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Characterisation of an *exo*-(α -1,3)-3,6-anhydro-D-galactosidase produced by the marine bacterium *Zobellia galactanivorans* Dsij^T; insight into enzyme preference for natural carrageenan oligosaccharides and kinetic characterisation on a novel chromogenic substrate

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Abstract

Flavobacteriia are important degraders in the marine carbon cycle, due to their ability to efficiently degrade complex algal polysaccharides. A novel *exo*-(α -1,3)-3,6-anhydro-D-galactosidase activity was recently discovered from a marine *Flavobacteriia* (*Zobellia galactanivorans* Dsij^T) on red algal carrageenan oligosaccharides. The enzyme activity is encoded by a gene found in the first described carrageenan-specific polysaccharide utilisation locus (CarPUL) that codes for a family 129 glycoside hydrolase (GH129). The GH129 family is a CAZy family that is strictly partitioned into two niche-based clades: clade 1 contains human host bacterial enzymes and clade 2 contains marine bacterial enzymes. Clade 2 includes the GH129 *exo*-(α -1,3)-3,6-anhydro-D-galactosidase from *Z. galactanivorans* (ZgGH129). Despite the discovery of the unique activity for ZgGH129, finer details on the natural substrate specificity for this enzyme are lacking. Examination of enzyme activity on natural carrageenan oligomers using mass spectrometry demonstrated that ZgGH129 hydrolyses terminal 3,6-anhydro-D-galactose from unsulfated non-reducing end neo- β -carrabiose motifs. Due to the lack of chromogenic substrates to examine *exo*-(α -1,3)-3,6-anhydro-D-galactosidase activity, a novel substrate was synthesised to facilitate the first kinetic characterisation of an *exo*-(α -1,3)-3,6-anhydro-D-galactosidase, allowing determination of pH and temperature optimums and Michaelis-Menten steady state kinetic data.

1. Introduction

Macroalgae (seaweed) are main primary producers of coastal ecosystems and support a large coastal food web [1]. Unlike land plants, the extracellular matrices of macroalgae are dominated by the presence of sulfated polysaccharides [2]. These highly complex biopolymers can also vary in structure through alternating linkages, additional modifications (e.g. glycosylation, pyruvation and methylation) and sulfation isoforms [2]. Due to their diverse chemical structures, these sulfated algal polysaccharides are

exploited in the alimentary and cosmetic industry for their rheological properties [3]. Furthermore, sulfated polysaccharides and the oligosaccharides derived from them often demonstrate bioactivities *in vitro*, such as immunostimulatory and anti-viral activities [4]. This is presumably due to their resemblance to metazoan sulfated sugars such as those found in the heavily glycosylated mucosal material lining the entryways to the body and the connective tissue glycoaminoglycans. Thus, the ability to tailor the physiochemical properties of these algal carbohydrates enzymatically is of strong biotechnological interest.

Coastal organic matter produced by marine macrophytes is largely recycled by marine heterotrophic bacteria. These bacteria have developed exquisite machinery to process the highly complex seaweed polysaccharides found within this material. For example, diverse bacterial species have grouped together genes encoding proteins involved in the processing of defined polysaccharides into genetic loci called polysaccharide utilisation loci (PULs) [5-11]. PULs were first classified in *Bacteroides* [12, 13]; however, recently the definition has been expanded to other bacterial phyla [6]. Within a ‘typical’ PUL the genes encode highly specialised proteins involved in recognition, catalysis, transport and regulation of PUL function. PULs are a rich enzymatic resource, unlocking an unknown PUL specificity for a defined polysaccharide can result in the discovery of novel CAZyme families and/or novel activities within already created CAZyme families (<http://www.cazy.org/>) [14]. Recently several marine bacterial PULs have been described [15-17], including PULs specific for the complex red algal extracellular matrix polysaccharide carrageenan [6, 8, 18, 19].

Carrageenan is a complex sulfated galactan with alternating α -1,3- and β -1,4-linkages. β -, κ - and ι -carrageenan differ in their degree of sulfation (none, one and two sulfates, respectively) but all have the repeating disaccharide unit -[3,6-anhydro-D-galactose-(α -1,3)-D-galactose-(β -1,4)]- (Figure 1A). The monosaccharide 3,6-anhydro-D-galactose is a bicyclic sugar, unique to red algae; the 3,6-anhydro-bridge

is formed through the action of sulfurylases [20] a group of enzymes with biochemistry also unique to red algae which utilise the D-galactose-6-sulfate variant as a substrate for ring closure. Within the carrageenan chain the β -1,4-linked D-galactose is maintained in a 4C_1 conformation [2]. However, the 3,6-anhydro-bridge introduces strict structural constraints on the α -1,3-linked 3,6-anhydro-D-galactose monosaccharide resulting in a ring inversion to the 1C_4 conformation and consequentially its α -linkage and C4 hydroxyl group are maintained equatorially within the carrageenan chain [2] (Figure 1A).

