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RESEARCH ARTICLE

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Structural elements determining the transglycosylating activity of glycoside hydrolase family 57 glycogen branching enzymes

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Abstract

Glycoside hydrolase family 57 glycogen branching enzymes (GH57GBE) catalyze the formation of an α-1,6 glycosidic bond between α-1,4 linked glucooliogosaccharides. As an atypical family, a limited number of GH57GBEs have been biochemically characterized so far. This study aimed at acquiring a better understanding of the GH57GBE family by a systematic sequence-based bioinformatics analysis of almost 2500 gene sequences and determining the branching activity of several native and mutant GH57GBEs. A correlation was found in a very low or even no branching activity with the absence of a flexible loop, a tyrosine at the loop tip, and two β-strands.

KEYWORDS
amylose, glycogen, glycogen branching enzymes, glycoside hydrolase family 57

1 | INTRODUCTION

Glycogen branching enzymes (GBE) (EC 2.4.1.18) play a key role in the biosynthesis of glycogen, a dendrimeric polyglucose carbon and energy storage molecule present in many prokaryotic microorganisms, fungi, yeast, and animals.[1-4] GBE catalyze the formation of α-1,6 glycosidic linkages in glycogen by transglycosylating a cleaved-off α-glucan chain from a growing α-1,4-linked anhydroglucopyranose chain to the same or a different chain.[1,5-7] In addition to the transglycosylating or branching reaction, GBEs also catalyze the hydrolysis of an α-1,4 glycosidic linkage using water as an acceptors,[8] or perform a cyclization reaction resulting in branched cyclic glucans.[9]

Based on the primary amino acid sequence and conserved motifs, all known GBEs are categorized in either the glycoside hydrolase (GH) family 13 or 57.[10-13] The GBEs from GH13 have a substantially high activity on the model substrate amylose, a typical linear α-glucan.[14-17] GH13 GBEs are involved in the classical glycogen biosynthetic pathway by the tandem action of glucose-1-phosphate adenylyltransferase (*glgC*)—glycogen synthase (*glgA*)—glycogen branching enzyme (*glgB*).[2,18] In contrast to GH13 GBEs, the role of GH57 GBEs is much less clear. So far, the biochemical properties of only five GH57 GBEs have been reported, while the crystal structure of only four of these five GH57 GBEs has been solved.[6,10,19,20] The activity of these five GH57 GBEs on amylose is relatively low to almost zero, ranging from a few mU/mg (*Thermotoga maritima* SMB8) to a few hundred mU/mg (*Thermus thermophilus* HB8, *Thermococcus kodakarensis* KOD1).[8,21] The GH57 GBE of *Mycobacterium tuberculosis*, having all the key features of GH57 GBEs, was reported to have no detectable activity on a range of α-glucans.[22] Based on the genomic organization, it is assumed that this GBE branches glucosylglycerate and plays a role in the biosynthesis of polymethylated polysaccharides.[22-24]

GH57 GBEs have a triangular three-dimensional shape consisting of three domains including a catalytic $(\beta/\alpha)_7$ barrel containing the two catalytic residues, a glutamate nucleophile, and an aspartate acid/base catalyst.[6,10,19,20] All GH57 GBEs have five conserved sequence regions (CSR), with the nucleophile located in CSRIII and the acid/base catalyst located in CSRIV.[25] Similar to GH13 GBEs, GH57 GBEs employ a double-displacement reaction mechanism resulting in retention of the α-configuration in the products.[6] In the crystal structures of the *Pyrococcus horikoshii*, *T. kodakarensis*, and *T. thermophilus* GBE, a flexible loop with a conserved tyrosine at the tip was

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identified.[6,10,19] Mutational analysis showed that the flexible loop and the tyrosine play a key role in the branching activity; replacing the tyrosine with an alanine in the *T. thermophilus* GBE or shortening the loop in *P. horokoshii* GBE resulted in a loss of the branching activity.[6,19]

The simultaneous presence of a gene encoding a GH13 GBE and one encoding a GH57 GBE in the genomes of a large number of bacteria makes the physiological role of GH57 GBEs even more puzzling.[21] To gain more insight into the enzymatic activity and possible physiological role of GH57 GBEs, an in-depth sequence-based bioinformatics analysis of almost 2500 GH57 GBE sequences and a basic biochemical characterization of a number of carefully selected GH57 GBEs overexpressed in *Escherichia coli* was conducted. Surprisingly, the flexible loop covering the active site was absent in the vast majority of the GH57GBE sequences analyzed in this study. Several of these loop-deficient GBEs displayed very low to no activity on amylose. Besides the flexible loop, an additional structural element, with two adjacent beta strands, was identified to play a key role in the branching activity. It is proposed that GH57 GBEs devoid of the flexible loop and the two beta strands are not glycogen-branching enzymes and do not play a role in glycogen biosynthesis but in one or more yet to be identified metabolic pathways.

