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A predominant β-CGTase G1 engineered to elucidate the relationship between protein structure and product specificity

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ABSTRACT

Low reaction yields and the high cost of obtaining a single type of pure CD make γ-CD costly. Using rational design and with the aid of 3D modeling structures, recombinant CGTase from *Bacillus* sp. G1 was molecularly engineered with the aim of producing a higher percentage of γ-CD. A single mutation at subsite −3, denoted H43T, was found to increase γ-CD production from 10% to approximately 39% using tapioca starch. This novel increment was probably the result of reduced steric hindrance to the formation of γ-CD because of the shortened side chain together with the shortened loop at positions 86–89, at substrate-binding subsite −3. A mutation (Tyr188 → Trp) and a deletion at loop 139–144 showed little effect on product specificity; however, mutagenesis at these sites affected cyclization, coupling and hydrolysis activities as well as the kinetic properties of the mutant CGTase. Based on rational design, three further mutations of the mutant H43T (denoted H43T/Δ(139–144)/S134T/A137V/L138D/V139I, H43T/S85G and H43T/Y87F) were constructed and produced γ-CD with yields of 20%, 20% and 39%, respectively. The mutant H43T/Δ(139–144)/S134T/A137V/L138D/V139I had very low cyclization and coupling activities, however their hydrolysis activity was retained. Double mutation (H43T/S85G) caused the enzyme to exhibit higher starch hydrolysis activity, approximately 26 times higher than the native CGTase G1. Although the mutants H43T and H43T/Y87F could produce the same percentage (39%) of γ-CD, the latter was more efficient as the total amount of CD produced was higher based on the V_{max} and k_{cat} values.

1. Introduction

Cyclodextrin glycosyltransferase (CGTase) is an industrial enzyme used for the production of cyclodextrins (CDs) from starch. The mechanism responsible for the formation of CDs is a cyclization reaction and the reverse reaction is called a coupling reaction. In a disproportionate reaction, starch is cleaved by CGTase and the linear maltooligosaccharide is then transferred to a second linear acceptor substrate. CGTase can also break down starch via a weak hydrolytic reaction.

CGTase produces mainly α-, β- and γ-CDs, which have closed ring structures with six, seven and eight glucose units, respectively, joined by α-1,4-glycosidic bonds. The CDs easily form inclusion complexes with many organic substances that can change the physicochemical properties of the guest molecule, such as its solubility and stability [1,2].

Most of the reported CGTases produce a mixture of α-, β-, and γ-CD, and the product ratio can vary depending on the source of the bacteria, reaction time, and conditions [3,4]. CGTases can be classified into three main groups, α-CGTase, β-CGTase and γ-CGTase, depending on the main CD produced. Most of the numerous reports on CGTase strains involve those that produce β-CD as the main product or α-, β-, and γ-CD in almost equal proportions. However, there have been few literature reports of predominant β-CGTases, a unique subgroup of β-CGTase [5]. A predominant β-CGTase is defined as an enzyme that produces very high percentage of β-CD while the remaining percentage is γ-CD and usually little or no α-CD is formed. As for γ-CGTase, this group is very rare; only a few wild-type CGTases and their sequences are known [6,7]. A major disadvantage of γ-CD production via a common CGTase is that α-, β-, and γ-CD are produced in a mixture where the amount of γ-CD is generally lower.

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Mutagenesis and X-ray crystallography studies of CGTase have resulted in three regions being proposed that may affect product specificity: (a) the centre active cleft [8,9], (b) subsite −3 and (c) subsite −7 [6,10–12]. Alteration of the amino acids in these regions may change the proportion of each CD produced. However, some of the amino acid substitutions in these regions were reported to have a beneficial effect on the product specificity while contradictory results have been reported in other publications [13–15]. At present, the roles of these sites remain unresolved.

Therefore, the aim of this paper is to investigate the mutation of a predominant β-CGTase strain G1 in order to enhance γ-CD specificity. A mutant CGTase H43T (mutagenesis at subsite −3 of the protein) is known to increase the percentage of γ-CD from 10% to approximately 39% [16]. The current paper elucidates that the increment in γ-CD production is probably due to reduced steric hindrance at the active site provided by the shortened side chain after mutagenesis in synchronization with the presence of a shortened loop at positions 86–89. Three further modifications of the mutant H43T were made. A double mutation H43T/Y87F mutant produced a similar CD profile as H43T, but it had a better kinetic performance. Both mutants, CGTase H43T and H43T/Y87F, could potentially be used for γ-CD production.

2. Experiments

2.1. Bacterial strains, plasmids and growth conditions

The alkalophilic bacteria identified as *Bacillus* sp. G1 was isolated from local soil [17], and the DNA fragment that encodes the signal peptide and mature gene of CGTase was cloned into plasmid pUC19 and expressed in *E. coli* JM109 [endA1, recA1, gyrA96, thi, hsd R17 (rB_{kB}^-, m_k^+), relA1, supE44, Δ(lac-proAB), (F′, traD36, proAB, $lacI^qZ\Delta$ M15)]. The transformed cells were grown overnight in Luria-Bertani (LB) medium containing 100 μg/ml ampicillin at 37 °C. Crude enzymes were then concentrated by salting them out with 70% ammonium sulphate and purifying via an α-CD Sepharose column using the same protocol published by Sian et al. [18].

2.2. DNA manipulations

Site-directed mutagenesis was carried out using the PCR method. The megaprimer-PCR method [19] was used to construct mutant H43T. Overlapping-extension PCR [20] was used to create mutants Y188W, Δ(139–144), H43T/(Δ(139–144)/S134T/A137V/L138D/V139I, H43T/S85G, and H43T/Y87F. In all approaches, Pfu polymerase was used because of its proofreading ability. The following oligonucleotides were used, respectively, in PCR amplifications: 5′-CCACCACAATACTTGGTAAGATCTATACAG-3′ (mutant H43T), 5′-CATTTACAGAAACTTATGGGATCTGGCAGA-3′ and 5′-GTCTGCCAGATCCCATAAGTTTCTGTAAAT-3′ (mutant Y188W), 5′-CCCATTTTCAACaagtgccggtgatgaatgatttgg-3′ and 5′-catcaccggcacttGTTGAAAATGGGGCGATATATGAT-3′ (mutant Δ(139–144)), 5′-ACCTCACCGGTTGACatcgaaaatggggcg -3′ and 5′-ccattttcgatGTCAACCGGTGAGGTATGATTTGG-3′ (H43T/Δ(139–144)/S134T/A137V/L138D/V139I), 5′-CCTACACCCAAGCGGCGGTACCTCCTACCATGG-3′ and 5′-CGTTTATGCCCTACACCCAGGCGGCTATACCTCCTACC-3′ (H43T/S85G), 5′-CCTACACCCAAGCGGCTTCACCTCCTACCATGG-3′ and 5′-CCATGGTAGGAGGTGAAGCCGCTTGGGTGTAGG-3′ (H43T/Y87F). Underlined nucleotides are the mutagenized codons and lower case nucleotides are overlapping regions that necessitate generation of the complete gene in the overlapping-extension PCRs. Six mutants were constructed; recombinant wild-type CGTase G1 gene was used as a template for single point mutation and single deletion in the respective mutants. Plasmid mutant H43T was used as a template for double point and multiple point mutations with deletion. Mutant genes were cloned into pUC19 and expressed using *E. coli* JM109. All mutants were confirmed by DNA sequencing.

