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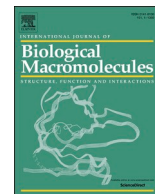
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Family GH157 enzyme exhibits broad linkage tolerance and a dual endo/exo- β -glucanase activity on β -glucans

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ABSTRACT

The structural and chemical diversity of β -glucans is reflected on the variety of essential biological roles tackled by these polysaccharides. This natural heterogeneity requires an elaborate assortment of enzymatic mechanisms to assemble, degrade or modify, as well as to extract their full biotechnological potential. Recent metagenomic efforts have provided an unprecedented growth in potential new biocatalysts, most of which remain unconfirmed or uncharacterized. Here we report the first biochemical and structural characterization of two bacterial β -glucanases from the recently created glycoside hydrolase family 157 (*LaGH157* and *BcGH157*) and investigate their molecular basis for substrate hydrolysis. Structural analysis by X-ray crystallography revealed that GH157 enzymes belong to clan GH-A, possessing a $(\beta/\alpha)_8$ -barrel fold catalytic domain, two β -sandwich accessory domains and two conserved catalytic glutamates residues, with relative positions compatible with a retaining mechanism of hydrolysis. Specificity screening and enzyme kinetics suggest that the enzymes prefer mixed-linkage glucans over β -1,3-glucans. Activity screening showed that both enzymes exhibit pH optimum at 6.5 and temperature optimum for *LaGH157* and *BcGH157* at 25 °C and 48 °C, respectively. Product analysis with HPAEC-PAD and LC-MS revealed that both enzymes are endo-1,3(4)- β -glucanases, capable of cleaving β -1,3 and β -1,4-linked glucoses, when preceded by a β -1,3 linkage. Moreover, *BcGH157* needs a minimum of 4 subsites occupied for hydrolysis to occur, while *LaGH157* only requires 3 subsites. Additionally, *LaGH157* possesses exohydrolytic activity on β -1,3 and branching β -1,6 linkages. This unusual bifunctional endo-1,3(4)/exo-1,3-1,6 activity constitutes an expansion on our understanding of β -glucan deconstruction, with the potential to inspire future applications.

1. Introduction

β -glucans are a diverse set of natural polysaccharides found in yeast, fungi, bacteria, seaweed, and cereals, where they carry out varied essential biological functions such as energy storage, structural support, and protective roles. The term β -glucan is mostly used to refer to β -1,3 and mixed-linkage β -(1,3-1,4) glucans (MLGs), whose molecular structures can vary depending on their origin and extraction procedure. Generally, β -1,3 glucans are composed of linear backbones of β -1,3 linked glucose units, typically adorned with β -1,6 linked branches, while MLGs are unbranched chains of β -1,3- and 1,4-linked glucose units in

varying proportions [1–3]. Besides the nutritional benefits arising from the conversion of diet β -glucans into short-chain fatty acids by the gastrointestinal microbiota, β -glucans have also been associated with other health-promoting immunomodulatory and metabolic effects [4–8]. Metabolic effects are mostly associated with MLGs and include modulation of the gut microbiome and improved lipidic and glycemic metabolism, which can help control diabetes and metabolic syndrome. β -1,3-glucans are more often associated with immunomodulatory properties such as anti-cancer, wound healing, and inflammation-controlling effects [9–14]. However, the underlying molecular mechanisms governing these effects remain to be fully understood.

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Additionally, the solubility and rheological properties of β -glucans also make them attractive components for many industrial processes, particularly in the food and cosmetic industries, where they are used as thickening, soothing, and moisturizing agents [15,16].

Given the biological and industrial relevance of β -glucans, there is an inherent interest in understanding the action of β -glucan modifying enzymes but, in comparison to other major polysaccharides, such as cellulose, xylans and mannans, they are not as extensively defined in terms of their structural, specificity and mechanistic profile. β -glucan deconstruction is mostly dependent on the action of β -1,3-glucanases, a group of glycoside hydrolases mainly expressed by bacteria, fungi, plants and some invertebrates. These enzymes take part in multiple physiological processes, such as energy storage, cellular remodelling and development, defence against pathogens, seed germination, digestion and reproduction, and are therefore crucial molecules for the maintenance of homeostasis [17–20]. There are three major activities within β -1,3-glucanases, namely *exo*- β -1,3-glucanases (EC 3.2.1.58), which cleave D-glucose from the nonreducing end of a glucan molecule, *endo*- β -1,3-glucanases (EC 3.2.1.39), which hydrolyse β -1,3 glycosidic bonds randomly within the polysaccharide chain, releasing glucan oligosaccharides, and the less specific *endo*-1,3(4)- β -glucanases (EC 3.2.1.6), which are able to hydrolyse both β -1,3 and β -1,4 bonds, as long as the glucose residue whose reducing group is involved in the linkage to be hydrolysed is itself substituted at the C-3 position [3,21]. The majority of the characterized β -1,3-glucanases are distributed between five glycoside hydrolase (GH) families in the CAZY database (<http://www.cazy.org/>), namely GH5, 16, 17, 55 and 81 [22]. Structure wise, β -1,3-glucanases can display different type of folds, depending on which family they belong to: GH16 family members adopt a β -jelly-roll fold, GH5 and 17 β -1,3-glucanases display a $(\beta/\alpha)_8$ fold, GH55 members have a β -helix conformation, while GH81s have a $(\alpha/\alpha)_6$ -barrel structural fold. Regarding active site conformation, most characterized β -1,3-glucanases have two conserved glutamate catalytic residues that function as the catalytic dyad, while their substrate-binding site is composed of tryptophan-rich sequences. However, there are still several β -1,3-glucan active families such as GH64, GH128, GH152 and, particularly, the recently created GH157 and GH158 families, which have very limited or no structural and mechanistic information at all, hampering our understanding of β -glucan deconstruction and limiting the development and enhancement of biotechnological and biomedical applications for β -glucanases.

Recently, in an effort to establish sequence-to-function relationships among a myriad of predicted carbohydrate-active enzymes, a large activity screening was performed on unassigned sequences too distant to allow their classification into any CAZY family, using a wide collection of carbohydrates substrates [23]. This study established two new β -1,3-glucan active families, including GH157, whose founding member (GenBank accession number WP_029429093.1) was able to depolymerize the linear β -1,3-glucan curdlan. Currently (June 2024), there are 13 other putative enzymes assigned to family GH157 on the CAZY database, all of bacterial origin. These include proteins from mesophilic organisms such as the human gut genus *Bacteroides*, as well as from psychrophilic marine bacteria. At present, this is the full extent of knowledge available for family GH157, as there hasn't been any progress made towards providing a structural and mechanistic characterization of this new family. Aiming to fill this gap and provide new insights into the natural bacterial toolbox for β -glucan depolymerization, the work here presented reports the first biochemical and structural characterization of two GH157 members, one from the marine psychrophilic bacterium *Labilbaculum antarcticum* (*LaGH157*) and one from the human gut bacterium *Bacteroides cellulosilyticus* (*BcGH157*). Substrate specificity and product analysis performed with LC-MS and HPAEC-PAD identified both enzymes as β -1,3(4)-glucanases with an *endo* mode of action, capable of cleaving β -1,3 and β -1,4-linked glucoses, when preceded by a β -1,3 linkage. *LaGH157* also showed *exo*-like activity on a β -1,3 linked glucan substrate as well as on the β -1,6 linked

branches of laminarin. We also describe the 3D structure of *LaGH157*, determined by X-ray crystallography, whose catalytic module adopts a classic $(\beta/\alpha)_8$ barrel fold topology. Moreover, through ligand complexes and homology analysis, we have identified the catalytic residues and overall arrangement of the substrate binding and catalytic centre, with two glutamates functioning as the nucleophile and general acid/base, defining the catalytic mechanism that acts with retention of the anomeric configuration. Together, these structural features place GH157 enzymes within the GH-A clan.

Overall, this study provides the first insight into the catalytic mechanism of GH157 enzymes, contributing to a better understanding of β -glucan processing mechanisms both in psychrophilic marine environments and the human gut.

2. Results and discussion

The work by Helbert *et al* provided an unprecedented expansion in the number of CAZY families [23]. By resorting to a high-throughput strategy for gene expression and activity screening, the authors were able to attribute function to several putative CAZymes too distantly related to be assigned to existing families. This led to the creation of 7 new glycoside hydrolase families and 7 new polysaccharide lyase families [24–26]. One of such families was GH157, whose founding member is overexpressed by the gut bacteria *B. cellulosilyticus* during growth on β -glucan and was found to be active on linear β -1,3-glucans [23]. Several other enzymes, originating from a taxonomically varied bacterial population, have since been added to the family but no information on the structural and mechanistic processes governing catalysis by GH157 enzymes was available until now.

2.1. Substrate specificity

Substrate specificity of *LaGH157* and *BcGH157* was evaluated with natural polysaccharides (Table 1). *LaGH157* showed the highest specific activity against barley β -glucan (325 ± 23.1 U/mg), followed by lichenan (168 ± 7.0 U/mg), laminarin from *Laminaria digitata* (65 ± 0.8 U/mg) and yeast β -glucan (11 ± 0.3 U/mg), indicating that *LaGH157* efficiently hydrolyses both MLGs and β -1,3-glucans. No apparent activity was detected against the linear β -1,3-glucans CM-curdlan and CM-pachyman over the course of the assay. Nonetheless, the overnight digests made for the product analysis revealed some hydrolysis of CM-curdlan (see below) suggesting that *LaGH157*'s kinetics on the chemically modified linear β -1,3-glucans are slow, likely due to a low tolerance for the bulky carboxymethyl substitutions. *BcGH157* displayed maximum activity against lichenan (994 ± 39.4 U/mg), followed by laminarin (608 ± 12.3 U/mg), CM-curdlan (569 ± 15.9 U/mg), yeast β -glucan (402 ± 32.5 U/mg), CM-pachyman (309 ± 42.6 U/mg) and barley β -glucan (201 ± 31.2 U/mg). Thus, *BcGH157* efficiently hydrolyses both branched and unbranched β -1,3-glucans, as well as MLGs. Both enzymes were unable to depolymerize 4-O-methyl-D-glucurono-D-xylan, birchwood xylan, lichenan, xylan, carboxymethyl cellulose, guar galactomannan, polygalacturonic acid, pullulan, rye flour arabinoxylan, xyloglucan, hydroxyethyl cellulose and pustulan. The higher apparent activity on MLGs in comparison to laminarins suggests that GH157 enzymes are β -1,3(4)-glucanases, rather than specific β -1,3-glucanases, as previously suggested.

2.2. Activity range

Both *LaGH157* and *BcGH157* revealed typical optimal pH values for β -1,3-glucanase activity, which lay on the slightly acidic range of pH 5.0 to 6.5 [3,27–31] with maximum activity at pH 6.5 (Fig. 1a,b). *LaGH157* retained over 60 % activity between pH 5.7 and 7.0, while *BcGH157* retained similar activity between pH 6.0 and 7.0. As for pH stability, *LaGH157* maintains stability between pH 5.0 and 8.0, quickly losing stability below pH 5.0 while seemingly being more tolerant of alkaline

Table 1

DNSA-measured enzymatic activity of BcGH157, LaGH157 and its catalytic residue mutants after 10-minute incubations with different polysaccharide substrates.

Substrate	LaGH157 (U/mg)	BcGH157 (U/mg)	LaGH157_E132A (U/mg)	LaGH157_E224A (U/mg)
Laminarin	65 ± 0.8	608 ± 12.3	ND	ND
CM-curdlan	ND	569 ± 15.9	–	–
CM-pachyman	ND	309 ± 42.6	–	–
Lichenan	168 ± 7.0	994 ± 39.4	ND	ND
Barley β-Glucan	325 ± 23.1	201 ± 31.2	ND	ND
Yeast β-Glucan	11 ± 0.3	402 ± 32.5	–	–
Other polysaccharides ^a	ND	ND	–	–

ND: not detected.

Values are presented as mean ± standard deviation from triplicate experiments.

