Purification and characterization of α-agarase produced from a marine bacterium, *Pseudomonas* sp. AP5333

해양으로부터 분리한 *Pseudomonas* sp. AP5333으로부터 생산되는 한천분해효소의 정제와 그 특성

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Abstract

An agarolytic bacterium was isolated from *Capsosi phon fulvecense* (C. agardh) collected in the Southern coast of Korea and the strain was identified as *Pseudomonas* sp. AP5333 by phylogenetic studies based on analysis of the 16s rDNA gene sequence. The optimum medium composition for the agarase production of the isolated strain was determined to be marine medium containing 0.2% (w/v) agar, 0.5% (w/v) beef extract, and 2.0% (w/v) NaCl. Optimal culture conditions for the production of agarase was estimated to be pH 7.5, 25℃, and 72 hr incubation. The enzyme was purified 13.5-fold purity against the culture supernatant by a series of ionic exchange chromatographies and gel filteration. The molecular weight of the enzyme was estimated to be 27 kDa by sodium dodesylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The optimum pH and temperature for hydrolysis of agarose were determined to be 7.5 and 50℃, respectively. The enzyme was identified as α-agarase

according to the degradation fashion of synthetic substrates. The enzyme showed a specific activity for agar, agarose, however, did not shown on sodium alginate, or κ , ι , and λ -carrageenan. The enzyme was inactivated by phenylmethylsulfonyl fluoride (PMSF), iodoacetate. However, it was not affected by leupeptin, ethylene diamine teteraacetate (EDTA), and pepstatin. The enzyme was inhibited by Mn^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, and Zn^{2+}. However, bivalent metal cations such as Mg^{2+}, Li^{2+} did not changed enzyme activity.

Introduction

Agar being the most well known marine polysaccharide, is found in the cell walls of red algae such as *Gelidium* (44%), *Gracilarlia* (53%), and considered to be a mixture of agarose and agaropectin (Sugano et al., 1984). It is a complex molecule consisting of α-1,3 linked β-D-galactosyl unit and β-1, 4 linked 3, 6-anhydro-α-L-galactosyl unit and additionally contains the residues of sulfate, pyruvate, and methoxyl in the both residues (Percival et al., 1967). Agar has a broad range of applications, especially in food, pharmaceuticals, and cosmetic products (Marinho-Soriano et al., 2003) and widely used as a gelling agent in the preparation of microbiological culture media. Agarose is also used as an anti conversion agent in electrophoresis and in the preparation of matrices used in chromatography (Hassairi et al., 2001).

Agar can be degraded by several bacterial strains from marine environments and other sources. These hydrolytic enzymes are classified into two groups according the mode of action on agarose. The α-agarase cleaves the α-L-(1, 3) linkage between 3, 6-anhydro-L-galactopyranose and D-galactopyranose units yielding oligosaccharides with 3, 6-anhydro-L-galactose at the reducing end (Buttner et al., 1987). The β-agarase cleaves the β-D-(1, 4) linkage between the D-galactopyranose and the 3, 6-anhydro-L-galactopyranose, yielding oligosaccharides with D-galactose residues at the reducing terminal (Sugano et al., 1983).

