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Enhancing the Thermostability of a Novel β-agarase AgaB through Directed Evolution

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Abstract To increase the thermostability of β-agarase AgaB by directed evolution, the mutant gene libraries were generated by error-prone polymerase chain reaction (PCR) and deoxyribonucleic acid (DNA) shuffling. Mutants with high thermostability were screened by a simple method based on agarase-degrading agar to generate a clear zone on the agar plate. A mutant S2 was obtained through two rounds of error-prone PCR and a single round of DNA shuffling and selection. It has higher thermostability and slightly increased catalytic activity than wild-type AgaB. Melting temperature (T_m) of S2, as determined by circular dichroism, is 4.6 °C higher than that of wild-type AgaB, and the half-life of S2 is 350 min at 40 °C, which is 18.4-fold longer than that of the wild-type enzyme. Saturation mutagenesis and hydrophobic cluster analysis indicated that hydrophobic interaction might be the key factor that enhances the enzyme stability.

Keywords Agarase · Thermostability · Directed evolution · Clear zone screening · DNA shuffling

Introduction

Agarase (EC3.2.1.81) belongs to glycoside hydrolase (GH) that hydrolyzes agar and agarose-generating oligosaccharides. Agarose is defined as having a linear chain structure

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composed of alternating residues of 3-O-linked β-D-galactopyranose and 4-O-linked 3, 6-anhydro-α-L-galactopyranose [1]. According to the mode of action on the agarose, agarases are classified into two groups: α-agarases, which cleave the α-1,3 linkage of agarose to produce a series of agarooligosaccharides, and β-agarases, which cleave the β-1,4 linkage of agarose to produce a series of neoagarooligosaccharides [2]. Based on the amino acid sequence similarity, known agarases are classified into three GH families: GH-16, GH-50, and GH-86 (http://afmb.cnrs-mrs.fr/CAZY/). The reported β-agarases generally degrade agarose and agar to give oligosaccharides with low degrees of polymerization (DP) as major products, for example, neoagarobiose, neoagarotetraose, or neoagarohexaose [3–6]. Agarases have potential applications in the food, cosmetic, and medical industries for the production of oligosaccharides from agar [7–9]. Moreover, agarases can be used to degrade the cell walls of marine algae for the preparation of protoplasts and to isolate deoxyribonucleic acid (DNA) from agarose gels in the biotechnology field [10, 11].

Recently, we have cloned and characterized a novel extracellular β-agarase-encoding gene, *agaB*, from marine *Pseudoalteromonas* sp. CY24 [12]. The *agaB* gene has no significant sequence similarity with any known proteins, including currently known GHs, and this enzyme hydrolyzes agarose and agar to generate neoagarooctaose and neoagarodecaose as the main products. This is quite different from other agarases. Moreover, AgaB has a higher specific activity of 5,000 U/mg on agarose. However, the low thermostability restricts its wide industry application as a tool enzyme.

In this study, we reported the application of error-prone polymerase chain reaction (PCR) and DNA-shuffling methods combined with the clear zone-screening method to identify AgaB mutants with improved thermostability. At last, we obtained a mutant that is more thermostable than the wild-type enzyme.

Materials and Methods

Bacterial Strains, Plasmids, and Reagents

Escherichia coli BL21 (DE3) (Novagen) was used as the expression host strain for enzyme purification and mass production. Plasmid pBluescript II KS (+) (Stratagene) and the modified pBS-ks (sv) vector were transformed to the *E. coli* DH5α strain and plasmid pET-24a (+) (Novagen) to the BL21 (DE3) strain. All the chemicals used in this study were of the purity of analytical grade. All restriction enzymes, DNA-modifying enzymes, and related reagents used for DNA manipulation were purchased from TakaRa.

Expression of the *agaB* Gene in pBS-ks(sv)

The plasmid pBS-ks (sv) was derived from pBluescript II KS (+). Initially, promoter and SD sequence of *agaB* gene was amplified and ligated with pBluescript II KS (+). The targeting plasmids that the transcription orientation of the inserted *agaB* promoter was the same as the T3 promoter were identified using *Nde*I and *Xho*I double-enzyme mapping. The resulting vector was verified by DNA sequencing and named pBS-ks (sv).

