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Structure basis of a mutant α-CGTase tyrosine167histidine from *Bacillus* sp. 602-1 with enhanced α-CD productions

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ABSTRACT

The crystal structure of Y167H mutant α-CGTase was determined at 2.1Å. The overall structure was like that of the Y195I mutant α-CGTase, with minor difference in flexible domains and the shift of one Ca^{2+}. The H167 in Y167H mutant α-CGTase moved slightly to surface of the enzyme. In the static models of enzyme-sugar complex, the side chain of H167 is above 5Å far from the sugar chain instead of 3Å of the Y167 in the Y195I mutant. H167 could bind the sugar chain at initial stage better due to its charges and location. This work helps explain the mechanism of the enhanced production of α-CD by Y167H mutant α-CGTase.

Keywords

α-CGTase, *Bacillus* sp. 602-1, α-CD, Crystal Structure, Product Specificity

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Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides composed of six, seven, or eight glucopyranose residues, nominated as α, β, and γ-CD, respectively [1]. The obtention of CDs relies on the starch conversion conducted by Cyclodextrin glucanotransferase (CGTase) *via* its cyclization reaction [2]. All known CGTase produce a mixture of α, β, and γ-CD, and CGTase is classified into three kinds (α, β, and γ-CGTase) based on its product specificity [3]. CD can encapsulate guest molecule, and are widely used in food, cosmetics pharmaceutical, and chemical industries, etc. [1,4,5]. α-CDs have better water-solubility and smaller cavity, which make them more needed. Many constructed mutations alter the cyclization of CD products. The nine sugar-binding subsites (labeled from -7 to +2) are predominant domain, especially the donor subsites -3, -6 and -7 [3], which affect CD formation. Most of the mutagenesis based on the subsites -3 and -7 changed the diversity of CDs. However, -6 subsite was also considered as a critical domain determining the product specificity of CGTase, although research on this subsite was not that numerous. In addition, mutagenesis of Tyr167 position appeared only once, which changed the α/β ratio slightly.

In order to elucidate the cyclization mechanism of CGTase, some x-ray structures of the CGTases have been determined from *Bacillus circulans* strain 8 and 251, Thermoanaerobacterium thermosulfurigenes strain EM1, alkalophilic *Bacillus* sp.1011 and *Bacillus stearothermophilus* [6-9]. Nevertheless, the structure of α-CGTase from *Bacillus macerans* has not been studied yet. In the previous study, we conducted site-saturation mutagenesis concerning tyrosine167 position at -6 subsite. The result demonstrated that when this position was substituted by histidine, the mutant displayed the best α-CD specificity (84.2%) compared with the wild-type CGTase (74.8%) [10]. Here, we reported the structure of a mutant α-CGTase from *Bacillus* sp. 602-1, to better understand its cyclization mechanism and the structure determinant of product specificity.

Materials and methods

■ Expression of Y167H mutant α-CGTase

The Y167H mutant was obtained based on the recombinant α-CGTase which came from *Bacillus* sp. 602-1 (stored in our lab). The mutant plasmid was cloned in competent *E. coli* DH5α. For protein expression, the mutant plasmid was transformed into *E. coli* BL21 (DE3) cells and spread over a LB medium with 50 μg/mL ampicillin. About 5 mL of the LB cultures inoculated with the single colony were grown at 37˚C in the presence of 50 μg/mL ampicillin. The overnight cultures were then transferred to 5 mL of TB medium (1.2% tryptone, 2.4% yeast extract, and 0.4% glycerol) with 50 μg/mL ampicillin inoculated at 1%. Until the OD_{600} value reached 0.6 to 0.8, the TB medium was cooled for 10 min. Subsequently, the mutant α-CGTase was induced with 0.01 mM isopropyl β-D-thio-galactoside (IPTG) at 17˚C for 96 h. Meanwhile, 150 mM glycine and 20 mM $CaCl_2$ were supplemented during the 24 h cultivation.

■ Protein purification and crystallization

The harvested culture was centrifuged at 8,000 rpm for 30 min at 4˚C and the supernatant was collected. The CGTase went through ammonium sulfate fractionation precipitation from 25% to 50%. The precipitation was gathered and resuspended in cold buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 140 mM NaCl, and 2.7 mM KCl, pH 7.0), proceeded with centrifugation at 8,000 rpm for 30 min at 4˚C. Subsequently, the resultant supernatant was subjected to Ni-NTA resin (GE Healthcare). The α-CGTase was eluted with buffer (50 mM Na_2HPO_4, 300 mM NaCl, and 200 mM imidazole liquid, pH 7.0). The enzyme purity was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and fractions with high purity were collected [10].

The promising single crystal was gained in the hanging drop consisting of 2 μL of 8 mg/mL protein solution mixed with equal amount of reservoir solution (15% PEG4000, 0.05 M Tris-HCl, 0.1 M sodium acetate buffer, 25 mM Na_2HPO_4, 150 mM NaCl, and 10 mM imidazole, pH 8.5), followed by incubation at room temperature for one week [11].

