FEBS Letters 579 (2005) 1124–1128 FEBS 29250

Mutational analysis of the role of calcium ions in the *Lactobacillus reuteri* strain 121 fructosyltransferase (levansucrase and inulosucrase) enzymes

L.K. Ozimek[a,b], G.J.W. Euverink[a,b], M.J.E.C. van der Maarel[a,c], L. Dijkhuizen[a,b,*]

[a] *Centre for Carbohydrate Bioengineering, TNO – University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands*
[b] *Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands*
[c] *Innovative Ingredients and Products Department, TNO Nutrition and Food Research, Rouaanstraat 27, 9723 CC Groningen, The Netherlands*

Received 24 September 2004; revised 16 November 2004; accepted 23 November 2004

Available online 19 January 2005

Edited by Judit Ovádi

Abstract Bacterial fructosyltransferase enzymes belonging to glycoside hydrolase family 68 (GH68) are not known to require a metal cofactor. Here, we show that Ca^{2+} ions play an important structural role in the *Lactobacillus reuteri* 121 levansucrase (Lev) and inulosucrase (Inu) enzymes. Analysis of the *Bacillus subtilis* Lev 3D structure [Meng, G. and Futterer, K. (2003) Nat. Struct. Biol. 10, 935–941] has provided evidence for the presence of a bound metal ion, most likely Ca^{2+}. Characterization of site-directed mutants in the putative Ca^{2+} ion-binding sites of *Lb. reuteri* Lev and Inu revealed that the Inu Asp520 and Lev Asp500 residues play an important role in Ca^{2+} binding. Sequence alignments of family GH68 proteins showed that this Ca^{2+} ion-binding site is (largely) present only in proteins of Gram-positive origin.

Keywords: Fructosyltransferase; Levansucrase; Inulosucrase; Mutagenesis; Calcium ion; *Lactobacillus reuteri*

1. Introduction

Bacterial fructosyltransferase (FTF) enzymes are found in Gram-negative and Gram-positive bacteria (see CAZY database: http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). They convert sucrose into fructan polysaccharides, in most cases with β2,6 glycosidic bonds (a levan). A few FTF enzymes of Gram-positive bacteria synthesize inulin, with β2,1 glycosidic bonds [1–3]. Previously, we have characterized the *Lactobacillus reuteri* strain 121 levansucrase (Lev; E.C. 2.4.1.10) [4] and inulosucrase (Inu; E.C. 2.4.1.9) [5] enzymes, and identified the catalytic triad in both enzymes [6]. These two FTF enzymes are very closely related (86% similarity and 56% identity over 768 amino acids), both depending on Ca^{2+} ions for activity (albeit to a different extent) and displaying unusually high temperature optima (about 50 °C) (see Section 3). They differ most clearly in fructans synthesized, levan and inulin. Fructan synthesis by FTF enzymes of this (probiotic) *Lb. reuteri* strain is of strong interest for food and nutrition applications.

*Corresponding author. Fax: +31 50 363 2154.
E-mail address: l.dijkhuizen@biol.rug.nl (L. Dijkhuizen).

Analysis of the first high-resolution (1.5 Å) 3D structure of a *Bacillus subtilis* Lev [7] has provided evidence for the presence of a bound metal ion, most likely Ca^{2+}. Amino acid residues involved in this putative Ca^{2+} ion-binding site are conserved in most of the FTF proteins from Gram-positive bacteria, but not in all members of family GH68 (bacterial FTF and invertase enzymes) [7] (see Section 3). In *B. subtilis* Lev, Asp339 was suggested to make a most important contribution to Ca^{2+} binding. Data for FTF Asp339 mutant enzymes have not been reported yet. In the present study, we have analyzed the (differences in) sensitivity of the *Lb. reuteri* strain 121 Lev and Inu enzymes for Ca^{2+} ions and EDTA. Moreover, the suggested role in Ca^{2+} binding of residues Asp500 (Lev) and Asp520 (Inu), equivalent to Asp339 in *B. subtilis* Lev, was probed by site-directed mutagenesis.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Escherichia coli strain Top10 (Invitrogen) was used for expression of wild type (WT) and mutant *Lb. reuteri* 121 *ftf* genes, inulosucrase (*inu*; GenBank Accession No. AF459437) and levansucrase (*lev*; GenBank Accession No. AF465251). Plasmid pBAD/myc/his/C (Invitrogen) was used for cloning purposes. Plasmid carrying *E. coli* strains were grown at 37 °C on LB medium [8] supplemented with 100 μg/ml ampicillin and 0.02% (w/v) arabinose for *ftf* gene induction. WT and mutant proteins were expressed in *E. coli* as constructs with a C-terminal truncation of 32 amino acid residues, and a C-terminal poly-histidine tag [2].

