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ORIGINAL ARTICLE

Biochemical and crystallographic characterization of a glucansucrase from *Lactobacillus reuteri* 180

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Abstract
Glucansucrases are large extracellular transglycosidases secreted by lactic acid bacteria. Using sucrose as a substrate they synthesize high molecular mass α-glucans or, in the presence of suitable acceptor molecules, low molecular mass oligosaccharides. Although about 60 glucansucrases have been classified in glycoside hydrolase family GH70, no three-dimensional structure has been reported for any. With the aim of solving the first structure of a GH70 glucansucrase, purification and crystallization experiments were performed with a fully active, 117 kDa N-terminally truncated fragment of glucansucrase GTF180 from *Lactobacillus reuteri* 180 (residues 742-1772). Crystallization experiments yielded crystals that belong to two different triclinic crystal forms (space group $P1$) and one orthorhombic crystal form (space group $P2_12_12_1$). Native data sets for both triclinic and the orthorhombic crystals were collected at 1.7 and 2.0 Å resolution, respectively. Enzyme activity assays, pH and temperature optima show comparable values for both the full-length and the N-terminally truncated GTF180.

Keywords: *Lactic acid bacteria, glucansucrase, crystallization*

Introduction

Glucansucrases (EC 2.1.4.5) are transglycosidases secreted by lactic acid bacteria that catalyse the synthesis of high molecular mass glucose polymers (α-glucans). They use the disaccharide sucrose as their substrate, cleaving its glycosidic bond and transferring its glucosyl unit to the growing glucan chain. In addition, if other (small) acceptor molecules are present, these can be glucosylated as well to form oligosaccharides (Monchois et al. 1999).

The α-glucan polysaccharides are produced extracellularly, where they form a viscous exopolysaccharide (EPS) layer around the bacteria. Their function is not fully known, but they have been proposed to protect the bacteria from, e.g. desiccation, engulfment by phagocytes or predatory microorganisms and/or to serve as a reserve of carbohydrates (van Hijum et al. 2006). The EPS may also participate in adhesion and the formation of biofilms (Sutherland 2001). In this respect, it is of interest to note that α-glucans produced by oral streptococcal glucansucrases play an important role in the formation of dental plaque and cariogenesis by attaching bacteria as well as food debris to tooth enamel (Kato & Kuramitsu 1991; Matsumoto-Nakano et al. 2007; Mooser et al. 1985).

On the other hand, α-glucans may also have health-promoting properties by facilitating the colonization of the gastrointestinal tract by probiotic bacteria (German 1999; Welman & Maddox 2003). This quality has made α-glucan exopolysaccharides of substantial interest to the food industry, beside their application as thickening, water-binding, solubilizing, and emulsifying agents (van Hijum et al. 2006). These latter properties depend on the physicochemical characteristics of the exopolysaccharides. Among the factors that determine these characteristics are the type of glycosidic linkage

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[e.g. α-(1,2), α-(1,3), α-(1,4) or α-(1,6) or combinations thereof], the degree and type of branching, the length of the branched chains, and the mass of the glucan (van Hijum et al. 2006).

Since the se properties of the glucans are determined by the enzymes that produce them, a significant interest in glucansucrases from various sources has developed. Already over 60 glucansucrases are presently classified in glycoside hydrolase family 70 (GH70) of the CAZy database [see http://www.cazy.org/fam/GH70.html (Henrissat & Davies 1997)], but no three-dimensional structure is available yet. Glucansucrases are large enzymes (~110–310 kDa), which have been proposed to consist of three domains, an N-terminal variable domain, a central conserved catalytic $(\beta/\alpha)_8$-barrel domain and a C-terminal putative glucan binding domain (Figure 1; Monchois et al. 1999; van Hijum et al. 2006). The catalytic domain of the GH70 family enzymes is related to that of enzymes belonging to the GH13 family (or α-amylase family, which mainly contains starch hydrolysing/modifying enzymes) and to the GH77 family of amylomaltases. These three families have therefore been grouped together in the α-amylase superfamily or GH-H clan (Henrissat & Davies 1997). They share high sequence similarity in four short stretches of amino acid residues involved in catalysis and substrate binding. These enzymes are therefore believed to share a common reaction mechanism, according to which cleavage of the glycosidic bond occurs via a double displacement mechanism, involving a covalent β-glucosyl enzyme intermediate and with the retention of the α-anomeric configuration in the product (MacGregor et al. 2001). The difference between the catalytic domains of GH70 glucansucrases and GH13/GH77 enzymes is that the $(\beta/\alpha)_8$-barrel elements of the GH70 enzymes are circularly permuted (MacGregor et al. 1996).