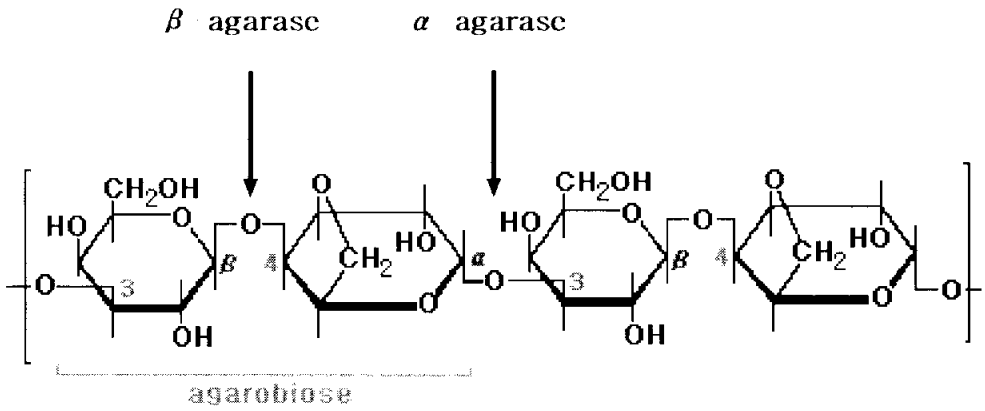

Fig. 1. Schematic diagram of agarose

Agar-decomposing bacteria were first isolated by Gran in 1902 (Yaphe et al., 1957). Yaphe and Co-workers were the first to describe an agar-degrading enzyme system from a marine bacterium (Day et al., 1975). Several β agarases producing microorganisms have been isolated and identified from marine and terrestrial environments. β agarase have been purified and characterized in the past decade from *Cytophaga* (Meulen et al., 1975), *Vibrio* sp. AP-2 (Aoki et al., 1990), and *Pseudomonas atlantica* (Morrice et al., 1983). Most reported agarase are endo type enzymes that hydrolyze agar and agarose to give, in order, neoagarotetaose, neoagarohexaose, neoagarobiose as the main hydrolysis products. On the other hand, α agarase have been reported from *Vivrio* sp. JT0107 (Sugano et al., 1994), and *Alteromonas agarlyticus* (Hassairi et al., 2001). Agaro oligosaccharides obtained from enzyme hydrolysis or acid treatment showed improved functionalities.

Especially, agarooligosaccharides obtained from enzyme hydrolysis

showed higher functional properties than that from acid hydrolysis. Agaro oligosaccharides has been reported its improved functionalities such as suppression of the inducible nitric monoxide synthetase (iNOS) expression, suppression of prostaglandin E2 production and TPA-induced edema, suppression of TNF-α production (Kato et al., 2001). Several studies have shown that agar yield depends upon species (Lignell et al., 1989), season (Hoyle et al., 1978), environmental parameters (Bird et al., 1988), method of extraction (Durairatnam et al., 1987) and stage of the life cycle (Whyte et al., 1981). It is interesting that oligosaccharide have received increased attention, especially because they have shown to be effective in stimulating the growth of bifidobacteria and lactobacilli in human large intestine. These oligosaccharide can be isolated from plant materials or can be enzymatically produced (Crittenden et al., 1996). Oligosaccharides have been used as bifidogenic substances and some infant products contain them in the hope that this might provide some of the benefits attributed to oligosaccharides in human milk (Rivero-Urgell et al., 2001).

In this work, a marine bacterium producing agarase was isolated and identified as *Pseudomonas* sp. AP5333, and the optimum culture conditions for the production of agarase have been determined with regard to carbon, nitogen, temperature, initial pH, and salt concentration. Furthermore, purification procedure of α- agarase from *Pseudomonas* sp. AP5333 was established and purified agarase was characterized on the biochemical properties.

MATERIALS AND METHODS

1. Materials

Agarose was purchased from FMC Co. Ltd (MA, U.S.A) for the substrate of agarase activity assay. Q and S-Sepharose, mono-Q HR 5/5 column, and Superose 6 column for FPLC system were purchased from Pharmacia Biotech (Uppsals, Sweden). Synthetic sbstrate such as ρ nitrophenyl β-D-mannopyranoside, ρ nitrophenyl α-D-mannopyranoside, ρ nitrophenyl α-L-fucopyranoside, ρ nitrophenyl β-D-fucopyranoside, ρ nitrophenyl β-D-galactopyronoside, ρ nitrophenyl α D galactopyronoside were purchased from SIGMA (MO, USA). Enzyme inhibitors such as phenylmethylsulfonyl fluoride (PMSF), leupeptin, iodoacetate and ethylene diamine teteraacetate (EDTA) were purchased from Sigma, and pepstatin was supplied from Cambridge Research Biochemicals Ltd. (Cheshire, U.K.). Low molecular weight markers for SDS PAGE and standard proteins for gel filtration chromatography were obtained from Bio-Rad (CA, U.S.A). All other reagents were of analytical or microbiological grade.

2. Isolation and identification of marine bacterium producing agarase

Screening and cultivation

Agarolytic bacteria were isolated from *Capsosi phon fulvecense* (*C.*

agardh) collected in the Southern coast of Korea. The screening medium for agarolytic bacteria was composed of 2.0% agar, 1.0% beef extract, 1.0% peptone and 2.5% NaCl (pH 7.5). The plates were incubated at 25℃ for three days. Colonies forming pits or clearing zones on agar plate were picked up and isolated further by the same plating method. The isolated strain was cultured in the modified marine medium (NaCl 23.0 g, KCl 0.7 g, $MgCl_26H_2O$ 10.6 g, $CaCl_2$ 1.1 g, Na_2SO_1 3.9 g, $NaHCO_3$ 0.2 g, $(NH_4)_2SO_4$ 1.0 g, K_2HPO_4 0.01 g, Tris-base 6.05 g, bacto peptone 5 g, yeast extract 1 g, ferric citrate 0.1 g, ammonium nitrate 0.0016 g, disodium phosphate 0.008 g and 1000 ㎖ distilled water, pH 7.8) to produce agarase. For liquid cultures, agar (0.3%) was added before sterilization. The stock culture preserved in 20% (v/v) glycerol solution at -80℃ was routinely used as a preinoculum to inoculate a 100 ㎖ culture flask containing 20 ㎖ of modified marine medium. The inoculum preparation was incubated for 18 hr at 200 rpm and 25℃. The resulting inoculum (500 ㎕) was transferred to a 250 ㎖ flask containing 50 ㎖ of the same medium and cultivated under constant conditions for 3 days.