2.2. Molecular techniques

Alignments of FTF and invertase enzymes of family GH68 (CAZY, URL: http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html) were constructed using the Clustal X (1.5b) program. Using site-directed mutagenesis, the *Lb. reuteri* 121 Lev Asp500 and Inu Asp520 amino acids were replaced by Asn and Ala residues. Single mutations were introduced in the *inu* and *lev* genes using the "megaprimer" method [9] and were confirmed by sequencing. PCRs with *Pwo* polymerase (Roche biochemicals) used plasmid pBAD/myc/his/C containing the *lev* or *inu* genes as templates. All PCR products were digested with *Nco*I and *Bgl*II and ligated into the pBAD/myc/his/C 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 PCRs: AD520A, 5′-CCAATGGTAAGCGCTGAAATTGAG-3′ (Inu D520A); BD500A, 5′-GGCTAGTGCTGAAGTTGAACGAC-3′ (Lev D500A); BD500N-I, 5′-CGTTTGGTCGTTCAACTTCATTACTAGCCATC-3′ (Lev D500N); AD520N, 5′-CAATGGTAAGCAATGAAATTGAG-3′ (Inu D520N).

0014-5793/$30.00
doi:10.1016/j.febslet.2004.11.113

Additionally, two flanking primers were used in all reactions: pBADRV-I, 5′-TCTGAGATGAGTTTTTGTTCGG-3′; pBADFD, 5′-TCCTACCTGACGCTTTTTATCG-3′. The underlined codons indicate mutations introduced; -I: antisense primer.

2.3. Purification of FTF proteins

All proteins produced were expressed in His-tag versions and purified by Ni–NTA affinity chromatography as described [2]. MilliQ water was used in all purification steps to minimize the calcium concentration in protein samples. However, no metal ion chelators were added. Purity was checked by SDS–PAGE. Enzyme concentrations were determined using the Bradford reagent (Bio-Rad, Munich, Germany) with bovine serum albumin as standard.

2.4. FTF enzyme activity assays

Activity variations with temperature (22–57 °C) were determined in 25 mM sodium acetate buffer, pH 5.4, with 100 mM sucrose (and Ca^{2+} ions as indicated), using purified *Lb. reuteri* 121 enzymes, WT Inu (2.9 μg/ml protein) and Inu mutants D520N, A (7.2 and 104 μg/ml, respectively), WT Lev (4.5 μg/ml protein) and Lev mutants D500N, A (13.8 and 21.6 μg/ml, respectively). At the highest temperatures (Lev mutants 45–55 °C, Inu mutants 50–57 °C), 5-fold higher protein concentrations were used. After preincubation of the assay mixture at the assay temperature for 5 min, reactions were started by enzyme addition. Samples were taken every 3 min and used to determine the amount of glucose released from sucrose [10]. The amount of glucose formed reflects the total amount of sucrose utilized during the reaction (V_G). Experiments were performed in duplicate. Effects of EDTA were analyzed by determining temperature optima of Inu and Lev enzyme activity using the standard assay but with the reaction buffer containing 1 mM EDTA instead of Ca^{2+} ions.

2.5. FTF affinity for calcium-ion binding

Lev and Inu enzyme activities were determined as described above with $CaCl_2$ at concentrations of 0 (calcium-free buffer, prepared with MilliQ water) to 10 mM. The data obtained were used to estimate the Ca^{2+} binding affinity of the proteins. The Sigma Plot (version 8.0) program was used for curve fitting of data, with the standard Michaelis–Menten equation: $[y = (ax)/(b + x)]$. In this formula, y is the specific enzyme activity [U/mg], x is the calcium concentration [mM], a is the V_{max} and b is the apparent K_d (K'_d) [mM of calcium].

2.6. Thermostability of Inu and Lev enzymes

The WT Inu (2.9 μg/ml protein) and WT Lev (4.5 μg/ml protein) enzymes were incubated at a range of temperatures (37–60 °C) for 30 min with 1 mM calcium or in calcium-free buffer. The remaining enzyme activities were assayed at 37 °C according to the standard procedure (see Section 2.4). Samples preincubated in the presence and absence of calcium were assayed in buffer containing 1 mM calcium or in calcium-free buffer, respectively.

