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The hydrogen-bond network around Glu160 contributes to the structural stability of chitosanase CsnA from *Renibacterium* sp. QD1

Yujuan Han [1], Rilei Yu [1], Peixin Gao, Xinzhi Lu *, Wengong Yu *

Key Laboratory of Glycoscience & Glycotechnology of Shandong Province, Key Laboratory of Marine Drugs, Chinese Ministry of Education, Laboratory for Marine Drugs and Bioproducts, Qingdao National Laboratory for Marine Science and Technology, School of Medicine and Pharmacy, Ocean University of China, 5 Yushan Road, Qingdao 266003, China

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ABSTRACT

CsnA, a chitosanase from *Renibacterium* sp. QD1, has great potential for industrial applications due to its high yield and broad pH stability. In this study, a specific Glu160 in CsnA was identified by sequence alignment, and structural analysis and MD simulation predicted that Glu160 formed a hydrogen-bond network with Lys163 and Thr114. To evaluate the effect of the network, we constructed four mutants, including E160A, E160Q, K163A, and T114A, which partially or completely destroy this network. Characterization of these mutants demonstrated that the disruption of the network significantly decreased the enzyme thermostability. The underlying mechanisms responsible for the change of thermostability analyzed by circular dichroism spectroscopy revealed that the hydrogen-bond network conferred the structural stability of CsnA. Moreover, the length of the side chain of residue at 160 impacted conformational stability of the enzyme. Taken together, the hydrogen-bond network around Glu160 plays important roles in stabilization of CsnA.

1. Introduction

Chitosanases (EC 3.2.1.132) are glycoside hydrolases that degrade chitosan to chitosan oligosaccharides. The majority of chitosanases were isolated from chitosan-utilizing microorganism [1–4] and few were detected in plants [5] and viruses [6]. Based on structure and amino acid sequence similarity, chitosanases were classified into six glycosyl hydrolase (GH) families, including GH5, 7, 8, 46, 75 and 80, among which families 5, 7 and 8 were the collection of enzymes with similar structures but having different substrate (mannan, cellulose, glucan and chitosan) specificities. However, all members of GH46, GH75 and GH80 characterized so far are specific for chitosan hydrolysis. Compared with GH75 and GH80, enzymes of GH46 have been the most intensively studied in terms of structure and function [7]. In this family, crystal structures of four chitosanases, including Csn (Ch01) from *Bacillus circulans* MH-K1 [8,9], OU01 from *Microbacterium* sp. OU01 [10], Csn (CsnN174) from *Streptomyces* sp. N174 [11,12] and SACTE_5457 from *Streptomyces* sp. SirexAA-E [13], have been determined. The enzymes are dumbbell shaped and composed of two globular lobes (the major and the minor). A long helical hinge has been implicated in allosteric communication between the two lobes, which is required for domain motion into an appropriate configuration for catalysis [13]. Enzymatic hydrolysis of the glycosidic bond takes place via general acid/base catalysis that requires two critical residues: a proton donor and a nucleophile/base. Previous studies indicated that the chitosanases of GH46 acted in a non-processive hydrolysis mode, and structural features about the mechanisms of the non-processive chitosanase OU01 were described [14].

As described above, the catalytic hydrolysis mechanism of GH46 chitosanases has been well illustrated but little information is available on the stability, especially with respect to the temperature. However, considerable efforts are being made to improve the understanding of the mechanisms underlying the protein stability, which are not only significant theoretically, but also have important implications for protein engineering to satisfy the demands in industrial application [15–20]. Numerous studies have revealed that intramolecular interactions such as hydrogen bonds and ionic bonds [21] contribute to maintaining protein structural stability. Besides, several studies have reported that residues located in some structures such as the N termini of β-sheets [22], dimer interface [23], hydrophobic cores [24], vicinity of specific loop region [25], play essential roles in favoring protein conformational stability.

CsnA, a new member of GH46, is a highly chitosan specific enzyme isolated from bacterium *Renibacterium* sp. QD1 and it

* Corresponding authors.
E-mail addresses: luxinzhi@ouc.edu.cn (X. Lu), yuwg66@ouc.edu.cn (W. Yu).
[1] Y. Han and R. Yu contributed equally to this work.

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hydrolyzes *N*-deacetylated polymeric glucosamines into chitobiose and –triose in an endo-type manner [26]. The enzyme displayed maximal activity at 55 °C and pH 5.6 and it was continuously stable at pH of 5.0–10.0. This enzyme is abundantly secreted into the medium by wild as well as recombinant *E. coli* strain. Because of its broad pH stability and abundant secretion, it becomes a target of interest for further study.

In this study, a hydrogen-bond network around the specific Glu160 on the helical hinge of CsnA was identified by sequence alignment combined with structural analysis and molecular dynamic simulations. The objective of this study was to examine the effect of the hydrogen-bond network on the enzyme function.

2. Materials and methods

2.1. Strains and plasmids

Genomic DNA of strain *Renibacterium* sp. QD1 was used as the template for cloning of CsnA gene (*csnA*). *E. coli* DH5α was used for routine plasmid propagation. The chitosanase gene was cloned into vector pET-24a(+) and transformed into *E. coli* BL21(DE3) for over-expression.