^a 4-O-methyl-D-glucurono-D-xylan, birchwood xylan, lichenan, xylan, carboxymethyl cellulose 4 M, guar galactomannan, polygalacturonic acid, pullulan, rye flour arabinoxyylan, xyloglucan, hydroxyethyl cellulose and pustulan.

pHs (Fig. 1a). BcGH157 is stable in a much narrower pH range of 5.5–7.5 and quickly lost stability outside these values (Fig. 1b). The optimal temperatures for LaGH157 and BcGH157 activity were 25 °C and 48 °C, respectively, reflecting their psychrophilic and mesophilic origin (Fig. 1c,d). The temperature ranges in which the enzymes displayed at least 60 % activity (at pH 6.5) were 10 °C – 32.5 °C for LaGH157 (Fig. 1c) and 32.5 °C – 52 °C for BcGH157 (Fig. 1d). Regarding the enzymes' thermostability, LaGH157 retained at least 80 % of its activity after 1 h incubation at temperatures between 4 and 32.5 °C, while it was unable to recover its function after incubation at temperatures above 35 °C (Fig. 1c). On the other hand, BcGH157 retained 80 % of activity between 4 and 48 °C and lost 50 % of its activity after 1 h at 49 °C (Fig. 1d).

The enzymes thermostability was also assessed at pH 5.0, 6.5 and 8.0 (Fig. 1e). The apparent melting temperatures (T_m) of LaGH157 were 51.5, 51.0 and 50.3 °C, respectively. BcGH157 T_m values were 51.9, 51.3 and 50.4 °C, respectively (Supplemental Table 1). Interestingly, the T_m of both enzymes is higher than their respective optimum temperature (LaGH157, 25 °C and BcGH157, 48 °C). This likely means that the active sites of the enzymes undergo structural changes, losing their ability to catalyse the hydrolysis of the glycosidic bonds, prior to unfolding of the overall protein structure. This effect is often observed and is usually more pronounced in the psychrophilic (LaGH157) and mesophilic (BcGH157) enzymes than in thermophilic ones [32]. Moreover, a slight decrease in thermal stability at pH 8.0 was also observed, which is in accordance with the pH stability curve.

2.3. Enzyme kinetics

Kinetic parameters of LaGH157 and BcGH157 were determined for the chromogenic laminaribioside substrate pNPLAM2 (Fig. 1f and Table 2). LaGH157 yielded a V_{max} of $0.298 \pm 0.064 \mu\text{M}\cdot\text{s}^{-1}$ and K_m of $1.40 \pm 0.650 \text{ mM}$. Although saturation was not observed in BcGH157 at substrate concentrations up to 3.2 mM, the curve suggests a V_{max} of $2.47 \pm 0.142 \mu\text{M}\cdot\text{s}^{-1}$ and K_m of $2.79 \pm 0.278 \text{ mM}$. These results revealed that LaGH157 presented greater affinity for pNPLAM2, since its V_{max} and K_m values were respectively 8 and 2 times lower than those for BcGH157. LaGH157 and BcGH157 displayed a k_{cat} of 1.19 s^{-1} and 9.9 s^{-1} and catalytic efficiency (k_{cat}/K_m) of $0.85 \text{ s}^{-1}\cdot\text{mM}^{-1}$ and $3.54 \text{ s}^{-1}\cdot\text{mM}^{-1}$, respectively. This suggests that, even though LaGH157 has a higher affinity for the substrate, BcGH157 was more efficient, since its k_{cat}/K_m ratio was 4 times higher. Kinetics on natural MLG substrates reveal a similar pattern, with LaGH157 having higher affinity for the substrates but lower catalytic efficiency (Table 2). It also shows that BcGH157 has significantly higher activity on lichenan than on barley MLG, suggesting it prefers substrates with a higher number of β 1–4 linkages.

2.4. Product analysis by HPAEC-DAD and LC-MS

The hydrolysis of overnight digests of MLG, lichenan, laminarin, and CM-curdlan by either BcGH157 or LaGH157 yielded low apparent

average molecular weight (MW_{app}) mixtures of oligosaccharides (Supplemental Table 2). The low MW_{app} of the overnight digests indicates that the digestions went to completion and confirmed that the bonds targeted by GH157 enzymes represent a significant fraction of the glycosidic linkages found in MLG and β -1,3-glucans.

Comparing HPAEC-PAD analyses of the GH157 MLG digests to digests by VvEG16 [33] and lichenase [34] shows that the hydrolytic products from the action of GH157 enzymes on MLG match those generated by lichenase (Fig. 2a, b). LC-MS analysis confirms the presence of lichenase-generated trisaccharide (G4G3G using the nomenclature of [33]) and tetrasaccharide (G4G4G3G) in the BcGH157 and LaGH157 digests (Fig. 2c,d). Similarly, digests of lichenan by BcGH157 and LaGH157 yield the same products (Supplemental Fig. 1) in differing quantities, typical of the differing underlying structures of MLG and lichenan [34]. The lichenase-like recognition of MLG by GH157 enzymes indicates selective recognition of laminaribiose (Lam2) motifs in the enzymatic negative subsites followed by cleavage of the subsequent β -1,4 linkage. Thus, the enzyme shows flexibility towards linkage type in the active site and in the positive subsites, but favours β -1,3 linkages in the negative subsites.

In line with its low MW_{app} , HPAEC-PAD analysis of the LaGH157 digest of laminarin showed that the major products are glucose and laminaribiose (Fig. 3b). In contrast, BcGH157 yielded a more limited digest of laminarin, containing only laminaribiose, laminaritriose, and two additional major oligosaccharide peaks that could not be identified conclusively (Fig. 3a). LC-MS analysis of the BcGH157 laminarin digest confirmed the presence of laminaribiose and laminaritriose and revealed the presence of some laminaritetraose but did not show the presence of any additional resolved disaccharide or trisaccharide peaks (Fig. 3c). Considering that both enzymes require a Lam2 motif at the negative subsites to hydrolyse the subsequent bond, this suggests that LaGH157 only requires occupancy across the –2 to +1 subsites while BcGH157 appears to require occupancy of the –2 to +2 subsites for activity on β -1,3-glucans. This reducing end exo-hydrolytic activity of LaGH157 is supported by the crystal of the LaGH157 in complex with laminaritetraose (Lam4) in which the Lam4 substrate is seen hydrolysed at the reducing end into Lam3 and glucose (*vide infra*). Interestingly, since the underlying linear structure of laminarin contains both β -1,6 and β -1,3 linkages [35], the near-complete hydrolysis of laminarin to glucose and laminaribiose by LaGH157 indicates that the enzyme has significant hydrolytic activity towards β -1,6 linkages. We speculate that the additional peaks observed in the HPAEC-PAD analysis of the BcGH157 laminarin digest are derived from oligosaccharides containing β -1,6 linkages.

The ligand promiscuity of LaGH157 is reminiscent of family GH131, which is exclusively comprised of fungal enzymes displaying a bifunctional exo- β -(1,3)/(1,6) and endo- β -1,4 activity towards β -glucans [36]. Nonetheless, GH131 are able to hydrolyse glycosidic bonds independently of the presence of an adjacent β -1,3 linkage, as shown by their ability to depolymerize β -1,4-linked substrates such as cellohexaose. Furthermore, GH131 enzymes are also able to fully hydrolyse β -1,6

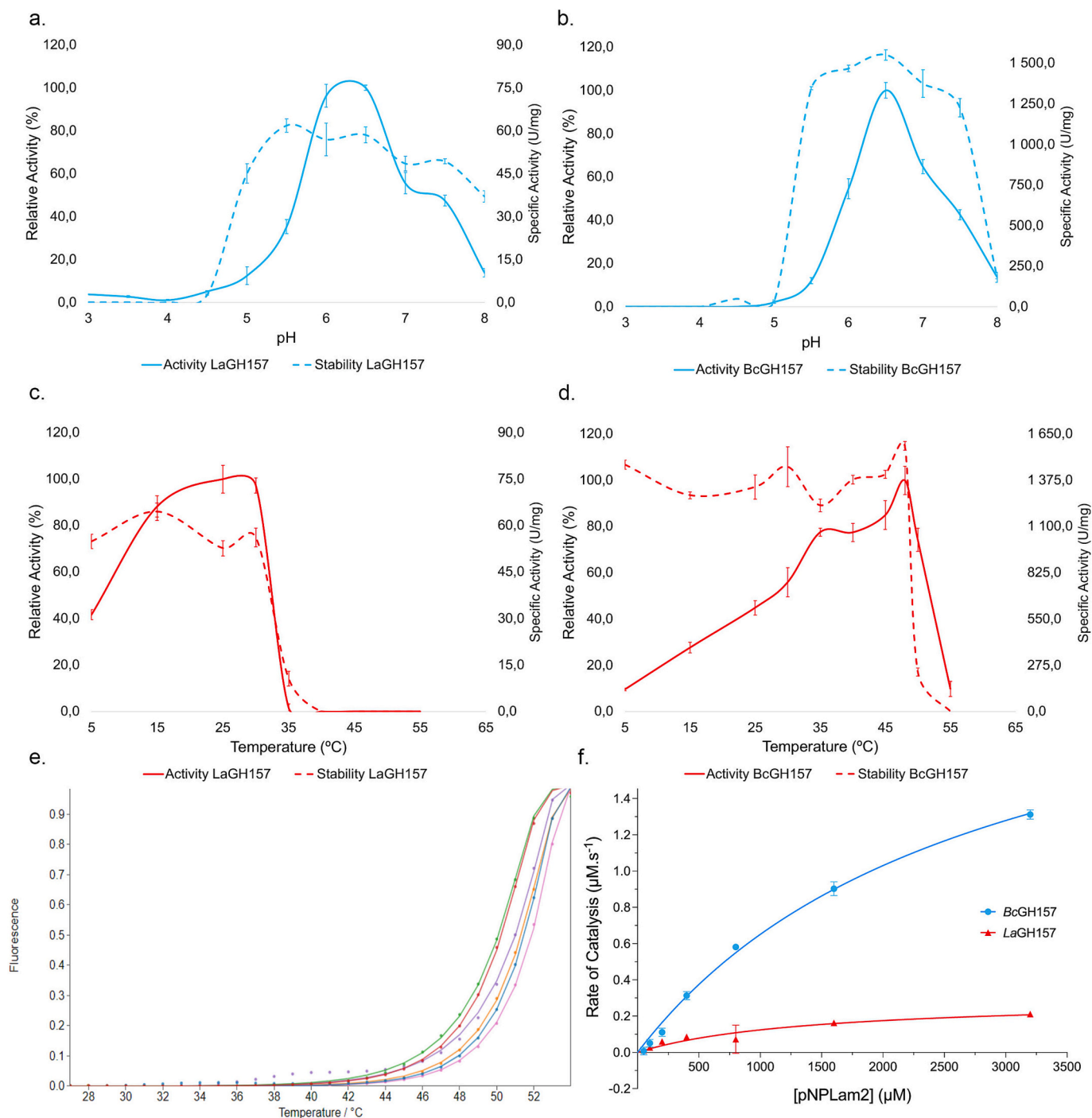


Fig. 1. GH157 enzymes' activity and stability. **a, b.** pH dependence of *LaGH157* (**a.**) and *BcGH157* (**b.**) activity using laminarin as substrate to monitor hydrolysis. Continuous blue lines show relative activity measured at each pH. The activity is shown as a percentage of the highest specific activity obtained. Dashed blue lines show the specific enzyme activity at pH 6.5 after preincubating the enzymes for 60 min at the several pH values. **c, d.** Temperature dependence of *LaGH157* (**c.**) and *BcGH157* (**d.**) activity using laminarin as substrate to monitor hydrolysis. Continuous red lines show relative activity measured at each temperature. The activity value is shown as a percentage of the highest specific activity obtained. Dashed lines show specific enzyme activity at pH 6.5 and 25 °C (**c.**) or 48 °C (**d.**), after preincubating the enzymes for 60 min at the several temperature values. **e.** Differential scanning fluorimetry curve of *LaGH157* (red, purple and brown) and *BcGH157* (blue, orange and green) at pH 5.0, 6.5 and 8.0, respectively. **f.** Michaelis–Menten plot of pNP-Laminaribiose hydrolysis by *LaGH157* and *BcGH157*. All experiments were performed in triplicate.

glucans to glucose, while the β -1,6 hydrolytic activity of *LaGH157* seems to be limited to branching sites on laminarin, as suggested by the absence of any detectable activity on the β -1,6 glucan pustulan (Table 1). This is suggestive of a more elaborate binding mode for *LaGH157*, likely due to an unexplored underlying specificity towards certain glycan motifs present on its natural substrate.

