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Overexpression and characterization of a thermostable β-agarase producing neoagarotetraose from a marine isolate *Microbulbifer* sp. AG1

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Abstract

An agarase gene containing 1 302 bp was cloned from *Microbulbifer* sp. AG1. It encoded a mature protein of 413 amino acids plus a 20-residue signal peptide. The recombinant enzyme without the signal peptide was expressed and purified from *Escherichia coli* BL21 (DE3). When agarose was used as a substrate, the optimal temperature and pH for the enzyme were 60°C and 7.5, respectively. The recombinant agarase showed excellent thermostability with 67% and 19% of residual activities after incubation at 50°C and 60°C for 1 h, respectively. Except SDS, the recombinant agarase had a relatively good resistance against the detected inhibitors, detergents and urea denaturant. Thin layer chromatography analysis and enzyme assay using *p*-nitrophenyl-α/β-D-galactopyranoside revealed that the recombinant agarase was a β-agarase that degraded agarose into neoagarotetraose as the main end product. The enzymatic hydrolysis products with different degree of polymerization exhibited the antioxidant activities.

Key words: thermostable, β-agarase, neoagarotetraose, *Microbulbifer* sp.

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1 Introduction

Agar, the main cell wall component of red algae, is a kind of polysaccharide which consists of agarose and agaropectin (Duckworth and Yaphe, 1971). Agarose is a linear chain composed of alternating residues of 3-O-linked β-D-galactopyranose and 4-O-linked 3,6-anhydro-α-L-galactose (Duckworth and Yaphe, 1971; Hamer et al., 1977). Agaropectin has the same basic disaccharide-repeating units as agarose, while some hydroxyl groups of 3,6-anhydro-α-L-galactose residues are substituted by sulfoxy or methoxy and pyruvate residues (Duckworth and Yaphe, 1971).

Agarases are specific glycoside hydrolases that can break down agar into oligosaccharides. They were classified into two groups by their cleaving mechanisms: α-agarases (EC 3.2.1.158), which cleave α-1,3 linkages to produce agaro-oligosaccharides, and β-agarases (EC 3.2.1.81), which cleave β-1,4 linkages to produce neoagaro-oligosaccharides (Fu and Kim, 2010). Based on the amino acid sequence similarity, agarases are classified into different families of glycoside hydrolases (GHs): Families GH96 and GH117 for α-agarases, and Families GH16, GH50, GH86 and GH118 for β-agarases (Chi et al., 2012). Among them, GH16 is the largest family which includes many characterized β-agarases. For some of these agarases, the catalytic mechanisms are comprehensively studied (Allouch et al., 2004; Hehemann et al., 2010; Takagi et al., 2015).

Agarases can be used to prepared oligosaccharides which exhibit many biological activities such as prebiotic effect (Hu et al., 2006), whitening effect (Lee et al., 2008), moisturizing effect (Kobayashi et al., 1997), and antioxidative effect (Chen and Yan, 2005). They have prospective applications in the food, pharmaceutical and cosmetic industries. In addition to preparation of oligosaccharides, agarases are also utilized as tools for recovery of DNA from agarose gels (Yu et al., 2008), preparation of seaweed protoplasts (Araki et al., 1998), and extraction of biological substances (Kim et al., 2013; Yun et al., 2011). To date, a number of agarases have been identified from various microorganisms. Among them, many agarases have been cloned and characterized from bacterial species (Fu et al., 2009; Lin et al., 2012; Tawara et al., 2015; Xie et al., 2013). Because the gelling temperature of agar is 43–45°C, it is desirable that agarases have good thermal stability. Agarases with excellent thermostability have

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great potential in industrial applications. In the past several years, only a few thermostabe agarases have been characterized (Chi et al., 2014b; Cui et al., 2014; Hou et al., 2015; Li et al., 2014; Minegishi et al., 2013; Ohta et al., 2004a, b).

Marine bacterium *Microbulbifer* sp. AG1 was isolated from a mangrove soil sample in Xiamen, China. Cloning, expression, purification and biochemical characterization of a thermostable β-agarase from that bacterium was described in this paper.

2 Materials and methods

2.1 *Isolation and identification of agarase-producing bacterial strain*

Mangrove soil sample (approximately 30 g) collected from Xiamen in China was suspended with 50 mL buffer A (NaCl 30.0 g/L, KNO_3 5.0 g/L, $MgSO_4·7H_2O$ 5.0 g/L, $CaCl_2$ 0.2 g/L, K_2HPO_4 0.1 g/L, $FeSO_4·7H_2O$ 0.02 g/L, pH 7.5). The suspension was cultured on the selection solid medium (Buffer A containing 2% (w/v) agar) at 28°C for 48 h. Pits on agar plates were formed around the individual colonies of agarase-producing strains. The plates were dyed with Lugol's iodine solution to confirm the agar-degrading ability at room temperature (Temuujin et al., 2012). Among them, isolate AG1 had the highest ratio of clear halo around colony to colony size and was selected for further studies.

Genomic DNA of Strain AG1 was isolated using the bacterial genomic DNA isolation kit (BBI, Canada) according to the manufacturer's instructions. The 16S rRNA gene was amplified from this genomic DNA using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Moreno et al., 2002). The PCR product was sequenced. Homology was analyzed in GenBank using the BLAST program (Altschul et al., 1997).

2.2 *Cloning of the agarase gene*

The agarase gene was amplified from Strain AG1 genomic DNA with a pair of degenerate primers F1 (5′-ATGAKRAAAAYMACY-3′) and R1 (5′-TTWCAGYTKCWCAAMG-3′), which were designed on the basis of the consensus sequences of *Microbulbifer* agarase genes available in the GenBank database. The PCR product was cloned into pMD-18T vector (TaKaRa, Japan) and then confirmed by sequencing.

2.3 *Sequence and structure analysis*

The gene and amino acid sequence was analyzed using DNAMAN 5.1 software (Lynnon BioSoft, Canada). Homology search of the deduced amino acid sequence was performed using BLAST (Altschul et al., 1997) against GenBank database. Alignment of the protein sequences was generated with ClustalW program. A bootstrapped phylogenetic tree was built using the neighbor-joining method (Saitou and Nei, 1987) with MEGA 6.0 software (Tamura et al., 2013). The signal peptide sequence of the deduced protein sequence was predicted using SMART (Letunic et al., 2012). Domain search was performed using BLAST against NCBI's conserved domain database (Marchler-Bauer et al., 2015).

2.4 *Overexpression of the agarase gene*

To clone the agarase gene into pGEX-6p-1 expression vector (GE Healthcare Life Sciences, USA), the agarase gene without its signal peptide-coding sequence was amplified by PCR using the following primers F2 (5′-CGCGGATCCGCAGATTGGGATGGAGTT-3′) and R2 (5′-CCGCTCGAGCAGCTTCACAAAGCGGAT-3′) (Incorporated *Bam* HI and *Xho* I restriction sites are underlined). After the amplicon was digested with *Bam* HI and *Xho* I, it was inserted into the pGEX-6P-1 vector. The resulting construct was named pGEX-6p-aga. After confirmation by sequencing, the recombinant plasmid was transferred into *E. coli* BL21 (DE3). The transformants were grown in 300 mL of Luria-Bertani (LB) medium containing 100 μg/mL ampicillin at 37°C until the OD_{600} reached 0.8, and then the cells were induced with 0.075 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG) at 22°C for 16 h. The induced cells were harvested by centrifugation at 6 500× *g* for 10 min.

