The evolutionary advantage of an aromatic clamp in plant family 3 glycoside exo-hydrolases

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In the barley β-D-glucan glucohydrolase, a glycoside hydrolase family 3 (GH3) enzyme, the Trp286/Trp434 clamp ensures β-D-glucosides binding, which is fundamental for substrate hydrolysis during plant growth and development. We employ mutagenesis, high-resolution X-ray crystallography, and multiscale molecular modelling methods to examine the binding and conformational behaviour of isomeric β-D-glucosides during substrate-product assisted processive catalysis that operates in GH3 hydrolases. Enzyme kinetics reveals that the W434H mutant retains broad specificity, while W434A behaves as a strict (1,3)-β-D-glucosidase. Investigations of reactant movements on the nanoscale reveal that processivity is sensitive to mutation-specific alterations of the tryptophan clamp. While wild-type and W434H utilise a lateral cavity for glucose displacement and sliding of (1,3)-linked hydrolytic products through the catalytic site without dissociation, consistent with their high hydrolytic rates, W434A does not adopt processive catalysis. Phylogenomic analyses of GH3 hydrolases disclose the evolutionary advantage of the tryptophan clamp that confers broad specificity, high catalytic efficiency, and processivity.

The barley (Hordeum vulgare L.) β-D-glucan glucohydrolase HvExoI is the only plant family 3 glycoside hydrolase (GH3) for which the atomic structure is available1-3, although the Carbohydrate-Active Enzymes CAZy database4 lists nearly 48,400 GH3 entries (August 2022) from archaea, bacteria, fungi, and eukaryotes, including economically important plants Triticum, Oryza, Glycine, Zea, Brassica, Medicago, Phaseolus, and Solanum.

HvExoI folds into a two-domain structure consisting of an (α/β)8 barrel (domain 1) and an (α/β)6 sandwich (domain 2) with a 13 Å-deep active site pocket embracing the subsites −1 and +1, positioned at the interface between the two domains. At the −1 subsite, the pocket contains the catalytic nucleophile Asp285 and the acid/base catalyst Glu491, while the +1 subsite is shaped in a clamp with a coin slot formed by the Trp286 and Trp434 residues that hold β-D-glucosides entering the active site in a productive mode for hydrolysis1-3 (Fig. 1a, b). HvExoI catalyses the hydrolytic removal of nonreducing glucose (Glc) moiety from a broad spectrum of poly- and oligomeric β-D-glucans. This hydrolytic reaction underpins a vital role of HvExoI during plant growth and development, to participate during e.g. primary root extension and the recycling of mixed-linkage (1,4;1,3)-β-D-glucans during germination and root elongation1-5. The capacity of HvExoI to hydrolyse a panel of β-D-glucosides was rationalised from the observation that non-reducing Glc at the −1 subsite is locked into a...
fixed position through a network of hydrogen bonds (H-bonds), while the reducing Glc moiety at the +1 subsite is constrained through stacking interactions, and thus the flexibility of attached substrates and their projection is unencumbered allowing HvExoI to accommodate isomeric (1,2)-, (1,3)-, (1,4)- and (1,6)-linked β-D-glucosides. It was estimated that the strength of binding through aromatic residues could be in the range of 2-5 kcal/mol.

Since the HvExoI structure was elucidated, many prokaryotic, fungal and unclassified metagenome GH3 structures have become available but provided little information on the substrate specificity of β-D-Glucan glucohydrolases, β-D-glucosidases/β-N-acetylhexosaminidases, β-D-xylosidases, and α-L-arabinofuranosidases.

Fig. 1 | Exo-hydrolytic enzymes of the GH3 family with an entrapped Glc molecule and a ligand-free form, and their phylogeny. a *Hordeum vulgare* HvExoI with entrapped Glc (PDB 3WLH; atomic colours), *Bacteroides ovatus* β-D-glucosidase with perfused Glc (PDB 5JP0; green cpk), and a ligand-free form of *Pseudoalteromonas* sp. exo-(1,4;1,3)-β-glucanase (PDB 3UTO; yellow cpk). Distances ache of HvExoI residues to Glc in the −1 subsite (black dashes) are within 3.3 Å. HvExoI and structurally equivalent residues in 3UTO and 5JP0 superpose with respective RMSD values of 0.7 Å and 1.2 Å. b Surface morphology of the active site pocket of HvExoI coloured by an electrostatic potential: white, neutral; red, −5 kT e⁻¹. Separations (dashed lines) between Glc and key interacting residues are within 3.3 Å. The positions of the −1 subsites in panels a and b are indicated. c Unrooted phylogenetic tree of representative GH3 family members (accession numbers listed in Supplementary Table 1) revealed the presence of four major clades with five activities [β-D-glucan glucohydrolases (EC 3.2.1.-), β-D-glucosidases (EC 3.2.1.21), β-N-acetylhexosaminidases (EC 3.2.1.52), β-D-xylosidases (EC 3.2.1.37), and α-L-arabinofuranosidases (EC 3.2.1.55)]. In β-D-glucan glucohydrolases, three subclades were identified with residue variability in the aromatic clamp (residue numbering corresponding to HvExoI; PDB 1EXI). The percentage of replicate trees with taxa clustering together in the bootstrap test (1000 replicates) are shown at node bipartitions.
this of enzymes. These include two-domain bacterial β-N-acetylglucosaminidases, for example, from Bacillus subtilis, and a β-N-acetylhexosaminidase from Salmonella typhimurium, while a β-hexosaminidase from Burkholderia concolor and Salmonella enterica subspp. enterica are known to have single-domain α/β sandwich structure. Several three-domain architectures consisting of an (α/β)n barrel, an (α/β)m sandwich, and another β-sandwich domain were identified in β-glucosidases from Thermotoga neapolitana, Hypocrea jecorina, and Aspergillus aculeatus. In a β-N-acetylglucosaminidase from Thermotoga neapolitana, in a (1,4;1,3)-β-exo-β-glucanase from Pseudomonas sp. BBI, and in a β-glucosidase JMB19063 isolated from metagenome. These additional β-sandwich domains are described as fibronectin type III or Ig-like β-sandwich folds and have similar architectures. Four-domains structures consisting of (α/β)n barrel, an (α/β)m sandwich, and two β-sandwich domain folds (PA14 and fibronectin type III) were identified in the β-glucosidases from Kluyveromyces marxianus, Aspergillus fumigatus, A. oryzae, and Streptomyces venezuelae. The precise roles of these additional β-sandwich domains have not been fully clarified. However, in some GH3 enzymes these domains are thought to be linked to substrate binding and specificity, protein structure stability, and oligomerisation, meaning that dimerisation is required for catalytic activity.

All of the factors are crucial to the catalytic mechanism in HvExoI. The precise information on the binding of positionally isomeric β-D-glucosides at the atomic level remains limited. Recently, we envisaged that β-D-glucan glucohydrases, such as HvExoI, adopt so-called substrate-product assisted processive catalysis, where an incoming substrate binds in a productive mode, the Glc product is displaced after a new substrate is attached. The productive binding of carbohydrates in HvExoI involves the participation of Trp286 and Trp434 residues to Ala, His, Phe, and Tyr to generate single (and in some instances) double mutants, and investigated their substrate specificity, steady-state and inhibition kinetics and substrate binding in atomic structures (Figs. 2–4; Supplementary Figs. 2–4; Supplementary Tables 2–6).

Substrate specificity of WT, and Trp286 and Trp434 mutants
We compared relative hydrolytic rates of β-D-linked poly- and oligosaccharides, and aryl-glycosides under optimal physico-chemical conditions (Supplementary Fig. 1; Supplementary Table 2; Supplementary Note 1). Of the tested mutants, W434H was the only mutant that exhibited broad substrate specificity similar to WT, contrary to W286F and W286Y, and W434F, W434Y, W286F/W434F, and W286F/W434A that lacked activities of at least with one carbohydrate substrate (Supplementary Table 2). The absence of hydrolytic activities was observed with (1,4;1,3)-β-D-glucans (barley glucan and lichenin) and 4NP-cellobioside in all mutants except W434H and a decrease in hydrolytic rates with oligomeric substrates and aryl β-D-glucosides except in W286Y with 4-nitrophenyl β-D-glucopyranoside (4NP-Glc) (Supplementary Table 2). Remarkably, W434A lost all hydrolytic activities with poly- and oligosaccharides, except (1,3)-β-D-linked β-D-glucosides, turning this mutant into a strict (1,3)-β-glucosidase, albeit with low specific activities (Supplementary Table 2). Our findings suggest that although the mutations of Trp286 to Ala and His led to folded but inactive enzymes, enzyme activity retention is more sensitive to alterations of Trp286 than Trp434, likely due to the proximity of Trp286 to Asp285, the catalytic nucleophile (Supplementary Table 2).
Table 3). This trend continued with the catalytic efficiency of the double W286F/W434F and W286F/W434A mutants with poly- and disaccharides, but by an about 21-fold decrease of inhibition constants (except G2SG-OMe) thio-analogues showed a competitive inhibition with WT, whereas the W434H and W434F mutants showed higher catalytic efficiency compared to WT, whereas the W434H and W434F mutants showed increased catalytic efficiency values with (1,3)-, (1,4)-, and (1,6)-linkages to quantify apparent inhibition constants ($K_i$) of the HvExoI:inhibitor complexes (Supplementary Table 4). In WT HvExoI, the (1,3)-β-glucosidase (4NP-G3SG; (1,4)-β-glucosidase (G6SG-OMe; green) moieties are bound across the -1 to +2 subsites. Panels a to d contain derived difference 2$F_o$-2$F_i$ electron density maps (blue) of thio-analogues (also shown as insets rotated by around -90° via x-axes relative to main panels) contoured at 1.0 σ levels. Glycerol (Gol) or polyethylene glycol (PEG) and water molecules are shown in cpk orange sticks and red spheres, respectively. Distances at separations within 3.3 Å are shown in dashed lines. Ligand designations above structural images indicate thio-analogues that were perfused in crystals.