2.2. Sequence alignment

Amino acid sequences of chitosanases in GH46 were retrieved from the CAZy database and aligned by online multiAlin (http://multalin.toulouse.inra.fr/multalin/). The alignment was subsequently submitted to ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) analysis, using the crystal structure of chitosanase from *Streptomyces* sp. SirexAA-E as the template to predict the secondary structure of the query.

2.3. Homology modeling

For homology modeling, the amino acid sequence of CsnA was subjected to BLASTP analysis against protein data bank (PDB) to identify the suitable template. Then the three-dimensional model of CsnA was built based on the X-ray crystallographic structure of *Streptomyces* sp. SirexAA-E chitosanase using SWISS-MODEL Workspace (http://swissmodel.expasy.org/). A Ramachandran plot (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) was used for structure validation.

2.4. Molecular dynamics (MD) simulation

The AMBER 2016 [27] software package was used for MD simulation of the target protein, using the method similar to previous study [28]. In brief, the complex was solvated into an octagon box of TIP3P water molecules and neutralized using Na^+. Then, it was minimized to remove unfavorable van der Waals interactions. After minimization, MD was performed. The systems were equilibrated in the NVT and NPT ensemble, respectively. Then in the production process, the whole system was relaxed and a 50-ns molecular dynamics process was carried out. For all MD steps, the time step was set to 0.002 ps, and the particle mesh Ewald (PME) method [29] was applied to deal with long-range electrostatic interactions and the lengths of the bonds involving hydrogen atoms were fixed with the SHAKE algorithm [30].

2.5. Gene cloning of CsnA and site directed mutagenesis

The gene of CsnA was amplified by polymerase chain reaction (PCR) with the genome DNA of bacterium *Renibacterium* sp. QD1 as the template and then inserted into pET-24a(+) using the restriction enzyme NdeI and HindIII. The recombinant plasmid was transformed into *E. coli* BL21(DE3). The recombinant chitosanase contained a hexahistidine tag at C-terminus for purification. Site-directed mutations were performed with the Site-directed Gene Mutagenesis Kit (Beyotime Biotechnology). Mutation conditions were 18 cycles of 95 °C for 40 s, 51 °C for 1 min and 68 °C for 3 min. The final PCR products were digested with DpnI at 37 °C for 1 h to eliminate the parental plasmid DNA. The digestion DNA was then transformed into *E. coli* BL21(DE3) and a number of colonies containing mutant plasmids were obtained and confirmed by sequencing. The primers used for gene cloning and mutagenesis were listed in Supplementary Table S1.

2.6. Purification of recombinant proteins

Single-colony of wild-type or mutant CsnA was picked in Luria-Bertani (LB) medium containing 50 μg/ml kanamycin. Once the optical density at 600 nm (OD600) reached to approximately 2.5, the protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) and the cultures were allowed to grow at 25 °C for 60 h. The culture supernatant was harvested by centrifuging at 10,000 × *g* for 30 min at 4 °C and then applied onto a Ni^{2+} column pre-equilibrated with 20 mM Tris-HCl buffer (pH 8.0) plus 0.3 M NaCl. The recombinant protein was eluted by the same buffer with 300 mM imidazole. Fractions with high chitosanase activity were collected and dialyzed at 4 °C against 10 mM phosphate buffer (pH 7.0) for three times then used for further experimentation. The purity and molecular weight of purified proteins were determined using SDS-PAGE.

2.7. Enzyme activity assay

Chitosanase activity was determined using the 3,5-dinitrosalicylic acid (DNS) method [31] with colloidal chitosan as the substrate. The reaction mixture consisted of 100 μl suitable concentration of purified enzyme and 900 μl of 0.5% (m/v) colloidal chitosan in 0.2 M sodium acetate buffer (pH 5.6). The reaction mixture was incubated at 55 °C for 10 min and then terminated by adding 750 μl of DNS reagent. After boiling at 100 °C for 10 min, the mixture was centrifuged at 12,000*g* for 10 min. The amount of the reducing sugar produced was determined by measuring the OD at 520 nm using D-glucosamine as a standard. One unit of chitosanase was defined as the amount of enzyme that generated reducing sugars corresponding to 1 μmol of glucosaminehydrochloride per minute under standard conditions.

2.8. Thermostability measurements

The purified CsnA variants were incubated at 30 °C or 40 °C for different time intervals. Their residual activities were assayed under standard conditions. Activities were each denoted as a percentage of the residual activity in comparison with the activity at the zero-time point.

2.9. Circular dichroism measurements

Far ultraviolet circular dichroism (CD) spectra of the CsnA variants were measured at a protein concentration of 0.15–0.2 mg/ml in 10 mM sodium phosphate buffer (pH 7.0), using a Jasco J-810 spectropolarimeter with a 0.1 cm path-length quartz cuvette. Ellipticity data were obtained between 190 and 250 nm, and the spectrum of a buffer blank was subtracted. The content of the protein secondary structure was calculated from Secondary Structure Estimation program on Spectra Manager software according to the method described previously [32,33].

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