Marine bacterial glycoside hydrolase activities hydrolysing the β -1,4 linkage in carrageenan have been well described in the literature [21-24]; however, only recently has an enzyme activity been described as active on the α -1,3 linkage [6]. Recent experiments revealed a novel *exo*-3,6-anhydro-D-galactosidase activity [6] in a family 129 glycoside hydrolase (GH129) member coded by the gene *zgal_3152* in *Zobellia galactanivorans* Dsij^T, a marine model *Flavobacteriaceae* (bacterial phylum *Bacteroidetes*) isolated from a healthy red alga [25, 26]. The enzyme, termed ZgGH129, cleaves 3,6-anhydro-D-galactose residues from the non-reducing end of neo-carrageenan oligosaccharides to produce novel oligosaccharides with D-galactose on the non-reducing terminus [6]. The elucidation of the function of *zgal_3152* led to the discovery of a polysaccharide utilization locus (PUL) and complex regulon responsible for carrageenan degradation in *Z. galactanivorans* [6]. Further experiments also identified 3,6-anhydro-D-galactosidase activity in three family 127 glycoside hydrolase (GH127) members located within the same carrageenan PUL (CarPUL) [6]. In marine *Pseudoalteromonas* species the GH127 and GH129 family members are absent [8, 18] though certain *Pseudoalteromonas* species with CarPULs display growth on κ - and ι -carrageenan suggesting that the source of the (α -1,3)-3,6-anhydro-D-galactosidase activity in these species remains to be discovered [8].

The GH129 family was first identified based on distant sequence similarity to family 101 glycoside hydrolases [27]. The first characterised GH129 enzyme, *BbGH129*, termed NagBb, is an *exo*- α -N-

acetylgalactosaminidase produced by the human gut bacterium *Bifidobacterium bifidum* JCM 1254 and is implicated in mucin degradation [27]. Catalytically, *BbGH129* proceeds using a retaining mechanism [27] and the catalytic nucleophile (*BbAsp435*) and acid/base residue (*BbGlu478*) have been identified through crystallographic analyses [pdb id 5wzn [28], Figure 1B]. Consistent with this data, *ZgGH129* [pdb id 5opq [6]] shares the crystallographically predicted nucleophile and acid/base catalytic residues with *BbGH129* (Figure 1B).

Despite the discovery of the unique *exo*-(α -1,3)-3,6-anhydro-D-galactosidase activity for *ZgGH129* [6], detailed information on the subsite preferences is lacking and thus more experiments to examine the specificity of this enzyme on natural substrate are needed. Here we investigate the unusual *exo*-(α -1,3)-3,6-anhydro-D-galactosidase enzyme activity of *ZgGH129* on its natural carrageenan substrate and on a novel artificial chromogenic substrate, thus allowing for determination of the fine substrate specificity, pH optimum, temperature optimum and steady state Michaelis-Menten kinetics data.

2. Results and Discussion

2.1 Phylogeny of the GH129 enzymes

A phylogenetic tree using 100 enzyme sequences from different bacterial species was constructed for the GH129 family of enzymes using a maximum likelihood approach and the Whelan and Goldman model [29]. Three characterised GH101 enzymes were used as an outgroup based on their distant sequence similarity to the GH129 family [30-32]. The tree with the highest log likelihood (-16026.33) is shown (Figure 1C) and the percentage of trees in which the associated taxa clustered together is shown next to the branches. The GH129 family consists only of bacterial members and the family divides into two distinct clades (Figure 1C and Supplementary File 1) with excellent bootstrap values of 99. Clade 1

contains bacterial members that have animal, including human, hosts and/or members that were sourced from human feces. Clade 2 contains bacteria from the marine niche and includes one member that has an animal host: *Flavobacterium faecale*, isolated from the stools of Antarctic penguins [33]. The clade 1 bacterial members are more diverse than their clade 2 counterparts and contain members from three Gram-positive *Firmicutes* from the Class *Clostridia*, several Gram-positive *Bifidobacterium* species and one Gram-negative *Victivallales* isolated from sewage sludge [34]. All of the marine clade 2 bacterial members are found in the Gram-negative *Bacteroidetes* phyla, either in Class *Flavobacteriia* or *Cytophagia*. The strict phylogenetic divide along niches within the GH129 family suggests another evolutionary example of a horizontal gene transfer event from a marine bacterium to a gut bacterium [6, 35]. Within clade 1 (Figure 1C) is the first characterised GH129 enzyme from the gut bacterium *Bifidobacterium bifidum* (NagBb), which is an *exo-α-N*-acetylgalactosaminidase active on mucosal glycans [27]. Clade 2 accommodates the novel *exo-(α-1,3)-3,6*-anhydro-D-galactosidase activity discovered on carrageenan oligosaccharides that is encoded in a PUL in the marine bacterium *Z. galactanivorans* [6].