■ Data collection and structure determination

The crystals were soaked in reservoir solution supplemented with 17% (v/v) glycerol, followed by flash-cooling to 100 K. The data was collected using MM-007HF rotating-anode

X-ray generator (a Rigaku R-AXIS IV ++ image-plate system mounted on a rotating-anode X-ray source operating at 40 kV and 20 mA) at wavelength 1.5418Å. A total of 360 images were collected with 0.5°oscillation per image. All data were processed with HKL2000. The structures were determined by the molecular replacement method using Phaser with previously reported structure (PDB: 3BMW) as the search model. The atomic models were completed with Coot and refined with phenix.refine in Phenix, and the stereochemical qualities of the final models were validated with MolProbity. Data collection, processing, and refinement statistics are summarized in **Table 1**. All figures represented here were also prepared using PyMOL.

Result

■ The overall structure of Y167H mutant α-CGTase

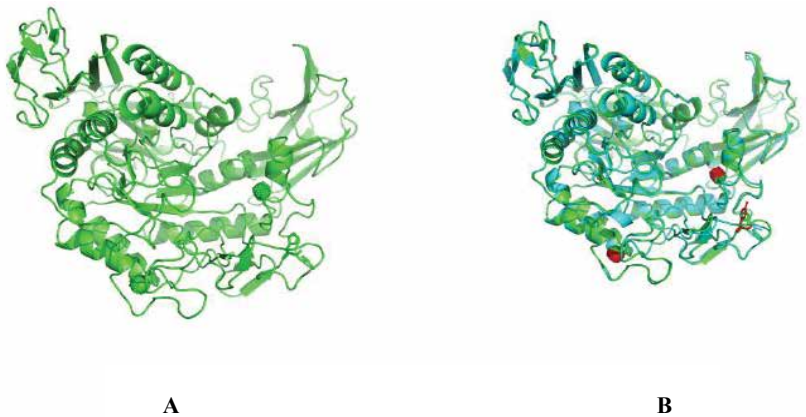
A 2.1Å diffraction data set used for solution and refinement of the structure was gathered at cryogenic temperature. The crystals are orthorhombic, belonging to the space group $P2_12_12_1$, with unit-cell parameters a=65.94, b=78.45, c=135.81Å, α=β=γ=90° **(Table 1)**.

The crystal structure of mutant Y167H α-CGTase was determined at 2.1Å. An N-terminal TIM barrel domain and three C-terminal IgG-like beta-barrel domains, it has like the Y195I mutant α-CGTase and other α-amylase family members **(Figure 1A)**. However, some differences were observed in comparison with the Y195I mutant α-CGTase in the flexible domains. Especially, the Ca^{2+} shifted from the original site of the Y195I mutant α-CGTase, which may be responsible for the stability change of Y167H mutant α-CGTase **(Figure 1B)**.

■ Comparison of 167 subsite between the Y167H mutant α-CGTase and Y195I mutant α-CGTase

Table 1. Data collection and refinement statistics.

	Structure
Data collection	
Space group	P212121
Cell dimensions	
a, b, c (Å)	65.94, 78.45,135.81
a, b, g (°)	90.00, 90.00, 90.00
Resolution (Å)	50-2.10 (2.18-2.10)
R_{merge}	0.065 (0.250)
I / σ I	20.5 (5.3)
Completeness (%)	99.6 (99.9)
Redundancy	4.0 (4.0)
Refinement	
Resolution (Å)	39.21-2.10
No. reflections	41763
R_{work} / R_{free}	0.1565/0.1948
No. atoms	
Protein	5233
Ligand/ion	2
Water	516
B-factors	
Protein	27.4
Ligand/ion	24.7
Water	35.3
R.m.s. deviations	
Bond lengths (Å)	0.007
Bond angles (°)	0.965
Ramachandran plot (%)	
Favored	97.08
Allowed	2.92
Outlier	0



A B

Figure 1. (A) An overall structure of the Y167H mutant α-CGTase; **(B)** The overlap view of the Y167H mutant α-CGTase (green) and the mutant Y195I α-CGTase (blue).

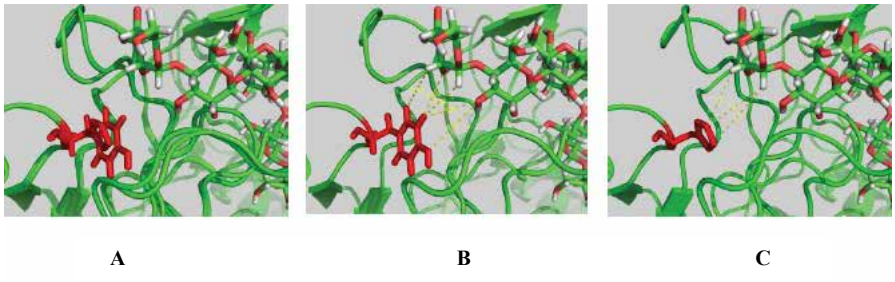


Figure 2. The location and the distance between the 167 site and the sugar chain; **(A)** The location of 167 sites of the solved mutant Y167H and Y195Iα-CGTases, **(B)** The distance between the Y167 site and the sugar chain in the Y195I α-CGTase structure, **(C)** The distance between the H167 site and the sugar chain in the Y167H α-CGTase structure.