2.5 *Purification of recombinant agarase*

Purification of the glutathione-S-transferase (GST)-tagged agarase was conducted using glutathione sepharose 4B (GE Healthcare Life Sciences, USA) affinity chromatography at 4°C according to the manufacturer's instructions. The above cell pellet was resuspended in 15 mL of PBS buffer (140 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, 1.8 mmol/L KH_2PO_4, pH 7.3). The cells were disrupted by sonication on ice for 10 min with pulsed mode of 10 s on and 10 s off, and the frequency of sonication was 25 kHz. After centrifugation at 13 000× *g* for 20 min, the resins were added to the cleared supernatant. After shaking at 4°C for 30 min, the lysate-resins mixture was loaded in a column. The resins were washed with PBS buffer. The binding protein was eluted off the column with elution buffer (50 mmol/L Tris-HCl, 10 mmol/L reduced glutathione, 5 mmol/L DTT, pH 8.0). The eluted proteins were dialyzed in 50 mmol/L Tris-HCl buffer (pH 7.5) for 36 h. The protein concentration was determined by Bradford method (Bradford, 1976) with bovine serum albumin as the standard. The homogeneity of the purified enzyme and its molecular mass were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following the standard procedures (Laemmli, 1970).

2.6 *Enzyme activity assay*

Unless otherwise noted, the standard assay of agarase activity was determined by measuring the amount of released reducing sugar equivalent using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). The reaction was initiated by adding 20 μL of the purified enzyme solution (0.2 mg/mL) to 580 μL of 50 mmol/L Tris-HCl (pH 7.5) containing 0.5% (w/v) agarose (Amresco) and carried out at 60°C for 15 min. It was stopped by adding 900 μL of DNS reagent and heating at 100°C for 10 min. After cooling to room temperature, the release of reducing sugar was monitored at 540 nm using a Cary 50 spectrophotometer (Varian, USA). One unit of agarase activity was defined as the amount of enzyme that liberated 1 μmol of the reducing sugar (D-galactose equivalent) from agarose per minute under the assay conditions.

2.7 *Substrate specificity*

Substrate specificity of the recombinant agarase was measured by using the artificial chromogenic substrates, *p*-nitrophenyl-α-D-galactopyranoside and *p*-nitrophenyl-β-D-galactopyranoside. The assay was based on the method of Temuujin et al. (2012) with some minor modifications. The reaction was initiated by adding 200 μL of enzyme solution (0.2 mg/mL) to 500 μL of the corresponding substrate solution (2 mg/mL) and carried out at 60°C for 30 min. The reaction was stopped by adding 500 μL of 1 mol/L Na_2CO_3 stop solution. The activity was measured spectrophotometrically at 420 nm using a Cary 50 spectrophoto-

meter (Varian, USA) by determining the release of *p*-nitrophenol due to hydrolysis of the artificial chromogenic substrates.

2.8 ***Effect of temperature on agarase activity***

In order to investigate the optimum temperature of the enzyme, reactions were performed at different temperatures (30, 40, 50, 60, 70 and 80°C) in 50 mmol/L Tris-HCl (pH 7.5). The thermal stability of agarase was determined after incubating the enzyme at 30, 40, 50, 60 and 70°C over the period of 10–60 min, respectively. After the heat treatment, the samples were cooled on ice immediately. The residual enzyme activity was measured by the standard method as described above. The activity of the enzyme without the treatment was defined as 100%.

2.9 ***Effect of pH on agarase activity***

The optimum pH of agarase was examined by assaying enzyme activity at 60°C in the following buffers with different pH values: 50 mmol/L citrate phosphate buffer (pH 4.0–7.0), 50 mmol/L Tris-HCl buffer (pH 7.0–9.0), and 50 mmol/L glycine-NaOH buffer (pH 9.0–10.0). The pH stability assay of agarase was performed by measuring the residual activity after keeping the enzyme in buffers with different pH values (ranging from 4.0 to 10.0) at 25°C for 1 h. The activity of the enzyme without the treatment was defined as 100%.

2.10 ***Effects of metal ions on agarase activity***

The effects of metal ions on agarase activity were examined by using various metal salts (NaCl, KCl, LiCl, $CaCl_2$, $MgCl_2$, $BaCl_2$, $MnCl_2$, $NiCl_2$, $CoCl_2$, $CuCl_2$, $ZnCl_2$, $FeCl_2$, $AlCl_3$ and $FeCl_3$) at final concentrations of 1 mmol/L or 10 mmol/L. The enzyme was incubated with each metal ion at 25°C for 30 min, respectively. Residual activity was measured by the standard method as described above. Reaction mixture without metal ion was used as a reference.

2.11 ***Effects of inhibitor, detergent and denaturant on agarase activity***

The effects of inhibitor, detergent and denaturant on agarase activity were investigated by using various reagents, including ethylenediaminetetraacetic acid (EDTA), β-mercaptoethanol (β-ME), dithiothreitol (DTT), and phenylmethylsulfonyl fluoride (PMSF) at final concentrations of 1 mmol/L or 10 mmol/L; sodium dodecyl sulfate (SDS), Tween-20, Tween-80, Triton X-100, and 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (Chaps) at final concentrations of 0.1% (w/v or v/v) or 1% (w/v or v/v); urea at final concentration of 1 mol/L. The enzyme was incubated with each reagent at 25°C for 30 min, respectively. Residual activity was measured by the standard assay as described above. Reaction mixture without additives was used as a control.

2.12 ***Determination of enzyme kinetic parameters***

Enzyme activity was assayed with agarose concentration ranging from 0.5 to 8 mg/mL by the DNS method (Miller, 1959). Michaelis–Menten substrate affinity constant (K_m) and maximum velocity (V_{max}) values were calculated by linear regression analysis of Lineweaver-Burk double-reciprocal plot.

2.13 ***Preparation and analysis of agarase hydrolysis products***

The recombinant agarase (20 U) was added to 10 mL of 50 mmol/L Tris-HCl (pH 7.5) containing 0.5% (w/v) agarose, and the reaction was performed at 40°C for 96 h. Some of the reaction mixture was withdrawn at different incubation periods. The enzyme activity was stopped by heating the mixture in boiling water for 10 min and then cooling on ice for 5 min. After centrifugation at 12 000× *g* for 10 min at 4°C, the supernatant was harvested as the hydrolysis products. The samples were applied to a Silica Gel 60 thin-layer chromatography (TLC) plate (Puke, China), and developed using n-butanol-acetic acid-water solution (2:2:1, v/v/v) as a solvent system. The separated products were visualized by spraying with 10% (v/v) H_2SO_4 in ethanol and heated at 110°C for 10 min. Galactose and neoagaro-oligosaccharides (NA) (Shanghai ZZBIO Co., Ltd., China) were used as the standards. Neoagaro-oligosaccharides standards included neoagarotetraose (NA4), neoagarohexaose (NA6) and neoagarooctaose (NA8).