2.3. Homology modeling

All computational methodology was carried out using Accelrys Discovery Studio (DS) Modeling 1.1 *Windows*-based modules. Blasting to NCBI database was done using the BLAST program, and automated sequence alignment between CGTase G1 and the template sequence was carried out. Adjustment on the gap was done manually to improve the alignment. Homology modeling was performed using Modeler software, and the internal algorithm for the optimization level option for model building was set to high where the program used a thorough molecular dynamic simulation annealing step when building the first model. The 3D structure of CGTase G1 obtained was further optimized by gradual energy minimization in two stages. Constraints in both stages were of positional harmonic constraints of 20 kcal/mol A, applied to all of the backbone atoms. The steepest descents method was set at 500 steps for first minimization, followed by 1000 steps of the conjugate gradient method. The refined model was used as the final structure for further analysis and comparison. The 3D structures for mutant CGTases were constructed using the Build Mutants program in Modeler's package. Display and comparison of structures were achieved using the DS Modeling Visualizer.

2.4. β-CD cyclization assay

The β-CD cyclization activity of CGTase was measured according to the method established by Kaneko et al. [21] with modification. The reaction mixture containing 40 mg of soluble starch in 1.0 ml of 0.1 M phosphate buffer (pH 6.0) and 0.1 ml of enzyme solution was incubated at 60 °C for 10 min. The reaction was stopped by adding 3.5 ml of 30 mM NaOH solution. Then, 0.5 ml of 0.02% (w/v) phenolphthalein in 5 mM Na_2CO_3 was added to the mixture and mixed well. After leaving the mixture to stand for 15 min at room temperature, the reduction in colour intensity was measured at 550 nm. One unit of enzyme activity is defined as the amount of enzyme that formed 1 μmol of β-CD per minute under the conditions mentioned above. Approximately 0.1 mg/ml of purified enzymes were used to determine the specific β-CD producing activity.

2.5. γ-CD cyclization assay

The γ-CD forming activity was determined by the bromocresol green (BCG) method of Kato and Horikoshi [22] with modification. Samples of 0.2 ml of enzyme solution and 1 ml of 4% soluble starch in phosphate buffer (pH 6) were incubated for 20 min at 60 °C. The reaction was stopped by adding 0.5 ml of 0.2 M HCl and subsequently 0.2 ml of 0.05% (w/v) BCG in 20% ethanol. After leaving the mixture for 20 min at room temperature, 2 ml of 1 M sodium acetate buffer containing 30 mM citric acid (final pH 4.2) were added and the colour intensity was measured at 630 nm. One unit of γ-CD forming activity is defined as the amount of enzyme that produced 1 μmol of γ-CD per minute under these conditions. Approximately 0.1 mg/ml of purified enzymes were used to determine the specific γ-CD producing activity.

2.6. CGTase coupling activity

The assay was performed by using β-CD as the donor and methyl-α-D-glucopyranoside as the acceptor, and the method described by Nakamura et al. [23] was used with slight

modification. To a solution containing 10 mM CD and 20 mM methyl-α-D-glucopyranoside, a total volume of 1 ml of CGTase was added. After 20 min of incubation at 60 °C, the reaction was stopped by boiling for 10 min. Then, 0.1 ml of reaction mixture was incubated with 4 U of glucoamylase in 0.1 ml of 0.4 M sodium acetate buffer, pH 5, for 1 h at 40 °C, to convert linear oligosaccharides into single glucose units. The amount of glucose was accurately detected with a glucose/GOD-Perid kit (Sigma). One unit of activity was defined as the amount of enzyme able to convert 1 μmol of CD to glucose per minute under corresponding conditions. Approximately 0.1 mg/ml of purified enzymes were used to determine the specific coupling activity.

2.7. CGTase hydrolysis activity

Hydrolytic activity was assayed using 1 ml of 4% soluble starch as substrate, measuring the increase in starch reduction after incubation at 60 °C for 10 min. The reducing power was measured with dinitrosalicylic acid using the DNS method [24]. One unit of activity was defined as the amount of enzyme that is able to release 1 μmol of reducing end per minute under the corresponding conditions at 540 nm. Approximately 0.1 mg/ml of purified enzymes were used to determine the specific starch hydrolysis activity.

2.8. Protein determination

Protein concentration was quantified by the method of bicinchoninic acid assay using the Pierce BCA Protein Assay™ (Rockford, IL) with bovine serum albumin as the standard.

2.9. Determination of enzyme kinetics parameters

The kinetics parameters for the purified enzyme were determined by incubating 0.2 ml of the samples with soluble starch in 1 ml of 0.1 M phosphate buffer (pH 6.0). The protein concentrations for the samples used were 15–20 μg/ml. The data were produced according to the γ-CD cyclization assay. K_m and V_{max} values were then determined from a Hanes-Woolf plot. The turnover number (k_{cat}) for CGTase G1 was calculated by dividing V_{max} by the molar concentration of CGTase ($k_{cat} = V_{max}/(E)_0$).

2.10. Analysis of CDs by HPLC

Diluted purified recombinant and mutant CGTases with 1 ml of 1% (w/v) tapioca starch in 0.1 M phosphate buffer (pH 6) were incubated at 60 °C for different periods of time, and the enzymatic reaction was then stopped by boiling for 10 min. Insoluble particles were filtered through a 0.45 μm syringe Nylon-filter (Whatman). The proportions of the different CDs produced were analyzed using a Waters HPLC system with an Econosphere NH2 (5 μm, 250 mm × 4.6 mm) column, and CDs were detected using an RI detector. The isocratic flow rate was set at 1 ml/min with 70:30 acetonitrile–water used as the mobile phase. The standards used for calibration were glucose, maltose, maltotriose, maltotetraose, and the CDs and were of high purity grade, purchased from Sigma and Supelco.