2 | MATERIALS AND METHODS

2.1 | Sequence collection, alignment, and structural modeling

Sequences were collected by searching the key word "DUF1957" at the National Center for Biotechnology Information (NCBI) on February 04, 2020. To filter redundant and short sequences, the source database and the sequence length were set at RefSeq and 400-1200 amino acid residues, respectively. Partial and hypothetical sequences were deleted manually. Finally, 2497 sequences with the title "DUF1957 domain-containing protein" or "Glycoside hydrolase family 57 protein" were used in the sequence alignment and phylogenetic analysis. ClustalW multiple alignment was conducted with the software package MEGA-X[26] by setting gap opening penalty at 10, the gap extension penalty at 0.2, the negative matrix at off, and a 30% delay divergent cutoff. Five conserved sequence regions were analyzed by calculating possibilities of residues within conserved sequence regions based on the multiple sequence alignment, as described previously.[27] The 3D models of GH57GBEs were predicted with an HHsearch homology detection method by uploading primary sequences of GH57GBEs in the Phyre2 server.[28]

2.2 | Identification of flexible loop and two beta strands

Protein structures were visualized using the Pymol software.[29] The three-dimensional structures of *T. thermophilus* (PDB ID: 3P0B),[6] *T. kodakarensis* (PDB ID: 3N8T),[10] *P. horokoshii* (PDB ID: 5WU7),[19] and *T. maritima* GBE (AmyC) (PDB ID: 2B5D)[20] were downloaded from the Protein Data Bank (PDB). The distances between two residues were measured with the measurement command in Pymol. The flexible loop and the two beta strand regions were defined based on the crystal structures.

2.3 | Overexpression and enzyme production

Various putative GH57 GBEs were overexpressed in *E. coli* (Table 1), and several mutants were also constructed (Table 2). Genes with optimized codons for overexpression in *E. coli* were synthesized and then ligated in the pRSET B (*T. kodakarensis* GBE, *T. thermophilus* GBE, and *T. maritima* GBE) or pET 28a(+) (the remaining GBEs used in this study) vector by Genscript. Plasmids were transformed into *E. coli* BL21 (DE3) by subjecting competent cells to a heat shock at 42°C for 90 s. A single colony was selected from ampicillin or kanamycin-containing agar plate for further cultivation. *E. coli* strains carrying the target gene were stored in 25% glycerol stock at −80°C. *E. coli* BL 21(DE3) carrying the native or mutant *gbe* was grown in Luria-Bertani (LB) medium (Becton, Dickinson and Company) at 37°C and 150 rpm. When the optical density reached 0.6 to 0.8 (600 nm), protein

TABLE 1 Information and sequence similarity of various putative GH57GBEs used in this study

Organism	Abbreviation	Accession number	Length (aa)	Sequence identity (%)
Thermus thermophilus HB8	Tt GBE	WP_011228999.1	520	100.00
Calidithermus timidus DSM 17022	Ct GBE	WP_018467494.1	708	66.2
Meiothermus sp. PNK-Is4	Ms GBE	WP_129865543.1	520	62.9
Thermoflexus hugenholtzii JAD2	Th GBE	WP_088572346.1	558	46.3
Thermococcus kodakaraensis KOD1	Tk GBE	WP_011250387.1	675	44.6
Thermoanaerobaculum aquaticum MP-01	Ta GBE	WP_053334947.1	522	39.4
Thermosyntropha lipolytica DSM 11003	Tl GBE	WP_073092191.1	519	36.4
Kosmotoga pacifica SLHLJ1	Kp GBE	WP_047754759.1	531	36.0
Petrotoga mexicana DSM 14811	Pme GBE	WP_103077822.1	538	33.6
Thermotoga maritima SMB8	Tm GBE	WP_004081707.1	528	32.8

TABLE 2 Mutants of tyrosine residue, flexible loop, and two beta strands used in this study

Mutant enzyme code	Mutant type	Sequence
Tk GBE Y233A	Single site mutation	221 SRLID EGPASNVAGEVLIADTEKT TLRP248
Tm GBE LS	Loop swap	208 SHAFW EGPASNVYGEVLIADTEKT GVYR236
Tm GBE LE	Loop extension	208 SHAFW FADEQPRYGEVLIADTEKT GVYR236
Tk GBE β-strand deletion	β-strand deletion	182 PECAY QGIE190