2.14 ***Antioxidant activity assay of the enzymatic hydrolysates***

2.14.1 *DPPH radical scavenging assay*

The samples of 40 min, 6 h and 96 h hydrolysis treatment were respectively applied on Millipore centrifugal filter 3 K devices (3 000 nominal molecular weight limit) (Millipore, USA). The filtrates were collected and further concentrated into powders by Labconco FreeZone 6 plus (ThermoFisher, USA), respectively. The prepared hydrolysis product powders were dissolved in distilled water yielding samples for the following antioxidant activity assays. The scavenging effect was determined by the half inhibitory concentration (IC50) value. The DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) radical scavenging activity assay was conducted according to the modified method of Zhu et al. (2011). One hundred microliters of the hydrolysis product was mixed with 100 μL of 0.12 mmol/L DPPH radical (ethanol as solvent). After the mixture was incubated in the dark for 30 min at room temperature, the absorbance of the resulting solution was measured at 517 nm. The DPPH radical scavenging capacity of the sample was calculated by the following formula:

$$R_s = (A_0 - A_1)/A_0 \times 100,$$

where R_s is scavenging rate (%), A_0 is the absorbance of the control without sample, and A_1 is the absorbance in the presence of the sample.

2.14.2 *ABTS radical cation scavenging assay*

The ABTS (2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical cation scavenging activity was assessed by the method according to a previously reported protocol (Zhou and Yu, 2004) with some modifications. A total of 7 mmol/L ABTS solution and 2.4 mmol/L potassium persulfate solution were mixed with equal volume, and then they were incubated at room temperature for 12–16 h in the dark to yield a dark colored solution containing ABTS radical cation. The working solution was prepared by diluting the above solution with distilled water until the absorbance was 0.700±0.02 at 734 nm. Five hundred microliters of the hydrolysis product was added into 1.5 mL of the working solution. After incubation at 37°C for 1 h, the absorbance of the solution was determined at 734 nm. The ABTS radical cation scavenging capacity of the sample was calculated by the formula above.

2.14.3 *Reducing power assay*

Reducing power of the hydrolysis products was assessed by the modified potassium ferricyanide reduction method of Ardestani and Yazdanparast (2007). Reaction solution consisted of 0.5 mL of 0.2 mol/L sodium phosphate buffer (pH 6.6), 0.5 mL of 1%

(w/v) potassium ferricyanide, and 0.2 mL of the hydrolysis product. The reaction was incubated at 50°C for 30 min and cooled at 4°C for 2 h. Then 0.5 mL of 10% (w/v) trichloroacetic acid was added to the reaction solution. After centrifugation at 7 000× *g* for 10 min, 0.5 mL of the supernatant was mixed with 0.5 mL of distilled water, and then 0.1 mL of 0.1% (w/v) ferric chloride was added to the mixture. The increased absorbance at 700 nm indicated the increased reducing power of the sample (Sahreen et al., 2010).

3 Results

3.1 *Identification of Strain AG1*

Thirteen agarase-producing strains were found to produce pits on the agar plates. After the iodine staining, the isolate AG1 had the high ratio of clear halo around colony to colony size (data not shown). The 16S rRNA gene sequence of Strain AG1 (GenBank accession number KU500639) showed 100% similarity with that of *Microbulbifer thermotolerans* Strain JAMB A94 (GenBank accession number NR_040989). Therefore, Strain AG1 was classified as genus *Microbulbifer*.

3.2 *Nucleotide and amino acid sequences analysis of agarase*

The agarase gene of 1 302 base pairs (GenBank accession number ALN70307) was amplified by PCR from genomic DNA of *Microbulbifer* sp. AG1. This DNA fragment contained an open reading frame encoding a protein of 433 amino acid residues of which the first 20 residues were predicted to be a signal peptide. The putative cleavage site of the signal peptide was located between Ala20 and Ala21. The theoretical molecular weight and pI value of the protein were 48.22 kDa and 5.42, respectively. The deduced protein sequence was compared with available agarase protein sequences from Non-redundant protein sequences database of NCBI. It shared 99% identity with an agarase (BAD29947) from *Microbulbifer thermotolerans*. Alignment of the deduced amino acid sequence of *Microbulbifer* sp. AG1 agarase with some agarase protein sequences suggested that Glu147 and Glu152 could be the putative catalytic residues (Fig. 1). *Microbulbifer* sp. AG1 agarase was predicted to contain a cellulose binding domain (CBD) Type IV located between 338 and 430 (Fig. 1). It was also predicted to be a β-agarase, member of glycoside hydrolase (GH) family 16, containing a carbohydrate binding module 6 (CBM6, 297–432).

In order to classify Strain AG1 agarase, a phylogenetic tree (Fig. 2) was constructed by using 25 agarolytic enzymes representing the previously classified six agarase families (Chi et al., 2012). It formed a distinct group with a β-agarase from *Microbulbifer thermotolerans* JAMB-A94 (BAK08910), which was the representative member of GH family 16. The result suggested that Strain AG1 agarase was closely related to GH family 16.

3.3 *Expression and purification of recombinant agarase*

The agarase gene was inserted into pGEX-6P-1 vector and expressed in *E. coli* as a GST-tagged fusion protein. The induced samples were analyzed on 12% SDS-PAGE and stained with Coomassie brilliant blue G250 (Fig. 3). A band with expected size of the fusion protein was observed in the induced *E. coli* containing pGEX-6p-aga (Fig. 3, Lane 4), whereas it was absent in the induced *E. coli* containing pGEX-6P-1 vector without DNA insert (Fig. 3, Lane 2). The fusion protein was purified using glutathione sepharose 4B affinity chromatography under the native conditions. A single band with the molecular mass between 70 and 85 kDa was detected on the SDS-PAGE (Fig. 3, Lane 5).

3.4 *Effect of temperature on agarase activity*

The optimal temperature of the recombinant agarase was determined at different temperatures. As shown in Fig. 4a, the enzyme exhibited the maximum activity at 60°C, and presented over 75% of relative activity in temperatures ranging from 40°C to 70°C. It had no activity at 80°C. The thermostability of agarase was investigated by measuring the residual activity after incubating the enzyme at 30°C, 40°C, 50°C, 60°C and 70°C for different times, respectively (Fig. 4b). The agarase was relatively stable at 30°C and 40°C. It retained 67% and 19% of residual activities after incubation at 50°C and 60°C for 1 h, respectively. The enzyme was not stable at 70°C. These results indicated that the recombinant agarase from *Microbulbifer* sp. AG1 had good thermal stability.

3.5 *Effect of pH on agarase activity*

The optimal pH of agarase activity was determined at various pH values at 60°C. As shown in Fig. 5a, the agarase had the optimal pH at 7.5, and displayed over 50% of its maximal activity in the pH range of 6.0–9.5. There was nearly no detectable enzyme activity at pH 4.0. The enzyme still had 22% of relative activity at pH 10.0. The pH stability of agarase was determined by pre-incubating the enzyme in a series of buffers with different pH values (4.0–10.0) for 1 h and then measuring the residual activity (Fig. 5b). The results showed that the recombinant agarase from *Microbulbifer* sp. AG1 exhibited good stability in a broad pH range of 6.0–9.0, maintaining more than 80% of its original activity. It could also retain 35% of residual activity at pH 4.0.

3.6 *Effects of metal ions on agarase activity*

The effects of metal ions on agarase activity were investigated by using various metal ions at final concentrations of 1 or 10 mmol/L (Table 1). The agarase was slightly inhibited by Ba^{2+}, and was obviously inhibited by Mn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Al^{3+} and Fe^{3+}. No effects of K^+ and Ca^{2+} were observed at both concentrations. Na^+, Li^+ and Mg^{2+} at 1 mmol/L had no influences on agarase activity, but showed negative effects at 10 mmol/L.

