Improvement in the Catalytic Activity of β-Agarase AgaA from Zobellia galactanivorans by Site-Directed Mutagenesis

Lee, Seungwoo, Dong-Geun Lee, Min-Kyung Jang, Myong-Je Jeon, Hye-Ji Jang, and Sang-Hyeon Lee*

Department of Pharmaceutical Engineering, College of Medical Life Sciences, Silla University, Busan 617-736, Korea

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In this study, site-directed mutagenesis was performed on the β-agarase AgaA gene from Zobellia galactanivorans to improve its catalytic activity and thermostability. The activities of three mutant enzymes, S63K, C253I, and S63K-C253I, were 126% (1,757.78 U/mg), 2.4% (33.47 U/mg), and 0.57% (8.01 U/mg), respectively, relative to the wild-type β-agarase AgaA (1,392.61 U/mg) at 40°C. The stability of the mutant S63K enzyme was 125% of the wild-type up to 45°C, where agar is in a sol state. The mutant S63K enzyme produced 166%, 257%, and 220% more neoagarohexaose, and 230%, 427%, and 350% more neoagarotetraose than the wild-type in sol, gel, and non-melted powder agar, respectively, at 45°C over 24 h. The mutant S63K β-agarase AgaA may be useful for the production of functional neoagarooligosaccharides.

Keywords: β-Agarase AgaA, activity improvement, site-directed mutagenesis, Zobellia galactanivorans

Materials and Methods

Bacterial Strains and Culture Conditions

Z. galactanivorans (KCTC No. 12921) was purchased from the Korean Collection of Type Cultures (Daejeon, Korea). Escherichia coli DH5α (F' supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 leuB6 thi-1) was used as the host for cloning, and E. coli BL21 (DE3) (leuA8 metB5 hsrM1) was used as the host for the expression of β-agarases. E. coli cells were routinely grown at 37°C in Luria–Bertani (LB) broth (Difco, USA), supplemented with ampicillin (100 µg/ml) when required.

Molecular Cloning and DNA Sequencing of the β-Agarase AgaA Gene

The methods used for molecular cloning were based on those reported by Sambrook et al. [17]. Genomic DNA of Z. galactanivorans was isolated using a Wizard Genomic DNA Purification Kit (Promega, USA). Plasmid DNA was isolated by the
alkaline lysis method [17]. The β-agarase AgaA gene of \textit{Z. galactanivorans} was amplified using PCR primers, which were designed based on the β-agarase AgaA gene of \textit{Z. galactanivorans} Dsj [9], and the genomic DNA of \textit{Z. galactanivorans} was used as a template with Pyrobest DNA polymerase (Takara, Japan). The forward primer was \textit{Z}. \textit{gal} \_\textit{b}-AgaAE1-F (5'-GAATTCATATGCGCACAGAATCTGAAGCAGGATCCTG-3'), which contained EcoRI and NdeI restriction sites (underlined) on the 5'-end. The reverse primer was \textit{Z}. \textit{gal} \_\textit{b}-AgaAE1-R (5'-AAGCTTGTTTACCGACAAGGCTTGA-3'), which contained HindIII and XhoI restriction sites (underlined) on the 5'-end. Amplified DNA was ligated to the pGEM-T easy vector (Promega), resulting in pGEMTe-Z\_gal\_b-AgaAE11. The recombinant plasmid was introduced into \textit{E. coli} DH5α cells. The integrity of the construct was verified by restriction analysis and sequencing.