expression was induced at 20°C for 12 h by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) (Thermo Fisher Scientific) to a final concentration of 0.2 mM. In order to enhance the solubility of the target protein, the chaperone plasmid pG-KJE8 (Takara, Japan) was coexpressed in *E. coli* by following the manufacturer's protocol. Cells were harvested by centrifugation at 8000*g* for 10 min at 4°C, washed cells twice with 50 mM phosphate-buffered saline (PBS) buffer (pH 7.0), and finally resuspended in 5 mM PBS (pH 7.0). Cells were disrupted by high-pressure homogenizer (Avestin, Canada) at room temperature, with an air pressure of 8 bar and a valve pressure of 6 bar. Heat treatment was performed at 50°C (*Calidithermus timidus* GBE, *Meiothermus* sp GBE, *Petrotoga mexicana* GBE) or 65°C (the remaining GBEs used in this study) for 15 min to denature *E. coli* host proteins. Then the supernatant was incubated with nickel metal resin, followed by removal of nonspecifically bound host protein by applying a washing buffer containing 25 mM imidazole (Sigma-Aldrich) and elution of the target protein with 250 mM imidazole. Imidazole was removed using a desalting column (Thermo Fisher Scientific) and a storage buffer to 5 mM PBS (pH 7.0). Purity of the target protein was checked by odium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was assayed by Bradford (Bio-Rad) using bovine serum albumin as the standard.

2.4 | Determination of catalytic activity

The branching reaction was performed at 50°C in 5 mM PBS buffer (pH 7.0) using 0.125% amylose V (Avebe, The Netherlands) as substrate, and a final concentration of enzyme ranging from 0.03 to 0.13 mg/ml was used depending on the total activity of enzyme. Samples were taken at time intervals to monitor the reaction progress. The reaction was stopped by boiling samples at 100°C for 10 min. Branched samples were treated with *Pseudomonas* sp. isoamylase (0.4 U/ml) and *Klebsiella planticola* pulullanase M1 (1.4 U/ml) (Megazyme, Ireland) at 40°C for 24 h with constant mild shaking. Reducing ends were quantified by the 2,2′-bicinchoninic acid (BCA) method using glucose as the standard.[30] The hydrolytic and branching activity units were calculated based on reducing end profiles of samples before debranching and net increase in reducing end profiles, respectively. One unit of activity is defined as 1 μmol reducing ends released or transferred per minute under aforementioned reaction conditions.

3 | RESULTS AND DISCUSSION

3.1 | Sequence-based analysis of putative GH57 GBE sequences

Using the key words "DUF1957 domain-containing protein" or "Glycoside hydrolase family 57 protein," 2497 amino acid sequences were retrieved from the NCIB database. These sequences varied in length between 418 and 1184 amino acids. Except for 50, all sequences had the nucleophile and acid/base catalyst and contained the five conserved sequence regions typical for GH57 members (Figure 1). The exception was sequences that missed one or both catalytic residues and showed a large variation in four of the five conserved sequence regions. These sequences were excluded from further analysis as it was assumed that they are not active. The first four conserved sequence regions are positioned within the A-domain containing the $(\beta/\alpha)_7$ barrel. Conserved region 5 is located in the C domain on the second α-helix.

When comparing the sequence logo for all the GH57 GBEs of this study with the logos recently published on 1602 GH57 sequences,[25] two GBE-specific fingerprints become clear; the first is a quintet of amino acids with the combination HxHLP, with x being A, S, or T, found in CSRI of almost all GBE sequences; in a small number of GBE sequences the L at position 4 is replaced by an I or M. In all other GH57 enzymes, a Q is present instead of an L at position 4 whereas in α-galactosidases and -related proteins there is an L at position 4, but this is followed by a Q or A/M and not a P as is the case for GH57 GBEs. The second GBE fingerprint is the sextet ELF(Y)GHW present in CSRIV. The first position of this fingerprint, the E, is conserved among all proteins assigned to a functional GH57 enzyme subfamily. This E is not conserved in the proteins that are categorized as -like proteins. These proteins miss one or both of the catalytic residues and are very likely not active. In the 4-α-glucanotransferase of *Thermococcus litoralis* the E is only 5.1 Å from the acid-base catalyst D (Figure 2A) and is involved in binding the −1 subsite residue through a water molecule.[31] In the other GH57 crystal structures, the conserved E is 4 Å (*T. maritima* GBE) to 7 Å (*T. thermophilus* GBE) from the acid-base catalyst (Figure 2B,C). In GH13 enzymes, a catalytic triad of a catalytic nucleophile D, a general acid base catalyst E, and a transition state stabilizer D play a key role in catalysis.[32-34] As the CSRIV E is completely conserved in all GH57 proteins assigned to a functional subfamily and is positioned close to the acid–base catalyst

in all available GH57 crystal structures, it is not unlikely to assume that this E plays a similar role as the transition state stabilizer D in GH13.

The other positions of the sextet are completely conserved in all GBE proteins analyzed in this study, with the exception of the third position, the F which is in *Kosmotoga pacifica* GBE replaced by another hydrophobic side chain containing amino acid, a Y. Previously it was reported that the C at position 16 (CSRIII) is conserved among GH57 GBEs,[6] whereas this position is not absolutely invariant, as 72 out of the 2497 (2.9%) sequences have a different amino acid in this position, a feature also noticed by Janeček and Martinovičová[25]; the majority of these have an M (56; 2.2%), nine have an S, five have an L, and two have an F. This almost fully conserved C can still be seen as a fingerprint as none of all the other GH57 enzymes and -like proteins have a C at this position.