3.7 *Effects of inhibitor, detergent and denaturant on agarase activity*

The effects of various inhibitors on agarase activity were determined by using EDTA, β-ME, DTT and PMSF (Table 2). The enzyme activity was inhibited by EDTA at 1 mmol/L and 10 mmol/L. With β-ME and DTT, the enzyme activity was stimulated at both concentrations tested. PMSF at 1 mmol/L had no influence on agarase activity, whereas at 10 mmol/L, its effect became negative. The influences of some detergents on the agarase activity were determined by using SDS, Tween 20, Tween 80, Triton X-100 and Chaps (Table 2). The recombinant agarase exhibited a good tolerance towards Tween 20, Tween 80, Triton X-100 and Chaps. When it was treated with 1% SDS, 30% of residual activity was maintained. The denaturant urea showed inhibitory effect on the enzyme activity at 1 mol/L, retaining about 83% of residual activity (Table 2).

3.8 *Kinetic parameters of K_m and V_{max}*

The kinetic parameters of Strain AG1 recombinant agarase were determined using agarose as the substrate. The K_m and V_{max} values obtained by Lineweaver-Burk plot were 5.0 mg/mL and 84.7 U/mg, respectively.

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agarase (Microbulbifer sp. AG1)                    1  MRKITSILLTCVMGCTATYAADWDGVPVPANPGSGKTWELHP-LSDD
agarase (Microbulbifer thermotolerans)             1  MRKITSILLTCVMGCTATYAADWDGVPVPANPGSGKTWELHP-LSDD
agarase (Simiduia agarivorans)                     1  -MNKLPLALAAIALASQTHAADWDGIPVPADPGAGNKWELHP-LSDD
agarase (Gilvimarinus agarilyticus)                1  -MKHTISTLTALLLCSSSFAADWDGLPVPADAGSGNTWQLQSNVSDD
agarase (Saccharophagus degradans)                 1  -MKTTKCALAALFFSTPLMAADWDGIPVPADPGNGNTWELQS-LSDD
agarase (Gilvimarinus polysaccharolyticus)         1  -MKHSISTLAALLLSSSSLAADWDDLPVPANAGSGNTWQLQSNVSDD
agarase (Microbulbifer agarilyticus)               1  -MKTTSLTLAALALSSSALAADWDNIPVPADAGAGNTWELHS-LSDD

agarase (Microbulbifer sp. AG1)                   60  FYERWKEGFINPWTGPGLTEWHPHYSYVSGGKLAITSGRKPGTNQVY
agarase (Microbulbifer thermotolerans)            60  FYERWKEGFINPWTGPGLTEWHPHYSYVSGGKLAITSGRKPGTNQVY
agarase (Simiduia agarivorans)                    59  FFERWNEGFINPWLGPGLTEFTASQSQVANGTLQLKASRKAGTNKVL
agarase (Gilvimarinus agarilyticus)               60  FYDRWSEGFINAWQGPGLTDYHNPNSRVENGELVIQATRKPGTNQVY
agarase (Saccharophagus degradans)                59  FYSRWSEGFINAWLGPGQTEFYGPNASVEGGHLIIKATRKPGTTQIY
agarase (Gilvimarinus polysaccharolyticus)        60  FYDRWSEGFINAWQGPGLTDYHNPNSRVENGELVIQATRKPGTNQVY
agarase (Microbulbifer agarilyticus)              59  FFERWSEGFINPWLGPGETEYYAPNSYVEGGNLVIKASRKPGTIKVH

                                                                                ▼     ▼
agarase (Microbulbifer sp. AG1)                  120  VYMEARAKLSNMVLASDFWFLSADSTEEIDVIEAYGSDRPGQEWYAE
agarase (Microbulbifer thermotolerans)           120  VYMEARAKLSNMVLASDFWFLSADSTEEIDVIEAYGSDRPGQEWYAE
agarase (Simiduia agarivorans)                   119  LYMEARTKITNLTAANAFWLLSSDSTQEIDVQESYGSDRPDQVWFDE
agarase (Gilvimarinus agarilyticus)              120  VYIETSSKIMDQVLANAVWMLSSDSTEEIDIVEAYGSSRPDQTWFAE
agarase (Saccharophagus degradans)               119  LYLEARTKITNLTLANAFWLLSSDSTEEIDVLESYGSDRATETWFDE
agarase (Gilvimarinus polysaccharolyticus)       120  VYIETSSKIMDQVLANAVWMLSSDSTEEIDIVEAYGSSRPDQTWFAE
agarase (Microbulbifer agarilyticus)             119  LFMEARVKITNLTLANAFWLLSSDSTEEIDVLESYGSDRPSETWFDE

agarase (Microbulbifer sp. AG1)                  180  FQDYQPTDAGSWYADGKGTKWRDAFHRVGVYWRDPWHLEYYVDGKLV
agarase (Microbulbifer thermotolerans)           180  FQDYQPTDAGSWYADGKGTKWRDAFHRVGVYWRDPWHLEYYVDGKLV
agarase (Simiduia agarivorans)                   179  FQDYQPKDEGSWYKKQGQSTWRDAYHTIGVYWIDPWNLEYYVDGVHV
agarase (Gilvimarinus agarilyticus)              180  FQDYQPKDAGAWYADG--RLWRDQYSRVGVYWRDPWHLEYYIDGQLV
agarase (Saccharophagus degradans)               179  FQDYQPKDAGSWYPNPDGGTWRDQFFRIGVYWIDPWTLEYYVNGELV
agarase (Gilvimarinus polysaccharolyticus)       180  FQDYQPKDVGAWYADG--RLWRDQYSRVGVYWRDPWHLEYYIDGELV
agarase (Microbulbifer agarilyticus)             179  FQDYQPKDAGSWYPNPDGGHWRDQFFRIGVYWIDPWTLEYYVNGEHV

agarase (Microbulbifer sp. AG1)                  240  FTGGTGLSKPMYAIINMEDQNWRSDNG-ITPTDAELADPNRNTYYVD
agarase (Microbulbifer thermotolerans)           240  FTGGTGLSKPMYAIINMEDQNWRSDNG-ITPTDAELADPNRNTYYVD
agarase (Simiduia agarivorans)                   239  YTGGTGLSKPMQAIFDVEDQDWRSDNG-ITATDAELADPSKNTYFVD
agarase (Gilvimarinus agarilyticus)              238  YTNGNGLSKPMQIIVDAEDQDWRSDNG-IIATDADLADSSKNQFYVD
agarase (Saccharophagus degradans)               239  YTNGTGLSKPMQVIFDAEHQPWRDEQGTAPPTDAELADSSRNQFLID
agarase (Gilvimarinus polysaccharolyticus)       238  YTNGNGLSKPMQIIVDAEDQDWRSDNG-IVATDTELADNSKNQFYVD
agarase (Microbulbifer agarilyticus)             239  YTNGNGLSKPMQVIFDAEHQPWRDAQGTAPPTDEELADPSRNKFLVD

agarase (Microbulbifer sp. AG1)                  298  ---------------------NATTVELGNFHNTGKDGANVTGDTVL
agarase (Microbulbifer thermotolerans)           298  ---------------------NATTVELGNFHNTGKDGANVTGDTVL
agarase (Simiduia agarivorans)                   298  TTPPPANG-------------ETVVKEMADFTATGKTGAAVAGDTIT
agarase (Gilvimarinus agarilyticus)              297  GGDNGGDNGGDNGGDNGGDNDITSSVDFDSFFATGKDGSAVAGDSVN
agarase (Saccharophagus degradans)               299  GGDPGNGGTPGNGG-----SGDTVVVEMANFSATGKEGSAVAGDTFT
agarase (Gilvimarinus polysaccharolyticus)       297  GGGN----------------DITSSVDFDNFFATGKDGSAVAGDSFN
agarase (Microbulbifer agarilyticus)             299  GGDPDNGGDPGNGGNPG--SGETIRVEMGSFSATGKAGAAVAGDTVA