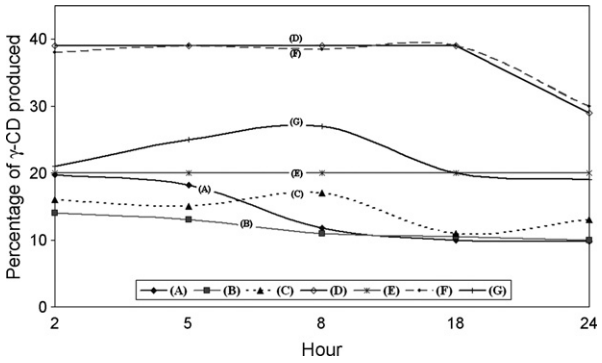


Fig. 1. Product specificity time plot for recombinant and mutant CGTases. A: Recombinant CGTase G1, B: mutant Y188W, C: mutant Δ(139–144), D: mutant H43T, E: mutant H43T/Δ(139–144)/S134T/A137V/L138D/V139I, F: mutant H43T/Y87F, and G: mutant H43T/S85G. In all cases, only β-CD and γ-CD were detected, with negligible α-CD.

3. Results and discussion

In this paper, the predominant β-CGTase G1 was used to elucidate the relationship between protein structure and product specificity. All of the mutant CGTases were purified using the α-CD Sepharose column. Based on the SDS-PAGE gels stained with silver staining kit, at least 98% in purity was achieved for the samples. Besides that, single symmetrical absorbance peak was detected when the purified sample was eluted from Superdex 75 gel filtration column (data not shown). Results from the CD spectra produced by the mutant CGTases constructed in this study are shown in Table 1 and Fig. 1. Table 1 summarized the ratio of β- to γ-CD produced at maximum quantity which occurred at the 18 h incubation, while Fig. 1 indicates the γ-CD ratios that were produced at various reaction periods. Three or more runs were conducted for all the enzymatic reactions studies. The data presented here are the average values.

Blasting of CGTase G1 protein sequence to NCBI database was done using the BLAST program. CGTase from *Bacillus stearothermophiles* (1CYG) was chosen as template for homology modeling due to its highest *E* value of $6.3\,e^{-227}$. Besides that, CGTase G1 has sequence identity of 62% and similarity of 78% to CGTase *Bacillus stearothermophiles*. The final 3D structure of CGTase G1 was obtained by several rounds of optimizations. The ERRAT score for CGTase G1 structure was further improved to 98.07 after three rounds of energy minimization processes resulting in a high

Table 1
Ratio of β-CD to γ-CD produced during the 18th reaction period for recombinant and mutant CGTases. Mutant CGTase H43T and H43T/Y87F both have the highest ratio of γ-CD compared to other CGTases. In all situations, α-CD was not detected.

CGTase	Description	Product specificity (β-CD:γ-CD ratio)
Control	Native recombinant CGTase G1	90:10
Y188W	Center of active site	90:10
H43T	Subsite −3	61:39
Δ(139–144)	Loop deletion at subsite −7	90:10
H43T/Δ(139–144)/S134T/A137V/L138D/V139I	Combination of subsite −3 and −7 mutations, plus amino acid substitutions in the subsite −7 region	80:20
H43T/Y87F	Subsite −3 double mutation	61:39
H43T/S85G	Subsite −3 double mutation	80:20

quality of CGTase G1 model. The score for the final model was 90.991% (data not shown).

3.1. Site-directed mutagenesis at subsite −3 (mutant H43T)

Subsite −3 (residue 43 according to CGTase G1 numbering) was reported to be important for the product specificity of CGTase. There is a clear discrimination pattern at this residue between different groups of CGTase. For α-CD and α-/β-CD producers, lysine (K) or arginine (R) is mainly found whereas, for the β-CD producer without or with minor α-CD (the predominant β-CGTase), histidine (H) is found. Histidine has a relatively shorter imidazole group side chain than lysine and arginine. α-CGTase, α-/β-CGTase, β-CGTase and predominant β-CGTase all carry positively charged amino acid residues at this site. In comparison, CGTase from *Bacillus firmus/lentus* 290-3, *Bacillus clarkii* 7364 and alkalophilic *Bacillus* sp. G-825-6 are γ-CD producers and have threonine (T) at the corresponding locations, as shown in Fig. 2. Threonine has a polar, uncharged and shorter side chain compared to lysine, arginine and histidine.

Wild-type CGTase G1, a member of the predominant β-CGTase category, has histidine at subsite −3 (H43). Based on rational design, mutant H43T was constructed. The proportion of γ-CD produced increased from 10% using the original native recombinant CGTase G1 to 39% for the mutant H43T (Table 1, Fig. 1). Virtually no α-CD was detected within the 24-h reaction period.

CGTase from *Bacillus circulans* 251 and G1 have residue Arg and His, respectively at position 43 (CGTase G1 numbering) as shown in Fig. 2. From the computational modeling for CGTase G1 (and mutant H43T), it was found that His43 and Thr43 (before and after mutation, respectively) have shorter side chains and no strong interactions with ligands, either substrate or product (Fig. 3).

It had previously been proposed that the relatively short side chain of the residue at subsite −3 is the reason for the enhanced formation of the larger CD [12] simply because the forming of the latter compound requires larger cavity volume in the active cleft as compared to smaller size CD. In an earlier report on subsite −3 mutagenesis [12], the mutation from arginine 47 to glutamine in CGTase *Bacillus circulans* 251 produced only a slight increment in the yield of γ-CD. Therefore, this hypothesis seems to be imperfect based on the mutant R47Q of CGTase *Bacillus circulans* 251 [12].

In the case of mutant R47Q of CGTase *Bacillus circulans* 251, having the shorter aliphatic side chain glutamine alone probably seems inadequate to provide a large enough space for γ-CD formation because of the hindrance caused by the loop 87–93 in the region of subsite −3, which is absent in the case of G1 CGTase (Figs. 2 and 4) and other CGTases that produce larger CD, namely γ-CGTases as shown in Fig. 2. The missing loop is actually part of subsite −3 [10] located just below the amino acid 43 (H or T, CGTase G1 numbering). It was deduced that this shorter loop creates extra space in the active site cleft, particularly at subsite −3 in the protein structure. It is therefore hypothesized here that the ability of mutant CGTase H43T to enhance the specificity of γ-CD production is due to the extra space provided by both the shorter side chain of residue 43 and the missing loop (residues 86–89) in CGTase G1 mentioned earlier. In other words, these two factors must occur in concert to maximize the production of γ-CD. This hypothesis seems rational as only predominant β-CGTases and γ-CGTases share this unique characteristic of the missing loop.

