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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Purification and characterization of a novel β-agarase, AgaA34, from *Agarivorans albus* YKW-34

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Abstract An extracellular β-agarase (AgaA34) was purified from a newly isolated marine bacterium, *Agarivorans albus* YKW-34 from the gut of a turban shell. AgaA34 was purified to homogeneity by ion exchange and gel filtration chromatographies with a recovery of 30% and a fold of ten. AgaA34 was composed of a single polypeptide chain with the molecular mass of 50 kDa. N-terminal amino acid sequencing revealed a sequence of ASLVTSFEEA, which exhibited a high similarity (90%) with those of agarases from glycoside hydrolase family 50. The pH and temperature optima of AgaA34 were pH 8.0 and 40°C, respectively. It was stable over pH 6.0–11.0 and at temperature up to 50°C. Hydrolysis of agarose by AgaA34 produced neoagarobiose (75 mol%) and neoagarotetraose (25 mol%), whose structures were identified by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy and ^{13}C NMR. AgaA34 cleaved both neoagarohexaose and neoagarotetraose into neoagarobiose. The k_{cat}/K_m values for hydrolysis agarose and neoagarotetraose were 4.04×10^3 and 8.1×10^2 s^{-1} M^{-1}, respectively. AgaA34 was resistant to denaturing reagents (sodium dodecyl sulfate and urea). Metal ions were not required for its activity, while reducing reagents (β-Me and dithiothreitol, DTT) increased its activity by 30%.

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Introduction

Agar, the cell wall matrix of red algae, consists of two different components of agarose and agaropectin (Araki 1937). Agarose is a neutral linear polysaccharide composed of alternating residues of 3-*O*-linked β-D-galactopyranose and 4-*O*-linked 3,6-anhydro-α-L-galactopyranose (Hamer et al. 1977). Agarases, catalyzing the hydrolysis of agar, were characterized as α-agarases (E.C. 3.2.1.158) that cleave α-1,3 linkage to produce agarooligosaccharides (Potin et al. 1993) and β-agarases (E.C. 3.2.1.81) that cleave β-1,4 linkage to produce neoagarooligosaccharides (Kirimura et al. 1999).

Hydrolysis of agar or agarose by β-agarase produces neoagarooligosaccharides, which have various special chemical properties and biological activities. They inhibit the growth of bacteria, slow down the degradation of starch, reduce the calorific value of food, and present anticancer and antioxidation activities (Giordano et al. 2006). The low polymerization degree (DP) product, such as neoagarobiose (NA2), has a moisturizing effect on skin and a whitening effect on melanoma cells (Kobayashi et al. 1997). Owning to these characteristics, neoagarooligosaccharides have potential applications in food, pharmaceutical, and cosmetic industries. So far, several agarases have been isolated from different genera of bacteria in seawater or marine sediment, including *Cytophaga* (Van der Meulen and Harder 1975), *Pseudomonas* (Groleau and Yaphe 1977), *Vibrio* (Araki et al. 1998), *Alteromonas* (Kirimura et al. 1999; Wang et al. 2006), *Agarivorans* (Ohta et al. 2005a), and *Acinetobacter* (Lakshmikanth et al. 2006).

Agarivorans albus YKW-34 was a marine bacterium capable of degrading the cell wall of brown algae *Laminaria japonica* (Yi and Shin 2006). An alginate lyase produced by this strain was purified and characterized in our previous study (Fu et al. 2007). Strain YKW-34 was formerly classified as the genus *Vibrio* based on phenotypic features (Yi and Shin 2006), while it was identified as *A. albus* based on 16S rRNA gene sequence in this study. An interesting phenomenon was observed in the present study that the strain formed deep depressions on agar plate, which supposed that the strain secreted agarase with high activity. It was reasonable because *Agarivorans* is a novel genus which was named for its agar devouring ability (Kurahashi and Yokota 2004). In the present study, the agarase produced by this strain was purified and characterized, and its enzymatic products were analyzed. This is the second report on the purification of agarase derived from genus *Agarivorans*, and the molecular mass differentiated this agarase from the first agarase purified from the strain of this genus by Ohta et al. (2005a).

Materials and methods

Microorganism and culture condition

The microorganism used in this study was *A. albus* YKW-34 isolated from the gut of a turban shell *Turbinidae batillus cornutus* (Yi and Shin 2006). The inoculum was prepared by incubating the glycerol seed at 20°C in a marine broth medium (Difico, Detroit, MI, USA) with shaking at 120 rpm for 12 h to reach a cell density of 1.0×10^7 CFU/ml. For agarase production, the prepared inoculum was transferred to a fermentation medium and incubated at 25°C with shaking at 120 rpm for 12 h. The fermentation medium was prepared by artificial seawater (2.5% NaCl, 0.5% $MgSO_4{\cdot}7H_2O$, 0.1% KCl, 0.02% $CaCl_2$, 0.01% K_2HPO_4, 0.002% $FeSO_4{\cdot}7H_2O$) supplemented with 0.23% agar and 0.27% yeast extract with an initial pH 7.8 adjusted by 1 N NaOH.

