Structural and biochemical characterization of the laminarinase ZgLamCGH16 from Zobellia galactanivorans suggests preferred recognition of branched laminarin

Aurore Labourel, Murielle Jam, Laurent Legentil, Balla Sylla, Jan-Hendrik Hehemann, Vincent Ferrières, Mirjam Czjzek, Gurvan Michel

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Laminarin is a β-1,3-β-glucan displaying occasional β-1,6 branches. This storage polysaccharide of brown algae constitutes an abundant source of carbon for marine bacteria such as Zobellia galactanivorans. This marine member of the Bacteroidetes possesses five putative β-1,3-glucanases [four belonging to glycosyl hydrolase family 16 (GH16) and one to GH64] with various modular architectures. Here, the characterization of the β-glucanase ZgLamC is reported. The catalytic GH16 module (ZgLamCGH16) was produced in Escherichia coli and purified. This recombinant enzyme has a preferential specificity for laminarin but also a significant activity on mixed-linked glucan (MLG). The structure of an inactive mutant of ZgLamCGH16 in complex with a thio-β-1,3-hexaglucan substrate unravelled a straight active-site cleft with three additional pockets flanking subsites −1, −2 and −3. These lateral pockets are occupied by a glycerol, an acetate ion and a chloride ion, respectively. The presence of these molecules in the vicinity of the O6 hydroxyl group of each glucose moiety suggests that ZgLamC accommodates branched laminarins as substrates. Altogether, ZgLamC is a secreted laminarinase that is likely to be involved in the initial step of degradation of branched laminarin, while the previously characterized ZgLamA efficiently degrades unbranched laminarin and oligo-laminarins.

1. Introduction

Found on rocky seashores in cold and temperate regions, brown seaweeds represent an estimated 70% of the primary biomass in these coastal areas (Duarte et al., 2005). This abundant resource mainly consists of polysaccharides, either constituting cell walls (e.g. alginate, cellulose and sulfated fucoidans; Michel et al., 2010b; Popper et al., 2011) or carbon storage (laminarin; Michel et al., 2010a). Laminarin represents up to 35% of the algal dry weight (O’Sullivan et al., 2010). This small vacuolar β-1,3-β-glucan contains 25 linearly linked glucosyl residues on average and occasional β-1,6-linked branches (Percival & Ross, 1951). It is composed of two series: the minor G-series, which contains only glucose residues, and the more abundant M-series, which displays a β-D-mannitol residue at the reducing end (Read et al., 1996). The presence of mannitol in laminarin is owing to a major horizontal gene-transfer event between the common ancestor of brown algae and an actinobacterium, which resulted in the acquisition of the bacterial biosynthetic pathway for mannitol (Michel et al., 2010a; Rousvoal et al., 2011; Groisillier et al., 2014) and alginate (Michel et al., 2010b). Moreover, an insoluble laminarin fraction has been characterized in some species such as Laminaria hyperborea and Saccharina longicirrus. In both cases, these insoluble β-1,3-glucans are essentially unbranched (Nelson & Lewis, 1974; Rioux et al., 2010).
Altogether, the different forms of laminarin constitute an abundant carbon source for seaweed-associated bacteria and other heterotrophic microbes living in coastal waters. However, knowledge of the degradation mechanisms of genuine laminarin by the relevant marine bacteria remains limited. Several β-1,3-glucanases from bacteria and fungi have been studied, but these organisms essentially originate from terrestrial environments (http://www.cazy.org; Lombard et al., 2014) and degrade other types of β-1,3-glucans such as the fibrillar callose of plants or the insoluble β-1,3,1,6-glucans of fungal cell walls (Stone, 2009). Among the exceptions, the β-glucanase from *Rhodothermus marinus*, which belongs to family 16 of glycoside hydrolases (GH16), has been well studied (Krah et al., 1998; Bleicher et al., 2011), but this marine bacterium was isolated from an oceanic hot spring and this biotope does not contain algal laminarin. In contrast, *Zobellia galactanivorans* is a model bacterium for the bioconversion of algal polysaccharides. This flavobacterium was isolated from the red alga *Delesseria sanguinea* in Roscoff, Brittany (Barbeyron et al., 2001) and has mostly been studied for the degradation of sulfated galactans from red seaweeds (agars, carrageenans and porphyrans; for reviews, see Michel & Cezjak, 2013; Martin et al., 2014). Nonetheless, Z. *galactanivora*ns can also assimilate polysaccharides from brown algae, such as alginates (Thomas et al., 2012, 2013). After alginates, laminarin is the second most abundant polysaccharide from brown algae and this storage compound can be also used as a sole carbon source by Z. *galactanivorans*. Its genome contains five putative laminarinases: four from family 16 of glycoside hydrolases (GH16; ZgLamA–ZgLamD) and one from the GH64 family (ZgLamE). While to date the GH64 family contains only β-1,3-glucanases (Lombard et al., 2014), the GH16 family is a large polyspecific family with at least 11 different known EC numbers. Interestingly, the GH16 family includes several enzymes specific for algal polysaccharides: κ-carrageenases (Michel et al., 2001), β-agarases (Jam et al., 2005), β-porphyranases (Hehemann et al., 2010) and of course laminarinases. Based on phylogenetic and structural evidence, laminarinase has been proposed to be the ancestral activity in the GH16 family (Barbeyron et al., 1998; Michel et al., 2001), consistent with the ancient nature of β-1,3-glucans as storage polysaccharides in eukaryotes (Michel et al., 2010a). The catalytic residues of the GH16 enzymes are the two glutamate residues found in the conserved signature EXDXX(E)X. The first glutamate acts as a nucleophile, while the second glutamate is the acid/base catalyst (Keitel et al., 1993; Juncosa et al., 1994). The putative laminarinases from Z. *galactanivorans* possess various additional modules, such as carbohydrate-binding modules (e.g. CBM6 and CBM42) and PKD domains. The complexity of this enzymatic system suggests that each enzyme may have a different biological function. We have recently reported the first characterization of an enzyme from this laminarinolytic system, ZgLamA <sub>GH16</sub> (Labourel et al., 2014). To deepen our understanding of the complementary functions of the β-glucanases from Z. *galactanivorans*, we have undertaken extensive characterization of the GH16 catalytic module of ZgLamC. Notably, the structure of an inactive mutant of ZgLamC<sub>GH16</sub> was determined in complex with a thio-β-1,3-glucan analogue.

