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Molecular characterization of the thermostability and carbohydrate-binding module from a newly identified GH118 family agarase, AgaXa

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ABSTRACT

AgaXa is a thermostable β-agarase from the agar-degrading bacterium *Catenovulum* sp. X3. To further understand the mechanism of protein stabilization of AgaXa, several mutants were generated by random and site-directed mutagenesis, and they were subsequently screened by analysing their enzymatic activity and thermostability. Four mutants (V197D, P259H, C442S and C528S) were found for which the enzyme activity and thermostability were significantly decreased. Moreover, secondary structures determined by circular dichroism (CD) showed that mutants V197D and P259H presented a higher percentage of α-helix but fewer β-strands than wild-type (WT) AgaXa. On the contrary, no significant changes were observed in the secondary structures of mutants C442S and C528S. Through the treatment by proteinase K, different digested profiles were generated from mutants V197D and P259H when compared to WT, and mutants C442S and C528S was observed with more digested protein fragments. These results indicate that the enzymatic activity and stability of AgaXa is mainly related to its hydrophobic structure and disulphide bonds. Furthermore, carbohydrate-binding ability was also analysed for the mutants of N- and C-terminal deletions, and the results showed that agarose binding capability was not changed, indicating that the carbohydrate-binding module is probably located in the middle of the β-agarase AgaXa.

1. Introduction

Agar, the main component of red algae cell walls (Rhodophyta), is composed of agarose and agaropectin. Unlike agaropectin, agarose is a neutral polymer with a linear polymer of repeat 1,4-linked 3,6-anhydro-α-L-galactose and 1,3-linked β-D-galactose [1]. Agarases, a group of glycoside hydrolases (GH) that catalyse the hydrolysis of agarose, can be grouped into α-agarases (EC 3.2.1.158, agarotetraose as the major product) and β-agarases (EC 3.2.1.81, neoagarobiose as the major product) according to whether they degrade α- or β- linkages in agarose [2]. The majority of the agarases are β-agarases, which can be further classified into four GH groups (GH16, GH50, GH 86 and GH118) based on the sequence similarity from the carbohydrate-active enzyme database (CAZy) [3].

Different types of agarases demonstrated various enzymatic characterization; however, most of them could not tolerate high temperature, which greatly limits their industrial application. Direct evolution provides a good choice to improve the enzymatic performance, and it has been applied to create some enzyme mutants [4–7]. A limited number of agarases were observed with both high activity and thermostability, but the mechanism for the changes, such as protein structure, are still not clear. Polysaccharides can be recognized and bind to the enzyme by the carbohydrate-binding module (CBM). The distance between the active site and the substrate decreases, which promotes the breakage of glycosidic bonds by the catalytic module (CM) [8–10]. Agarases were usually found with one or more CBMs, which also enhanced the enzymatic catalysis [11]; however, some agarases without CBMs were found to display a relatively higher enzymatic activity [10,12]. Therefore, the location of CBMs is also recognized as another important factor for enzymatic activity.

A novel and thermostable β-agarase AgaXa of the GH118 family from *Catenovulum* sp. X3 has been previously reported by our research group [13]. To further investigate the structural features of

Abbreviations: WT, wild-type; CD, circular dichroism; PCR, polymerase chain reaction; GH, glycoside hydrolase; CBM, carbohydrate-binding module; CM, catalytic module; DNS, 3,5-dinitrosalicylic acid; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

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Table 1
Primer sets used in this study.

Primers	Position	Sequence (5′–3′)
Xaf	Forward	GCGCGGATCCATCAAACTATTGAAT
Xar	Reverse	GGCCTCGAGAATTACAACCCAT
V197D	Forward	GGAGCTTTTACGTGACGATGACCGTGTAGATGAT
	Reverse	ATCATCTACACGGTCATCGTCACGTAAAAGCTCC
V197T	Forward	GGAGCTTTTACGTGACACTGACCGTGTAGATGAT
	Reverse	ATCATCTACACGGTCAGTGTCACGTAAAAGCTCC
I217E	Forward	AAGTAAACGGCAATATTGAGGGTTTGTATGGCGGG
	Reverse	TTCCCGCCATACAAACCCTCAATATTGCCGTTTACT
I217V	Forward	AAGTAAACGGCAATATTGTTGGTTTGTATGGCGGG
	Reverse	CCCGCCATACAAACCAACAATATTGCCGTTTACTT
P259H	Forward	CTCAGGCTTTTTAAACCATTGGTCTGAAAAACGCC
	Reverse	GGCGTTTTTCAGACCAATGGTTTAAAAAGCCTGAG
C442S	Forward	AGTATTCGGCTCTGGTGGTGTGGT
	Reverse	ACCACACCACCAGAGCCGAATACT
C528S	Reverse	GGCCTCGAGAATTAGAACCCATAATACTG
N529K	Reverse	GGCCTCGAGACTTACAACCCATAATACTG

Forward: forward primer; Reverse: reverse primer.

AgaXa and their effects on the enzymatic activity, random and site-directed mutageneses were adopted to generate agarase mutants with significant changes in their biochemical characteristics. Circular dichroism (CD), a method to determine the secondary structural of a protein [14], and digested protein profile analysis using proteinase K were also utilized to measure structural changes after amino acid substitution in AgaXa. Furthermore, C-terminal and N-terminal deleted mutants of AgaXa were generated for identifying the tentative substrate binding locations.

