving the expected restriction patterns were purified using the Wizard[™] Minipreps DNA Purification System (Promega, Australia). DNA sequencing confirmed the nature and sites of the site-directed mutations [19] (Amrad Biotech T7

sequencing kit or automated sequencing by Sydney University and Prince Alfred Macromolecular Analysis Centre, Sydney University, Sydney, Australia).

Purification of recombinant Ftf activities

Parental and mutated proteins were expressed in *E. coli* NM522, extracted and purified as previously described [1]. At each stage of the purification, the specific polymer-forming activity of the Ftf proteins was quantified using [U-¹⁴C]fructosyl-labelled sucrose [20] and the degree of purity monitored by SDS/PAGE [21]. The final products contained only Ftf as determined by silver staining [22].

Sucrose hydrolysis and fructan production by parental and mutated Ftfs

The kinetic properties of the mutated Ftfs were determined as previously described following the detection of the amount of glucose and fructose formed at a given time with TC D-Glucose/D-Fructose kits supplied by Boehringer–Mannheim GmbH (Mannheim, Germany) [1]. The nature of the fructans produced in these reactions were analysed by thin layer chromatography [1].

Tryptophan emission fluorescence spectroscopy

Fluorescence spectroscopy was performed on a LS50B Luminescence Spectrometer (Perkin–Elmer Applied Biosystems, Foster City, CA, U.S.A.). The excitation wavelength was 290 nm and the emission was scanned from 300 nm to 400 nm every 0.1 nm. Measurements were made using 1 cm path-length acrylic fluorescence cuvettes (Sarstedt Inc, NC, U.S.A.). Samples of parental and mutated Ftfs at 5.0 µg/ml were prepared in 10 mM potassium phosphate buffer pH 6.0.

Multiple sequence alignment

Protein amino acid sequences were aligned using the default parameters of ECLUSTALW [23] accessed on WebAngis (<http://www.angis.org.au>).

Table 2 Primers used in mutagenesis

Primer type	Altered restriction site	Oligonucleotide primer (5' → 3')*
Selection primer	<i>Mlu</i> I → <i>Bsr</i> I	TC CGT CGT GAG GAG ACG CGT GGC AGG CAA GCT T
D312N	<i>Dpn</i> I	TC CGT CGT GAG GAG <u>ACT GGT</u> GGC ATG CAA GCT T Ser Ala Thr Val Asn Ser Asp Gly Ser Ile Gln Leu Tyr Tyr TCA GCG ACT GTT AAC TCT GAT GGT AGT ATC CAA CTT TAT TAC A GCG ACT GTT AAC TCT <u>AAT GGA TCT</u> ATC CAA CTT TAT TAC Asn
D312E	<i>Dpn</i> I	CG ACT GTT AAC TCT <u>GAA GGA TCT</u> ATC CAA CTT TAT TAC Glu
D312S	<i>Ava</i> I	GCG ACT GTT AAC <u>TCC TCG</u> GGT AGT ATC CAA CTT Ser
D312K	<i>Sty</i> I	GCG ACT GTT AAC <u>TCC AAG</u> GGT AGT ATC CAA CTT Lys
D312I	<i>Dpn</i> I	GCG ACT GTT AAC <u>TGG ATC</u> GGT AGT ATC CAA CTT Ile
D397S	<i>Bgl</i> II	Asp Asn Tyr Cys Leu Arg Asp Pro His Ile Ile Glu Asp GAT AAC TAC TGT CTT <u>CGT GAC</u> CCA CAC ATC ATT GAA GAT GAT AAC TAC TGT CTT <u>AGA TCT</u> CCA CAC ATC ATT GAA G Ser

* The double underline shows the position of the restriction site. For the selective primer, the original DNA sequence is shown (top line), while for the mutagenic primers the original DNA sequence and its amino acid translation are shown for D312N and D397N only. Changed bases are shown in bold.

RESULTS AND DISCUSSION

Expression and purification of the site-directed mutated Ftf

The site-directed mutated Ftf of *S. salivarius* were expressed at 37 °C in *E. coli* NM522 and their specific activities determined at pH 6.0 before and after purification to electrophoretic homogeneity (Table 3). Replacement of Asp-312 with Ile (D312I) resulted in an enzyme devoid of polymer-forming activity while the changes D312E, D312N, D312S and D312K gave enzymes of lesser activity. Mutation of Asp-397 to serine (D397S) also abolished enzyme activity, indicating that both Asp-312 and Asp-397 play an important role in Ftf activity.