The DNA fragment encoding mature AgaB (1,437 bp) was amplified and inserted into pBS-ks (sv). This ligation mixture was transformed to *E. coli* DH5α cells and plated on Luria–Bertani (LB) agar plates containing 50 μg/ml of ampicillin and 1.5% agar. Positive clones were identified by observing the size of clear zone around the colony.

Error-prone PCR

Error-prone PCR was carried out in a 100-μl reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 6 mM $MgCl_2$, 0.05 mM $MnCl_2$, 0.2 mM deoxyadenosine triphosphate, 0.2 mM deoxyguanosine triphosphate, 1 mM deoxycytidine triphosphate, 1 mM deoxythymidine triphosphate, 25 pmol of each *agaB* primers, 5 ng of template DNA, and 3 U of *Taq* DNA polymerase. The PCR was carried out on PTC-200 thermocycler (MJ Res) by denaturing at 94 °C for 2 min followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2.5 min and a final extension at 72 °C for 10 min. The PCR product was purified, ligated with pBS-ks (sv), and transferred into *E. coli*. DH5α.

Shuffling of the Mutant Library

The thermostable AgaB mutants from error-prone PCR-based mutant libraries were pooled, amplified, and digested with diluted DNaseI at 20 °C for 8 min. DNA fragments of 100–250 bp were purified from 2% (*w*/*v*) agarose gel and subjected to self-assembly PCR using *Taq* DNA polymerase. The thermocycling reaction consisted of an initial denaturation step at 94 °C for 2 min, followed by 60 cycles of denaturation at 94 °C for 30 s, annealing and extending at 55 °C for 30 s, and a final extension at 72 °C for 8 min. In the following amplification step, 1–3 μl of the self-assembled DNA products was used as the template in 100 μl PCR reaction mixture with 25 pmol of each *agaB* primers. The PCR procedure was the same as the amplification of error-prone PCR but with 22 cycles. The products were purified, ligated with pBS-ks (sv), and transferred into *E. coli* DH5α, generating a library covering all mutations generable in this way.

Thermostable Mutant Screening and Activity Assaying

To screen mutants with enhanced thermostability, transformants were inoculated onto 1.5% (*w*/*v*) solid agar LB plates containing 50 μg/ml of ampicillin. The plates were incubated at 37 °C for 6 h and then placed at 40 °C for 10 h. Colonies with a clear zone larger than that of the wild-type AgaB were considered positive with their improved thermostabilities further confirmed by determining their remaining agarase activity using the 3,5-dinitrosalicylic acid (DNS) method [13]. One unit of enzymatic activity was defined as the amount of protein that produced 1 μmol of reducing sugar per minute as D-galactose under the conditions of the assay. All measurements were performed in triplicate parallels and repeated at least thrice for each sample. All statistical analyses were performed using the SPSS statistical software package version 10.0.1. Probability values of less than 0.05 were considered statistically significant.

CD Spectrum

To determine the melting temperature (T_m) of enzymes, the far-UV circular dichroism (CD) spectra of wild-type AgaB and mutant S2 were profiled in a JASCO 810 spectropolarimeter, which was equipped with a liquid nitrogen intracooler system. The enzymes were concentrated to 0.2–0.3 mg/ml in 20 mM phosphate buffer (pH 6.5) and placed in a differential scanning calorimetry aluminum pan. After being sealed, the pan was heated from 10 to 80 °C at a heating rate of 1 °C/min, and the change was measured in ellipticity at 222 nm. The data were analyzed with Origin7.0 software, and curves were fitted using Fit Sigmoidal function [14].

The Properties of Mutant Enzymes

To evaluate the effect of mutation and elevated melting temperature on the biochemical properties of enzymes, pH, temperature, and product profiles of mutant enzyme S2 were constructed and compared with the wild-type enzyme. The procedure is same with that of Ma et al. [12]. To identify the final hydrolysis products of mutant S2, 50 ml 0.2% agarose was hydrolyzed by 50 U of purified recombinant mutant enzyme. Hydrolysis products were analyzed using the fluorophore-assisted carbohydrate electrophoresis (FACE) method.