### Site-Directed Mutagenesis and Screening of Mutants

Site-directed mutagenesis of the β-agarase AgaA gene was carried out using the Quikchange site-directed mutagenesis kit (Stratagene, USA) according to the manufacturer’s instructions. The PCR reaction was performed using 25 ng of pGEMTe-Z\_gal\_b-AgaAE11 as a template with primers \textit{Z}. \textit{gal} \_\textit{b}-AgaAE11(C253I), and pGEMTe-Z\_gal\_b-AgaAE11(S63K) (5'-CCACTGCCTTTACGAAACAGGCTTGA-3'), which contained HindIII and XhoI restriction sites (underlined) on the 5'-end. Amplified DNA was ligated to the pGEM-T easy vector (Promega), resulting in pGEMTe-Z\_gal\_b-AgaAE11(S63K-C253I). After digestion with \textit{Dpn}I, the resulting plasmids containing the mutated β-agarase AgaA gene were transformed into \textit{E. coli} XL10-Gold ultracompetent cells (Stratagene). The transformed cells were plated on an LB ampicillin (100 μg/ml) agar plate and incubated at 37°C overnight. The cells were collected by centrifugation at 5,000 \( \times \) \( g \) for 5 min and then subjected to plasmid preparation. Three mutants were confirmed by sequencing analyses, and designated as pGEMTe-Z\_gal\_b-AgaAE11(S63K), pGEMTe-Z\_gal\_b-AgaAE11(C253I), and pGEMTe-Z\_gal\_b-AgaAE11(S63K-C253I).

### Enzyme Assay

Agarase activity was determined by the enzymatic production of reducing sugars from agarose [20]. The enzyme was incubated in 50 mM MOPS (pH 7.5) buffer containing 300 mM NaCl and 0.2% (w/v) melted agar at 25–55°C for 1 h, and the thermostability of the enzyme was regarded as the activity at 25–55°C for 1 h after preincubation at each specific temperature for 30 min. The enzyme reaction was stopped by the addition of the \( \text{Cu}^{2+} \) reagent. The mixture was then boiled for 10 min and cooled, followed by the addition of the arsenomolybdate reagent. The amount of reducing sugar liberated was measured using \( \text{n}-\text{galactose} \) as a standard. One unit of the enzyme activity was defined as the amount of protein needed to produce 1 μmol of reducing sugar per minute under the assay conditions.

### Chromatographic Analysis of the Hydrolyzed Products

The hydrolyzed products of agar and agarose produced by β-agarase were identified by TLC. Enzymatic hydrolysis was carried out at 25°C and 45°C in 100 ml of 50 mM MOPS (pH 7.5) buffer containing 300 mM NaCl and 0.125 g of agar or agarose. Melted and non-melted substrates (agar and agarose) were enzymatically treated. Melted substrates were heated at 95°C for 30 min and used at 25°C and 45°C for 24 h, whereas non-melted substrate was used at 25°C and 45°C without heating at 95°C. The reaction mixture was applied to silica gel 60 TLC plates (Merck, Germany) [6, 8]. The plates were developed using a solvent system that was composed of \( n-\text{butanol/acetate acid/H}_2\text{O} \) (2:1:1, by vol.). The spots were visualized by spraying with 10% (v/v) \( \text{H}_2\text{SO}_4 \) and heating at 80°C. \( \text{n}-\text{Galactose (Sigma), neoagarotetraose (V-Labs Inc.), and neoagaroheptaose (Sigma) were used as standards. The products of the enzyme reaction were quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).}"

### Result

#### Amino Acid Sequence Analysis of β-Agarases AgaA and AgaB

In a previous study, randomly mutated enzymes of β-agarase AgaB (E99K, T307I, and E99K-T307I) were shown to display improved activities and elevated thermostabilities [10]. To determine the corresponding amino acids for glutamate 99 and threonine 307 of β-agarase AgaB in β-agarase AgaA of \textit{Z. galactanivorans} that had been equilibrated with column buffer. The column was washed with the same buffer and then equilibrated with cleavage buffer (column buffer with 30 mM DTT) at 4°C overnight. Proteins were eluted with column buffer to a total volume of 50 ml. The protein concentration was determined using the BCA protein assay reagent (Pierce Biotechnology, USA), where bovine serum albumin was used as the standard protein.
agarase AgaA, the amino acids sequences of β-agarases AgaA and AgaB were aligned using MacVector 8.1.1 software (Accelrys, USA). In this analysis, glutamate 99 and threonine 307 of β-agarase AgaB were shown to correspond to serine 63 and cysteine 253 of β-agarase AgaA, respectively (Fig. 1). Thus, site-directed mutagenesis was performed on serine 63 and cysteine 253 of β-agarase AgaA to generate mutants S63K, C253I, and S63K-C253I.