Identification of the microorganism

Chromosomal DNA of *A. albus* YKW-34 was isolated as described by Wilson (1997). 16S ribosomal RNA (rRNA) gene fragment was amplified by polymerase chain reaction (PCR) with the universal primers 27F and 1492R. The amplification was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The PCR fragment was purified using a Solgent PCR purification kit (Solgent, Daejeon, Korea). Sequencing was performed on an ABI Prism 3730 DNA analyzer using an ABI BigDye cycle sequencing kit with Solgent EF-Taq DNA polymerase (Solgent). The 16S rRNA gene sequence (accession number: EU084496) was compared with sequences in nucleotide database by using the BLAST algorithm at the NCBI site (http://www.ncbi.nlm.nih.gov/BLAST/).

Enzyme assay

The agarase activity was quantified by spectrometric determination of reducing sugars by Nelson method. After incubating 50 μl supernatant of the culture fluid with 200 μl of 0.1% agarose in 20 mM Tris–HCl (pH 8.0) at 40°C for 30 min, the amount of formed reducing sugar was determined by Nelson reagent using D-galactose as a standard. Enzyme activity (U/ml) was defined as the amount of enzyme required to liberate 1 μmol D-glucose per minute. Protein concentration was determined by the method of Bradford using bovine serum albumin as the calibration standard. The relative protein contents of chromatography fractions were estimated by absorbance at 280 nm.

Purification of agarase

All the purification procedures were carried out at 4°C. Culture fluid (100 ml) was centrifuged at 12,000×*g* for 15 min, and the cell-free supernatant was concentrated by ultrafiltration (10-kDa cutoff membrane, Millipore, Bedford, MA, USA) to 40 ml followed by dialysis against buffer A (20 mM Tris–HCl buffer, pH 8.0) overnight. The dialyzed solution was applied to a DEAE Sepharose FF column (2.6×30 cm; Amersham, Uppsala, Sweden) pre-equilibrated with buffer A. Bound proteins were eluted with 480 ml linear gradient of 0.3–1.0 M of NaCl in buffer A at a flow rate of 1.5 ml/min (8 ml per tube). There are two enzyme peaks, and the fractions of the major peak (fraction numbers 39–46) were pooled, concentrated to 2 ml by ultrafiltration, and dialyzed against buffer A containing 0.15 M NaCl. The dialyzed enzyme solution was applied to a Sephacryl S-100 HR column (1.6×60 cm; Amersham) pre-equilibrated with buffer A containing 0.15 M NaCl. Bound proteins were eluted with the equivalent buffer at a flow rate of 0.5 ml/min (2 ml per tube). Active fractions (fraction numbers 38–43) were pooled, dialyzed against buffer A, and used for further study. The molecular mass was determined using the latter column with a range of molecular mass markers (Sigma, St. Louis, MO, USA): β-galactosidase (116 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and trypsin inhibitor (20.1 kDa).

Electrophoresis

The purified agarase was applied to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel by the method of Laemmli. After electrophoresis, zymogram analysis for agarase was performed as described by Ohta et al. (2004). SDS in the gel was removed by rinsing the gel three times with 20 mM Tris–HCl buffer (pH 8.0) for each 10 min. Thereafter, the gel was overlaid onto a plate sheet containing 2% agar and incubated at 37°C for 3 h. Finally, the gel was stained by Lugol's iodine solution (5% I_2 and 10% KI in distilled water). The molecular mass markers described above were used to determine the molecular mass. Protein bands were detected by staining the electrophoresed gel with Coomassie Brilliant Blue R-250.

N-terminal amino acid sequence

The purified agarase was transferred onto a polyvinylidene fluoride membrane using a Trans-Blot SD semi-dry transfer cell (Bio-Rad, Hercules, CA, USA) from the electrophoresed gel. The N-terminal amino acid sequence of the agarase was determined using an automated protein sequencer (Procise sequencer 492, Applied Biosystems).