Site-directed Mutagenesis and Saturation Mutagenesis

To investigate the effect of mutant amino acids on thermostability, Leu^{122} and Asn^{446} of wild-type agarase AgaB were substituted, respectively, using the QuikChange™ site-directed mutagenesis kit (Stratagene). The primers 5′-CGTAATTTGCAGCTAATTGATG-3′ and 5′-CATCAATTAGCTGCAAATTACG-3′ were used for the substitution of Leu^{122} with Gln^{122}, and the resulting mutant was named L122Q. Similarly, primers 5′-GGTACTAACATCAATATTCGT-3′ and 5′-ACGAATATTGATGTTAGTACC-3′ were used for the substitution of Asn^{446} with Ile^{446}, yielding N446I. The mutated gene was cloned into the expression vector pET-24a (+) and expressed in the *E. coli* BL21 (DE3) strain. The mutations were confirmed by DNA sequencing.

To investigate the effect of amino acid at poison 446 on thermostability of AgaB, the residue Asn^{446} of AgaB was replaced with all possible amino acids using primers 5′-GCACGCGGTACTAACNNNAATATTCGTTACAAC-3′ and 5′-GTTGTAACGAATATTNNNGTTAGTACCGCGTGC-3′. The mutations were identified by DNA sequencing.

Sequence Comparison and HCA Analysis

The mutant genes were sequenced by BioAsia (Shanghai, China). Sequence comparison was carried out using ClustalW and BioEdit programs. The secondary structures of enzymes were analyzed using a hydrohobic cluster analysis (HCA) software (program DRAWHCA at http://www.smi.snv.jussiou.fr/hca).

Results and Discussion

Screening of Mutants for Increased Thermostability

The mutant libraries of the *agaB* gene were constructed by error-prone PCR and DNA shuffling using a constitutive expression vector, pBS-ks (sv), which generated by inserting the promoter and SD sequence of the *agaB* gene into pBluescript II KS (+). This vector omits the step of inducement and facilitates high-throughput screening.

Screening is one of the most important factors in directed evolution. The screening strategy used in this study is based on clear zones around colonies, which is formed by extracellular agarase-hydrolyzing agar. The larger clear zone represents higher enzyme activity or an increased quantity of enzyme. In addition, wild-type AgaB has low thermostability when temperature is above 35 °C; therefore, after incubation at 40 °C for 10 h, clones that show larger clear zone than wild-type AgaB were presumed to have either higher thermostability or higher activity or both than that of the parental enzyme. Moreover,

the thermostability and activity of each positive mutant were verified further by measuring the residual agarase activity using DNS method.

The mutation condition was selected to achieve a nucleotide mutational rate of ~0.15% (determined by sequencing 12 randomly selected clones) resulting in a library consisting of 30–40% of mutants with less than 10% activity of the parent enzyme. In the first round of error-prone PCR, approximately 6,000 clones were screened, and five positive clones that had larger clear zones were obtained. Three of their mutants whose culture supernatant activities were 2.3–3.2-fold higher than that of the wild-type enzyme were used as parental clones in the second round of error-prone PCR. The higher activities of another two clones were not caused by the mutant sequence. With the same procedure, more than 5,000 clones from the second-generation mutant library were screened. Eight positive clones were identified, and their residual activities were 2.5–4.1-fold higher than that of the wild-type enzyme using the DNS method. To obtain the beneficial mutations and delete the harmful mutations of these sequences, these eight clones were used as parental templates in DNA shuffling. After 5,000 transformants were screened as described above, two clones S2 and S21 with higher residual agarase activity were obtained, and their residual activities were 7.1±0.1- and 6.3±0.15-fold higher than that of wild-type enzyme (data not shown). DNA sequence analysis revealed that S2 contained two amino acid transitions (L122Q and N446I) and two synonymous mutations. S21 has one N446I substitution and three synonymous mutations (two of them are different from S2).