Expression and Purification of Wild-Type β-Agarase AgaA and Its Mutants
Production of wild-type β-agarase AgaA and its mutants was performed using E. coli BL21 (DE3) as a host and pTXB1 as a vector. E. coli BL21 (DE3) cells harboring pTXB1-Z_sp-b-AgaBE11 and its mutant (S63K, C253I and S63K-C253I) plasmids produced high amounts of β-agarases (Fig. 2). The SDS-PAGE of the purified enzyme contained a single band with an apparent molecular mass of 29,810 Da (Fig. 2).

Optimum Temperatures and Thermostability of β-Agarase AgaA Mutants
The wild-type and mutant S63K enzymes displayed optimal activities at 45°C, whereas mutant C253I and S63K-C253I enzymes showed optimal activities at 35°C (Fig. 3A). The activity of the mutant S63K enzyme was 126% higher than that of the wild-type enzyme, whereas mutant C253I and S63K-C253I enzymes display an activity of 2.4%
and 0.57% of the wild-type enzyme at 40°C, respectively (Fig. 3A). When the enzymes were subjected to heat treatment, the wild-type and mutant S63K enzymes retained their activities between 35 and 45°C, but showed steep declines of activities at 50°C (Fig. 3B). Mutant C253I and S63K-C253I enzymes showed a severe loss of activity even at 40°C. The thermostability of the mutant S63K enzyme was 128% higher than the wild-type enzyme when treated at 45°C for 1 h (Fig. 3B). Thus, mutant S63K enzyme showed the overall highest activity and was thermally stable up to 45°C for over 1 h.

Production of Neoagarooligosaccharides Using Recombinant β-Agarase AgaA and Its Mutant

The enzymatic products of agarose produced by wild-type and mutant S63K β-agarases AgaA were analyzed as a function of time by TLC (Fig. 4). In the initial stage, both enzymes hydrolyzed agarose and generated neoagarohexaose and neoagarotetraose with various degrees of polymerization. Both wild-type and mutant S63K β-agarases AgaA produced neoagarohexaose after 0.1 h, and the highly polymerized substrate disappeared at 0.25 h. After 1 h of incubation, the mutant S63K enzyme produced more neoagarohexaose (280%) and neoagarotetraose (190%) than the wild-type enzyme. This same trend was observed after 24 h, where the mutant S63K enzyme produced more neoagarohexaose (110%) and neoagarotetraose (340%) than the wild-type enzyme (Fig. 4). The main products of the wild-type and mutant S63K enzymes after 24 h were neoagarohexaose (49.5% and 49% of total products, respectively) and neoagarotetraose (50.5% and 51% of total products, respectively).

![Fig. 3. Effects of temperature and heat treatment on catalytic activities of wild-type β-agarase AgaA and its mutants.](image)

(A) Effect of temperature on the catalytic activities of the enzymes. The values are shown as the percentages of wild-type enzyme activity observed at 40°C, which was taken as 100%. (B) Effect of heat treatment on the catalytic activities of the enzymes. Values are the means ± SEM for three samples. Factorial ANOVA with Fisher's PLSD post-hoc test; *p<0.001 compared with the activity of the wild-type enzyme at 40°C (100%, 1,392.61 U/mg).

![Fig. 4. TLC of degraded agarose by purified wild-type β-agarase AgaA and its mutant S63K.](image)

The reaction was carried out at 40°C in 50 mM MOPS buffer (pH 7.5) with enzymes. NA6, neoagarohexaose; NA4, neoagarotetraose; Gal, α-galactose.
The reaction was carried out at 25°C in 50 mM MOPS buffer (pH 7.5) for 24 h. NA6, neoagarohexaose; NA4, neoagarotetraose; Gal, β-galactose.