The relatively high MW_{app} of the CM-curdlan digests shows that the presence of the carboxymethyl group prevents complete digestion of the substrate. Due to the anionic nature of the carboxymethyl group, the incomplete digestion, and the lack of standards for carboxymethylated oligosaccharides, HPAEC-PAD analysis was not run on the CM-curdlan digests. However, LC-MS analysis of the CM-curdlan digests show that

Table 2Kinetic parameters of *LaGH157* and *BcGH157* on pNP-Lam2 and MLGs.

Enzyme	Substrate	K_m (mM)	V_{max} ($\mu\text{M}\cdot\text{s}^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1}\cdot\text{mM}^{-1}$)
<i>LaGH157</i>	pNP-Lam2	1.40 ± 0.650	0.298 ± 0.064	1.19	0.85
<i>BcGH157</i>	pNP-Lam2	2.79 ± 0.278	2.47 ± 0.142	9.9	3.54
<i>LaGH157_E132A</i>	pNP-Lam2	ND	ND	ND	ND
<i>LaGH157_E224A</i>	pNP-Lam2	ND	ND	ND	ND

Enzyme	Substrate	K_m (mg. $\cdot\text{mL}^{-1}$)	V_{max} ($\mu\text{M}\cdot\text{s}^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m (mg. $\cdot\text{s}^{-1}\cdot\text{mL}^{-1}$)
<i>LaGH157</i>	bMLG	1.55 ± 0.419	0.119 ± 0.012	0.072	0.046
<i>LaGH157</i>	Lich	1.03 ± 0.357	0.146 ± 0.022	0.089	0.087
<i>BcGH157</i>	bMLG	4.4 ± 2.452	0.103 ± 0.036	0.62	0.14
<i>BcGH157</i>	Lich	1.92 ± 0.761	0.166 ± 0.019	1.00	0.52

ND: not detected. bMLG: barley β -glucan. Lich: Lichenan.Kinetic parameters are reported as fitted values \pm standard deviation from triplicate experiments.

BcGH157 and *LaGH157* have differing tolerances towards carboxymethyl groups (Supplemental Fig. 2), revealing the presence of laminaribiose, and little else, in the *BcGH157* digest (Supplemental Fig. 2a). In contrast, LC-MS analysis of the *LaGH157* digest of CM-curdlan showed both laminaribiose and laminaritriose as well as an abundance

of carboxymethylated oligosaccharides, including Glc₃, Glc₄ and Glc₅ peaks (Supplemental Fig. 2b). The presence of significant quantities of *LaGH157* carboxymethylated Glc₃ and Glc₄ oligosaccharides without carboxymethylated Glc₂ shows that *LaGH157* excludes carboxymethyl groups from the -2 to $+2$ subsites. The dominance of laminaribiose in the *BcGH157* CM-curdlan digest shows much less tolerance of carboxymethyl groups along a longer active site. We speculate that the laminaribiose generated by *BcGH157* has been excised from the longest stretches of unmodified β -1,3-glucan.

2.5. Overall structure analysis of *LaGH157*

To understand the molecular determinants supporting the proposed catalytic mechanism of family 157 glycoside hydrolases, the structure of *LaGH157* was determined free and in complex with Lam2 and Lam4. Due to a limited access to AI assisted structure prediction tools at the time of crystallization and data collection, the absence of closely related structures required the use of a selenium single-wavelength anomalous diffraction (Se-SAD) approach for phasing. As such, a selenomethionine (SeMet) derivative was produced and crystallized and diffraction data was collected at the selenium X-ray absorption edge wavelength. The resulting refined structure was then used as a model to solve the complex with Lam2 and Lam4 using molecular replacement. After refinement, all datasets resulted in good quality $2\text{mF}_o\text{-DF}_c$ maps, with clearly visible amino acid side chains and ligands in the difference maps. Data collection and refinement parameters and statistics are given in Table 3.

The asymmetric unit is comprised of four copies of the protein, each with three distinct domains, which appear to be related by non-crystallographic symmetry. The resulting tetramer will be further

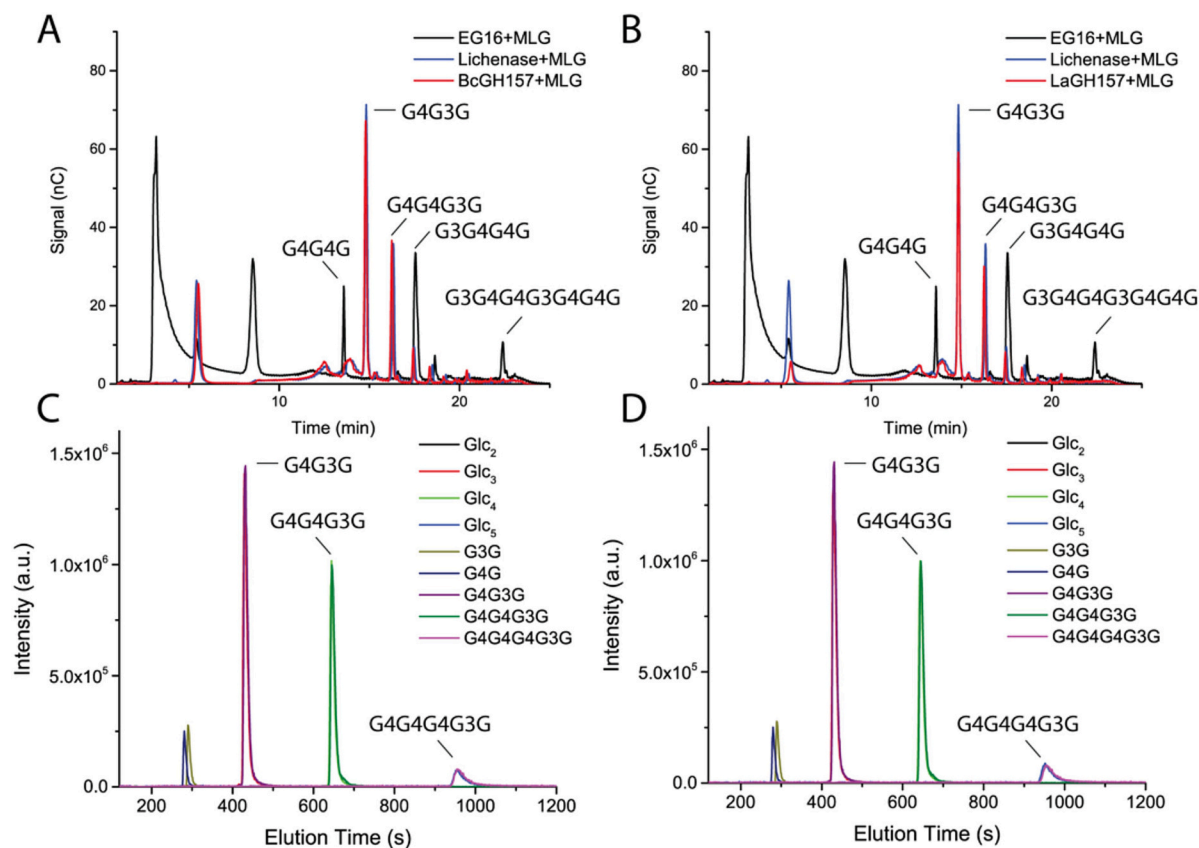


Fig. 2. Analysis of GH157 limit digests of MLG. A) HPAEC-PAD trace for the digest of MLG by *BcGH157* superimposed on the EG16 and lichenase MLG digests. B) HPAEC-PAD trace for the digest of MLG by *LaGH157* superimposed on the EG16 and lichenase MLG digests. LC-MS analysis of the *BcGH157* and *LaGH157* MLG digests are shown in (C) and (D) respectively. Each differently coloured trace corresponds to an extracted ion chromatogram. Representative MS1 compound spectra for the observed peaks can be found in fig. S3. Peaks matching carbohydrate standards are annotated. Where purified standards were not available, peaks matching those observed in the EG16 or lichenase digests are annotated based on previously reported products from these digests.

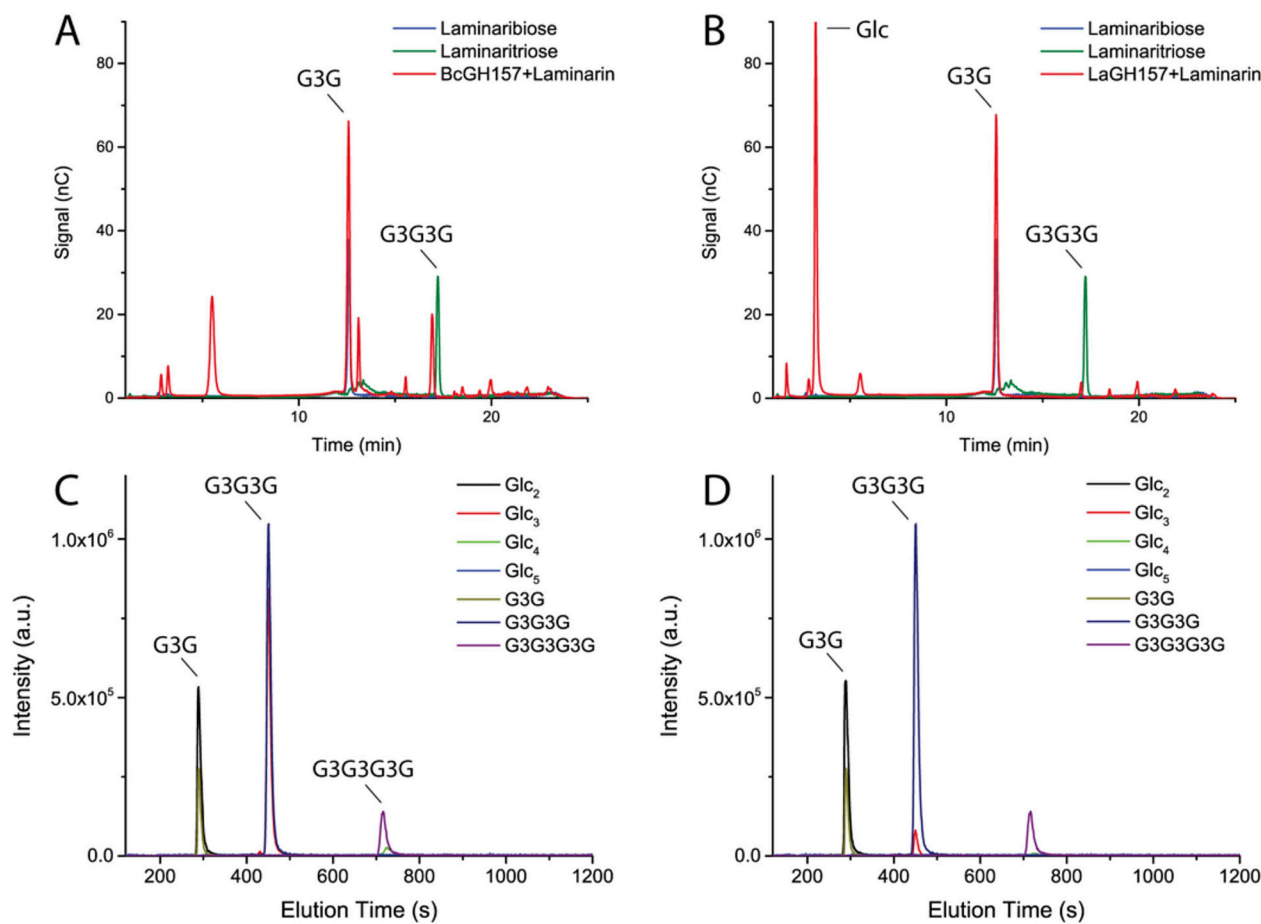


Fig. 3. Analysis of GH157 limit digests of laminarin. A) HPAEC-PAD trace for the digest of laminarin by BcGH157 superimposed on the laminaribiose and laminaritriose standard traces. B) HPAEC-PAD trace for the digest of laminarin by LaGH157 superimposed on the laminaribiose and laminaritriose standard traces. LC-MS analysis of the BcGH157 and LaGH156 laminarin digests are shown in (C) and (D) respectively. Each differently coloured trace corresponds to an extracted ion chromatogram. Representative MS¹ compound spectra for the observed peaks can be found in supplemental fig. 4. Carbohydrate standard peaks are annotated based on known laminarin oligosaccharide standards.

discussed below, in light of the interface analysis and SEC-MALLS results. The catalytic domain (domain 1 - residues 3–319) adopts a (β/α)8-barrel fold, while the middle domain (domain 2 - residues 320–407) and C-terminal domain (domain 3 - residues 415–529) consist of two seven-stranded β -sandwiches (Fig. 4). A particularly extensive loop is formed by residues 267–309, connecting β -strand 8 to α -helix 8, which, together with the loops connecting β -strand 1 to α -helix 1 (residues 27–47) and β -strand 7 to α -helix 7 (residues 224–242) form most of the substrate binding groove. The remaining wall of the substrate cleft is defined by β -strands 3 and 4, and a loop from the C-terminal β -sandwich domain. The two β -sandwich domains are very similar in their overall conformation, being comprised of seven antiparallel β -strands organized into two β -sheets, with one sheet formed by strands 1, 2, and 5 and the other by strands 3, 4, 6 and 7. Both domains make extensive contacts with the catalytic domain, contributing for structural stabilization. Interestingly, the AlphaFold 3 model of LaGH157 shows remarkable similarity to the experimental model, with both aligning with an r.m.s.d. of 0.535 Å over 526 aligned Ca atoms (Supplemental Fig. 4). More importantly, there is a near perfect matching of the substrate binding residues, with only small variations on the side-chains, demonstrating the potential of current generation AI assisted protein prediction.

