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# The crystal structure of *Erwinia amylovora* levansucrase provides a snapshot of the products of sucrose hydrolysis trapped into the active site

Jochen Wuerges [a], Lorenzo Caputi [b], Michele Cianci [c], Stephane Boivin [c], Rob Meijers [c], Stefano Benini [a,*]

[a] Bioorganic Chemistry and Bio-Crystallography laboratory ($B_2Cl$), Faculty of Science and Technology, Free University of Bolzano, 39100 Bolzano, Italy
[b] Department of Biological Chemistry, John Innes Centre, Norwich Research Park, Norwich NR4 7UH, United Kingdom
[c] European Molecular Biology Laboratory (EMBL), Hamburg Outstation, Notkestrasse 85, 22603 Hamburg, Germany

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ABSTRACT

Levansucrases are members of the glycoside hydrolase family and catalyse both the hydrolysis of the substrate sucrose and the transfer of fructosyl units to acceptor molecules. In the presence of sufficient sucrose, this may either lead to the production of fructooligosaccharides or fructose polymers. Aim of this study is to rationalise the differences in the polymerisation properties of bacterial levansucrases and in particular to identify structural features that determine different product spectrum in the levansucrase of the Gram-negative bacterium *Erwinia amylovora* (Ea Lsc, EC 2.4.1.10) as compared to Gram-positive bacteria such as *Bacillus subtilis* levansucrase. Ea is an enterobacterial pathogen responsible for the Fire Blight disease in rosaceous plants (e.g., apple and pear) with considerable interest for the agricultural industry. The crystal structure of Ea Lsc was solved at 2.77 Å resolution and compared to those of other fructosyltransferases from Gram-positive and Gram-negative bacteria. We propose the structural features, determining the different reaction products, to reside in just a few loops at the rim of the active site funnel. Moreover we propose that loop 8 may have a role in product length determination in *Gluconacetobacter diazotrophicus* LsdA and *Microbacterium saccharophilum* FFase. The Ea Lsc structure shows for the first time the products of sucrose hydrolysis still bound in the active site.



## 1. Introduction

The Gram-negative bacterium *Erwinia amylovora* is a plant pathogen of Rosaceae, such as apple and pear, causing the disease known as fire blight. *E. amylovora* synthesizes two exopolysaccharides, amylovoran and levan which are both required for full pathogenicity. Production of amylovoran requires the action of several enzymes whose expression is regulated by the *ams* operon while levan is produced by a single enzyme, levansucrase (Lsc, EC 2.4.1.10). Levan is required for the formation of a protective biofilm and represents one of several virulence factors of the bacterium (Zhao et al., 2005; Geier and Geider, 1993). Levansucrases catalyse two reactions, the hydrolysis of sucrose yielding fructose and glucose, and the transfructosylation whereby a fructosyl unit is transferred from an enzyme-fructosyl intermediate to an acceptor, which can either be an oligomer or a polymer of fructose (fructan) or sucrose in a ping-pong mechanism as proposed already in 1974 by Chambert et al. (1974). Levansucrases join the fructosyl units through the β(2 → 6)-linkage, while the related enzymes inulosucrases (EC 2.4.1.9) do so through the β(2 → 1)-linkage. According to the linkage type, the fructan polymers are either called levan or inulin. The enzymes are members of the glycoside hydrolases (GH), with bacterial enzymes being grouped into family 68, and structurally related plant and fungal enzymes into family 32 (Cantarel et al., 2009).

An interesting aspect of levansucrases from different bacteria is their specificity in the formation of either fructooligosaccharides (FOS) or high molecular weight polymers. Generally, levansucrases from Gram-positive bacteria produce polymers and those from Gram-negative bacteria produce FOS. Structurally characterised examples for enzymes from the first group are SacB from *Bacillus subtilis* (Meng and Fütterer, 2003) and *Bacillus megaterium*

*Abbreviations:* Bs, *Bacillus subtilis*; Bm, *Bacillus megaterium*; Ea, *Erwinia amylovora*; Gd, *Gluconacetobacter diazotrophicus*; Ms, *Microbacterium saccharophilum*.