Steady-state kinetic constants of WT, and Trp286 and Trp434 mutants

Consistent with our predictions, catalytic rate $k_{cat}$ (first-order rate) constants and in some instances, Michaelis-Menten $K_M$ values of single W286A, W286H, W286F, W434A, W434H, W434F, and W434Y, and double W286F/W434F and W286F/W434A mutants with poly- and oligomeric substrates decreased compared to WT (Supplementary Table 3). This trend continued with the catalytic efficiency $k_{cat}/K_M$ (second-order rate) constants, where these values corresponded to those of relative hydrolytic rates (Supplementary Table 2). These values confirmed the strict (1,3)-β-oligosaccharide specificity of W434A, with little change in the apparent strength of binding of laminaribiose, but by an about 21-fold decrease of $k_{cat}$ (compared to WT), consequently yielding a low $k_{cat}/K_M$ value. A similar profile was noted for W286F/W434A, although in this case, the kinetic parameters decreased even more, pointing to a negative effect of the non-conservative (radical) W434A mutation on substrate binding and hydrolysis (Supplementary Table 3). Notable was the behaviour of W286Y that showed increased catalytic efficiency values with (1,3,1,6)-β-β-glucan (laminarin), laminaribiose (G3OG), and 4NP-Glc, while these values in W434Y were considerably lower (Supplementary Table 3). In W286F/W434F, the catalytic efficiency values were also drastically reduced compared to WT, whereas the W434H and W434F mutants showed higher catalytic efficiency than the double mutants, with W434F being more active except with laminaribiose. These data signal that although the mutations of Trp434 affected the catalytic properties of HvExoI more substantially compared to Trp286, both Trp residues are required for binding the isomeric β-glucosides to achieve productive binding modes required for high catalytic efficiency.

Inhibition constants $K_i$ of WT, and Trp286 and Trp434 mutants

To further examine binding modes of β-glucosides, we used the thio-analogues with (1,2)-, (1,3)-, (1,4)- and (1,6)-linkages to quantify apparent inhibition constants ($K_i$) of the HvExoI:inhibitor complexes (Supplementary Table 4). In WT HvExoI, the (1,3)-β-glucosidase (4NP-G3SG), (1,4)-β-glucosidase (G4SG4OG4S; cyan); and (1,6)-β-glucosidase (G6SG-OMe; green) moieties are bound across the -1 to +2 subsites. Panels a to d contain derived difference 2$F_o$-2$F_i$ electron density maps (blue) of thio-analogues (also shown as insets rotated by around -90° via x-axes relative to main panels) contoured at 1.0 σ levels. Glycerol (Gol) or polyethylene glycol (PEG) and water molecules are shown in cpk orange sticks and red spheres, respectively. Distances at separations within 3.3 Å are shown in dashed lines. Ligand designations above structural images indicate thio-analogues that were perfused in crystals.

WT and mutant HvExoI:thio-β-glucoside complexes reveal the details of binding at the atomic level

We observed that the thio-analogue $K_i$ constants (except G2SG-OMe) measured with the Trp286 and Trp434 mutants, correlated with their capacity to hydrolyse (1,3)-, (1,4)- and (1,6)-linked β-glucosides. To decipher their precise binding modes at the atomic levels, we pursued structural studies (Figs. 2–4; Supplementary Figs. 2–4; Supplementary Tables 5 and 6).

![Figure 2](https://example.com/figure2.png)
G2SG-OMe binding. The G2SG-OMe thio-analogue bound weakly to native and WT recombinant HvExol with the respective $K_i$ of 2 $\times 10^{-3}$ M and 4 $\times 10^{-3}$ M (Supplementary Table 4), reflecting its ability to attach at the +1 and +2 subsites instead of the −1 and +1 subsites (Fig. 2a)\(^{22}\), which was most likely due to the rigidity of this thio-ligand. On the other hand, in W286F the electron density map of G2SG-OMe was defined at the −1 and +1 subsites (Supplementary Fig. 2c), where this thio-ligand formed H-bonds with Asp95, Lys206, His207, Arg158, Tyr253, Asp285, Arg291, and Glu491, and stacking interactions with W286F and Trp343, whereas G2SG-OMe did not bind to W286A and W286Y. The dispositions of G2SG-OMe at the −1 and +1 subsites in the W434A, W434F, W434H, and W434Y mutants (Figs. 3a, 4a; Supplementary Figs. 3a and 4a) revealed similar structural features of the ligand binding to those seen in W286F (Supplementary Fig. 2c).

4NP-G3SG and 4NP-G3SG3OG binding. 4NP-G3SG bound tightly to native and WT recombinant HvExol with the $K_i$ constant of 0.7 $\times 10^{-3}$ M (Supplementary Table 4); the $K_i$ value of 0.2 $\times 10^{-3}$ M was previously observed with 4NP-G3SG3OG in WT HvExol.\(^{1}\) These relatively tight binding constants suggested that these thio-analogues should be seen in the active sites, as observed with WT, and the W286F, W434A, W434F, W434H, and W434Y mutants. The well-defined electron density maps of the (1,3)-linked thio-ligands illustrated that the saccharide moieties were attached through the networks of H-bonds formed between nine to ten residues in each case (Figs. 2b, 3d, 4b; Supplementary Figs. 2d, 3b, 4b). The 4NP-G3SG thio-analogue in WT was located at the +1 to +2 subsites, with the 4NP group clearly discernible at the +2 subsite (Fig. 2b). However, in W286A, and W286Y, 4NP-G3SG was not observed, and in W286F, the reducing-end Glc moiety of 4NP-G3SG at the +1 subsite remained disordered (Supplementary Fig. 2d). This emphasised that the Trp286 mutations to Ala, Tyr, and Phe disturbed the binding of (1,3)-linked thio-β-glucosides and that this residue was necessary for inhibitor binding.

G4SG-OMe and G4SG4OG4SG4OG4SG binding. G4SG-OMe bound to native and WT recombinant HvExol with the $K_i$ values of 1.0 $\times 10^{-3}$ M (Supplementary Table 4). This compares favourably to the hexasaccharide G4SG4OG4SG4OG4SG, where we previously observed the $K_i$ constant of 0.6 $\times 10^{-3}$ M with native HvExol.\(^{1}\) These binding constants suggested that the (1,4)-linked thio-analogues should locate in the enzyme’s active sites. This occurred with WT, and the W434A, W434F, W434H, and W434Y mutants, while no binding was observed in the W286A, W286Y, and W286F mutants. The well-defined electron density maps in W434A (Fig. 3c) and W434F (Supplementary Fig. 3c) revealed three saccharide moieties at the −1 to +2 subsites with the reducing thio-saccharide moiety protruding beyond the environment of the active site pocket at the +2 subsite. In W434A, we observed that the tri-thio-cellobioside moiety was somewhat misplaced (compared to WT) and that the W434A substitution unexpectedly allowed Lys493 to be rotated outward and become engaged in the binding of this thio-ligand; here, 11 residues participated in forming H-bonds with tri-thio-cellobioside moiety (Fig. 3c). To generate the W434H and W434Y complexes, we perfused the G4SG-OMe ligand, consequently, only two
thio-saccharide moieties were observed in active sites (Fig. 4c; Supplementary Fig. 4c). But with W286H, and W286Y, we could not see the binding of G4SG-OMe, which correlated with their weaker \( K_i \) values compared to WT (Supplementary Table 4), hinting that this thio-analogue should be observed in the active sites of WT and mutants (Figs. 2d, 3d, 4d; Supplementary Figs. 2a, b, e, 3d, 4d). This is what we indeed observed in WT, and the W286A, W286F, W434A, W434F, W434H, and W434Y mutants, where well-defined electron density maps were formed through eight to ten residues (Asp95, Lys206, His207, Arg158, Tyr253, Gly57, Arg291, Glu491, W434H, Trp286). In W434A we noticed approximately 120° rotation (through the x-axis) of the reducing-end \(-\text{D-glucopyranose}'s\) aromatic residues) of Trp286 or Trp434. Although these mutations may have been perturbed. These findings reinforce the conclusion that G6SG-OMe binds in HvExol regardless of mutations (particularly into aromatic residues) of Trp286 or Trp434. Among these mutations impact the precise orientation of the glucopyranoside moieties, as observed in W434A, they permit G6SG-OMe binding.