Even though all obtained crystal structures displayed four copies of LaGH157 in the asymmetric unit, they do not all share the same non-crystallographic symmetry pattern. An assembly analysis on the LaGH157-Lam2 complex with PISA calculated that the larger interaction surfaces occur between chains C and B, with an interaction surface area

of 1246.9 Å² and a predicted solvation energy (SE) gain (ΔG) of -7.9 kcal/mol, and between chains D and A, with an interaction surface area of 1203.4 Å² and a predicted SE gain (ΔG) of -5.8 kcal/mol. The interaction between chains B and D, and A and C have much smaller interaction surfaces (around 400 Å²) and less favourable SE gains (around -3.5 kcal/mol). This suggests that the natural biological assembly of LaGH157 is the homodimer formed by chains B:D and A:C, and that the tetrameric arrangement in the asymmetric unit is a dimer of dimers. Curiously, despite the different tetrameric arrangements, the dimer suggested by PISA as the natural biological assembly is present in all obtained crystal structures of LaGH157 with similar interaction parameters, except in the nucleophile mutant + Lam4 complex, suggesting that either the single mutation or substrate binding may cause the dimer to dissociate. Surprisingly, a SEC-MALLS analysis of the wild-type protein revealed that the enzyme exists primarily as a tetramer in solution. Two peaks were eluted from the size-exclusion column, with the dominant peak having an estimated MW of 226 kDa, which is approximately the size of a tetramer, and the smaller, late-eluting peak having an estimated MW of 66 kDa, which corresponds to a monomer (Supplemental Fig. 5). The continuous variation in apparent MW between the two peaks as well as the tailing of the main peak are indicative of slow dissociation of the tetramer during the SEC run, likely due to a weak intermolecular association with a slow k_{off} value.

A structural similarity search on the DALI server [37] using the catalytic domain of LaGH157 returned members of clan GH-A as structural homologues, the top two being *FsGUS* and *EtGalAse* from glycoside

Table 3

X-ray crystallography parameters and validation statistics for data collection, 3D structure solution and refinement. Values for the high-resolution shell are shown in parentheses.

	LaGH157 SeMet	LaGH157-Lam2	LaGH157E224-Lam4
Data collection	ESRF ID30A-1	ESRF ID30A-3	ESRF ID30A-3
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>H</i> 3	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	103.15, 112.58, 251.46	208.63, 208.63, 165.29	114.85, 127.55, 171.41
α , β , γ (°)	90, 90, 90	90, 90, 120	90, 90, 90
Resolution (Å)	49.41–2.44 (2.47–2.44)	40.65–2.71 (2.75–2.71)	42.68–2.32 (2.35–2.32)
<i>R</i> _{merge}	0.126 (0.967)	0.166 (1.726)	0.185 (2.084)
<i>R</i> _{pim}	0.038 (0.474)	0.074 (0.860)	0.067 (0.731)
<i>CC</i> _{1/2}	0.998 (0.515)	0.995 (0.375)	0.992 (0.498)
<i>I</i> / σ <i>I</i>	12.43 (1.16)	9.6 (0.8)	7.43 (0.68)
Completeness (%)	93.43 (57.10)	98.40 (71.81)	99.82 (99.89)
Refinement			
No. reflections	1,105,108 (9573)	71,478 (2023)	929,727 (32124)
<i>R</i> _{work}	0.249 (0.353)	0.197 (0.314)	0.186 (0.452)
<i>R</i> _{free}	0.262 (0.376)	0.260 (0.356)	0.228 (0.425)
No. atoms	17,407	17,324	18,190
Protein	16,993	17,000	16,959
Ligands	37	135	262
Water	377	189	969
<i>B</i> -factors	57.73		
Protein	58.14	85.41	36.94
Ligands	53.90	77.14	41.91
Water	39.39	69.32	31.85
R.m.s deviations			
Bond lengths (Å)	0.010	0.010	0.011
Bond angles (°)	1.50	1.77	1.76
Ramachandran plot residues			
In most favourable regions (%)	97.34	93.63	96.67
In allowed regions (%)	2.19	5.66	3.00
PDB code	9FZ9	9G5G	9G4N

hydrolase family 2. *FsGUS* (PDB code 6NCY) is an hexameric hybrid β -glucuronidase/ β -galacturonidase (GUS/GalAse) from *Fusicatenibacter saccharivorans*, presenting 18 % identity, a Z score of 30.7 and RMSD of 2.3 Å over 276 aligned residues with LaGH157, while *EtGalAse* is a tetrameric β -galacturonidase (GalAse) from *Eisenbergiella tayi* (PDB code 6NCX) presenting 19 % identity, a Z score of 30.5 and RMSD of 2.5 Å over 276 aligned residues with LaGH157 [38]. The two homologues are sugar scavenging hexuronidases from family GH2 secreted by human gut bacteria, that cleave the sugar conjugates of the epimers glucuronate and galacturonate. Beyond the general fold, there is also some conservation of the active site residues between LaGH157 and the GH2 enzymes, which assisted in the identification of LaGH157's catalytic residues (*vide infra*). Nevertheless, the overall conformation of their active sites is substantially different, with the hexuronidases having a much smaller exo-like substrate binding groove that does not allow accommodation of any extra sugars at the non-reducing end of the uronic acid moiety, while the GH157 has a wide binding cleft which is able to accommodate a larger portion of the polysaccharidic substrate, allowing for an endo mode of catalysis.

LaGH157's domain 2 shows distant homology to the third domain of *Vibrio cholerae*'s GbpA protein (PDB code 2xwx, Z = 9.9, RMSD = 2.2 Å and 10 % identity over 84 aligned residues) [39]. GbpA is a four domain *N*-acetylglucosamine binding protein used by *V. cholerae* to interact with the intestinal epithelium and chitinous exoskeleton of its hosts and domain 3 seems to be responsible for binding to the *V. cholerae* bacterial surface. The C-terminal domain of LaGH157 shows distant homology to a fibronectin type III (FnIII) fold domain of the RbmA protein, also expressed by *V. cholerae* (PDB code 2xwx, Z = 10.1, RMSD = 3.4 Å and

11 % identity over 107 aligned residues) [40]. FnIII domains have been described in many cell surface receptors and cell adhesion proteins and are known to mediate protein-protein interactions [41] as well as to provide resistance to mechanical tension through elasticity and recoil [42]. To probe for possible CBM activity on both β -sandwich domains, a β -glucan affinity gel electrophoresis screen was performed. No binding was detected to any of the tested polysaccharides (laminarin, barley β -glucan and lichenan) with any of the accessory domains (Supplemental Fig. 6). These results, together with the homology analysis, are highly suggestive that the modules do not possess a CBM-like function. It is possible that the two accessory domains exist to provide structural stability to the enzyme, contributing to proper folding, while domain 3 also seems to be essential for catalysis, as it folds onto the substrate binding cleft and participates in the formation of the +1 subsite, contributing to substrate recognition at the active site, as discussed below.

2.6. Active site and complexes with ligands

To identify the substrate binding site and catalytic centre, a multiple sequence alignment was performed using the top LaGH157 homologue sequences obtained with the Basic Local Alignment Search Tool (BLAST) (Supplemental Fig. 7). The resulting alignment was used to colour a model of LaGH157 according to sequence conservation across homologues, which allowed identification of the substrate binding groove (Fig. 5a, b). By performing a structural comparison of LaGH157 with its closest structural homologue, *FsGUS*, residues Glu132 and Glu224 were identified as the catalytic acid/base and nucleophile, respectively (Fig. 5c, d). This was then confirmed by site-directed mutagenesis, swapping either Glu132 or Glu224 with an alanine. Both resulting mutants (LaGH157_E132A and LaGH157_E224A) showed complete loss of detectable catalytic activity on all tested substrates (pNPLam2, laminarin, barley β -glucan and lichenan), confirming the two glutamates as the essential catalytic residues (Tables 1 and 2). This catalytic dyad, where the acid/base and nucleophile are present on loops following β -strands 4 and 7 of the versatile (β/α)₈-barrel fold is characteristic of clan GH-A members. Additionally, the distance between Glu132 and Glu224 is approximately 5 Å both in the unbound and oligosaccharide bound LaGH157 structures, which is consistent with the average distance between the two catalytic residues in retaining glycoside hydrolases, the characteristic catalytic mechanism of clan GH-A [43]. A phylogenetic analysis with β -glucanases from different families further confirms GH157 as part of the GH-A clan by placing its members in the same clad as GH17, GH5 and GH158 proteins, all of which belong to the GH-A clan (Supplemental Fig. 8). GH-A is the largest known GH clan and, in addition to β -glucosidases, is comprised by enzymes that catalyse the hydrolysis of a variety of equatorially linked substrates such as β -D-galactosides and β -D-mannosides [43]. Interestingly, the topology of the active sites in the immediate vicinity of the cleavage site is conserved across all clan members, with the two catalytic glutamates in structurally identical positions [44].

To define the active site residues and their interaction with the substrate, the structure of LaGH157 in complex with Lam2 was determined. The structure revealed one unit of Lam2 bound to the predicted catalytic site of LaGH157. The β -1,3 linked disaccharide occupies the –1 and –2 subsites and is coordinated by a complex network of 12 direct hydrogen bonds and several hydrophobic interactions (Fig. 6). At the –1 subsite it is possible to identify the catalytic pair Glu132/Glu224 strategically adjacent to the anomeric carbon of the –1 glucose. Immediately preceding the acid/base residue a conserved asparagine (Asn131) is also establishing an important H-bond with the hydroxyl group at the C2 position, while the C4 hydroxyl is held by glutamate Glu289 and Lys276. At the –2 subsite residues Glu29, Tyr39, Tyr269 and Lys276 are also seen establishing six more H-bonds with the non-reducing end glucose unit of Lam2.