Three-dimensional structures are known for several enzymes from GH13 and GH77 families including structures with bound substrates and covalent intermediates (Barends 2007; Skov et al. 2002; Uitdehaag et al. 1999), but for GH70 enzymes no three-dimensional structure has been reported to date. To study the molecular basis of the reaction mechanism of GH70 glucansucrases and the determinants of their linkage type specificity, we have investigated the glucansucrase GTF180 from *Lactobacillus reuteri* 180. This 198 kDa enzyme produces a glucan of ≈36 MDa with α-1,6 and α-1,3 glycosidic linkages (Kralj et al. 2004a). Our crystallization attempts include both the full length GTF180, as well as a 117 kDa fragment of the enzyme, which lacks the predicted N-terminal variable domain (GTF180-ΔN, residues 742–1772, see Figure 1). By omitting this domain we expected to avoid protein flexibility and therefore to increase the chances of successful crystallization. The truncated enzyme is fully active, and produces an α-glucan with a similar mass and linkage profile as the full length enzyme (Kralj et al. 2004a). So far, only crystallization experiments with the truncated enzyme yielded well-diffracting crystals, in three different crystal forms.

Experimental procedures

Protein expression and purification

Both recombinant full-length GTF180 (MG-39-1772-His_6) lacking the signal sequence and the N-terminally truncated GTF180-ΔN (MG-742-1772-His_6) were over-expressed in *E. coli* BL21star (DE3) and purified as described previously for the homologous GTFA-ΔN from *Lactobacillus reuteri* 121 (Kralj et al. 2004a).

Purified protein was concentrated by ultrafiltration using a MacroSep-30 device (Pall Life Sciences, New York, USA). Purity and homogeneity were analysed by SDS-PAGE and native PAGE (Laemmli 1970), and the protein concentration was estimated by UV-absorption using a calculated specific absorption coefficient of 1.595 mg ml^{-1} cm^{-1} at 280 nm. Dynamic light scattering (DLS) experiments were carried out at 293 K with 2.0 mg ml^{-1} GTF180-ΔN in 25 mM sodium acetate buffer, pH 4.7, 1 mM $CaCl_2$, 14 mM NaCl, using a DynaPro MSTC-800 instrument and Dynamics software (Wyatt Instruments, Santa Barbara, USA).

Activity assays

Enzymatic activities of GTF180 and GTF180-ΔN were determined by measuring glucose and fructose release from sucrose (van Geel-Schutten et al. 1999). The amount of released fructose corresponds to the total enzyme activity (the initial formation of leucrose or other sucrose isomers was negligible,

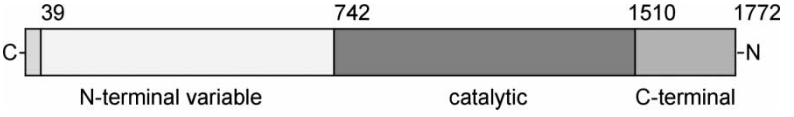

Figure 1. Predicted domain arrangement for GTF180 of *L. reuteri* 180. The first 38 residues constitute the signal peptide, followed by an N-terminal variable domain, a central catalytic domain and a C-terminal putative glucan binding domain.

