Introduction

Agar is a polysaccharide that is present in the cell wall of some red algae [1]. It is composed of 3,6-anhydro-\(\alpha\)-galactose and D-galactose, forming a linear chain linked by alternating \(\beta\)-1,4- and \(\alpha\)-1,3-glycosidic bonds [1, 2]. Agar can be degraded by agarase enzymes that are classified into two distinct types: \(\beta\)-agarases and \(\alpha\)-agarases. \(\beta\)-Agarases specifically cleave the \(\beta\)-1,4-glycosidic bonds of agar [3], whereas \(\alpha\)-agarases hydrolyze only \(\alpha\)-1,3-glycosidic bonds [4]. When \(\alpha\)-agarases depolymerize agar, they produce agarooligosaccharides with 3,6-anhydro-\(\alpha\)-L-galactose at the reducing end [1]. On the other hand, \(\beta\)-agarases degrade agar into neoagarooligosaccharides with D-galactose at the reducing end [1]. The major components of neoagarooligosaccharides are neoagarobiase (NA2) composed of two sugars, neoagarotetraose (NA4) with four sugars, and neoagarohexaose (NA6) with six sugars [1]. NA2 can be cleaved into monomeric sugars, 3,6-anhydro-L-galactose and D-galactose, by neoagarobiase hydrolase [1, 5]. Among the two monomeric sugars, D-galactose can be more easily used as a carbon source for bacterial cells [6]. The other sugar, 3,6-anhydro-L-galactose, requires additional enzymatic degradation into 2-keto-3-deoxygalactonate for utilization as a carbon source. This is accomplished by the sequential process of two catalytic enzymes; an NADP\(^+\)-dependent 3,6-anhydro-\(\alpha\)-L-galactose dehydrogenase and a 3,6-anhydrogalactonate cycloisomerase [7]. An understanding of the catabolic enzymes involved in agar degradation is necessary for engineering the bioconversion of agar into industrial chemicals or biofuels. Notably, neoagarooligosaccharides produced by agarase are known to have many biological activities, such as antibacterial activity [8], moisturizing the skin [9], whitening of melanoma cells [9], anti-obesity activity [10], and antidiabetic activity [10].

\(\beta\)-Agarases have been found in various bacteria, including...
Among them, two agarase genes belonging to GH16, GH39, GH42, and GH86 were identified. The whole-genome sequence revealed 12 putative glycoside hydrolase (GH) families, including GH86, few of which were studied by biochemical analyses [1, 11, 15, 27]. In other GH families, such as GH86, few β-agarases have been biochemically characterized [28, 29].

Gayadomonas joobiniege G7 is a rod-shaped, aerobic, non-motile, and non-pigmented gram-negative marine bacterium that was isolated from a seawater sample from Gaya Island, and has strong agar-degrading activity [30]. The 16S rRNA gene sequence of G. joobiniege G7 shows 95.5% similarity with that of Catenovulum agarivorans YM01 [30, 31]. The whole-genome sequence revealed 12 putative agarase genes belonging to GH16, GH39, GH42, and GH86 [30, 31]. Among them, two β-agarases were biochemically characterized. The β-agarase AgaJ9, a member of GH39, is a cold-tolerant enzyme having more than 80% of its activity at 5°C [3]. Another GH16 β-agarase, AgaJ11, is an acidic β-agarase that has agarase activity only at the acidic pH range of 4–5 [27].

In this study, we analyzed the biochemical features of AgaJ5, a novel agarase from G. joobiniege G7. The following experiments show that the GH86 member AgaJ5 was an acidic endo-type β-agarase with the highest activity at an acidic pH range of 4.5–5.5. We also observed that AgaJ5 was a cold-adapted enzyme retaining 40% of enzymatic activity at 10°C. The main hydrolysis product of AgaJ5 was NA6, with relatively minor amounts of NA4 and NA2 produced.

Materials and Methods

Bacterial Strains and Culture Conditions

Escherichia coli strain ER2566 was used for cloning and expression of AgaJ5 and was grown at 37°C in Luria-Bertani (LB) medium. The pET-28a plasmid was present in ER2566 cells, kanamycin (50 μg/ml) was added.