In addition, five residues in the vicinity of the active sites were identified to be fully conserved in all sequences; three tryptophans (W274, W404, and W413), one histidine (H146), and one arginine (R265) (*T. thermophilus* numbering). In *T. kodakarensis*, the three tryptophans and the one at position 28 have been defined as the aromatic gate keepers.[8] This group of four aromatic gate keeper tryptophans is highly conserved in all GH57 GBEs except W28, which is a threonine in the GBEs of *Thermus* and *Meiothermus* species. In the *T. thermophilus* GBE, the W274 is positioned to the side and the W404 at the bottom of the positive subsites.[6] Both are involved in substrate binding by aromatic stacking (W274) and hydrogen bonding (W404). In the *T. maritima* GBE, the W274 equivalent (W246) is buried such that aromatic stacking is very unlikely to occur while the position of the W413 equivalent (W411) is difficult to predict.[6] The role of the H146 and the R265 is not clear.

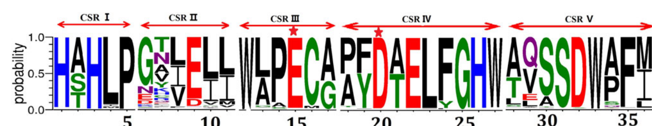

FIGURE 1 Fingerprint logos of five CSRs among 2447 glycoside hydrolase family 57 glycogen branching enzyme homologues; the two catalytic residues are indicated by a red star symbol

In the *P. horokoshii* GBE, a tryptophan (W22) at the bottom of the active site groove is involved in substrate recognition. Changing this W into an A resulted in almost complete loss of activity.[19] This W is also found at the same position in the crystal structure of *T. thermophilus* and *T. kodakarensis*. In GH57GBEs from *T. maritima*, *P. mexicana*, *P. mobilis*, and *K. pacifica*, this W is replaced by D or E or P. Besides the bottom W, four other aromatic amino acids are found in close vicinity of the active sites of *T. kodakarensis*, *T. thermophilus*, *P. horokoshii*, or *T. maritima*; F23, F289, W360, and F461 (*T. thermophilus* numbering). In all the other sequences of this study, three of these four aromatic amino acids are functionally conserved while the F23 is not conserved. Zhang et al.[8] reported another three important amino acids near the active site, H11, S462, and D463 (*T. thermophilus* numbering). These are all conserved at the respective positions in all the 2497 sequences.

3.2 | The flexible loop and tyrosine

From the sequence alignment, two major gaps became visible. The first was a sequence of 19 amino acids from position 229 to 247 (*T. thermophilus* GBE numbering) in between CSR III and IV. In the crystal structures of *T. thermophilus*[6] *T. kodakarensis*[10] and *P. horokoshii*[19] GBE, these amino acids make up a flexible loop covering the catalytic cleft (Figure 3). In 201 other amino acid sequences, a flexible loop of varying length is present (Figure 4). The average loop size is 26 amino acids with the shortest being 13 amino acids, while the longest is 50 amino acids. At the tip of the flexible loop of *T. thermophilus*, *T. kodakarensis*, and *P. horokoshii* GBE, a tyrosine is present.[6,10,19] Part of the flexible loop including the tyrosine was invisible in the crystal structure of *Thermus thermophilus* GBE.[6] The *T. thermophilus* tyrosine was shown to play a prominent role in the branching activity. Replacing this tyrosine with an alanine resulted in a loss of branching activity.[6] A minority of GBEs with a flexible loop do not have a tyrosine at the tip (35; 17.2%). Instead, an alanine,