2. Materials and methods

Except where mentioned otherwise, all chemicals were purchased from Sigma. The thio-β-1,3-hexaglucan was synthesized according to a known procedure (Sylla, 2010).

2.1. Cloning and site-directed mutagenesis of ZgLamC<sub>GH16</sub>

The gene encoding the putative laminarinase ZgLamC was cloned as described previously (Groisillier et al., 2010). Briefly, primers were designed to amplify the coding region corresponding to the GH16 catalytic module of ZgLamC, referred to as ZgLamC<sub>GH16</sub> (forward primer, GGGGGGGGATCC-CAAAGATATCAACTTGTCTGCAAG; reverse primer, CCCCCCAATTGTATCATTTGGTTAGACCCCTAGTAACTTCT), by PCR from Z. *galactanivorans* genomic DNA. After digestion with the restriction enzymes *Bam*HI and *Mfe*I, the purified PCR product was ligated using T4 DNA ligase into the expression vector pFO4 pre-digested with *Bam*HI and *Eco*RI, resulting in a recombinant protein with an N-terminal hexahistidine tag (plasmid pZgLamC<sub>GH16</sub>). This plasmid was used to transform *Escherichia coli* DH5α strain for storage and *E. coli* C43(DE3) strain for protein expression. Site-directed mutagenesis was performed using the QuikChange II site-directed mutagenesis kit (Stratagene) and the plasmid pZgLamC<sub>GH16</sub>. The two putative catalytic residues G1U137 and Glu142 were replaced by either a serine or an alanine (mutant E137A, forward primer TGGCCTGCTGCGGGA- GCAATAGATATCAGG, reverse primer CTCCATGATATCATGGCC, forward primer TGGCCTGCTGCGGGTCATAGATATCATGGAG, reverse primer CTCCATGATATCTATGCCCCGGCACGCCAGG; mutant E137S, forward primer TGGCCTGCTGCGGGTCATAGATATCATGGAG, reverse primer CTCCATGATATCTATGCCCCGGCACGCCAGG; mutant E142A, forward primer GAAATAGATATCATGCGCGCGCCATCAATAACGCT, reverse primer AGCGTTATGTAGTGGCGGCCCATGATATC- TATTT; mutant E142S, forward primer GAAATAGATATCATGCGCGCATCAATAACGCT, reverse primer AGCGTTATGTAGTGGCGGCCCATGATATCATT(TTC)). The plasmids were sequenced to confirm that the mutation occurred at the correct position. These variant plasmids were also used to transform *E. coli* DH5α strain for storage and *E. coli* C43(DE3) strain for protein expression.

2.2. Overexpression and purification of ZgLamC<sub>GH16</sub> and ZgLamC<sub>GH16-E142S</sub>

The *E. coli* C43(DE3) strain containing the plasmid pZgLamC<sub>GH16</sub> was used to inoculate 3 ml Luria–Bertani (LB) broth medium supplemented with 100 μg ml<sup>−1</sup> ampicillin. This preculture was incubated overnight at 37°C and 1 ml was transferred to inoculate 11 of the auto-inducible ZYP 5052 medium (Studier, 2005). The culture was incubated at 20°C and 180 rev min<sup>−1</sup> until the stationary phase was reached and was then harvested by centrifugation at 3000g and 4°C for 35 min. The cell pellet was stored at −20°C. The cells were
resuspended in 20 ml buffer A (20 mM Tris–HCl pH 7.5, 200 mM NaCl, 30 mM imidazole). An anti-protease mixture (Complete EDTA-free, Roche) and 0.1 mg ml\(^{-1}\) DNase were added. The cells were disrupted in a French press. After centrifugation at 12 500g for 2 h at 4°C, the supernatant was loaded onto a 10 ml Chelating Sepharose Fast Flow column (GE Healthcare) previously charged with 100 mM NiSO\(_4\) and equilibrated with buffer A. The column was washed with buffer A (110 ml) and the protein was eluted with a 60 ml linear gradient from buffer A to buffer B (20 mM Tris–HCl pH 7.5, 200 mM NaCl, 500 mM imidazole) at a flow rate of 1 ml min\(^{-1}\). The different fractions (1 ml each) were analyzed by SDS–PAGE. The fractions corresponding to a single band at the expected size (26 kDa) were pooled (13 ml) and were concentrated by ultrafiltration on an Amicon membrane (10 kDa cutoff; 4 ml at 7.5 mg ml\(^{-1}\)). Two aliquots of 2 ml (7.5 mg ml\(^{-1}\)) were loaded onto a 120 ml Superdex 75 column previously equilibrated with buffer C (20 mM Tris–HCl pH 7.5, 200 mM NaCl). The protein was eluted using between 70 and 80 ml buffer C and the purity of the fractions was checked by SDS–PAGE. 24 fractions of 1 ml each were pooled and a concentration of 1.25 mg ml\(^{-1}\) was determined using a NanoDrop spectrophotometer. A calibration curve was also used to determine the oligomerization state of ZgLamCGH16. The mutant protein ZgLamCGH16-E142S was produced using the same procedure, but the buffers were different: buffer A’, 50 mM HEPES pH 7.5, 150 mM NaCl, 30 mM imidazole; buffer B’, 50 mM HEPES pH 7.5, 150 mM NaCl, 500 mM imidazole; buffer C’, 50 mM HEPES pH 7.5, 100 mM NaCl. ZgLamCGH16-E142S was concentrated by ultrafiltration on an Amicon membrane (10 kDa cutoff) to 13.6 mg ml\(^{-1}\). The protein was filtrated on an Ultrafibre Durapore PVDF 0.1 µm membrane before crystallization screening.