agarase (Microbulbifer sp. AG1)                  336  TKGDWADYTVNLPAAGEYRVDLVIASPMSSGLGAELTFAGNAAKTVI
agarase (Microbulbifer thermotolerans)           336  TKGDWADYTVNLPAAGEYRVDLVIASPMSSGLGAELTFAGNAAKTVT
agarase (Simiduia agarivorans)                   344  TLGDYGDYTVNLPSAGSYKIELVAASPSSGNLGADISIDGSYVGSIN
agarase (Gilvimarinus agarilyticus)              357  TVGDWAEYSINLPEAGEYRLELDTASTVSTGLGADISIDDVFVGTVA
agarase (Saccharophagus degradans)               354  TLGDWADYTVNFPAAGNYTVNLIAASPVTSGLGADILVDSSYAGTIP
agarase (Gilvimarinus polysaccharolyticus)       341  TVGDWAEYSINLPEDGEYRLELDTASTVSSGLGADISIDGVFVGTVA
agarase (Microbulbifer agarilyticus)             356  TLGDWGDYTVNFPEAGNYNVELLAASPTTSGIAADVQVDGSYVGTIP

agarase (Microbulbifer sp. AG1)                  396  TLPQTISVSSPGNYNFRLKSTGSSNWQWNGDEIRFVKL---------
agarase (Microbulbifer thermotolerans)           396  TLPQTISVSSPGNYNFRLKSTGSSNWQWNGDEIRFVKL---------
agarase (Simiduia agarivorans)                   404  TLATNVYVASAGNHSVRVQSSGTASWQWNGDEIRFVKIDDGNT--QP
agarase (Gilvimarinus agarilyticus)              417  SLANTINIG-AGTHTLRVQSAGSSPWQWNGNAIRMVKVGEGSSNNQT
agarase (Saccharophagus degradans)               414  SLPSSIYIASAGNHTIRVQSSGGSAWQWNGDELRFTQTDADTG--TN
agarase (Gilvimarinus polysaccharolyticus)       401  SLANTINIG-AGTHTLRVQSAGSSQWQWNGEAIRMVKMGEGSSNNQT
agarase (Microbulbifer agarilyticus)             416  TLPSTIYIASAGNHTIRVQSAGGSAWQWNGDEIRFTKTEDDNT--PP

agarase (Microbulbifer sp. AG1)                       -----------------------------------------------
agarase (Microbulbifer thermotolerans)                -----------------------------------------------
agarase (Simiduia agarivorans)                   462  ENFDAVGGTYADGQAQKISTYTTGGVTAINYVNKGDYADYTLNVSAA
agarase (Gilvimarinus agarilyticus)              476  ESFNSTGGPYDG-----FQTYTQSGITATNYNQRGDYAEYTLSVPTA
agarase (Saccharophagus degradans)               470  ENFNAVGGTFSDGQAQPVSVYTVNGNTAINYVNQGDYADYTIAVAQA
agarase (Gilvimarinus polysaccharolyticus)       460  ESFNNTGGPYGG-----FQTYTQSGITATNYNQRGDYAEYSLSVPVA
agarase (Microbulbifer agarilyticus)             474  ESFASVGGTYADGQAQPISVYTTNGSTAINYVNAGDFADYTINVADA

agarase (Microbulbifer sp. AG1)                       -----------------------------------------------
agarase (Microbulbifer thermotolerans)                -----------------------------------------------
agarase (Simiduia agarivorans)                   522  VGGQVDFLVNTNGSWVNQSQTTVPNNGWNNFQALNGGVVSLPAGSVK
agarase (Gilvimarinus agarilyticus)              531  SGAAMTLTLNGNALVSLDVPSTGGWNTFTEVNASGAVALPAGTHTLR
agarase (Saccharophagus degradans)               530  TGGSIEFLVNENGSWASKTVTAVPNQGWDNFQPLNGGSVYLSAGTHQ
agarase (Gilvimarinus polysaccharolyticus)       515  SGAAMTLTLNGSALVSLDVPSTGGWNTFAEVSASGGVVLPAGTHTLR
agarase (Microbulbifer agarilyticus)             534  TGGSIEFLVNEGGSWNSKTATPVPNQGWDNFQPLDGGSVYLEAGTHQ

agarase (Microbulbifer sp. AG1)                       ----------
agarase (Microbulbifer thermotolerans)                ----------
agarase (Simiduia agarivorans)                   582  NMDKFVLTPQ
agarase (Gilvimarinus agarilyticus)              591  NADRFIFTPQ
agarase (Saccharophagus degradans)               590  NLDKFTLSN-
agarase (Gilvimarinus polysaccharolyticus)       575  NADRFAFTPQ
agarase (Microbulbifer agarilyticus)             594  NLDKFVLSN-
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Fig. 1. Alignment of the deduced amino acid sequence of *Microbulbifer* sp. AG1 agarase with other agarase protein sequences. Arrowheads indicate two catalytic Glu residues. The cellulose binding domain (CBD) type IV of Strain AG1 agarase is boxed. The multiple sequence alignment was performed with ClustalW program. Genbank accession numbers: *Microbulbifer thermotolerans*, BAD29947; *Simiduia agarivorans*, WP_015048661; *Gilvimarinus agarilyticus*, WP_041522726; *Saccharophagus degradans*, WP_011467657; *Gilvimarinus polysaccharolyticus*, WP_049721028; and *Microbulbifer agarilyticus*, BAE06228. Identical and similar amino acids are shaded in black and grey, respectively, and white background indicates unrelated amino acids.