Gene Cloning

For making pHis-AgaJ5, the fragment of AgaJ5 (NCBI Reference Sequence: WP_017446675.1) without the predicted signal peptide (amino acids 1–30) was cloned into the pET-28a plasmid. To amplify the 2,328 bp gene fragment, the polymerase chain reaction (PCR) was performed using a forward primer (5'-GCCGACTGACATACAAAGAAACAACTGCCGT-3') and a reverse primer (5'-GGCTGAAATTCCGATCCGTAAAAGGAGTGAGTCCAAA-3'). These primers contained adaptor sequences (underlined) corresponding to the terminal 15 bp sequences of the pET28a fragment digested by the Ndel and BamHI restriction enzymes for the In-Fusion reaction. PCR was performed using Q-Cycler (Quanta Biotech, UK). PCR amplicons were inserted into the pET28a fragment digested by the Ndel and BamHI restriction enzymes (New England Biolabs, USA) using the In-Fusion HD cloning kit (Clontech, USA).

Purification of AgaJ5

ER2566 cells harboring pHis-AgaJ11 were inoculated in LB medium at 37°C. When the OD₆₆₀ reached 0.5, 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the LB medium to induce expression of AgaJ5. The cells were then cultured for 12 h at 16°C. The cells harvested by centrifugation were resuspended in lysis buffer (50 mM Tris-HCl, 250 mM NaCl, pH 7.5). The resuspended cells were lysed using a French pressure cell at 12,000 psi. Cellular debris and supernatants were separated by centrifugation at 12,000 × g for 20 min at 4°C. Only the supernatant was used for protein purification through BD TALON metal affinity resin (Clontech). After washing with binding buffer, proteins bound to the affinity resin were eluted using lysis buffer containing 250 mM imidazole. To dilute imidazole in buffer, the eluted sample was dialyzed in 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM KCl for 10 h at 4°C. The concentration of purified AgaJ5 was determined through the Bradford assay [32].

Zymogram Assay

The unboiled purified AgaJ5 was separated using a 12% polyacrylamide gel containing 0.1% agarose. After immersion in 2% Triton X-100 for 30 min and washing twice with 20 mM Tris-HCl buffer (pH 8.0), the protein gel was incubated in 10 mM sodium citrate buffer (pH 4.5) at 30°C overnight. To identify the agarase activity of AgaJ5, the protein gel was stained using Lugol’s iodine solution [3].

Agarase Assay

The agarase activity of purified AgaJ5 was determined by the 3,5-dinitrosalicylic acid (DNS) method measuring the amount of reducing sugars, as described previously [11]. The reaction mixture was composed of 20 μl of purified AgaJ5 and 480 μl of 10 mM sodium citrate buffer (pH 4.5) containing 0.1% agarose. After incubation at 30°C for 30 min, 500 μl of DNS solution (0.65 g of DNS, 4.5 ml of glycerol, and 32.5 ml of 2 N NaOH in 100 ml of distilled water) was added to the reaction mixture. After boiling at 100°C for 10 min, the sample was monitored at 540 nm using a spectrophotometer to determine the concentration of reducing sugars. The reaction mixture containing 20 μl of distilled water instead of purified AgaJ5 was used as a control. To determine the optimum temperature of AgaJ5 activity, the enzyme assay was performed at temperatures between 10°C and 60°C. Similarly, the optimum pH was determined through the enzyme reactions under various pH conditions: 3, 3.5, 4, 4.5, 5, 5.5, and 6 in 10 mM
sodium citrate; 6 and 7 in 10 mM MOPS; 7, 8, and 9 in 10 mM Tris-HCl; and 9 and 10 in 10 mM glycine-NaOH. The effect of metal ions was determined through the enzyme reactions in 10 mM sodium citrate buffer (pH 4.5) containing 5 mM of each metal ion, NaCl, KCl, CoCl₂, KCl, ZnCl₂, CuCl₂, CaCl₂, MnCl₂, NiCl₂, and FeCl₂.