Effects of pH and temperature on agarase stability and activity

The effect of pH on agarase activity was assayed by replacing Tris–HCl buffer (pH 8.0) with 10 mM of each buffer: acetic acid buffer (pH 4.0–6.0), phosphate buffer (pH 7.0 and 8.0), and glycine–NaOH buffer (pH 9.0–12.0) at 40°C. The effect of temperature on agarase activity was determined by carrying out the enzyme activity assay at each temperature (10–90°C) at pH 8.0. The pH stability of the agarase was determined by pre-incubating the enzyme solution at each pH (4.0–12.0) at 40°C for 1 h and then measuring the residual enzyme activity after adjusting the pH with Tris–HCl (0.1 M, pH 8.0). The thermal stability of the agarase was determined by pre-incubating the enzyme solution at each temperature (10–90°C) at pH 8.0 for 1 h and then measuring the residual enzyme activity. The relative activity was defined as the percentage of activity determined with respect to the maximum agarase activity.

Substrate specificity and cleavage pattern

Various polysaccharides including alginate, CM-cellulose, fucoidan, and κ- and λ-carrageenan (Sigma) were used instead of agarose in the enzyme activity assay to identify the substrate specificity of the enzyme. Neoagarooligosaccharides (Dextra, Reading, UK) including neoagarohexaose (NA6) and neoagarotetraose (NA4) were used instead of agarose to identify the cleavage pattern. Thin layer chromatography (TLC) was performed to determine the enzymatic products as described by Aoki et al. (1990).

Effects of various reagents on agarase activity

The effects of metal ions, chelators, denaturants, and reducing reagents on enzyme activity were determined by adding 100 μl of each metal ion and reagent, respectively, to the reaction mixture. The relative activity was defined as the percentage of activity determined with respect to that measured under the standard condition described previously.

Estimation of kinetic parameter

K_m and V_{max} values for the agarase acting on agarose (final concentration of 0.05–0.5 mg/ml, molecular mass of 10 kDa) and NA4 (final concentration of 0.04–0.4 mg/ml, molecular mass of 630 Da) were calculated by linear regression analysis of Lineweaver–Burke, double-reciprocal, plots of initial velocity data obtained under the condition described above. Values of k_{cat} (turnover number) and k_{cat}/K_m (catalytic efficiency) were calculated based on K_m, V_{max}, and $[E]$ (concentration of agarase) values.

Enzymatic product analysis

Agarose (200 mg) was suspended in 20 ml distilled water and heated in boiling water until it was completely dissolved, and then the solution was cooled down in a water bath at 40°C. The purified agarase (20 ml, 0.5 U/ml) was incubated with the agarose solution at 40°C with shaking at 120 rpm overnight. The hydrolyzate was centrifuged at 12,000×g for 15 min, and the supernatant was applied to ultrafiltration (10-kDa cutoff membrane, Millipore). The filtrate was lyophilized and collected as oligosaccharide products. Molecular mass distribution of the products was determined using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (QSTAR Pulsar I, Applied Biosystems). Carbon 13 nuclear magnetic resonance (^{13}C NMR) spectrum of the products was recorded on a Jeol 300M (Jeol, Tokyo, Japan) apparatus with 2,2-dimethyl-2-silapentane-5-sulfonate as the internal standard.

Nucleotide sequence accession number

The nucleotide sequence of 16S rRNA of the strain was submitted to GenBank nucleotide sequence database. The

sequence is available in the DDBJ/EMBL/GenBank nucleotide sequence database with the accession number of: EU084496.

Results

Identification of the bacteria

16S rRNA sequence (1,185 bases) of strain YKW-34 was determined and compared to the known 16S rRNA sequences in the NCBI database. The 16S rRNA gene sequence of strain YKW-34 showed highest similarity (97%) to that of *A. albus* MKT112. As reported by Embley and Stackebrandt (1994), the bacteria sharing with 97% similarity in 16S rRNA sequence were classified into the same species. Some phenotypic features of strain YKW-34 previously described by Yi and Shin (2006) were also agreed well to those of six strains belonging to *A. albus* (including strain MKT112) reported by Kurahashi and Yokota (2004). Based on these data, strain YKW-34 was identified as *A. albus*.

Purification of AgaA34

AgaA34 was purified from the cell-free culture supernatant of *A. albus* YKW-34 by DEAE Sepharose and Sephacryl S-100 chromotographies (Fig. 1). The purification procedure was summarized in Table 1. AgaA34 was purified by tenfold with a yield of 30%. The specific activity of the purified agarase was 25.54 U/mg. AgaA34 showed a single band in SDS-PAGE (Fig. 2) with molecular mass of 50 kDa, which coincided with the result estimated by Sephacryl S-100 gel filtration chromatography. A clear zone on agar sheet was formed by active staining due to the degradation of agar by AgaA34 (Fig. 2).