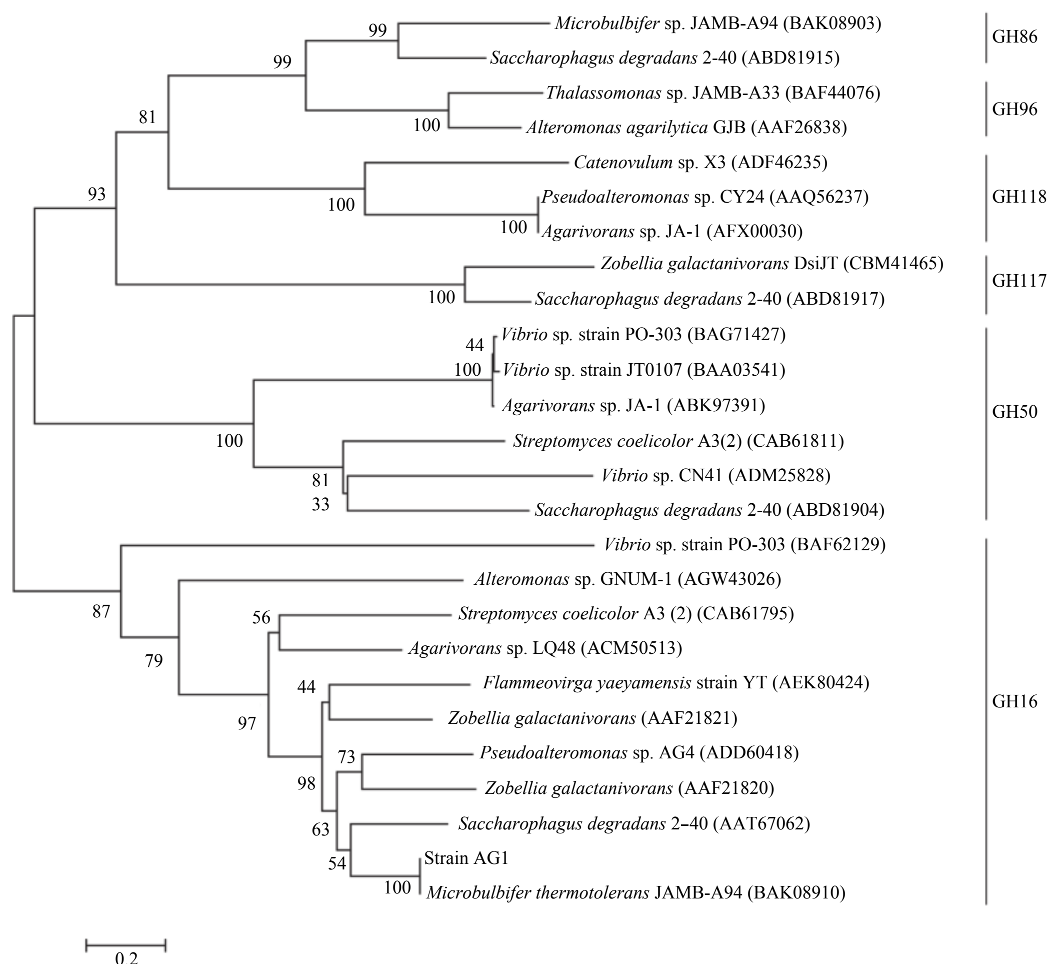


Fig. 2. Unrooted neighbor-joining phylogenetic tree of Strain AG1 agarase. The amino acid sequences of the relative agarases were referred to the previously classified six agarase families. Sequence alignment was performed by ClustalW and the tree was built by MEGA 6.0 software. Analysis was bootstrapped and bootstrap values are indicated at respective nodes (1 000 replications). The scale indicates the number of amino acid substitutions per site.

3.9 *Substrate specificity*

The substrate specificity of the recombinant agarase was investigated using the artificial chromogenic substrates. The enzyme could actively hydrolyze *p*-nitrophenyl-β-D-galactopyranoside (OD_{420}=0.624) but not *p*-nitrophenyl-α-D-galactopyranoside (OD_{420}=0.011), suggesting that it recognizes the β-linkage but not the α-linkage. These results indicated that this agarase was a β-agarase that could specifically hydrolyze the β-glycosidic bond, producing neoagaro-oligosaccharides from agarose as a substrate.

3.10 *Primary structure comparison with other agarases*

Primary structure comparison of thermostable β-agarase from Strain AG1 to a thermostable agarase from *Pseudoalteromonas* sp. AG4 (Oh et al., 2010) (GenBank accession number ADD60418) as well as two mesophilic agarases from *Thalassomonas agarivorans* (Liang et al., 2014) (GenBank accession number AGT98631) and *Agarivorans gilvus* WH0801 (Liu et al., 2014) (GenBank accession number AFP32918) was made with respect to various factors involved in the protein thermal stability (Table 3). In comparison to their counterparts from *Thalassomonas agarivorans* and *Agarivorans gilvus* WH0801, *Microbulbifer* sp. AG1 agarase and *Pseudoalteromonas* sp. AG4 agarase showed the presence of a high percentage of proline and aromatic residues (Table 3). Furthermore, Strain AG1 agarase possessed higher number of arginine and lower number of labile residues compared with the two mesophilic agarases (Table 3). However, other potential stabilizing factors, including high proportion of aliphatic, hydrophobic and charged residues as well as low percentage of glycine, were not found in agarase from *Microbulbifer* sp. AG1.

3.11 *Analysis of enzymatic hydrolysis products*

The hydrolysis products produced by the recombinant agarase were investigated by TLC. As shown in Fig. 6, the enzyme hydrolysed agarose to generate a series of neoagaro-oligosaccharides in the initial stage. At 40 min post hydrolysis, the main products were neoagaro-oligosaccharides larger than NA8, NA6 and NA4. As time went on, the amount of the oligosaccharides larger than tetramers decreased, whereas the amount of neoagarotetraose increased. When hydrolysis time reached 6 h, the main products included NA6 and NA4. After incubation for 96 h, the main enzymatic product was neoagarotetraose.

3.12 *Antioxidant activity of the hydrolysis products*

Assays of reducing power, scavenging DPPH and ABTS radic-

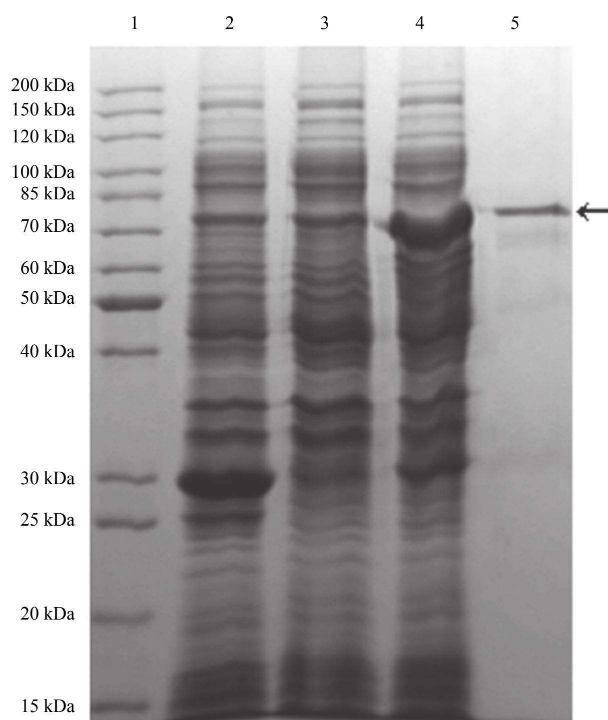


Fig. 3. Expression of the agarase gene from *Microbulbifer* sp. AG1 in *E. coli*. Lane 1 represents molecular weight protein marker; Lane 2 total proteins from *E. coli* BL21 (DE3) containing pGEX-6P-1 vector without DNA insert after induction with IPTG; Lane 3 total proteins from *E. coli* BL21 (DE3) containing pGEX-6P-aga recombinant plasmid before induction; Lane 4 total proteins from *E. coli* BL21 (DE3) containing pGEX-6P-aga recombinant plasmid after induction with IPTG; and Lane 5 purified fusion protein. The arrow indicates the position of the recombinant agarase.