**G6SG-OMe binding.** G6SG-OMe bound tightly to native and WT recombinant HvExol with the respective \( K_i \) values of 0.1 ×10^{-3} M and 0.2 ×10^{-3} M (Supplementary Table 2a, b, e, 3d, 4d). This is what we indeed observed in WT, and the W286A, W286Y, W286F, W434A, W434F, W434H, and W434Y mutants, where well-defined electron density maps of G6SG-OMe and tight H-bond networks that were formed through eight to ten residues (Asp95, Lys206, His207, Arg158, Tyr253, Asp285, Arg291, Glu491, W434H, and Trp286). In W434A we noticed approximately 120° rotation (through the x-axis) of the reducing-end moiety of G6SG-OMe compared to its disposition in WT (or in-solution structure of G6OG or gentiobiose), due to the presence of a rotatable \( C_1-O-C_6 \) glycosidic bond of G6SG-OMe; this did not occur in WT or other mutants. To find out, if the Glc molecule alone adopts similar or different dispositions at the −1 and +1 subsites, we perfused Glc into WT, W434A, and W286Y, and compared their positions, which at the −1 subsite were identical in all instances, but at the +1 subsite of W434A the Glc molecule was disordered (data not shown). We noted that the quality of the G6SG-OMe electron density map slightly declined in W286Y (Supplementary Fig. 2b) but that these maps were well-formed in W286A and W286F (Supplementary Fig. 2a, e), suggesting that in the W286Y mutant the process of ligand-binding in (or near) the active site may have been perturbed. These findings reinforce the conclusion that G6SG-OMe binds in HvExol regardless of mutations (particularly into aromatic residues) of Trp286 or Trp434. Although these mutations impact the precise orientation of the glucopyranoside moieties, as observed in W434A, they permit G6SG-OMe binding.

**Conformational behaviour of S- and O-linked \( \beta-\)glucosides.** To determine the conformational landscape of \( \beta-\)glucosides bound in HvExol, we analysed enzyme-thio-ligand-binding modes in the crystal structures before and after cMD simulations. As described above, the \( \beta-\)glucopyranose rings of thio-saccharide analogues at the −1 subsites in the WT crystal structure complexes were engaged in H-bond networks and observed in the \( C_1 \) conformation for G2SG-OMe and G6SG-OMe, the \( H_1 \) conformation for 4NP-G3SG, and the \( E/H_1 \) conformations for G4SG4OG (Supplementary Figs. 5 and 6; Supplementary Table 7), while the \( \beta-\)glucopyranose moieties of thio-ligands at the +1 subsites adopted the \( C_1 \) conformation. To analyse the dynamic behaviour of \( \beta-\)glucosides, a total of eight models were generated for G2(S or O)G, G3(S or O)G, G4(S or O)G, and G6(S or O)G systems, wherein we reverted mutations (if present) in HvExol, removed ions and other molecules from thio-analogues, and for the O-linked systems, replaced S-atoms with O-atoms. While cMD simulations revealed that \( \beta-\)glucopyranose rings in thio-analogues adopted predominantly the \( C_1 \) conformations in both subsites, alternate

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**Fig. 4 | Thio-saccharide analogues are bound in the active site of the HvExol W434H mutant.** a Thio-sophoroside (G2SG-OMe, pink); b thio-laminaribioside (4NP-G3SG, magenta); c thio-celllobioide (G4SG; cyan); and d thio-gentiobioside (G6SG-OMe, green) moieties are bound across the −1 to +2 subsites. Panels a to d contain derived difference 2\(F_c\) −\(|DF_c|\) electron density maps (blue) of thio-

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[Image of molecular structures with labels and annotations related to the text content.]
conformations also participated, such as $^4H_3/E/^4H_5$ or $^1S_3/1,4B$, depending on a type of the glycosidic linkage (Fig. 5; Supplementary Fig. 6). The cluster analysis of the trajectory of the WT HvExoI:G4SG4OG complex with G4OG3OG was generated based on the WT HvExoI:G4SG4OG complex (Fig. 2c), upon replacing G4 with G3. The analysis of cMD simulations of this complex showed that the Glc moieties of G4OG3OG adopted the stable $^4C_1$ conformations at the +1 and +2 subsites (Supplementary Fig. 10). However, Glc at the −1 subsite adopted a state, that was nearly identical to the Glc moiety in G4SG4OG, and not entirely a perfect chair, thus we defined it as a pseudo-chair (Supplementary Fig. 10b). Again, this is consistent with the conformational FEL of G4OG3OG at −1 subsite, computed by QM/MM MD metadynamics (Fig. 6a; Supplementary Fig. 11), which shows a wide free energy minimum that extends from $^4C_1$ towards $^4H_4$.

Additionally, we investigated the binding of the G3OG product originating from the G4OG3OG substrate in WT and the W434A mutant, after the non-reducing Glc moiety was cleaved off, using cMD simulations (Figs. 6b, c). The cluster analysis of the trajectory of the G3OG product in WT indicated that the Glc moieties adopted at least two poses: ~96% of the populations located in the +1 and +2 subsites, and ~4% to the intermediary poses at the −1 and +1 subsites (Fig. 6b). Conversely, the cluster analysis of the G3OG product in W434A indicated a dramatic shift in poses to ~15% of the populations located in the +1 and +2 subsites, and ~74% to the intermediary (midway) poses at the −1 and +1 subsites (Fig. 6c). These observations imply that Trp434 plays a key role in the sliding of the (1,3)-β-D-glucan hydrolytic products through the active site during catalysis and that Trp434 prevents the sliding of mixed-linkage substrates.

**Binding and hydrolysis of the G4OG3OG natural substrate were explored via cMD and QM/MM MD metadynamics simulations**

To understand the binding of one of the natural substrates of HvExoI, we selected the kinked G4OG3OG oligosaccharide (Fig. 6; Supplementary Figs. 10 and 11), which is the key hydrolytic product originating from (1,4;1,3)-β-D-glucans; these polymers fulfi the role of structural cell wall components in cereals, such as barley. The complex with G4OG3OG was generated based on the WT HvExo1:G4SG4OG complex (Fig. 2c), upon replacing G4 with G3. The analysis of cMD simulations of this complex showed that the Glc moieties of G4OG3OG adopted the stable $^4C_1$ conformations at the +1 and +2 subsites (Supplementary Fig. 10). However, Glc at the −1 subsite adopted a state, that was nearly identical to the Glc moiety in G4SG4OG, and not entirely a perfect chair, thus we defined it as a pseudo-chair (Supplementary Fig. 10b). Again, this is consistent with the conformational FEL of G4OG3OG at −1 subsite, computed by QM/MM MD metadynamics (Fig. 6a; Supplementary Fig. 11), which shows a wide free energy minimum that extends from $^4C_1$ towards $^4H_4$.

Additionally, we investigated the binding of the G3OG product originating from the G4OG3OG substrate in WT and the W434A mutant, after the non-reducing Glc moiety was cleaved off, using cMD simulations (Figs. 6b, c). The cluster analysis of the trajectory of the G3OG product in WT indicated that the Glc moieties adopted at least two poses: ~96% of the populations located in the +1 and +2 subsites, and ~4% to the intermediary poses at the −1 and +1 subsites (Fig. 6b). Conversely, the cluster analysis of the G3OG product in W434A indicated a dramatic shift in poses to ~15% of the populations located in the +1 and +2 subsites, and ~74% to the intermediary (midway) poses at the −1 and +1 subsites (Fig. 6c). These observations imply that Trp434 plays a key role in the sliding of the (1,3)-β-D-glucan hydrolytic products through the active site during catalysis and that Trp434 prevents the sliding of mixed-linkage substrates.

