Kinetic properties of an inulosucrase from *Lactobacillus reuteri* 121

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Abstract **Inulosucrases catalyze transfer of a fructose moiety from sucrose to a water molecule (hydrolysis) or to an acceptor molecule (transferase), yielding inulin. Bacterial inulin production is rare and a biochemical analysis of inulosucrase enzymes has not been reported. Here we report biochemical characteristics of a purified recombinant inulosucrase enzyme from *Lactobacillus reuteri*. It displayed Michaelis–Menten type of kinetics with substrate inhibition for the hydrolysis reaction. Kinetics of the transferase reaction is best described by the Hill equation, not reported before for these enzymes. A C-terminal deletion of 100 amino acids did not appear to affect enzyme activity or product formation. This truncated form of the enzyme was used for biochemical characterization.**

Key words: Fructosyltransferase; Inulosucrase; Sucrose; *Lactobacillus reuteri*

1. Introduction

Bacterial fructosyltransferase (FTF) enzymes perform three types of reactions with sucrose: (i) polymerization, in which fructose units are linked to a fructan polymer; (ii) hydrolysis, in which sucrose is split into fructose and glucose; and (iii) transferase of fructose to an acceptor forming an oligosaccharide. FTF enzymes producing fructan polymers with β(2→1) linked fructosyl units (inulin) are referred to as inulosucrases (sucrose: 2,1-β-D-fructan 6-β-D-fructosyltransferase; E.C. 2.4.1.9). Bacterial inulin production is rare and has been reported only for *Streptococcus mutans* [1–3], and recently *Lactobacillus reuteri* 121 [4] and *Leuconostoc citreum* [5]. No detailed biochemical studies have been performed on the inulosucrase enzymes involved. Levansucrase enzymes (E.C. 2.4.1.10), synthesizing levan polymers with β(2→6) linked fructosyl units, occur more widespread in bacteria and have been studied in more detail. The biochemical characteristics of the levansucrases from *Bacillus subtilis* [6], *Gluconacetobacter diazotrophicus* [7], and *Streptococcus salivarius* [8] have been determined. These studies have shown that levansucrase enzymes display Michaelis–Menten type of kinetics for the transferase and hydrolysis reactions.

L. reuteri is an inhabitant of the gastrointestinal tract of a broad spectrum of hosts, including mammals and birds. In these hosts it forms the major component of gut lactobacilli [9]. The health-promoting effects of fructo-oligosaccharides have been studied extensively [10,11]. Fructans produced by FTFs from oral streptococci are thought to enhance the cariogenicity of dental plaque formation [12]. The important roles of these (producers of) FTF enzymes, and/or their products in the colonization of the gastrointestinal tract and dental plaque formation justify a thorough investigation of the mode of action of these enzymes.

Previously [4], we have reported the successful expression in *Escherichia coli* of two Inu variants, InuHis (the full-length Inu protein, with a C-terminal His tag), and InuΔ699His (an Inu protein with a 100 amino acid truncation at the C-terminus, from amino acid 699 onwards, with a C-terminal His tag). Both Inu variants, when incubated with sucrose, produced fructan polymers that were identical with respect to binding type of fructosyl units (β(2→1)) and molecular weight distribution. Furthermore, both Inu variants produced considerable amounts of fructo-oligosaccharides (95% 1-kestose and 5% nystose) from sucrose [4]. InuHis protein produced in *E. coli* showed smearing on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels, and a His tag could not be detected [4]. These observations may suggest aberrant protein folding in *E. coli* or that the InuHis protein in *E. coli* is in fact truncated at its C-terminus. Because InuHis protein could not be purified, and enzyme activity and product formation did not appear to be affected by the C-terminal truncation, InuΔ699His was used for biochemical characterization (this paper). The kinetics of its transferase reaction is unique, and is best described by the Hill equation.

2. Materials and methods

2.1. Strains, plasmids, media and growth conditions

E. coli strain Top10 (Invitrogen, Carlsbad, CA, USA) was used for expression of the *L. reuteri* 121 inulosucrase gene (*inu*; GenBank accession number AF459437). The *E. coli* strain was grown aerobically at 37°C in Luria–Bertani medium [13], supplemented with 50 μg ml^{-1} ampicillin for maintaining plasmids, and with 0.02% arabinose (w/v) for enzyme induction.

