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## Y93F Substitution of Cyclodextrin Glucanotransferase from *Bacillus* sp. A2-5a and its Enzyme Characterization

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### ABSTRACT

In this recent study, Tyr93 of *Bacillus* sp. A2-5a (BA2-5a) cyclodextrin glucanotransferase (CGTase) was replaced with Phe residue by site-directed mutagenesis to study its role on the product specificity and kinetic properties. Molecular docking approach was also applied to acquire a detailed analysis of enzyme-substrate interaction. The Y93F was overproduced in *Escherichia coli* BL21 (DE3) and purified by Ni-NTA resin. The purified Y93F CGTase was 76.39 kDa based on 10% SDS-PAGE analysis and showed both  $\beta$ -cyclization and starch hydrolysis activities in a zymography assay. There was no major structural conformational change in the Y93F since, its optimum and stability of temperature and pH were the same as the wild type. The substitution did not alter the cooperativity property of the enzyme. Our work provided the new evidence that Y93F substitution caused no  $\alpha$ -cyclodextrin (CD) formation in BA2-5a CGTase. In addition, the Y93F produced more  $\beta$ -CD and decreased its  $k_{cat}$  and  $k_{cat}/K_m$ . In conclusion, Y93F substitution of BA2-5a CGTase alters the enzyme kinetic property and product specificity.

**Key words:** *Bacillus* sp. A2-5a, Y93F CGTase, site-directed mutagenesis, kinetic, product specificity

### INTRODUCTION

The cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) catalyzes a conversion of starch into cyclodextrin (CD) through a cyclization (Kelly *et al.*, 2009). The CGTase usually generates a composite of  $\alpha$ -CD,  $\beta$ -CD and  $\gamma$ -CD from starch and product ratio can vary depending on the source of bacteria (Tonkova, 1998). The CDs containing six, seven and eight glucose molecules are called  $\alpha$ -CD,  $\beta$ -CD and  $\gamma$ -CD, respectively. Mechanism of CD formation involves at least nine sugar binding substitutes, labeled -7 to +2 in the active site of *Bacillus circulans* strain 251 (BC251) CGTase (Strokopytov *et al.*, 1996). Cleavage of the starch chain between subsites -1 and +1 results in formation of the

covalently linked intermediate in the substituted-enzyme complex (Uitdehaag *et al.*, 1999) and is followed by the non-reducing end of this intermediate to the acceptor site (circularization) to form a CD (Strokopytov *et al.*, 1996). Cyclodextrin is a unique molecule, possessing cavity with feature hydrophobic inside and hydrophilic outside as of be able to form inclusion complex with hydrophobic compounds. The inclusion complex protects hydrophobic molecule from light degradation, hydrolysis reaction and forming aggregate besides increasing bioavailability of active compound (Qi and Zimmermann, 2005). Therefore, CDs are extensively used in industrial pharmacy, cosmetic, food and textile industry (Del Valle, 2004; Qi and Zimmermann, 2005; Li *et al.*, 2007). The CGTase produces a mixture of  $\alpha$ -CD,  $\beta$ -CD and  $\gamma$ -CD,

therefore, a selective purification step based on dissimilarity in solubility property of different CDs and the different complexing agents are required to obtain the pure  $\alpha$ -CD,  $\beta$ -CD and  $\gamma$ -CD (Biwer *et al.*, 2002). Complexing agents such as organic solvents are usually used and since they are hazardous for human consumption, their presence in final product should be avoided. Generation of CGTase producing only one type of CD is in demand to prevent an expensive procedure and harmful substances. The current effort is to obtain improved enzyme performance by site-directed mutagenesis for better product specificity of CGTase (Penninga *et al.*, 1995; Leemhuis *et al.*, 2010).