In one unit of the tetramer, a glucose molecule, likely originating

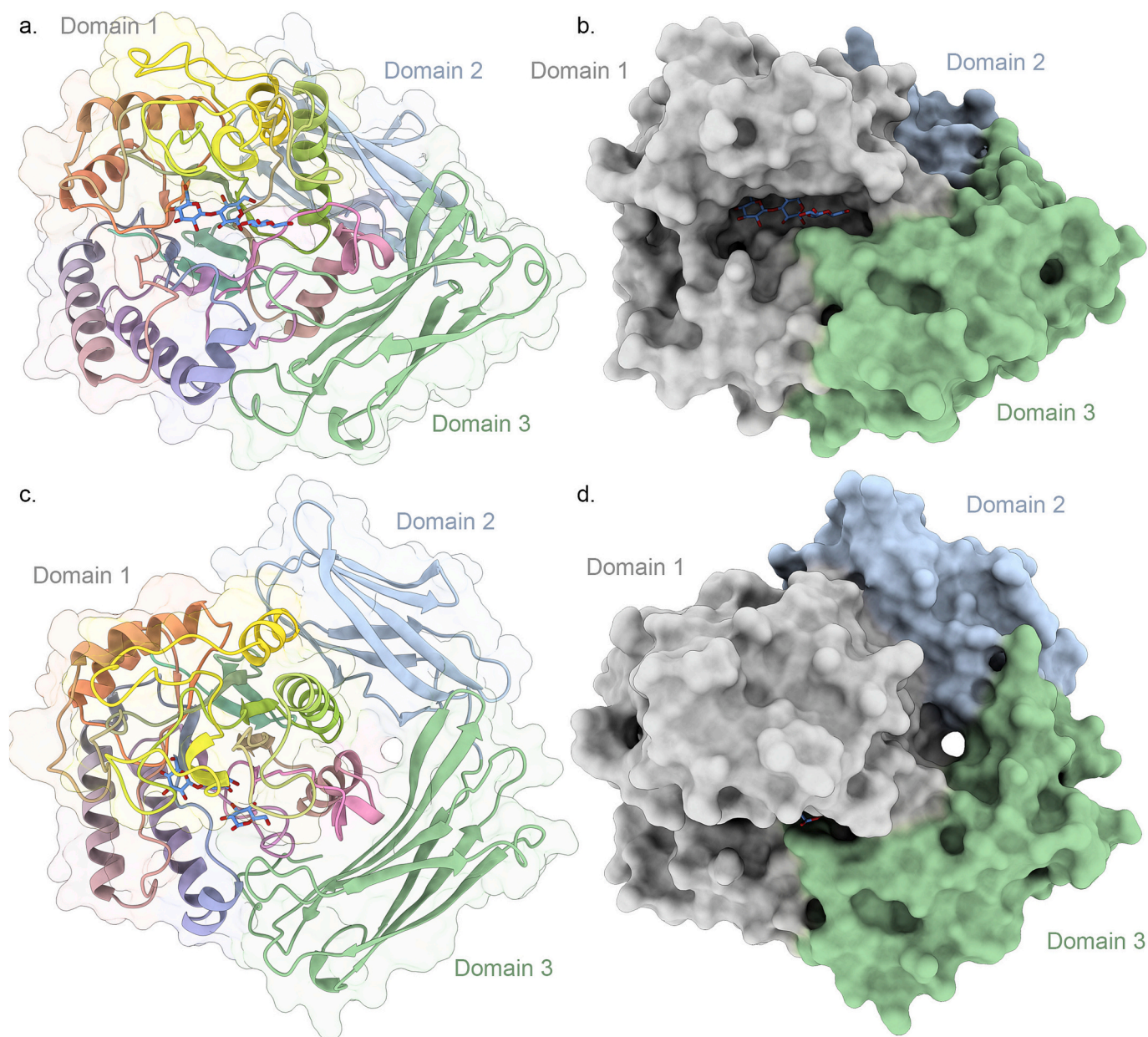


Fig. 4. The 3D structure of *LaGH157*. **a, c.** Ribbon representation of the structure of *LaGH157* with the catalytic domain coloured in rainbow (N-terminus in blue, C-terminus in red) and domains 2 and 3 coloured blue and green, respectively. It is possible to observe the $(\beta/\alpha)_8$ -barrel fold of the catalytic module and the seven stranded β -sandwich structure of domains 2 and 3. **b, d.** Van der Waals' surface of a *LaGH157* monomer with the catalytic module coloured grey and modules 2 and 3 coloured blue and green, respectively. Blue coloured laminaribiose and glucose molecules in stick representation can be seen bound in the active centre.

from substrate contamination, can be modelled adjacent to the -1 subsite, held in place by two aglycone-binding aromatic residues, namely Tyr238 and Trp460, possibly defining the $+1$ subsite. Interestingly, Trp460 belongs to domain 3, which seems to participate in the catalytic mechanism by contributing to hold the aglycone leaving group in the active site. An overlay with a GH128 *endo*- β -1,3-glucanase in complex with laminaripentaose (PDB 6UAS) further suggests the Tyr238/Trp460 clamp as the likely $+1$ subsite (Supplemental Fig. 9) [45]. To evaluate the contribution of domains 2 and 3 for the catalytic activity of *LaGH157*, two truncated versions were designed, one with the isolated catalytic module (*LaGH157*₁₋₃₁₉) and another missing domain 3 (*LaGH157*₁₋₄₀₇). Their catalytic ability was tested by overnight incubations with laminarin, barley β -glucan and lichenan. No detectable activity was observed with any of the truncated forms. Additionally, *LaGH157*₁₋₃₁₉ also displayed reduced solubility. This confirms that the

accessory domains are contributing to protein stability and that domain 3 is essential for substrate hydrolysis by *LaGH157* by positioning the leaving aglycone at the $+1$ subsite. Although accessory modules on CAZymes are generally structurally independent from the associated catalytic domain, they can sometimes directly contribute to substrate binding at the active site, as observed here. Some notable examples are the C-terminal CBM46 from *Bacillus halodurans* Cel5B endoglucanase, which potentiates the enzyme's activity against purified β -1,3-1,4-glucans by defining an extra positive subsite [46], and the CBM3c from *Thermomonospora fusca*, which extends the catalytic site of the associated GH9 catalytic domain [47]. The clamp-like $+1$ subsite is probably an essential feature for the ligand promiscuity displayed by *LaGH157*, much like in the *Candida albicans* exo- β -1,3-glucanase derived glycosynthase, whose phenylalanine clamp allows several different sugar positions and orientations, including a 180° flip, which in turn permits

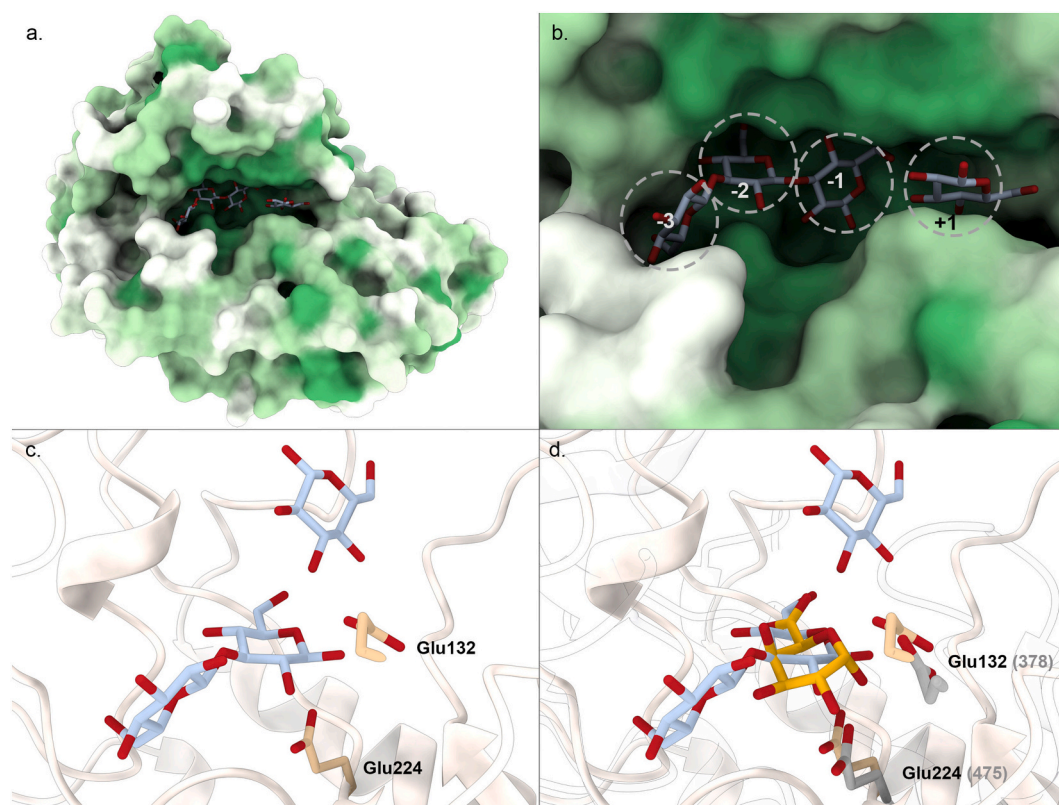


Fig. 5. *LaGH157* homology and binding subsites. **a.** Birdseye view of an *LaGH157* dimer with the Van der Waals' surface coloured according to sequence conservation, calculated by aligning sequences (ClustalOmega) with the closest primary structure homologues found in GenBank using the BLASTP tool. The colour pattern matches the alignment used to render the structure (Supplemental Fig. 7), with darker shades of green corresponding to full conservation and white to no conservation. **b.** Detailed view of the catalytic site of *LaGH157* with -3 to $+1$ binding sub-sites highlighted with dashed circles. **c, d.** Overlay of *LaGH157* with *EtGalAse* (PDB code 6NCY, in grey) highlighting the two catalytic residues (Glu132 and Glu224).

the formation of either β -1,3, β -1,4 or β -1,6 linkages (PDB 4M82) [48].

Despite extensive co-crystallization and soaking efforts with laminaritriose (Lam3), – tetraose (Lam4), – pentaose (Lam5), – hexaose (Lam6), and MLG oligosaccharides, it was not possible to obtain a Michaelis-complex structure with any of the catalytically impaired mutants (*LaGH157_E132A*, *LaGH157_E224A*, and a double mutant variant). However, co-crystallization of the nucleophile mutant (*LaGH157_E224*) with Lam4 resulted in a good-quality structure, with the ligand clearly defined by the electron density maps, identified as hydrolysed into laminaritriose and glucose. The electron density around the glucose was poor, likely due to low occupancy, which hampered the positioning of the $+1$ sugar. Nonetheless, attempts to model water molecules as well as other buffer components within this density led to unsatisfactory results during refinement. The best outcome was obtained by lowering the glucose occupancy to 75 %. Substrate hydrolysis could have resulted from residual catalytic activity where an activated water replaced the mutated nucleophilic residue and attacked the anomeric carbon, or from translation misincorporation leading to trace amounts of protein with the original amino acid. These phenomena could have been facilitated by the long incubation period and high substrate concentration used for co-crystallization. This complex allowed the identification of the -3 subsite where the extra glucose is stabilized by residues Tyr39 and Asp91 through hydrogen bonding and stacking interactions (Fig. 6b, c). The laminaritriose is seen occupying subsites -3 to -1 , while the glucose molecule is held at the $+1$ subsite by the Tyr238/Trp460 clamp, which is consistent with the reducing end exohydrolytic activity suggested by the product analysis.