2.2. Inu enzyme purification

Two versions of the *inu* gene were expressed in *E. coli* Top10 harboring the plasmid pBAD/Myc-His C, (i) a full-length *inu* (InuHis; encoding the full-length Inu protein, with a C-terminal His tag), and (ii) a truncated *inu* (InuΔ699His; encoding an Inu protein with a 100 amino acid truncation at the C-terminus, from amino acid 699 onwards, with a C-terminal His tag) [4]. Both recombinant proteins were extracted from *E. coli* cells and InuΔ699His was purified by Ni-NTA and Resource-Q column chromatography steps as described previ-

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ously [4]. The InuHis and InuΔ699His proteins were dialyzed overnight against a sodium acetate buffer (25 mM, pH 5.4) and stored at 4°C for further analysis.

2.3. Biochemical characterization of the recombinant Inu enzyme

Sucrose conversion by inulosucrase yields (a) fructose, which is (partly) built into the growing fructan polymer, and (b) glucose, which is in a 1:1 ratio to the amount of sucrose converted. The amount of free fructose is a measure for the hydrolytic activity of the enzyme. The amount of glucose minus the amount of free fructose reflects the transferase activity. Glucose and fructose were measured enzymatically as described before [14]. Inu enzyme activity was measured in a sodium acetate buffer (25 mM; pH 5.4) with 250 mM sucrose and 1 mM calcium chloride at 50°C, unless stated otherwise. One unit of enzyme activity is defined as the release of 1 μmol of product per min. All experiments were performed in triplicate and, where appropriate, the results are presented as the means ± standard error of means (S.E.M.). All activity measurements showed linear increases in time, and were proportional to the amount of enzyme added to the assay. The 'Sigma Plot' program (version 4.0) was used for curve fitting of the data, either with the standard Michaelis–Menten formula: $y=(a\times x)/(c+x)$, the three-parameter Hill formula: $y=(a\times x)^b/(c^b+x^b)$, or a Michaelis–Menten formula with a substrate inhibition constant: $y=(a\times x)/(c+x+(x^2/d))$. In these formulas, y is the specific activity (U mg^{-1}), x is the substrate concentration (mM sucrose), a is the V_{max} (U mg^{-1}), b is the Hill factor, c is the K_m or K_{50} (mM sucrose; K_m in case of Michaelis–Menten type of kinetics; K_{50} in case of Hill type of kinetics), and d is the substrate inhibition constant (mM sucrose).

3. Results

3.1. Effect of pH, temperature, and metal ions on Inu enzyme activity

InuΔ699His had a broad pH spectrum with highest enzyme activity (measured as the release of glucose from sucrose; total activity) at pH 5–5.5 (Fig. 1A) at 50°C (Fig. 1B). Its activity decreased rapidly at temperatures higher than 60°C (Fig. 1B). A 10 times diluted enzyme solution remained stable at 4°C for more than one month. Undiluted enzyme preparations stored at 4°C remained stable for several days. When frozen in 10% glycerol at −20°C, no enzyme activity was lost, not even after storage for several months. The cations (1 mM) Hg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+} and Fe^{3+} as well as ethylenediamine tetraacetic acid (EDTA) significantly reduced enzyme activity. The cations (1 mM) Mg^{2+}, K^+, and Na^+ had no significant effect on enzyme activity. Enzyme activity increased with almost 30% upon addition of Ca^{2+} ions (Table 1).

Table 1
Effects of various compounds on InuΔ699His enzyme activity

Compound (1 mM)	Activity (%)
–	100.00 ± 1.2
EDTA	0.17 ± 0.04
HgCl	3.62 ± 0.17
KCl	104.6 ± 0.7
NaCl	98.1 ± 1.1
$CaCl_2$	127.4 ± 0.7
$CuCl_2$	1.81 ± 0.27
$FeCl_2$	46.4 ± 1.8
$MgCl_2$	98.3 ± 1.5
$ZnCl_2$	9.3 ± 0.1
$FeCl_3$	28.1 ± 1.5

Enzyme measurements were done with 3.6 μg ml^{-1} enzyme (final concentration) in a sodium acetate buffer (25 mM; pH 5.4) with 250 mM sucrose. The amount of glucose released by the enzyme during 15 min was taken as a measure for enzyme activity. Results are given as means ± S.E.M.; $n=3$, relative (in %) to the enzyme activity without addition of cations.