The BA2-5a CGTase was the most specific  $\beta$ -CD producer (77%  $\beta$ -CD) with the highest ratio of cyclization per hydrolysis reaction (Kelly *et al.*, 2009). Until now, crystal structure of BA2-5a CGTase had not been determined. The CGTase of BA2-5a shares more than 80% similarity with that of *Bacillus* sp. I-5 in their amino acid sequence, so their higher order structures are expected to be similar (Sin *et al.*, 1994). In *Bacillus* sp. I-5 CGTase, the amino acid residue at position 100 is located near the putative active centre, substitution at position 100 (Y100F) had yielded more product ratio of  $\beta$ -CD. The variations in product specificity between the various CGTases can be ascribed to the relationship between their similar catalytic centre and different subsite structure (Kim *et al.*, 1997). Therefore, mutant BA2-5a CGTase was designed by site-directed mutagenesis which would result in Y93F (100 in *Bacillus* sp. I-5 numbering) and then its biochemical characteristics were analyzed. Molecular docking approach was also carried out to show the role of active site residues interaction with the substrate at the +1, -1, -2, -3, -4, -5, -6 and -7 subsites.

## MATERIALS AND METHODS

**Materials:** *Escherichia coli* Top10 and *E. coli* BL21(DE3) (Invitrogen, USA) were maintained at the Laboratory of Pharmaceutical Biotechnology, School of Pharmacy, Institute of Technology Bandung and used for DNA manipulations and for intracellular production of Y93F CGTase, respectively. pJexpress401 vector (www.dna20.com) carrying the wild type *cgtase*-synthetic Open Reading Frame (ORF) was used as a template for site-directed mutagenesis. Some pairs of primer for mutant ORF generation were designed based on the nucleotide sequences of *cgtase* ORF *Bacillus* sp. A2-5a (EMBL accession number BAA31539.1) using DNASTAR program (DNASTAR, Inc., USA).

### Methods

**Site-directed mutagenesis:** The Y93F CGTase ORF was constructed by site-directed mutagenesis according to Single-Primer Reactions In Parallel (SPRINP) method using *Pwo* DNA polymerase (Roche, Germany) as previously described (Edelheit *et al.*, 2009). The following

primers containing the mutated codons (underlined) were used to introduce the mutation: forward 5'-GTCCTACCACGGTTTTTGGGCTCGC-3'-and reverse 5'-GCGAGCCCAAAAACCGTGGTAGGAC-3-. The product of site-directed mutagenesis was treated by *DpnI* (Promega, USA) and transformed into *E. coli* Top10. The mutation was confirmed by DNA sequencing of mutant CGTase ORF carried by recombinant plasmids obtained from several *E. coli* transformants.

**Production and purification of Y93F CGTase:** Plasmid pJexpress401 carrying positively characterized mutant *cgtase* ORF was transformed into *E. coli* BL21 (DE3) for protein overproduction. The *E. coli* BL21 (DE3) culture was grown in Luria Bertani broth in the presence of kanamycin (25  $\mu\text{g mL}^{-1}$ ) at 37°C in an incubator shaker, 200 rpm. The next step was done following as described with the addition of CaCl to a final concentration of 10 mM at mid-log phase (Imaniar *et al.*, 2012). The Y93F was produced as a fusion protein containing 6 histidine residues at its amino terminus. The Y93F was purified by a Ni-NTA affinity chromatography, according to the manufacturer instruction (Novagen, Germany). After washing, bound protein was gradually eluted by Lysis-Equilibration-Wash buffer containing several imidazole concentrations (15, 50, 100, 150, 200 and 250 mM). Protein purity and molecular weight were determined by a 10% SDS-PAGE analysis. Protein concentration was measured using the Bradford reagent (Bio-Rad, Germany) and Bovine Serum Albumin (BSA) (Sigma-Aldrich, Germany) was used as a standard.

### Activity assays of CGTase

**Cyclization assay:** Cyclization activity was determined by reacting 1 mL of 10% (w/v) pregelatinized soluble starch in 50 mM Tris-HCl buffer (pH 7) and 0.1 mL enzyme solution at 60°C for 30 min. The reaction was stopped by heating in boiling water for 5 min. The formation of  $\beta$ -CD was measured by a colorimetric method through its ability to form inclusion complex with phenolphthalein (Horikoshi, 2004). One unit of activity was defined as amount of enzyme resulting 1  $\mu\text{mol}$  of  $\beta$ -CD per min (Goel and Nene, 1995).

