

Engineering Novel S-Glycosidase Activity into Extremo-Adapted β -Glucosidase by Rational Design

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ABSTRACT

The breakdown of sulphur glycosidic bonds in thioglycosides can produce isothiocyanate, a chemoprotective agent linked to the prevention of cancers, however only a handful of enzymes have been identified that are known to catalyse this reaction. Structural studies of the myrosinase enzyme, which is capable of hydrolysing the thioglycosidic bond, has identified residues that may play important roles in sulphur bond specific activity. Using rational design, two extremophile-adapted β -glycosidases from the species *Thermus nonproteolyticus* (*TnoGH1*) and *Halothermothrix orenii* (*HorGH1*) were engineered towards thioglycoside substrates. Twelve variants, six for *TnoGH1* and six for *HorGH1*, were assayed for activity. Remarkable enhancement of the specificity (k_{cat}/K_M) of *TnoGH1* and *HorGH1* towards β -thioglycoside was observed in the single mutants *TnoGH1*-V287R ($2500 \text{ M}^{-1}\text{s}^{-1}$) and *HorGH1*-M229R, ($13260 \text{ M}^{-1}\text{s}^{-1}$) which showed a 3-fold increase with no loss in turnover rate when compared to the wild type enzymes. Thus, the role of arginine is key to induce β -thioglycosidase activity. Thorough kinetic investigation of the different mutants has shed light on the mechanism of β -glycosidases when acting on the native substrate.

KEY POINTS

Key residues were identified in the active site of *Brevicoryne brassicae* myrosinase.
Rationally designed mutations were introduced into two extremophile-adapted β -glycosidases.
 β -glycosidases mutants exhibited improved activity against thioglycosidic bonds.
The mutation to arginine in the active site yielded the best variant.

KEYWORDS

Enzyme engineering, Site directed mutagenesis, glycoside hydrolase, *Thermus nonproteolyticus*, *Halothermothrix orenii*, myrosinase

INTRODUCTION

The glycoside hydrolase family 1 of enzymes (GH1; EC 3.2.1.21) is characterised by the ability to catalyse the hydrolysis of glycoside linkages in a variety of sugars (β -glucosides) (Davies and Henrissat 1995; Park et al. 2017). In terms of structure, it has been demonstrated that GH1 are $(\beta/\alpha)_8$ barrel folded enzymes, which consist of eight twisted, parallel β -strands, located in the internal part of the protein, surrounded by eight α -helices in the external part. The C-terminal (in the β strand 8) of all known $(\beta/\alpha)_8$ barrel proteins hosts the active site residues within the $\beta \rightarrow \alpha$ loop (Henn-Sax et al. 2001; Silverman et al. 2001). The hydrolytic ability of GH1 is dependent on two critical glutamic acid residues (Fig. 1), E164 (*Thermus nonproteolyticus* glycoside hydrolase, *TnoGH1* numbering), located in the T¹⁶¹LNEP¹⁶⁵ motif (β -strands 4), is the acid catalyst, and a second one, E338, in the I³³⁶TENG³⁴⁰ motif (β -strands 7) is the nucleophile (Wang et al. 2003) (Fig. 1a). E164 plays an important role in the formation of the intermediate (enzyme-saccharide) of classical glycosidases as an activator of the glycosidic oxygen.

Thioglycosides are among the most stable glycosidic molecules. In these structures, the glycoside is bridged to the aglycon moiety by a sulphur bond. The breakdown of S- glycosidic bonds in glucosinolates (GSL) can release molecules with activity against pests and herbivores (part of the plant defence mechanism) and isothiocyanate, a chemoprotective agent linked to the prevention of cancers (Dufour et al. 2015; Halkier and

38 Gershenzon 2006; Rakariyatham et al. 2005 ; Samec et al. 2016; Winde and Wittstock 2011).

39 Myrosinases (EC 3.2.3.147) are unique members of the GH1 family able to hydrolyse thioglycosides. Unlike
40 β -glycosidases which are ubiquitous, myrosinases have been identified only in a handful of species such as
41 *Sinapis alba* (Burmeister et al. 1997), *Brevicoryne brassicae* (Jones et al. 2002), *Verticillium longisporum* (Witzel
42 et al. 2015), *Arabidopsis thaliana*, and *Brassica napus* (Nong et al. 2010). The active site of *Sinapis alba*
43 myrosinase (*SaMYR*), a plant species, differs from that of classical β -glycosidases, as it lacks the catalytic
44 glutamic acid residue in the T¹⁸⁴INQL¹⁸⁸ motif (equivalent to T¹⁶¹LNEP¹⁶⁵ in *TnoGH1*) (Bourderieux et al. 2005),
45 while it maintains the second one (E409 in motif T⁴⁰⁸ENG⁴¹¹) (Burmeister et al. 1997). (Fig. 2a). In addition,
46 *SaMYR* requires ascorbic acid as a cofactor to catalyses the hydrolysis of thioglycosidic substrates.