3.2. Inulosucrase kinetic properties

In the present study we observed that InuHis obtained from *E. coli* cell extracts (results not shown) and purified InuΔ699His showed similar kinetic behavior, with virtually identical ratios of hydrolytic and transferase activities. For InuΔ699His, the optimal temperature for the release of glucose from sucrose was 50°C. At 20 and 50°C, however, InuΔ699His enzyme activity (measured as glucose release from sucrose) increased with sucrose concentrations up to 1 M (Fig. 2A and B), indicating that even at these high sucrose concentrations the enzyme was not saturated by its substrate. Kinetic constants were determined at both temperatures, based on enzyme activity versus substrate concentration curves (Fig. 2A and B), measuring (i) glucose, (ii) fructose and calculating (iii) glucose minus fructose release from sucrose. Curve fitting was done for each of the three curves (V_G, V_F, and V_{G-F}). Michaelis–Menten type of kinetics with sucrose substrate inhibition was observed for the hydrolysis reaction (V_F), at both 20°C and 50°C. For the hydrolysis reaction the enzyme showed higher affinity for sucrose at 20°C (10.5 mM) than at 50°C (16.4 mM). Catalytic turnover rates were at 50°C (88.9 s^{-1}) approximately 10 times higher than at 20°C (10.0 s^{-1}; Table 2). The hydrolysis reaction showed at both temperatures minor substrate inhibition effects, reflected by the com-

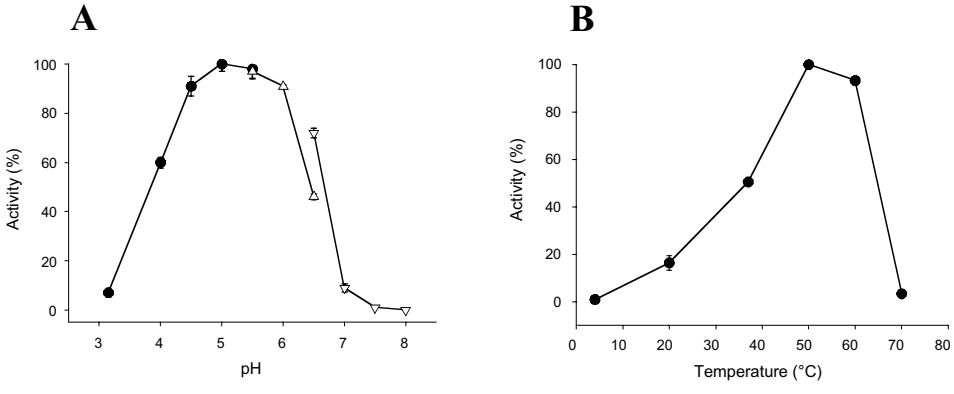

Fig. 1. Effect of pH (A) and temperature (B) on InuΔ699His enzyme activity. For the pH gradient (A) assay buffers contained (i) 25 mM buffer (●) sodium acetate, (△) MES, and (▽) MOPS, (ii) 250 mM sucrose, and (iii) 1 mM $CaCl_2$. The reaction was started by the addition of 3.6 μg ml^{-1} enzyme. Glucose formation from sucrose was followed for 15 min. Results are given proportional (in %) to the enzyme activity at pH 5.0 (A) or at 50°C (B), with a mean ± S.E.M.; $n=3$.