data not shown). The amount of free glucose represents the hydrolytic activity; the transferase activity is represented by the amount of released fructose minus the amount of free glucose. Unless indicated otherwise, reactions were performed at 50°C (which is the temperature optimum for full length as well as N-terminally truncated enzyme, see below) in 25 mM sodium acetate buffer, pH 4.5, containing 1 mM $CaCl_2$, 50 mM sucrose and 5 nM purified GS180-ΔN enzyme. To determine the pH-optimum, Michaelis barbital-acetate buffer was used (BDH, Poole, England). Data points were taken every 5 min over a period of 30 min. One unit of enzyme activity is defined as the release of 1 μ mol of monosaccharide per min.

Kinetic assays were performed as described previously (Kralj 2004b), with the following modifications: twelve different sucrose concentrations ranging from 0.25 to 100 mM were used and during a 18 min incubation period, samples of 25 μl were withdrawn every 3 minutes and inactivated with 2.5 μl 1M NaOH.

Crystallization

Crystallization experiments for GTF180-ΔN were performed using the hanging drop vapour diffusion method at 293 K. Drops were prepared by mixing 1.0–1.5 μl of protein solution (6.9 mg/ml GTF180-ΔN in 25 mM sodium acetate buffer, pH 5.0, 1 mM $CaCl_2$, 50 mM NaCl) with an equal volume of reservoir solution. Crystals were grown from two different crystallization conditions (I and II).

Crystallization condition I corresponds to solution P2-22 from the PACT Premier HT-96 screen (Molecular Dimensions, Newmarket, UK). The reservoir contained 20% (w/v) PEG 3350, 0.1 M Bis-Tris- propane-HCl buffer, pH 6.5, 0.02 M sodium potassium phosphate. Reproduction of these crystals turned out to be very difficult; the drops often showed phase separation or even large aggregates of precipitated protein. The only way to obtain useful crystals was via the streak-seeding method (Stura & Wilson 1991). To this end, hanging drop experiments were set up using 16–17% (w/v) PEG 3350 in the reservoir; after 7 days of equilibration