**Binding and displacements of laminaritriose (G3OG3OG) and laminaribiose (G3OG3OG3OG3OG) hydrolytic products**

These studies were performed in the WT, W434H, and W434A enzymes with active sites accommodating hydrolytic products of laminaritriose, i.e. Glc in the −1 subsite and laminaribiose (G3OG) in the +1 and +2 subsites, or laminaribiose, i.e. Glc at the −1 subsite and laminaritriose (G3OG3OG3OG3OG) in the +1 and +2 subsites, and exposed to bulk solvent. The selection of these mutants was based on relative hydrolytic rates of poly- and oligosaccharide substrates, where these mutants either retain (W434H) or lose (W434A) their broad substrate

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**Fig. 5** | Conformational behaviour of O-linked oligosaccharides bound in the active site of HvExo1 calculated by cMD simulations plotted as a function of $\phi$ and $\psi$ puckering coordinates. a) Sophorose (G2OG; pink); b) laminaribiose (G3OG; magenta); c) cellulobiose (G4OG4OG; cyan); and d) gentiobiose (G6OG; green) ligands are bound at the −1 to +2 subsites (colour gradients from purple at 0 ns to yellow at 200 ns in Mercator projections are indicated). Complexes of WT with G3SG, G4SG4OG, G6SG, and W434A in complex with G2SG were used to predict binding modes of O-linked oligosaccharides. The β-D-glucopyranose moieties at the −1 to +2 subsites, and displacements of Asp285 and Glu491 catalytic residues are also shown.
specificity (Supplementary Table 2). To assess the effect of saccharides on HvExoI behaviour, cMD simulations were also performed with the WT apo-form, lacking bound Glc or β-D-glucosides.

The cMD and GaMD simulations with laminaritriose and laminarihexaose hydrolytic products indicated that the WT structure was stable (Supplementary Fig. 12), as indicated by backbone Root-Mean-Square-Deviation (RMSD) values, although high mobilities in some loops were detected. Conversely, cMD and GaMD simulations of W434H and W434A with laminaritriose and laminarihexaose hydrolytic products showed contrasting behaviour compared to WT (Supplementary Fig. 12). cMD simulations of the mutants with the Glc and the G3OG products (run for 1000 ns) revealed that the protein backbones remained stable (respective RMSD values: 1.7 Å for W434H, and 1.25 Å for W434A) and that Glc was attached at the −1 subsite, but, after around 100 ns in W434H and 200 ns in W434A, G3OG moved to bulk solvent, indicating that the mutants were ineffective in retaining the G3OG product (Supplementary Fig. 13). Such behaviour was
not observed with laminarhexaose hydrolytic products, within the time-frames of cMD simulations (600 ns), whereby these products remained attached in WT and both mutants (Supplementary Fig. 13). These simulations indicate that the dynamics of ternary complexes of mutants presented variabilities compared to WT, where these differences depend on the length of hydrolytic products bound, i.e. longer products were more stable.

To resolve variability with the length of hydrolytic products in mutants, GaMD simulations (800 ns) with enhanced sampling were conducted. We found that the protein scaffold of the W434A mutant was more flexible (RMSD value 2.5 Å) than that of WT or W434H (RMSD values between 1.0 Å – 1.5 Å) (Supplementary Fig. 12), and that the Gly210-Met230 loop in the vicinity of the catalytic site exhibited Root-Mean-Square-Fluctuation (RMSF) values of up to 7 Å in both mutants compared to WT (RMSF value ≤ 2 Å). While Glc remained highly restrained in the –1 subsite in WT and W434H, it was mobile in W434A, hinting that the overall flexibility of the catalytic site was little affected in W434H, but strongly affected in W434A. During GaMD simulations, the G3OG and laminaripentaose products remained bound in WT, but G3OG vacated the catalytic site in both mutants, in contrast to laminaripentaose, which initially remained bound (~25% and ~50% of simulation time in W434A and W434H, respectively), but eventually it traversed to bulk solvent. These findings suggested that the release of Glc in catalytic cavity (and bulk solvent) in WT, W434H, and W434A, we identified path 3 (we termed it vertical) for Glc displacement (Fig. 8), where Glc utilised the space vacated by laminaripentaose and exited via the Trp286/Trp434 aromatic clamp, and through the +2 subsite into bulk solvent. This path could only be available when no products or substrates are bound. This scenario for Glc egress is, however, only a hypothetical option, also challenging our previous study22, where in native HvExol, two Glc molecules were bound at the –1 and +1 subsites, effectively blocking path 3.

In summary, GPathFinder simulations revealed that path 1 is the preferred route in WT and W343H with bound (1,3)-linked hydrolytic products, and is independent of a substrate length, contrary to W434A, which utilises path 2, where the essential structural attributes of substrate-product assisted processivity (toll-like barrier, salt bridge, loop motions, lateral cavity) do not participate.

**Discussion**

In nature, processive (non-dissociative) catalysis is omnipresent in enzymes that synthesise, modify, and degrade biopolymers such as nucleic acids, proteins, and polysaccharides, and has been described in nucleases, peptideases, proteases, proteasomes, and endo- and exo-acting polysaccharide hydrolases22,25,26–31. Processive polysaccharide hydrolases have certain common characteristics that stem from the architectures of their catalytic sites that dictate spatial arrangements governing substrate specificity and binding (including directionality) and the dispositions of catalytic residues to ensure a high efficiency of catalysis. Processive endo- and exo-acting cellulases25,29–31 and exo-acting chitinases23,24,29,30 are known to be driven by processive hydrolysis or substrate-assisted catalysis, and the GH3 exo-acting β-glucan glycohydrolases with a pocket-shaped active site (such as HvExol) by substrate-product assisted processive catalysis23. The difference between these two types is in the role of a hydrolytic product, that in the latter group evokes the formation of a lateral cavity, which serves as a conduit for Glc egress to allow for the next catalytic cycle using the retaining catalytic mechanism (Fig. 9). This type of catalysis enables multiple events that proceed without an enzyme losing contact with oligo- or polymeric substrates, contrary to distributive catalysis, where the catalyst follows a conventional association-conversion-dissociation pattern.

The crucial role of substrate binding in HvExol implies a pair of the aromatic Trp286 and Trp434 residues that form a clamp at the –1 subsite, which is positioned next to a pocket-shaped site at the –1 subsite, housing the Asp285 and Glu491 catalysts1. These residues, typically around 5.5–6.5 Å apart locked in a rigid configuration via a dense H-bond network, are responsible for the hydrolysis of oligo- and polymeric β-glucosides to release Glc from non-reducing ends of substrates (Figs. 1a, b, and 9). To understand the mechanism that drives the unidirectional motion of the Glc product in HvExol during substrate-product assisted processive catalysis, and specifically the roles of Trp286 and Trp434, we analysed substrate specificity in the context of elementary steps of Glc movement. We created HvExol mutants replacing Trp286 and Trp434, with Ala, Phe, Tyr, and His residues, and compared their substrate specificity, steady-state
We examined the conformational behaviour of thio-green algae assisted processivity, where data were corroborated with phylogenomic analyses of GH3 family laminarihexaose hydrolytic products in the W434H and W434A catalytic site or binding and displacements of laminaritriose and (1,6)-linked poly-, and oligosaccharides, and aryl-glycosides, were substantially altered. The W434A mutant lost its hydrolytic activities under a variety of pH and temperature conditions was relatively small compared to WT, except for W286H with its pH optimum shifted to the alkaline region (from 5.25 for WT to 6.7 for W286H) (Supplementary Fig. 1) due to a proximity effect to Asp285. However, the relative hydrolytic rates and specificities of mutants with (1,2)-, (1,3)-, (1,4) and (1,6)-linked poly-, and oligosaccharides, and aryl-glycosides, were substantially altered. The W434A mutant lost its hydrolytic activities with polymers and oligomers, except (1,3)-linked oligosaccharides.

 kinetics, and inhibition parameters relative to WT. We conducted atomic resolution (1.5 Å–2.5 Å) X-ray crystallographic analyses of 25 structures of WT and mutant forms in complex with positional isomers of thio-analogues, revealing the details of β-N-glucoside binding. We examined the conformational behaviour of thio-analogues and O-linked β-N-glucosides, and explored the binding and hydrolysis of the G4OG3OG natural substrate via cMD and QM/MM MD metadynamics simulations. To define the atomistic details and dynamics of reactant movements, we examined the unidirectional motion of Glc and its egress, and substrate sliding through the catalytic site or binding and displacements of laminaritriose and laminarihexaose hydrolytic products in the W434H and W434A mutants through cMD, GaMD, and GPathFinder simulations. These data were corroborated with phylogenomic analyses of GH3 family members, to trace the evolutionary origin of substrate-product assisted processivity, where β-N-glucan glucohydrolases are expected to retain an aromatic Trp286/Trp434 clamp. In the unrooted phylogenetic tree (Fig. 1c; Supplementary Table 1), we observed the clustering of plant β-N-glucan glucohydrolases to subclades 1-3 with the participation of the Arg158-Asp285-Glu491 toll-like barrier (triangles in dashed lines; distances indicated in Å). Surface morphologies of structures are coloured by electrostatic potentials: white, neutral; blue, −5 kT e⁻¹; red, −5 kT e⁻¹. Separations in Å between the positions of C1 carbons of Glc molecules, as they move from the −1 subsite in initial and final structures are indicated. The lateral cavity (a, b, c indicated in black dotted ellipsoids) forms transiently, is partly exposed to bulk solvent, and facilitates Glc displacement. In panel c, Glc is displaced to bulk solvent through the opening formed by the W434A mutation.