Biochemical tests and 16s rDNA sequencing

Total genomic DNA was prepared using Genomic DNA extract kit (iNtRon, Kyungki-Do, Korea) according to manufactures manual. PCR of 16S rDNA gene was conducted by GeneAmp PCR Systems 2700 (Applied Biosystems. CA, USA) using primer 49f: 5 AGAATTCTNANACATGCAAGTCGANCG-3, 1510r: 5-

GTGGATCCGGYTACCTTGTTACGACTT 3 (N: degenerate including 4 nucleotide, Y: degenerate including pyrimidines, Moyer, 1994) in 16S rDNA of *E. coli*. PCR was performed as follows; 5 min of preincubation at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 61°C, 1 min at 72°C. The PCR product was extracted with GENECLEAN Turbo kit (Q·BIO gene, CA, USA) from separated band in 1% agarose gel. The PCR product was ligated into the plasmid vector pGEM-T (Promega, WI, USA) overnight at room temperature using the pGEM-T Easy Vector System Kit (Promega, WI, USA) and transformed in *E. coli* XL-1 Blue strain as a competent cell. Plasmid DNA was isolated using the Plasmid Extraction Kit (AtmanBio, Korea). PCR products using pGEM T vector were directly analyzed with ABI 377 XL upgrade DNA sequencer (Perkin elmer, MA, USA). The resulting sequences were manually aligned with GenBank databases.

Determination of culture conditions

Optimum incubation conditions for the growth of isolate and agarase activity were determined with modified marine medium. To determined the effect of carbon source for the production of cell growth and agarase production, several carbon sources were tested with the same concentration by incubating at 25℃ for 3 days. Effect of several nitrogen sources such as yeast extract, casein, bacto peptone, beef extract, trypton, gelatin, and urea on cell growth and agarase activity was determined by incubation at 25℃ for 3 days

with modified marine medium containing the same concentration. The effect of incubation temperature on the growth and enzyme activity was performed at 20, 25, 30, and 37℃ incubation at pH 7.5 for 3 days. The initial pH of the medium was determined the pH range from 4 to 10, which was adjusted prior to sterilization The influence of salt concentration on the growth and enzyme activity was determined by incubation at 25℃ for 3 days with the modified marine medium (pH 7.5) containing different concentration 0-9% (w/v) of NaCl. At least three tests were carried out in parallel for each experiment. Cell growth was determined by measuring the absorbance at 660 nm.

Enzyme activity

Agarase activity was determined by the enzymatic production of reducing sugars from agarose (Somogyi Nelson, 1952). The enzyme reaction mixture, containing 100 $\mu\ell$ of the enzyme and 900 $\mu\ell$ of 0.1% (w/v) agarose in 10 mM sodium phosphate buffer (pH 7.5), was incubated at 40℃ for 30 min. The reaction was stopped by the addition of Somogyi reagent used for the determination of reducing sugars. Reaction mixture was boiled for 10 min and arsenomolybdate reagent was added after cooling. One unit of activity was defined as the amount of enzyme that produces 1 μmol of D galactose equivalents per minute.

Determinztion of galatose contens

The amount of galactose during enzymatic reaction was determined by the method of Somogyi and Nelson (1952). A 1 ㎎/㎖ stock solution of D-galactose in water were taken 0, 10, 20, 40, 60, 80 ㎕. and mixed with 1.0 ㎖ of somogyi reagent. Reaction mixture was boiled for 10 min and arsenomolybdate reagent was added after cooling. The mixture was subjected to the measurement of absorbance at 510 nm (Fig. 1). The amount of galactose in samples was determined by the comparison with the standard curve.

3. Purification and characterization of agarase

Purification of agarase

All the following steps were carried out in chilled temperature unless specified otherwise. After a 3 day culture of *Pseudomonas* sp. AP5333, culture medium was centrifuged at 12000×g 30 min. The supernatant was diluted with 2 volume of 20 mM Tris-HCl buffer (pH 7.5) and loaded onto a S-Sepharose colume (2.5×12 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.5). Unbound fraction of the S-Sepharose column was load onto a Q-Sepharose column (2.5 ×12 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The enzyme was eluted with 400 ㎖ of linear gradient ranging from 0.1 to 1.0 M NaCl in the same buffer. Enzyme fractions were collected and diluted with 2 volume of equilibration buffer and loaded onto a Mono Q (Phamacia, HR 5/5) column equilibrated with 20 mM

Tris HCl buffer (pH 7.5). The elution was performed with a linear gradient of 0.5 to 1.0 M NaCl in 20 mM Tris HCl buffer (pH 7.5). The flow rate and fraction size were 30 ㎖/h and 1 ㎖, respectively. The enzyme fraction was further purified by the Mono Q column chromatography with previous conditions. The enzyme fractions from Mono-Q column were pooled and loaded onto Superose-6 column attained in FPLC system. With the GPC chromatography, the agarase was purified and stored at 20℃ for characterization studies.