2.7. Structure of *BcGH157*

The AlphaFold 3 predicted structure of *BcGH157* (<https://alphafoldserver.com/>) revealed a very similar conformation to that of *LaGH157*. It is composed of a $(\beta/\alpha)_8$ -barrel fold catalytic domain and two seven stranded β -sandwich domains, with the C-terminal domain folding onto the predicted active site. The constellation of substrate-binding residues between the -2 and $+1$ subsites, including the two catalytic residues, is completely conserved between the two GH157 enzymes. Nonetheless, the overall shape of the substrate binding groove shows notable differences. In *LaGH157*, the binding groove curves towards the -3 subsite, following the natural curvature of the bound β -1,3 oligosaccharide (Fig. 7a, d). On the other hand, in *BcGH157* the -3 site defining Tyr39 and Asp91 are replaced by an asparagine (Asn41) and a tryptophan (Trp91), respectively. The Trp residue, which is reminiscent of the characteristic “hydrophobic knuckle” at the -3 subsite of family GH128 β -glucanases [45], “pushes” the ligand cleft up, and the Tyr to Asp substitution opens up a pocket, resulting in a more linear groove (Fig. 7b, e). This could mean that *LaGH157* favours β -1,3 over β -1,4 linkages between the -3 and -2 subsites, while *BcGH157* favours β -1,4 over β -1,3, which would explain the more limited digestion of laminarin by *BcGH157* and its higher catalytic efficiency on MLGs with a high β -1,4 linkage content. For *BcGH157* to be able to accommodate a β -1,3 linked glucose at the -3 subsite it would have to function in a substrate flattening binding mode, similarly to the GH128 *endo*- β -1,3 glucanase of *Lentinula edodes* (*LeGH128_IV*), in which the ψ angle of the β -1,3-linkage between the glucosides -2 and -3 rotates 180° to adopt an unusual but stereochemically favourable flattened conformation [45]. Unfortunately, despite several attempts to crystallize *BcGH157* in the presence of MLG and β -1,3 oligosaccharides to further explore this hypothesis, no

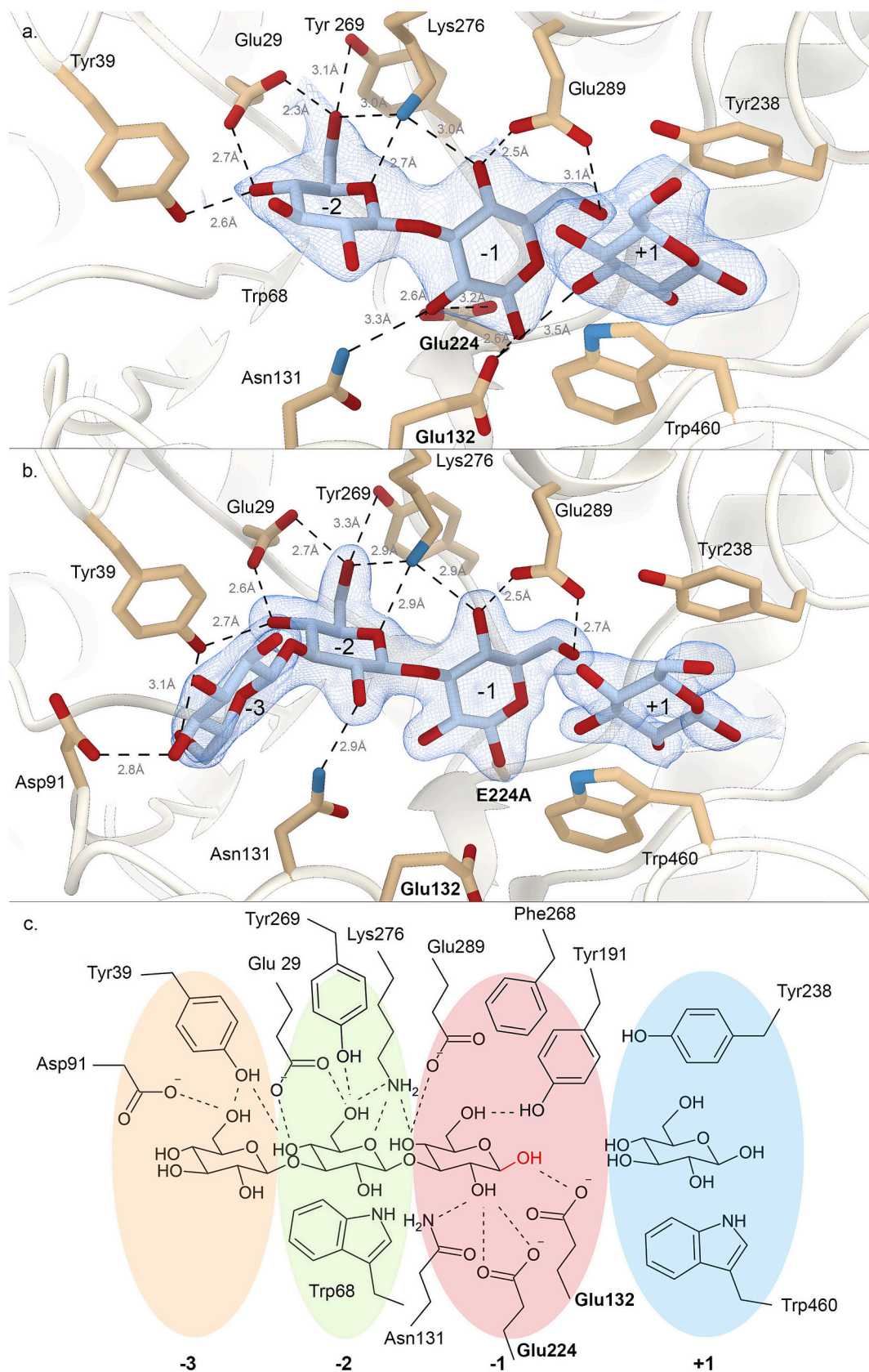


Fig. 6. *LaGH157* binding site – substrate interaction. **a, b.** Detailed view of the crystallized *LaGH157*–Lam2 and *LaGH157E224A*–Lam4 complexes within the active centre. The enzyme residues making hydrogen-bond contacts (black dashed lines) with the ligands are displayed in stick representation. The ligands are coloured blue and surrounded by a mesh representation of the Refmac5 maximum-likelihood σ_A -weighted $2F_o - F_c$ electron density map contoured at 1.5 σ . **c.** Schematic representation of the Lam3-Glu ligand bound to *LaGH157* active centre, showing all the hydrogen bond interactions between both molecules. Binding sub-sites are coloured yellow (–3), green (–2), red (–1), and blue (+1).

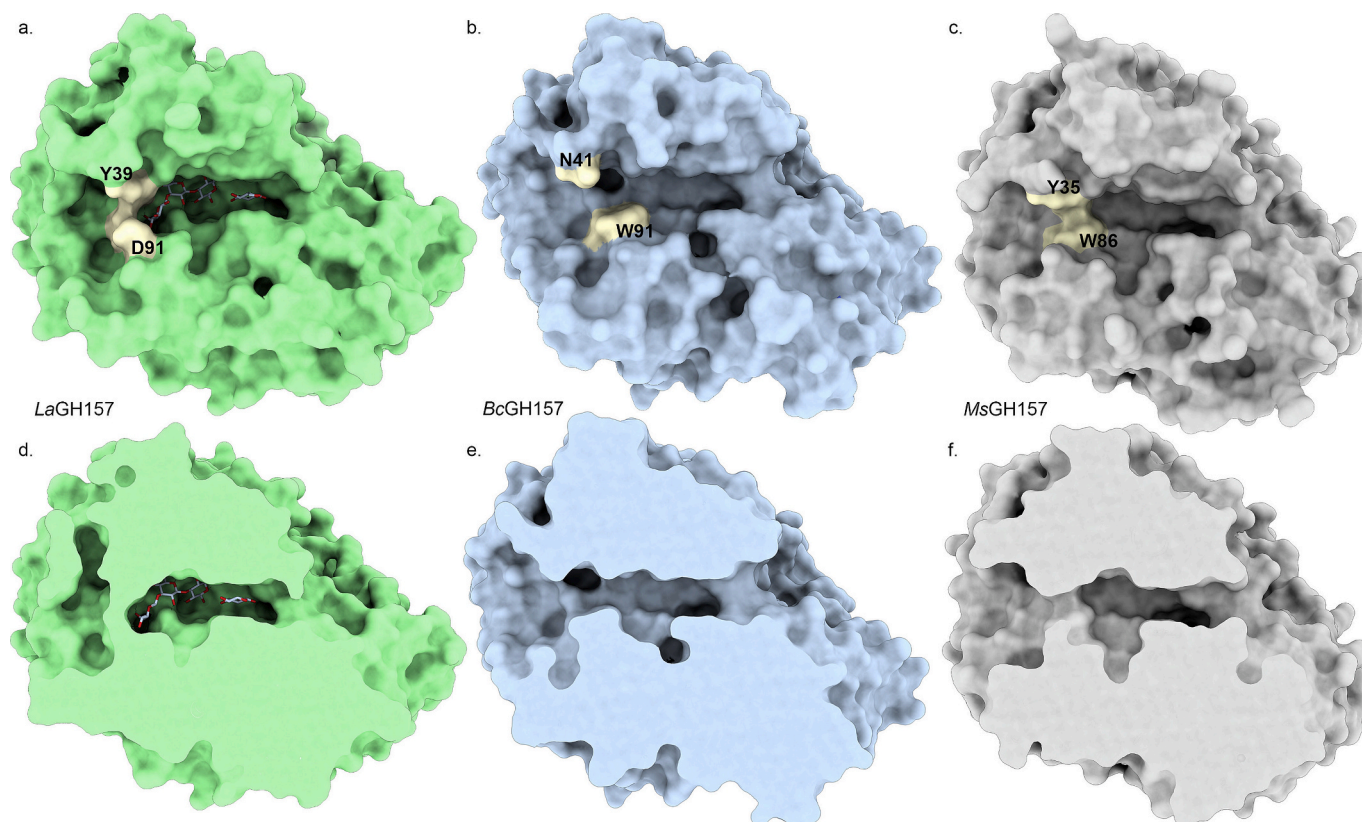


Fig. 7. Topology of family GH157 binding cleft. **a, b, c.** Van der Waals surface of *LaGH157* (green), *BcGH157* (blue) and *MsGH157* (grey) with the major differences at the -3 subsite highlighted in yellow. **d, e, f.** Capped structure highlighting the shape of the substrate binding cleft of the same enzymes. *BcGH157* and *MsGH157* 3D structures were predicted using the AlphaFold 3 server.

crystals have been obtained. This structural divergence at the -3 subsite is also observed across all GH157 members, dividing the family into groups: those, like *LaGH157*, with the aspartate (or glutamate) and tyrosine pair (Tyr39/Asp91) that curves the binding cleft outwards, and those, like *BcGH157*, with the asparagine and tryptophan pair (Asn41/Trp91), which form a more linear binding site. GH157 enzymes from the genus *Mesoterricola* seem to be an exception, having both the Tyr and Trp residues, that leads to an intermediate conformation in which the binding groove is somewhat linear but shallower than in *BcGH157* (Fig. 7c, f). *BcGH157*'s binding cleft also appears to extend further towards the positive subsites than *LaGH157*. This is consistent with the product analysis data suggesting that *BcGH157* requires occupation of subsites -2 to $+2$ for substrate hydrolysis, while *LaGH157* only requires binding between -2 and $+1$, supporting its reducing end exohydrolytic activity.

3. Conclusion

In summary, family GH157 is comprised of β -glucan depolymerizing enzymes supported by the versatile $(\beta/\alpha)_8$ -barrel fold and the pair of glutamic acid residues that act as the acid/base catalyst and nucleophile, characteristic of clan GH-A. Analyses of the limit digests of MLG and lichenan generated by *BcGH157* and *LaGH157* indicate exclusion of β -1,4 linkages between the -1 and -2 subsites, but not from the cleavage site or positive subsites. Analysis of the *BcGH157* laminarin digest shows that a minimum of 4 subsites must be occupied for hydrolysis to occur. In contrast, *LaGH157* only requires 3 subsites to be occupied for hydrolysis to occur, suggesting exo-hydrolytic activity in addition to its *endo*-hydrolytic mode of action. Furthermore, *LaGH157* also shows a broad linkage tolerance, accepting β -1,3, β -1,4, or β -1,6 linkages in the catalytic centre. The broader substrate tolerance of

LaGH157 extends to CM-curdlan, allowing it to cleave carboxymethylated oligosaccharides into fragments as small as Glc_3 . *LaGH157*'s ability to hydrolyse β -1,6 linkages in laminarin means that, due to their branched nature, the β -1,6 linkages must only occupy the -1 subsite. The non-reducing-end exo-hydrolytic activity suggests that *LaGH157* possesses an alternative mode of binding where it forgoes occupancy of the -2 subsite for the hydrolysis of β -1,6 linkages. *LaGH157*'s ligand promiscuity might be explained by an underlying specificity towards certain glycan motifs present on the enzyme's natural substrate, which remains to be explored. Nonetheless, this bifunctional endo- β 1,3(4)/exo- β (1,3)/(1,6) activity is unusual among bacterial glycoside hydrolases and constitutes an expansion on current knowledge about existing natural strategies for β -glucan deconstruction, which, given the significant biological relevance of these polymers, can inspire future efforts to understand β -glucan biology and develop new biotechnological solutions.

4. Materials and methods

4.1. Bacterial strains and substrates

Escherichia coli strains DH5 α and BL21 (DE3) were respectively used for gene cloning and expression. The expression vector pET29b (Sigma-Aldrich, USA) was used for both cloning and expression.