* Corresponding author.
*E-mail addresses:* jochen.wuerges@unibz.it (J. Wuerges), Lorenzo.Caputi@jic.ac.uk (L. Caputi), michele.cianci@embl-hamburg.de (M. Cianci), Stephane.Boivin@embl-hamburg.de (S. Boivin), r.meijers@embl-hamburg.de (R. Meijers), Stefano.Benini@unibz.it (S. Benini).

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(Strube et al., 2011), and the inulosucrase InuJ from *Lactobacillus johnsonii* (Pijning et al., 2011) whereas enzymes from Gram-negative bacteria are *Gluconacetobacter diazotrophicus* LsdA (Martinez-Fleites et al., 2005) and *E. amylovora* Lsc from this study. The β-fructofuranosidase Ms FFase from the Gram-positive *Microbacterium saccharophilum* appears to be an exception as in the presence of sucrose even at high concentration it is mainly hydrolytic, while in the presence of a mixture of sucrose and lactose it produces mainly lactosucrose (Tonozuka et al., 2012). We have shown in a previous study that with substrate concentrations up to 0.5 M sucrose (similar to the one found in the nectar during flower infection), *E. amylovora* Lsc predominantly produces short-chain FOS of three to six units (Caputi et al., 2013b).

Extensive biochemical and structural work has already been done to rationalise the wide range of products formed by glycoside hydrolases of the GH 32 and 68 families, all of which feature a five-bladed β-propeller fold as catalytic core (Lammens et al., 2009). Active site residues are located at the inside of a funnel-like cavity and are conserved in all members of the two families, giving rise to the notion that the mechanism of their hydrolase activity is the same. On the contrary, residues lining the outer rim of the cavity appear far less conserved, as multiple sequence alignments have indicated (Pons et al., 2004). However, those at the surface were reported to influence the transfructosylation reaction and thus, to play an important role in defining the product spectrum of levansucrases (Strube et al., 2011). We compared the *E. amylovora* Lsc crystal structure to those of fructosyltransferases from Gram-positive and Gram-negative bacteria with the aim of contributing to the identification of determinants of the diverse product spectrums displayed by these related enzymes. We were also interested in investigating whether structural features of levansucrases, from different *Erwinia* species, could correlate with the potency of these enzymes as a virulence factor. The structure of Lsc from *E. amylovora* presented here is the first to be determined for a levansucrase from an *Erwinia* species. This opens the possibility to reliably transfer the biochemical information of the key residues from the well-characterised *B. subtilis* and *B. megaterium* levansucrases SacB onto the *Erwinia* levansucrases. The structural comparison revealed that loop 8 may have a role in determining product length in Gd LsdA and Ms FFase.