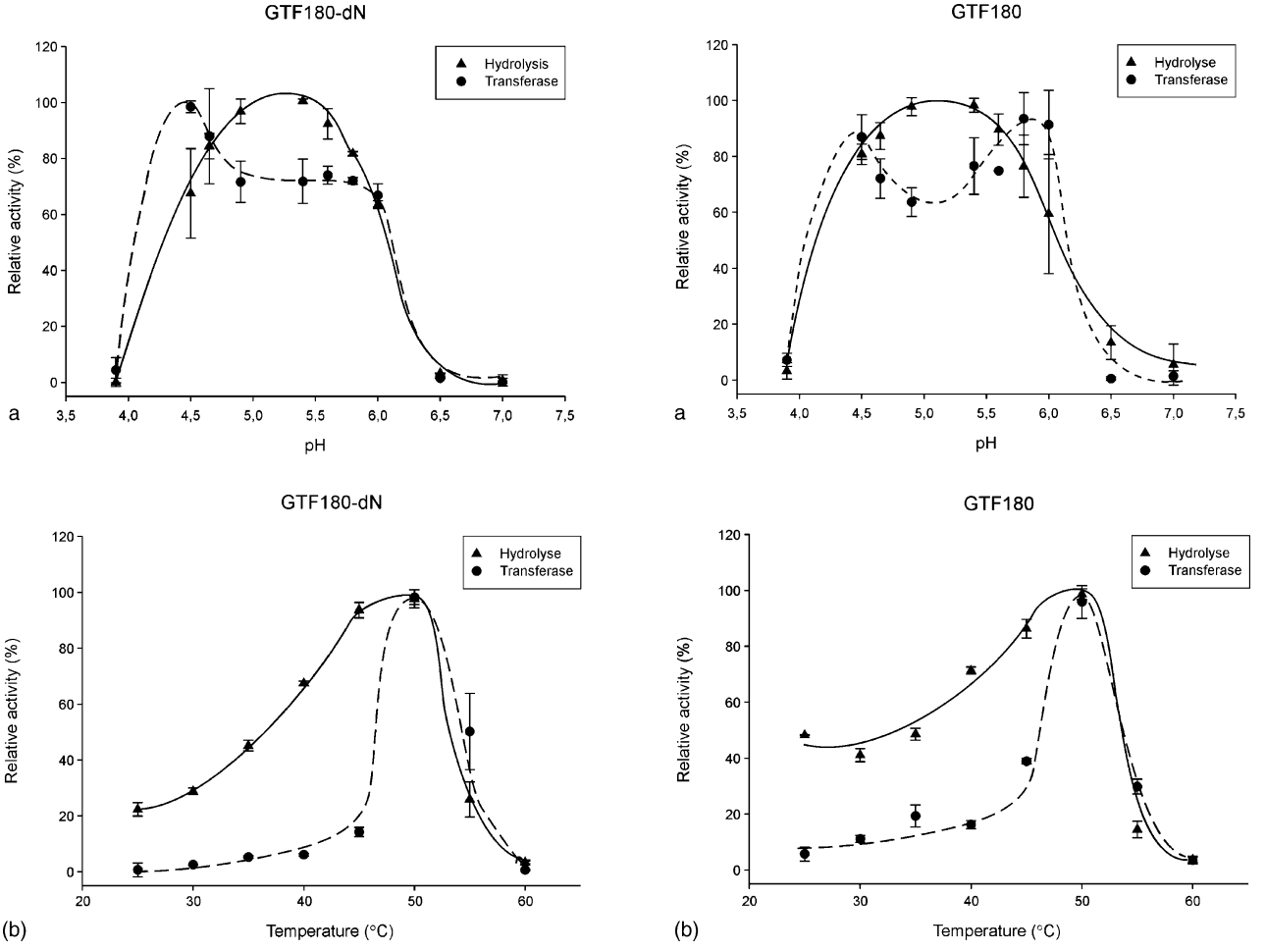

Figure 2. Effect of (a) pH and (b) temperature on the activity of N-terminally truncated (GTF180-dN) and full length GTF180 (GTF180) from *L. reuteri* 180.

the drops were streak-seeded with a cat's whisker. Crystals were cryoprotected in a solution similar to the reservoir solution but with 25% (w/v) PEG 3350 and 15% (v/v) glycerol, and flash-cooled in liquid nitrogen. In later experiments it was shown that glycerol could be left out of the cryoprotectant by increasing the PEG 3350 concentration to 35% (w/v).

The second crystallization condition (condition II) was solution I-44 of the Structure Screen (Molecular Dimensions, Newmarket, UK), in which the reservoir contained 2.0 M $(NH_4)_2SO_4$ and 5% (v/v) 2-propanol. The crystal was cryoprotected using 2.2 M $(NH_4)_2SO_4$, 5% (v/v) 2-propanol, 25% glycerol and flash-cooled in liquid nitrogen. Attempts to reproduce crystallization based on this condition were successful in only a very limited number of experiments, and also required the streak-seeding method (Stura & Wilson 1991).

Data collection and processing

All diffraction data sets were collected at 100 K. Diffraction data for crystals grown under condition I and II were collected at beam line ID29 at the ESRF (Grenoble, France) and beam line X12 at the EMBL-Hamburg outstation (at DESY, Hamburg, Germany), respectively. All data sets were indexed and integrated using MOSFLM (Leslie 1992) and scaled using SCALA from the CCP4 suite (Collaborative Computational Project Number 4 1994).