The strong divide of the GH129 clades along the bacterial niches likely represents the different biochemistries of the GH129 enzymes between these niches. The conformations of the non-reducing end D-galactose substrate moieties differ significantly - 4C_1 for *N*-acetyl-D-galactosamine (GalNAc, clade 1) and 1C_4 for 3,6-anhydro-D-galactose (clade 2), as the 3,6-anhydro-bridge found in the latter monosaccharide inflicts strict conformational constraints on the structure. The ring flip from 4C_1 to 1C_4 also results in the hydroxyl group at C4 and the anomeric carbon flipping from axial to equatorial positions. Furthermore, the sugar decorations change the chemical nature of the monosaccharides, an *N*-acetyl group at C2 of GalNAc (clade 1) introduces a polar group whereas the 3,6-anhydro-bridge (clade 2) introduces a hydrophobic surface to the C6 face of the monosaccharide. These major differences between sugar structures is supported by the divergent enzyme activities and different bacterial niches described for the two clades.

The structure of the clade 1 *BbGH129* [pdb id 5wzn [28]] was determined in complex with GalNAc in the -1 subsite, allowing crystallographic prediction of the nucleophile (*BbAsp435*) and acid/base (*BbGlu478*) catalytic residues (Figure 1B). The predicted catalytic residues are shared within the GH129 family. The structure of the clade 2 *ZgGH129* [pdb id 5opq [6]] has only been determined in complex with a bound Tris molecule in the predicted -1 site to date. Other than the critical catalytic residues, the active site architectures of the two characterised GH129 enzymes are quite different (Figure 1B). One major difference is the coordination of a divalent cation within the active site of *BbGH129* which is absent in *ZgGH129*. There is also a perpendicular shift of the hydrophobic tryptophan platform between the two active sites. Another key difference is the participation of both monomers in the *ZgGH129* active site which does not occur in *BbGH129* as *ZgGH129* exists as a homodimer and *BbGH129* is monomeric. Overall, these variances in -1 active site sub-structure likely play an important role in defining the differences in substrate recognition between the two GH129 enzymes.

2.2 Deeper insight into *ZgGH129* substrate specificity

Initial biochemical analyses of *ZgGH129* indicated *ZgGH129* could hydrolyse the 3,6-anhydro-D-galactose moiety carrageenan oligosaccharides [6]. One question that remained unclear from the study was whether *ZgGH129* is capable of hydrolysing the 3,6-anhydro-D-galactose moiety from a non-reducing neo- β -carrabiose motif or a neo- κ -carrabiose motif. To investigate, and described in detail below, mass spectrometry methods were used to characterise the *ZgGH129* activity on a natural carrageenan oligosaccharide substrate initially predicted to be neo- κ -DP6 based on mass.

Furcellaran, from the extracellular matrix of the red alga *Furcellaria lumbricalis*, is composed primarily of β - and κ -carrageenan motifs [36]. To obtain carrageenan oligosaccharide substrates for *ZgGH129* with the 3,6-anhydro-D-galactose moiety on the non-reducing terminus (the neo-series of oligosaccharides),

furcellaran was treated with the κ -carrageenase from *Pseudoalteromonas carrageenovora*. As described, this endolytic enzyme hydrolyses the β -1,4-linkage between the D-galactose-4-sulfate and 3,6-anhydro-D-galactose motifs [23]. From this knowledge, all oligosaccharides released enzymatically using this methodology have 3,6-anhydro-D-galactose on the non-reducing end and D-galactose-4-sulfate on the reducing end [36].

The reactions obtained from the κ -carrageenase from *Pseudoalteromonas carrageenovora*, with and without a consecutive action of ZgGH129 were analysed using MALDI mass spectrometry (Figure 2). Before the action of the ZgGH129 enzyme (Figure 2A), signals attributed to five oligo-carrageenans with a degree of polymerisation (DP) from 6 to 8 were found in the mass range m/z 800-1500. Species at m/z 1423.295 and m/z 1117.192 respectively correspond to a DP8 containing 2. κ +2. β motifs (κ - β - β - κ or β - κ - β - κ or β - β - κ - κ) and a DP6 containing 2. κ +1 β motifs (either κ - β - κ or β - κ - κ) as $[M-2H+Na]^-$ ions for both species. The species at m/z 1321.352 and m/z 1015.241 were attributed to sulfate losses during the experiment as $[M-H]^-$ ions (Figure 2A). Sulfate losses (corresponding to a SO_3Na loss) are commonly observed upon MALDI ionisation even when using 2,5-dihydroxybenzoic acid/*N,N*-dimethylaniline (DMA-DHB) as a matrix, which strongly reduces the loss of labile functionalities [37]. After the action of the ZgGH129 enzyme (Figure 2B) additional species were observed. Species observed at m/z 1279.248 and m/z 973.150 were attributed to hydrolysis of the non-reducing α -1,3-linked 3,6-anhydro-D-galactose as $[M-2H+Na]^-$ ions. The species attributed to sulfate losses of these products were also detected at m/z 1177.308 and m/z 871.199 respectively as $[M-H]^-$ ions (Figure 2B). These results correlate with the expected *exo*-3,6-anhydro-D-galactosidase activity of ZgGH129. Conversely, the activity of ZgGH129 on the unknown DP6 species released by the use of the κ -carrageenase required additional experiments (Figure 2). In accordance with the ‘ideal’ composition of the carrageenan furcellaran, the species at m/z 1219.118 (Figure 2A) could be attributed to a neo- κ -DP6 oligosaccharide (κ - κ - κ) as a $[M-3H+2Na]^-$ ion. After incubation of this DP6 with ZgGH129 an ion at m/z 1075.087 appeared (Figure 2B) with the

corresponding loss of the peak at m/z 1219.118. This unexpected result could correspond to the loss of a 3,6-anhydro-D-galactose residue on a pure neo- κ -DP6; however, ZgGH129 has not demonstrated activity when tested previously on pure neo- κ -carrageenan oligosaccharide standards [6].