Fig. 7 | Glc displacement routes in WT, and the W434H, and W434A mutants of HvExoI with Glc and laminariptaenose, calculated through cMD and GPath-Finder. a WT; b W434H; c W434A – Four steps along Glc displacement routes based on converged structures in complex with Glc (−1 subsite; carbons in cpk yellow lines, and spheres) and laminariptaenose (+1 and +2 subsites and bulk solvent; carbons: magenta sticks) obtained by docking. Selected residues (carbons: atomic sticks), and positions of Glc, and laminariptaenose are indicated. Glc in the −1 subsite is separated from the lateral cavity that is evolving during Glc egress by the participation of the Arg158-Asp285-Glu491 toll-like barrier (triangles in dashed lines; distances indicated in Å). Surface morphologies of structures are coloured by electrostatic potentials: white, neutral; blue, −5 kT e⁻¹; red, −5 kT e⁻¹. Separations in Å between the positions of C1 carbons of Glc molecules, as they move from the −1 subsite in initial and final structures are indicated. The lateral cavity (a, b, c indicated in black dotted ellipsoids) forms transiently, is partly exposed to bulk solvent, and facilitates Glc displacement. In panel c, Glc is displaced to bulk solvent through the opening formed by the W434A mutation.

Dicystostelium, and have emerged with the evolution of land plants – these enzymes are present in liverworts and mosses (Embryophyta), ferns and conifers (Tracheophyta), a basal Angiosperm Amborella trichopoda, and dicotyledons (Eudicots) and monocotyledons (including grasses) (Fig. 1c). The β-N-glucan glucohydrolases with the Trp286/Trp434 signature are also richly represented in bacteria, e.g. the sludge Chloroflexi bacterium fermenting carbohydrates, and a plant-pathogenic Xanthomonas signature in bacteria, e.g. the sludge Chloroflexi bacterium fermenting carbohydrates, and a plant-pathogenic Xanthomonas signature in bacteria, e.g. the sludge Chloroflexi bacterium fermenting carbohydrates. The β-N-glucan glucohydrolases from Rhodothermus marinus and Saccharopolyspora erythraea have the Tyr286-Trp434 signature.

During the investigations of physicochemical properties and substrate specificity in HvExoI and its mutants, we detected that the impact of Trp286 and Trp434 mutations on the hydrolysis of 4NP-Glc under a variety of pH and temperature conditions was relatively small compared to WT, except for W286H with its pH optimum shifted to the alkaline region (from 5.25 for WT to 6.7 for W286H) (Supplementary Fig. 1) due to a proximity effect to Asp285. However, the relative hydrolytic rates and specificities of mutants with (1,2)-, (1,3)-, (1,4) and (1,6)-linked poly-, and oligosaccharides, and aryl-glycosides, were substantially altered. The W434A mutant lost its hydrolytic activities with polymers and oligomers, except (1,3)-linked oligosaccharides,
and acted as a strict (1,3)-β-D-glucosidase (Supplementary Table 2). In this mutant, no activity was observed with laminarin [(1,3)-β-D-linked polymer with (1,6)-β-D-branching at the 3:2 ratio] suggesting that the binding of branched substrates may be influenced by steric effects. These modified hydrolytic rates in the Trp286 and Trp434 mutants were followed by apparent first- and second-order rate kinetic (Supplementary Table 3) and apparent inhibition constants (Supplementary Table 4). Changes in these parameters suggested that Trp286 and Trp434 play key roles in orienting positionally isomeric β-D-glucosides towards the formation of productive HvExoI:substrate complexes.

To address the atomic details underlying substrate binding and hydrolysis, the crystalline WT and mutant HvExoI forms were perfused with (1,2)-, (1,3)-, (1,4)- and (1,6)-linked thio-β-D-glucosides followed by X-ray crystallography analyses (Figs. 2–4; Supplementary Figs. 2–4; Supplementary Tables 5 and 6) to assess the contributions of residues that interacted with glycone/aglycone moieties and thus their involvements in catalysis. Our interpretations of the electron density maps of these complexes (25 in total) led us to postulate that it was primarily the aromatic clamp, that binds variously linked thio-glucosides at the +1 subsite, and mediates broad substrate specificity in HvExoI. In the bound (1,2)- and (1,6)-linked thio-glucosides, we observed C1 conformations for Glc moieties at the −1 subsite, or H3 or H2/E respective conformations for the (1,3)- and (1,4)-linked thio-glucosides (Supplementary Table 7), suggesting that certain conformational flexibility of glucopyranose rings was present. This was supported by our previous observations that β-glucono-1,5-lactone (transition state analogue) with inherently a half-chair conformation was an effective inhibitor (Ki = 2.1 × 10−6 M)21. This is acceptable because the active site must be able not only to recognise the ground state of saccharides in C1 conformation but also distorted conformations43,44. We concluded that the electron density maps observed in the complexes could represent snapshots of time-space averaged conformations, that are formed at different time intervals on the substrate-binding trajectory from the ground to transition states.

One significant observation stemmed from the comparative analyses in terms of binding of (1,2)-, (1,3)-, (1,4)- and (1,6)-linked thio-glucosides by HvExoI (Figs. 2–4; Supplementary Figs. 2–4). In these WT complexes, the Glc moiety at the −1 subsite was oriented almost identically, contrary to the Glc moiety at the +1 subsite that adopted various orientations. In G6SG the apolar β face of Glc moiety was exposed to Trp286, while in the HvExoI:G2SG,:G4SG, and:G6SG complexes, the apolar thio-analogue sides mostly faced Trp434. It is known that in these stacking interactions, the saccharide surface encounters a side-chain aromatic moiety when an apolar face of a saccharide exposes three or more C–H groups close in space45. Concerning the substrate specificity from another angle, it is important to compare the G6SG position in enzyme forms. While the Glc moieties of G6SG at the −1 subsite were oriented nearly identically in WT and mutant

![Fig. 8](https://example.com/fig8.png)

**Fig. 8** | Glc egress paths 1-3, illustrated on the top of WT HvExoI with bound Glc (carbons in cpk yellow lines, and spheres) and laminaripentaose (cpk magenta sticks). Path 1 (WT and W434H), path 2 (W434A), and path 3 (vertical) are depicted in orange, cyan, and magenta spheres, respectively, and aligned next to each other. Path 3 is found when no ligand is bound at the +1 and +2 subsites. Illustrated trajectories represent frames that correspond to consecutive stages of Glc egress with conforming coordinates for Glc in each frame. Glc egress paths were identified by cMD simulations and GPathFinder. Surface morphology is coloured by electrostatic potentials: white, neutral; blue, +5 kT·e−1; red, −5 kT·e−1. Positions of Trp286 and Trp434 delineating the +1 subsite, are indicated in grey sticks. The right panel depicts the detailed view of paths 1-3, with HvExoI being rotated by around −80° via the y-axis relative to the left panel.
structures, the position of the Glc moiety at the +1 subsite varied – it was identical in WT and W286A, while in W434A it was rotated by 120° through the x-axis. This comparison underscored the pivotal role of Trp434 in correctly orienting saccharide substrates engaged in stacking interactions, also known as dispersion-driven C-H-π forces (consisting of London dispersion forces)\(^4\). The geometries of these interactions with Trp, Phe, Tyr, and His involved face-to-face geometries, although the edge-face and offset stacked geometries also occur in protein complexes\(^5\). These stacking interactions are mediated by Trp residues in 40%, trailed by Tyr, Phe, and His\(^6\).