Results and conclusions

Protein expression and purification

GTF180-ΔN could be over-produced in *E. coli* and purified to a final yield of about 12 mg L^{-1} of culture. The purity of the GTF180-ΔN solution was checked on SDS-PAGE silver stained gels, which showed a main band at $\approx$120 kDa; on native PAGE silver stained gels the protein appeared as a single band (data not shown). In dynamic light scattering experiments, GTF180-ΔN showed a monomodal distribution with an apparent molecular mass of 100–110 kDa and low polydispersity (14%) using the regularization model. Together these results indicate a pure and monodisperse sample, and monomeric behaviour of GTF180-ΔN in solution.

Activity assays

The pH-optimum for hydrolytic activity was in a range from pH 4.5 to 6.0 for both GTF180 and GTF180-ΔN (Figure 2a). The transglycosylase activity for both enzymes was highest at pH 4.5 and this pH was used for all subsequent assays. For both enzymes and both hydrolase and transglycosylase reactions, the optimal temperature was 50°C (Figure 2b), with the transferase reaction in particular showing a sharp optimum at this temperature.

Table I. Kinetic parameters for the N-terminally truncated (GTF180-ΔN) and full length (GTF180) enzymes determined using 12 different concentrations of sucrose (0.25–100 mM).

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
Total activity			
GTF180-ΔN	7.0±2.0	358.9	51
GTF180	3.2±0.2	101.8	32
Hydrolysis activity			
GTF180-ΔN	9.2±2.2	106.0	12
GTF180	2.7±0.4	50.2	19
Transglycosylase activity			
GTF180-ΔN	6.4±1.1	254.9	40
GTF180	3.8±0.5	50.2	13

Both GTF180 and GTF180-ΔN displayed Michaelis–Menten type kinetics for the total transglycosylase and hydrolytic activity (data not shown). The N-terminally truncated enzyme shows a lower affinity for substrate (higher K_m), which is compensated by a higher turnover rate (k_{cat}), resulting in a catalytically more efficient enzyme, see Table I. Therefore the activities of the two enzymes are comparable, the most pronounced difference being the significant increase in transglycosylase activity of GTF180-ΔN. A similar effect has been observed for the N-terminally truncated GTFA-ΔN from *Lactobacillus reuteri* 121 (Kralj et al. 2004a). Whether the N-terminal domain interacts with the catalytic domain in the full length enzyme and obstructs for example easy entry/departure of the growing glucan chain to/from the active site, will be the subject of future investigations.

X-ray data diffraction analysis

Crystals grown under crystallization condition I (see Figure 3a) belong to two different crystal forms (Ia and Ib), both growing to maximum dimensions of about $0.10 \times 0.02 \times 0.01$ mm over several weeks. Crystals of form Ia belong to the triclinic space group *P*1. Assuming that there is one molecule per unit cell, the solvent content was $\sim$52% and the Matthews coefficient, V_M, is 2.5 $Å^3$ Da^{-1}.

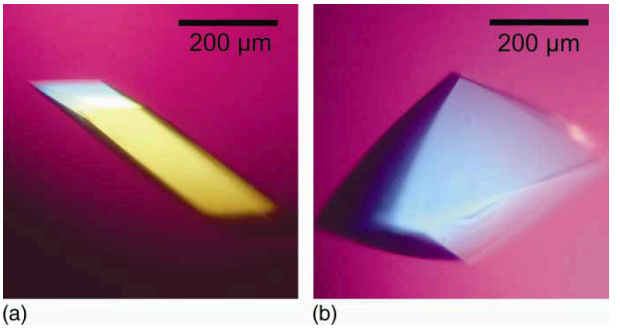

Figure 3. Crystals of *L. reuteri* 180 GTF180-ΔN grown as described in the text from condition I (*a*) and condition II (*b*).

Only about 10% of the crystals from condition I belonged to form 1b; these crystals also belong to space group *P*1, but have different unit cell parameters. Assuming a Matthews coefficient of 2.3 Å^3 Da^{-1}, the presence of two protein molecules in the unit cell is likely, corresponding to a solvent content of ~46%.