**Zymography assay:** The  $\beta$ -cyclization and starch hydrolysis activities were determined by zymography assay (Pakzad *et al.*, 2004; Imaniar *et al.*, 2012). Native PAGE was performed with 10% polyacrylamide gels. This assay used two wells to separate 15  $\mu\text{L}$  CGTase each.

**Assay of optimal pH and temperature:** Optimal pH was determined by reacting 0.05 U of enzyme with 10% (w/v) pregelatinized soluble starch dissolved in several buffers with varying pH (Rahman *et al.*, 2006). Optimal temperature was determined by incubating the same amount of enzyme with 10% (w/v) pregelatinized soluble starch at different temperature from 30-100°C in pH 7. Reaction was performed using the same treatment represented in cyclization assay (Rahman *et al.*, 2006).

**Assay of pH and temperature stability:** The pH stability was determined by incubating 0.05 U enzyme in several buffers at different pH (Rahman *et al.*, 2006) without substrate at 60°C for 30 min. Subsequently, the residual activity of the enzyme was measured after reacting 0.05 U enzyme mixture with 10% (w/v) pregelatinized soluble starch in 0.05 M Tris-HCl buffer (pH 7) and incubated at 60°C for 30 min. Temperature stability was determined by incubating 0.05 U enzyme with 0.2 mL of 0.05 M Tris-HCl buffer (pH 7) at several temperatures from 30-100°C, without substrate for 30 min. Subsequently, the residual activity of the enzyme was measured after reacting 0.05 U enzyme mixture with 10% (w/v) pregelatinized soluble starch in 0.05 M Tris-HCl buffer (pH 7) and incubated at 60°C for 30 min (Rahman *et al.*, 2006).

**Assay of protein thermal shift:** The protein stability to thermal exposure was analyzed in a protein thermal shift assay (Applied Biosystem, USA) as described (Ericsson *et al.*, 2006) with slight modification in solution composition, temperature setting as well as excitation and emission wavelengths using Real-Time PCR CFX96 (Bio-Rad Laboratories, USA). The composition of each reaction in this work was 5 µL dye buffer, 15 µL of 1mM CGTase and 5 µL dye 50X Sypro Orange dye (ROX reporter) in each (96-well thin-wall PCR plate) (Applied Biosystem, USA). Temperature was set at range of 25-95°C with temperature increment of 0.5°C sec<sup>-1</sup>. The excitation and emission wavelength used were 589±10 and 623±14 nm, respectively.

**Kinetic parameters:** Kinetic parameters were measured by reacting 0.05 U of pure enzyme in solution of pregelatinized soluble starch at concentration from 0.01-10% (w/v) at 60°C for 30 min. The values of K<sub>m</sub> and k<sub>cat</sub> were determined by the nonlinear least-squares method with Hill-equation (Kim *et al.*, 1997).

**Analysis of CDs by High-Performance Liquid Chromatography (HPLC):** The product specificity of Y93F CGTase was analyzed by determining the concentration of α-CD, β-CD and γ-CD measured by HPLC (L-2490 RI Detector, Hitachi, USA) with Spherisorb NH column (10 µm, 4.6×250 mm) (Waters, USA) with acetonitrile and water (70:30) as the mobile phase and a flow of 1 mL min<sup>-1</sup>, at room temperature (Kelly *et al.*, 2009). The product ratio from the reaction of mutant CGTase on 10% soluble starch was determined after 24 h of incubation at 60°C in 0.1 M Tris base buffer pH 7.