3. Results and discussion

3.1. Effects of Ca^{2+} ions and EDTA on Lb. reuteri 121 FTF enzyme activity

Plots of *Lb. reuteri* 121 Inu and Lev specific activity (initial rates) versus temperature in the presence or absence of Ca^{2+} (or EDTA, data not shown) revealed interesting similarities and differences between the two proteins (Fig. 1). Without addition of EDTA or Ca^{2+}, the *E. coli* produced and purified Lev enzyme lost activity at temperatures above 40 °C and was completely inactive at 50 °C, whereas Inu activity decreased above 45 °C and was completely inactive at 55 °C. Addition of 1 mM EDTA strongly reduced the activities of both enzymes. At room temperature, there was no effect of EDTA on Inu activity whereas a 60% reduction in Lev activity occurred. The negative effect of EDTA strongly increased with temperature, reaching 65% (Lev) and 40% (Inu) reduction in activities at 30 and 37 °C, respectively, the temperature optima for activity in the presence of EDTA (data not shown). With additional Ca^{2+} ions (1 mM) present, the Lev and Inu enzymes both showed considerably enhanced activities at higher temperatures, Inu now displaying an optimum at 55 and Lev at 45 °C (Fig. 1). The presence of (extra) Ca^{2+} ions thus appeared essential, especially at higher temperatures, to prevent inactivation of both the Lev and Inu enzymes (Fig. 1), most likely by temperature-dependent unfolding (see below). These effects of calcium ions and EDTA on both *Lb. reuteri* FTF proteins are in agreement with previously published observations for *B. subtilis* Lev [11–14] and *Streptococcus salivarius* FTF [15–17]. Our observations thus suggest that Ca^{2+} ions play an important structural role in these bacterial FTF enzymes and promote the *Lb. reuteri* Lev and Inu enzyme activity at elevated temperatures. Both *Lb. reuteri* WT FTF enzymes showed a high-affinity for Ca^{2+} binding, with K'_d values of 6.9 μM (Lev at 45 °C) and 0.48 μM (Inu at 50 °C). By comparison, a somewhat lower affinity for Ca^{2+} binding (K_d of 18 μM) has been reported for the *S. salivarius* Lev [18].

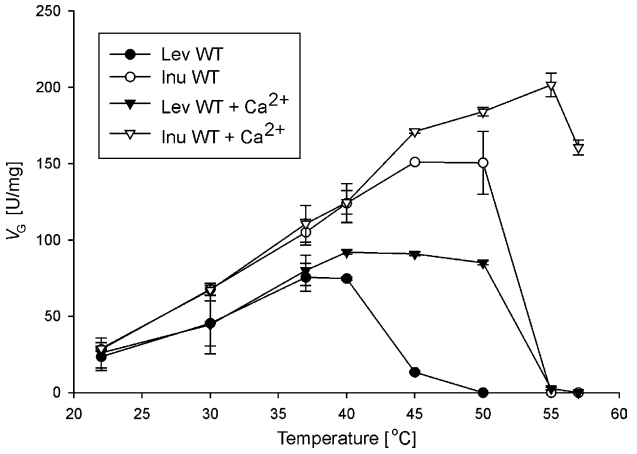

Fig. 1. Relationship between the specific activities (V_G) of the *Lb. reuteri* 121 Inu and Lev wild type enzymes and incubation temperature, measured in the absence or presence of 1 mM Ca^{2+} ions.

Chambert and Petit-Glatron [11] have shown previously that proper folding of Lev of *B. subtilis* depends on the presence or absence of a metal chelator. Our data show that also the two FTF enzymes of *Lb. reuteri* are affected in a similar way by the presence and absence of calcium ions and the chelator EDTA.

3.2. Effects of Ca^{2+} ions on thermostability of Lb. reuteri 121 FTF enzymes

The Inu and Lev proteins were incubated at a range of temperatures for 30 min, followed by determination of the remaining activity at 37 °C. A drastic loss of Inu activity was observed at temperatures above 45–50 °C, the presence of Ca^{2+} ions providing clear protection (Fig. 2A). Rather different profiles were obtained for the Lev protein (Fig. 2B). Following incubation of Lev at temperatures of 50–60 °C, 50–80% of activity was recovered at 37 °C. Also in this case the presence of Ca^{2+} ions provided protection. A clear reduction in Lev activity (initial rates) was observed at higher temperatures (Fig. 1), but the Lev protein apparently suffered no irreversible damage and recovered activity upon subsequent incubation at 37 °C