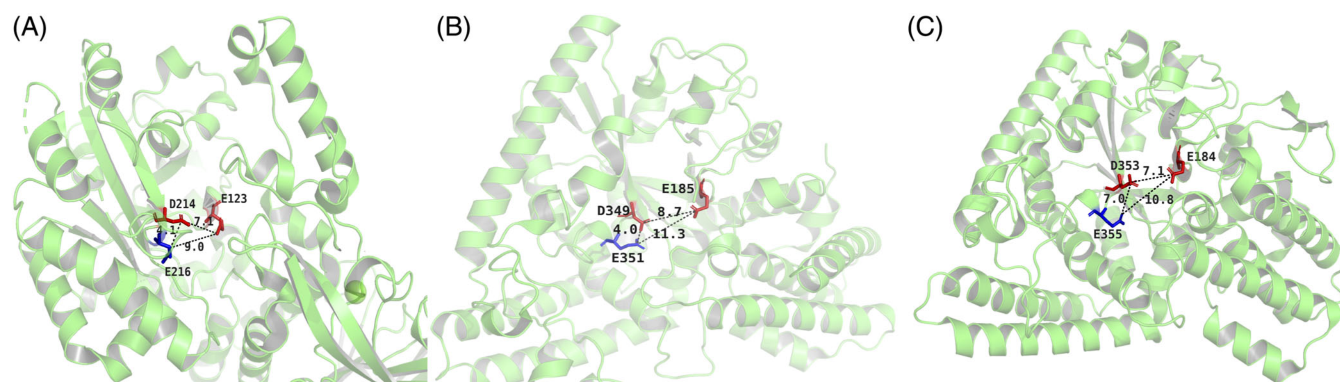

FIGURE 2 Distance in Å between the conserved E of CSRIV (blue) and catalytic residues E in CSR III and D in CSR IV (red). (A) GH57 4-α-glucanotransferase of *Thermococcus litoralis*; (B) GH57 glycogen branching enzyme of *Thermotoga maritima*; (C) GH57 glycogen branching enzyme of *Thermus thermophilus*

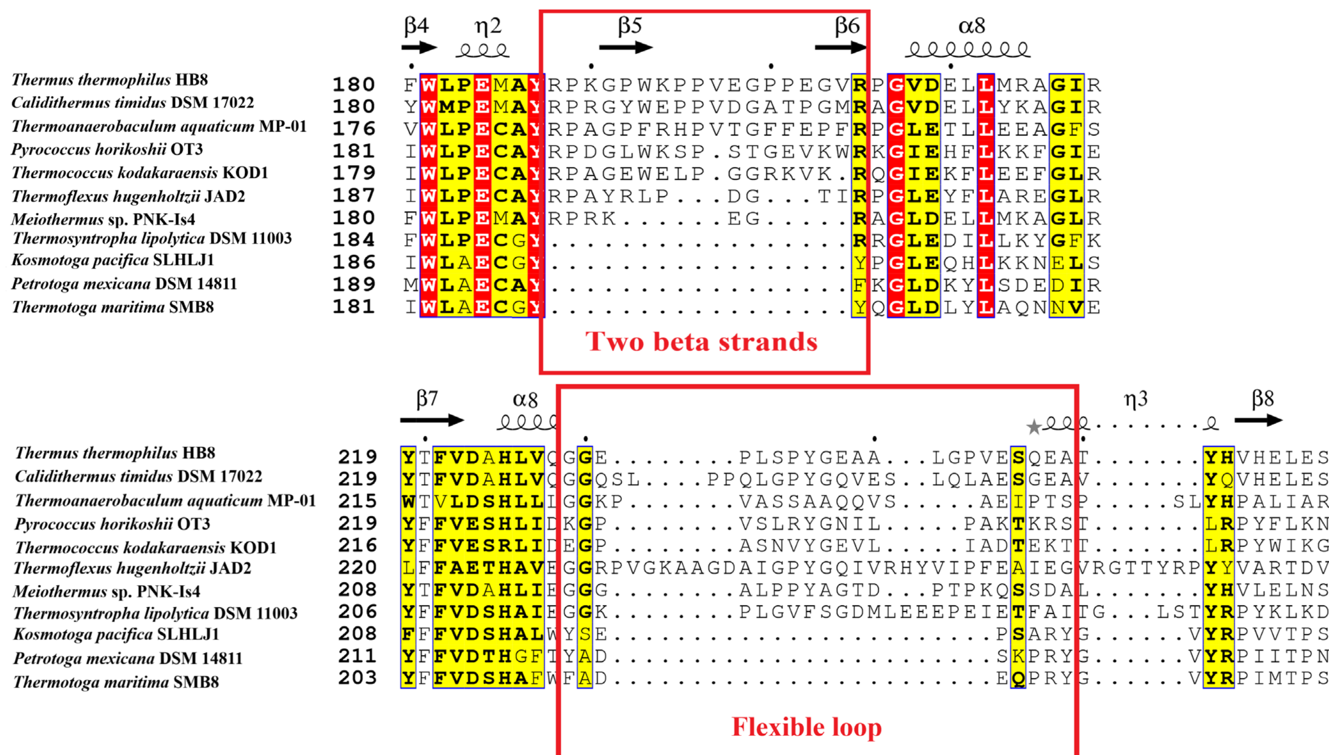

FIGURE 3 Partial alignment of the regions containing the β5 and β6 strands and the flexible loop plus tyrosine of glycoside hydrolase family 57 glycogen branching enzymes. Similar residues are written with black bold characters and boxed in yellow; identical residues are highlighted in red