**Homology modeling and molecular docking in 3D structure:** The homology identity of BA2-5a CGTase to BC251 CGTase was 59%. BC251 CGTase had crystal structure which was deposited in Protein Data Bank (PDB) with PDB ID: 1CDG (2.0 Å resolutions) (Lawson *et al.*, 1994). The three dimensional structure of Y93F CGTase was built using this crystal structure by the SWISS-MODEL version 4.1 program at the ExpASY server. The Y93F CGTase was

modeled using wild type BA2-5a CGTase as a template by replacing the suitable amino acid and maltonaose (PDB 1CXX) was used as substrate (Uitdehaag *et al.*, 1999). Autodock vina in PyRx v 8.0 PyRx was applied to study the interaction between CGTase and its substrate (Jacob *et al.*, 2012). The result of docking complex was analyzed using Pymol version 1.6.0.0 and Ligplot (Wallace *et al.*, 1995). SuperPose version 1.0 was used to calculate the Root Mean Square Deviation (RMSD) between the template-model and the wild type-mutant model of alpha carbon backbones were used (Maiti *et al.*, 2004). Homology modeling and molecular docking of Y100F CGTase from *Bacillus* sp. I-5 were also built by using the same template and substrate. The identity of *Bacillus* sp. I-5 CGTase to BC251 CGTase was 88%.

## RESULTS AND DISCUSSIONS

The Y93F CGTase was generated by site directed mutagenesis of wild type *cgtase* ORF of BA2-5a, confirmed by DNA sequencing to show that the proper base substitution (TTA93<sup>®</sup>TTT) at codon 93 and no unintended mutations were introduced. The Y93F was overproduced as a His-tag fusion protein in *E. coli* BL21 (DE3) and purified by Ni-NTA chromatography. When elution was done by LEW buffer containing low concentrations of imidazole (50, 100 and 150 mM), a protein impurity of 40 kDa was identified together with Y93F CGTase (data not shown). The impurity was probably Lac repressor (produced by the *E. coli* BL21 (DE3) (Owens *et al.*, 2001). The repressor protein contains three histidine residues at 163, 173 and 201 positions; therefore, it can bind nonspecifically to Ni-NTA resin (Owens *et al.*, 2001). The Lac repressor could be separated from Y93F CGTase when higher concentrations of imidazole were used for elution. A single protein of Y93F CGTase (76.36 kDa) was obtained after elution using imidazole concentrations of 200 and 250 mM (Fig. 1). The size of the purified protein was in

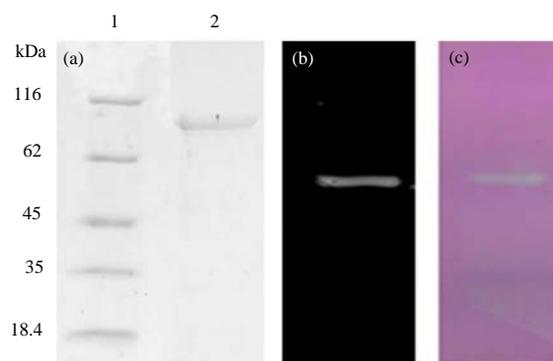


Fig. 1(a-c): SDS-PAGE analysis of purified Y93F CGTase and its zymography assay results (a) 10% SDS-PAGE, Lane 1: protein marker, Lane 2: pure Y93F CGTase protein, (b) Iodine staining with clear band showing the result of starch hydrolysis and (c) Phenolphthalein staining with a sharp clear band showing β-CD production

agreement with its theoretical size. The purified protein was active for both  $\beta$ -cyclization and starch hydrolysis activities as determined by zymography assay (Fig. 1).

**Effect of Y93F substitution on CGTase conformation:** To determine whether Y93F substitution affected major three dimensional conformation of CGTase, several enzymatic parameters of the mutant protein were investigated and compared to those of wild type enzyme. The Y93F exhibited

optimum activity at 60°C and pH 7.0. The mutant had half-life temperature about 65.4°C and was stable from pH 7.0-9.0 with a gradual decrease of activity at higher and lower pH values (Fig. 2). A thermo-shift assay was also performed to define the melting point ( $T_m$ ) of Y93F. The result showed that the  $T_m$  of Y93F was 69°C (Fig. 2). From previous work, wild type CGTase had optimum activity at (60°C, pH 7.0),  $T_{50}$  about 65.4°C, pH stability from pH 6.0-8.0 and  $T_m$  (69°C). All enzymatic characteristics i.e., the optimum temperature