Thermostability of the Evolved AgaB Mutants

To compare their thermostability precisely at the enzyme level, recombinant AgaB and mutants were expressed and purified by ammonium sulfate precipitation, weak anion-exchange chromatography, hydrophobic interaction chromatography, and gel filtration chromatography. At last, mutant S2 was purified about 476.5-fold, and a final yield of 20.9% was achieved, which is similar with the wild-type enzyme [12]. The thermostibility of enzymes was determined by measuring the residual activities after heat treatment for 30 min at various temperatures. As shown in Fig. 1, S2 and wild-type AgaB have the same pattern of

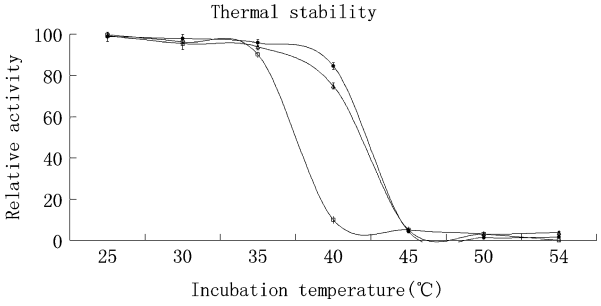

Fig. 1 Effect of temperature on the stability of wild-type AgaB, mutant S2 and N446I. The purified enzymes were diluted to similar concentration with 20 mM sodium phosphate buffer (pH 6.0) and treated at different temperatures for 30 min. The thermostability was expressed as the percentage of the remaining activity to the original. Three independent experiments were carried out, and values whose error ranges were within 5% were averaged. Symbols: *empty squares* for wild-type enzyme; *empty triangles* for mutant N446I, and *filled circles* for mutant S2

thermostability, but the thermostability of S2 was about 5 °C higher than that of wild-type enzyme. The half-life of mutant S2 was 350 min at 40 °C, which was 18.4-fold longer than that of wild-type AgaB.

The melting temperature (T_m) is the midpoint temperature at which an enzyme denatures its structure. Any enzyme that has a high T_m value means it has a high thermostability. T_m values of AgaB and mutant S2 were determined by CD. The T_m of AgaB and S2 were calculated with 49.2 and 53.8 °C, respectively (Fig. 2). As expected, S2 had a higher melting temperature, which was 4.6 °C higher than that of wild-type AgaB.

To identify the mutation amino acid responsible for thermostability, two mutants L122Q and N446I that contain a sole mutation were constructed, and their thermal stabilities were also investigated. It is interesting to note that N446I has the similar enhanced thermostability with S2 (Fig. 1), whereas the mutant L122Q enzyme showed similar thermostability with wild-type AgaB (data not shown). This result demonstrated that the substitution of Ile for Asn was critical to the thermostability of S2.

To further investigate the effect of amino acid at position 446 on the thermostability of AgaB, saturation mutagenesis was carried out. Six mutants with high thermostability were obtained; three of them were substituted by valine, two by isoleucine, and one by leucine. The half-life of mutant N446V and N446L at 40 °C were 18.2- and 12.9-fold longer than that of wild-type AgaB, respectively, and mutant N446I was described as above. These results indicated that the introduction of isoleucine at position 446 is most effective for enhancing the thermostability of AgaB.

Properties of the Evolved AgaB Mutants

To evaluate the effect of mutations on the enzyme's biochemical properties, specific activity profiles of the mutants were constructed and compared to the wild-type enzyme. The introduction of Gln^{122} (L122Q) can raise the specific activity 1.3±0.12-fold ($P<0.05$) higher than that of wild-type enzyme, whereas the introduction of Ile^{446} (N446I) can decrease 10–20% of the specific activity than the wild-type enzyme. This made the specific activity of mutant S2 slightly higher than that of wild-type AgaB (about 1.1±0.10-fold; $P<0.05$). Both mutant enzyme S2 and wild-type AgaB had similar initial rate/temperature