Biochemical analyses were carried out for mutant CGTase H43T, and the specific activities are summarized in Table 2. By changing the histidine residue to threonine, the β-CD cyclization activity dropped from 1443 to 217 U/mg while the γ-CD cyclization activity increased from 1.7 to 2.2 U/mg. The starch hydrolysis activity increased tremendously from 3.5 to 40.1 U/mg, a 10-fold enhancement in activity. Mutant CGTase H43T also showed lower affinity for CDs in the coupling reaction where the breakdown of CD was slower compared to the native enzyme. Even though the mutant CGTase H43T was able to enhance the proportion of γ-CD that was produced, the efficiency of this mutant in converting starch to CDs is relatively low compared to the original enzyme. One possible reason is that mutant H43T displays an increased hydrolysis activity, which causes an accumulation of short oligosaccharides in the reaction mixture that can inhibit the cyclization reaction. In addition, the drop in cyclization efficiency might be caused by γ-CD inhibition. It was observed by Hirano et al. [7] that the yield of total CDs produced by γ-CGTase is remarkably lower than that of the other CGTase groups, probably due to inhibition by γ-CD itself.

3.2. Site-directed mutagenesis at the centre active site (mutant Y188W)

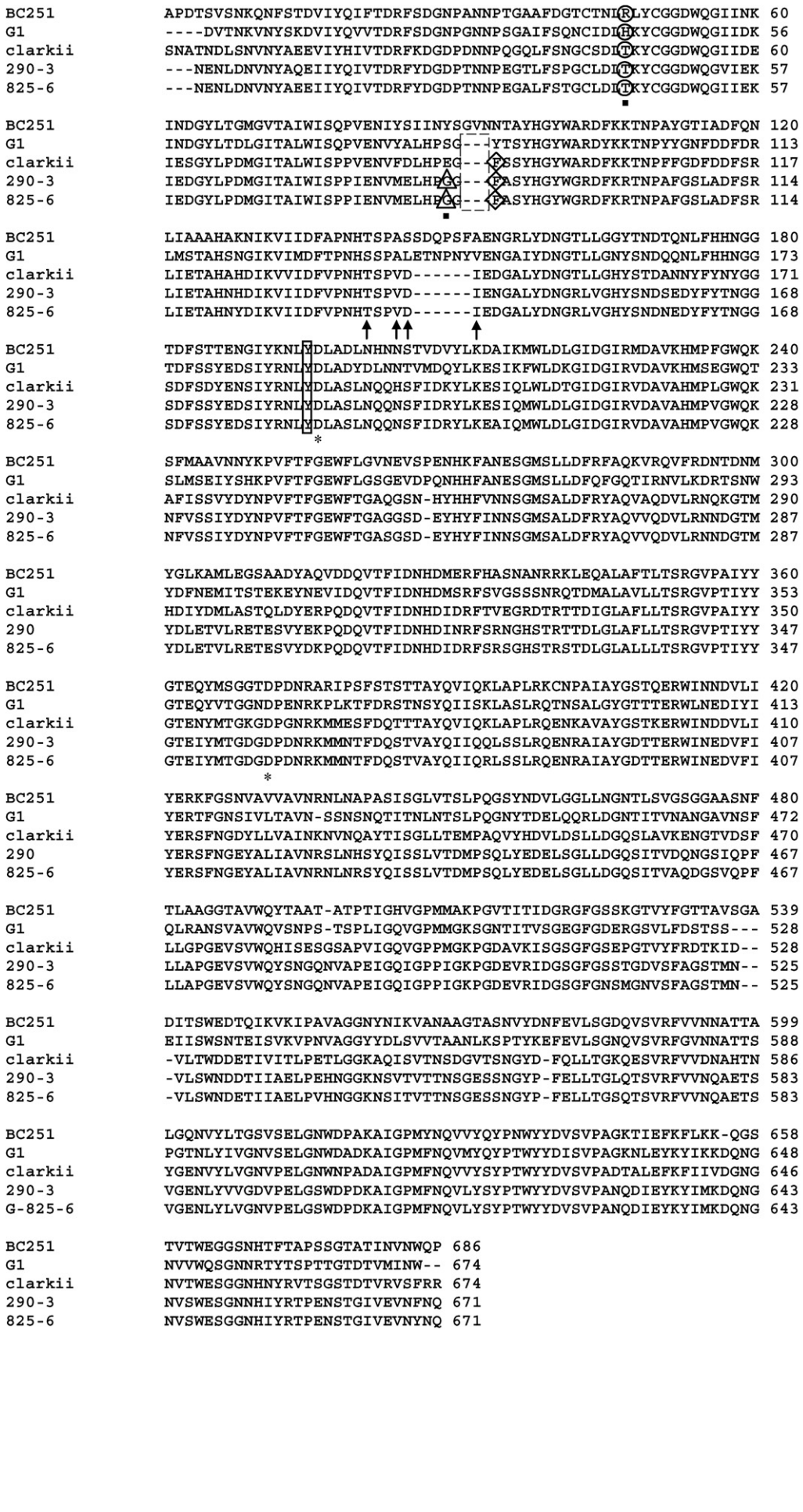
Mutant Y188W was designed based on the theory of the active site cleft [8]. The results in Table 1 and Fig. 1 show that no significant changes in product specificity were observed. Sin et al. [8] suggested that the size of the aromatic amino acid (Phe or Tyr), present in a dominant position in the centre of the active site cleft of CGTases, influences the preferred CD size. It was reported that a double increment in γ-CD yield was observed. The theory of the centre active site cleft was generally accepted at that time and was consistent with the results for CGTase from *Bacillus circulans* No. 8 [9], where the percentage of γ-CD increased from 21% to 55% of the total CDs produced due to the mutant Y195W. However, the theory of the centre active site cleft does not always work for all CGTase strains where mutation at this location did not really determine specificity of the CDs produces [13,14].

In our study, the initial cyclization to form β-CD and γ-CD via mutant CGTase Y188W is significantly less efficient than reaction via the original recombinant enzyme (Table 2). Reduced cyclization activity was also reported earlier for variants from two different CGTases strains where the tyrosine residue was also substituted by tryptophan [9,13]. However, the specific activity of starch hydrolysis for the in-house mutant Y188W was insignificantly higher than that of native CGTase G1. The coupling activity, which is the CD degradation activity for mutant Y188W, was reduced approximately by threefold compared to the control CGTase.

3.3. Double mutation at subsite −7 (mutant Δ(139–144))

The third location that is commonly accepted as important in influencing the product specificity of CGTase is subsite −7 [6,10–12]. Mutant CGTase denoted Δ(139–144) was constructed using the overlapping-extension PCR method. The nucleotides that encoded amino acids numbered 139–144 were successfully removed. The product specificity for mutant CGTase Δ(139–144) is shown in Table 1 and Fig. 1.