47 A myrosinase from the cabbage aphid *Brevicoryne brassicae* (*BbMYR*) on the other hand, relies on the typical
48 catalytic acid/base system found in β -glycosidases (E167 and E374) (Fig. 2b) and it more closely aligns with
49 classic β -glycosidases than *SaMYR* in term of structure and amino acid residues present in the active site. *BbMYR*
50 has however unique structural features, not observed in either β -glycosidases or other myrosinases. K173 and
51 R312 (Fig. 3) play a critical role in the hydrolysis of GSL, as they are directly involved in its recognition (Jones
52 et al. 2002). Y180 may also play a role due to its proximity to the thioglycosidic linkage in the substrate (Husebye
53 et al. 2005). Figure S1 shows full sequence alignment of *Thermus nonproteolyticus* glycoside hydrolase
54 (*TnoGH1*), *Halothermothrix orenii* (*HorGH1*), cabbage aphid *Brevicoryne brassicae* (*BbMYR*), and *Sinapis alba*
55 myrosinase (*SaMYR*).

56 A thermophilic GH1 from the extremophilic bacterium *Thermus nonproteolyticus* (*TnoGH1*) and a
57 halotolerant GH1 isolated from *Halothermothrix orenii* (*HorGH1*) have been previously described in the literature
58 (He et al. 2001; Kori et al. 2011). Enzymes from extremophile organisms have significantly higher tolerance
59 than the mesophilic counterparts to temperature and/or pHs, making them attractive for industrial applications
60 (Yin et al. 2015), however, no known examples have been reported of extremophilic GH1 with myrosinase
61 activity. Engineering extemo-adapted GH1 to broaden their substrate scope towards β -thioglycosidase activity
62 could significantly increase their potential applications in an industrial setting.

63 Here we report how rational design aided the introduction of amino acid mutations by mapping the *BbMYR*
64 active site onto the extremophilic *TnoGH1* enzyme first, and then onto *HorGH1* to further confirm the key role
65 played by selected residues in the recognition and hydrolysis of thioglycosides. In *TnoGH1*, the mutations L171K,
66 V287R, and H178Y were introduced as single and double mutants (generating 3 additional variants with all
67 possible permutations). The equivalent single and double mutants at positions E173K, M299R, and H180Y were
68 then introduced into *HorGH1*. The kinetic properties of all variants and wild type enzymes with test substrates β -
69 D-thioglucopyranoside (pNT-Glc) and 4-nitrophenyl- β -D-glucopyranoside (pNP-Glc) have been carried out and
70 the role of arginine (*TnoGH1*-V287R and *HorGH1*-M299R) has been found to be pivotal as a marked
71 improvement of activity towards the β -thioglucoside substrate has been observed in both mutant proteins. These
72 are the first examples of extremophilic GH1s in which myrosinase activity has been introduced.

MATERIALS AND METHODS

73

74 **Reagents and bacterial strains**

75 Substrates 4-nitrophenyl β -D-thioglucopyranoside (pNT-Glc) and 4-nitrophenyl- β -D-glucopyranoside (pNP-
76 Glc) were purchased from Carbosynth. *Escherichia coli* bacterial strains and QuikChange II Site-Directed
77 Mutagenesis Kit were from Agilent Technologies. The plasmid miniprep kit was from Macherey-Nagel. Growth
78 media and assay components were procured from Fisher Scientific. All other chemicals were purchased from
79 Sigma.

80 **DNA preparation and site-directed mutagenesis**

81 Mutations were introduced using the QuikChange Site-Directed Mutagenesis kit. pCH93b hosting the ds-DNA
82 of *TnoGH1* (GenBank accession number [AF225213](#)) and pET45b hosting the ds-DNA of *HorGH1* (GenBank
83 accession number WP_012636460) respectively were used as templates in the PCR reactions (Heckmann 2017).
84 The mutagenic primers were designed using QuikChange Primer Design Program
85 (www.agilent.com/genomics/qcpd). Primers are summarised in Table S1.

