Biotechnol Lett (2006) 28:1925–1932
DOI 10.1007/s10529-006-9171-y

ORIGINAL RESEARCH PAPER

Cloning, expression, and characterization of a glycoside hydrolase family 50 β-agarase from a marine *Agarivorans* isolate

Dong-Geun Lee · Geun-Tae Park · Nam Young Kim · Eo-Jin Lee · Min Kyung Jang · Young Gyun Shin · Gwang-Seok Park · Tae-Min Kim · Jae-Hwa Lee · Jung-Hyun Lee · Sang-Jin Kim · Sang-Hyeon Lee

Received: 9 June 2006 / Revised: 21 July 2006 / Accepted: 24 July 2006 / Published online: 23 September 2006
© Springer Science+Business Media B.V. 2006

Abstract The gene for a thermostable β-agarase from *Agarivorans* sp. JA-1 was cloned and sequenced. It comprised an open reading frame of 2,988 base pairs, which encode a protein of 109,450 daltons consisting of 995 amino acid residues. A comparison of the entire sequence showed that the enzyme has 98.8% sequence similarities to β-agarase from *Vibrio* sp. JT1070, indicating that it belongs to the family glycoside hydrolase (GH)-50. The gene corresponding to a mature protein of 976 amino acids was inserted and expressed in *Escherichia coli*. The recombinant β-agarase was purified to homogeneity. It had maximal activity at 40°C and pH 8.0 in the presence of 1 mM NaCl and 1 mM $CaCl_2$. The enzyme hydrolyzed agarose as well as neoagarohexaose and neoagarotetraose to yield neoagarobiose as the main product. Thus, the enzyme would be useful for the industrial production of neoagarobiose.

Keywords β-agarase · *Agarivorans* · Cloning · Expression

D.-G. Lee · N. Y. Kim · E.-J. Lee · M. K. Jang · Y. G. Shin · G.-S. Park · T.-M. Kim · J.-H. Lee · S.-H. Lee (✉)
Department of Pharmaceutical Engineering, College of Medical Life Sciences, Silla University, Busan 617-736, Korea
e-mail: slee@silla.ac.kr

G.-T. Park
Research and University-Industry Cooperation, Pusan National University, Busan 609-735, Korea

J.-H. Lee · S.-J. Kim
Microbiology Laboratory, Korea Ocean Research and Development Institute, PO box 29, Ansan 425-600, Korea

Introduction

Agar is found in the cell walls of some red algae and is composed of agarose and agaropectin. Agarose consists of a linear chain of alternating residues of 3-*O*-linked β-D-galactopyranose and 4-*O*-linked 3,6-anhydro-β-galactose (Duckworth and Yaphe 1971). Agarases are classified into two groups based on their mode of action; namely, α-agarase and β-agarase, which hydrolyze α-1,3 linkages and β-1,4 linkages in agarose, respectively. Agaro-oligosaccharides, which have 3,6-anhydro-α-L-galactose residues at their reducing ends, are produced from agarose; both by α-agarase and acids (Araki 1959). In contrast, neoagaro-oligosaccharides, which have D-galactose residues at their reducing ends, are produced only by enzymatic depolymerization of agarose by β-agarase (Araki 1959).

Because neoagaro-oligosaccharides exhibit many functions which have applications for the food, cosmetic and medical industries, they have