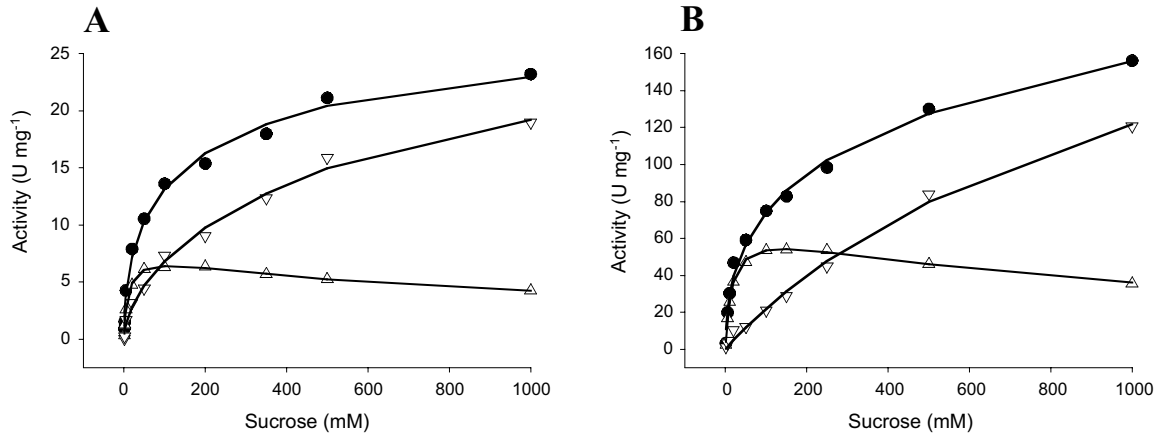

Fig. 2. *V–S* relationships for the InuΔ699His enzyme. Enzyme was incubated at 20°C (A; with 22 μg ml^{-1} enzyme; final concentration) and 50°C (B; with 3.6 μg ml^{-1} enzyme; final concentration). (●) V_G (total activity), (△) V_F (hydrolytic activity), and (▽) V_{G-F} (transferase activity).

parable high inhibition constants (1.3 and 1.2 M, respectively). Both transferase (V_{G-F}) and total enzyme activity (V_G) did not follow normal Michaelis–Menten kinetics but could best be fitted with the Hill equation, at both 20°C and 50°C. Even at higher concentrations the V_G and V_{G-F} activities of the enzyme were not saturated by sucrose, resulting in high standard errors (exceeding 30%) obtained with curve fits. At 50°C, the catalytic turnover rate was 504 ± 198 s^{-1} (Table 2) for total enzyme activity. A 10-fold lower rate was observed at 20°C (43.6 ± 6.4 s^{-1}). The Hill factors were comparable at 20°C and 50°C (0.55 ± 0.07 and 0.46 ± 0.06, respectively) and were indicating a negative cooperativity for the total activity. Also the catalytic turnover rate of the transferase reaction showed high standard errors. At 50°C (362 ± 124 s^{-1}) an approximately 10 times higher turnover rate was observed than at 20°C (55.2 ± 20.3 s^{-1}). At 20°C the transferase reaction showed negative cooperativity (reflected by the lower Hill factor); at 50°C the Hill factor was close to 1, indicating that there were neither negative nor positive cooperativity effects.

4. Discussion

Very little is known about the biochemical and mechanistic properties of the bacterial inulosucrases reported to be present in *L. reuteri* 121 [4], *S. mutans* GS-5 [3,15], and *L. citreum* [5]. Specific activity values reported for the recombinant *S. mutans* GS-5 FTF enzyme were over 4000 U mg^{-1}, indicating a K^G_{cat} value of 6000 s^{-1}. This K^G_{cat} value is 10–100 times higher than those reported for any other levansucrase enzyme and Inu (Table 2). No data are available about the affinity of the enzyme for its substrate sucrose.

The basic biochemical properties of *L. reuteri* 121 Inu are comparable to those reported for levansucrase enzymes of *S. salivarius* and *G. diazotrophicus* (Table 2). These levansucrase enzymes all display Michaelis–Menten type of kinetics for the

Table 2
Comparison of apparent kinetic constants for some bacterial FTFs

Kinetic parameter	*L. reuteri* Inu		*G. diazotrophicus* LsdA[b]	*S. salivarius* FTF[c]
	20°C[a]	50°C[a]		
$K^G_{50/m}$ (mM)	–[d]	–[d]	11.8 ± 1.4	5.0 ± 0.3
K^G_{cat} (s^{-1})	43.6 ± 6.4	504 ± 198	n.d.	63.5 ± 3.6
$K^G_{cat} \times (K^G_m)^{-1}$ (mM^{-1} s^{-1})	–[d]	–[d]	n.d.	12.7
Hill factor^G	0.55 ± 0.07	0.46 ± 0.06	n.d.	n.d.
$K^F_{50/m}$ (mM)	10.5 ± 1.0	16.4 ± 1.0	11.8 ± 1.4	4.9 ± 0.2
K^F_{cat} (s^{-1})	10.0 ± 0.3	88.9 ± 1.9	60 ± 7	28.9 ± 1.2
$K^F_{cat} \times (K^F_{50/m})^{-1}$ (mM^{-1} s^{-1})	0.95	5.4	5.2	6.5
K^F_i (M)	1.3 ± 0.1	1.2 ± 0.1	n.d.	n.d.
$K^{G-F}_{50/m}$ (mM)	–[d]	–[d]	n.d.	n.d.
K^{G-F}_{cat} (s^{-1})	55.2 ± 20.3	362 ± 124	n.d.	n.d.
Hill factor^{G-F}	0.64 ± 0.09	0.97 ± 0.12	n.d.	n.d.