86 **Protein expression and purification**

87 For protein expression, BL21(DE3) *E. coli* strain was used. Chemically competent *E. coli* cells were
88 transformed with each plasmid. A 300 mL LB flask was inoculated with starter culture and grown at 37 °C (200
89 rpm) to an OD₆₀₀ of ~ 0.6, prior to induction with 1 mM IPTG. Induced cultures were then incubated at 37 °C
90 overnight. Cells were harvested at 3,500 \times g, 4°C, 20 min. The pellet was resuspended in loading buffer (50 mM
91 HEPES pH 7.5, 150 mM NaCl, 10 mM imidazole) and lysed by sonication for 20 minutes on ice (1 min on, 30
92 sec off; 20 cycles). The soluble fraction was decanted following centrifugation at 22,800 \times g, 4 °C for 1 hour, and
93 filtered with a 0.45 μ m filter. Filtered supernatant was loaded onto a 1 mL HisTrap FF crude[®] column, using an
94 AKTA[™] Start. The column was washed with eight column volumes of loading buffer, followed by fifteen column
95 volumes of loading buffer with 10 % elution buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 300 mM
96 imidazole). The protein was then eluted with eight column volumes of 100% elution buffer. Pure fractions were
97 pooled and dialysed for 20 hours at room temperature with dialysis buffer (50 mM HEPES pH 7.5, 150 mM
98 NaCl), with one buffer exchange after 2h.

99 **Enzyme quantification**

100 The concentration of the purified enzymes was determined by absorbance at 280 nm. The extinction coefficient
101 ϵ was estimated using the EXPasy ProtParam tool (Gasteiger et al. 2005) (Table S2). Proteins were analysed with
102 a 12% SDS PAGE, by staining with InstantBlue (Fig. S2).

103 **Kinetics assays**

104 Enzyme activity was measured spectroscopically in triplicate by monitoring the change in absorbance at 420
105 nm of the p-nitrophenol or p-nitrothiophenol. The extinction coefficients for the products was determined using a
106 calibration curve (Fig. S3).

107 Assays were conducted in 200 μ L at 50°C for *TnoGH1* and at 25°C for *HorGH1*. A typical reaction mixture
108 contained 100 mM HEPES buffer pH 7.5, 500 mM sodium chloride. pNT-Glc was dissolved in 30% DMSO, the
109 concentration of DMSO was controlled at 9 % across pNT-Glc assays. All assay components were filtered with a

110 0.45 μm filter prior to use. Assays were initiated with the addition of enzyme. Primary non-linear regression plots
111 are described in Fig S5, S6, S7 and S8.

112 **Data analysis**

113 In silico modelling of crystal structures was performed using the UCSF Chimera software (Pettersen et al.
114 2004). Sequence alignments were determined with ENDscript server software (Robert and Gouet 2014).
115 Similarities and identities, including homology modelling, between sequences were calculated using EMBOSS
116 software (Rice et al. 2000). Kinetic parameters were evaluated by nonlinear regression analysis in Igor Pro
117 (Babonneau 2010). Bar graphs were produced in GraphPad Prism (Swift 1997). The primary plots were analysed
118 using equation 1. For consensus analysis of amino acids, β -glycosidase sequences were taken from the Pfam
119 protein family's database (El-Gebail et al. 2019), ~4000 sequences of representative proteome were used for
120 amino acid analysis. The representative proteome at 15% comembership threshold, as defined by Pfam which is
121 an even sampling of the sequences of the glycosyl hydrolase family PF00232 (Chen et al. 2011), was aligned with
122 the wild type sequences of the β -glycosidase used in this study with the ClustalW tool with the MEGA X software
123 (Kumar et al. 2018). Sequence logo analysis was performed with WebLogo (Crooks et al. 2004).

$$\frac{v_i}{[E]_T} = \frac{k_{cat}^{app} [S]}{K_M^{app} + [S]} \quad (\text{Equation 1})$$

124 **RESULTS**

125 **Computational study of *TnoGH1*, *HorGH1*, *BbMYR*, and *SaMYR***

126 A comparative study between the amino acid sequences of the four glycosyl hydrolases (*TnoGH1*, *HorGH1*,
127 *BbMYR*, and *SaMYR*) was carried out (Fig. S1). Both *TnoGH1* and *HorGH1*, as expected, present a greater
128 sequence similarity to the *BbMYR* than *SaMYR*. *TnoGH1* and *BbMYR* show a 53.6% similarity (34.3% identity),
129 greater than that observed between *TnoGH1* and *SaMYR* (46.3% similarity and 30.0% identity). *HorGH1* has a
130 58.1 % similarity (37.2 % identity) with *BbMYR* and a 51.9% (33.1 % identity) with *SaMYR*. The active site is
131 highly conserved across all enzymes.