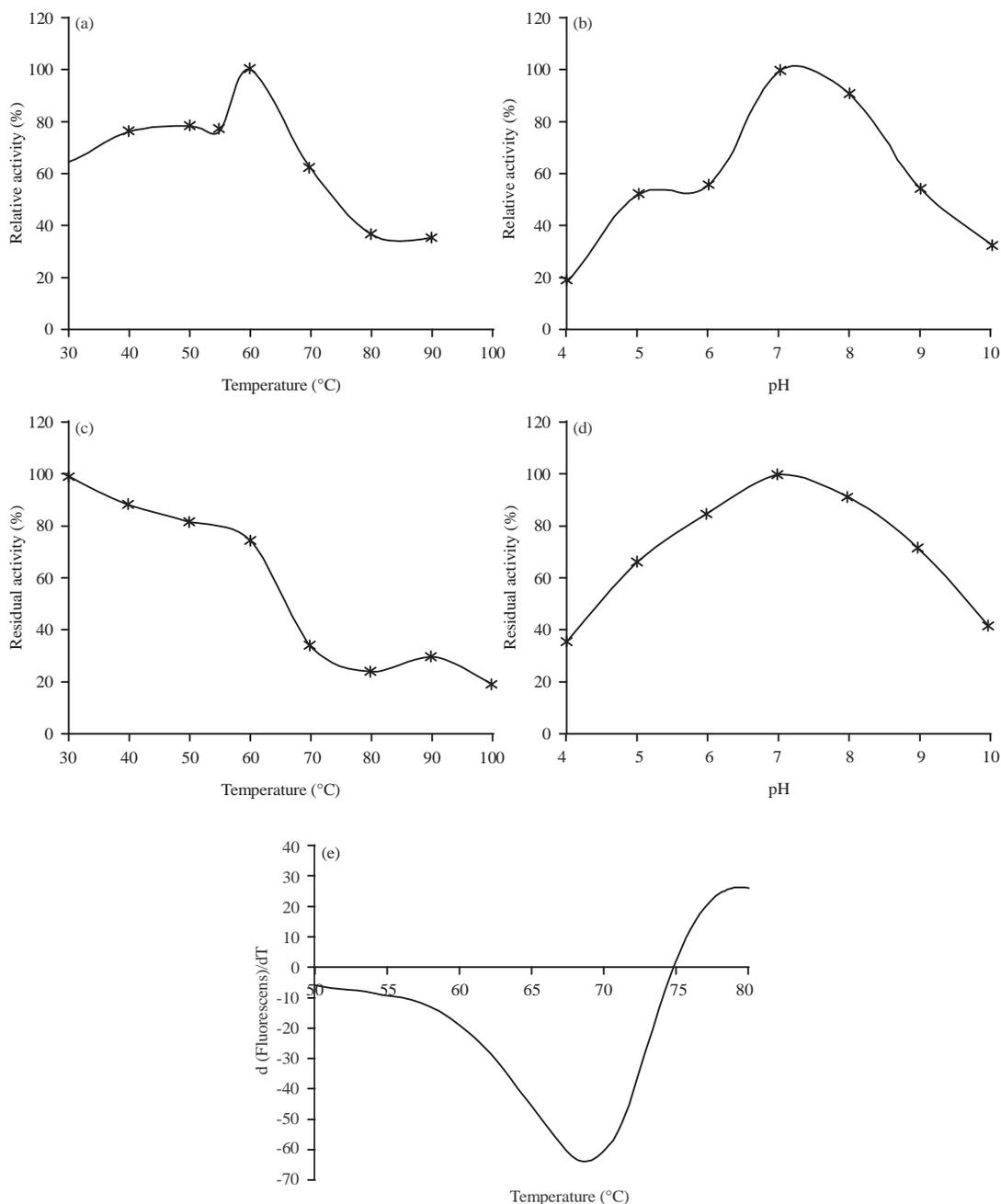


Fig. 2(a-e): Properties of Y93F CGTase showing (a) Optimum temperature, (b) Optimum pH, (c) Thermostability, (d) pH stability and (e) Thermal-stability of Y93F CGTase