N-terminal sequence

The N-terminal sequence analysis of the first ten amino acid residues of AgaA34 revealed a sequence of ASLVTSFEEA. Similarity search using BLAST algorithm in the NCBI database showed a high match (90%) with a sequence of ATLVTSFEEA, which was the N-terminal sequence of four other agarases, i.e., agarases from *Agarivorans* sp. JA-1, *Agarivorans* sp. QM38, *Agarivorans* sp. JAMB-A11, and *Vibrio* sp. JT0107. These four agarases all belong to glycoside hydrolase family 50 (GH-50; http://www.cazy.org/). The GH-16 family agarases such as those from *Vibrio* sp. PO-303, *Pseudoalteromonas* sp. CY24, *Microbulbifer* sp. JAMB-A3, *Pseudomonas* sp. ND137, *Microbulbifer* sp. JAMB-A7 shared a similar N-terminal sequence of ADWDNIPIPA (70–100% homology). It is evident that the N-terminal sequences of agarases belonging to different GH families showed only 20% similarity, while those belonging to the same GH family possessed high similarity.

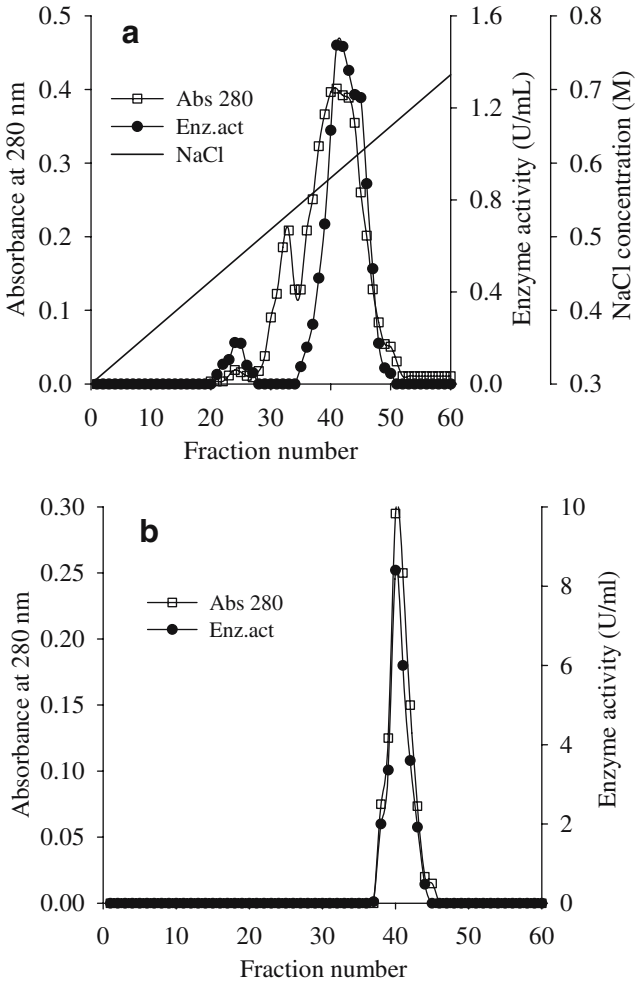

Fig. 1 Purification of the AgaA34 produced by *Agarivorans albus* YKW-34. **a** DEAE Sepharose FF column chromatography. **b** Sephacryl S-100 HR column chromatography

Table 1 Purification of AgaA34 from *Agarivorans albus* YKW-34

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold
Culture fluid	32.05	83.00	2.59	100	1
DEAE Sepharose	2.76	37.88	13.72	46	5
Sephacryl S-100 HR	0.99	25.28	25.54	30	10

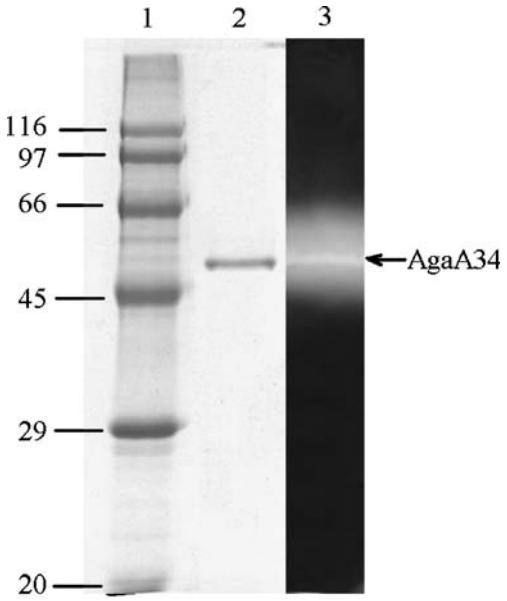

Fig. 2 SDS-PAGE analysis of AgaA34 produced by *Agarivorans albus* YKW-34. *Lane 1* molecular mass markers, *lane 2* purified AgaA34, *lane 3* zymogram for purified AgaA34