attracted increasing interest. Neoagaro-oligosaccharides slow the rate of degradation of starch and inhibit the growth of bacteria (Kohno et al. 1990; Kono and Hidaka 1989). The polysaccharides fractions prepared from marine algae by β-agarase also display macrophage-stimulating activity (Yoshizawa et al. 1995). Furthermore, neoagarobiose has both a moisturizing effect on skin and a whitening effect on melanoma cells (Kobayashi et al. 1997). For the production of neoagaro-oligosacchrides, several β-agarases have been purified and characterized from *Vibrio* sp. AP-2 (Aoki et al. 1990); *Pseudomonas* sp. W7 (Ha et al. 1997); *Pseudoalteromonas gracilis* B9 (Schroeder et al. 2003); *Bacillus cereus* ASK202 (Kim et al. 1999); *Zobellia galactanivorans* Dsij (Allouch et al. 2003); and *Microbulbifer* sp. JAMB-A94 (Ohta et al. 2004). Based on the amino acid sequence similarity, β-agarases are classified into three families of glycoside hydrolase (GH)-16, GH-50 and GH-86 (Ohta et al. 2004). β-Agarase A and B from *Zobellia galactanivorans* Dsij, which belongs to the family GH-16, degrade agarose and agarose oligosaccharides comprised of at least six sugars to yield neoagarotetraose as a main product (Allouch et al. 2003). β-Agarase (AgaA) from *Vibrio* sp. JT0107, which belongs to the family GH-50, degrades not only agarose but also agarose oligosaccharides which are comprised of at least four sugars to yield neoagarobiose (Sugano et al. 1993). β-Agarase I from *Pseudoalteromonas atlantica* T6c, which belongs to the family GH-86, is an agarase that diffuses around colonies on solid agar and confers a survival advantage compared with an agarase-deficient mutant (Belas et al. 1988). Among the β-agarases of the GH families, AgaA of family GH-50 is the only one to produce neoagarobiose, which possesses a whitening effect and can be applied for the cosmetic industry (Kobayashi et al. 1997), as its major product.

We have analyzed the molecular cloning, sequencing, and expression of the encoding gene of β-agarase, which belongs to the family GH-50, from *Agarivorans* JA-1. We also analyzed the enzymatic properties of the recombinant enzyme expressed in *Escherichia coli* cells.

Materials and methods

Bacterial strains and culture conditions

Agarivorans sp. JA-1 was originally isolated from the sea at the northeast coast of Cheju Island, Korea. Sea water was spread on Marine agar 2216 (Difco, Detroit, USA), and incubated at 25°C for 36 h. Bacteria exhibiting agarolytic activities were then selected. *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 *Escherichia coli* BL21(DE3) (*leuA8 metB5 hsrM1*) was used as the host for expression of β-agarase. *E. coli* cells were routinely grown at 37°C in Luria–Bertani (LB) broth (Difco), supplemented with 100 μg ampicillin/ml when required.

Molecular cloning and DNA sequencing of the β-agarase gene

The methods used for molecular cloning were based on those of Sambrook et al. (1989). Genomic DNA of *Agarivorans* sp. JA-1 was isolated using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Plasmid DNA was isolated by the alkaline lysis method (Sambrook et al. 1989). The β-agarase gene of *Agarivorans* sp. JA-1 was amplified using PCR primers, which were designed based on the β-agarase A gene of *Vibrio* sp. JT0107 (Sugano et al. 1993), in addition to the genomic DNA of *Agarivorans* sp. JA-1 as a template with Pyrobest DNA polymerase (Takara Bio Inc., Otsu, Japan). The forward primer was A_sp_b-agaE1-F (5′-CATATGGCTGCTACCTTAG TCACCTC-3′) incorporating a *Nde*I restriction site (underline) into the 5′-end. The reverse primer was A_sp_b-agaE1-R (5′-CTCGAGCACTTTACGACGTCTTAG-3′) having a *Xho*I restriction sequence (underline) into the 5′-end. Amplified DNA was ligated to pGEM-T Easy vector (Promega), resulting in pGEMTe-A_sp_b-agaE11. DNA sequencing was carried out by BioNex inc. (Seoul, Korea). Sequence analysis was carried out using the DS_Gene ver. 1.5 program (Accelrys Inc., San Diego, CA, USA).

