Site-directed mutagenesis study of the three catalytic residues of the fructosyltransferases of *Lactobacillus reuteri* 121

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Abstract Bacterial fructosyltransferases (FTFs) are retaining-type glycosidases that belong to family 68 of glycoside hydrolases. Recently, the high-resolution 3D structure of the *Bacillus subtilis* levansucrase has been solved [Meng, G. and Futterer, K., Nat. Struct. Biol. 10 (2003) 935–941]. Based on this structure, the catalytic nucleophile, general acid/base catalyst, and transition state stabilizer were identified. However, a detailed characterization of site-directed mutants of the catalytic nucleophile has not been presented for any FTF enzyme. We have constructed site-directed mutants of the three putative catalytic residues of the *Lactobacillus reuteri* 121 levansucrase and inulosucrase and characterized the mutant proteins. Changing the putative catalytic nucleophiles D272 (inulosucrase) and D249 (levansucrase) into their amido counterparts resulted in a 1.5–4×10^5 times reduction of total sucrase activity.

Key words: Fructosyltransferase; Inulosucrase; Levansucrase; Catalytic residue; Mutagenesis; *Lactobacillus reuteri*

1. Introduction

Fructosyltransferase (FTF) enzymes belong to the glycoside hydrolase family 68 (GH68) [1] and synthesize either inulin, composed of β(2-1)-linked fructose residues (inulosucrase, Inu; EC 2.4.1.9) or levan, composed of β(2-6)-linked fructose residues (levansucrase, Lev; EC 2.4.1.10) [2,3]. They are β-retaining enzymes employing a double displacement mechanism that involves the formation and hydrolysis of a covalent glycosyl–enzyme intermediate, with an oxocarbenium ion-like transition state [4]. The active site of members of the GH68 family is composed of a catalytic triad: a catalytic nucleophile, a general acid/base catalyst, and a transition state stabilizer. The catalytic nucleophile attacks the anomeric center of the sugar, generating the enzyme–substrate intermediate. In a subsequent step this intermediate undergoes either transglycosylation or hydrolysis. Both steps require assistance of a general acid/base catalyst and a transition state stabilizer.

Very recently, the 3D structure of the *Bacillus subtilis* levansucrase with sucrose bound in the active site was resolved [5], the first of the GH68 family. The crystallographic data showed that D86 and E342 were in the proper position and in close contact with the sucrose to represent the catalytic nucleophile and general acid/base catalyst, respectively. Alignments showed that residue E342 is equivalent to the invariant Glu of yeast invertase and *Zymomonas mobilis* levansucrase (E278), which are vital to catalysis according to site-directed mutagenesis studies [6,7]. Residue D247 was identified as a transition state stabilizer based on the observation that it forms strong hydrogen bonds with the C3′ and C4′ hydroxyls of the fructosyl unit, but it is too far away from either the C2′ hydroxyl or the glycosidic oxygen to be one of the residues directly involved in catalysis. Additional evidence for the role of D247 comes from mutational studies on the equivalent residue in the levansucrases of *Z. mobilis* (D194), *Streptococcus salivarius* (D397) and *Gluconacetobacter diazotrophicus* SRT4 (D309) [7–9]. With respect to the catalytic nucleophile, no experimental mutagenesis studies have been reported yet.

Lactobacillus reuteri 121 possesses two fructosyltransferase (*ftf*) genes encoding an inulosucrase (Inu; GenBank accession number AF459437) and a levansucrase (Lev; GenBank accession number AF465251). We characterized site-directed mutants of the catalytic triad in the two *L. reuteri* FTFs. This is the first report on mutagenesis data of the catalytic nucleophile of levansucrases and inulosucrases, showing that when this residue is changed into its amido counterpart the sucrase activity is lost completely.

2. Materials and methods

2.1. General

Plasmid pBAD/myc/his/C (Invitrogen) was used for cloning purposes. *Escherichia coli* strain Top10 (Invitrogen) was used for gene expression. Plasmid-carrying *E. coli* strains were grown at 37°C on LB medium [10] with 100 μg/ml ampicillin and 0.02% (w/v) arabinose. Proteins were produced with a C-terminal truncation of 32 amino acid residues and a C-terminal poly-histidine tag, and purified, as described [11]. Protein concentrations were determined using the Bradford reagent (Bio-Rad, Munich, Germany) with bovine serum albumin as standard.