pH dependence of parental and mutated Ftf

In order to examine whether the pH dependence of the Ftf activity was modulated by the site-directed mutations, the activities of the various purified mutated Ftf of *S. salivarius* expressed in *E. coli* at 37 °C were determined over the pH range

4.5–8.0 (results not shown). While the parental Ftf possessed a maximum activity around pH 6.0 [1], there was a slight acid-shift to pH 5.5 for the mutated Ftf, D312E, D312S, D312N and D312K. A steady decline in activity was noted when the pH was increased from 5.5 to 8.0, except in the case of D312K, where 80 % of the activity was rapidly lost as the pH increased to 6.5. Mutated Ftf, D312I and D397S, remained inactive over the entire pH range of 4.5–8.0. As a compromise, all subsequent assays were performed at pH 6.0 irrespective of the form of the enzyme so as to allow direct comparison with the parental Ftf.

Kinetic and product analysis of the mutated Ftf

The relationship between sucrose concentration and the initial formation of the three products, glucose, fructose or fructan, was determined for the active forms of the purified enzymes (results not shown). Unlike the levansucrases of *B. subtilis* and *A. diazotrophicus*, which have been reported as being unable to synthesize fructan at a sucrose concentration < 50 mM [4], each mutated Ftf of *S. salivarius* synthesized fructan at a sucrose concentration of 1 mM. In all instances, both sucrose hydrolysis and polymer formation were apparent irrespective of the concentration of sucrose used. All of the 'sucrose box'-mutated Ftf that were active when expressed at 37 °C synthesized high M_r fructans (degree of polymerization > 15) from 50 mM sucrose within 20 s in a similar manner to that observed with the parental enzyme [1]. No short chain oligofructans were accumulated by any of the mutated Ftf (results not shown), indicating that similar reaction mechanisms to those found with the parental Ftf were occurring in each of the mutated enzymes [1].

To further compare the Asp-312-mutated Ftf with the parental Ftf, a kinetic analysis of the transfructosylation from sucrose to sucrose and water was undertaken for each mutated form of the enzyme. Each form exhibited a maximum velocity at around 40 mM sucrose, although the value of the maximum velocity differed. Double reciprocal plots of the initial rate of glucose formation, v_g , and the rate of fructose formation, v_f , versus sucrose concentration were linear ($r^2 \geq 0.910$ [results not shown]). On the basis of the known Ping Pong mode of catalysis for the parental enzyme [1], various kinetic parameters were determined

Table 3 Specific polymer-forming activity of mutated Ftf expressed at 37 °C

Ftf	Specific activity (units per mg of protein)*	
	Cell lysate	Purified enzyme
Parental	1.7	58
D312E	1.2	27
D312N	0.6	16
D312S	0.9	20
D312K	0.7	17
D312I	0.0	0.0
D397S	0.0	0.0

* The activity was determined at 37 °C using [U-¹⁴C]fructosyl-labelled sucrose [20]. One unit of activity was defined as the amount of Ftf that catalysed the incorporation of 1 μ mol of the fructose moiety of sucrose into 75% (v/v) ethanol-insoluble fructan per min at pH 6.0.

Table 4 Kinetic parameters for the transfructosylation reaction from sucrose to sucrose at 37 °C

Parameter	Form of Ftf*						
	D312	D312E	D312N	D312K	D312S	D312I†	D397S
K_m (mM)	5.0 ± 0.3	6.4 ± 0.1	5.7 ± 0.2	9.4 ± 0.3	6.7 ± 0.2	n.a.	n.a.
k_{cat}^G (min ⁻¹)	3809 ± 217	1691 ± 44	1024 ± 33	2667 ± 218	1892 ± 145	n.a.	n.a.
k_{cat}^G/K_m (M ⁻¹ · min ⁻¹)	7.6 × 10 ⁵	2.7 × 10 ⁵	1.5 × 10 ⁵	2.9 × 10 ⁵	3.3 × 10 ⁵	—	—
k_{cat}^F (min ⁻¹)	1733 ± 70	711 ± 35	469 ± 14	1145 ± 30	617 ± 19	n.a.	n.a.
k_{cat}^F/K_m (M ⁻¹ · min ⁻¹)	3.9 × 10 ⁵	1.2 × 10 ⁵	0.7 × 10 ⁵	1.2 × 10 ⁵	1.3 × 10 ⁵	—	—
k_{AS}/k_{H_2O}	1.3	1.2	1.2	1.3	1.8	—	—
$Y_{\ddagger}^{\ddagger}$ (%)	57	55	55	57	65	—	—

* Data are expressed as mean ± S.E.M. ($n = 3$).