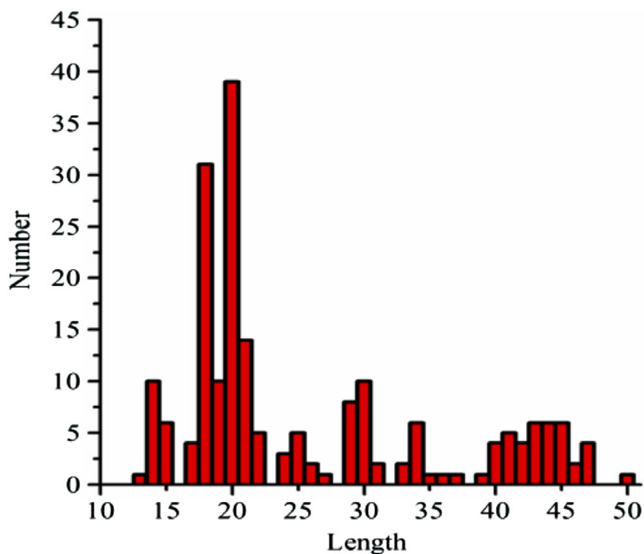

FIGURE 4 Length distribution of the flexible loop among the 201 loop-containing glycoside hydrolase family 57 glycogen branching enzymes

serine, or threonine is found. The *Thermoanaerobaculum aquaticum* GBE, which has a medium-sized loop of 22 residues with an alanine at the tip, has a relatively high activity toward amylose, dominated by the branching activity of 168 mU/mg and a ratio of branching over hydrolytic activity of 38.1 (Table 3). On the contrary, the *Calidithermus timidus* GBE with a flexible loop of 23 amino acids and a tyrosine at the tip has a branching activity of 356.9 mU/mg, being twice that *T. aquaticum* GBE. This result indicates that the tyrosine at the tip of the flexible loop does not play a determining role in the branching activity. This was confirmed by a tyrosine to alanine mutation in the *T. kodakarensis* GBE, which has a flexible loop of 19 amino acids, and the same size and configuration as that of the *T. thermophilus* GBE (Table 3; Figure 3). The wild type *T. kodakarensis* GBE has a dominant branching activity of 480 mU/mg and a branching over hydrolysis ratio of 41, whereas the Y/A mutant retains the dominant branching activity (426 mU/mg). The hydrolytic activity of the Y/A mutant doubled from 12.6 to 26.3 mU/mg, resulting in a branching over hydrolysis ratio of 16.2. These results suggest that not only the tyrosine at the tip of the loop but also the size and configuration of the flexible loop play a role in the branching activity.

In contrast to the *T. thermophilus*, *T. kodakarensis*, and *P. horokoshii* GBE, the flexible loop is absent in the *T. maritima* GBE (Figures 3 and 5), as was already noted by Zhang and coworkers.[8] It has been reported that *T. maritima* GBE, in spite of the lack of a flexible loop, has a low but reproducible branching activity toward amylose.[8] Shortening of the flexible loop in the *P. horokoshii* GBE from 19 to 9 amino acids resulted in a twofold increase in total activity and a considerable reduction of the branching activity.[19] The flexible loop is not only absent in the *T. maritima* GBE but also in 2296 of the 2497 sequences (92%) used in this study (Table 4). A comparable low branching activity was found for the GBE of *P. mexicana* and

TABLE 3 Branching and hydrolytic activity of various glycoside hydrolase family 57 glycogen branching enzymes. The activity was determined using amylose as substrate

	Structural element					
	Loop	Tyrosine	β strands	Branching (mU/mg)	Hydrolytic (mU/mg)	*B/H*[a]
Wild-type enzyme						
Thermus thermophilus HB8	+	+	+	480.0 ± 3.5[b]	11.7 ± 1.5	41.0 ± 2.1
Thermococcus kodakaraensis KOD1	+	+	+	550.0 ± 5.5	12.6 ± 1.7	43.6 ± 3.3
Calidithermus timidus DSM 17022	+	+	+	356.9 ± 10.6	15.3 ± 2.5	24.1 ± 4.6
Thermoanaerobaculum aquaticum MP-01	+	−	+	168.0 ± 9.8	4.4 ± 0.2	38.3 ± 4.1
Meiothermus sp. PNK-Is4	+	+	−	29.1 ± 3.5	6.5 ± 1.5	4.7 ± 1.7
Thermosyntropha lipolytica DSM 11003	+	−	−	NA[c]	NA	/
Thermoflexus hugenholtzii JAD2	+	+	+	13.9 ± 0.1	0.5 ± 0.1	30.6 ± 3.5
Petrotoga mexicana DSM 14811	−	+	−	14.1 ± 3.4	1.4 ± 0.3	10.0 ± 0.5
Kosmotoga pacifica SLHLJ1	−	+	−	9.1 ± 0.8	2.4 ± 0.1	3.8 ± 0.8
Thermotoga maritima SMB8	−	+	−	10.5 ± 0.3	1.8 ± 0.5	5.7 ± 0.1
Mutant enzyme						
Tk GBE Y233A				426.2	26.3	16.2
Tm GBE LS				24.4 ± 3.9	6.2 ± 0.1	4.0 ± 0.6
Tm GBE LE				22.6 ± 3.1	5.6 ± 0.4	4.0 ± 0.8
Tk GBE β-strand deletion				NA	NA	/

[a]Ratio branching activity to hydrolysis activity.
[b]Average of three independent measurements with SD.
[c]NA = no activity.