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Fig. 9 | Mechanistic view of the catalytic function of HvExoI, a family GH3 enzyme. a HvExoI uses the retaining catalytic mechanism, where nucleophile Asp285 (magenta) and acid/base catalyst Glu491 (green) play key roles in substrate hydrolysis\(^7\). b Reaction kinetics indicates the formation of Michaelis complexes (E-S and E-S), and enzyme-product complexes before (E-P1-Glc) and following the first Glc egress (E-P1), and after hydrolysis of P1 (E-Glc)\(^8\). S and P1 are trimeric and dimeric molecules, respectively. First-order rate constants are shown. Entrapped Glc product from E-Glc is displaced after a new substrate is attached. The hydrolytic cycle with a (1,3)-linked substrate is repeated until it is hydrolysed to Glc (indicated by vertical and horizontal lines). c Simplified mechanism of Glc displacement with a disaccharide\(^9\). After the disaccharide (empty blue and filled grey squares) bound in the −1 and +1 subites (step 1) is hydrolysed and the reducing end Glc (aglycon) diffuses away from +1 subsite, the non-reducing end Glc (cyan square) remains non-covalently trapped. After a new dimer binds (step 3) and advances to the catalytic site, Glc modifies its binding patterns and egresses (large arrow) via a transient lateral cavity (cylinder in dotted lines) formed near the Trp clamp (step 4)\(^10\). Curved arrows indicate dimer entries into the catalytic site. d Glc displacement paths in WT (left panels), and the W434A mutant (right panels) with Glc and the G3OG dimer, calculated through cMD and GPathFinder. Large arrows indicate Glc positions at the initial (step 1) and after the final (step 4 – following Glc departure from −1 subites) stages of catalysis (this work).
To address the structural features of β-β-glucosidases binding (or Michaelis complexes) in HvExol at the atomic level, we compared the conformational behaviour of thiо-analogues bound in crystalline forms (Supplementary Figs. 5 and 6) and after cMD simulations (Supplementary Fig. 8). These analyses revealed C1 conformations of the glucopyranose moieties in the +1 subsite (Fig. 5; Supplementary Fig. 8; Supplementary Table 7), and the pseudo-C1 conformation for the sugar moiety bound at the −1 subsite, although previous cMD analyses of a fungal GH3 enzyme did not find evidence of sugar distortion.

These findings supported the analyses of puckering conformations of glucopyranose rings in Michaelis complexes of other exo-acting GH enzymes23,28, and suggested that these exo-acting enzymes differ from endo-acting enzymes, where substrate distortions were systematically observed29–31.

Additionally, via cMD simulations and QM/MM MD metadynamics, we investigated the trajectory of the G3OG product originating from the G4OG3OG substrate, where the Gc moieties in C1 conformations and FEL maps shared properties (Fig. 6; Supplementary Figs. 10 and 11). The cluster analysis of the G3OG product in WT and W434A showed positions in markedly different proportions (Fig. 6b). This led us to conclude that the G3OG product could not overcome a high energy barrier to achieve the productive binding mode, due to an unfavourable orientation of the Gc moiety in the −1 subsite. Here, the CH2OH substituent faced the acid/base Glu491 residue, while the anomeric carbon was positioned away from the Asp285 nucleophile. A change in the ring puckering of the Gc moiety could eventually cause the anomeric carbon to approach Asp285, but such a possibility was eliminated based on the calculated FEL map (Fig. 6a). In W434A the G3OG product disposition allowed more flexibility, but G3OG still faced a high energy barrier to achieving a productive binding mode.

These findings have profound implications for the hydrolysis of mixed-linkage β-β-glucosidoglycosaccharides by HvExol, where the G4OG3OG natural substrate cannot slide through the active site from the +2 and +1 to the +1 and −1 subsites, although it could utilise the lateral cavity exit mechanism for Gc egress. The binding and hydrolysis of the G4OG3OG natural substrate for HvExol proceed in two steps: (i) after the 1st glycosidic linkage (G4OG) from the non-reducing end is cleaved off, and Gc egresses through the lateral cavity; (ii) the G3OG product diffuses out of the active site and rebinds in the correct orientation to form a productive enzyme-substrate complex. This distributive catalytic process can occur with trimERIC or longer substrates that are (1,2)-, (1,4)-, (1,6)- or (1,4,1,3)-linked, but not with (1,3)-linked substrates, which utilise both assets of processivity, i.e. Gc egress and substrate single-chain sliding movement forward through the catalytic site without dissociation. Notably, in HvExol, the processive character is paramount to the catalytic efficiency and is correlated with high 1 values of the (1,3)-linked substrates (Supplementary Table 3). Similar (i–ii) steps can be used by G4OG4OG3G, which with G4OG3OG are the key low-molecular hydrolytic products generated from mixed-linkage (1,4,1,3)-β-β-glucans – polysaccharide components in cereal cell walls32,33.

We alluded to the pivotal role of Trp434 in HvExol that through stacking interactions participated in the orientations of substrates to positions amenable for hydrolysis through substrate-product assisted processivity. To extend our knowledge of the role of Trp434 in these processes, and why the evolution converged to Trp434, we applied cMD, GaMD55, and GPathFinder37 simulations, where cMD simulations safeguard the stability of complexes in geometries close to those in crystal structures, GaMD provide an enhanced sampling of conformational spaces, and GPathFinder explores dynamic traits of reactants binding or unbinding, via backbone motions and side-chain optimisations of discrete rotameric states. For these studies, we selected two mutants, which retained broad substrate specificity (W434H), or behaved as a strict (1,3)-β-β-glucosidase (W434A).

Summarising these findings, we revealed that: (i) the stability and time occupancy of the laminaribiose or laminaripentaose products in the +1 subsites +1 and −1 were affected by W434H and W434A mutations; (ii) in WT and W434H, the lateral Gc egress path was used during substrate-product assisted processivity (path 1; Fig. 8); (iii) in W434A, Glc exited through the W434A–Trp286 clamp and a gap formed by the non-conservative W434A mutation, and without engaging substrate-product assisted processivity (path 2; Fig. 8); (iv) in WT and both mutants, Glc egressed through the aromatic Trp286/Trp343 clamp, when no oligosaccharides are present at this site (path 3; Fig. 8), which was energetically most favoured; (v) substrate-product assisted processivity can only be utilised, when the unbinding of the product at the +1 subsite is slower than Gc egress through the lateral cavity (Figs. 7–9; Supplementary Fig. 14; Supplementary Movies 1–3). Finally, our unconstrained MD simulations of HvExol complexed with laminaripentaose, and Glc removed from the −1 subsite revealed that the sliding of laminaripentaose proceeded spontaneously, even in W434H or W434A, meaning that all enzymes position Glc moieties in the active sites correctly due to the structural complementarity.

Importantly, the combined experimental and computational approaches addressed the key role of the evolutionary conserved Trp286/Trp343 clamp in the GH3 family, which secures Gc unbinding and through C-Hπ interactions guarantees the optimal binding of hydrolytic products while preventing their attachments by reducing-terminals. These approaches also revealed another important function, which is to assure that when 1,3-β-link linked polymeric substrates bind to the active site and are hydrolysed with Gc egressing through an ad-hoc formed lateral cavity, the hydrolytic products could slide through the catalytic site and are rectified via substrate-product assisted processivity to warrant a next hydrolytic event. This process is accompanied by a cork-like motion between domains 1 and 2, which associates with an active site opened and closed states (Supplementary Movies 1–3).

Our studies provide a blueprint for emerging studies of reactant movements to advance the knowledge of catalysis in hydrolases and demonstrate that specificity and efficiency could be engineered via residue alterations that lock substrates into active sites. Particularly, the work through our multiscale strategy contributes to the understanding of catalysis in GH3 exo-hydrolases by defining structural trajectories and mechanisms for the breakdown of oligo- and polysaccharides (Fig. 9). We suggest that our findings could be utilised in enzyme design, as they are correlated with the statistical energy inferred from a maximum entropy model of homologous sequences36. Further, this knowledge can be exploited in the biomolecular design of artificial molecular motors77,78, and more broadly in biotechnology and drug design, where enzymes are sought as environmentally friendly and cost-effective alternatives in bioremediation, and applications in medicine and pharmacetics.

**Methods**

**Generation of HvExol mutants, protein expression, and purification**

Mutant HvExol forms were prepared by site-directed mutagenesis using the pPICZαBNH8 vector containing an optimised cDNA fragment encoding mature HvExol as a template22,29,30. Trp286 and Trp434 were singly replaced with alanine (Ala), histidine (His), phenylalanine (Phe), and tyrosine (Tyr). Doubly mutated W286F/W434F and W286F/W434A forms were also prepared to yield a total of ten mutants. Site-directed mutagenesis was carried out as described in the QuikChange® Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) using primers specified in Supplementary Table 8. DNA constructs were transformed in *Pichia pastoris* strain SMD1168H to express and purify proteins as described29,79,80.
Enzyme stability, substrate specificity, kinetic analyses, and inhibition

Dependency of activities of WT and mutant HvExo1 on pH (McIlvaine buffers in the pH range of 3.5–8.5 at 30 °C) and temperature (100 mM sodium acetate buffer, pH 5.25, temperature range 0–80 °C, 15 min incubations) were determined using 0.2% (w/v) 4-NP-Glc\textsuperscript{26,59}. Relative rates of hydrolysis of β-linked poly-oligosaccharides, and α- and β-glucosides were determined using near-homogenous HvExo1 at 30 °C. Apparent first-order rate parameters (k\textsubscript{M} and k\textsubscript{cat}) were determined with (1,3;1,6)-β-D-glucan (laminarin), barley (1,4;1,3)-β-D-glucan, laminaribiose, cellobiose, and 4NP-Glc with substrate concentrations ranging between 0.3-3 μM levels in duplicate as described\textsuperscript{59,61}. Apparent inhibition constants of enzyme-inhibitor complexes (K\textsubscript{i}) using the 4NP-Glc substrate were determined at six concentrations with G2SG-OMe, 4NP-G3S, G4SG-OMe, and G6SG-OMe at 0.4-5 times the K\textsubscript{M} values in duplicate as described\textsuperscript{62,63}. Kinetic parameters were calculated from the analyses of Michaelis-Menten plots with Grafit 7.0.3.3\textsuperscript{62} and processed by a proportional weighted fit, using nonlinear regression\textsuperscript{64}. The relative rates of hydrolysis, kinetic, and inhibition data were rounded to one or two decimal points (Supplementary Tables 2–4).