132 ***TnoGH1* and *HorGH1* mutants construction**

133 Three residues in the amino acid sequence of *BbMYR* - K173, R312, and Y180, were identified to be important
134 in the hydrolysis of thioglucosides (Jones et al. 2002; Husebye et al. 2005). The sequence alignment between
135 *BbMYR* and *TnoGH1* (Fig. 3) reveals that *TnoGH1* presents a leucine, a valine, and a histidine at the equivalent
136 positions (L171, V287, and H178 - *TnoGH1* numbering). Likewise, in *HorGH1* (Fig. 3) three residues (E173,
137 M299 and H180) were selected to be replaced by K, R, and Y, respectively. Three single mutants were generated
138 in both the thermotolerant enzyme *TnoGH1* (L171K, V287R, and H178Y) and the halotolerant *HorGH1* (E173K,
139 M299R, and H180Y). Double mutant permutations were then created (L171K/V287R, L171K/H178Y, and
140 V287R/ H178Y in *TnoGH1*, and E173K/ M299R, E173K/ H180Y, and M299R/ H180Y in *HorGH1*) to elucidate
141 any synergistic effect among these amino acids. All mutants were expressed and purified (Fig. S2).

142 **Wild type *TnoGH1* and *HorGH1* substrate scope**

143 *BbMYR* activity against sinigrin (Fig. 4), the native substrate, was reported with $k_{cat} = 36 \text{ s}^{-1}$, $K_M = 0.41 \text{ mM}$
144 (Pontoppidan et al. 2001). Both *TnoGH1* and *HorGH1* exhibited some activity against pNT-Glc, used here as

145 substrate mimic. All determined kinetic parameters are summarised in Table 1 and Table 2. For *TnoGH1*, k_{cat} of
146 1.34 s^{-1} , K_M of 1.43 mM and k_{cat}/K_M $940 \text{ M}^{-1}\text{s}^{-1}$ were observed (Fig. S4). For *HorGH1*, k_{cat} of 37.20 s^{-1} , K_M of
147 4.10 mM and k_{cat}/K_M $9083 \text{ M}^{-1}\text{s}^{-1}$ were measured (Fig. S4), identifying the latter as the better catalyst towards
148 pNT-Glc (k_{cat} of ~ 28 -fold higher than *TnoGH1*).

149 ***TnoGH1* and *HorGH1* single mutant kinetic studies (pNT-Glc)**

150 When compared to the wild type, the *TnoGH1*-V287R single mutant shows the greatest increase in specificity,
151 k_{cat}/K_M , and a retention of turnover rate, k_{cat} (Table 1) with pNT-Glc.

152 The mutant *TnoGH1*-L171K shows a near retention in specificity ($1200 \text{ M}^{-1}\text{s}^{-1}$) and turnover rate (1.10 s^{-1}),
153 while *TnoGH1*-H187Y shows a 3-fold loss in specificity ($300 \text{ M}^{-1}\text{s}^{-1}$) and a 10-fold loss in turnover rate (0.15 s^{-1}).

155 *TnoGH1*-V287R shows an improvement in specificity with no loss of turnover rate. The combined
156 improvement in k_{cat}/K_M and retention of k_{cat} identifies *TnoGH1*-V287R as the best mutant for practical application.
157 The kinetic parameters of *TnoGH1* are summarised in Table 1.

158 A similar pattern is observed in the *HorGH1* mutants. *HorGH1*-M299R shows over 30% increase in specificity
159 and a retention of turnover number (Table 2). While little change is observed in *TnoGH1*-L171K, *HorGH1*-E173K
160 shows a 3-fold loss in specificity and a 2-fold loss in turnover rate. The *HorGH1*-H180Y mutant again shows a
161 3-fold decrease in specificity and a 4-fold decrease in turnover compared to the wild type, similar to the change
162 observed in the respective mutant in *TnoGH1*. All kinetic parameters of *HorGH1* are summarised in Table 2.