Effects of pH and temperature on activity and stability of AgaA34

The pH profile of AgaA34 is shown in Fig. 3a. The enzyme exhibited the maximum agarase activity at pH 8.0 and showed more than 80% of the maximum activity in a pH range of 6.0–9.0. The enzyme was most stable at pH 8.0 and retained more than 80% activity after incubation at a wide pH range of 6.0–11.0 for 1 h. The temperature profile of AgaA34 is shown in Fig. 3b. The optimal temperature for AgaA34 was 40°C. The enzyme exhibited 90% of the maximum activity at 30°C and lower than 80% of the maximum at temperature below 30°C or above 40°C. The agarase possessed 95% activity after incubation at 50°C for 1 h and inactivated as temperature increased. These results indicated that AgaA34 was thermostable up to 50°C.

Substrate specificity and cleavage pattern

Various polysaccharides from brown and red algae as well as neoagarooligosaccharides were used to determine the substrate specificity. AgaA34 could not hydrolyze alginate, CM-cellulose, fucoidan, and carrageenan (κ and λ) because reducing sugar was not detected in the reaction mixture after incubation for up to 2 h. It was confirmed that AgaA34 selectively hydrolyzed agarose. The result of TLC indicated that AgaA34 cleaved both NA6 and NA4 into NA2 (data not shown).

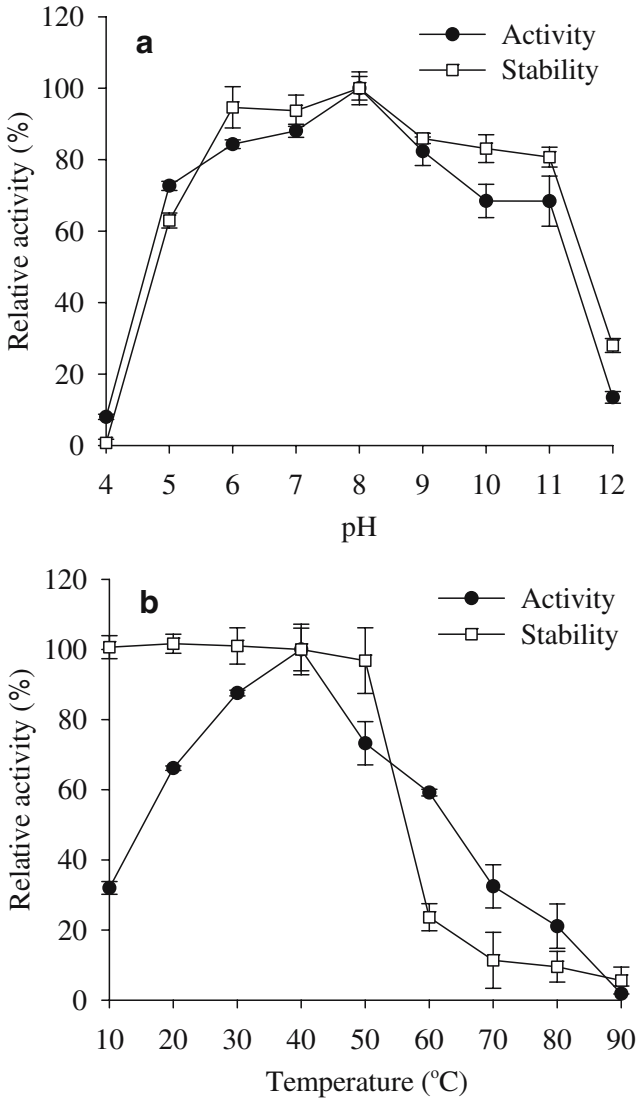

Fig. 3 Effects of temperature and pH on stability and activity of AgaA34. **a** pH profiles were checked at 40°C in different buffers (pH 4.0–12.0). **b** Temperature profiles were checked at different temperatures (10–90°C) in 0.1 M Tris–HCl (pH 8.0)

Effects of various reagents on the activity of AgaA34

Effects of metal ions and other organic reagents on the activity of AgaA34 were investigated (Table 2). No significant activation or inhibition of AgaA34 was observed by metal ions (Na^+, K^+, Mg^{2+}, and Ca^{2+}) and chelators (ethylenediaminetetraacetic acid, EDTA and ethyleneglycoltetraacetic acid, EGTA), as well as detergents (SDS and urea). The activity of AgaA34 was strongly inhibited by Cu^{2+}, Mn^{2+}, Fe^{3+}, or Al^{3+}. On the other hand, the reducing reagents (β-Me and DTT), even at a low concentration of 2 mM, were found to increase the activity of AgaA34 by more than 30%.