The structures of the unknown DP6 and the corresponding product species were further examined using ESI-MSMS. Species were isolated at m/z 598.1 as an $[M+Na-3H]^{2-}$ ion for the DP6 and m/z 526.0 as an $[M-2H]^{2-}$ ion for the hydrolysed product and each were fragmented using collision energy optimisation. The fragmentation results demonstrate that the structure of the ambiguous DP6 corresponds to a neo- β - ι - κ hexasaccharide (Figure 3A), this is not completely unexpected as a ‘non-negligible’ amount of ι -carrabiose moieties are present in furcellaran [36]. The *exo*-3,6-anhydro-D-galactosidase activity of ZgGH129 is therefore on the neo- β -carrageenan motif at the non-reducing end as confirmed by the MSMS analysis of the m/z 526.0 species (Figure 3B). This is in consensus with the enzyme’s previously described inactivity on κ -carrageenan oligosaccharides [6]. These mass-spectrometry experiments, along with the previous experiments demonstrating inactivity on neo- κ -oligosaccharides, suggest ZgGH129 is specific for neo- β -carrageenan motifs at the non-reducing end. Furthermore, these results also demonstrate that ZgGH129 is capable of accommodating a sulfate group on the 2-hydroxyl of 3,6-anhydro-D-galactose in its +2 sub-site and a 4-sulfo group on the D-galactose moiety in its predicted +3 sub-site (the neo- ι -carrabiose motif) [6]. These results align with the suggested pathway of carrageenan degradation of *Z. galactanivorans*, where the GH129 enzyme acts on neo- β -carrabiose motifs after the action of the CarPUL carrageenases and sulfatases [6, 38].

2.3 Synthesis of 4-nitrophenyl 3,6-anhydro- α -D-galactopyranoside

In general, synthetically derived substrates, possessing a leaving group that when hydrolysed can be followed spectrophotometrically, are ideal materials to identify and characterise enzymes of interest. A

common class of substrates that are used to study glycosidases are the 4-nitrophenyl-based glycosides due to the release of 4-nitrophenolate which can be monitored spectrophotometrically. Therefore, the putative substrate 4-nitrophenyl 3,6-anhydro- α -D-galactopyranoside (3,6-ADG-*p*NP) was prepared as an artificial substrate for ZgGH129 (Scheme 1). The most efficient route to 3,6-ADG-*p*NP would be via a ring closure of 4-nitrophenyl α -D-galactopyranoside (Gal-*p*NP), as this material is readily available commercially. One previously utilised method to form a 3,6-anhydro-bridge is to first prepare the 6-*O*-tosyl-glycoside, followed by treatment with a strong base [39, 40]. With this knowledge 4-nitrophenyl 6-*O*-tosyl- α -D-galactopyranoside (6-tosyl-Gal-*p*NP) was successfully obtained from Gal-*p*NP using the conditions of Siewert and Westphal [41]. However, when 6-tosyl-Gal-*p*NP was exposed to the strong basic conditions needed for the ring closure, (sodium methoxide) as done in previous 3,6-anhydro bond-forming reactions [39], the starting material was consumed but no formation of the desired 3,6-ADG-*p*NP was observed. Instead the reaction mixture possessed a strong yellow colour which is conducive to the presence of 4-nitrophenolate, indicating the 4-nitrophenyl group was being displaced under the reaction conditions. This is likely due to the 3,6-anhydro moiety which once installed results in additional ring strain and increased reactivity, causing instability of the compound under basic conditions [40]. An alternative method which does not expose the product to strongly basic conditions is a direct route which has previously been applied to methyl galactopyranosides [42, 43] which were subjected to Appel bromination reaction conditions and resulted in methyl 3,6-anhydro-galactopyranoside being formed as well as methyl 6-bromo-6-deoxy-galactopyranoside as a significant side product. Pleasingly, using these conditions, but with Gal-*p*NP, resulted in the successful isolation of the desired 3,6-ADG-*p*NP substrate. The yield was low (45%) and 4-nitrophenyl 6-bromo-6-deoxy- α -D-galactopyranoside was also formed (26%, 6-bromo-Gal-*p*NP) (Scheme 1). The NMR and MS details for the novel compounds are described in the experimental section (4.4) and Supplementary File 2.