**Enzymatic Kinetics**

The kinetic parameters (Kₘ and Vₘₐₓ) of AgaJ5 were determined by the enzyme reactions in 10 mM sodium citrate buffer (pH 4.5) with various amounts of agarose (from 0.1 to 8 mg/ml). To limit substrate utilization below 5%, the samples were incubated at 30°C for 7 min only [33]. The Kₘ and Vₘₐₓ values were calculated from the Lineweaver-Burk plot.

**Measurement of Viscosity**

The enzymatic reaction of AgaJ5 was performed in 10 mM sodium citrate buffer (pH 4.5) containing 0.5% agarose at 30°C. The viscosity of the reaction mixture was measured using a DV2T viscometer (Brookfield AMETEK, USA), at various reaction times, ranging from 0 to 60 min.

**Thin-Layer Chromatography Analysis**

The enzymatic reaction of AgaJ5 was performed at 30°C in 10 mM sodium citrate buffer (pH 4.5) with 0.1% agarose or neoagarooligosaccharides, and was stopped by boiling for 5 min. Oligosaccharides of the reaction samples were separated using a Silica Gel 60 plate (Merck, USA) and an n-butanol:acetic acid:H₂O solution (2:1:1, by volume). After spraying with 20% H₂SO₄ in methanol, oligosaccharides spots on the silica gel plate were visualized by heating at 120°C for 2 min. The plates were imaged using a digital camera (EOS 100D; Canon Inc., Japan).

**Mass Spectrometry Analysis**

The unstained regions on the TLC plates corresponding to hydrolysis products were scraped out and dissolved in 100% methanol. After evaporation of the methanol, the molecular mass of the extracted oligosaccharides was determined using a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Autoflex III, Germany). 2-(4’-Hydroxybenzeneazo)benzoic acid was used as the matrix [27].

**Results**

**A Novel Agarase (AgaJ5) of G. joobiniege G7**

There are 12 putative agarase genes in G. joobiniege, a marine bacterium with strong agar-degrading ability [30]. Among them, we have characterized two novel β-agarases, AgaJ9 and AgaJ11 [3, 27]. AgaJ9 has cold-adapted agarase activity, and AgaJ11 is an acidic β-agarase. To study the novel agarase in G. joobiniege, we cloned the agaJ5 gene of G. joobiniege encoding 805 amino acids (NCBI Reference Sequence: WP_017446675.1). Comparative sequence analyses using BLAST showed that AgaJ5 showed 51% identity with a β-agarase (NCBI Reference Sequence: WP_035014943.1) from the agarolytic marine bacterium *Catenovulum agarivorans* DS-2 [34], and 48% identity (64% similarity) with the characterized agarase AgaA (NCBI Reference Sequence: WP_062064961.1) from the agarolytic bacterium *Cellulibrio* sp. [28]. AgaJ5 also had 35% identity to the agarase AgrA (GenBank Accession No. AAA25696.1) from *Pseudoalteromonas atlantica* T6c [35, 36], and 28% identity to another characterized β-agarase, AgaO, (GenBank Accession No. BAK08903.1) from the deep-sea bacterium *Microbulbifer thermotolerans* JAMB-A94 [29]. Because AgaA, AgrA, and AgaO all belong to the GH86 family, the similar AgaJ5 may also belong to this family. The SignalP program (http://www.cbs.dtu.dk/services/SignalP) predicted that AgaJ5 has a signal sequence peptide at the N terminus (amino acids 1–30).

To analyze the biochemical characteristics of AgaJ5, the agaJ5 gene fragment, encoding the mature form without the predicted signal sequence, was inserted into a pET28a vector. The His-tagged recombinant AgaJ5 protein was overexpressed and purified using TALON metal affinity chromatography (Fig. 1A). The molecular mass of His-tagged recombinant AgaJ5 was predicted to be 89,278 Da,

![Fig. 1. Purification of AgaJ5.](image-url)

(A) ER2566 cells containing pHis-AgaJ5 were used for overexpression of the hexahistidine-tagged AgaJ5. After purification of AgaJ5 using metal affinity chromatography, purified AgaJ5 was subjected to SDS-PAGE. Lane M, size marker; lane 1, extract of cells before induction; lane 2, extract of cells after induction with 1 mM IPTG; lane 3, soluble fraction of crude extract; lane 4, purified AgaJ5. (B) Zymogram analysis of AgaJ5. Purified AgaJ5 was separated on a 12% polyacrylamide gel containing 0.1% agarose. After the enzymatic reaction, the polyacrylamide gel was stained using Lugol’s iodine solution. The stained gel was imaged using a digital camera.
which is approximately consistent with the experimental molecular mass on SDS-PAGE (Fig. 1A). Zymogram analysis was performed to determine whether purified AgaJ5 has agarase activity. The activity staining of purified AgaJ5 showed an apparent clear zone at a molecular mass of 89kDa (Fig. 1B), indicating that AgaJ5 has agarase activity.