Cloning and sequencing of the complete β-agarase gene

The complete gene and its regions of the β-agarase gene were cloned using a LA PCR *in vitro* Cloning kit (Takara Bio Inc.), according to the manufacturer's instructions. To determine the upstream and downstream regions of the β-agarase gene, two primers each for the upstream region, A_sp_b-aga51-R (5′-GGAACTCGTTGC CGCCATTT-3′) and A_sp_b-aga52-R (5′-CCATTGGTTTGAAA GCCGC-3′), and two primers for the downstream region, A_sp_b-aga31-F (5′-ACCACCAACAGG TGGCGTAAC-3′) and A_sp_b-aga32-F (5′-TG GCGGTGGCGGTAGCGCAGG-3′), were synthesized based on the cloned mature β-agarase gene. The genomic DNA of *Agarivorans* sp. JA-1 was digested with *Sau*3AI and ligated to the *Sau*3AI cassette of the kit. The upstream or downstream region of the β-agarase gene was amplified by PCR using the *Sau*3AI cassette-ligated genomic DNA as a template with primers C1 in the kit and A_sp_b-aga51-R or A_sp_b-aga31-F, respectively. The PCR products were used for the second round of PCR with primers C2 in the kit and A_sp_b-aga52-R or A_sp_b-aga32-F for the upstream or downstream region, respectively. A 0.6-kb DNA fragment for the upstream region and a 0.3-kb DNA fragment for the download region were ligated into pGEM-T easy vector (Promega) and then sequenced.

Expression and purification of recombinant β-agarase

pGEMTe-A_sp_b-agaE11 carrying the β-agarase gene was digested with *Nde*I and *Xho*I. Also, a 2.9-kb DNA fragment was ligated to corresponding sites of an *E. coli* expression vector, pTXB1 (New England Bio-labs Inc., Beverly, MA, USA). The recombinant plasmid was introduced into *E. coli* DH5α cells. *E. coli* DH5α cells harboring the recombinant plasmid were grown overnight, collected by centrifugation at 5,000 × *g* for 5 min, and subjected for plasmid preparation. Integrity of the recombinant plasmid was confirmed by restriction digestion using *Nde*I and *Xho*I and designated pTXB1-A_sp_b-agaE11. *E. coli* BL21(DE3) cells were transformed by β-agarase expression plasmid, pTXB1-A_sp-b-agaE11 and grown in 1 l of LB broth supplemented with 100 μg ampicillin/ml at 37°C for 3 h. IPTG (final concentration: 0.3 mM) was added into the medium in order to induce the T7 promoter. Three h after IPTG induction, the cells were collected by centrifugation at 5,000 × *g* for 5 min, and suspended in 30 ml of ice-cold column buffer [20 mM Tris/HCl (pH 7.4), 0.5 M NaCl, 0.2% Triton X-100, 2 mM EDTA]. After cell disruption by sonication, the sample was centrifuged at 20,000× *g* for 20 min, and the supernatant was put on a chitin bead column (20 ml of set volumes) (New England Biolabs Inc.) equilibrated with column buffer. The column was washed with the same buffer, and then equilibrated with a 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 amount of protein was measured using BCA protein assay reagent (Pierce Biotechnology, IL, USA), utilizing bovine serum albumin as the standard protein.

Enzyme assay

Agarase activity was determined by the enzymatic production of reducing sugars from agarose (Somogyi 1952). The enzyme was incubated in 50 mM TAPS (Sigma) (pH 8.0) buffer containing 1 mM NaCl, 1 mM $CaCl_2$, and 0.2% (w/v) molten agar at 40°C for 30 min. Enzyme reaction was ended by the addition of the Cu^{2+} reagent and used for the determination of reducing sugars. The mixture was boiled for 10 min and cooled, with arsenomolybdate reagent added afterwards. The amount of reducing sugar liberated was measured using D-galactose as a standard. One unit of the enzyme activity was defined as the amount of protein that produces 1 μmol of reducing sugar per min under these assay conditions.

SDS-PAGE

SDS-PAGE was performed by the Laemmli method with an 11% (w/v) polyacrylamide gel. The enzyme solution was mixed with the sample buffer and boiled for 5 min before being placed on the gel. The gels were stained for protein with

GelCode Blue Stain Reagent (Pierce, Rockford, IL, USA).

Analysis of effects of temperature and pH on enzyme activity

The optimal temperature of β-agarase was examined in the buffer used in the standard assay condition at various temperatures. The optimal pH of β-agarase was determined in various buffers. The buffers used were 50 mM sodium acetate buffers, pH 3.5–5.0; 50 mM sodium phosphate buffers, pH 5.0–8.0; 50 mM TAPS buffers, pH 8.0–10.0.