![Fig. 5](image.png)

**Fig. 5.** Activity of wild-type and mutant S63K β-agarases AgaA to sol, gel, and non-melted powder agars and agaroses. The reaction was carried out at 25°C (A) and 45°C (B) in 50 mM MOPS buffer (pH 7.5) for 24 h. NA6, neoagarohexaose; NA4, neoagarotetraose; Gal, β-galactose.

The enzymatic products of sol, gel, and non-melted powder-state agars and agaroses were examined by TLC (Fig. 5). Enzymatic reactions were conducted for 24 h, and the activities between wild-type and S63K enzymes were similar, which is in contrast to the results observed after 1 h (Fig. 4). Wild-type and mutant S63K enzymes displayed enzymatic activity on sol, gel, and non-melted powder agars and agaroses. The products in the mutant S63K enzyme reactions with sol, gel, and non-melted powder agars were 166%, 257%, and 220% higher for neoagarohexaose, respectively, and 230%, 427%, and 350% higher for neoagarotetraose, respectively, and 230%, 427%, and 350% higher for neoagarotetraose, respectively, than the wild-type enzyme at 45°C. In addition, higher levels of neoagarohexaose (116%, 175%, and 118%, respectively) and neoagarotetraose (121%, 233%, and 139%, respectively) were produced in sol, gel, and non-melted powder agaroses than those of the wild-type enzyme at 45°C. In contrast, wild-type and mutant S63K enzymes produced similar levels of neoagarooligosaccharides on melted and non-melted powder substrates (agar and agarose) at 25°C (Fig. 5). The mutant S63K enzyme had the same or higher activities than wild-type enzyme in gel-, sol, and powder-state at 25 and 45°C. In addition, the productivity of neoagarooligosaccharides was higher from agarose than agar; however, the productivity from agar was more than 50% of that from agarose.

**DISCUSSION**

Jam et al. [9] reported two β-agarases (AgaA and AgaB) of *Z. galactanivorans*, and AgaA was shown to be more efficient in the degradation of agarose gels. This hinted us that β-Agarase AgaA would show high activity on the non-melted powder and gel substrate, as confirmed in Fig. 5, and β-agarase AgaA could be useful for the production of functional neoagarooligosaccharides from agar at a lower cost. In addition, we previously reported that a β-agarase AgaB (E99K-T307I) mutant displayed an increase in activity (260%) when compared with the wild-type enzyme [10]. To identify the corresponding amino acids of glutamate 99 and threonine 307 of β-agarase AgaB in β-agarase AgaA, amino acid alignment between β-agarases AgaA and AgaB was performed using analysis software. Based on this analysis, serine 63 and cysteine 253 of β-agarase AgaA were chosen for the replacement by site-directed mutagenesis (Fig. 1).

In our previous report, all three β-agarase AgaB mutant (E99K, T307I, and E99K-T307I) enzymes displayed an increased activity and thermostability relative to the wild-type protein. For the β-agarase AgaA mutant enzymes, only the S63K mutant, which had a serine residue substituted to a lysine at the 63th amino acid position, displayed increased catalytic activity and thermostability (Fig. 3). However, it is still not clear why the S63K mutant enzyme showed increased activity and thermostability, where a neutral serine residue was substituted to a positively charged lysine. Tyrosine 69, asparagine 71, and tryptophan 73 residues are the nearest amino acids in primary structure of β-agarase AgaA among the residues in the active site; however, serine 63 is not near the active site of the enzyme [1, 2]. Tryptophan 65 is a conserved residue in the glycoside hydrolase-16 family, namely β-agarases AgaA and AgaB of *Z. galactanivorans*, carrageenase of *P. carrageenovora*, and lichenases of *B. licheniformis* and *P. macerans* [1]. Tryptophan 65 is the start amino acid of the β-strand and serine 63 is located in an alpha helix [1]. Recent studies have indicated that the effect of mutations on properties such as activity and thermostability appear to be uncorrelated with their distance from the active site [15], and mutations distal from the active site have been shown to improve the catalytic efficiency of enzymes [19].