and pH,  $T_m$ ,  $T_{50}$  and pH stability of Y93F were the same as those of wild type CGTase strongly indicating that the mutant did not undergo any significant structural changes. Our homology model of Y93F was built using the crystallographic structure of BC251 CGTase as the template because they shared 59% identity. This model showed that the overall structure of Y93F BA2-5a and BC251 CGTase was quite similar (RMSD of 0.9Å). This comparative model had a quite high accuracy, with the RMSD value i.e., <1 Å and the sequence identity percentage was more 50% (Marti-Renom *et al.*, 2000; Baker and Sali, 2001). Moreover, RMSD value of mutant with wild type was 0.07 Å. This showed that structural alignment of Y93F was almost the same as that of the wild type. The result of this modeling also revealed that major structural alteration did not occur in Y93F CGTase.

**Homology modeling and molecular docking of Y100F *Bacillus* sp. I-5 CGTase:** To compare Y93F BA2-5a-substrate interaction with Y100F *Bacillus* sp. I-5-substrate, model and molecular docking of Y100F were built. The RMSD value of Y100F *Bacillus* sp. I-5 CGTase with BC251 CGTase was 0.25 Å with the identity >80%. The model of Y100F *Bacillus* sp. I-5 CGTase had also a quite high accuracy with those values as analyzed (Marti-Renom *et al.*, 2000; Baker and Sali, 2001). Molecular docking data was explained in kinetic parameter and product specificity section.

**Kinetic Parameters of Y93F:** The kinetic parameters  $K_m$ ,  $k_{cat}$ ,  $k_{cat}/K_m$  and the Hill coefficient- $n_H$  for Y93F CGTase were 0.53 mg mL<sup>-1</sup>, 216 sec<sup>-1</sup>, 408 mL mg<sup>-1</sup> sec<sup>-1</sup> and  $n_H=1$ , respectively. Those parameters for wild type CGTase from earlier study were 0.52 mg mL<sup>-1</sup>, 653 sec<sup>-1</sup>, 1257 mL mg<sup>-1</sup> sec<sup>-1</sup> and  $n_H=1$ , respectively. Our data showed that replacement of Tyr at position 93 with Phe in BA2-5a

CGTase did not alter the  $K_m$  and non-cooperativity manner but decreased  $k_{cat}$  and  $k_{cat}/K_m$  almost three-fold. These data demonstrated that Tyr93 did not play a crucial role in affinity between substrate and enzyme as well as the cooperativity property of BA2-5a CGTase. This observation was in agreement with Y100F substitution in *Bacillus* sp. I-5 CGTase (93 in BA2-5a) did not change its  $K_m$  (Kim *et al.*, 1997). Using the molecular docking approach, there was no interaction type change of Phe93 with substrate observed in Y93F CGTase compared to Tyr93 in wild type. Then, the inclusion of Phe residue at positions 93 may also not redesign the enzyme conformation and consequently did not affect flexibility of mutant to bind substrate (Monod *et al.*, 1965). The  $k_{cat}$  states distinction energy level between the substrate bound ground state and the transition state or the activation energy (Van der Veen *et al.*, 2000a). The  $k_{cat}/K_m$  is a postulate for catalytic efficiency, represents the energy level of the transition state, with case to the free enzyme and free substrate (Van der Veen *et al.*, 2000a). An enzyme with low  $k_{cat}/K_m$  has high transition state energy so less efficiently in utilization of soluble starch. In our research, Y93F substitution caused a significantly increased activation energy and energy level of transition state. The impact of lower  $k_{cat}$  of Y93F was the reduction of  $\beta$ -CD product formation from soluble starch by one molecule of the mutant CGTase active site in a condition of enzyme saturation by substrate. This explains that Y93F cannot utilize soluble starch efficiently. Our results are in agreement with those observed in CGTase of *Bacillus* sp. I-5 that Y100F (93 in BA2-5a) substitution resulted in the decrease of  $k_{cat}$  and  $k_{cat}/K_m$  (Kim *et al.*, 1997). Based on our molecular docking analysis result, no interaction was observed between substrate and Asp364 due to the loose of one hydrogen bond present in the wild type (Fig. 3). This interaction in wild type was presented in the prior