Chromatographic analysis of the products of hydrolysis of agar and neoagaro-oligosaccharide substrates

Hydrolyzed products of agarose, neoagarohexaose and neoagarotetraose by β-agarase were identified using thin-layer chromatography (TLC). Enzymatic hydrolysis of agarose (USB Inc., Cleveland, OH, USA), neoagarohexaose (Sigma), or neoagarotetraose (V-Labs Inc., St. Covington, LA, USA) were carried out at 40°C in 50 mM TAPS (pH 8.0) buffer containing 1 mM NaCl, 1 mM $CaCl_2$, and 1.0% (w/v) of each substrate. The agarose had molten due to heating at 95°C and was used as molten substrate at 40°C. The reaction mixtures were applied to silica gel 60 TLC plates (Merck, Darmstadt, Germany) (Duckworth and Yaphe 1970; Groleau and Yaphe 1977). The plates were developed using a solvent system composed of n-butanol/acetic acid/H_2O (2:1:1, by vol.). The spots were visualized by spraying with 10% (v/v) H_2SO_4 and heating (80°C). D-Galactose (Sigma), neoagarotetraose, and neoagarohexaose were used as standards.

Results

Taxonomic analysis of the isolate

To determine the phylogenetic position of the agar-degrading bacterium strain JA-1, its 16S rRNA sequence was determined and analyzed using comparative sequence analysis against known 16S rRNA sequences. The 16S rRNA sequence of JA-1 was 98% identical to *Agarivorans albus* (Kurahashi and Yokota 2004) (data not shown). The G + C content of the 16S rRNA sequence of JA-1 agreed well to those of *A. albus* strains (49–50 mol%) (data not shown).

Cloning of the β-agarase gene from *Agarivorans* sp. JA-1

The β-agarase gene from *Agarivorans* sp. JA-1 was cloned and sequenced as described in Materials and methods. The gene is comprised of 2,988 bp with a G + C content of 45.9%. The gene begins with ATG and ends with TAA (Fig. 1). The gene encodes a protein of 995 amino acids with a molecular mass of 109,450 daltons (Da) (Fig. 1). The amino acid sequence was 98.8% identical to the *agaA* gene from *Vibrio* sp. JT0107 (data not shown) (Sugano et al. 1993). This result indicates that β-agarase from *Agarivorans* sp. JA-1 should be classified as part of the GH family-50, which can only produce neoagarobiose (Sugano et al. 1993).

Expression and purification of recombinant β-agarase

Production of recombinant β-agarase was examined using *E. coli* BL21(DE3) as a host and pTXB1 as a vector. *E. coli* BL21(DE3) cells harboring pTXB1-A_sp-b-agaE11 produced a high amount of β-agarase (Fig. 2). The recombinant β-agarase was purified 88-fold after affinity chromatography, with a specific activity of 167 U/mg and a final yield of 41.8% (Table 1). The SDS-PAGE of the purified enzyme exhibited a single band with an apparent molecular mass of 109 kDa (Fig. 2). This value agreed with that estimated from the DNA sequence.

Effects of temperature and pH on enzyme activity and stability

The optimal temperature for activity of β-agarase was 40°C (Fig. 3A). Enzyme activity was more than 70% at 60°C and more than 50% at 70°C, compared with the enzyme activity (100%) at 40°C. The optimal pH for activity of β-agarase was approximately 8.0 (Fig. 3B).