On the other hand, the cysteine to isoleucine amino acid substitution at the 253th amino acid of β-agarase AgaA negatively impacted the activity and thermostability (Fig. 3). Allouch *et al.* [1] suggested that glutamate 308 and glutamine 310 of β-agarase AgaB may participate in substrate binding, both of which are adjacent to the mutated threonine 307. Jang *et al.* [10] suggested that amino acids near T307I might be not only involved in substrate binding but are also important to the rigidity of β-agarase AgaB. Allouch *et al.* [2] reported that glutamate 254 and glutamine 256 of β-agarase AgaA were in the catalytic active channel and formed hydrogen bonds between the agarose units and enzyme residues, both of which are adjacent to the mutated cysteine 253. Both threonine and isoleucine are C-beta branched amino acids, whereas cysteine is not. C-Beta branched amino acids are...
more bulky than cysteine. Thus, it is possible that configurative changes occurred at glutamate 254 and 256 in the mutant C253I β-agarase AgaA, which could have weakened the hydrogen bond between mutant C253I β-agarase AgaA and the substrate, and decreased the enzymatic activity. Shi et al. [18] reported that hydrophobic interactions might play a major role in improving the thermostability of β-agarase from *Pseudomonas* sp. CY24. The structure of the amino acid, besides its hydrophobicity, might also play an important role in enhancing the activity and thermostability of mutant C253I β-agarase AgaA.

TLC analysis of the degradation of agarose as a function of time demonstrated that wild-type and mutant S63K β-agarases AgaA could produce a mixture of neoagarohexaose and neoagarotetraose (Fig. 4). Wild-type and mutant S63K enzymes produced neoagarohexaose (49.5% and 49%, respectively) and neoagarotetraose (50.5% and 51%, respectively) from agarose after 24 h. Using β-agarase from *Agarivorans* sp. JA-1, we previously produced a mixture of neoagarobiose (58%) and neoagarotetraose (42%) from agarose [14].

Allouch *et al.* [1] suggested that there was a calcium binding region on the β-agarases AgaA and AgaB of *Z. galactanivorans*. One mM Ca\(^{2+}\) ions specifically increased the thermostabilities of the wild-type and mutant β-agarases AgaB [10]; however, Ca\(^{2+}\) ions decreased the activities and thermostabilities of the wild-type and all three mutant β-agarases AgaA in general at 1 and 10 mM concentrations (data not shown).

Jam *et al.* [9] reported that β-agarase AgaA was more efficient than β-agarase AgaB in the degradation of agarose gels. Thus, it may be possible to use non-melted or powder substrate (agarose or agar) for the production of bioactive neoagarooligosaccharides such as neoagarotetraose [13] and this would result in a cost reduction. Although the enzyme displayed lower activity on non-melted powder agarose than melted agarose for both the wild-type and mutant S63K β-agarases AgaA, both enzymes produced 40% more products after 24 h (Fig. 5). The productivity of the mutant S63K enzyme on melted agarose was 117% for neoagarohexaose and 141% for neoagarotetraose relative to the wild-type enzyme on melted agarose. In addition, the productivity of the mutant S63K enzyme on non-melted powder agar was 18% for neoagarohexaose and 47% for neoagarotetraose relative to the wild-type enzyme on melted agarose. A lower production of neoagarooligosaccharides was observed on melted agar than agarose, regardless of melting and mutation (Fig. 5). However, agarose is 10 times more expensive than agar, and the mutant S63K β-agarase AgaA produced approximately 50% more neoagarooligosaccharide from agar when compared with wild-type β-agarase AgaA under the same condition (Fig. 5). Hence, it would be economical to produce neoagarooligosaccharides from agar using the mutant S63K β-agarase AgaA.

In this study, three β-agarase AgaA mutant enzymes were successfully produced by site-directed mutagenesis, and the mutant S63K enzyme was shown to have an enhanced activity and thermostability. The mutant S63K enzyme was almost optimally active at 45°C, which is above the gelling temperature of agar, and could produce functional neoagarooligosaccharides from agarose and agar without melting. Thus, the mutant S63K β-agarase AgaA enzyme could be used for the industrial production of biologically functional neoagarooligosaccharides from agar at low cost.

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**References**