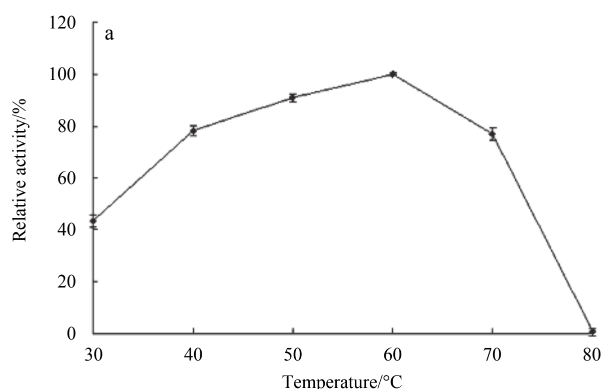
als were used to assess the antioxidant activity. The enzymatic hydrolysis products of different reaction time had the inhibitory effects on DPPH and ABTS radicals, and the scavenging activities increased with the concentration of the samples (Figs 7a and b). The IC_{50} values of scavenging DPPH radical were 5.12 mg/mL (40 min), 2.96 mg/mL (96 h) and 2.43 mg/mL (6 h), respectively. The IC_{50} values of scavenging ABTS radical cation were 0.67 mg/mL (96 h), 0.58 mg/mL (40 min) and 0.47 mg/mL (6 h), respectively. As is shown in Fig. 7c, the hydrolysis products showed increased ferric reducing power with the increased sample concentration. The samples of 96 h and 6 h had nearly the same effects. At the same concentration, they had better reducing power than the sample of 40 min. All these results indicated that the agarase hydrolysis products had the antioxidant activity, and the activity of the products did not correlate with the degree of polymerization regularly. The sample of 6 h which mainly contained neoagarohexaose and neoagarotetraose had good reducing power, scavenging effects of DPPH and ABTS radicals.

4 Discussion

In this study, an agarase gene was cloned from *Microbulbifer* sp. AG1 isolated from marine mud. After expression in *E. coli*, this recombinant enzyme was biochemically characterized.

The optimum reaction temperature of Strain AG1 agarase was 60°C, which coincides with that for agarase from *Catenovulum agarivorans* YM01^T (Cui et al., 2014). Comparisons of thermostability and pH stability between Strain AG1 agarase and other agarases were shown in Table 4. Although less stable than its counterparts from *Catenovulum agarivorans* YM01^T and *Flammeovirga pacifica* WPAGA1, agarase form *Microbulbifer* sp. AG1 is substantially more stable than the agarases from *Alteromonas* sp. GNUM-1, *Catenovulum* sp. X3, *Agarivorans* sp. JA-1 and *Streptomyces coelicolor* A3(2). Like agarases from *Catenovulum agarivorans* YM01^T, *Flammeovirga pacifica* WPAGA1 and *Catenovulum* sp. X3, Strain AG1 agarase also displayed good pH stability. In addition, the recombinant agarase in this study exhibited good resistance towards the tested inhibitors, detergents (except SDS), and urea denaturant. These properties convert Strain AG1 agarase into a very attractive enzyme in the food, cosmetic and medical industrial applications.

In comparison to the two mesophilic agarases from *Thalassomonas agarivorans* and *Agarivorans gilvus* WH0801, Strain AG1 agarase was found to have higher percentage of proline, arginine and aromatic residues, and lower percentage of labile residues in the primary structure. Proline and arginine may contribute to protein stability (Vieille and Zeikus, 2001). Aromatic residues have been reported to contribute to thermal stability as a result of



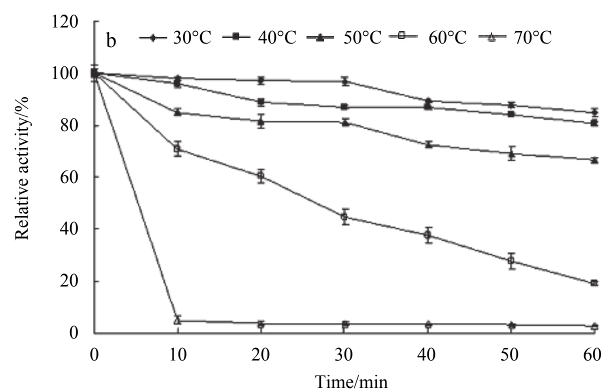


Fig. 4. Effect of temperature on activity and stability of *Microbulbifer* sp. AG1 agarase. a. Optimum temperature of the agarase. The enzymatic activity at different temperatures was measured. Activity at 60°C was taken as 100%. b. Thermostability of the agarase. The enzyme thermostability was investigated by measuring the residual activity after keeping the enzyme at the indicated temperatures for different times (10 to 60 min), respectively. Activity of the enzyme before incubation was defined as 100%. Data are presented as mean±SD from three independent experiments.

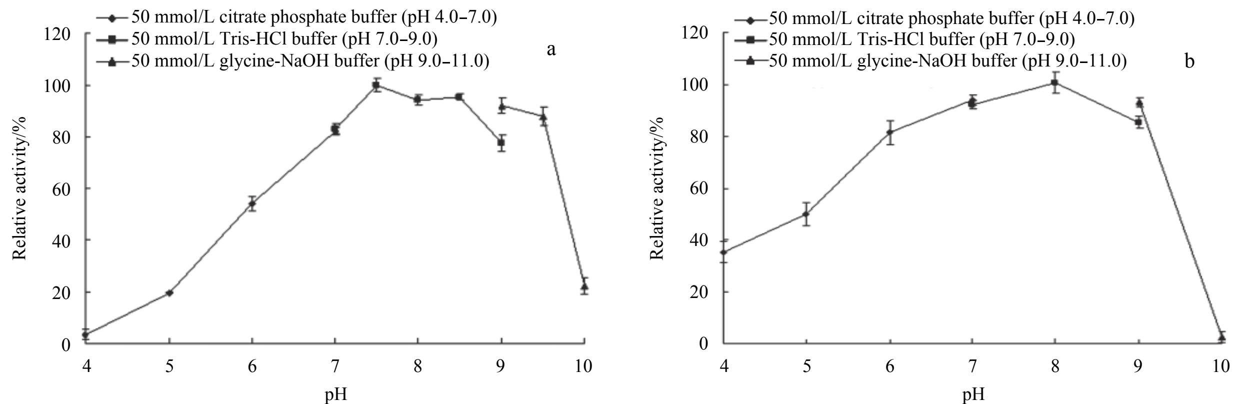


Fig. 5. Effect of pH on activity and stability of *Microbulbifer* sp. AG1 agarase. a. Optimum pH of the agarase. The enzyme activity in buffers with different pH values was measured. Activity at pH 7.5 was taken as 100%. b. pH stability of the agarase. The pH tolerance profile of the enzyme was determined by measuring the residual activity after keeping the enzyme in buffers with different pH values at 25°C for 1 h. Activity of the enzyme without the treatment was defined as 100%. Each value represents the mean of three replicates±SD. The buffer systems used were 50 mmol/L of citrate phosphate buffer (pH 4.0–7.0), Tris-HCl buffer (pH 7.0–9.0), and glycine-NaOH buffer (pH 9.0–11.0).

the ring interaction that occurs in the protein core (Vieille and Zeikus, 2001). In conclusion, factors contributing to the thermostablility of Strain AG1 agarase might be due to high percentage of proline, arginine, and aromatic residues as well as low percentage of labile residues.