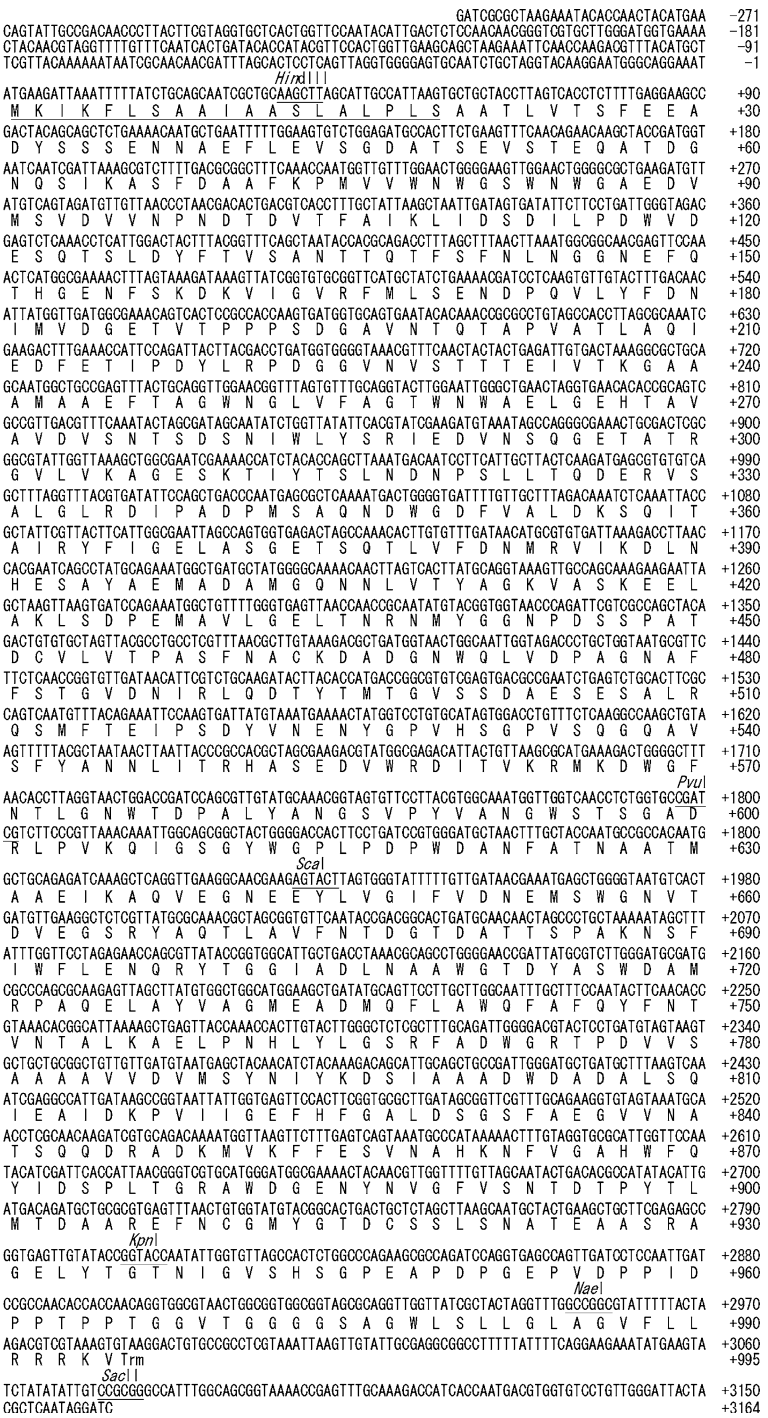

Fig. 1 Nucleotide sequence of the β-agarase of *Agarivorans* sp. JA-1 and deduced amino acid sequence of the enzyme. The nucleotide sequence of the β-agarase gene and its flanking regions are shown. The deduced amino acid sequence of the gene product is indicated by the single-letter codes under the nucleotide sequence. The putative signal peptide sequence is underlined. Some unique restriction sites are shown

Chromatographic analysis of the products of hydrolysis

The products of the enzyme reactions in the course of time were analyzed by TLC (Fig. 4A) and quantified by NIH image software. In the initial stage, the enzyme hydrolyzed agarose to generate many oligosaccharides with various degrees of polymerization. After 1 h of incubation, the main products were neoagarotetraose (45%