Effects of pH, Temperature, and Metal Ions on the Enzymatic Activity of AgaJ5

To determine the optimal pH for AgaJ5 agarase activity, the enzymatic reaction was performed within a pH range of 3–10 (Fig. 2A). The maximum activity of AgaJ5 was observed at pH 4.5 and AgaJ5 had more than 80% of maximum enzymatic activity only at a narrow pH range from 4.5 to 5.5, suggesting that AgaJ5 is an acidic agarase. In a similar way, the optimal temperature for AgaJ5 activity was determined by measuring the enzymatic activities at various temperatures of 10°C to 60°C (Fig. 2B). The maximum activity of AgaJ5 was observed at 30°C, and more than 40% of enzymatic activity was retained at 10°C. These cold-adapted features were similar to the previously

![Fig. 2](image)

**Fig. 2.** Effects of pH, temperature, and metal ions on AgaJ5 activity. (A) The enzymatic activity of AgaJ5 was measured under various pH conditions: diamonds, 3, 3.5, 4, 4.5, 5, 5.5, and 6 in 10 mM sodium citrate; squares, 6 and 7 in 10 mM MOPS; triangles, 7, 8, and 9 in 10 mM Tris-HCl; circles, 9 and 10 in 10 mM glycine-NaOH. (B) The enzymatic activity of AgaJ5 was measured at various temperatures (10°C to 60°C) in 20 mM sodium citrate buffer (pH 4.5). (C) The effect of metal ions was estimated in 10 mM sodium citrate buffer (pH 4.5) containing 5 mM metal ions; KCl, ZnCl$_2$, CuCl$_2$, CaCl$_2$, MnCl$_2$, NiCl$_2$, NaCl, KCl, CoCl$_2$, and FeCl$_2$.

![Fig. 3](image)

**Fig. 3.** Measurement of kinetic parameters for AgaJ5. The $K_m$ and $V_{max}$ were determined by the enzyme reactions in 10 mM sodium citrate buffer (pH 4.5) with various amounts of agarose (from 0.1 to 8 mg/ml). The kinetic parameters of AgaJ5 were determined using reciprocal Lineweaver-Burk plots. All data are mean values from two replicate experiments.
characterized agarase AgaJ9 of *G. joobiniege* [3]. It is therefore possible that the cold-adapted feature is a general characteristic of agarases from *G. joobiniege*.

Metal ions also affected the agarase activity of AgaJ5 (Fig. 2C). The enzymatic activity of AgaJ5 was strongly inhibited by some metal ions, such as CuCl$_2$ (1% remaining), FeCl$_2$ (11% remaining), and MnCl$_2$ (11% remaining), whereas it was slightly activated by monovalent ions, such as those supplied by NaCl and KCl. The addition of EDTA moderately inhibited the agarase activity of AgaJ5. Therefore, these results indicate that monovalent ions are important for the maximum enzymatic activity of AgaJ5. The $K_m$ and $V_{max}$ of AgaJ5 were 8.9 mg/ml and 188.6 U/mg, respectively (Fig. 3). Previous studies showed that AgaJ9 has the $K_m$ and $V_{max}$ of 0.68 mg/ml and 17.2 U/mg, respectively, and the $K_m$ and $V_{max}$ of AgaJ11 are 21.42 mg/ml and 25 U/mg, respectively [3, 27]. Therefore, AgaJ5 has a significantly high value of $V_{max}$ when compared with AgaJ9 and AgaJ11 agarases from *G. joobiniege*.