4-O-methyl-D-glucurono-D-xylan, birchwood xylan, laminarin (from *Laminaria digitata*), lichenan, xylan, and cellobiose (G4G) were purchased from Sigma-Aldrich (USA). Medium-viscosity barley β -glucan, yeast β -glucan, carboxymethyl cellulose, carboxymethyl curdlan, carboxymethyl pachyman, guar galactomannan, polygalacturonic acid, pullulan, rye flour arabinoxylan, xyloglucan, laminaribiose (G3G), laminaritriose (G3G3G), laminaritetraose (G3G3G3G),

laminaripentaose (G3G3G3G3G), laminarihexaose (G3G3G3G3G3G), 3¹-β-D-cellobiosyl-glucose, and cellotriose were purchased from NEOGEN/Megazyme International (Ireland). Hydroxyethyl cellulose was procured from Honeywell Fluka™ (Swiss). Pustulan was procured from Calbiochem® (USA).

4.2. Gene synthesis and cloning

The protein sequences from the putative uncharacterized family GH157 enzymes *LaGH157* (Accession Number WP_096433028) and *BcGH157* (Accession Number ALJ59177) were retrieved from the CAZY database (<http://www.cazy.org/>) [49] and the catalytic module boundaries were predicted by employing a combination of multiple sequence alignment with automated carbohydrate-active enzyme annotation using the dbCan server (<https://bcbl.unl.edu/dbCAN2/blast.php>) [50]. Signal peptides were predicted using the SignalP 5.0 server (<http://www.cbs.dtu.dk/services/SignalP/>) [51].

The genes encoding *LaGH157* and *BcGH157* were synthesized *in vitro* (NZYTech, Portugal) and codon optimised for expression in *E. coli*, using NZYTech's codon optimization software ATGenium. The recombinant primary sequence of each protein is shown in Supplemental Table 3. The synthetic genes were cloned into the pET29b vector (Sigma-Aldrich, USA), under the control of an inducible T7 promoter. The constructs contained a C-terminal His₆ tag, which allowed for recombinant protein purification through immobilized metal-ion affinity chromatography (IMAC). Successful cloning was confirmed by Sanger sequencing of the recombinant plasmids.

4.3. Generation of mutant derivatives of *LaGH157* by site-directed mutagenesis

The single-site mutants, *LaGH157_E132A* and *LaGH157_E224A* (Supplemental Table 3) were generated using site-directed mutagenesis with plasmid pET29b:*LaGH157* as a template. Site-directed mutagenesis was performed by PCR amplification using the NZYmutagenesis Kit (NZYTech, Portugal) and the primers presented in Supplemental Table 4. The incorporation of the desired mutation was confirmed by Sanger sequencing of the plasmid derivatives.

4.4. Generation of truncated versions of *LaGH157*

The synthetic genes encoding the truncated derivatives *LaGH157*_{320–407}, *LaGH157*_{415–529} and *LaGH157*_{320–529} were synthesized *in vitro* (IDT, USA). The truncated forms *LaGH157*_{1–319} and *LaGH157*_{1–407} were generated using PCR amplification with plasmid pET29b:*LaGH157* as a template and the primers presented in Supplemental Table 5. All derivatives were cloned into the pHTP1 vector (NZYTech, Portugal), under the control of an inducible T7 promoter. The constructs contained an His₆ tag at the N-terminus of the derivatives, which is required for recombinant protein purification by IMAC. Sequences of all recombinant plasmids were confirmed by Sanger sequencing. The recombinant primary sequence of each protein is shown in Supplemental Table 3.

4.5. Expression and purification of recombinant proteins

Competent *E. coli* strain *BL21 (DE3)* cells were transformed with the recombinant plasmids encoding *BcGH157* or *LaGH157*. The cells were used to inoculate Luria-Bertani (LB) media supplemented with kanamycin (50 µg/mL) and grown at 37 °C to an OD₆₀₀ of 0.4 to 0.6. Recombinant protein expression was induced by the addition of 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubation for a further 16 h at 19 °C. Cells were harvested by a 15 min centrifugation at 5000 xg at 4 °C and lysed by ultra-sonication in IMAC binding buffer (50 mM HEPES, pH 7.5, 10 mM imidazole, 1 M NaCl, 5 mM CaCl₂). The cell-free supernatant fluids were recovered by 30 min centrifugation at

15000 xg and filtered through 0.45 µm membrane. Then, the soluble fraction was loaded into a HisTrap nickel-charged Sepharose column (GE Healthcare, UK) and purified by IMAC in an AKTA Purifier fast protein liquid chromatography system (GE Healthcare, UK) using a 35 mM imidazole wash and a 35–300 mM imidazole elution gradient (in 50 mM HEPES, pH 7.5, 1 M NaCl, 5 mM CaCl₂). Protein fractions were buffer exchanged in PD-10 Sephadex G-25 M gel filtration columns (GE Healthcare, UK) into 50 mM HEPES, pH 7.5, containing 200 mM NaCl, 5 mM CaCl₂. The proteins were further purified by gel-filtration chromatography by loading the protein fractions onto a HiLoad 16/60 Superdex 75 (GE Healthcare, UK) at a flow rate of 1 mL min⁻¹. Purified samples were concentrated with an Amicon Ultra-15 centrifugal filter with a 10-kDa cutoff membrane (Millipore, USA) and washed three times with molecular biology grade water (Sigma, USA). The final concentrations for *BcGH157* and *LaGH157* were approximately 8 mg/mL. Each protein concentration was respectively estimated from a molar extinction coefficient (ε) of 99,950 and 97,305 M⁻¹ cm⁻¹ using a NanoDrop One Microvolume UV-Vis spectrophotometer (ThermoFisher Scientific, USA). Protein fractions were analysed by 12 % (w/v) SDS-PAGE to determine their molecular mass and purity.

A seleno-methionine *LaGH157* derivative was expressed in the methionine auxotroph B834 strain of *E. coli*, using the growth conditions described by Ramakrishnan et al. 1993 [52]. Protein purification was conducted as described above, except that a reducing agent was added to all the buffers: 5 mM of 2-mercaptoethanol in affinity-chromatography buffers, 5 mM DTT in gel-filtration chromatography buffer and 1 mM TCEP in storage buffer. The final concentration of the seleno-methionine *LaGH157* derivative was 10 mg/mL.

4.6. Substrate specificity

The substrate specificity of *LaGH157* and *BcGH157* was evaluated against various polysaccharides including 4-O-methyl-D-glucurono-D-xylan, birchwood xylan, laminarin (from *Laminaria digitata*), lichenan, xylan, medium viscosity barley β-glucan, yeast β-glucan, carboxymethyl cellulose, carboxymethyl curdlan, carboxymethyl pachyman, guar galactomannan, polygalacturonic acid, pullulan, rye flour arabinoxylan, xyloglucan, hydroxyethyl cellulose and pustulan. Assays were performed by incubation at 25 °C for 10 min in citrate/phosphate buffer pH 6.5, containing 0.1 % (w/v) bovine serum albumin (BSA). The 100 µL reaction mixtures contained 1 % (w/v) substrate and 10 µL purified *LaGH157* enzyme (0.1 mg/mL) or *BcGH157* enzyme (0.01 mg/mL). The specific activity was determined by measuring the reducing sugar released in the mixture by using the dinitrosalicylic acid method [53,54]. All assays were performed in triplicate. Blank experiments without enzyme were performed and background controls were subtracted. Glucose (0 to 11 mM) was used to generate a standard curve for quantitation.

The activities of the mutant derivatives, *LaGH157_E132A* and *LaGH157_E224A* on laminarin, lichenan and medium viscosity barley β-glucan were also determined as described above, using both a 10 min and overnight incubation at 25 °C.

4.7. Biochemical characterization of *LaGH157* and *BcGH157*

The optimal pH for *LaGH157* and *BcGH157* activities was determined by incubating 0.1 mg/mL *LaGH157* or 0.01 mg/mL *BcGH157* with 1 % (w/v) laminarin, for 10 min at 25 °C in a pH range of 3–8 in citrate/phosphate buffer, containing 0.1 % (w/v) BSA. The reducing sugar released was quantified as described above. The effect of temperature was evaluated by incubating *LaGH157* (0.1 mg/mL) or *BcGH157* (0.01 mg/mL) in a 100 µL reaction mixture containing 1 % (w/v) laminarin, in citrate/phosphate buffer pH 6.5 with 0.1 % BSA (w/v) at different temperatures ranging from 4 °C to 55 °C for 10 min. The released reducing sugars were determined as described above.

To determine the effect of pH on the stability of *LaGH157* and

BcGH157, the enzymes were incubated in citrate/phosphate buffer with 0.1 % (w/v) BSA, in a pH range of 3 to 8 for 60 min at 25 °C. Following incubation, enzymatic activity was determined under optimal conditions: pH 6.5 at 25 °C for LaGH157 and pH 6.5 at 48 °C for BcGH157. The thermostability of the enzymes was evaluated by incubating LaGH157 or BcGH157 in citrate/phosphate buffer pH 6.5 with 0.1 % (w/v) BSA at a range of temperatures (4–55 °C) for 60 min, followed by determination of specific activity at optimal conditions.

4.8. Enzyme kinetics

Kinetic assays with 4-nitrophenyl- β -laminaribioside (pNPLAM2) were performed in triplicate at room temperature for 10 min in citrate/phosphate buffer pH 6.5, containing 0.1 % (w/v) BSA. The reaction was initiated by the addition of 50 μ L of pNPLAM2 to 50 μ L of the enzymes, for a final substrate concentration range of 50–3200 μ M and final enzyme concentration of 250 nM. Released 4-nitrophenol (pNP) was measured in an iMark™ plate reader (Bio-Rad laboratories, Inc., USA) at 405 nm. All data obtained were processed using GraphPad Prism 10 data analysis software (GraphPad Software). Initial rates of catalysis were calculated by linear fit of free pNP concentrations measured between 0 and 95 s. Kinetic parameters (K_m and V_{max}) were determined by plotting the initial rates of catalysis as a function of substrate concentration and fitting the data using the Michaelis-Menten model, as follows:

$$v = \frac{V_{max} [S]}{K_m + [S]}$$

Kinetic parameters were also determined on lichenan and medium viscosity barley β -glucan, using a polysaccharide concentration range of 0.125–4 mg/mL and either 1.63 μ M (LaGH157) or 0.165 μ M (BcGH157) of enzyme. The reducing end formation was quantified using a bicinchoninic acid (BCA) reducing-sugar assay according to established protocols [55]. Kinetic parameters were calculated as above, using the initial rates of catalysis determined with the concentration of reducing ends formed between 10 and 50 s of reaction.

4.9. Differential scanning fluorimetry

Differential scanning fluorimetry (DSF) experiments were performed in triplicate with a final volume of 20 μ L. The sample mixture contained LaGH157 or BcGH157 (at 25, 50 or 100 μ M) and Sypro Orange dye (final concentration 5 \times) in citrate/phosphate buffer at pH 5.0, 6.5 and 8.0. Samples were temperature ramped from 25 to 95 °C at 60 s per degree, on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, USA). Fluorescence was measured each time before the temperature changed. Data were analysed using the JTSA server (<http://paulsbond.co.uk/jtsa>). Data points to the right of the highest fluorescence and to the left of the lowest fluorescence were discarded. The remaining points were fitted with a five-parameter sigmoid equation (Sigmoid-5) model using the Levenberg–Marquardt algorithm.

4.10. HPAEC-PAD and LC-MS analysis of hydrolysates

Solutions of medium-viscosity barley β -glucan (MLG), lichenan (Lich), laminarin (Lam), and carboxymethyl curdlan (CM-Curd) were prepared at 5 mg/mL in deionized water (diH₂O) following manufacturer instructions. The BcGH157 digests were prepared by combining 200 μ L of 5 mg/mL substrate, 790 μ L of 20 mM HEPES buffer pH 7.5, 200 mM NaCl and 10 μ L of 1 mg/mL enzyme in the same buffer. The reactions were incubated overnight at 30 °C. The LaGH157 digests were prepared by combining 200 μ L of 5 mg/mL substrate, 50 μ L of 1 M sodium MES buffer pH 6.0, 650 μ L of diH₂O and 100 μ L of 1 mg/mL enzyme in 20 mM HEPES buffer pH 7.5, 200 mM NaCl. The reactions were incubated overnight at 22 °C. The lichenase MLG digest was prepared by combining 200 μ L of 5 mg/mL substrate, 50 μ L of 1 M sodium

acetate buffer pH 6.0, 790 μ L of diH₂O and 10 μ L of 230 U/mL lichenase (NEOGEN/Megazyme International (Ireland)). The EG16 MLG digest was prepared by combining 200 μ L of 5 mg/mL substrate, 50 μ L of 1 M sodium acetate buffer pH 6.0, 789 μ L of diH₂O, 1 μ L of 1 M dithiothreitol, and 10 μ L of 1 mg/mL VvEG16 (prepared as described in McGregor et al. [33]). The lichenase and VvEG16 digests were incubated overnight at 30 °C.