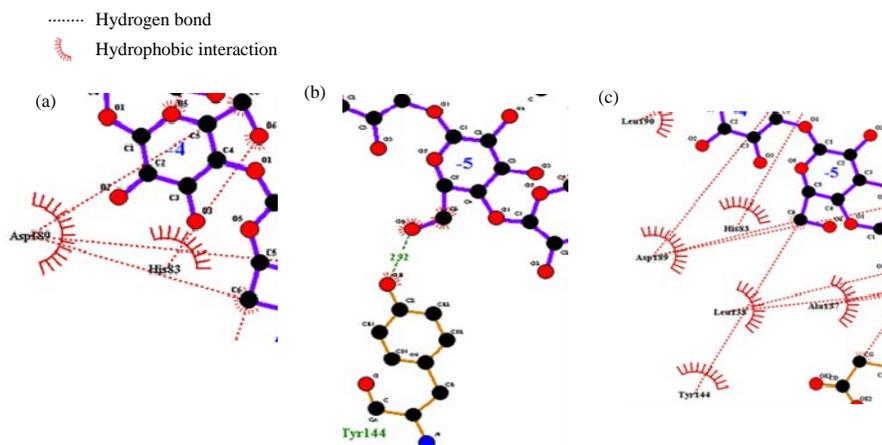


Fig. 3(a-c): A view of the interactional types of wild type and Y93F CGTase residues with sugar at subsite -4 and -5. The figure was constructed by Autodock Vina and visualized by Ligplot. (a) Hydrophobic interactions between Asp189 and His83 of Y93F CGTase to sugar at subsite -4, (b) A hydrogen bond between Tyr144 wild type BA2-5a CGTase with sugar at subsite -5 and (c) A hydrophobic interaction between Tyr144 of Y93F CGTase with sugar at subsite -5

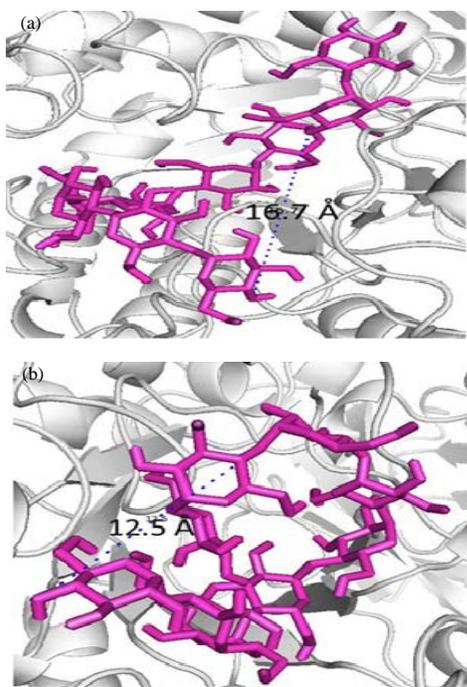


Fig. 4(a-b): Modelling structure of Y93F *Bacillus* sp. A2-5a and Y100F *Bacillus* sp. I-5 with a bound maltononaose inhibitor. Enzymes are shown in light grey, ligands in magenta. The distance between O1(-1) and O4 (-7) in substrate-CGTase interaction (Å) (a) Y93F *Bacillus* sp. A2-5a CGTase-substrate and (b) Y100F *Bacillus* sp. I-5 CGTase-substrate

work. Previous study showed that Asp371 in BC251 (equal to position in 364 BA2-5a) served to stabilize at subsite -3 in a circularization process (Kelly *et al.*, 2009). The Y93F may lack stabilization of the transition state and this may influence the increase of activation energy and the decrease of catalytic efficiency. This finding is supported by the fact that Y100F substitution in *Bacillus* sp. I-5 CGTase (equal position as Y93F) had decreased the cyclization activation (Kim *et al.*, 1997).