2.3. Thermostability analysis

The thermostability of ZgLamCGH16 was studied by dynamic light scattering (DLS). 50 µl of a solution of ZgLamCGH16 at 7.5 mg ml\(^{-1}\) was filtrated on a 0.2 µm membrane. Using a Zetasizer Nano (Malvern), the protein solution was heated from 10 to 70°C in steps of 1°C over a total period of 12 h and the hydrodynamic gyration radius (R\(_g\)) was measured at each step. The denaturation temperature was determined as the point of sharp change in R\(_g\).

2.4. Enzymatic activity assays on β-glucans

The hydrolytic activities of the purified ZgLamCGH16 and ZgLamCGH16-E142S were measured by the ferricyanide reducing-sugar assay (Kidby & Davidson, 1973) on different β-glucans: laminarin from L. digitata [0.1% (w/v)], carboxymethyl cellulose (CMC), mixed-linked glucan (MLG) from barley, curdlan from Alcaligenes faecalis and paramylon from Euglena gracilis [all at 0.2% (w/v)]. Since laminarin is a small polysaccharide, this substrate was reduced prior to its usage as previously reported (Laboureul et al., 2014). Reduced laminarin was hydrolyzed by 10 nM purified enzyme in 1 ml buffer C at 40°C for 30 min. Aliquots of the reaction mixture (40 µl) were taken at T\(_0\), 10 min and 30 min and were added to 200 µl 5× ferricyanide reagent. The samples were boiled at 95°C for 15 min and cooled to 20°C before absorbance measurements at 420 nm. All experiments were undertaken in triplicate. A calibration curve with 0–3.33 mM glucose (0.278, 0.556, 1.11, 1.67, 2.22, 2.78 and 3.33 mM) was used to calculate the amount of released reducing ends as glucose reducing-end equivalents. The activity of ZgLamCGH16 on MLG, CMC, curdlan and paramylon was similarly measured, except that the reactions were monitored for 15 h. Aliquots were taken at T\(_0\), 10 min, 1 h and 15 h.

The pH optimum for laminarin hydrolysis was determined as follows: 0.1% (w/v) laminarin was hydrolyzed by 10 nM ZgLamCGH16 in a 500 µl reaction mixture at 40°C for 10 min. Different buffers (at 100 mM) were tested at a pH varying from 3 to 9 in 0.5 pH-unit increments: phosphate–citrate (pH 3–6), MOPS (pH 6–7.5), Tris–HCl (pH 7.5–8.5) and glycine (pH 8.5–9). Released reducing ends were measured as described above, except that aliquots of the reaction mixture (40 µl) were taken every 2 min.

The kinetic parameters of ZgLamCGH16 on reduced laminarin and MLG were determined using 10 nM enzyme in 500 µl reaction mixture at 40°C in 100 mM phosphate–citrate pH 5.0. The amount of released reducing ends was measured as above. For each substrate, five concentrations were used: 0.06, 0.12, 0.24, 0.48, 0.96 and 1.28% (w/v) for laminarin and 0.05, 0.1, 0.15, 0.2 and 0.25% (w/v) for MLG. Aliquots of the reaction mixture (40 µl) were taken every 2 min for 10 min for laminarin and every 5 min for 25 min for MLG. For each substrate, K\(_m\) and k\(_cat\) were determined from a Lineweaver–Burk plot.

2.5. Fluorophore-assisted carbohydrate electrophoresis (FACE) analysis

0.5% (w/v) laminarin was hydrolyzed using 100 nM ZgLamCGH16 in a reaction mixture consisting of 500 µl phosphate–citrate buffer pH 5.0 at 20°C. The temperature of 20°C was chosen to slow down the reaction in order to be able to determine the mode of action of ZgLamCGH16. An aliquot of 20 µl (100 µg oligosaccharides) was taken at 2 min, 10 min, 30 min and 1 h. The samples were boiled to inactivate the enzyme and then dried in vacuum (SpeedVac). The FACE experiment was undertaken as described previously (Jackson, 1990). Briefly, the oligosaccharides were mixed with 2 µl 0.15 M 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and 5 µl 1 M NaBH\(_4\)CN. The reaction mixtures were incubated at 37°C for at least 3 h and dried in vacuum (SpeedVac). The oligosaccharides were resuspended in 20 µl 25% glycerol and 10 µl (50 µg) was loaded onto a 36% acrylamide gel. The migration was undertaken at 200 V and 4°C with 1× migration buffer (192 mM glycine, 25 mM Tris pH 8.5). The experiment was repeated using 0.5% (w/v) MLG (from barley) and 100 nM ZgLamCGH16. The reaction mixture was incubated at 37°C and an aliquot of 20 µl was taken at 1, 2, 3, 4, 5 and 30 min. 100 µg of different commercial linear β-1,3-4-glucans (from laminaritriose to laminarihexaose; Megazyme) were


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hydrolyzed using 4.5 μM ZgLamCGH16 in a reaction mixture consisting of 100 μl phosphate–citrate buffer pH 5.0 at 37°C for 12 h. For each sample, an aliquot containing 50 μg oligosaccharides was treated as mentioned above. 5 μl (12.5 μg) were loaded onto a 36% acrylamide gel.