163 ***TnoGH1* and *HorGH1* double mutant kinetic studies (pNT-Glc)**

164 All the *TnoGH1* double mutants show a lower turnover rate with the target pNT-Glc compared to the wild
165 type and, with the exception of the *TnoGH1*-V287R/H178Y, to the single mutants. In particular, *TnoGH1*-
166 L171K/V287R shows a 2-fold increase in specificity ($1820 \text{ M}^{-1} \text{ s}^{-1}$) but a 3-fold decrease in turnover rate. The
167 *TnoGH1*-L171K/H178Y mutant shows a 3-fold decrease in specificity ($380 \text{ M}^{-1} \text{ s}^{-1}$) as well as a 10-fold decrease
168 in turnover rate. The *TnoGH1*-V287R/H178Y mutants shows a 2.5-fold increase in specificity and a 1/3-fold
169 decrease in turnover rate. *TnoGH1*-V287R/H178Y is the only variant in this series with a specificity ($2420 \text{ M}^{-1} \text{ s}^{-1}$)
170 comparable to that observed in the single mutant *TnoGH1*-V287R ($2500 \text{ M}^{-1} \text{ s}^{-1}$).

171 With *HorGH1* double mutants, the *HorGH1*-E173K/M299R shows a 3-fold decrease in specificity and a 4-
172 fold decrease in turnover rate compared to the wild type (Table 2). The *HorGH1*-E173K/H180Y mutant shows
173 an 8-fold decrease in specificity and an 8-fold decrease in turnover. The *HorGH1*-M299R/H180Y mutant shows
174 a near 3-fold decrease specificity and a 4-fold decrease in turnover rate, similar to that seen in *HorGH1*-E173K/
175 M299R.

176 As with single mutants, the pattern observed in *TnoGH1* double mutants is closely mapped in the *HorGH1*
177 variants. All double mutants show a lower turnover rate compared to that of the single mutants or wild type.
178 Double mutants containing the arginine mutation (*TnoGH1*-V287R and *HorGH1*-M299R) show an increase in
179 specificity. Kinetic parameters summarised in Table 1 and 2.

180 ***TnoGH1* and *HorGH1* variants: substrate analysis**

181 The *TnoGH1*-V287R mutant shows a significant shift in specificity towards pNT-Glc which corresponds to
182 30% loss in specificity towards pNP-Glc without any loss in turnover rate (Table 1). In the analogous *HorGH1*
183 mutant, this observation is pronounced, as there is a 35-fold loss in specificity towards the O-glycosidic substrate,
184 as well as a 2-fold decrease in turnover number (Table 2). When comparing substrates, kinetic parameters for both
185 enzymes appear to diverge to some extent in this case.

186 With pNT-Glc, *TnoGH1*-L171K shows a retention of kinetic parameters however with pNP-Glc, there is a
187 2.5-fold loss in specificity and a retention of turnover (Table 1). In *HorGH1*-E173K a decrease in kinetic
188 parameters is observed with pNT-Glc, with pNP-Glc this is more pronounced, a 10-fold decrease in specificity is
189 observed with a 2-fold decrease in turnover rate (Table 2). This mutation results in little perturbation in turnover
190 rate for both substrates in *TnoGH1*, however a decrease in parameters is observed for both substrates in *HorGH1*
191 (Table 1).

192 *TnoGH1*-H178Y mutant shows the lowest single mutant turnover rate with pNP-Glc, representing a 2-fold
193 loss (Table 1). Similarly, *HorGH1*-H180Y also shows a 2-fold decrease in specificity and turnover rate with the
194 same substrate (Table 2). Kinetics parameters change in the same direction for both substrates in both enzymes
195 on mutation at this position (Table 1 and Table 2).

196 Double mutants also show a complex relationship with respect to the native substrate. *TnoGH1*-L171K/V287R
197 shows a 3-fold loss in specificity and a 1/3-loss in turnover rate (Table 1) compared to the wild type. Whereas
198 with pNT-Glc, this variant shows an increase in specificity (Table 1). The *TnoGH1*-L171K/H178Y mutant shows
199 similar result with pNP-Glc as with pNT-Glc compared to the wild type, a 3-fold loss in specificity is observed in
200 both specificity and turnover rate for pNP-Glc (Table 1). *TnoGH1*-V287R/H178Y shows a similar specificity
201 towards pNP-Glc as the wild type enzyme, but a 1/3 - loss in turnover rate compared to wild type, while with
202 pNT-Glc a gain in specificity is observed compared to the wild type. (Table 1).