Table 2 Effects of various reagents on the activity of Aga34

Reagents	Concentration (mM)[a]	Relative activity (% ± S.D.)[b]
None		100±0.29
Na^+	100	100±0.59
K^+	100	100±0.24
Mg^{2+}	100	96±0.39
Ca^{2+}	100	97±0.24
Cu^{2+}	100	15±0.10
	20	20±0.34
Mn^{2+}	100	9±0.22
	20	13±0.13
Fe^{3+}	100	5±0.01
	20	8±0.21
Al^{3+}	100	11±0.23
	20	14±0.38
EDTA	10	100±0.22
EGTA	10	99±0.16
SDS	10	95±0.24
Urea	10	107±0.48
β-Me	10	145±0.15
	2	132±0.23
DTT	10	148±0.14
	2	130±0.20

[a] The concentration is the final concentration of the reagents in the enzyme activity assay system.
[b] The activity measured under the standard condition described in the text was defined as 100%.

Kinetic parameter

The Lineweaver–Burke plots of AgaA34 acting on agarose and NA4 are shown in Fig. 4. K_m, V_{max}, k_{cat}, and k_{cat}/K_m values for agarose were 1.17 mg/ml (1.17×10^{-4} M), 529 U/mg, 0.46 s^{-1}, and 4.0×10^3 s^{-1} M^{-1}, respectively, while those for NA4 were 0.21 mg/ml (3.33×10^{-4} M), 313 U/mg, 0.27 s^{-1}, and 8.1×10^2 s^{-1} M^{-1}, respectively. These results indicated that the DP value of the substrate affected the kinetic parameter of the enzyme. AgaA34 displayed lower K_m value and higher V_{max}, k_{cat}, and k_{cat}/K_m values toward polysaccharide (agarose) than those toward oligosaccharide (NA4). Thus, AgaA34 exhibited superior property toward the substrate of agarose.

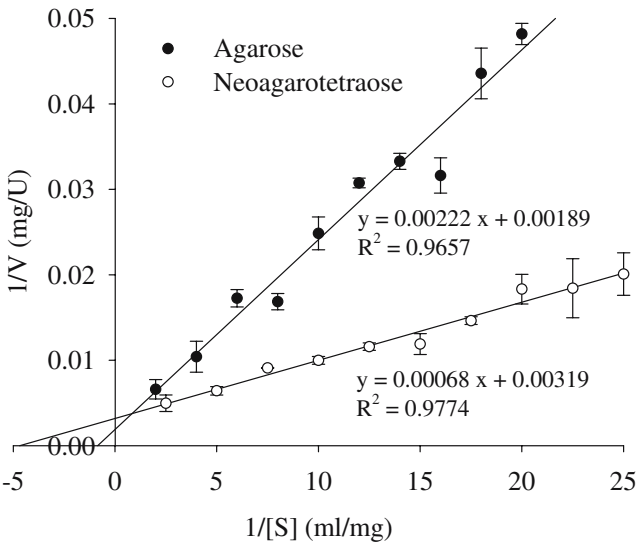

Fig. 4 Lineweaver–Burke plots to determine the kinetic parameters of AgaA34 acting on agarose and neoagarotetraose (NA4)

Enzymatic product

As shown in the MALDI-TOF mass spectrum (Fig. 5), the dominant peaks with molecular masses of around 347 and 676 Da corresponded most probably to molecules of $[M+Na]^+$and $[M+2Na]^+$, respectively. Thus, the enzymatic products of AgaA34 were a dimer (324 Da, $C_{12}H_{20}O_{10}$) composed of one β-D-galactopyranose residue (G-unit) and one 3,6-anhydro-α-L-galactopyranose residue (A-unit), and a tetramer (630 Da, $C_{24}H_{38}O_{19}$) composed of two G-units and two A-units. It could be seen that the dimer (75 mol%) was three times more than the tetramer (25 mol%) based on the relative peak intensities.

The structure information of the products was further obtained by ^{13}C NMR. A typical pattern for neoagarooligosaccharides was observed according to the NMR spectrum (Fig. 6). The resonances at 97.047 and 93.058 ppm were assigned to be β and α anomeric carbon of the G-unit at the reducing end of the neoagarooligosaccharides, respectively. No resonance was observed at around 90.7 ppm which is the characteristic signal of the A-unit at the reducing end of the agarooligosaccharides. These results confirmed that the dimer and tetramer produced by AgaA34 were NA2 and NA4, respectively, and AgaA34 was, hence, classified as a β-agarase.