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2.2. Site-directed mutagenesis

Single mutations were introduced using the 'megaprimer' method [12] and confirmed by sequencing. *Pwo* polymerase (Roche biochemicals) was used for all polymerase chains reactions (PCR) using plasmid pBAD containing the *L. reuteri* 121 *lev* or *inu* genes as templates. PCR products were digested with *Nco*I and *Bgl*II and ligated into the pBAD vector, downstream of an inducible arabinose promoter and upstream of a His tag. For site-directed mutagenesis (synthesis of 'megaprimers') the following oligonucleotides were used in PCR reactions: Ad272n-I, 5′-GCCATGAATTCCATACATCTAAAGG-3′ (Inu D272N); Ad424n, 5′-CGATAATATAGCAATGCGTAATG-CTCATG-3′ (Inu D424N); Ae523q, 5′-GCGATGAAATTCAGC-GGCCGAATGTAG-3′ (Inu E523Q); Bd249n-I, 5′-GTACGGGCC-AAGAGTTCCAGACATC-3′ (Lev D249N); Bd404n, 5′-CTGCTT-ACGTAACCCACACGTTGTTC-3′ (Lev D404N); Be503q, 5′-GT-GATGAAGTTCAACGGCCGAACGTAG-3′ (Lev E503Q). Additionally, two flanking primers were used in all reactions: pBADRV-I, 5′-TCTGAGATGAGTTTTTGTTCGG-3′; pBADFD, 5′-TCCTAC-CTGACGCTTTTTATCG-3′. The underlined codons indicate mutations introduced; -I: antisense primer.

2.3. Activity measurements

Total activity of purified enzymes was determined by measuring the amount of glucose released from sucrose [2]. The amount of glucose formed reflects the total amount of sucrose utilized during the reaction (V_G). Assays were performed at 37°C in 25 mM sodium acetate buffer pH 5.4 and 1 mM $CaCl_2$ with 250 mM (Inu) or 100 mM (Lev) sucrose, respectively. For the wild-type enzyme samples were taken every 3 min. Because Inu and Lev mutants had low residual activity the assay conditions were modified as follows: measurements were done with 200 mM sucrose as substrate over a period of several hours with samples taken every 60 min (modified conditions). Although the FTFs are optimally active at 50°C [3], activity measurements over longer time periods were performed at 37°C to avoid abiotic hydrolysis of sucrose. All activity measurements showed linear increases in time and were proportional to the amount of enzyme added. The SigmaPlot 2001 version 7.0 was used for curve fitting of the data with the standard Michaelis–Menten formula. The kinetic parameters were determined at 37°C using sucrose concentrations ranging from 1 mM to 1 M. Enzyme activity assays were performed in triplicate.

2.4. Circular dichroism (CD) spectra

Multiple far-UV CD spectra (195–260 nm) were recorded at pH 7 and 37°C on a Jasco J-715 spectropolarimeter (Jasco, Japan) in quartz cells (optical path length 1 mm); scan speed 100 nm/min; data interval 0.2 nm; bandwidth 1.0 nm; sensitivity 20 mdeg; response time 0.125 s. Samples were first equilibrated for 6 min. All recorded spectra were corrected by subtraction of the spectrum of a protein-free sample. Spectra were analyzed for their secondary structure content according to [13]. Multiple near-UV CD spectra (250–350 nm) were recorded at pH 7 and 37°C using a cell with an optical path length of 1.0 cm; scan speed 50 nm/min; data interval 0.5 nm; bandwidth 1.0 nm; sensitivity 10 mdeg; response time 0.25 s. Samples were equilibrated for 15 min. All recorded spectra were corrected by subtracting a spectrum of a protein-free sample.

3. Results and discussion

3.1. Site-directed mutagenesis of the catalytic triad

Very recently, the 3D structure of the *B. subtilis* levansucrase was published [5]. The residues D86, D247, and E342 were identified as respectively the catalytic nucleophile, the transition state stabilizer and the general acid/base catalyst. Mutations in these residues, introducing alanine residues at these positions, resulted in loss of activity. The enzyme activity assay used measured levan production by monitoring changes in optical density over time of a reaction mixture with buffer, sucrose and enzyme. Earlier studies have reported mutagenesis data for the putative general acid/base catalyst or the transition state stabilizer in FTF enzymes, but the individual mutations were made in different enzymes. No mutagenesis data on the catalytic nucleophile of FTF enzymes have been reported. Thus, a comparative site-directed mutagenesis study combined with a determination of the resulting catalytic activity and the kinetic properties for one and the same enzyme has not been published.