‡ Yield of fructan (%) = 100 ($v_G - v_F$)/ $v_G = 100 [1 - (1 + k_{AS}/k_{H_2O})^{-1}]$.

† Expressed at 37 °C.

n.a., no activity.

—, not applicable.

for each of the mutated forms (Table 4). Analysis of these data showed that, even though the parental and mutated Ftf, D312E, D312N, D312S and D312K, possessed similar K_m values for sucrose, the efficiency of the transfructosylation reaction was markedly modulated by the nature of the amino acid at position 312. The values of k_{cat}^G/K_m and k_{cat}^F/K_m of the mutated Ftf, D312E, were reduced by 65 and 69%, respectively (Table 4), indicating a significant effect on enzyme activity of a one carbon increase in the length of the acidic side chain at position 312. However, the fact that the mutated Ftf, D312S and D312K, retained 43 and 38% of their total activity (k_{cat}^G/K_m), respectively, indicated that a carboxy group or negative charge at position 312 was not essential for catalysis.

The ratio of the reaction rates, v_G/v_F , for each mutated Ftf was pseudo-constant, irrespective of the sucrose concentrations used (results not shown), as was the case for the parental enzyme [1]. The values of the pseudo-rate constants, k_{AS}/k_{H_2O} , were thus calculated by extrapolating the values of the ratio of the reaction rates as the concentration of sucrose [S], approached zero [1] (Table 4). In contrast to the parental and other mutated Ftf, the mutated Ftf, D312S, possessed a significant increase in the value of k_{AS}/k_{H_2O} . The yield of fructan also increased nearly 10% compared with the other forms of Ftf (Table 4). These results suggested that the hydroxy group at position 312 might be involved in the binding between the Ftf and fructosyl acceptors, since the constant ratio, k_{AS}/k_{H_2O} , reflects the competition between sucrose (and oligofructan) and water to act as fructosyl acceptor [1]. Irrespective of whether or not this is the case, the possibility of Asp-312 serving as a catalytic nucleophile in the formation of the fructosyl-enzyme intermediate could be excluded.

Neither glucose nor fructose was detected in the reaction catalysed by the mutated Ftf, D397S or D312I, expressed at 37 °C, indicating that these amino acid substitutions created significant changes in the kinetic properties of the enzyme (Tables 3 and 4).

Effect of expression temperature on the activity of the mutated Ftf, D312I and D397S

The mutated Ftf, D312I, which lacked any detectable activity when expressed at 37 °C (Table 3), was active when expressed in *E. coli* at the lower growth temperatures of 25 or 30 °C; the specific activities of the cell lysates being 400 and 600 m-units per mg of protein respectively. Heating at 37 °C for 2 h did not

inactivate the enzyme expressed at these lower temperatures. In contrast, incubating the inactive mutated Ftf, D312I, that had been expressed at 37 °C at either 25 or 30 °C for 2 h did not lead to any recovery of activity. These results indicated that inactivation of the mutated Ftf, D312I, occurred during protein expression in *E. coli* at 37 °C.

In contrast to the 'sucrose box'-mutated Ftf, D312I, the mutated Ftf, D397S, remained inactive irrespective of whether it was expressed at 25, 30 or 37 °C, suggesting that the mechanism of inactivation of this mutated enzyme was different to that of the mutated Ftf, D312I.

Effects of site-directed mutations on the folding of the parental and mutated Ftf

To exclude the possibility that incorrectly folded proteins were expressed by *E. coli* at 37 °C, tryptophan emission fluorescence spectra were obtained from the purified parental and mutated Ftf, D312E, D312S, D312K and D397S, which contain 12 Trp residues [11]. The parental Ftf and mutated enzymes, D312E, D312S, D312K and D397S, possessed approximately the same tryptophan emission spectra with maximum intensity of 59–139 at 341 nm (Figure 2). These data indicated that the loss of enzyme activity in the mutated enzymes (Table 4) was not due to major structural changes in the proteins. However, the tryptophan emission spectra of the mutated Ftf, D312N and D312I, differed. An increase in intensity of 4.8- and 4.5-fold to values of 662 and 630 respectively and a red-shift in the maximum emission wavelength to 345 nm was observed (Figure 2). These changes in protein conformation most likely account for the 80% decrease in the values of k_{cat}^G/K_m and k_{cat}^F/K_m for the mutated Ftf, D312N (Table 4), while supporting a role for misfolding in the inactivation of the mutated Ftf, D312I, when expressed at 37 °C.