A glucan tetrasaccharide containing two 1,3-linkages separated by one 1,4-linkage (G4G3G4G) was also purchased from Megazyme. Three samples of this substrate at 50 μg were labelled with ANTS and was used as a control. 10 μl (25 μg) of each sample was loaded onto a 36% acrylamide gel.

2.6. Crystallization and structure refinement

Crystallization screening was undertaken with a Honeybee nanodrop robot (Cartesian) using the commercial screens The PACT and JCSG+ Suites (Qiagen). Using the sitting-drop vapour-diffusion method, 300 nL protein solution was mixed with 150 nL reservoir solution. The best initial crystallization condition was further optimized in 24-well Linbro plates by the hanging-drop vapour-diffusion method at 20°C. Single crystals of ZgLamCGH16-E142S were obtained by mixing 2 μl enzyme/oligosaccharide mixture with 1 μl reservoir solution and equilibrating against 750 μl reservoir solution. In the first case (corresponding to PDB entry 4crq), the 2 μl drop consisted of 8.6 mg ml⁻¹ ZgLamCGH16-E142S and 1 ml purified laminaritetraoses produced by ZgLamACG16. The reservoir solution comprised 14% PEG 6000, 100 mM sodium acetate pH 5.0, 220 mM MgCl₂, 10% glycerol. In the second case (corresponding to PDB entry 4cte), the 2 μl drop consisted of 12.2 mg ml⁻¹ ZgLamCGH16-E142S and 1 ml thio-β-1,3-hexaglucan substrate. This substrate was synthesized as described previously (Sylla, 2010). The reservoir solution consisted of 11% PEG 6000, 100 mM sodium acetate pH 5.0, 220 mM MgCl₂, 4% 2-propanol, 3% glycerol. Prior to flash-cooling in a nitrogen stream at 100 K, single crystals were quickly soaked in their reservoir solution supplemented with 30% ethylene glycol (for both types of crystals). Diffraction data for the crystals of ZgLamCGH16-E142S obtained in the presence of laminaritetraoses (hereafter referred to as the ‘apo’ form of ZgLamCGH16-E142S) were collected on the PROXIMA1 beamline at the SOLEIL synchrotron, Saint-Aubin, France. The diffraction data for the ZgLamCGH16-E142S–thio-β-1,3-hexaglucan complex (PDB entry 4cte) were collected on beamline BM14 at the ESRF synchrotron, Grenoble, France. X-ray diffraction data were integrated using MOSFLM (Leslie, 2006) and scaled with SCALA (Evans, 2006).

The structure of ZgLamCGH16-E142S was determined by molecular replacement with MOLREP (Vagin & Teplyakov, 2010) using chain A of the laminarinase from Thermotoga maritima MSB8 (PDB entry 3azx; Jeng et al., 2011) as a starting model. The structure of ZgLamCGH16-E142S was built using Coot (Emsley et al., 2010) by modifying and completing this starting model. For the ZgLamCGH16-E142S–inhibitor complex, the structure was also determined by molecular replacement but using the coordinates of chain A of ZgLamCGH16-E142S (the ‘apo’ form). For all of the structures, the initial molecular-replacement solutions were further refined with REFMAC5 (Vagin et al., 2004) alternating with cycles of manual rebuilding using Coot. A subset consisting of a randomly selected 5% of the reflections was excluded from computational refinement to calculate the Rfree factors.
throughout refinement. The addition of the ligand sugar units for the complex structure was performed manually using Coot. Water molecules were added automatically with REFMAC–ARP/wARP and were visually verified. The final refinement was carried out using REFMAC with TLS, isotropic B factors, automatic NCS restraints and Babinet solvent scaling for the two ZgLamC$_{GH16-E142S}$ structures. Data-collection and refinement parameters are presented in Table 1.