2.4 Kinetic analyses of ZgGH129

Determination of the pH optimum was done at the isosbestic point of 4-nitrophenol/4-nitrophenolate (348 nm) first in universal buffer (Figure 4A), followed by a finer sampling in citrate buffer (Figure 4B) [44, 45] using 3,6-ADG-*p*NP as the substrate. Both buffer systems confirmed the optimal activity of ZgGH129 to be pH 5 and the enzyme activity was stable for the duration of these experiments. Of note though is that ZgGH129 is not stable stored in the citrate buffer at pH 5 for extended periods as it precipitates almost entirely after an overnight dialysis. On the other hand, the enzyme can be stored refrigerated for months at a time in 1 x PBS or 50 mM Tris pH 8 and still retain its activity.

The predicted localisation of the ZgGH129 enzyme is periplasmic [6] (SignalP-5.0 [46], data not shown). In *E. coli* the pH in the periplasm is in the order of that outside the cell; challenges using rapid acid shift showed the periplasm reflected a similar pH as the extracellular milieu [47]. Conversely, in the cytoplasm there was a sharp drop in the pH upon acid treatment followed by a slow return towards baseline [47]. This suggests that the outer membrane is not a significant impediment to proton movement. If this hold true in *Bacteroidetes*, then the pH of the periplasm should be that of seawater. The surface seawater in the English channel has been measured at pH 8.28±0.01 in March 2008 and 8.09±0.01 in July 2008 [48], seawater pH can also vary with both location and temperature [49, 50]. The pH optimum performed both in citrate and in universal buffer shows a slow decline in activity above pH 5. The universal buffer pH curve shows activity at pH 9, albeit considerably lower than at pH 5. Furthermore, initial ZgGH129 assays on furcellaran oligosaccharides were successful at pH 7.5 [6]. These results indicate that ZgGH129 maintains significant activity even several pH units away from the optimum. Altogether, these results suggest that ZgGH129 would be active within the periplasm of *Z. galactanivorans* at the pH of seawater.

Initial optimal temperature tests between 28 °C and 51 °C indicated that ZgGH129 preferred a higher temperature range (data not shown) thus a finer optimal temperature range was also performed in triplicate between 45 °C to 52 °C with the optimum temperature determined to be 49 °C (Figure 4C). This

result was unexpected as *Z. galactanivorans* was isolated from a red macroalga, *Delesseria sanguinea*, in the waters off Roscoff, France, on the English Channel [26]. The waters off Roscoff range in temperature from 9 °C in winter to a maximum of 18 °C in the summer [51]. At low tide, however, macroalgae are exposed to the elements and in sunny conditions the temperature on the surface of the algae rises appreciably [52]. An enzyme (e.g. ZgGH129) capable of activity at higher temperatures would be useful to the bacterium under these conditions. Stability of the enzyme was not investigated at the optimal temperature, though the enzyme remains viable for months at 4 °C when the appropriate buffer system is chosen.

To complement the temperature and pH optimum data obtained for ZgGH129, the Michaelis-Menten parameters were also determined (at the temperature and pH optimum) using 3,6-ADG-*p*NP as a substrate (Figure 4D). The K_m was determined to be 0.7 ± 0.1 mM, the k_{cat} 38.9 ± 2.4 s⁻¹ and the specificity constant $5.6 \pm 0.9 \times 10^4$ M⁻¹s⁻¹. Analysis of the same data by Lineweaver-Burk plot produced similar results (not shown). Thus, ZgGH129 shows robust activity on the artificial substrate 3,6-ADG-*p*NP and demonstrates that this compound could have wider utility in elucidating *exo*-3,6-anhydro-D-galactosidase activity in biochemical assays. Overall, these kinetic characterisations provide a first mechanistic glimpse into a newly discovered enzyme activity. More experimentation is certainly needed to determine optimal parameters if the enzyme is to be exploited industrially to modify neo-carrageenan oligosaccharides.

3. Conclusion

Sulfated polysaccharides demonstrate a wide variety of bioactivities presumably due to their resemblance to metazoan sulfated sugars. The ability to tailor the physiochemical properties of these algal carbohydrates enzymatically is of strong biotechnological interest. The *exo*-(α -1,3)-3,6-anhydro-D-galactosidase activity is an activity only recently discovered [6], therefore there is strong fundamental

interest to describe the biochemistry behind this newly discovered enzymatic activity within the GH129 family. The phylogenetic analyses demonstrate the GH129 family is divided into two niche-distinct clades, likely an example of a horizontal gene transfer event. Using natural oligo-carrageenan substrates and detailed mass spectrometry experiments, we have shown the marine *Flavobacterium* clade 2 member ZgGH129 cleaves the unique red algal 3,6-anhydro-D-galactose moiety from non-reducing end neo- β -carrabiose motifs. Furthermore, we have begun to decipher the sub-site preferences of the enzyme using these structural methods. Finally, we have developed a novel molecular tool, 3,6-ADG-*p*NP, allowing us to perform a fine kinetic characterisation of *exo*-(α -1,3)-3,6-anhydro-D-galactosidase enzyme activity. This artificial substrate will be useful for characterisation of other enzymes from clade 2 of the GH129 family and for the newly discovered *exo*-(α -1,3)-3,6-anhydro-D-galactosidase activity within the GH127 family, whose characterised members are encoded in the same *Flavobacterium* CarPUL as ZgGH129 [6].