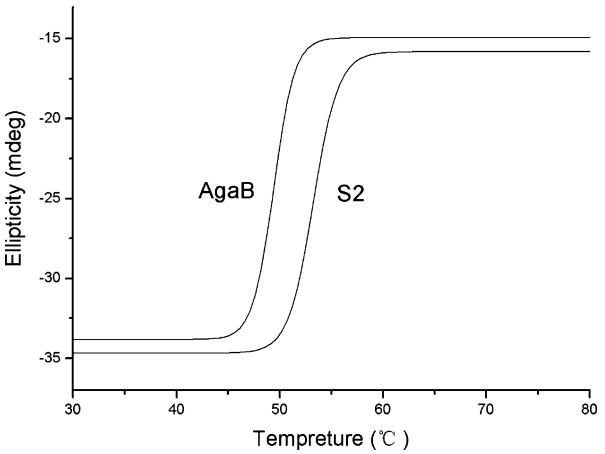

Fig. 2 Unfolding curves of wild-type AgaB and mutant S2. The enzyme was heated from 10 to 80 °C (the curve started from 30 °C) at the speed of 1 °C/min, its ellipticity recorded at 222 nm by the CD spectrum. The T_m values of AgaB and S2 were calculated by a Sigmoidal fit of the transition temperature

optimum at 40 °C, and they had comparable pH stability and optimum pH profiles (data not shown). Under an ordinary hydrolysis condition, AgaB and S2 had the same pattern giving neoagarooctaose and neoagarodecaose as the main products (Fig. 3). Therefore, the evolved enzyme retained the temperature, pH, and product specificity in comparison with the wild-type enzyme.

HCA Analysis

HCA is an efficient method to analyze and compare protein sequences by detecting the secondary structural segments constitution and hydrophobic core of globular protein domains [16, 17]. From the HCA pattern (Fig. 4), short peptide LLLI, which was located at position 121–124, forms a β/α structure ({1111}; following the hydrophobic cluster nomenclature of Lemesle-Varloot et al. [17], 0 represents the hydrophilic residue and 1 the hydrophobic residue). The structure is shorter after substitution of Gln for Leu at position 122. Judging from the rich threonine and proline at the 440–460-amino acid region, it can be deduced that it is a hinge domain in this region. When Ile is substituted for Asn at position 446, peptide INIRY (position 446–450) forms a classical β/α structure ({10101}), and the hydrophobic interaction was increased.

In summary, we have created a thermostable mutant S2 of β-agarase AgaB by directed evolution. Its half-life at 40 °C is 18.4-fold longer than that of the wild-type enzyme. Although the temperature stability of S2 improved only by 5°, it has been fully able to meet

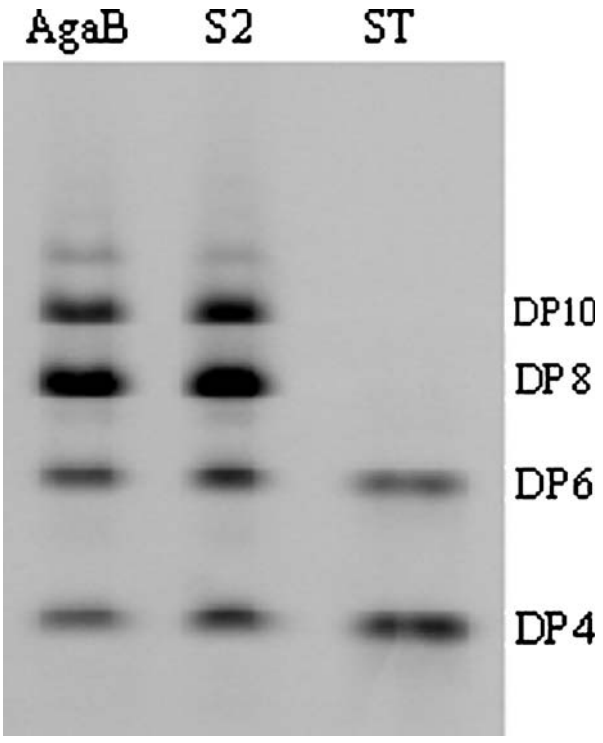

Fig. 3 FACE analysis of final hydrolysis products of wide-type AgaB and mutant S2. After hydrolysis for 12 h, the main products of mutant S2 were neoagarooctaose (*DP8*) and neoagarodecaose (*DP10*), which was the same as wide-type AgaB. The lane marked *ST* was loaded with neoagarotetraose (*DP4*) and neoagarohexaose (*DP6*; Sigma) as the standard