3. Results

3.1. ZgLamC$_{GH16}$ is a monomeric β-glucanase active on laminarin and MLG

The putative laminarinase ZgLamC (GenBank CAZ95067) features an N-terminal cleavable signal peptide followed by a catalytic module of family 16 of the glycoside hydrolases (GH16), a central carbohydrate-binding module of family 6 (CBM6) and a C-terminal PorSS module (Fig. 1a). The Por secretion system (PorSS) is a recently described protein-secretion machinery that is unique to the Bacteroidetes phylum (Sato et al., 2010), and the PorSS modules are conserved C-terminal domains that are likely to be involved in the targeting of proteins to the PorSS (Karlsson et al., 2004; McBride & Zhu, 2013). The GH16 catalytic module of ZgLamC has 37% sequence identity to the homologous domain of ZgLamA (Fig. 1b), which we have recently characterized (Labourel et al., 2014). The nucleotide sequence of this module was cloned in the pFO4 vector and expressed in E. coli C43(DE3) cells as a soluble protein referred to as ZgLamCGH16. Two purification steps [immobilized metal-affinity chromatography (IMAC) and size-exclusion chromatography (SEC)] were needed to purify this recombinant protein and we obtained 30 mg pure protein per litre of culture. This sample was divided into two aliquots: the first for biochemical characterization (1 ml at 1.25 mg ml$^{-1}$) and the second for crystallization assays (23 ml at 1.25 mg ml$^{-1}$). The SEC experiment and DLS analysis indicate that ZgLamCGH16 is a monomer in solution. DLS was also used to study the thermostability of the protein. A sharp increase in $R_g$ was observed above 40°C, corresponding to the beginning of protein denaturation. The enzyme activity was tested by the ferricyanide reducing-sugar assay (Kidby & Davidson, 1973) on various β-glucans: soluble laminarin, MLG and CMC, and crystalline curdlan and paramylon. Activity was only detected in the presence of laminarin and MLG. ZgLamCGH16 is active over a wide range of pH (from 3 to 9) and its optimal activity is observed in 100 mM phosphate citrate pH 5.0 (Fig. 2). Although the inhibitory effect of Tris–HCl on ZgLamCGH16 is less drastic than that on ZgLamA$_{GH16}$
Labourel et al. (2014), an inhibitory effect is observed for this buffer at pH 7.5 (and to a lesser degree with phosphate–citrate buffer pH 6.0) in comparison to MOPS buffer (Fig. 2). This inhibitory effect of Tris–HCl buffer has been reviewed previously (Roberts & Davies, 2012). The activity of ZgLamA<sub>GH16</sub> was also assayed by a reducing-sugar assay at different temperatures, and 40°C was determined to be the optimal temperature for kinetic characterization (data not shown). The kinetic parameters of ZgLamC<sub>GH16</sub> were thus determined at 40°C and in phosphate–citrate buffer pH 5.0 on reduced laminarin and on MLG. While the Michaelis constant for laminarin is better than that for MLG (K<sub>m</sub> of 4.83 ± 0.43 and 36.7 ± 4.2 mM, respectively), the turnover of ZgLamC<sub>GH16</sub> is surprisingly lower for laminarin than for MLG (k<sub>cat</sub> of 286 ± 14 and 795 ± 134 s<sup>−1</sup>, respectively). Nonetheless, the catalytic efficiency of ZgLamC<sub>GH16</sub> remains three times higher for laminarin than for MLG (k<sub>cat</sub>/K<sub>m</sub> of 59 213 and 21 662 M<sup>−1</sup>s<sup>−1</sup>, respectively).

Based on the knowledge of the catalytic residues in GH16 lichenases (Keitel et al., 1993; Juncosa et al., 1994) and on sequence comparison, one can predict that Glu137 and Glu142 are the nucleophile and acid/base catalyst of ZgLamC<sub>GH16</sub>.

Figure 3
Mode of action and terminal products of ZgLamC<sub>GH16</sub>. The hydrolysis of laminarin (a, c) and mixed-linked glucan (MLG) (b, d) by ZgLamC<sub>GH16</sub> was monitored by fluorophore-assisted carbohydrate electrophoresis (FACE). (a) 0.5%(w/v) laminarin was hydrolyzed by 100 nM ZgLamC<sub>GH16</sub> at 20°C. (b) 0.5%(w/v) MLG was hydrolyzed by 4.5 µM ZgLamC<sub>GH16</sub> at 37°C. (c) Standard laminarin oligosaccharides are labelled from DP2 to DP6 (lanes 1, 2, 4, 6 and 8). 100 µg of the oligosaccharides from DP3 to DP6 at 0.1% were incubated with 4.5 µM ZgLamC<sub>GH16</sub> at 37°C for 12 h (lanes 3, 5, 7 and 9). (d) The reaction mixtures contain 0.1%(w/v) of the tetrasaccharide G4G3G4G and 4.5 µM active (lane 3 and 5) or inactive (lane 4) ZgLamC<sub>GH16</sub> in 100 mM phosphate–citrate pH 5.0 at 37°C for 30 min. An asterisk indicates that the G4G3G4G oligosaccharides were labelled before the enzymatic reaction, while the absence of an asterisk indicates that the oligosaccharides were labelled after the reaction.
In order to obtain the structure of an inactive form of ZgLamCGH16 in complex with laminarin or MLG oligosaccharides, we undertook the mutagenesis of these putative catalytic residues (E137A, E137S, E142A and E142S). Among these four site-directed mutations, only the replacement of the codon for Glu142 by a serine codon was confirmed by sequencing of the extracted plasmids. The protein corresponding to this mutated plasmid was expressed in soluble form in E. coli C43(DE3) cells and is hereafter referred to as ZgLamCGH16-E142S. Like ZgLamCGH16, a yield of 30 mg pure protein per litre of culture was obtained after two steps of chromatography (IMAC and SEC). The purification buffers were changed (HEPES instead of Tris–HCl for ZgLamCGH16) to avoid protein precipitation during the concentration process (see §3.3). The hydrolysis of laminarin and of MLG by ZgLamCGH16-E142S was tested by the reducing-sugar assay, but no enzymatic activity was detected even after 24 h of hydrolysis, confirming the involvement of Glu142 in the catalytic machinery of ZgLamCGH16.

3.2. ZgLamCGH16 displays an endolytic mode of action

The hydrolysis of laminarin and MLG by ZgLamCGH16 was monitored by FACE for 1 h and 30 min, respectively (Figs. 3a
and 3b). For both substrates, oligosaccharides with a relatively high degree of polymerization (DP) were initially released, progressively followed by oligosaccharides of smaller sizes. These product patterns indicate that ZgLamC<sub>GH16</sub> proceeds according to an endolytic mode of action. The degradation products of ZgLamC<sub>GH16</sub> were further analyzed. For laminarin, four standard β-1,3-glucan oligosaccharides (DP from 3 to 6) were digested by ZgLamC<sub>GH16</sub>, to completion. The reducing end of the reaction products was labelled with ANTS and analyzed by FACE (Fig. 3c). Hydrolysis of the trisaccharide resulted in the release of two bands corresponding to a monosaccharide and a disaccharide. The released glucose was partially masked by the migration front of the fluorescent marker, but remained visible. The same degradation pattern was also observed for the other oligosaccharides (Fig. 3c).