203 When *HorGH1* double mutants are compared to the wild type with pNP-Glc substrate, all mutants show a loss
204 in specificity and turnover rate. This is similar to the pattern observed with these mutants and the pNT-Glc. Unlike
205 *TnoGH1*, the double mutants on *HorGH1* show the same change in kinetic parameters for both substrates. (Table
206 2).

207 **DISCUSSION**

208 Significantly enhanced activity towards a thiosaccharide substrate, pNT-Glc, was introduced in *TnoGH1* and
209 *HorGH1* through rational design. The *TnoGH1*-V287R and *HorGH1*-M299R variants yielded the greatest
210 increase in specificity towards pNT-Glc while retaining similar turnover number to the wildtype. It has been
211 shown previously that R312 play a critical role in aphid myrosinase for sulfur recognition (Jones et al. 2002). In
212 β -glycosidases, the amino acids at the equivalent position are hydrophobic in nature (valine in *Tno* and methionine
213 in *Hor*). A mutation to arginine introduces a guanidinium side chain in the active site capable of hydrogen bonding
214 to the thioglycosidic bond in the substrate. R312 may have a potential interaction that may stabilize the transition
215 state resulting in the observed increase in k_{cat}/K_M . *TnoGH1*-L171K and *HorGH1*-E173K, it also adds a positively
216 charged side chain into the active site, however an increase in the k_{cat}/K_M is not observed. The crystal structure of

217 *BbMYR* indicates K173 pointing away from the active site, possibly reducing its direct involvement in substrate
218 binding.

219 The introduction of a tyrosine in both *TnoGH1* and *HorGH1* to replace a histidine results in a dramatic decrease
220 in k_{cat}/K_M towards both pNP-Glc and pNT-Glc substrates compared to the wild types. Y180 (*BbMYR* numbering)
221 had been suggested to have a possible catalytic role due to the proximity of the sidechain to the thiosidic linkage
222 in glucosinolates. However, in both extremophilic enzymes, the histidine displayed at that position appears to be
223 highly conserved among β -glycosidases (Fig. 5), suggesting that a mutation at this position is poorly tolerated.
224 Specifically, tyrosine while not forbidden, has an incidence of less than 3% in the data set. The others two targeted
225 positions (L171 and V287, *TnoGH1* numbering) are not as highly conserved (Fig. S9)

226 A more complex relationship is observed when the double mutants are compared to the wild type and single
227 mutants. From the single mutant results, we observe a correlation between the introduction of polar residues in
228 the active site of *TnoGH1* and the specificity for the pNT-Glc substrate. This is supported by the change in
229 specificity observed between the *TnoGH1*-H178Y mutant and the *TnoGH1*-V287R/H178Y double mutant, where
230 the latter has a much-improved specificity when compared to the former. Likewise, when *TnoGH1*-V287R is
231 combined with *TnoGH1*-L171K, the double mutant enzyme has a higher specificity than the *TnoGH1*-L171K but
232 lower specificity than the *TnoGH1*-V287R variant.

233 We can also see that a similar effect was observed in *HorGH1* specificity of pNT-Glc substrate with the
234 *HorGH1*-M299R/H180Y double mutant. Unlike *TnoGH1*, double mutant *HorGH1*-E173K/M299R shows similar
235 specificity to the *HorGH1*-E173K single mutant. We see a larger K_M (pNT-Glc) in the double mutant when
236 compared to the *HorGH1*-M299R single mutant, and a large reduction in k_{cat} compared to both single mutants.
237 This would suggest that the same effect in the *TnoGH1* mutants may be playing a role in the *HorGH1* mutants.

238 The *HorGH1* and *TnoGH1* mutants all exhibited a decrease in turnover rate and specificity with the native
239 substrate pNP-Glc (Table 1 and 2). This indicates that the increase in specificity for pNT-Glc is at the expense of
240 the native substrate. The increase observed in k_{cat}/K_M (pNT-Glc) induced by the introduction of the arginine
241 residues, is not observed with pNP-Glc. Considering the catalytic efficiency of the *HorGH1*-M299R, this mutant
242 has a ratio of k_{cat}/K_M of glycoside to thioglycoside of 3:1, compared to the *HorGH1* wild type which has a ratio
243 of 11:1.