Table 1
Total activity (U/mg) of purified *L. reuteri* 121 inulosucrase and levansucrase wild-type and site-directed mutant proteins

Inu		Lev	
WT	47 ± 4	WT	71 ± 15
D272N	$3.2\times10^{-4} \pm 2.8\times10^{-4}$[a]	D249N	$1.8\times10^{-4} \pm 10^{-4}$[a]
D424N	$6.6\times10^{-4} \pm 1.1\times10^{-4}$[a]	D404N	$2.3\times10^{-4} \pm 3.04\times10^{-5}$[a]
E523Q	$2.8\times10^{-3} \pm 1.1\times10^{-3}$[a]	E503Q	$2.2\times10^{-3} \pm 1.4\times10^{-3}$[a]

[a]Remaining activity sampled every hour.

Based on a sequence alignment of *L. reuteri* 121 Lev and Inu and the *B. subtilis* levansucrase (Fig. 1), residues D249, D404 and E503 (Lev) and D272, D424, and E523 (Inu) were identified as the putative catalytic nucleophile, the transition state stabilizer and the general acid/base catalyst, respectively. These residues were changed into their amido counterparts by site-directed mutagenesis and the total activity, i.e. the release of glucose from sucrose, was measured. The activity of the mutants was reduced at least 10 000-fold compared to the wild-type enzymes (Table 1). This is the first report on mutagenesis of the catalytic nucleophile in FTF enzymes, showing that when this residue is changed into its amido counterpart, enzyme activity is lost virtually completely.

Kinetic properties could only be determined for the Lev site-directed mutants D404N and E503Q (Table 2). The k_{cat} for the D404N mutant had decreased 613 000-fold, with an almost unchanged K_m. A considerably decreased k_{cat} value (68 000-fold) also was determined for the E503Q mutant, whereas its K_m decreased about eight-fold (from 21 to 2.6 mM). Unfortunately, the assay sensitivity could not be increased enough to determine kinetic parameters for the four other mutants. The k_{cat} values of these four other mutants are at least 10^5-fold lower compared to the wild-type enzymes (Table 2).

The large reduction in V_G of *L. reuteri* 121 Inu mutants (D272N, D424N and E523Q) and Lev mutants (D249N,

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SACB_BACSU     82  LDVWDSWPLQ     244  TLRDPHY     337  VTDEIERA
INU_LACRE     268  LDVWDSWPVQ     421  AMRDAHV     518  VSDEIERP
LEV_LACRE     245  LDVWDSWPVQ     401  CLRDPHV     498  ASDEVERP
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Fig. 1. Multiple sequence alignment (created using Clustal X (1.5b) program) [14] of bacterial FTF proteins, revealing three conserved blocks. The first conserved block contains the so-called VWDS motif and the second conserved block contains the so-called RDP motif. Asterisks indicate fully conserved residues. Residues subjected to mutagenesis are shown in bold. SACB_BACSU, *B. subtilis* SacB; INU_LACRE, *L. reuteri* inulosucrase; LEV_LACRE, *L. reuteri* levansucrase.

Table 2
Kinetic parameters of purified *L. reuteri* 121 levansucrase wild-type and site-directed mutant proteins

	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
WT	184 ± 17	21 ± 4.1	8.7
D249N	n.d.	n.d.	n.d.
D404N	$3\times10^{-4} \pm 2.1\times10^{-5}$	28 ± 11	1.07×10^{-5}
E503Q	$2.7\times10^{-3} \pm 1.3\times10^{-3}$	2.6 ± 0.75	1.0×10^{-3}

n.d., not determined.

D404N and E503Q) suggests that these residues are essential for catalysis. The E503Q mutant showed a relatively small reduction in k_{cat}/K_m ratio (Table 2), suggesting that the E503 (Lev) and E523 (Inu) residues act as general acid/base catalysts, similar to E342 of *B. subtilis* levansucrase [1] (Fig. 1). Our data support the hypothesis [5] that the Asp residue located in the highly conserved family GH68 motif -V**WD**SW- (invariant residues in bold) acts as catalytic nucleophile (Fig. 1): D249 (Lev) and D272 (Inu) residues. Finally, we conclude that D404 (Lev) and D424 (Inu) in the highly conserved family GH68 RDP motif (Fig. 1) constitute the third residue (transition state stabilizer) of the catalytic triad in these *L. reuteri* 121 enzymes.

Mutations in the catalytic residues of the *B. subtilis* levansucrase caused minor, if any, structural changes in the protein [5]. To determine whether mutations in the catalytic residues of the *L. reuteri* 121 levansucrase and inulosucrase had caused structural changes, we recorded far- and near-UV CD spectra of the wild-type and the mutants enzymes. These spectra showed that minor, if any, structural changes had occurred and thus that a change in the chemical nature of the mutated residues was responsible for the inactivation of mutant proteins (data not shown).

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