4. Experimental procedures

4.1 Phylogeny

GH129 sequences were selected by Blastp and using the CAZy databank [53]. Three GH101 characterised enzymes were chosen for the outgroup [30-32]. The amino acid sequences were aligned using MAFFT [54] with the L-INS-i algorithm and the alignment was manually refined using Bioedit [55]. The evolutionary history was inferred by using the Maximum Likelihood method and the Whelan And Goldman model [29]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 4.2144)]. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis

involved 100 enzyme sequences from different bacterial species. There were a total of 526 amino acid positions in the final dataset. Evolutionary analyses were conducted in MEGA X [56].

4.2 Protein production and purifications

The pFO4 plasmid containing the *zgal_3152* gene fragment [6] was transformed into *Escherichia coli* BL21(DE3) and grown in 1 L of autoinduction Zyp-5052 media (200 $\mu\text{g mL}^{-1}$ ampicillin, 20 °C). Cells were harvested by centrifugation at 3063 $\times g$ for 30 min and lysed by chemical lysis [57]. The lysate was clarified at 13,865 $\times g$ for 45 min at 4 °C. The supernatant was loaded onto a 5 mL GE His Trap HP column (part of an ÄKTA FPLC), washed with 1x PBS pH 7.4 and eluted with an increasing gradient of 1–100% 1x PBS pH 7.4 containing 1 M imidazole. Fractions containing the protein of interest were pooled and concentrated (MWCO 5 kDa). It is necessary to maintain recombinant ZgGH129 in a buffer that is above pH 7 as the enzyme precipitates when stored at pH 5. The experimentally obtained pH optimum is not an ideal pH for the storage of recombinant ZgGH129. The ExPASy ProtParam tool [58] was used to generate the extinction coefficient for calculation of protein concentration using A280.

4.3 Mass spectroscopy

4.3.1 Enzyme reactions for mass spectroscopy experiments

Substrates were produced from the polysaccharide furcellaran, obtained as a generous gift from CP Kelco (Brian Rudolph), from the red alga *Furcellaria lumbricalis* [6]. Furcellaran oligosaccharides were produced as described previously [6, 36] using the recombinant κ -carrageenase from *Pseudoalteromonas carrageenovora* [59].

4.3.2 Matrix assisted laser desorption ionisation-Time of flight-MS analysis

MALDI-TOF-MS spectra were acquired in negative ionisation mode and reflector detection in the m/z range 500-1500 on an AutoflexSpeed TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany), equipped with a Smartbeam Laser (355 nm, 1000 Hz). Samples were analysed at a concentration of 500 $\mu\text{g}\cdot\text{mL}^{-1}$ using DMA-DHB as a matrix as described in Ropartz *et al.* [37]. An external calibration using a mixture of carrageenan standards was used. Acquisition parameters (laser power, pulsed ion extraction, etc.) were optimised on the sample without any enzyme process. Spectra were recorded using FlexControl 3.4.

4.3.3 Electrospray-quadrupole- Time of flight-collision induced dissociation-MSMS analysis

ESI-q-TOF Tandem MS experiments were performed in negative ion mode on a Synapt G2Si HDMS (Waters, Manchester, UK) in the m/z range 150-1600. Samples were diluted in MeOH/H₂O (1:1, v/v) at 5 $\mu\text{g mL}^{-1}$ and infused at a flow rate of 5 $\mu\text{L min}^{-1}$. The capillary voltage was set at 2.2 kV and the source temperature at 120 °C. Candidate ions were fragmented with an optimized collision energy by CID in the transfer cell of the instrument using Argon as collision gas. Data were recorded using MassLynx 4.1 (Waters, Manchester, UK).

4.3.4 MS data processing

MALDI MS and ESI-q-TOF tandem MS raw data were converted in mzML format using MSConvert 3.0.18261 (<http://proteowizard.sourceforge.net/downloads.shtml>) and peak peaking was performed using mMass 5.5.037. For MSMS spectra annotation, the nomenclature of Domon and Costello was used [60].

4.4 Synthesis of 4-nitrophenyl 3,6-anhydro- α -D-galactopyranoside (3,6-ADG-pNP)

General - All reagents and materials were purchased from commercial suppliers. Thin layer chromatography (TLC) used to monitor the reaction, was effected on Merck silica gel 60 F254 aluminium-backed plates and spots stained by heating with 5% conc. H₂SO₄ in ethanol. Flash column chromatography was performed on Merck silica gel using the specified solvents. NMR spectra were obtained on a Bruker Avance IIIHD 600 spectrometer, with DMSO-*d*₆ or CD₃OD as solvents with CD₃S(O)CD₂H (¹H, δ 2.50 ppm), (CD₃)₂SO (¹³C, δ 39.52 ppm), CD₂HOD (¹H, δ 3.31 ppm) or CD₃OD (¹³C, δ 49.00 ppm) used as an internal standard. Infrared spectra were obtained with neat samples on a PerkinElmer spectrum one FT-IR spectrometer fitted with a PerkinElmer Universal Attenuated Total Reflectance (ATR) sampling accessory. Infrared spectra annotated as strong (s), medium (m), weak (w) or broad (br) absorption band. High resolution mass spectra (HR-MS) were obtained on a Waters LCT Premier XE spectrometer, run in W-mode, using the ESI equipped ion source, in negative mode.