No improvement in γ-CD specificity was detected for mutant CGTase Δ(139–144). The biochemical analyses (Table 2) revealed that the overall cyclization activities for this mutant declined to a great extent. Drops of more than 90% and 29% in β-CD and γ-CD forming activities were detected, respectively, compared to the native recombinant CGTase G1. The hydrolysis activity was, however increased while the coupling activity for this mutant was the lowest compared to the parent CGTase, mutant Y188W and mutant H43T. These data reveal that loop 139–144 of CGTase G1 plays an important role in the cyclization and coupling reaction and has a lesser effect on hydrolysis. The importance of the loop at subsite −7 to cyclization and coupling activation was also noted by other researchers [15].


BC251 APDTSVSNKQNFSTDVIYQIFTDRFSDGNPANNPTGAAFDGTCTNLRLYCGGDWQGIINK 60
G1 ----DVTNKVNYSKDVIYQVVTDRFSDGNPGNNPSGAIFSQNCIDLHKYCGGDWQGIIDK 56
clarkii SNATNDLSNVNYAEEVIYHIVTDRFKDGDPDNNPQGQLFSNGCSDLTKYCGGDWQGIIDE 60
290-3 ---NENLDNVNYAQEIIYQIVTDRFYDGDPTNNPEGTLFSPGCLDLTKYCGGDWQGVIEK 57
825-6 ---NENLDNVNYAEEIIYQIVTDRFYDGDPTNNPEGALFSTGCLDLTKYCGGDWQGIIEK 57
BC251 INDGYLTGMGVTAIWISQPVENIYSIINYSGVNNTAYHGYWARDFKKTNPAYGTIADFQN 120
G1 INDGYLTDLGITALWISQPVENVYALHPSG---YTSYHGYWARDYKKTNPYYGNFDDFDR 113
clarkii IESGYLPDMGITALWISPPVENVFDLHPEG---FSSYHGYWARDFKKTNPFFGDFDDFSR 117
290-3 IEDGYLPDMGITAIWISPPIENVMELHPGG---FASYHGYWGRDFKRTNPAFGSLADFSR 114
825-6 IEDGYLPDMGITAIWISPPIENVMELHPGG---FASYHGYWGRDFKRTNPAFGSLADFSR 114
BC251 LIAAAHAKNIKVIIDFAPNHTSPASSDQPSFAENGRLYDNGTLLGGYTNDTQNLFHHNGG 180
G1 LMSTAHSNGIKVIMDFTPNHSSPALETNPNYVENGAIYDNGTLLGNYSNDQQNLFHHNGG 173
clarkii LIETAHAHDIKVVIDFVPNHTSPVD------IEDGALYDNGTLLGHYSTDANNYFYNYGG 171
290-3 LIETAHNHDIKVIIDFVPNHTSPVD------IENGALYDNGRLVGHYSNDSEDYFYTNGG 168
825-6 LIETAHNYDIKVIIDFVPNHTSPVD------IEDGALYDNGRLVGHYSNDNEDYFYTNGG 168
BC251 TDFSTTENGIYKNLYDLADLNHNNSTVDVYLKDAIKMWLDLGIDGIRMDAVKHMPFGWQK 240
G1 TDFSSYEDSIYRNLYDLADYDLNNTVMDQYLKESIKFWLDKGIDGIRVDAVKHMSEGWQT 233
clarkii SDFSDYENSIYRNLYDLASLNQQHSFIDKYLKESIQLWLDTGIDGIRVDAVAHMPLGWQK 231
290-3 SDFSSYEDSIYRNLYDLASLNQQNSFIDRYLKESIQMWLDLGIDGIRVDAVAHMPVGWQK 228
825-6 SDFSSYEDSIYRNLYDLASLNQQNSFIDRYLKEAIQMWLDLGIDGIRVDAVAHMPVGWQK 228
BC251 SFMAAVNNYKPVFTFGEWFLGVNEVSPENHKFANESGMSLLDFRFAQKVRQVFRDNTDNM 300
G1 SLMSEIYSHKPVFTFGEWFLGSGEVDPQNHHFANESGMSLLDFQFGQTIRNVLKDRTSNW 293
clarkii AFISSVYDYNPVFTFGEWFTGAQGSN-HYHHFVNNSGMSALDFRYAQVAQDVLRNQKGTM 290
290-3 NFVSSIYDYNPVFTFGEWFTGAGGSD-EYHYFINNSGMSALDFRYAQVVQDVLRNNDGTM 287
825-6 NFVSSIYDYNPVFTFGEWFTGASGSD-EYHYFINNSGMSALDFRYAQVVQDVLRNNDGTM 287
BC251 YGLKAMLEGSAADYAQVDDQVTFIDNHDMERFHASNANRRKLEQALAFTLTSRGVPAIYY 360
G1 YDFNEMITSTEKEYNEVIDQVTFIDNHDMSRFSVGSSSNRQTDMALAVLLTSRGVPTIYY 353
clarkii HDIYDMLASTQLDYERPQDQVTFIDNHDIDRFTVEGRDTRTTDIGLAFLLTSRGVPAIYY 350
290 YDLETVLRETESVYEKPQDQVTFIDNHDINRFSRNGHSTRTTDLGLAFLLTSRGVPTIYY 347
825-6 YDLETVLRETESVYDKPQDQVTFIDNHDIDRFSRSGHSTRSTDLGLALLLTSRGVPTIYY 347
BC251 GTEQYMSGGTDPDNRARIPSFSTSTTAYQVIQKLAPLRKCNPAIAYGSTQERWINNDVLI 420
G1 GTEQYVTGGNDPENRKPLKTFDRSTNSYQIISKLASLRQTNSALGYGTTTERWLNEDIYI 413
clarkii GTENYMTGKGDPGNRKMMESFDQTTTAYQVIQKLAPLRQENKAVAYGSTKERWINDDVLI 410
290-3 GTEIYMTGDGDPDNRKMMNTFDQSTVAYQIIQQLSSLRQENRAIAYGDTTERWINEDVFI 407
825-6 GTEIYMTGDGDPDNRKMMNTFDQSTVAYQIIQRLSSLRQENRAIAYGDTTERWINEDVFI 407
BC251 YERKFGSNVAVVAVNRNLNAPASISGLVTSLPQGSYNDVLGGLLNGNTLSVGSGGAASNF 480
G1 YERTFGNSIVLTAVN-SSNSNQTITNLNTSLPQGNYTDELQQRLDGNTITVNANGAVNSF 472
clarkii YERSFNGDYLLVAINKNVNQAYTISGLLTEMPAQVYHDVLDSLLDGQSLAVKENGTVDSF 470
290 YERSFNGEYALIAVNRSLNHSYQISSLVTDMPSQLYEDELSGLLDGQSITVDQNGSIQPF 467
825-6 YERSFNGEYALIAVNRNLNRSYQISSLVTDMPSQLYEDELSGLLDGQSITVAQDGSVQPF 467
BC251 TLAAGGTAVWQYTAAT-ATPTIGHVGPMMAKPGVTITIDGRGFGSSKGTVYFGTTAVSGA 539
G1 QLRANSVAVWQVSNPS-TSPLIGQVGPMMGKSGNTITVSGEGFGDERGSVLFDSTSS--- 528
clarkii LLGPGEVSVWQHISESGSAPVIGQVGPPMGKPGDAVKISGSGFGSEPGTVYFRDTKID-- 528
290-3 LLAPGEVSVWQYSNGQNVAPEIGQIGPPIGKPGDEVRIDGSGFGSSTGDVSFAGSTMN-- 525
825-6 LLAPGEVSVWQYSNGQNVAPEIGQIGPPIGKPGDEVRIDGSGFGNSMGNVSFAGSTMN-- 525
BC251 DITSWEDTQIKVKIPAVAGGNYNIKVANAAGTASNVYDNFEVLSGDQVSVRFVVNNATTA 599
G1 EIISWSNTEISVKVPNVAGGYYDLSVVTAANLKSPTYKEFEVLSGNQVSVRFGVNNATTS 588
clarkii -VLTWDDETIVITLPETLGGKAQISVTNSDGVTSNGYD-FQLLTGKQESVRFVVDNAHTN 586
290-3 -VLSWNDDTIIAELPEHNGGKNSVTVTTNSGESSNGYP-FELLTGLQTSVRFVVNQAETS 583
825-6 -VLSWNDETIIAELPVHNGGKNSITVTTNSGESSNGYP-FELLTGSQTSVRFVVNQAETS 583
BC251 LGQNVYLTGSVSELGNWDPAKAIGPMYNQVVYQYPNWYYDVSVPAGKTIEFKFLKK-QGS 658
G1 PGTNLYIVGNVSELGNWDADKAIGPMFNQVMYQYPTWYYDISVPAGKNLEYKYIKKDQNG 648
clarkii YGENVYLVGNVPELGNWNPADAIGPMFNQVVYSYPTWYYDVSVPADTALEFKFIIVDGNG 646
290-3 VGENLYVVGDVPELGSWDPDKAIGPMFNQVLYSYPTWYYDVSVPANQDIEYKYIMKDQNG 643
G-825-6 VGENLYLVGNVPELGSWDPDKAIGPMFNQVLYSYPTWYYDVSVPANQDIEYKYIMKDQNG 643
BC251 TVTWEGGSNHTFTAPSSGTATINVNWQP 686
G1 NVVWQSGNNRTYTSPTTGTDTVMINW-- 674
clarkii NVTWESGGNHNYRVTSGSTDTVRVSFRR 674
290-3 NVSWESGNNHIYRTPENSTGIVEVNFNQ 671
825-6 NVSWESGGNHIYRTPENSTGIVEVNYNQ 671