Thus, the smallest oligosaccharide that can be degraded by ZgLamC<sub>GH16</sub> is laminaritriose and the terminal products are glucose and laminaribiose. Finally, the degradation of a glucan tetrasaccharide containing two β-1,4 linkages separated by one β-1,3 linkage (G4G3G4G) was also monitored by FACE. ANTS labelling was undertaken either prior to or after the enzymatic reaction. When the tetrasaccharide was labelled first no cleavage was observed, indicating that the ANTS moiety hindered the action of ZgLamC<sub>GH16</sub>. When labelling was undertaken after hydrolysis the reaction products migrate as two new bands corresponding to a monosaccharide and a trisaccharide (Fig. 3d). Therefore, as observed for ZgLamA<sub>GH16</sub> (Labourel et al., 2014), ZgLamC<sub>GH16</sub> specifically cleaves β-1,4 linkages next to β-1,3 linkages, and the MLG trisaccharide and glucose are the terminal products.

3.3. Crystal structure of ZgLamC<sub>GH16-E142S</sub> (‘apo form’)

Prior to crystallization trials, ZgLamC<sub>GH16</sub> was submitted to a concentration step, but unfortunately the complete sample precipitated. Since we had already produced ZgLamC<sub>GH16-E142S</sub> in parallel, we decided to pursue the structural study using this mutated enzyme. Initially, we were not able to crystallize ZgLamC<sub>GH16-E142S</sub> alone. However, single crystals were obtained in the presence of purified laminaritetraoses produced by ZgLamA<sub>GH16</sub> (Labourel et al., 2014). These crystals had good X-ray diffraction quality and the structure of ZgLamC<sub>GH16-E142S</sub> was solved at 1.5 Å resolution by molecular replacement using chain A of the laminarinase TmLamCD from the hyperthermophilic bacterium T. maritima (47% sequence identity; PDB entry 5azx; Jeng et al., 2011). The crystal is orthorhombic (P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>) and two protein molecules are found in the asymmetric unit (from Asp24 to Lys254), as well as 426 water molecules. Surprisingly, there is no laminarin tetrasaccharide visible in the electron-density map, even though the presence of these oligosaccharides was essential in order to obtain crystals. The overall structure of ZgLamC<sub>GH16-E142S</sub> displays 13 β-strands and three small α-helices. The β-strands are organized into two twisted β-sheets typical of the jelly-roll fold of GH16 enzymes. ZgLamC<sub>GH16-E142S</sub> displays an open active-site cleft parallel to the inner β-sheet (Fig. 4a).

The following ions and solvent ligands have been modelled into electron density: for protein chain A one Ca<sup>2+</sup> ion, one Cl<sup>-</sup> ion, one Mg<sup>2+</sup> ion, two acetate ions and two ethylene glycols and for chain B one Ca<sup>2+</sup> ion, two Cl<sup>-</sup> ions, two Na<sup>+</sup> ions, one acetate ion and two ethylene glycols. In both chains the Ca<sup>2+</sup> ion is found in the calcium-binding site conserved in most GH16-family enzymes (Michel et al., 2001), with the exception of the xyloglucan endotransglycosylases (Johansson et al., 2004) and xyloglucan hydrolases (Baumann et al., 2007). Here, the calcium coordination displays identical pentagonal bipyramidal geometry in both chains. The base of the bipyramid is formed by bonds between the Ca<sup>2+</sup> ion and Glu32 O, Asp247 O, Asp247 OD1 and two ethylene glycols. In both chains the Ca<sup>2+</sup> ion is found in the calcium-binding site conserved in most GH16-family enzymes (Michel et al., 2001), with the exception of the xyloglucan endotransglycosylases (Johansson et al., 2004) and xyloglucan hydrolases (Baumann et al., 2007). Here, the calcium coordination displays identical pentagonal bipyramidal geometry in both chains. The base of the bipyramid is formed by bonds between the Ca<sup>2+</sup> ion and Glu32 O, Asp247 O, Asp247 OD1 and two ethylene glycols. In both chains the Ca<sup>2+</sup> ion is found in the calcium-binding site conserved in most GH16-family enzymes (Michel et al., 2001), with the exception of the xyloglucan endotransglycosylases (Johansson et al., 2004) and xyloglucan hydrolases (Baumann et al., 2007). Here, the calcium coordination displays identical pentagonal bipyramidal geometry in both chains. The base of the bipyramid is formed by bonds between the Ca<sup>2+</sup> ion and Glu32 O, Asp247 O, Asp247 OD1 and two ethylene glycols.
hydrogen-bonded to three residues, Lys92, Glu44 and Arg90; in chain B, the nature of this molecule is less clear. Since no clear electron density for any oligosaccharide could be identified, this structure (PDB entry 4crq) can be considered as an ‘apo’ structure of ZgLamCGH16-E142S.

3.4. Structure of ZgLamCGH16-E142S in complex with a thio-\(\beta-1,3\)-hexaglucan

In an additional attempt to obtain a complex structure, ZgLamCGH16-E142S was co-crystallized with a substrate analogue consisting of a \(\beta-1,3\)-glucan hexasaccharide displaying a benzyl group at the reducing end and in which the O-glycosidic bonds 3 and 4 were replaced by S-glycosidic linkages (Fig. 4c; Sylla, 2010). The X-ray structure was solved at 1.8 Å resolution (PDB entry 4cte) by molecular replacement using chain A of ZgLamCGH16-E142S. The crystal is orthorhombic (P2\(_1\)2\(_1\)2\(_1\)) and two protein molecules (Asp24–Lys254) and 333 water molecules were found in the asymmetric unit. Each protein molecule also bound one Ca\(^{2+}\) ion, one Cl\(^{-}\) ion, an ethylene glycol and a glycerol, and one additional acetate ion was modelled in chain B. As in the ‘apo’ structure of ZgLamCGH16-E142S, the Ca\(^{2+}\) ion was found in the binding site that is conserved in most GH16 enzymes, displaying a pentagonal bipyramidal geometry. In chain B, two of the base ligands (water molecules in chain A) were modelled as a bidentately binding acetate ion. Moreover, an oligosaccharide was clearly visible in the negative subsites of each protein (Fig. 5a). Additional electron density was also observed in the positive subsites, but was too disordered to be modelled as sugar units. In the more disordered chain A, only two glucose moieties were modelled spanning subsites \(-1\) and \(-2\). In chain B, three glucose moieties were modelled spanning subsites \(-1\) to \(-3\) (Fig. 5a). Superimposition of the ‘apo’ and