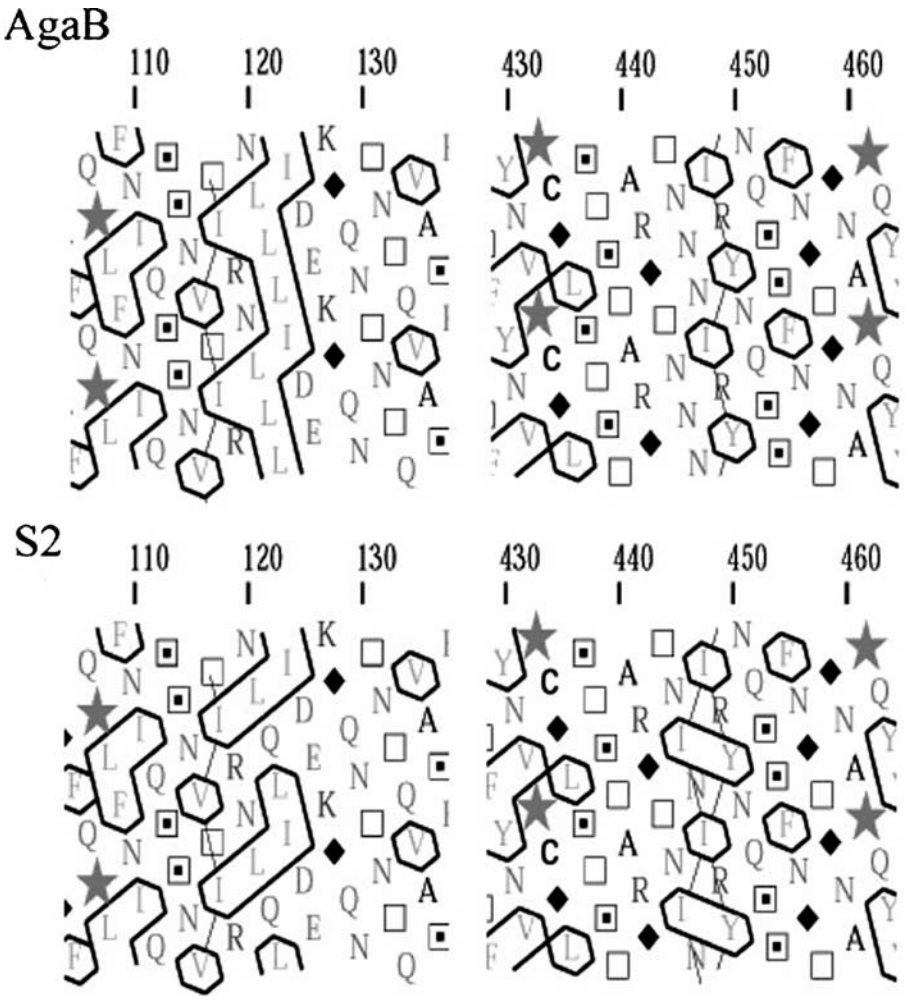

Fig. 4 Plots of HCA of wide-type AgaB and mutant S2 (partial). The amino acid sequences can be read on the near *vertical rows*, from the *top left* to the *bottom right*. Hydrophobic clusters are *boxed*. Standard *one-letter codes* for amino acids are used, except the symbol *filled star* is used for Pro, *filled diamonds* for Gly, ■ for Ser, and *empty squares* for Thr

the industrial application. On one hand, the optimal reaction temperature of wild agarase and mutant S2 is 40 °C. Being stable at 40 °C means a longer time of high-activity reaction, more products, and less cost. On the other hand, the gelling temperature of ordinary agarose is below 40 °C (37±1.5 °C); therefore, the reaction mixture can be kept in a liquid state at 40 °C; this will ensure that enzymatic reaction carried out effectively. More fortunately, we improved the thermostablity of wild-type AgaB without decreasing its activity. Instead, the activity of the modified enzyme is increased slightly, unlike some previous works that the stabilization is improved at the cost of losing activity [15]. Altogether, the higher thermostability of S2, in conjunction with its high specific activity and product specificity, will allow this enzyme to have potentials in industrial applications.

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