While the kinetic and protein conformational analyses of the mutated Ftf, D312I, point to a role for the involvement of the Asp-312 in the 'sucrose box' in binding to fructosyl acceptors, an alternative hypothesis is also plausible. Although the three-dimensional structure of the Ftf of *S. salivarius* is not known, the nature of the sequence, SGSAT-NSDGS that forms part of the 'sucrose box' strongly suggests that a 180° reverse turn (β -turn) may occur around the Gly or Ala residues and be stabilized by hydrogen bonding from the Ser, Thr, Asn and Asp residues in the sequence [24]. This proposal is consistent with the secondary structure prediction of the Ftf carried out at the ExPASy

Figure 2 Tryptophan fluorescence emission spectra of the parental and mutated Ftfs

Spectra obtained from 5 μg Ftf/ml. (—) D312; (—) D312N; (—) D312E; (—) D312S; (—) D312K; (—) D312L and (—) D397S.

Molecular Biology Server of the Swiss Institute of Bioinformatics (<http://www.expasy.ch/swissmod/SWISS-MODEL>) [25–27], where a loop structure is predicted around Asp-312 (results not shown). As reverse turns have been proposed as nucleation sites for protein folding [28], substituting Asp-312 with the bulky non-polar Ile residue could be envisaged as disrupting the putative reverse turn structure, causing protein misfolding, while mutations resulting in comparable hydrogen bonding donors such as Glu, Ser and Asn would allow the enzyme to fold and retain some activity. In this regard, it is perhaps pertinent that an Asp residue (Asp-176) has been found to play a key role in the activity of glucoamylase from *Aspergillus niger* by forming a hydrogen bond to stabilize a reverse turn that keeps the catalytic Glu residue (Glu-179) in an optimal orientation for catalysis [29]. Furthermore, multiple alignment of 60 family-32 glycosyl-hydrolases, of which the Ftf of *S. salivarius* is a member [9], including 19 Ftfs (10 bacterial and 9 plant), 6 bacterial levanases, 14 bacterial sucrases, 15 plant invertases, 3 fungal inulinases and 3 fungal invertases using the ECLUSTALW program [23] not only showed that the triplet motif, RDP, corresponding to the Arg-396/Asp-397/Pro-398 in the Ftf of *S. salivarius*, was 100% conserved but that the only other conserved residue was Gly-305 located in the putative β -turn-promoting motif that includes Asp-312. This suggests that Gly-305 could be the actual site of this β -turn.

As the mutated Ftf, D397S, remained inactive irrespective of the temperature of expression, a more detailed structural investigation was carried out as the parental and mutated Ftfs underwent denaturation in the presence of urea. During denaturation, the tryptophan fluorescence emission spectra of both proteins were similar (Figure 3), suggesting that they possessed similar folded structures and that the abolition of Ftf activity in the mutated Ftf, D397S, was due to the removal of the carboxy group at position 397 rather than any change in protein structure. The Asp residue in position 397 is thus an essential catalytic residue and could be the one that forms a covalent fructosyl-enzyme intermediate from sucrose. Substitution of Asp-309 with Asn in the equivalent RDP motif of the levansucrase from *A. diazotrophicus* did not change the K_m value for sucrose but did reduce the k_{cat} of the enzyme by 75-fold [13]. This observation adds support to our hypothesis that Asp-397 is the catalytic

Figure 3 Tryptophan fluorescence emission spectra of the parental Ftf and the mutated Ftf, D397S, during denaturation with urea

(A) parental Ftf (10 $\mu\text{g}/\text{ml}$); (B) mutated Ftf, D397S (10 $\mu\text{g}/\text{ml}$). Spectra from the top: no denaturing agent; 2, 6, 10, 20, 30, 40, 60 min after addition of 4 M urea; 2 min after addition of 8 M urea.

nucleophile in the Ftf of *S. salivarius*. In theory, both Asp and Asn residues can serve as nucleophiles to attack the C-2 of the fructosyl moiety of sucrose due to their resonance structures [3,30]. Consequently, the fructosyl-enzyme intermediate could be bound to the catalytic Asp-309 of the levansucrase of *A. diazotrophicus* by a glycosyl ester linkage involving the β -carboxyl group of the Asp residue or by an *N*-glycosyl linkage involving the amide group of the Asn residue in the mutated enzyme, D309N. Since the resonance effect in the amide group is weaker than that in the carboxy group [30], the observed reduction in enzyme activity by the mutated levansucrase, D309N, would be predicted [13]. In contrast, Ser does not possess a carbonyl group in the side chain to make the hydroxy group sufficiently electronegative to act as a nucleophile to attack the fructosyl moiety of sucrose. As a result, mutation of the presumptive catalytic Asp-397 of the Ftf of *S. salivarius* to Ser would be expected to abolish enzyme activity, as was the case.

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