**Figure 6**
Molecular basis for the recognition of \(\beta-1,3\)-glucan by ZgLamCGH16-E142S and ZgLamAGH16-E269S. (a), (b) and (c) correspond to ZgLamCGH16-E142S in complex with the thio-\(\beta-1,3\)-glucan analogue. Each panel focuses on a specific subsite (subsites \(-1\), \(-2\) and \(-3\), respectively). In (a), (b) and (c) a dashed circle highlights the additional compounds found in the vicinity of the C6 hydroxyl group of each glucose moiety. A glycerol molecule is found in subsite \(-1\) (a). An ethylene glycol molecule is found in subsite \(-2\) (b). A chloride ion is found in subsite \(-3\) (c). (d), (e) and (f) correspond to ZgLamAGH16-E269S in complex with a laminaritetraose (focusing on subsites \(-1\), \(-2\) and \(-3\), respectively). The amino acids involved in substrate binding are displayed as sticks. The labels of the residues specific to each enzyme are shown in bold italics. The labels of the conserved catalytic amino acids of the GH16 family are underlined and an asterisk indicates each mutated catalytic residue. In (c) and (f) a transparent molecular surface is displayed to highlight the presence of an open cavity next to subsite \(-3\) for ZgLamCGH16-E142S (c). ZgLamAGH16-E269S lacks such an open cavity (f).
the complexed structures of ZgLamC<sub>GH16-E142S</sub> shows that no major conformational change occurs between the two structures. Since the substrate was visible in the electron-density map, it seems to have a higher affinity than the natural terminal products and was most likely to be bound to the protein with a S-glycosidic bond between the cleavage subsites −1 and +1. Taking into account this point and the structure of the glucan, an S-glycosidic bond was also modelled between subsites −1 and −2 (Fig. 5a). No significant positive or negative peak in the region of the S-glycosidic linkages was detected in the F<sub>o</sub> − F<sub>c</sub> electron-density map (data not shown). In subsite −1 (Fig. 6a), the O4 hydroxyl group of the glucose residue is hydrogen-bonded to the nucleophile Glu137 OE2 (2.41 Å). Surprisingly, this glucose is found to be perpendicular to the two aromatic residues Trp117 and Glu137 OE2 (2.41 Å). These glycerol molecules make hydrogen bonds to three residues (Fig. 6b). O2 makes a hydrogen bond to Asn223 OD1 (2.64 Å), while O1 makes a hydrogen bond to Arg90 NH2, and Trp132 serves as a hydrophobic platform. In both chains, an ethylene glycol is also found in a pocket located next to subsite −2 (Fig. 5c), and is hydrogen-bonded to Lys329 NZ, Glu44 OE2 and Arg90 NH2. Subsite −3 of ZgLamC<sub>GH16-E142S</sub> is characterized by a hydrogen bond between O2 and Gly53 O (2.46 Å; Fig. 6c). The O6 is solvent-exposed and above this subsite a Cl<sup>−</sup> ion is found to make hydrogen bonds to Asn54 OD1 and Arg90 NH2, and Trp132 serves as a hydrophobic platform. In both chains, an ethylene glycol is also found in a pocket located next to subsite −2 (Fig. 5c), and is hydrogen-bonded to Lys92 NZ, Glu44 OE2 and Arg90 NH2. Subsite −3 of ZgLamC<sub>GH16-E142S</sub> is characterized by a hydrogen bond between O2 and Gly53 O (2.46 Å; Fig. 6c). The O6 is solvent-exposed and above this subsite a Cl<sup>−</sup> ion is found to be associated with two molecules of water (HOH2193 and HOH2035). The Cl<sup>−</sup> ion is close to the hydrophobic surface made up by the lateral and the main chain of Trp52. It also interacts with Tyr49 N. HOH2193 makes a hydrogen bond to O5 (3.07 Å) and O6 (3.35 Å) of the glucose unit in subsite −3. Strikingly, the O6 groups of the glucose moieties in subsites −1, −2 and −3 point towards pockets containing a glycerol, an ethylene glycol and a chloride ion, respectively. A conserved structural water molecule (HOH2030 in chain A and HOH2038 in chain B) was found to make hydrogen bonds to O5 (2.77 Å) and O6 (2.82 Å) of the glucose unit at subsite −2 and to Arg90 NH1 (2.87 Å) and Trp52 O (2.75 Å).