2. Materials and methods

2.1. Chemicals and reagents

Unless otherwise stated, all chemicals used were analytical reagent grade or higher. Enzymes for molecular biology analysis were purchased from Takara (Dalian, China). Oligonucleotides were synthesized by BGI (Beijing, China). Agarose was purchased from MDBio Company (Beijing, China).

2.2. Random mutagenesis and mutant screening

Error-prone polymerase chain reaction (PCR) with DNA polymerase lacking the 3′–5′ exonuclease activity was carried out to introduce random nucleotide substitutions. Recombinant plasmid pET-32a(+)-AgaXa [13] was used as the template for PCR with forward primer Xaf and reverse primer Xar (Table 1). The PCR amplifications were carried out on an RT-Gradient PCR thermal cycler (Biometra, Germany) by denaturing at 95 °C for 4 min followed by 30 cycles of 94 °C for 40 s, 50 °C for 40 s, and 72 °C for 90 s and a final extension at 72 °C for 10 min. The amplified fragments were ligated to pET-32α(+) plasmid and transformed into *E. coli* DH5α cells. The transformed cells were then spread onto Luria-Bertani (LB) agar plates supplemented with ampicillin (100 μg/mL). After 16–18 h of cultivation at 37 °C, 140 μL of ampicillin/IPTG mixture (20 mg/mL ampicillin and 10 mM IPTG) was evenly sprayed onto the surface of the agar plate for another 5–6 h of incubation. Finally, Lugol's solution was used to display the hydrolytic halo zone for positive colony identification, and the inserted DNA fragments were confirmed by DNA sequencing (BGI, China).

2.3. Site-directed mutagenesis

Point mutations of AgaXa were generated by utilizing PCR with designed oligo primers for introducing the change of amino acids (Table 1). The PCR mixture contained approximately 200 ng of pET-32a(+)-AgaXa plasmid DNA, 0.25 mM dNTP mixture, 0.2–0.3 μM aliquots of each primer and 1.25 U of PrimeSTAR® HS DNA Polymerase (TaKaRa, Dalian, China), and the PCR process was performed according to the instructions for this polymerase. The amplified vectors were digested with *Dpn*I (Beyotime, Shanghai, China) for 1 h and then transformed into *E. coli* DH5α. The mutants with desired mutations were confirmed by sequencing.

2.4. Expression and purification of mutant proteins

E. coli BL21 (DE3) with modified plasmids was induced with IPTG as described previously [13]. Cells and culture medium were separated by centrifugation, and proteins in the supernatant were precipitated by adding ammonium sulphate to a final concentration of 40% (w/v). After centrifugation, the protein pellet was dissolved in balance buffer (20 mM Tris-HCl, 0.25 M NaCl, 20 mM imidazole, pH 7.4) at 4 °C, and then loaded onto a Ni-sepharose Column (Beijing, China). After washing with binding buffer (20 mM Tris-HCl, 0.25 M NaCl, 100 mM imidazole, pH 7.4) to remove non-specific proteins, the target recombinant protein was eluted with elution buffer (20 mM Tris-HCl, 0.25 M NaCl, 400 mM imidazole, pH 7.4). The eluted proteins were successively concentrated and desalted by centrifugal filter devices (Millipore, USA), and the purity of protein was analysed by SDS-PAGE.

2.5. Enzymatic characterization of the mutants

The agarase activities of the wild-type (WT) and mutants were quantitatively analysed using the 3,5-dinitrosalicylic acid (DNS) method [15] by measuring the production of reducing sugar from agarose with the enzymatic treatment. The enzyme was incubated in 50 mM Tris-HCl (pH 7.4) containing 0.25% (w/v) melted agar at 40 °C for 20 min; 1 mL of DNS was then added, and the reaction mixture was boiled for 10 min before being diluted to 10 mL with distilled water. The absorbance of the reducing sugar was measured at a wavelength of 540 nm. To determine the thermostability of agarase, the enzyme was incubated at 47 °C for 1 h [13], and the residual enzyme activity was measured under standard conditions as described above. The kinetic parameters (K_m, V_{max} and K_{cat}) of proteins were estimated using Lineweaver-Burk plot calculations, and thin-layer chromatography (TLC) analysis on the enzymatic hydrolysates was also carried out according to the method of Xie et al. [13]. WT AgaXa was used as control and all the samples were tested in triplicate.

2.6. Analysis of protein structure changes

Protein secondary structure was analysed using Chirascan circular dichroism (CD) spectroscopy (Applied Photophysics, UK). The protein was dissolved in 10 mM phosphate buffer (pH 7.9), injected into a quartz cuvette (1 mm), and scanned at a wavelength from 200 to 260 nm at a speed of 1 nm/s. Each sample had three replicates and was analysed at sequential temperatures of 20, 35, 50 and 65 °C with an interval of 1 min between two adjacent temperatures. The percentages of each secondary structure (including α-helix, β-strand, turns and unordered) were calculated using the CDPro program (http://lamar.colostate.edu/~sreeram/CDPro/main.html). Changes in protein structure were further analysed by the fragment profiles with proteinase K digestion according to the method of Qin et al. [16]. WT and mutant proteins were incubated with 0.1 μg proteinase K (100 μg/mL, without Ca^{2+}) and 10 mM dithiothreitol at 25 °C for 20 min. The digestion was stopped by adding 5 mM phenylmethanesulphonyl fluoride (PMSF), and the digested protein profile was analysed by SDS-PAGE. Proteins without proteinase K treatment were used as negative controls.

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