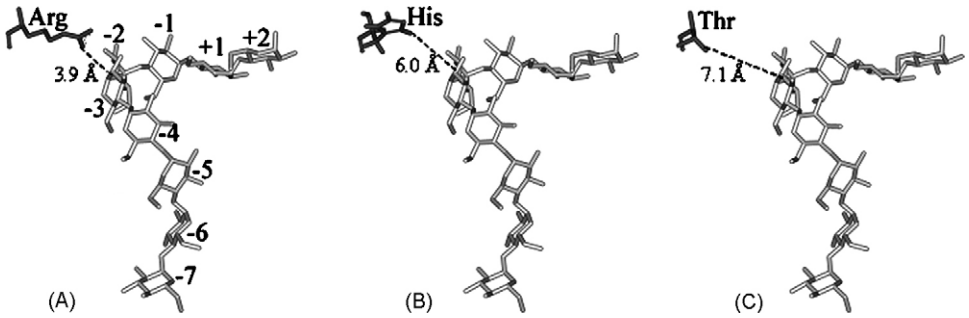


Fig. 3. Nine sugars are bound at the catalytic groove of the CGTase, namely +2 to −7. During the cyclization mechanism, the cleave of starch takes place between sugar −1 and +1. CGTase from *Bacillus circulans* 251 has Arg at subsite −3 position (A) and the distance between the side chain to sugar −3 was approximately 3.9 Å. In CGTase G1 (B), histidine 47 was found to have a distance of 6.0 Å and after mutating the residue to Thr, the distance increased to approximately 7.1 Å (C). The size of CD formed has inverse correlation with the distance between the side chain and the sugars.

Table 2
Specific activities for purified recombinant and mutant CGTases. The specific γ-CD forming activity for mutant H43T, H43T/Y87F and H43T/S85G were higher than the control. Mutant H43T/S85G exhibited extraordinary high starch hydrolysis activity. Mutation at multiple sites (mutant H43T/Δ(139–144)/S134T/A137V/L138D/V139I) has cause the enzyme to lose most of its transglycosylation (cyclization, coupling and disproportional) activities.

CGTase	β-CD cyclization (U/mg)	γ-CD cyclization (U/mg)	Starch hydrolysis (U/mg)	Coupling (U/mg)
G1	1443	1.7	3.5	0.067
Y188W	205	1.4	7.7	0.024
H43T	217	2.2	40.1	0.046
Δ(139–144)	115	1.4	11.0	0.010
H43T/Δ(139–144)/S134T/A137V/L138D/V139I	62	0.2	4.0	0.005
H43T/Y87F	1623	1.8	29.8	0.023
H43T/S85G	847	3.0	93.0	0.049

Parsiegla et al. [9] showed that a similarly constructed mutant Δ(145–151)D, derived from CGTase *Bacillus circulans* No. 8 with doubled the percentage of γ-CD was achieved compared to the wild-type CGTase [9]. It was mentioned that deletion at subsite −7 would probably open up one end of the active site cleft and provide additional space for saccharide binding. However, this thought was further clarified by van der Veen et al. [25] in a review paper when they proposed that the loop 145–151 is located at the end of the active site cleft, which is already opened up to the medium.