4-Nitrophenyl 3,6-anhydro-α-D-galactopyranoside (3,6-ADG-pNP) and 4-nitrophenyl 6-bromo-6-deoxy-α-D-galactopyranoside (6-bromo-Gal-pNP)

Carbon tetrabromide (110 mg, 0.33 mmol) and triphenylphosphine (174 mg, 0.66 mmol) were added to a stirred solution of 4-nitrophenyl α-D-galactopyranoside (100 mg, 0.33 mmol) in pyridine (2.5 ml) at 0 °C. The resulting yellow solution was then stirred at 60 °C for 2.5 h and after this time was quenched with methanol at 0 °C and concentrated. The resulting residue was purified by flash column chromatography (MeOH:CHCl₃ 1:19) to obtain 3,6-ADG-pNP as a white solid (42 mg, 45%). R_f = 0.43 (MeOH:CHCl₃ 1:9). ¹H NMR (600 MHz, (CD₃)₂SO): δ 8.22-8.18 (m, 2H), 7.28-7.23 (m, 2H), 5.57 (d, *J* = 2.6 Hz, 1H), 5.51 (*br s*, 1H), 5.29 (*br s*, 1H), 4.38 (*br s*, 1H), 4.25-4.22 (m, 1H), 4.22 (d, *J* = 5.2 Hz, 1H), 4.12 (d, *J* = 5.2 Hz, 1H), 3.99-3.95 (m, 1H), 3.91 (dd, *J* = 10.0, 2.9 Hz, 1H); ¹³C NMR (150.9 Hz, (CD₃)₂SO): δ 161.7, 141.7, 125.7, 116.6, 94.1, 81.1, 77.5, 69.2, 69.1, 68.3; FTIR (ATR): ν = 3310 (br), 1611 (w), 1591 (m), 1509 (m) cm⁻¹; HR-MS (ESI-): *m/z* [M+HCOO]⁻ calcd. for C₁₃H₁₄NO₉: 328.0669, found: 328.0668. Next to elute was 6-bromo-Gal-pNP as a white solid (31 mg, 26%). R_f = 0.34 (MeOH:CHCl₃ 1:9).

^1H NMR (600 MHz, CD_3OD): δ 8.23-8.19 (m, 2H), 7.35-7.32 (m, 2H), 5.68 (d, $J = 3.5$ Hz, 1H), 4.05-4.03 (m, 1H), 4.02-3.95 (m, 3H), 3.55 (dd, $J = 10.5, 5.2$ Hz, 1H), 3.47 (dd, $J = 10.5, 8.0$ Hz, 1H); ^{13}C NMR (150.9 Hz, CD_3OD): δ 163.5, 143.9, 126.5, 118.2, 99.4, 73.9, 71.0, 70.9, 69.3, 31.3; FTIR (ATR): $\nu = 3485$ (br), 1611 (w) 1591 (m), 1508 (s), 1493 (s) cm^{-1} ; HR-MS (ESI-): m/z $[\text{M-H}]^-$ calculated for $\text{C}_{12}\text{H}_{13}\text{NO}_7^{79}\text{Br}$: 361.9875, found: 361.9885.

4.5 Enzyme Kinetics

All reactions were done in triplicate and measured on a Spark microplate reader available through the Genomer platform at the Station Biologique de Roscoff. For determining Michaelis-Menten parameters reactions were set up using between 0 to 1.5 mM 3,6-ADG-*p*NP, 5 nM ZgGH129, 0.1 M NaOAc pH 5, 0.1 M CaCl_2 in a 49 °C water bath. An aliquot of 150 μL of the reaction was taken at the 1, 2 and 3-minute mark, the linear period on the curve, with the reaction stopped by the addition of 75 μL of 1 M glycine pH 10.88. Spectrophotometric reads were taken immediately at 405 nm to measure 4-nitrophenolate production. Steady state Michaelis-Menten parameters were determined by non-linear curve fitting using Origin version 7 and by Lineweaver-Burk plot (not shown).