Discussion

A. albus YKW-34 was formerly known as *Vibrio* sp. YKW-34 based on some phenotypic characterization (Yi and Shin 2006) according to Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). 16S rRNA sequencing revealed that the strain should be identified as *A. albus*, a kind of agar devouring bacterium, which was a novel genus of γ-*Proteobacteria* recently described by Kurahashi and Yokota (2004). A few bacteria belonging to this genus, such as *Agarivorans* sp. JAMB-A11 (Ohta et al. 2005a), JA-1 (Lee et al. 2006), and QM35 (Du et al. 2007), were found in recent years from marine sediment or seawater. Searching in UniProtKB/TrEMBL database (http://www.expasy.org/) indicated that four agarases from the strains of genus *Agarivorans* were found till now. Two of these

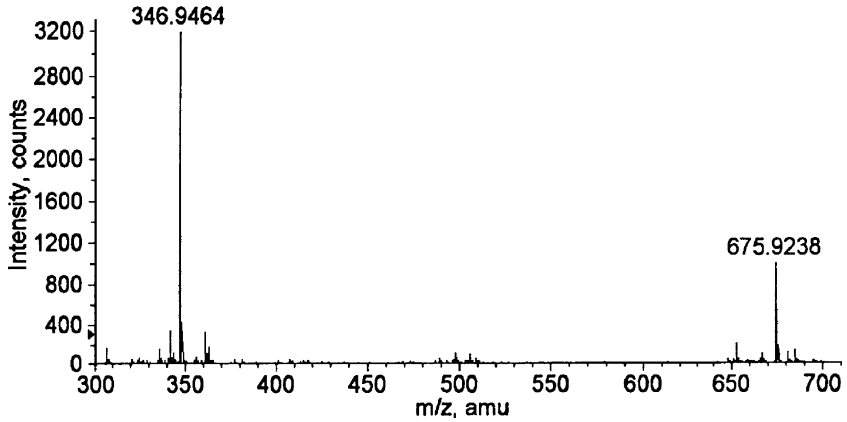

Fig. 5 MALDI-TOF mass spectrum of agarose hydrolysis fragments

agarases were described in literatures (Ohta et al. 2005a; Lee et al. 2006), but only one was purified (Ohta et al. 2005a). These four agarases in the database have molecular masses of 106–107 kDa. The molecular mass of 50 kDa of AgaA34 was novel and therefore distinguished it from the four agarases derived from this genus. Its molecular mass also differed from those of the agarases purified from strains of *Vibrio* genus (Aoki et al. 1990; Sugano et al. 1993; Araki et al. 1998).

β-Agarases are classified into three families of GH-16, GH-50 and GH-86 based on the amino acid sequence similarity (http://www.cazy.org/). Comparison of the N-terminal sequences in the NCBI database revealed that agarases in the same family possessed similar N-terminal sequence (70–100% homology) while the agarases from different families differed much in N-terminal sequence. Because the N-terminal sequence of AgaA34 exhibited high similarity with those of agarases from GH-50 (90% homology), this β-agarase was supposed to be a member of GH-50 family. The main product of NA2 by AgaA34 also supported this estimation because NA2-producing agarases are classified into GH family 50 (Ohta et al. 2005a; Lee et al. 2006). Further identification of AgaA34 based on the nucleotide sequence of its gene is underway.

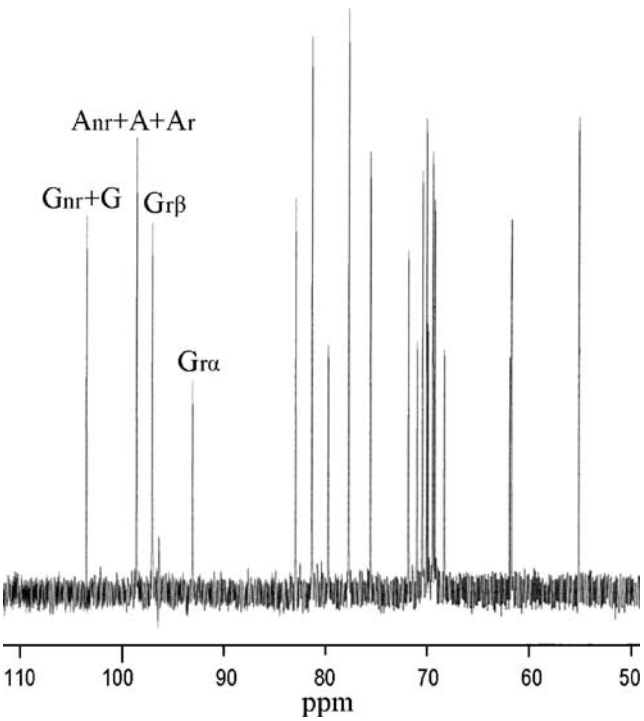

Fig. 6 ^{13}C NMR spectrum of the enzymatic products of agarose by AgaA34. *G*, β-D-galactopyranose, *A* 3,6-anhydro-α-L-aglactopyranose, *r* reducing end, *nr* non-reducing end, α α anomer, β β anomer