244 In this study, enhanced β -thioglycosidase activity was introduced by rational design in the extremophilic β -
245 glycosidases *TnoGH1* and *HorGH1* by in-silico modelling of the *B. Brassicae* myrosinase. A three-fold increase
246 in specificity for the thioglycosidic substrate with no loss in turnover number was observed by replacing of
247 hydrophobic residues of both enzymes by arginine. These mutants were seen to have the greatest increase in
248 specificity of all assayed mutants, including double mutants. Among the novel β -thioglycosidases addressed in
249 this study, *HorGH1*-M299R is the most promising mutant for the industrial application due to the larger turnover
250 number.

251 AUTHOR CONTRIBUTION STATEMENT

252 FP conceived and designed research. NA conducted experiments. FP, NA, and NRM analysed data. NA and NRM
253 drafted the manuscript and all authors read and approved the manuscript

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical standards

The authors conducted the research to the high ethical standards of the journal of submission.

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351

FIGURES

352 **Fig. 1.** Protein database structures of the active sites of glycoside enzymes. **a** *Thermus nonproteolyticus* glycoside
353 hydrolase (pdb:1NP2); the residues mutated in this study are indicated, L171, H178 and V287. Residues essential
354 for activity also indicated, E164 and E338. **b** *Halothermothrix orenii* glycoside hydrolase (pdb:3TA9); the
355 residues mutated in this study are indicated, E173, H180 and M299. Residues essential for activity also indicated,
356 E166 and E354. **c** diagram indicating the relationship between the substrate and the side chains of the residues of
357 wild type *Thermus nonproteolyticus* mutated in this study, essential residues also displayed. Distances between
358 the glycosidic substrate and sidechains predicted with UCSF Chimera. **d** diagram indicating the relationship
359 between the glycosidic substrate and the side chains of the residues of wild type *Halothermothrix orenii* mutated
360 in this study, essential residues also displayed. Distances between substrate and sidechains predicted with UCSF
361 Chimera.

362 **Fig. 2.** Protein database structures of the active sites of myrosinase enzymes. **a** *S. alba* myrosinases (pdb:1E4M);
363 residues essential for activity also indicated, Q187 and E409. **b** *B. brassicae* myrosinases (pdb:1WCG); target
364 residues of mutagenesis indicated, K173, Y180 and R312; residues essential for activity also indicated, E167 and
365 E374. **c** diagram indicating the relationship between the thioglycosidic substrate and the side chains of the residues
366 of wild type *B. brassicae* myrosinases targeted residues of mutagenesis, essential residues also displayed.
367 Distances between substrate and sidechains predicted with UCSF Chimera.

368 **Fig. 3.** Amino acid sequence alignment of *Thermus nonproteolyticus* glycoside hydrolase (*T. nonproteolyticus*),
369 *Halothermothrix orenii* glycoside hydrolase (*H. orenii*), *B. brassicae* myrosinases (*B. brassicae*), and *S. alba*
370 myrosinases (*S. alba*).

371 **Fig. 4.** Structures of the substrates, the native substrate of myrosinase is added to show the similarity in the
372 structure of the molecules. **a** 4-nitrophenyl β -D-thioglucopyranoside (pNT-Glc). **b** 4-nitrophenyl- β -D-
373 Glucopyranoside (pNP-Glc). **c** sinigrin, the native substrate of myrosinase.

374 **Fig. 5** Sequence logo generated for amino acid position 178 (*Thermus nonproteolyticus* glycoside hydrolase
375 numbering) of glycosyl hydrolase family 1, indicating that cystine and histidine are the most highly conserved at
376 position 178. Sequences were taken from Pfam, the representative proteome at 15% co-membership threshold
377 (approximately 3900 sequences) was aligned with the wild type sequences of the β -glycosidase used in this study.
378 Sequence logo was generated with WebLogo.