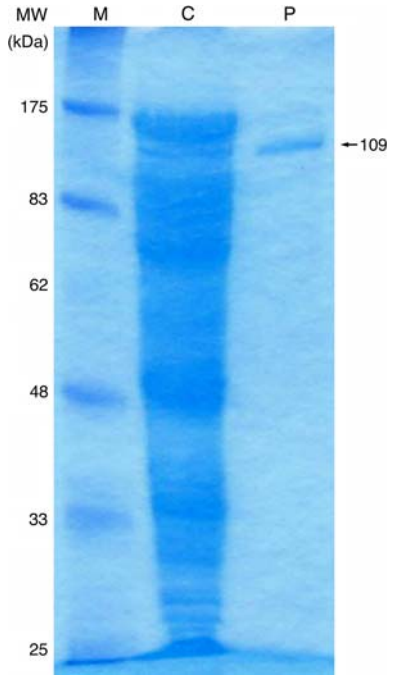

Fig. 2 SDS-PAGE of β-agarase from *E. coli* cells harbouring pTXB1-A_sp-b-agaE11. Lane M, size marker, lane C, cell-free extract, lane P, purified enzyme by affinity chromatography. The arrow indicates the position of β-agarase

total products) and neoagarobiose (55% total products). These results suggest that the enzyme is an endo-type β-agarase. With the passage of time (2–12 h), the amount of the tetramer corresponding to neoagarotetraose decreased (53–45% of total products), while the amount of the dimer corresponding to neoagarobiose increased (47–55% of total products). After 24 h of incubation, the main product was neoagarobiose (58%) with lesser amounts of neoagarotetraose (42%). The enzyme degraded neoagarohexaose and neoagarotetraose to generate neoagarobiose as the main product (Fig. 4B). These results indicate that the enzyme hydrolyzes β-1,4 linkages in agarose.

Discussion

We have cloned and sequenced the novel β-agarase gene from the marine bacterium *Agarivorans* sp. JA-1, isolated from the sea at the northeast coast of Cheju Island, Korea. The recombinant β-agarase was produced using pTXB1 as a vector and *E. coli* BL21(DE3) as a host. The amino acid sequence of the enzyme showed high homology (98.8%) with that of β-agarase (AgaA) from *Vibrio* sp. JT0107, which belongs to the family GH-50 (Sugano et al. 1993). The final main product of agarose hydrolysis by the enzyme is neoagarobiose. Many agarases reported to date produce neoagarotetraose as the predominant product of agarose hydrolysis (Allouch et al. 2003; Ohta et al. 2004; Schroeder et al. 2003). However, there are few reports about agarases, which produce neoagarobiose effectively. Neoagarobiose should be an attractive material for functional cosmetics, because it has both moisturizing and whitening effects on skin (Kobayashi et al. 1997). Therefore, β-agarase, which was analyzed here, has industrial applications in terms of selective neoagarobiose production in the cosmetic and medical industries.

The recombinant β-agarase had a molecular mass of 109 kDa and a specific activity of 167 U/mg. Maximal activity of the enzyme was observed at 40°C and pH 8.0. The enzyme is an endo-type β-agarase, and the final main product is neoagarobiose.

Recently, the three-dimensional structures of the two agarases from the family GH-16 were reported (Allouch et al. 2003). To date, there is no information available on the three-dimensional structure of any other agarases. To understand the mechanism of catalysis of the family GH-50 agarase, determination of the three-dimensional structures of this enzyme is required. Also, the structures should be compared with the two recently reported the family GH-16 β-agarases.

Table 1 Purification of β-agarase from *E. coli* cells harbouring pTXB1-A_sp-b-agaE11

Purification step	Total protein (mg/l)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Cell-free extract	292	560	1.9	100
Affinity chromatography	1.4	234	167	42