The degree of digestion for each reaction was estimated based on total reducing end content measured using the BCA assay [56]. Briefly, 10 μ L of each digest was diluted with 40 μ L of diH₂O and 10 μ L of the resulting solution was diluted with a further 40 μ L of diH₂O to give 5-fold and 25-fold dilutions. Each solution was mixed with 40 μ L of freshly prepared BCA reagent (990 μ L of BCA A [250 mM Na₂CO₃, 140 mM NaHCO₃, 2.5 mM bicinchoninic acid, stored at room temperature] supplemented with 10 μ L of BCA B [125 mM CuSO₄, 250 mM L-serine, stored at –20 °C]) and heated to 80 °C for 10 min. 75 μ L of the resulting solution was transferred to a clear 384-well plate and A₅₆₃ was determined using a Biotek Epoch plate reader. A water blank was subtracted from all readings. Reducing ends were quantified against a glucose calibration series from 0 to 120 μ M. Signal from DTT in the EG16 digest was subtracted by preparing a second BCA reaction without 80 °C treatment (DTT reacts with Cu(II) to form Cu(I) rapidly at room temperature) and subtracting the resulting A₅₆₃ from each measurement instead of the water blank.

Standards were prepared at 50–100 mM in diH₂O and diluted to 50 μ M with additional diH₂O. For HPAEC-PAD and LC-MS analysis, 10 μ L of each digest was diluted with 90 μ L of water. HPAEC-PAD analysis was performed using a CarboPac PA-10 column flowing at 0.5 mL/min and 30 °C. Solvent A was diH₂O, solvent B was 200 mM NaOH in diH₂O, solvent C was 500 mM NaOAc, 100 mM NaOH in diH₂O. The separation gradient was: 0–5 min 50 % A, 50 % B; 5–21 min linear gradient to 25 % A, 25 % B, 50 % C, 21–21.1 min linear gradient to 50 % A, 50 % B, 21.1–25 min equilibration with 50 % A, 50 % B. Sample injection volume was 5 μ L.

LC-MS analysis was performed using a Hypercarb PGC column (1 \times 100 mm, 3 μ m, Thermo) flowing at 100 μ L/min at 60 °C. Solvent A was 0.1 % LC-MS-grade NH₄OH in water, solvent B was 80 % MeCN in solvent A. The separation gradient was: 0–5 min linear gradient from 2 to 5 % B; 5–20 min linear gradient from 5 to 60 % B; 20–20.1 min linear gradient from 60 to 100 % B; 20.1–21.5 min isocratic 100 % B; 21.5–22.0 min linear gradient to 2 % B; 22–25 min isocratic 2 % B. Sample injection volume was 1 μ L.

4.11. Crystallization of LaGH157, LaGH157–Lam2 complex and mutant LaGH157E224A – Lam4 complex

Seleno-methionine LaGH157 (10 mg/mL) was tested against a range of commercial crystallization screens (Crystal Screen, Crystal Screen 2, PEG/Ion Screen I and II (Hampton Research, USA), JCSG+ HT96 (Molecular Dimensions, UK), by the sitting-drop vapor-diffusion method. Initial sitting-drop crystal screens were set up by adding an equal volume (0.8 μ L) of protein and reservoir solution on 96-well crystallization plates (SWISSCI 'MRC' 2-Drop Crystallization Plates, Douglas Instruments, UK) using a robotic nanodrop dispensing system (Oryx8, Douglas Instruments, UK). The resulting plates were stored at 293 K. Seleno-methionine LaGH157 first crystals were obtained with 4 % (v/v) tacsimate pH 6.0, 12 % (w/v) PolyEthylene Glycol 3350 (PEG 3350). An optimization screen was set up by varying pH (4.0–7.0) and PEG 3350 concentration (8–18 % (w/v)). Crystals were cryoprotected in harvesting solution supplemented with 25 % (v/v) glycerol and flash cooled in liquid nitrogen.

For the ligand soaking experiment, LaGH157 and the mutant LaGH157_E224A (8 mg/mL) were initially crystallized using the same protocol described for selenomethionine-labeled LaGH157 crystals. Crystals were observed with 0.2 M sodium citrate tribasic tetrahydrate and 20 % (w/v) PEG 3350; and 0.1 M sodium malonate pH 6.0 and 12 %

(w/v) PEG 3350. Optimization screens were set up by varying sodium citrate tribasic tetrahydrate (0.05–0.5 M) and PEG 3350 (10–30 % (w/v)) concentrations; and by varying the pH of sodium malonate solution (4.0–7.0) and PEG 3350 concentration (8–18 % (w/v)). After crystals formation on the optimization screens, 20 mM laminaribiose (G3G, Lam2), 20 mM laminaritetraose (G3G3G3G, Lam4), 20 mM laminaripentaose (G3G3G3G3G, Lam5), 10 mM laminarihexaose (G3G3G3G3G3G, Lam6) and 6 mM 3¹-β-D-cellobiosyl-glucose were introduced through soaking to the protein/reservoir solution drops at 293 K. Crystals were cryoprotected in crystallization solution supplemented with 25 % (v/v) glycerol and flash cooled in liquid nitrogen for data collection. Only the LaGH157-Lam2 and LaGH157E224A-Lam4 complexes produced good-quality crystals in 0.1 M sodium malonate pH 5.0 and 8 % (w/v) PEG 3350.

4.12. 3D structure determination and refinement

The seleno-methionine LaGH157 derivative structure was solved by single wavelength anomalous dispersion (SAD). X-ray diffraction data for selenomethionine-labeled LaGH157 crystals were collected at 100 K, using radiation at the selenium peak wavelength (0.97946 Å) in ID30A-1 beamline at the European Synchrotron Radiation Facility (ESRF) to a maximum resolution of 2.40 Å. While, for LaGH157 – Lam2 and LaGH157E224A – Lam4 complexes, data were collected in ID30A-3 beamline to a maximum resolution of 2.7 Å and 2.3 Å, respectively, using 0.9677 Å wavelength radiation. LaGH157 – Lam2 and LaGH157 – Lam4 structures were determined by molecular replacement. A systematic grid search was carried out on all crystals to select the best-diffracting part of each crystal. iMosflm [57] was used for strategy calculation during data collection. All data sets were processed using the Fast_dp and xia2 [58] packages, which use the programs XDS [59], POINTLESS and SCALA [60] from the CCP4 suite (Collaborative Computational Project, Number 4, 1994 [61]). Data-collection statistics are given in Table 3.

Se-methionine-labeled LaGH157 crystals indexed in space group $P2_12_12_1$, with a calculated Matthews coefficient of $2.42 \text{ \AA}^3 \text{ Da}^{-1}$ [62], a solvent content of 49.1 % and 4 molecules in the asymmetric unit. The best diffracting LaGH157 – Lam2 and LaGH157 – Lam4 complexes crystals belonged to space group $H3$ and $P2_12_12_1$, with 4 molecules in the asymmetric unit, a solvent content of 56.5 % and 52.1 % and a Matthews coefficient of $2.83 \text{ \AA}^3 \text{ Da}^{-1}$ and $2.56 \text{ \AA}^3 \text{ Da}^{-1}$, respectively [62].

The Se-methionine LaGH157 structure was solved by SAD experiment with AUTOSOL [63] from the PHENIX suite [64]. AUTOBUILD was used to build the initial model [65]. For the LaGH157 – Lam2 and LaGH157E224A – Lam4 complexes structure determination, Phaser MR [66] was used to carry out molecular replacement, using the structure of the seleno-methionine LaGH157 derivative as a search model. All models were adjusted and refined using REFMAC5 [67] and PDB REDO [68] interspersed with manual adjustment in COOT [69]. The final round of refinement was performed using the TLS/ restrained refinement procedure, using each module as a single group. The root mean square deviation of bond lengths, bond angles, torsion angles and other indicators were continuously monitored using validation tools in COOT and MOLPROBITY [70]. Final coordinates and structure factors for the Se-methionine-labeled LaGH157, LaGH157 – Lam2 complex and LaGH157E224A – Lam4 complex were deposited in Protein Data Bank (PDB) under accession codes 9FZ9, 9G4N and 9G5G, respectively. wwPDB Validation Service was used to validate the structures before deposition in the PDB. The structure of BcGH157 was predicted with AlphaFold 3 in the AlphaFold server (<https://alphafoldserver.com/>) [71]. 3D structure figures were generated using UCSF ChimeraX [72].

4.13. SEC-MALLS

Experiments were conducted on a system comprising a Wyatt

HELEOS-II multi-angle light scattering detector and a Wyatt rEX refractive index detector linked to a Shimadzu HPLC system with a Superdex 200 SEC column. Work was conducted at room temperature ($20 \pm 2 \text{ }^\circ\text{C}$). Sample injection volume was 100 μL except where stated; Shimadzu LabSolutions software was used to control the HPLC and Astra 7 software for the HELEOS-II and rEX detectors. The Astra data collection was 1 min shorter than the LC solutions run to maintain synchronisation. Blank buffer injections were used as appropriate to check for carry-over between sample runs. Data were analysed using the Astra 7 software. MWs were estimated using the Zimm fit method with degree 1. A value of 0.182 was used for protein refractive index increment (dn/dc). The running buffer was 20 mM HEPES pH 7.5, 200 mM NaCl, 0.2 μm filtered. Samples were supplied in similar composition buffer at $\sim 5 \text{ mg/mL}$ and used directly.

4.14. Affinity gel electrophoresis (AGE)

The AGE experiments were conducted following the method described by [73]. Tested polysaccharides, such as laminarin (from *Laminaria digitata*), lichenan and barley β -glucan were prepared at a concentration of 0.3 % (w/v). Then, electrophoresis was carried out at room temperature in native 10 % (w/v) polyacrylamide gels in the presence or absence of polysaccharides. The gels were also loaded with BSA, which acts as a non-interacting negative control. After electrophoresis, protein bands were visualized through staining with Coomassie Blue.

4.15. Phylogeny analysis

A phylogeny tree was constructed using GH157 enzymes, including BcGH157 and LaGH157, as well as β -glucanases from other families, spanning clans GH-A, B and M. After selection of the enzymes, the catalytic modules were identified using the dbcan3 server (<https://ccb.unl.edu/dbCAN2/>) [74] and isolated. Signal peptides were identified using signal P server [51] and removed. The resulting sequences are presented in Supplemental Table 6. A maximum likelihood tree was constructed in MEGA11 using the model with the lowest Bayesian Information Criterion score (Wheland and Goldman with frequencies) [75,76]. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The resulting tree is presented in Supplemental Fig. 8.

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CRedit authorship contribution statement

Catarina Caseiro: Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Nicholas G.S. McGregor:** Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Victor Diogo Alves:** Writing – review & editing, Investigation, Formal analysis. **Ana Luísa Carvalho:** Writing – review &

editing, Investigation, Formal analysis. **Maria João Romão:** Writing – review & editing, Funding acquisition. **Gideon J. Davies:** Writing – review & editing, Methodology, Formal analysis. **Carlos M.G.A. Fontes:** Writing – review & editing, Methodology. **Pedro Bule:** Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Catarina Caseiro reports financial support was provided by Foundation for Science and Technology. Carlos M.G.A. Fontes reports a relationship with NZYTech Lda that includes: board membership and employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2024.137402>.

Data availability

Coordinates and structure factors have been deposited in the Protein Data Bank under accession code PDB 9FZ9 [<https://www.rcsb.org/structure/9FZ9>], 9G4N [<https://www.rcsb.org/structure/9G4N>] and 9G5G [<https://www.rcsb.org/structure/9G5G>]. All further data supporting the findings of this study are available from the corresponding author, upon reasonable request.

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