The kinetic constants are K_{cat}, $K_{50/m}$, and $K_{cat} \times (K_{50/m})^{-1}$ for (a) formation of glucose (^{G}; total enzyme activity), (b) formation of fructose (^{F}; hydrolytic enzyme activity), and (c) glucose minus fructose ($^{G-F}$; transferase enzyme activity).
n.d., kinetic parameters have not been reported.
[a]Final enzyme concentrations were 22 μg ml^{-1} (at 20°C) and 3.6 μg ml^{-1} (at 50°C).
[b]Data from [7].
[c]Data from [8].
[d]These kinetic parameters could not be determined due to the fact that the enzyme was not saturated with sucrose, resulting in high standard errors with curve fits (Fig. 2A, B).

hydrolysis and transferase reactions. Inu displays a temperature optimum at 50°C and non-Michaelis–Menten type of kinetics for the transferase reaction. For levansucrases [6–8] and the *S. mutans* inulosucrase [3], temperature optima of 37°C have been reported. The Inu pH optimum of 5.0–5.5 is comparable to the values reported for other FTFs. Similar inhibiting effects of the compounds Hg^{+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+} and EDTA were reported for the various levansucrases. Non-standard Michaelis–Menten kinetics also has been reported for the plant *Helianthus tuberosus* 1-SST and 1-FFT enzymes [16]. As is the case for Inu, these enzymes cannot be saturated by their substrate sucrose. Bacterial FTFs, however, do not share a high sequence homology with plant FTFs (about 10%; results not shown). The hydrolysis reaction of the *L. reuteri* Inu enzyme displayed a mild substrate inhibition effect (K_i value of 1.2 ± 0.1 M; Table 2). This substrate inhibition effect can be explained by the competition of sucrose and water as acceptor for the fructosyl unit. Analysis of the FTF enzyme of *S. salivarius* [8] revealed Michaelis–Menten type of kinetics with strong substrate inhibition effects with all three reactions (V_G, V_F and V_{G-F}), but no inhibition constants have been reported. For the *Bacillus circulans* levansucrase [17] enzyme substrate inhibition effects were observed for V_G and V_{G-F}. Interestingly, the latter enzyme does not show substrate inhibition for the hydrolysis reaction.

The kinetics of the Inu transferase reaction is best described by the Hill equation. The Hill factors calculated for Inu were all lower than 1, indicating a negative cooperativity in the Inu enzyme. With Hill type of kinetics it is assumed that there is more than one binding site present in the enzyme and/or multimeric forms of the enzyme. A positive cooperativity indicates a positive interaction of binding sites present in the enzyme and/or multimers. Alternatively, a negative cooperativity indicates a negative interaction of enzyme binding sites and/or multimers. In FTF enzymes, it is not known how many binding sites are present for substrate and product binding due to the lack of detailed structural protein information. Furthermore, we have shown that the *L. reuteri* 121 levansucrase enzyme is monomeric [14]. Multimeric forms of FTF enzymes have been reported only for the levansucrase from *Actinomyces viscosus* T14 [18]. Only Michaelis–Menten type of kinetics has been observed for levansucrase enzymes. Further studies are required to draw mechanistical conclusions on the nature of kinetics observed in the Inu enzyme.

The Inu enzyme from *L. reuteri* 121 produced large amounts of the fructo-oligosaccharide 1-kestose. 1-Kestose production has been reported with FTFs from *Paenibacillus polymyxa* CF43 [19], *G. diazotrophicus* SRT4 [7], and *Zymomonas mobilis* ATCC 10988 [20]. Interestingly, the main products of these enzymes are levan polymers with $\beta(2\rightarrow6)$ linked fructosyl units. 1-Kestose carries a $\beta(2\rightarrow1)$ linked fructosyl unit on the fructose residue of sucrose. The fact that these levansucrase enzymes produce levan and 1-kestose might indicate the presence of two binding sites in FTF enzymes: one site generates the 1-kestose primer molecules, which are subsequently used by a second binding site in the polymerization process. A second binding site for the polymerization reaction has previously been proposed for the FTF enzyme of *S. salivarius* [21].

The kinetics of the *L. reuteri* inulosucrase for the transferase reaction is best described as Hill type of kinetics with a negative cooperativity. The enzyme could not be saturated by its substrate sucrose for the transferase and overall enzyme activities. These observations are very different from the standard Michaelis–Menten kinetics reported for levansucrase enzymes. It therefore remains unclear whether the catalytic properties of the *L. reuteri* inulosucrase enzyme are common for bacterial inulosucrase enzymes, or not.

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