Protein crystallisation and ligand perfusion

Near-homogenous WT and mutated HvExo1 were crystallised by macro- and cross-seeding with native HvExol microcrystals\textsuperscript{33,60,64}. Briefly, crystallisation was carried out at 4 °C in hanging drops consisting of 4-μL of HvExol (4.5–12 mg/mL) and 4 μL of precipitant solution A (100 mM HEPES-NaOH buffer, pH 7.0 containing 2.3% (w/v) polyethylene glycol 400 and 1.6 M ammonium sulfate). Drops were placed on 22 mm siliconised circular glass coverslips, 2.3% (w/v) polyethylene glycol 400 and 1.6 M ammonium sulfate). 2.3% (w/v) polyethylene glycol 400 and 1.6 M ammonium sulfate). 4 and 2 μL of precipitant solution A (100 mM HEPES-NaOH buffer, pH 7.0 containing 2.3% (w/v) polyethylene glycol 400 and 1.6 M ammonium sulfate). Drops were placed on 22 mm siliconised circular glass coverslips, onto which three to four 5-μL drops of native HvExol\textsuperscript{64} were transferred by a cat whisker. Coverslips were suspended over 1 mL mother liquor solutions (1.7 M ammonium sulfate in 46.9 mM sodium acetate buffer, pH 5.25, temperature range 0–80 °C, 15 min incubations) were determined with (1,3;1,6)-β-D-glucan (laminarin), barley (1,4;1,3)-β-D-glucan, laminaribiose, cellobiose, and 4NP-Glc with substrate concentrations ranging between 0.3-3 μM levels in duplicate as described\textsuperscript{59,61}. Apparent inhibition constants of enzyme-inhibitor complexes (K\textsubscript{i}) using the 4NP-Glc substrate were determined at six concentrations with G2SG-OMe, 4NP-G3S, G4SG-OMe, and G6SG-OMe at 0.4-5 times the K\textsubscript{M} values in duplicate as described\textsuperscript{62,63}. Kinetic parameters were calculated from the analyses of Michaelis-Menten plots with Grafit 7.0.3.3\textsuperscript{62} and processed by a proportional weighted fit, using nonlinear regression\textsuperscript{64}. The relative rates of hydrolysis, kinetic, and inhibition data were rounded to one or two decimal points (Supplementary Tables 2–4).

The iterative model building using REFMAC5\textsuperscript{66}, M IFIT\textsuperscript{68}, and COOT\textsuperscript{69} showed that 99.8% of the residues were found in the most favourable, additionally allowed, and generously allowed regions of the plot, with the well-defined density map for Ile432, the only residue located in disallowed regions. Structural images were prepared in PyMOL Molecular Graphics System v2.3.3 (Schrodinger LLC, Portland, OR, USA). The summary of 25 structures with thio-analogues and Glc and PDB accession numbers, data collection, and refinement statistics are reported in Supplementary Table 6. The (2 m\textsuperscript{c}|F\textsuperscript{c}-|F\textsuperscript{m}) difference electron density maps were calculated using Phenix\textsuperscript{70} with the mtz and PDB files after CCP4 REFMACS\textsuperscript{71} refinements. The maps were contoured at the 1.0 σ level. Ligands bound in active sites (Figs. 2–4; Supplementary Figs. 2–4) were visualised in PyMol Molecular Graphics System v2.3.3.

cMD simulations of Michaelis complexes derived from thio-ligand complexes

A total of eight models were generated for the following systems: W286F-G2(S or O)G in complex with G2SG-OMe; PDB 6JG7; WT-G3(S or O) in complex with 4NP-G3S; PDB 6JG2; WT-G4(S or O)G in complex with G4SG4OG4S; PDB 6JG5; and WT-G6(S or O)G in complex with G6SG-OMe; PDB 3WLP. The procedure consisted of (i) reverting the mutation in the W286f crystal structure; (ii) removing SO\textsubscript{4}\textsuperscript{2-} anions, glycerol (GOL), polyethylene glycol (PEG), N-acetyl glucosamine (NAG), and aglycon moieties of thio-analogues; (iii) neutralising the systems with three Na\textsuperscript{+} cations; and (iv) adding a cubic water box around proteins. For O-linked saccharide systems, an additional step was taken by replacing S-atoms in thio-analogues with O-atoms. The leap code, included in AMBER\textsuperscript{11} was chosen to parametrise systems using the ff99SB force field\textsuperscript{72} for the protein, Glycam06\textsuperscript{73} for saccharides, and the TIP3P force field\textsuperscript{74} for water molecules. The equilibration protocol consisted of a minimisation step (8 × 10\textsuperscript{3} steps), progressive heating to 300 K (700 ps), NPT equilibration under a pressure of 1 atm to ensure physiological density (100 ps), and a final NVT equilibration (with a time step value of 2 ps using the SHAKE\textsuperscript{75} algorithm to constrain bonds involving hydrogens) until the RMSD values of protein backbone atoms stabilised. Default parameters were used for non-bonding interactions and long-range electrostatics. Simulations at NVT were extended to 200 ns each.

cMD simulations of the Michaelis complex with a natural substrate G4OG3OG bound to WT and W434A, based on the WT-G4SG4OG4G4S complex

The natural substrate G4OG3OG was modelled, based on the WT HvExol crystal structure in complex with G4SG4OG4S (PDB 6JG7), where, S-atoms in G4SG4OG4S were replaced by O-atoms, the glycosidic linkage was re-aranged to be 3-O-linked between the 2\textsuperscript{nd} and 3\textsuperscript{rd} Glc moieties from the non-reducing end, and the atoms were carefully moved such that the system maintained β-anomeric conformation. Further, SO\textsubscript{4}\textsuperscript{2-} anions, GOL, PEG, and NAG were removed, and the system was neutralised with three Na\textsuperscript{+} cations and solvated in a cubic water box. From this structure, two more systems representing the product of the hydrolytic reaction of G4OG3OG were modelled. The Glc product at the -1 subsite was removed in both domains (domain 1, residues 1-357; domain 2, residues 374-559) as two independent anisotropic domains with translation-libration-screw (TLS) motion\textsuperscript{73}. Electron density maps of ligands were defined in the active site regions at 3σ levels in the m|F\textsuperscript{c}-|F\textsuperscript{m}| maps (m is the figure of merit and D is an estimated coordinate error) and the water molecules were located automatically with CCP4 ARP at levels higher than 2σ levels. Water molecules were retained if they satisfied H-bond criteria and if the 2 m|F\textsuperscript{c}-|F\textsuperscript{m}| electron density maps were confirmed. During model building and refinement, 5% of the data were flagged for cross-validation using R\textsubscript{free} statistics\textsuperscript{73,75}. PROCHECK\textsuperscript{76} was used to check the stereo-chemical quality of models. Ramachandran plots of structures showed that 99.8% of the residues were found in the most favourable, additionally allowed, and generously allowed regions of the plot, with the well-defined density map for Ile432, the only residue located in disallowed regions. Structural images were prepared in PyMOL Molecular Graphics System v2.3.3 (Schrodinger LLC, Portland, OR, USA). The summary of 25 structures with thio-analogues and Glc and PDB accession numbers, data collection, and refinement statistics are reported in Supplementary Table 6. The (2 m|F\textsuperscript{c}-|F\textsuperscript{m}) difference electron density maps were calculated using Phenix\textsuperscript{70} with the mtz and PDB files after CCP4 REFMACS\textsuperscript{71} refinements. The maps were contoured at the 1.0 σ level. Ligands bound in active sites (Figs. 2–4; Supplementary Figs. 2–4) were visualised in PyMol Molecular Graphics System v2.3.3.