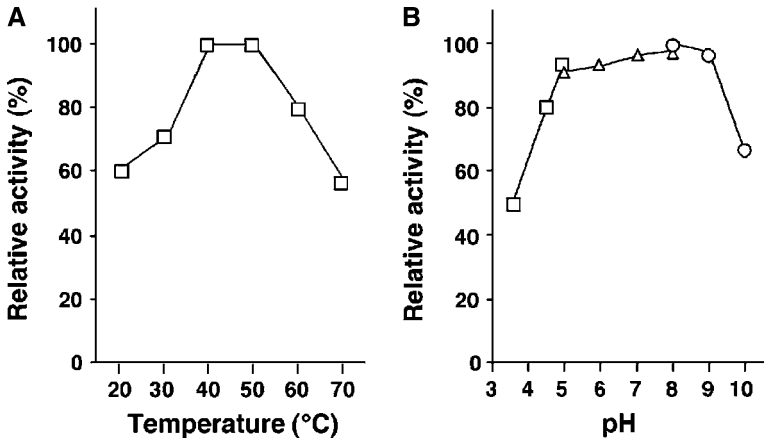

Fig. 3 Effects of temperature and pH on activity of recombinant β-agarase. (**A**) Temperature dependence of the enzyme activity. The values on the ordinate are shown as percentages of the enzyme activity (100%) observed at 40°C (**B**) pH dependence of the enzyme activity. The buffers used were sodium acetate buffers (open rectangles, pH 3.5–5.0), sodium phosphate buffers (open triangles, pH 5.0–8.0) and TAPS buffers (open circles, pH 8.0–10.0). The values on the ordinate are shown as percentages of the enzyme activity (100%) observed at pH 8.0

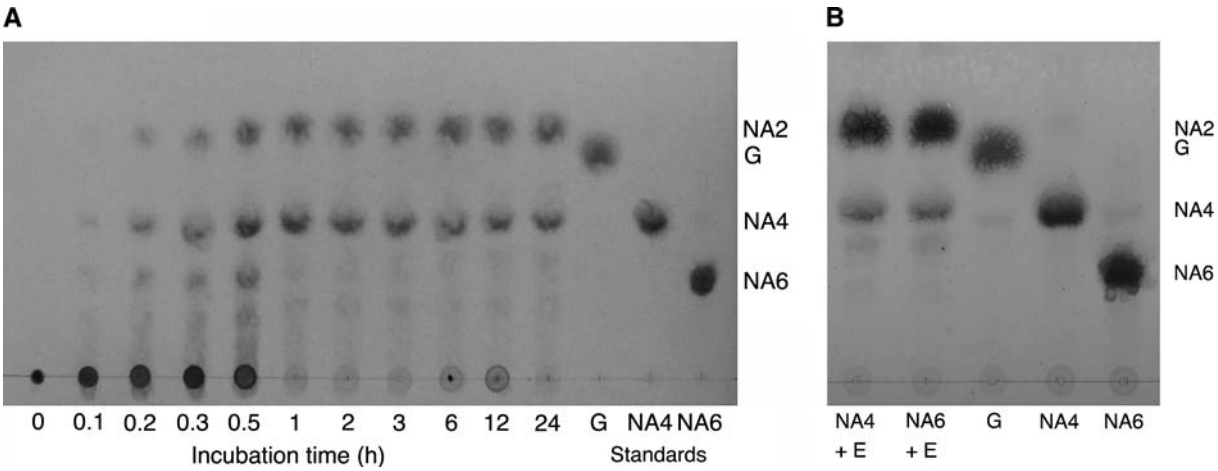

Fig. 4 TLC of the products of agarose hydrolysis by recombinant β-agarase. (**A**) The reactions were carried out at 40°C in 50 mM TAPS (pH 8.0) buffer containing 1 mM NaCl, 1 mM $CaCl_2$ and 1% agarose with 0.4 U/ml enzyme for indicated times. The reaction mixtures were developed by TLC. (**B**) The reaction was carried out at 40°C in 50 mM TAPS (pH 8.0) buffer containing 1 mM NaCl, 1 mM $CaCl_2$ and 1% neoagarotetraose (NA4) or neoagarohexaose (NA6) with 0.4 U/ml enzyme (+E) for 24 h. The reaction mixtures were developed by TLC. G, D-galatose; NA2, neoagarobiose; NA4, neoagarotetraose; NA6, neoagarohexaose

Acknowledgement This work was supported by the Marine and Extreme Genome Research Center Program, Ministry of Maritime Affairs & Fisheries, Republic of Korea.