K. pacifica of 14.1 and 9.1 mU/mg, respectively (Table 3). Although having a low activity, both enzymes introduced 6% branches in the final product when incubated with amylose V (data not shown), confirming that both enzymes clearly have branching activity. Introduction of the full or partial flexible loop of *T. kodakarensis* GBE, including the tyrosine, in the *T. maritima* GBE resulted in a twofold increase of the branching activity and a threefold increase of the hydrolytic activity (Table 3), being substantially lower than the activity of the wild-type *T. kodakarensis* GBE. The flexible loop appears not to be the only structural element that determines the overall activity of the GH57 GBEs.

The length of about 19 residues and configuration of the *T. thermophilus* and *T. kodakarensis* GBE flexible loop seems to be optimal with respect to the branching activity. One hundred three of the 201 sequences (51%) have a flexible loop of 17 to 22 amino acids (Figure 4). A smaller but still significant number of proteins (81; 40%) have a flexible loop of 24 or more amino acids while 17 proteins (9%) have a flexible loop of 13 to 15 amino acids. The models of the *Thermoflexus hugenholtzii* and *Thermosyntropha lipolytica* GBE, with 36 and 28 amino acids loop, respectively, show that only the first part of the flexible loop folds into the active site cleft while the rest folds next to or behind the part that covers the cleft (Figure 5). The *T. hugenholtzii* flexible loop contains a tyrosine at position 245, which is turned away from the cleft being far away from the two active site residues (E191 and D382). This GBE has a very low branching activity of only 13.9 mU/mg, while the *T. lypolytica* GBE did not show any activity (Table 3). Thus, for a GH57 GBE to act as a "true" glycogen branching enzyme, a flexible loop of 17 to 22 amino acids covering the active cleft and, when present, a tyrosine positioned deep into the active site close to the catalytic E and D is required. The absence of the flexible loop or a loop smaller or larger than 17-22 amino acids results in a significant reduction to complete loss of activity toward amylose. What the in vivo substrate for these loop-deficient and long-looped GH57 GBEs is remains to be established. It could be that these GBEs do show activity toward a growing α-glucan chain, the in vivo substrate of most GBEs.[1]

3.3 | The two beta strands

The second gap in the sequence alignment is in the β5-β6 region, where in the *T. thermophilus* GBE with two prominent beta strands (β5-β6; 188-205, *T. thermophilus* GBE numbering) are localized.[6] Without exception, all 2296 GBEs (92%) that miss the flexible loop also do not have the β5 nor β6 strand (Table 4). In contrast, the majority of the 201 sequences that have a flexible loop also possess the β5 to β6 region (159% or 79.1%). Four of the GBEs with a flexible loop and the β5 to β6 region were included in this study and showed considerable branching activity ranging from 168 to 550 mU/mg. Of those GBEs with a flexible loop but without the β5 to β6 region, one, that from *Meiothermus* sp. PNK-Is4, was overproduced and analyzed. It was active toward amylose but as other GBEs without the two beta