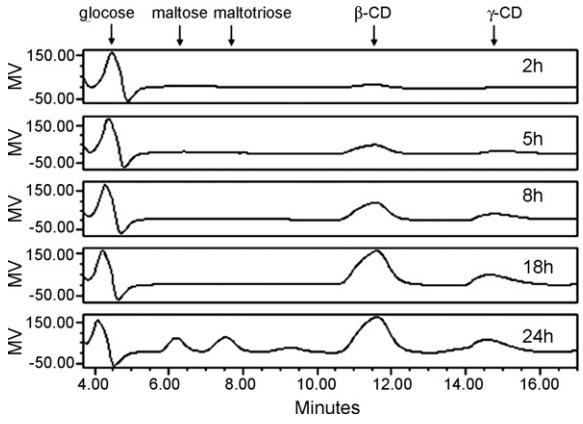


Fig. 4. HPLC chromatogram for mutant H43T/S85G at various reaction times.

This means a better explanation for the enhancement of γ-CD yield is the lack of residues involved in product specificity rather than increased exposure of the active site.

3.4. Further mutations at subsites −3 and −7 (mutant H43T/Δ(139–144)/S134T/A137V/L138D/V139I, H43T/S85G and H43T/Y87F)

Our work has demonstrated that 39% yield of γ-CD and 61% yield of β-CD could be produced by mutant H43T. Although the increment is novel, nevertheless, the proportion of γ-CD is still low compared to wild-type γ-CGTases such as CGTase from *Bacillus clarkii* [6] and *Bacillus* sp. G-825-6 [7] which produces up to 79% and 90% γ-CD, respectively. We demonstrated in another paper that the performance of mutant H43T could be improved by manipulating the reaction conditions [16]. However, at the protein structural level, the factors that can further boost γ-CD specificity are yet unclear.

Using mutant H43T as a template, three additional mutants were designed with aspirations to further enhance the γ-CD ratio. All three mutants are novel and were constructed based on the results of sequence alignment (Fig. 2) and comparison of 3D modeling structures for CGTase *Bacillus* sp. G1, CGTase *Bacillus firmus/lentus* 290-3, CGTase *Bacillus* sp. G-825-6 and CGTase *Bacillus clarkii* 7364.

Both subsites −3 and −7 were proposed to have an important role in determining the product specificity of CGTases [6,10–12]. We

Fig. 2. Mature amino acid sequence comparison for β-CGTase from *Bacillus circulans* 251 (denoted BC251), predominant β-CGTase *Bacillus* sp. G1 (denoted G1) and γ-CGTases from *Bacillus clarkii* 7364 (clarkii), *Bacillus firmus/lentus* 290-3 (290-3), and *Bacillus* sp. G-825-6 (825-6). (○) Arg is found for β-CGTase, His for predominant β-CGTase and Thr for γ-CGTase at position 43 of CGTase G1 numbering. (△) Non-conserved residue; however, two glycines are found alongside for γ-CGTases from *Bacillus firmus/lentus* 290-3 (290-3) and *Bacillus* sp. G-825-6 (825-6). (Dash-box) Unique shorter loop found only in predominant β-CGTase and γ-CGTases. (◊) Conserved Phe found in γ-CGTases. (↑) Locations where the residues adjacent to the gap in the subsite −7 were substituted, as in the case for mutant H43T/Δ(139–144)/S134T/A137V/L138D/V139I. (Box) Residue Y188 (G1 numbering), which was mutated to Trp. (*) Conserved Asp196 and Tyr371 (CGTase BC251 numbering) that stabilize the cyclization mechanism. (■) Arg47 and Tyr89 (CGTase BC251 numbering) that stabilize the product during the cyclization mechanism; however, neither residue is well conserved.

have already shown that only subsite −3 mutations (residue His 43) gave a positive result for CGTase G1. However, it was observed that the naturally existing wild-type γ-CGTases have both threonine and shortened loops at subsites −3 and −7, respectively. Therefore, a new CGTase with multiple site mutations—mutant H43T/Δ(139–144)/S134T/A137V/L138D/V139I—was constructed. This mutant CGTase carries the same amino acids typically found in subsites −3 and −7 of γ-CGTases. The aim of this modification was to recreate the pseudoactive site cleft environment that exists in γ-CGTases. When allowed to react with 1% tapioca starch, approximately 80% β-CD and 20% γ-CD were produced using this mutant (Table 1). Surprisingly, the percentage of γ-CD produced by mutant CGTase H43T was only half. The specific activities of mutant CGTase H43T/Δ(139–144)/S134T/A137V/L138D/V139I were greatly reduced (Table 2). Both β-CD and γ-CD cyclization activities for this mutant dropped to 62 and 0.2 U/mg, respectively, which correspond to 4% and 12% of the original activities. The reduction in cyclization performance was probably due to a weaker interaction between the mutant CGTase and the substrate. Minute changes in starch hydrolysis were detected using this mutant CGTase, as shown in Table 2. The results suggest that there are still some unclear factors affecting the active site cleft and, consequently, the product specificity of the predominant β-CGTase and γ-CGTase, which probably may not lie at subsite −7.

Therefore, two further experiments were carried out in an attempt to identify other amino acids along the active site cleft that may affect product specificity. At the equivalent residue for Ser85 (CGTase G1 numbering), glycine is found in the γ-CGTase from *Bacillus firmus/lentus* 290-3 and *Bacillus* sp. G-825-6 (Fig. 2) and, in fact, two glycines are actually placed side by side in the γ-CGTase protein sequence. Since Ser 85 is part of a loop in the 3D structure, it was thought that substituting Gly for Ser 85 would probably add flexibility to the loop, which is in fact positioned near subsite −3 in the active site cleft.

Interestingly, the percentage of γ-CD produced by mutant H43T/S85G dropped compared to that of mutant H43T, as shown in Table 1 and Fig. 1. Approximately 20% of γ-CD was detected after 2 h of reaction, a maximum of 27% γ-CD was detected after 8 h of incubation, and the number decreased to 19% γ-CD after 24 h of incubation. The action of CGTase H43T/S85G on starch produces a large quantity of glucose. The glucose peaks on the HPLC chromatograms were normally negligible for the native enzyme and mutant H43T. In Table 2, our results also show that replacement of serine 85 with glycine substantially increased CGTase starch hydrolysis activity (93 U/mg), which was obviously higher than that of the native and any other mutant CGTases. The area of the glucose peak in the HPLC chromatogram increased along with the reaction time, however the peak area started to diminish after 18 h (Fig. 4). Major peaks formed at running times specific for maltose and maltotriose were detected after 24 h of reaction. It is very likely that, at the early stage of reaction, hydrolysis activity that produces glucose seems to be enhanced. During prolonged incubation, the degradation of CD (via a coupling reaction) to longer linear products was accelerated and, almost at the same time, disproportionate activity increased, which converted the longer linear product to maltose and maltotriose.