References

Allouch J, Jam M, Helbert W, Barbeyron T, Kloareg B, Henrissat B, Czjzek M (2003) The three-dimensional structures of two β-agarases. J Biol Chem 278:47171–47180

Aoki T, Araki T, Kitamikado M (1990) Purification and characterization of a novel β-agarase from *Vibrio* sp. AP-2. Eur J Biochem 187:461–465

Araki C (1959) Seaweed polysaccharides. In: Wolfrom ML (ed) Carbohydrate chemistry of substances of biological interest. Pergamon Press, London pp 15–30

Belas R, Bartlett D, Michael S (1988) Cloning and gene replacement mutagenesis of a *Pseudomonas atlantica* agarase gene. Appl Environ Microbiol 54:30–37

Duckworth M, Yaphe W (1970) Thin-layer chromatographic analysis of enzymic hydrolysate of agar. J Chromatogr 49:482–487

Duckworth M, Yaphe W (1971) Structure of agar. I. Fractionation of a complex mixture of polysaccharides. Carbohydr Res 16:189–197

Groleau D, Yaphe W (1977) Enzymatic hydrolysis of agar: purification and characterization of β-neoagarotetraose hydrolase from *Peudomonas atlantica*. Can J Microbiol 23:672–679

Ha JC, Kim GT, Kim SK, Oh TK, Yu JH, Kong IS (1997) Beta-Agarase from *Pseudomonas* sp. W7: purification of the recombinant enzyme from *Escherichia coli* and the effects of salt on its activity. Biotechnol Appl Biochem 26:1–6

Kim BJ, Kim HJ, Ha SD, Hwang SH, Byun DS, Lee TH, Kong JY (1999) Purification and characterization of β-agarase from marine bacterium *Bacillus cereus* ASK202. Biotechnol Lett 21:1011–1105

Kobayashi R, Takisada M, Suzuki T, Kirimura K, Usami S (1997) Neoagarobiose as a novel moisturizer with whitening effect. Biosci Biotechnol Biochem 61:62–63

Kohno T, Kitagawa H, Hiraga T (1990) Production of hetero-oligosaccharides. In: Gijutsu Kenkyu Kukami (eds) Shokuhin sangyo bioreactor system, Jissen bioreactor, Shokuhin Kagaku Shimbunsa, Tokyo, Japan, pp 87–105

Kono T, Hidaka H (1989) Properties and production of neoagarooligosaccharide. Nippon Nogeikagaku Kaishi 63:1126–1129

Kurahashi M, Yokota A (2004) *Agarivorans albus* gen. nov., sp. nov., a gamma-proteobacterium isolated from marine animals. Int J Syst Evol Microbiol 54:693–697

Ohta Y, Nogi Y, Miyazaki M, Li Z, Hatada Y, Ito S, Horikoshi K (2004) Enzymatic properties and nucleotide and amino acid sequences of a thermostable beta-agarase from the novel marine isolate, JAMB-A94. Biosci Biotechnol Biochem 68:1073–1081

Sambrook E, Fritsch F, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Labarotory, Cold Spring Harbor, New York

Schroeder DC, Jaffer MA, Coyne VE (2003) Investigation of the role of a beta(1-4) agarase produced by *Pseudoalteromonas gracilis* B9 in eliciting disease symptoms in the red alga *Gracilaria gracilis*. Microbiology 149:2919–2929

Somogyi M (1952) Notes on sugar determination. J Biol Chem 195:19–23

Sugano Y, Matsumoto T, Kodama H, Noma M (1993) Cloning and sequencing of *agaA*, a unique agarase 0107 gene from a marine bacterium, *Vibrio* sp. strain JT0107. Appl Environ Microbiol 59:3750–3756

Yoshizawa Y, Ametani A, Tsunehiro J, Nomura K, Itoh M, Fukui F, Kaminogawa S (1995) Macrophage stimulation activity of the polysaccharide fraction from a marine alga (*Porphyra yezoensis*): structure–function relationships and improved solubility. Biosci Biotechnol Biochem 59:1933–1937