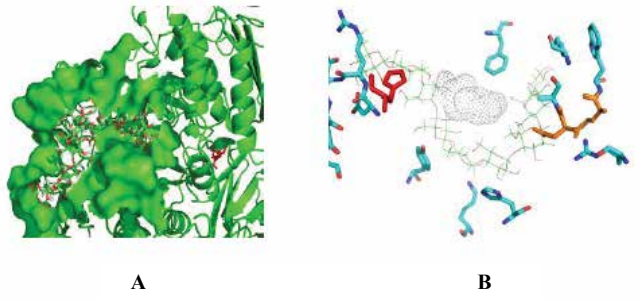


Figure 3. The close-up view of the active domain of the complex models; **(A)** Surface model of the Y167H α-CGTase-sugar complex, **(B)** Location of the crucial amino acids and the sugar chain.

The sugar-enzyme complex model was constructed using the cyclodextrin glucanotransferase (PDB ID 3BMW) as the search model. The molecules at -6 subsite were shown as sticks **(Figure 2A)**. Correspondingly, the side chain of histidine is about 5Å far from the sugar chain in the static model of enzyme-sugar complex, while the closest distance from Y167 to the sugar chain is about 3.6Å in the Y195I mutant enzyme-sugar complex **(Figures 2B and 2C).** The distance between histidine and the sugar chain let more sugar chains going into the substrate-binding groove, improving the α-CD cyclizing activity. The histidine at this site was 1.4Å away from the active domain compared with the tyrosine 167 in the Y195I mutant, which might result from the polar activity of the histidine. This small movement expand the space near the -6 subsite, thus enable the mutant to hold more sugar chains than the wild type enzyme. This may help to select the substrate with longer chain length and induce them into the active domain at the initial stage of reaction.

■ Overall interactions of the sugar chain and the Y167H mutant α-CGTase

As shown in **Figure 3A**, the sugar chain in the active domain is "V" shape-like. **Figure 3B** exhibites the interactions between the sugar chain and binding and catalyzing residues. It was proposed that a series of actions occurred sequentially in space. Also, in Y167H, longer sugar chains were transformed to the active domain through the -6/-7 subsites compared with wild type enzyme. This might result from the enhanced binding ability of histidine to sugar chains, thus improve the productivity of the α-CD.

Discussion

The main purpose of this study is to determine the mechanism of enhanced product specificity of Y167H through analysis of the crystal structure of Y167H mutant CGTase. In our previous studies [10,11], it was found that Y167H had better substrate binding ability than wild bacteria and the ability to increase the yield of α-CD. Moreover, stronger α-CD specificity was observed with the Y167HHH mutant, which also confirms that histidine does play an important role in enhancing the α-CD specificity of the -6 site [11]. But the mechanism was not clear.

In comparison of the crystal structure of a mutant CGTase Y195I with that of Y167H obtained in this study. It is found that the Ca^{2+} of the two enzymes cannot be completely coincided, and the change of Ca^{2+} position is likely to cause

the change of Y167H stability. The location of Tyr167 and His167 loci was shifted, and the distance from the sugar chain was changed, and the distance between the original Tyr167 site and the sugar chain was prolonged by the His167 site. After the 167 loci become histidine, the distance between the active center and the sugar chain is far away, which is likely to be related to the strong polarity of histidine, which makes it more attractive to the glucose molecule. At the same time, on the whole of the enzyme, the change of the 167-site amino acid also affects the whole -6 site in the active domain, which is likely to affect the ability to combine the active region with the sugar molecules at the initial stage of the reaction, thus screening more suitable substrate molecules into the active center. In the experiment, a surface model of sugar chain enzyme composite structure was established. It was found that the "V" structure of the enzyme active center was combined with the groove of the surface of the α-CGTase, and the interaction between the sugar chain structure of the α-CGTase and the important catalytic sites could be found to predict the distribution sequence of these sites.

In summary, after a series of analysis of the Y167H crystal structure, we have concluded that the Y167-histidine has a great influence on the production of specific α-CD, probably by affecting the distance between the α-CGTase and the substrate, as well ase the combination of the α-CGTase with more substrates because of the strong polarity of the histidine. We found that Ca^{2+} is an important cofactor for the enzyme and discussed the shift of Ca^{2+} in mutant structure. However, the function and importance of this cofactor is needing our further research. The date will be used to further investigate the structure of α-CGTase as well as to investigate the mechanism of product specificity of α-CGTase and its mutant enzymes.

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