On the other hand, Tyr87 of CGTase G1 has the equivalent conserved amino acid of phenylalanine in the other three γ-CGTases (Fig. 2). Phenylalanine has a slightly shorter side chain compared to tyrosine and it was thought that Tyr87Phe substitution would probably give slightly more room at subsite −3 for the formation of a larger CD. In the time-lapse experiments (Fig. 1), the CD spectra produced by mutant CGTase H43T/Y87F and mutant H43T were similar (39% γ-CD and 61% β-CD). No change in product specificity was observed, and this suggests that the impact of side chain shortening is too small and plays a negligible role in providing more space for the formation of a bigger CD.

Although mutation at H43T/S85G and H43T/Y87F failed to further improve the γ-CD ratio, these results at least suggested that these two amino acids might not be involved in determining the product specificity of CGTases, and particularly not in enhancing the percentage of γ-CD.

3.5. Kinetics parameters of CGTase G1 and mutants

Since the enhancement of γ-CD specificity is the main interest of this paper, the kinetics parameters of CGTase G1 and the mutant CGTases were adjudicated based on the experimental results obtained from the initial rate of formation of γ-CD. The kinetic properties for γ-CD cyclization are summarized in Table 3.

The turnover number (k_{cat}) for CGTase represents the maximum number of γ-CD cyclization reactions per active site per second and characterizes how efficiently CGTase works at saturating starch concentration. The value can also be linked to the activation energy: a high k_{cat} value indicates low activation energy. Recombinant CGTase G1 has a k_{cat} value of 2500 s^{-1}, while the mutants CGTase Y188W, Δ(139–144), H43T/Y87F and H43T/S85G have elevated values. The mutant CGTase H43T/S85G however has the highest turnover number (Table 3). Mutants H43T and H43T/Δ(139–144)/S134T/A137V/L138D/V139I have lower k_{cat} values of 2200 and 1900 s^{-1}, respectively. Mutagenesis at both subsites −3 and −7 of mutant H43T/Δ(139–144)/S134T/A137V/L138D/V139I caused an approximately 25% increase in activation energy.

The k_{cat}/K_m is an apparent second-order rate constant for the enzymatic conversion of starch into γ-CD at low starch and it describes the efficiency of CGTase when the starch concentration is not at saturation levels. A higher k_{cat}/K_m value indicates lower transition-state energy. As shown in Table 3, the original recombinant CGTase G1 has a k_{cat}/K_m value of 900 ml/(mg s). Only mutant CGTase H43T/Δ(139–144)/S134T/A137V/L138D/V139I has a lower catalytic efficiency of 800 ml/(mg s). The other mutant CGTases had higher k_{cat}/K_m values.

Table 3
Kinetic properties of recombinant and mutant CGTases as determined using γ-CD assay. Although the ratio of γ-CD produced by mutant H43T/Y87F was the same as mutant H43T, the former CGTase has higher V_{max} and k_{cat} values and this indicated that mutant H43T/Y87F can actually produce a higher amount of γ-CD. The comparison made was based on the same amount of protein used for each mutant.

CGTase	K_m (mg/ml)	V_{max} ($\times 10^{-3}$ μmol/(min mg protein))	k_{cat} (s)	k_{cat}/K_m (ml/(mg s))
G1	2.9	3.8	2500	900
Y188W	2.0	2.8	3100	1600
H43T	1.9	8.0	2200	1200
Δ(139–144)	1.8	3.1	2600	1400
H43T/Δ(139–144)/S134T/A137V/L138D/V139I	2.4	5.0	1900	800
H43T/Y87F	1.8	19.7	2700	1500
H43T/S85G	2.1	27.8	5000	2400

In the case of mutant H43T and H43T/Y87F, data shown in Table 2 indicates that the γ-CD cyclization, starch hydrolysis, and coupling activities for the latter mutant were lower than those of the former. The difference in overall catalysis preferences influenced the kinetics parameters. The K_m values for both mutants were the same (±1.85 mg/ml), but the V_{max} for mutant H43T/Y87F (19.7×10^{-3} μmol/(min mg protein)) was more than double than of mutant H43T (8.0×10^{-3} μmol/(min mg protein)). These data show that, in fact, mutant H43T/Y87F can actually produce a higher amount of γ-CD compared to mutant H43T although their CD ratios were the same for both mutant CGTases. Thus, the k_{cat} for mutant H43T/Y87F was higher ($2700\ s^{-1}$) than that of mutant H43T ($2200\ s^{-1}$). Nevertheless, the performance of both mutant CGTases at low starch concentrations was almost identical as there was no significant difference in the values of k_{cat}/K_m.

The equilibrium constant between enzyme (E) + substrate (S) (CGTase + starch) and the transition state $ES^{‡}$ is proportional to the activation energy $\Delta G_T^{‡}$ of k_{cat}/K_m [26]. Theoretical Gibbs energy changes during the reaction begin with the interaction of CGTase and starch (E + S) and track the formation of a CGTase–starch complex (ES complex) and the transition state of glycosidic bond cleavage ($ES^{‡}$). From the data of k_{cat} and γ-CD cyclization activity in Tables 2 and 3 for mutant H43T, it shows that at high starch concentrations the binding energy of reaction only stabilized the transition state of glycosidic bond cleavage ($ES^{‡}$) but not the ES binding, based on the theoretical explanation by Fersht [26]. Mutant H43T/Y87F, on the other hand, outperformed mutant H43T because the binding in ES and $ES^{‡}$ was stronger than in the case of the mutant CGTase H43T.

4. Conclusions

Studies of the functionality of CGTase with regard to product specificity need to be revisited. Although the hypotheses of the centre active site cleft and subsites −3 and −7 are generally accepted, some findings in the past were self-contradictory. Here we have produced mutants of a predominant β-CGTase from *Bacillus* sp. G1 to verify these hypotheses. Interestingly, single mutation at subsite −3 (mutant H43T) was able to enhance the yield of γ-CD by approximately fourfold compared to the wild-type enzyme, and this characteristic is novel. It was found, too, that the increment was probably due to the extra space in the active site cleft caused by the mutation itself and the natural existence of free space due to the shortened loop 86–89 adjacent to subsite −3. Residue Y188 and region 139–144 were found to be important for cyclization activity and were not involved in product specificity. Mutations of other amino acids at/near subsites −3 and −7 were also examined; however, we were unable to further improve the γ-CD ratio. Mutant H43T/Y87F outperformed mutant H43T as the former enzyme was able to produce a higher quantity of γ-CD although the CD spectra for both mutants were the same. This is because the binding in the ES and $ES^{‡}$ states was stronger for mutant H43T/Y87F than for mutant H43T.

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