**Analysis of Hydrolysis Products of AgaJ5**

To determine whether AgaJ5 is an α- or β-agarase, the enzymatic activity of AgaJ5 was estimated using two chromogenic substrates, $p$-nitrophenyl-α-D-galactopyranoside and $p$-nitrophenyl-β-D-galactopyranoside [3, 27]. Unexpectedly, AgaJ5 did not exhibit any activity on either substrate (data not shown). This was similar to the results obtained from other characterized agarases from *G. joobiniege*, AgaJ9 [3] and AgaJ11 [27]. To determine whether AgaJ5 is an endo- or exo-type agarase, the change of viscosity in the enzyme reaction mixture was examined. The viscosity of the reaction sample dropped rapidly for the first 20 min, but then decreased slowly until the end of the reaction (Fig. 4A), suggesting that AgaJ5 is an endo-acting agarase.

![Fig. 4. Hydrolysis products of AgaJ5.](image)

(A) The enzymatic reaction of AgaJ5 was performed in 10 mM sodium citrate buffer (pH 4.5) containing 0.5% agarose at 30°C. The viscosity of the reaction mixture was measured using a DV2T viscometer (Brookfield AMETEK, USA), at various reaction times of 0–60 min. (B) Hydrolysis products of AgaJ5 on agarose. The enzymatic reaction of AgaJ5 was carried out at 30°C in 10 mM sodium citrate buffer (pH 4.5) containing 0.1% agarose for the indicated time. Hydrolysis products were analyzed using a silica gel 60 plate. NA2, neoagarobiose; NA4, neoagartetraose; NA6, neoagarohexaose. (C) The enzymatic reactions of AgaJ5 using NA2, NA4, or NA6 as a substrate. The enzymatic reactions of AgaJ5 were carried out at 30°C for 24 h in 10 mM sodium citrate buffer (pH 4.5) containing NA2, NA4, or NA6 as a substrate. Reaction products were loaded onto a silica gel 60 plate and separated using an n-butanol:acetic acid:H$_2$O solution (2:1:1, by volume).
Fig. 5. MALDI-TOF mass spectra of oligosaccharides extracted from the TLC samples. Oligosaccharide spots corresponding to neoagarotetraose (NA4) (A) and neoagarohexaose (NA6) (B) on the TLC plate were extracted using methanol and their molecular weights were estimated by MALDI-TOF mass spectrometry.
The hydrolysis products of the AgaJ5 enzymatic reaction were measured by TLC (Fig. 4B). AgaJ5 hydrolyzed agarose mainly into NA6, and a smaller amount of NA2, NA4, and neoagarooligosaccharides larger than NA6 were produced. The neoagarooligosaccharides larger than NA6 disappeared at the later stages of the reaction, whereas NA2 and NA4 remained to the end of the reaction (Fig. 4B). TLC analyses were also performed using NA2, NA4, and NA6 as substrates (Fig. 4C). Interestingly, little NA2, NA4, and NA6 were hydrolyzed by AgaJ5 after an overnight incubation, implying that AgaJ5 cannot degrade neoagarooligosaccharides smaller than NA8. These results could help explain the lack of AgaJ5 activity using chromogenic substrates, which are much smaller molecules than NA8. The molecular masses of oligosaccharides extracted from the TLC plates were estimated by mass spectrometry (Fig. 5). The MALDI-TOF analysis showed that the hydrolysis products of AgaJ5 are molecular ions at $m/z$ of 653 (M+Na)$^+$ and 959 (M+Na)$^+$, which correspond to NA4 and NA6, respectively. In conclusion, these results suggest that AgaJ5 is an endo-type $\beta$-agarase producing NA6 from agarose.

**Discussion**

In this study, we characterized the biochemical characteristics of the novel GH86 $\beta$-agarase AgaJ5 from *G. joobiniege* G7. This agarase has maximum activity at pH 4.5 and 30°C (Fig. 2). The main final hydrolysis product of AgaJ5 is NA6 (Fig. 4).