**Product specificity of Y93F:** The product ratio of Y93F CGTase on 10% soluble starch was investigated after 24 h of incubation at 60°C in 0.1 M Tris base buffer pH 7.0 by using HPLC with the ratio of  $\alpha$ - $\beta$ - $\gamma$ -CD i.e 0:82:18, whereas the ratio of those of wild type from previous work was 2:77:21. In this condition, the Y93F produced no  $\alpha$ -CD, increased  $\beta$ -CD by 6% and decreased 16% of  $\gamma$ -CD. Hence, Y93F substitution altered the product specificity of B2-5a CGTase. The replacement of Tyr93 with Phe resulted in a significant change in the product ratio of  $\beta$ -CD and  $\gamma$ -CD. Interestingly, no  $\alpha$ -CD was produced by Y93F. The tendency toward  $\beta$ -CD formation was supported by the presence of hydrophobic interaction between Tyr144 of Y93F CGTase with sugar at subsite -5 in molecular docking model (Fig. 3). While this

position in the wild type CGTase-substrate interaction, a hydrogen bond was observed. The variable loop region located in positions 142-152 (136-145 in BA2-5a CGTase) is correlated with CD size specificity (Kelly *et al.*, 2009). A previous work showed that S146P substitution in BC251 was introduced to disturb this hydrogen-bonding network, caused a significant alteration in its product specificity with a strongly reducing  $\beta$ -CD ratio (Van der Veen *et al.*, 2000b). The property of Y93F in lowering the ratio of  $\alpha$ -CD and increasing the ratio of  $\beta$ -CD was similar with Y100F substitution in *Bacillus* sp. I-5 (Kim *et al.*, 1997). Unlike in Y100F *Bacillus* sp. I-5, corresponding substitution in BA2-5a, Y93F decreased  $\gamma$ -CD ratio. To our knowledge, the mechanism of lowering of  $\gamma$ -CD ratio in Y93F is unclear. Seven glucoses are required in the formation of  $\beta$ -CD, therefore the distance between the nonreducing end of O4 (-7) oxygen of the maltononaose inhibitor at subsite -7 and the reducing end O1 (-1) oxygen at subsite -1 should be closed and stabilized long enough to allow the  $\beta$ -CD ring (Strokopytov *et al.*, 1996). From molecular docking approach, the distance of O4 (-7) oxygen to O1 (-1) oxygen of the maltononaose for Y93F and Y100F were 16.7 and 12.5 Å, respectively (Fig. 4) with the result that Y93F may lack stabilization to allow the  $\beta$ -CD ring. These results may explain that Y93F was less optimal in  $\beta$ -CD formation compared in Y100F since the increased ratio of  $\beta$ -CD in Y93F BA2-5a was half (6%) than that of in *Bacillus* sp. I-5 CGTase which (12%) (Kim *et al.*, 1997). However, the interacting residues present in Y93F but not in wild type enzyme with substrate generated no  $\alpha$ -CD production.

## CONCLUSION

The results of experimental work as well as the molecular model of the *Bacillus* sp. A2-5a Y93F show that Phe93 affects the ratio of  $\alpha$ - $\beta$ - $\gamma$ -CD with the loss of a hydrogen bond between Tyr144 and substrate. This work reveals the new insight that no  $\alpha$ -CD formation and decreased  $\gamma$ -CD of this mutant that would be suitable to applications producing  $\beta$ -CD. Moreover, our result shows for the first time that Tyr93 in BA2-5a CGTase is pivotal to preserve enzymatic activity energy but not to maintaining cooperativity property and enzyme-substrate affinity.

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