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systems and for one system the WT structure was retained, while for the other,Tpl34 was mutated to Ala. These three systems were parametrised using the tleap program of AmberTools21 using the ff19SB protein force field90. Glycam06b for saccharides, and the TIP3P water field97 for water molecules. Amber20 was used to minimise and equilibrate all systems using the protocol described above, and applying restraints when necessary until the RMSD values of protein backbones stabilised. Simulations were extended to 150 ns each.

**QM/MM MD metadynamics simulations of G3OG and G4OG3OG substrates in WT**

For these studies or conformational Free Energy Landscape (FEL) simulations of Glc moieties of G3OG and G4OG3OG positioned in active sites of HvExol, we used the CPMD code (parallelised plane wave/pseudopotential implementation of Density Functional Theory, Copyright IBM Corp 1990-2005)90 patched with the open-source community-developed PLUMED2 library82,83. For the conformational FEL simulation of the G3OG disaccharide, the QM box (region) included 45 atoms corresponding to the disaccharide in the +1 and +1 subsites; the MM region included the protein and water molecules (85,478 MM atoms). For the conformational FEL simulation of the G4OG3OG trisaccharide, the Glc molecule was not included in the QM box (region) but instead, the Glc molecule in the +1 subsite and the half-ring of Glc in the +1 subsite (with two monovalent pseudopotentials to saturate the QM region) were included in the QM box, corresponding to 29 QM atoms in total. The other half-ring of the Glc molecule in the +1 subsite and the third Glc moiety placed in the +2 subsite were described as MM atoms, which also included protein and water molecules (11,5415 MM atoms)54,94 (Supplementary Fig. 15). The G3OG and G4OG3OG systems were enclosed in isolated supercells of 13.5 Å x 19.3 Å x 14.8 Å and 13.3 Å3 sizes. Kohn-Sham orbitals and water molecules (11,5415 MM atoms)84 (Supplementary Fig. 15). For the conformational FEL simulation of the G3OG disaccharide, the QM box (region) included 45 atoms corresponding to the disaccharide in the +1 and +1 subsites; the MM region included the protein and water molecules (85,478 MM atoms). For the conformational FEL simulation of the G4OG3OG trisaccharide, the Glc molecule was not included in the QM box (region) but instead, the Glc molecule in the +1 subsite and the half-ring of Glc in the +1 subsite (with two monovalent pseudopotentials to saturate the QM region) were included in the QM box, corresponding to 29 QM atoms in total. The other half-ring of the Glc molecule in the +1 subsite and the third Glc moiety placed in the +2 subsite were described as MM atoms, which also included protein and water molecules (11,5415 MM atoms)54,94 (Supplementary Fig. 15). The G3OG and G4OG3OG systems were enclosed in isolated supercells of 13.5 Å x 19.3 Å x 14.8 Å and 13.3 Å3 sizes. Kohn-Sham orbitals were expanded on a plane wave basis set with a kinetic energy cut-off of 70 Ry. Troullier-Martins ab initio pseudopotentials83 were used for all elements. The PBE functional83 in the generalised gradient-corrected approximation of DFT with a time step of 0.12 fs and a fictitious electron mass of 600 a.u. for the Car-Parrinello Lagrangian were used in both systems. The Nosé-Hoover thermostat87 was coupled to the systems to reach a constant temperature of 300 K. After the systems were equilibrated, metadynamics simulations were used in both systems. The Nosé-Hoover thermostat87 was coupled to the systems to reach a constant temperature of 300 K. After the systems were equilibrated, metadynamics simulations were used in both systems. The Nosé-Hoover thermostat87 was coupled to the systems to reach a constant temperature of 300 K. After the systems were equilibrated, metadynamics simulations were used in both systems. The Nosé-Hoover thermostat87 was coupled to the systems to reach a constant temperature of 300 K. After the systems were equilibrated, metadynamics simulations were used in both systems. 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GpathFinder simulations to explore Glc displacement routes in WT, and the W434H and W434A HvExol mutants
This method uses the multi-objective genetic algorithm of the GaudiMM engine\(^{100}\), wherein genes are structural operators (e.g. atomic positions and displacements), and objectives (fitness functions in evolutive algorithms) are structural and energetic descriptors (e.g. force field functions, distances, angles, etc.). The GaudiMM engine combines full-atom representations of a ligand and a protein, and allows diverse levels of structural flexibility including internal rotation, global rotation and translation of ligands, and the exploration of protein motions that are in a short-range (e.g. internal rotation, global rotation, and translation of ligands) or a long-range (e.g. rotameric exploration and normal mode analysis of the protein). Typically, in GpathFinder runs, fast energetic evaluations are needed, which are provided by a docking scoring function, i.e. using Autodock Vina and steric clashes. GpathFinder’s philosophy relies on the conformational selection hypothesis, as collective motions (provided by the sampling of the lowest energetic modes obtained from Normal Mode Analysis) are responsible for conformational changes of proteins associated with their function, including ligand binding. Ligand diffusion of Glc from the −1 subsite was calculated with Gpathfinder for six systems: WT, W434H, and W434A with or without laminaribiose (i-iii) or laminaripentaose (iv-vi) hydrolysis products bound in the +1 and +2 subsites and pointing to bulk solvent for laminaripentaose, since the Glc molecule occupied the −1 subsite. In each case, the starting structure calculations were chosen from the most representative clusters of CMD simulations described above, and Gpathfinder calculations were performed using standard genetic algorithm parameters\(^3\). Clashes and the scoring Autodock Vina function were used as objectives, together with genes representing rotamers and Normal Modes Parameters calculated with ProDi\(^{105}\). The pool of individuals consisted of a population of sixteen candidates that satisfied the genetic algorithm for 800 generations. Calculations were analysed using GaudiView for visual inspection, together with the movie plug-in in UCSF Chimera to inspect trajectories. Other analyses were performed through in-house UCSF Chimera built-in plug-ins, such as finding clashes/contacts and distances, to locally explore interactions of Glc with amino acid residues during egress pathways.

Unconstrained MD simulations to explore sliding of substrates in WT, and the W434H and W434A HvExol mutants
These simulations proceeded with laminaripentaose in the +1 and +2 subsites and pointing to bulk solvent, and Glc removed from the −1 subsite. The systems were generated from the structures obtained from GauMD simulations and were run as described above for ternary complexes, with production runs of 200 ns each.

Additional methods are available in Supplementary Methods (Materials, Phylogenetic analysis, Organic Syntheses of 4NP-G3SG and G4SG-OMe).

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data that support this study are available from the corresponding author upon request. The atomic coordinates and structure factors have been deposited in the Protein Data Bank (www.pdb.org) with the following PDB accessions: WT HvExol, and in complex with 4NP-G3SG, and (G4SG4O)\(_3\) are 6jg2, and 6jg1, respectively. The PDB accession of W286A in complex with G6SG-OMe is 6j66, and W286F in complex with G2O2G-OMe, 4NP-G3SG, and G6SG-OMe are 6jg7, 6jgA, and 6jGB. The PDB accession of W286Y in complex with Glc, G2SG-OMe, 4NP-G3SG30G, (G4SG4O)\(_3\), and G6SG-OMe are 6UKe, 6jGE, 6jJJ, 6LBB, and 6k6V. The PDB accession of W434F in complex with G2SG-OMe, 4NP-G3SG, (G4SG4O)\(_3\), and G6SG-OMe are 6jG, 6LC5, 6jGK, and 6LBV. The PDB accession of W434H in complex with G2SG-OMe, 4NP-G3SG, G4SG-OMe, and G6SG-OMe are 6jGL, 6jCN, 6jGO, and 6jGP. The PDB accession of W434Y in complex with G2SG-OMe, 4NP-G3SG, G4SG-OMe, and G6SG-OMe are 6jGQ, 6jGR, 6jGS, and 6jGT. Source data are provided with this paper.

Code availability
Software applications described in Methods with associated references were used without code modifications. For analyses of geometrical parameters and MD simulations, we used in-house scripts, which are available on GitHub (https://github.com/insilichem/utils; https://github.com/roviralab/utils) and ZENODO repositories\(^{106,107}\).

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Author contributions

S.P. and S.F. synthesised thio-ligands; S.L. and J.R.K.C. implemented mutagenesis; S.L. completed enzyme kinetics; S.L. and M.H. purified and crystallised proteins; V.A.S. and M.H. collected X-ray data; S.L., V.A.S., and M.H. refined crystal structures; M.H. and J.G.S. performed phylogeny analyses; A.N.H., S.A.G., and C.R. performed cMD, and QM/MM MD metadynamics calculations; X.F.L., J.-D.M., and L.M. implemented cMD, GaMD, and GPathFinder simulations; X.F.L., A.N.H., and M.H. generated animations and structural images. M.H. wrote the paper with contributions from S.L., S.F., X.F.L., A.N.H., J.-D.M., L.M., and C.R. M.H. oversaw the project.

Competing interests

The authors declare no competing interests.

Additional information

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