AgaJ5 is a novel $\beta$-agarase from the GH86 family. Previously, two $\beta$-agarases have been biochemically characterized (Table 1). Among them, AgaJ5 had biochemical similarity to AgaO from *M. thermotolerans* JAMB-A94 [29]. The main hydrolysis product of AgaJ5 and AgaO was NA6, whereas NA2 and NA4 were produced as minor products of agarose hydrolysis. In addition, the activity of both was activated by several ions. The AgaO activity was strongly activated by the divalent ion Ca$^{2+}$ [29], whereas the AgaJ5 activity was slightly activated by monovalent ions, such as Na$^+$ and K$^+$ (Fig. 2). However, the optimal pH and temperature of the two agarases were significantly different. The maximal activity of AgaO was observed at pH 7.5 and 45°C [29]. Conversely, AgaJ5 was an acidic agarase with the maximum activity at pH 4.5, and was a cold-tolerant agarase, having 40% of agarase activity at 10°C.

The biochemical features of two $\beta$-agarases from *G. joobiniege* G7, AgaJ9 and AgaJ11, were analyzed in previous studies [3, 27]. AgaJ5 shares several biochemical features with AgaJ9 and AgaJ11. Like AgaJ9, AgaJ5 also had the cold-adapted characteristic (Fig. 2). In addition, AgaJ5 had maximum enzymatic activity at acidic pH, like AgaJ11. Although AgaJ9 was active from pH 4 to 8, its optimal pH was 5 [3]. Therefore, these results imply that many agarases from *G. joobiniege* G7 may share the acidic or cold-adapted enzymatic features.

Various biological activities of neoagarooligosaccharides have been described, including melanoma cell whitening [9], skin moisturizing [9], and antibacterial [8], anti-obesity, and antidiabetic effects [10]. In particular, neoagarooligosaccharides, composed mainly of NA4 and NA6, can effectively suppress obesity and obesity-related metabolic syndromes, such as hyperlipidemia, steatosis, insulin resistance, and glucose intolerance [10]. It is thought to suppress these conditions by inducing adiponectin production in high-fat diet-induced obese mice [10]. Because AgaJ5 has a significantly high value of $V_{\text{max}}$, and produces NA6 as the predominant hydrolysis product, it may be useful to the food, pharmaceutical, and cosmetic industries.

**Acknowledgments**

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**Table 1.** Comparison of various biochemical features of characterized agarases belonging to glycoside hydrolase family 86.

<table>
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<tr>
<th>Agarase</th>
<th>Molecular mass (kDa)</th>
<th>End products</th>
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<th>Optimal temperature</th>
<th>Effect of metal ions</th>
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<td>NA2, NA4</td>
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<td>42.5°C</td>
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<td>NA6</td>
<td>7.5°C</td>
<td>45°C</td>
<td>Activated by Ca$^{2+}$</td>
<td>Not determined</td>
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<td>[29]</td>
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<tr>
<td>AgaJ5</td>
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<td>NA6</td>
<td>4.5°C</td>
<td>30°C</td>
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<td>8.9 mg/ml</td>
<td>188.6 U/mg</td>
<td>This study</td>
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The hydrolysis products of the AgaJ5 enzymatic reaction were measured by TLC (Fig. 4B). AgaJ5 hydrolyzed agarose mainly into NA6, and a smaller amount of NA2, NA4, and neoagarooligosaccharides larger than NA6 were produced. The neoagarooligosaccharides larger than NA6 disappeared at the later stages of the reaction, whereas NA2 and NA4 remained to the end of the reaction (Fig. 4B). TLC analyses were also performed using NA2, NA4, and NA6 as substrates (Fig. 4C). Interestingly, little NA2, NA4, and NA6 were hydrolyzed by AgaJ5 after an overnight incubation, implying that AgaJ5 cannot degrade neoagarooligosaccharides smaller than NA8. These results could help explain the lack of AgaJ5 activity using chromogenic substrates, which are much smaller molecules than NA8. The molecular masses of oligosaccharides extracted from the TLC plates were estimated by mass spectrometry (Fig. 5). The MALDI-TOF analysis showed that the hydrolysis products of AgaJ5 are molecular ions at $m/z$ of 653 (M+Na)$^+$ and 959 (M+Na)$^+$, which correspond to NA4 and NA6, respectively. In conclusion, these results suggest that AgaJ5 is an endo-type $\beta$-agarase producing NA6 from agarose.
Conflict of Interest

The authors have no financial conflicts of interest to declare.

References


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