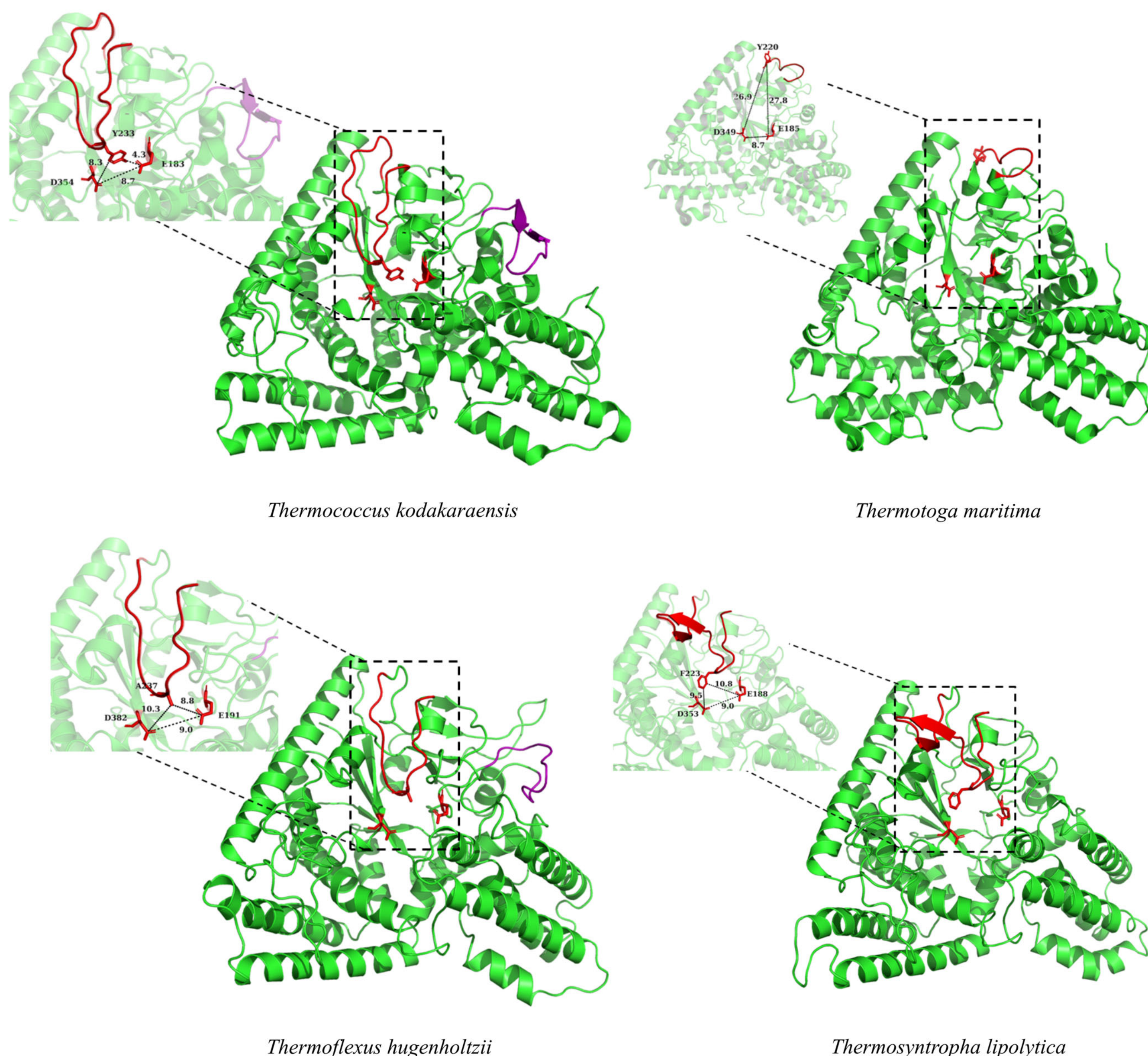

FIGURE 5 Configuration of the several glycoside hydrolase family 57 glycogen branching enzyme showing the position of the flexible loop, the tyrosine at the tip of the flexible loop, and the presence/absence of the β5 and β6 strands (purple). The insert shows the position and distance of the tyrosine at the tip of the flexible loop and the two catalytic residues

TABLE 4 Distribution of flexible loop and β5 to β6 strands among 2.497 glycoside hydrolase family 57 glycogen branching enzymes

Structural elements	# Sequences	% Total
No loop and no β5 to β6 strands	2296	92
Loop and β5 to β6 strands	159	5.3
Loop and no β5 to β6 strands	42	2.7

strands, it only had a very low branching activity (29.1 mU/mg). Also, the branching over hydrolysis ratio was very similar to those GBEs that missed the β5-β6 region; for example, *K. pacifica* and *T. maritima* GBE with a branching over hydrolysis ratio of 3.8 and 5.7, respectively. A similar result was found for the *T. hugenholtzii* GBE, having a long flexible loop of 36 amino acids with a tyrosine and a very short β5 to β6 region (Table 3). No activity could be detected for the *T. lipolytica* GBE, which has a long flexible loop of 28 residues without a tyrosine and no β5 to β6 region. Removing the β5 to β6 region from the *T. kodakarensis* GBE resulted in a complete loss of activity (Table 3).

Interestingly, the *M. tuberculosis* H37Rv GH57 GBE also misses the flexible loop as well as the β5 to β6 region. Gusthart over-expressed the *M. tuberculosis* GH57GBE but did not find any activity.[22] When incubating the *M. tuberculosis* GH57GBE with amylose, no activity was detected (Gang and van der Maarel, unpublished

results). The β5 to β6 region thus plays a determining role in the activity of GH57 GBEs, in particular in the branching activity. How exactly this beta-strand region exerts its influence on the branching activity remains unclear.

4 | CONCLUSIONS

Bioinformatics analysis reveals the sequence-based features of putative GH57GBE sequences, which are two GH57GBE-specific sequence logos, variations in flexible loop and presence/absence of two β-strands were noted in 2497 sequences, with a majority of them (92%) having no flexible loop and absence of two β-strands; Biochemical data verified that putative GH57GBE sequences can be defined by the former feature and true GH57GBEs can be designated by the latter two structural features, which were assigned as optimal flexible loop and presence of two β-strands. These results guide us to further investigate GH57GBEs or -like sequences in an explicit direction.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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