3.5. Comparison of the ZgLamC<sub>GH16-E142S</sub>–thioglycan and ZgLamA<sub>GH16-E269S</sub>–laminaritetraose complex structures

The laminarinases ZgLamA<sub>GH16</sub> and ZgLamC<sub>GH16</sub> are relatively divergent in sequence (37% identity; Fig. 1b), but superimposition of the ZgLamA<sub>GH16-E269S</sub>–laminaritetraose (Labourel et al., 2014) and ZgLamC<sub>GH16-E142S</sub>–inhibitor complexes results in a low root-mean-square deviation (0.91 Å over 199 matched Cα atoms). Both complex structures display sugar molecules bound to the negative subsites −1, −2 and −3 (except where mentioned, all of the amino acids are numbered as in ZgLamC<sub>GH16-E142S</sub>). At subsite −1 the glucose unit does not adopt the same position in the two structures (Figs. 6a and 6d). In ZgLamA<sub>GH16-E269S</sub> the glucose is typically found parallel to the two conserved tryptophans (Trp117 and Trp121), while this sugar binds perpendicularly to these aromatic residues in ZgLamC<sub>GH16-E142S</sub>. The glucose moiety establishes more hydrogen bonds with ZgLamA<sub>GH16-E269S</sub> with O6 interacting with Trp238 NE1 and O1 with His288 (ZgLamC<sub>GH16-E269S</sub> numbering), a histidine that is conserved throughout the GH-B clan (Michel et al., 2001). The pocket located above subsite −1 is also found in ZgLamA<sub>GH16-E269S</sub> and all residues forming this pocket are conserved (Asn223, Tyr60, Glu56 and Trp117). The glucose units in subsites −2 and −3 can be partially superimposed and they adopt a similar orientation in both enzymes. Three amino acids are conserved between subsites −2 (Figs. 6b and 6c): Arg90, Asn54 and Trp132. In ZgLamA<sub>GH16-E269S</sub> a fourth residue participates in subsite −2, Glu250, which belongs to the additional loop of this enzyme and makes a hydrogen bond to the glucose unit. This glutamate is absent in ZgLamC<sub>GH16-E142S</sub> which instead displays a pocket next to subsite −2 which is occupied by an ethylene glycol molecule. In ZgLamC<sub>GH16-E142S</sub> subsite −3 consists of a hydrogen bond between O2 of the glucose unit and the carbonyl of Gly53, while in ZgLamA<sub>GH16-E269S</sub> the carbonyl of Trp264 is hydrogen-bonded to the O6 hydroxyl group of the glucose (Figs. 6c and 6f; Labourel et al., 2014).

4. Discussion

Brown algae produce a variety of β-1,3-glucans with different biological functions: the M-series and G-series of branched laminarins (Read et al., 1996), linear insoluble laminarins (Nelson & Lewis, 1974; Rioux et al., 2010) and even semi-crystalline callose in the sieve tubes of kelps (Laminariales; Parker & Huber, 1965). To face this physicochemical diversity, the seaweed-associated bacterium Z. galactanivorans possesses a multi-enzymatic system of five putative β-1,3-glucanases (four GH16s, ZgLamA–ZgLamD, and one GH64, ZgLamE) with different modular architectures. The four GH16 modules are quite divergent, with sequence identity ranging from 29 to 37%. Moreover, these β-glucanases are predicted to have different cellular localizations. For instance, ZgLamA is predicted to be a lipoprotein located in the outer membrane (Labourel et al., 2014), while ZgLamC features an N-terminal signal peptide and a C-terminal Por secretion system (PorSS) domain. Such conserved C-terminal domains are also present in two enzymes from Z. galactanivorans already known to be secreted into the extracellular medium: the κ-carrageenase ZgCgkA (Barbeyron et al., 1998) and the β-agarase ZgAgaA (Jam et al., 2005). Therefore, ZgLamC is likely to be targeted to the periplasm by the Sec system and then exported across the outer membrane by PorSS (Sato et al., 2010). Altogether, the sequence divergences and the
Zgalactanivorans have distinct and/or complementary roles. Recently, we have characterized the first enzyme of this laminarolytic system, ZgLamAGH16. This enzyme is highly efficient and almost exclusively active on algal laminarin. The structure of ZgLamAGH16 in complex with laminaritetrose has revealed a unique topology within the GH16 family (a bent active site; Fig. 4b), which explains this exquisite adaptation to algal laminarin (Labourel et al., 2014).

In the present work, we have undertaken a first comparative analysis to test the hypothesis of the differing or complementary functions of the β-glucanases of Z. galactanivorans. Thus, we have overexpressed and purified the GH16 catalytic module of ZgLamC. The recombinant enzyme ZgLamCGH16 is active on both laminarin and MLG, with a catalytic efficiency (kcat/Km) three times higher for laminarin than for MLG. This enzyme acts according to an endolytic mode of action (Figs. 3a and 3b). Its minimal substrate is laminaritrose, releasing glucose and laminaribiose (Fig. 3c). ZgLamCGH16 also cleaves β-1,4-linkages next to β-1,3-linkages in MLG, giving the terminal products glucose and the trisaccharide G4G3G (Fig. 3d). Interestingly, ZgLamCGH16 is less efficient on linear laminarin than ZgLamAGH16 (Labourel et al., 2014; kcat/Km of 59 213 and 82 000 M⁻¹ s⁻¹, respectively), but approximately six times more active on MLG (kcat/Km of 21 662 and 3678 M⁻¹ s⁻¹, respectively). These differences in catalytic efficiency can be explained by the respective active-site topologies of ZgLamCGH16 and ZgLamAGH16. Indeed, the straight-cleft topology of ZgLamCGH16 (Fig. 4a) is a good compromise to provide significant activity on both MLG (straight shape) and laminarin (helical shape), while the bent active site of ZgLamAGH16 (Fig. 4b) is optimized for laminarin recognition but results in a much weaker activity on MLG (Labourel et al., 2014).

The lower affinity for linear β-1,3-glucan in comparison to ZgLamAGH16 was also highlighted by our difficulty in obtaining a complex structure of ZgLamC_{GH16-E142S} with native oligo-laminarins. Eventually, we succeeded in obtaining a complex structure in the presence of a thioglycosidic linkages in position of the glucose residue, which is perpendicular to molecule in subsite a complex structure in the presence of a thioglycosidic linkages in position of the glucose residue, which is perpendicular to molecule in subsite...