4.6 Determination of optimal pH and temperature

The pH optimum for ZgGH129 was determined first in Universal buffer [61, 62] between pH 2-8 using 20 mL of stock solution of 50 mM Na_2HPO_4 , 33 mM citric acid, 50.7 mM boric acid, 243 mM NaOH which was diluted down with appropriate amounts of 0.1 M HCl for the desired pH and topped up to 100 mL with water. The reactions were set up in triplicate at 25°C in a volume of 150 μL with 144.6 μL of the diluted stock solution at the desired pH, 1 mM 3,6-ADG-*p*NP, and 184.8 nM of ZgGH129. A second pH curve was determined with reactions set up in triplicate in a volume of 150 μL with 144.6 μL of 50 mM

citrate buffer between pH 3-7, 1 mM 3,6-ADG-*p*NP, and 177.6 nM of ZgGH129. Continuous reads were collected spectrophotometrically at the isosbestic point of 4-nitrophenol/4-nitrophenolate (348 nm). For determining optimal temperature reactions (150 μ L) contained 5 nM of ZgGH129, 0.25 mM 3,6-ADG-*p*NP, 1.3 mM CaCl₂ and 50 mM sodium acetate buffer pH 5. Reactions were stopped with 75 μ L 1 M glycine at pH 10.88. Stopped reads were read spectrophotometrically at 405 nm and the rate of reaction was determined using the 4-nitrophenolate extinction coefficient of 18.5 M⁻¹ cm⁻¹.

5. Data Availability

The detailed phylogenetic tree can be found in Supplementary File 1. The ¹H and ¹³C NMR spectra of synthesised compounds is also found in Supplementary File 2.

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Figures

Figure 1. Substrate specificity of *exo*-(α -1,3)-3,6-anhydro-D-galactosidases and their relationship to other enzymes in the GH129 family. (A) The structures of *exo*-neo- β -carrabiose and *endo*-neo- κ - and *endo*-neo- ι -carrabiose, which contain the individual monosaccharides 3,6-anhydro- α -D-galactose (3,6-ADG) and D-galactose (D-Gal). The molecules differ in structure due to the degree of sulfation (levels $\beta < \kappa < \iota$). The *exo*-neo- β -motif and bond that is cleaved by ZgGH129 is shown in neo- β -carrabiose. (B) Structural overlap of the active sites of the two characterised members of GH129. BbGH129 [pdb id 5wzn [28]] is shown in green in complex with GalNAc in teal. ZgGH129 [pdb id 5opq [6]] is shown in salmon for one monomer with the amino acids from the other monomer shown in yellow. Calcium from BbGH129 is depicted as a maroon sphere. (C) Phylogenetic tree of the GH129 family of glycoside hydrolases with three GH101 enzymes chosen for the outgroup [30-32]. The amino acid sequences were aligned using MAFFT [54] with the L-INS-i algorithm and the alignment was manually refined using BioEdit [55]. The evolutionary history was inferred by using the Maximum Likelihood method and Whelan And Goldman model [29] in MEGA X [56]. The conformations of the substrates, 3,6-ADG and GalNAc, for the different clade enzymes illustrates the differences between the two enzyme activities.

Figure 2. Characterization of the carrageenan oligosaccharides released from enzymatic digestion by MALDI-TOF-MS measurement in negative ionisation mode. (A) Initial oligosaccharides released by the κ -carrageenase enzymatic activity from *Pseudoalteromonas carrageenovora* [23] and (B) followed by the action of ZgGH129. The oligosaccharides resulting from the activity of ZgGH129 are indicated by arrows. The oligosaccharides attributed to sulfate loss during the experiment are labeled.

Figure 3. Structural characterization of β - ι - κ DP6 oligosaccharide and its enzymatic products obtained by ESI-q-TOF-MSMS analysis in negative ionisation mode. The upper spectrum (A) was obtained by

isolating the $[M-3H+Na]^{2-}$ ion at m/z 598.1 which corresponds to the neo- β -1- κ DP6 oligosaccharide. The bottom spectrum (B) was obtained by isolating the $[M-3H+Na]^{2-}$ ion at m/z 526.0 which corresponds to the loss of the non-reducing α -1,3-linked 3,6-anhydro-D-galactose from the neo- β -1- κ DP6 oligosaccharide after treatment with ZgGH129. Peaks annotated with a red star correspond to doubly charged fragments (based on the isotope pattern). Fragments annotated (Δ) correspond to water losses. Fragments indicated ($\dagger$) correspond to sulfate losses. All unambiguous fragments are reported on the structures.

Figure 4. Activity of ZgGH129 on the chromogenic substrate 3,6-ADG-*p*NP, all reactions were performed in triplicate. The pH optimum of ZgGH129 in (A) Universal buffer [61, 62] and (B) Citrate buffer, (C) Effect of temperature on the activity of ZgGH129 on 3,6-ADG-*p*NP at pH 5.0, (D) Michaelis-Menten non-linear curve fitting using Origin7. Reactions were performed with 5 nM ZgGH129, 0.1 M NaOAc pH 5.0, 0.1 M CaCl₂ at the temperature optimum of 49 °C.

Scheme 1. Chemical synthesis of 4-nitrophenyl 3,6-anhydro- α -D-galactopyranoside (3,6-ADG-*p*NP).

Supplementary File 1: Expanded GH129 phylogenetic tree. Only bootstraps above 70 are shown.

Supplementary File 2: ¹H and ¹³C NMR spectra of synthesised compounds.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Scheme 1.