Agarase from *Microbulbifer* sp. AG1 degraded agarose into neoagarotetraose as the main end product, which is similar with some other known agarases (Lin et al., 2012; Oh et al., 2010). Agarases can be used to hydrolyze agar into oligosaccharides which have potentially economic values because of their physiological and biological activities (Chen and Yan, 2005; Lee

Table 1. Effects of metal ions on the recombinant agarase activity

Metal ions	Concentration/mmol·L^{-1}	Relative activity/%
Control	—	100.0±1.8
Na^{+}	1	102.4±0.6
	10	90.3±6.6
K^{+}	1	98.2±1.4
	10	98.6±2.6
Li^{+}	1	98.5±3.2
	10	86.0±6.0
Ca^{2+}	1	97.9±0.8
	10	96.6±0.5
Mg^{2+}	1	99.4±0.5
	10	93.7±1.5
Ba^{2+}	1	88.7±1.6
	10	83.2±0.5
Mn^{2+}	1	66.8±2.0
	10	46.3±0.8
Ni^{2+}	1	48.8±4.7
	10	36.6±5.4
Co^{2+}	1	82.4±1.5
	10	59.6±1.1
Cu^{2+}	1	60.0±1.5
	10	NA
Zn^{2+}	1	1.8±0.3
	10	NA
Fe^{2+}	1	75.3±2.8
	10	NA
Al^{3+}	1	60.3±2.3
	10	NA
Fe^{3+}	1	39.9±0.8
	10	NA

Note: The enzyme was incubated with each metal ion with final concentrations of 1 mmol/L or 10 mmol/L at 25°C for 30 min, individually. Residual activity was determined by the standard assay described in Section Materials and Methods. The enzyme activity without addition of metal ion was defined as 100%. Values are presented as mean±SD (n=3). NA stands for no activity.

Table 2. Influences of inhibitor, detergent and denaturant on the recombinant agarase activity

Inhibitor, detergent and denaturant	Concentration	Relative activity/%
Control	—	100.0±2.7
EDTA	1 mmol/L	93.6±2.2
	10 mmol/L	88.1±0.6
β-ME	1 mmol/L	127.6±0.8
	10 mmol/L	169.8±1.2
DTT	1 mmol/L	107.8±1.0
	10 mmol/L	111.8±1.4
PMSF	1 mmol/L	103.9±3.4
	10 mmol/L	83.4±4.7
SDS	0.1%	74.8±2.0
	1%	30.1±2.8
Tween 20	0.1%	98.7±3.0
	1%	98.6±1.2
Tween 80	0.1%	101.3±2.0
	1%	103.3±1.0
Triton X-100	0.1%	99.0±0.4
	1%	98.6±1.2
Chaps	0.1%	104.5±0.6
	1%	101.1±0.6
Urea	1 mol/L	82.9±0.6

Note: The enzyme was incubated with each inhibitor, detergent and denaturant with different final concentrations at 25°C for 30 min, individually. Residual activity was measured by using the standard assay described in Section Materials and Methods. Reaction mixture without inhibitor, detergent and denaturant was used as a reference. Values are mean±SD from three independent experiments.

Table 3. Factors in the protein primary structure which may influence protein thermal stability

Factor	*Microbulbifer* sp. AG1	*Pseudoalteromonas* sp. AG4	*Thalassomonas agarivorans*	*Agarivorans gilvus* WH0801
P/%	5.77	5.17	3.62	4.25
R/%	4.16	3.45	3.75	4.03
G/%	9.93	6.90	6.72	7.32
Aliphatic residues (A+I+L+V)/%	23.32	25.52	25.84	26.65
Aromatic residues (F+H+W+Y)/%	15.48	15.17	14.86	14.21
Hydrophobic residues (A+F+I+L+V+W)/%	31.18	33.45	33.72	34.18
Charged residues (D+E+H+K+R)/%	21.49	21.03	25.13	22.08
Labile residues (C+M+N+Q)/%	10.86	15.17	13.32	14.23

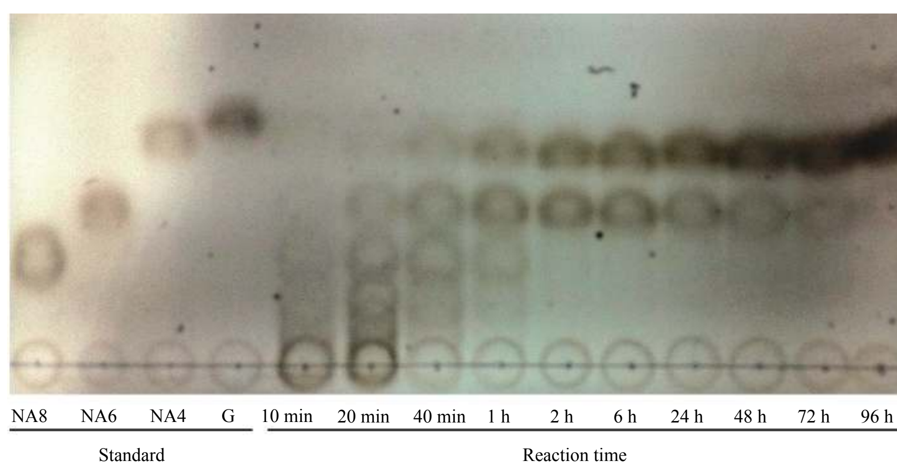


Fig. 6. TLC analysis of the recombinant agarase hydrolysis products depending on the reaction time. Standard markers include neoagarooctaose (NA8), neoagarohexaose (NA6), neoagarotetraose (NA4) and D-galactose (G).

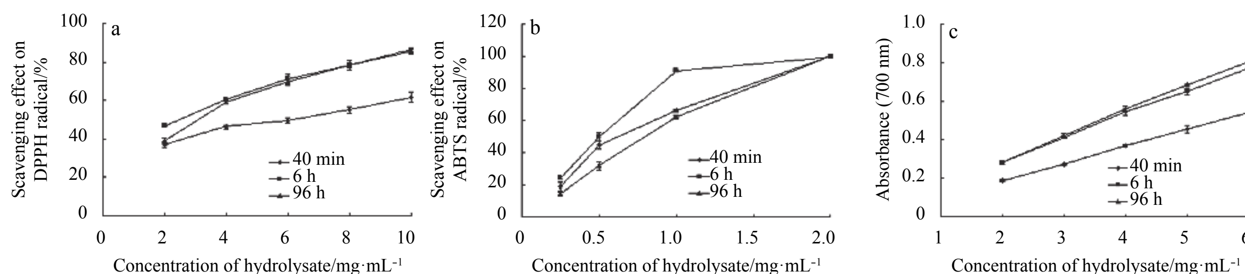


Fig. 7. Antioxidant activity of the hydrolysis products produced by the recombinant agarase. a. DPPH radical scavenging activity, b. ABTS radical cation scavenging activity, and c. reducing power of the hydrolysates. Values are presented as mean±SD from three independent experiments.

Table 4. Thermostability and pH stability of some agarases

Organism	Thermal stability	Stable pH	References
Microbulbifer sp. AG1	67% and 19% residual activities after 1 h at 50°C and 60°C, respectively	6.0–9.0	this study
Catenovulum agarivorans YM01^T	more than 80% residual activity after 1 h at 50°C	4.0–9.0	Cui et al. (2014)
Flammeovirga pacifica WPAGA1	no activity loss after 10 h at 50°C	5.0–10.0	Hou et al. (2015)
Alteromonas sp. GNUM-1	about 20% residual activity after 30 min at 50°C	–	Chi et al. (2014a)
Catenovulum sp. X3	about 40% residual activity after 1 h at 50°C	5.0–9.0	Xie et al. (2013)
Agarivorans sp. JA-1	no activity after 1 h at 55°C	–	Lee et al. (2012)
Streptomyces coelicolor A3(2)	45% residual activity after 1 h at 50°C	–	Temuujin et al. (2011)

Note: – means not reported.

et al., 2008). In this study, the hydrolysates produced by Strain AG1 agarase exhibited antioxidant activities by assays of reducing power, scavenging DPPH and ABTS radicals.

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