379 **Table 1:** Table summarizing the kinetic parameters of the wild type enzyme and mutants of *Thermus*
 380 *nonproteolyticus* (*TnoGH1*) against the 4-nitrophenyl β -D-thioglucopyranoside (pNT-Glc) and 4-nitrophenyl- β
 381 -D-Glucopyranoside (pNP-Glc). Experiments were conducted in triplicate. Standard errors are given, based on
 382 fitted theoretical curves.

	k_{cat}/K_M (pNT-Glc) / $\text{M}^{-1}\text{s}^{-1}$	k_{cat} (pNT-Glc) / s^{-1}	K_M (pNT-Glc)/ mM	k_{cat}/K_M (pNP-Glc)/ $\text{M}^{-1}\text{s}^{-1}$	k_{cat} (pNP-Glc) / s^{-1}	K_M (pNP-Glc)/ mM
WT <i>TnoGH1</i>	940 ± 70	1.34 ± 0.04	1.40 ± 0.10	$246 \times 10^3 \pm 3 \times 10^3$	132 ± 3	0.54 ± 0.06
<i>TnoGH1</i> -L171K	1200 ± 100	1.10 ± 0.04	0.90 ± 0.10	$92 \times 10^3 \pm 9 \times 10^3$	136 ± 4	1.50 ± 0.20
<i>TnoGH1</i> -V287R	2500 ± 400	1.39 ± 0.01	0.56 ± 0.01	$190 \times 10^3 \pm 20 \times 10^3$	117 ± 3	0.61 ± 0.07
<i>TnoGH1</i> -H178Y	300 ± 50	0.15 ± 0.01	0.27 ± 0.07	$230 \times 10^3 \pm 36 \times 10^3$	77 ± 2	0.34 ± 0.06
<i>TnoGH1</i> -L171K/V287R	1820 ± 320	0.08 ± 0.01	0.21 ± 0.04	$58 \times 10^3 \pm 5 \times 10^3$	116 ± 3	1.90 ± 0.20
<i>TnoGH1</i> -L171K/H178Y	380 ± 80	0.09 ± 0.04	0.22 ± 0.05	$110 \times 10^3 \pm 10 \times 10^3$	69 ± 2	0.70 ± 0.10
<i>TnoGH1</i> -V287R/H178Y	2420 ± 500	0.84 ± 0.04	0.35 ± 0.08	$260 \times 10^3 \pm 40 \times 10^3$	106 ± 3	0.41 ± 0.07

383

384 **Table 2:** Table summarizing the kinetic parameters of the wild type enzyme and mutants of *Halothermothrix*
 385 *oreni* (*HorGH1*) against the 4-nitrophenyl β -D-thioglucopyranoside (pNT-Glc) and 4-nitrophenyl- β -D-
 386 glucopyranoside (pNP-Glc). Experiments were conducted in triplicate. Standard errors are given, based on fitted
 387 theoretical curves.

	$k_{\text{cat}}/K_{\text{M}}(\text{pNT-Glc})$ / $\text{M}^{-1}\text{s}^{-1}$	$k_{\text{cat}}(\text{pNT-Glc})$ / s^{-1}	$K_{\text{M}}(\text{pNT-Glc})$ / mM	$k_{\text{cat}}/K_{\text{M}}(\text{pNP-Glc})$ / $\text{M}^{-1}\text{s}^{-1}$	$k_{\text{cat}}(\text{pNP-Glc})$ / s^{-1}	$K_{\text{M}}(\text{pNP-Glc})$ / mM
WT <i>HorGH1</i>	9083 \pm 529	37.2 \pm 0.7	4.1 \pm 0.3	102000 \pm 7 \times 10 ³	80 \pm 1	0.5 \pm 0.1
<i>HorGH1</i> -E173K	3331 \pm 153	19.1 \pm 0.3	5.7 \pm 0.3	11000 \pm 1 \times 10 ³	26 \pm 2	2.5 \pm 0.5
<i>HorGH1</i> -M299R	13260 \pm 170	33.7 \pm 1.2	2.5 \pm 0.4	36000 \pm 2 \times 10 ³	34 \pm 1	0.9 \pm 0.1
<i>HorGH1</i> -H180Y	2501 \pm 131	9.4 \pm 0.2	3.8 \pm 0.2	55000 \pm 5 \times 10 ³	38 \pm 1	0.7 \pm 0.1
<i>HorGH1</i> - E173K/M299R	2622 \pm 122	10.1 \pm 0.2	3.8 \pm 0.2	7000 \pm 1 \times 10 ³	12 \pm 1	1.6 \pm 0.1
<i>HorGH1</i> - E173K/H180Y	1080 \pm 61	5.2 \pm 0.1	4.8 \pm 0.3	15000 \pm 1 \times 10 ³	24 \pm 1	1.8 \pm 0.2
<i>HorGH1</i> - M299R/H180Y	3770 \pm 198	7.3 \pm 0.1	1.9 \pm 0.1	23000 \pm 3 \times 10 ³	25 \pm 2	1.1 \pm 0.2

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