AgaA34 was purified to homogeneity by ion exchange and gel filtration chromatographies. The molecular mass estimated by SDS-PAGE and gel filtration was 50 kDa, indicating that the enzyme was a monomer. Its molecular mass was similar to that of *Alteromonas* sp. C-1 (52 kDa; Leon et al. 1992), but was different from other purified agarases (Wang et al. 2006). However, AgaA34 differed in several properties from the agarase of *Alteromonas* sp. C-1 which was rapidly inactivated at temperatures above 30°C (Leon et al. 1992).

AgaA34 exhibited maximum activity against agarose at 40°C. Temperature optima of various agarases (Kirimura et al. 1999; Suzuki et al. 2003; Ohta et al. 2005b) were higher than the gelling temperature of agarose (around 38°C; Armisén et al. 2000) because compact bundles of gelled agarose hindered enzyme action (Van der Meulen and Harder 1975). The thermal stability of AgaA34 was superior to those of other agarases to the best of our knowledge. AgaA34 was stable up to 50°C, whereas other agarases were only stable up to 40°C (Van der Meulen and Harder 1975; Kirimura et al. 1999; Ohta et al. 2005b) or 35°C (Suzuki et al. 2003). AgaA34 was stable in a wide pH range of 6.0–11.0, which was similar to the α-agarase from *Thalassomonas* sp. JAMB-A33 (Ohta et al. 2005b) but superior to other β-agarases such as *Bacillus* sp. MK03 of 7.1–8.2 (Suzuki et al. 2003), *Alteromonas* sp. E-1 of 7.0–

9.0 (Kirimura et al. 1999), and *Cytophaga flevensis* of 6.0–9.0 (Van der Meulen and Harder 1975). The superior thermal and pH stabilities of AgaA34 to other β-agarases will benefit its industrial application.

Metal ions found in seawater, such as Na^+, K^+, Ca^{2+}, and Mg^{2+}, activated some agarases (Groleau and Yaphe 1977; Wang et al. 2006; Lee et al. 2006), but these metal ions did not affect the activity of AgaA34. But AgaA34 was inhibited by other mental ions, such as Cu^{2+}, Mn^{2+}, Fe^{3+}, and Al^{3+}, which was the same to other agarases (Suzuki et al. 2003, Araki et al. 1998). These results indicated that AgaA34 was not a metal-ion-dependent enzyme. EDTA and EGTA were poor inhibitors for AgaA34 because the divalent metal ions were not essential for its activity. AgaA34 was resistant to SDS and urea, while the agarase from *Bacillus* sp. MK03 was completely inhibited by SDS (Suzuki et al. 2003). In addition to this unique property, the activity of AgaA34 was enhanced by 30% in the presence of β-Me and DTT, which indicated the possible existence of thiol in the catalytic site, as the reducing reagent could protect thiol from being oxidized to disulfide bound.

AgaA34 could not hydrolyze the tested polysaccharides except agarose. The enzymatic products from agarose identified by MALDI-TOF MS and NMR were NA2 (75 mol%) and NA4 (25 mol%). Thus, AgaA34 was a β-agarase acting by hydrolyzing the β-(1,4) glucosidic linkages between D-galactose and 3,6-anhydro-L-galactose. Many reported β-agarases produced NA4 as the dominant product, whereas a few β-agarases produced NA2 efficiently (Lee et al. 2006). Consisting of agarases belonging to GH-50 family (Ohta et al. 2005a; Lee et al. 2006), AgaA34 produced NA2 as the dominant product, which was an attractive oligosaccharide for cosmetic and medical industries.

In conclusion, AgaA34, a new β-agarase, was purified from *A. albus* YKW-34 which belonged to a recently described genus of *Agarivorans*. Neoagarobiose was its main product. The molecular mass and thermal stability distinguished it from other reported agarases. Furthermore, the excellent characteristics, such as thermal and pH stabilities and satisfactory kinetic property, enlightened its potential application in the production of neoagarobiose. To facilitate large-scale production of AgaA34, further studies on cloning and expression of this enzyme are underway.

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