A GTF180-ΔN crystal appearing after ≈6 weeks in the Structure Screen (condition II), with dimensions of 0.15 × 0.12 × 0.05 mm, was used to collect a native data set to 2.0 Å resolution (GTF180-II). A diffraction image collected for this type of crystal is shown in Figure 4. Crystals grown from condition II (see Figure 3b) belong to the orthorhombic space group $P2_12_12_1$. Assuming there are two molecules per asymmetric unit, the Matthews coefficient, V_M, is 3.6 Å^3 Da^{-1} and the estimated solvent content is ~66%.

Cell parameters, data collection and processing statistics for all three crystal forms are listed in Table II.

In conclusion, three high resolution native X-ray diffraction data sets were collected for the C-terminal fragment of *L. reuteri* 180 GTF180 (GTF180-ΔN). The N-terminal deletion does not significantly interfere with the enzyme's activity, and the truncated enzyme shows similar pH and temperature optima as the full length enzyme. Therefore, structure determination of GTF180-ΔN using Se-Met substituted protein is the aim of further investigations.

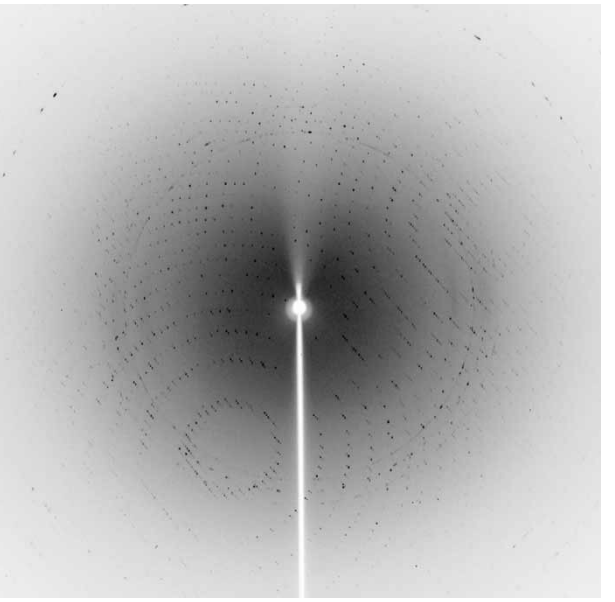

Figure 4. Diffraction image of a crystal grown from condition II, collected at beam line X12, EMBL-Hamburg outstation (at DESY, Hamburg, Germany), using a 0.5° oscillation angle. At the corners of the detector the resolution is 1.8 Å.

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Table II. Data collection and processing statistics. Values in parentheses are for the highest resolution shell.

Dataset	GTF180-ΔN Ia	GTF180-ΔN Ib	GTF180-ΔN II
Wavelength (Å)	0.9791	1.0653	1.1053
Oscillation angle/frame (°)	1	1	1
Space group	*P*1	*P*1	$P2_12_12_1$
No. of molecules in the asymmetric unit	1	2	2
Unit-cell parameters			
a, *b*, *c* (Å)	58.3, 66.0, 82.5	78.0, 83.3, 91.1	92.8, 147.4, 244.4
α, *β*, *γ* (°)	73.3, 78.5, 85.8	81.9, 68.1, 79.9	90.0, 90.0, 90.0
Resolution range (Å)	50.00–1.65 (1.69–1.65)	50.00–1.70 (1.79–1.70)	50.00–2.00 (2.07–2.00)
No. of unique observations	133 854	210 558	212 281
Redundancy	3.7 (3.7)	2.6 (2.5)	6.3 (2.9)
R_{sym} (%)[#]	7.7 (45.3)	4.3 (12.7)	9.9 (34.4)
Completeness (%)	96.6 (94.8)	91.8 (71.5)	94.1 (69.5)
$I/\sigma(I)$	12.3 (2.5)	17.3 (7.6)	16.7 (3.1)

[#] $R_{sym} = \Sigma_{hkl}\Sigma_i|I_i(hkl) - \langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i I(hkl)$.

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