## 2. Materials and methods

The production of recombinant levansucrase from *E. amylovora* (strain Ea273, ATCC 49946) and its crystallisation were described in detail previously (Caputi et al., 2013a). In summary, a plasmid for overexpression in *Escherichia coli* was produced by ligation of the PCR-amplified gene into the pETM-30 vector (Dümmler et al., 2005). The protein used for crystallisation comprised a Ser-to-Ala mutation in the second residue as a consequence of completing the restriction site for the NcoI endonuclease and, after TEV protease-cleavage of the N-terminal $His_6$-GST tag, a remaining Gly-Ala tail in front of the starting Met. Purified Lsc, with a yield of 25 mg per litre of cell culture, was concentrated to 10 mg $mL^{-1}$ and used for hanging-drop vapour diffusion set-ups in LINBRO plates. The structure described here was obtained from X-ray diffraction data collected on a crystal which grew in drops composed of 1 μL protein solution (containing 25 mM Tris–HCl, pH 7.5, 150 mM NaCl) and 1 μL crystallisation reagent (35% PEG 2000 MME, 0.1 M KSCN). The crystal was soaked in a solution corresponding to the crystallisation drop with PEG concentration raised to 40% and containing sucrose to a final concentration of 0.5 M, for 1 min immediately prior to flash-freezing the crystal in liquid $N_2$. Diffraction data were collected on beamline P13 at EMBL Hamburg c/o DESY, Germany, and processed with XDS (Kabsch, 2010) and SCALA (Evans, 2006). Phase information for the structure solution of Lsc was obtained by molecular replacement using *G. diazotrophicus* (Gd) levansucrase LsdA (PDB accession code 1W18) as a search model within the automated pipeline BALBES (Long et al., 2008). The resulting start model for the eight molecules of Lsc in the asymmetric unit was initially completed using the autobuild routine in PHENIX (Terwilliger et al., 2008) and iteratively refined with COOT (Emsley et al., 2010) and REFMAC5 (Winn et al., 2003). The last cycles of refinement were carried out using PHENIX (Adams et al., 2010). NCS restraints were employed throughout refinement for the protein atoms whereas ligands were refined independently and, in the last few cycles, also TLS (translation, libration and screw-rotation) refinement. $F_{obs} - F_{calc}$ and $2F_{obs} - F_{calc}$ electron density in the active site cavity, respectively at $3\sigma$ and $1\sigma$ contour level, was interpreted only at the end of protein refinement by filling the density tentatively with either sucrose, fructose or glucose molecules. The fit was assessed by analysing the residual $F_{obs} - F_{calc}$ density around the sugar unit and the refined B-factors of its atoms and those of the neighbouring protein atoms. The quality of the model was assessed with the help of MOLPROBITY (Chen et al., 2010). Final refinement statistics are reported in Table 1. Crystallographic figures were created using PyMOL. (The PyMOL Molecular Graphics System, Schroedinger, LLC.)

## 3. Results

### 3.1. Overall structural features of Ea levansucrase Lsc

Ea Lsc crystallised in space group $P2_12_12$ with eight molecules in the asymmetric unit. These molecules can be grouped in four crystallographic dimers, which are classified as metastable entities based on dissociation energies calculated with PISA (Krissinel and Henrick, 2007). However in solution (25 mM Tris–HCl, pH 7.5, 150 mM NaCl) and in normal conditions of temperature and concentration Lsc behaves as a monomer.

Ea Lsc shows the five-bladed β-propeller typical of glycoside hydrolase families 32 and 68 members (Lammens et al., 2009) (Fig. 1A). Each β-sheet consists of four twisted strands with the outer β-strand being oriented almost perpendicularly to the inner strand. A short strand at the C-terminus is part of the N-terminal β-sheet, thereby closing the fold at both ends of the polypeptide chain. No electron density was observed due to disorder for the N-terminal residues up to Asp3 and the last residue Lys415 which were not included in the final protein model. A forty residue long 'clamp', beginning at the N-terminus and stretching up to the first blade, wraps around half the circumference of the propeller fold and forms short β-sheet interactions with the outer strands of blades III and IV.

### 3.2. Hydrolysis products in the active site

The inner part of the β-propeller creates a funnel which hosts the active site and substrate recognition residues. The bottom of the funnel is formed by a loop between the first two β-strands in blade I. This feature forces product molecules to exit the active site through the entrance. The surface of the funnel is predominantly lined with acidic residues, a feature observed in all structures of GH families 32 and 68 available to date. Sequence alignments show that the catalytically active triad (Asp46, Asp203 and Glu287 in *Ea* Lsc numbering) as well as most other residues shaping the hydrolytically active site (termed −1 and +1 subsites) are conserved among levansucrases (Martinez-Fleites et al., 2005). Differences will be analysed further below.

In both the $2F_{obs} - F_{calc}$ (contoured at 1 sigma level) and $F_{obs} - F_{calc}$ (contoured at 3 sigma level) volumes of electron density