Determination of protein concentration

The protein concentration of fraction from chromatographies were estimated spectophotometrically at 280 nm. The protein concentration of pooled enzyme solution from each purification step were determined according to the method of Bradford (1976). Bovine serum albumin was used as a standard protein.

Determination of the molecular weight of agarase

The molecular weight of the purified agarase was determined by sodium dodesyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) and by Superose 6 gel filtration column according to the method of Andrews (1964). For the preparation of protein samples for SDS PAGE, sample was concentrated by precipitation with trichloroacetic acid (TCA) and cold acetone. phosphorylase (148 kDa), bovine serum albumin (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50

kDa), carbonic anhydrase (36 kDa), and myoglobin red (22 kDa) were used as the SDS-PAGE standards. Tyroglobulin (670 kDa), bovine gamma globulin (158 kDa), ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B-12 (1.35 kDa) were used as standard proteins for gel filtration.

pH dependence of agarase activity

Buffer solutions for the determination of the enzyme activity were prepared to four sequences as follows; 10 mM citric acid - sodium citrate buffer (pH 4.0 to 6.0), 10 mM sodium phosphate buffer (pH 6.0 to 7.5), 10 mM Tris HCl buffer (pH 7.0 to 9.0), and 10 mM sodium bicarbonate buffer (pH 9.0 to 10.0).

Temperature dependence of agarase activity

The effects of temperature on agarase activity were examined under the standard assay conditions except that the temperature was varied from 20℃ to 60℃ with the same concentration of enzyme solution used in pH dependence.

pH stability of agarase activity

For the measurement of the pH stability, 10 ㎕ of enzyme solution was incubated with 390 ㎕ of buffer with different pH values for 12 hr at 4℃ and the remaining activity was measured under the standard assay conditions.

Temperature stability of agarase activity

To determine the temperature stability of the enzyme, the residual

activity after incubating the enzyme solution at different temperature (20℃ to 60℃) for 30 min was measured under the standard assay conditions.

Natural substrate specificity of the agarase

In order to determine whether or not selectively hydrolyzed the bond between D-galctose and 3,6-anhydro-L-galctose of the agarose structure from agarase. Substrate specificities were determined by using different polysaccharides, such as agar, agarose, alginate, cellulose, κ-carrageenan, λ carrageenan, and ι carrageenan.

Synthetic substrate specificity of the agarase

Sbstrate such as ρ nitrophenyl β D mannopyranoside, ρ nitrophenyl α-D-mannopyranoside, ρ nitrophenyl α -L-fucopyranoside, ρ nitrophenyl β D-fucopyranoside, ρ nitrophenyl β-D-galactopyronoside, ρ nitrophenyl α-D-galactopyronoside were used for the hydrolytic activity of the agarase. Synthetic substrate specificity for synthetic substrate was determined by measuring the amount of ρ-nitrophenyl released from synthetic substrate. The assay mixture containing 1 ㎖ of enzyme solution and 250 ㎕ of 1 mM synthetic substrate was incubated at 50℃ for 60 min. The reaction was stopped by adding 1 ㎖ of 0.5 M NaOH solution, and released ρ nitrophenol was quantified spectrophotometrically at 410 nm.

Effect of enzyme inhibitors on agarase activity

All inhibitor stock solutions were prepared at the concentration of 10 mM. Phenylmethylsulfonyl fluoride (PMSF) was dissolved in 5% isopropanol. Leupeptin and pepstatin were dissolved in a small amount of DMSO and the final concentration was adjusted with distilled water. Iodoacetate and ethylene diamine teteraacetate (EDTA) were directly dissolved in distilled water. Ten $\mu\ell$ of enzyme solution was incubated at room temperature for 30 minutes with 10 or 100 $\mu\ell$ of each inhibitor solution and 380 $\mu\ell$ or 290 $\mu\ell$, respectively, of 20 mM Tris-HCL buffer (pH 7.5). After 600 $\mu\ell$ of 0.1% (w/v) agarose in 10 mM sodium phosphate buffer (pH 7.5) was added, and remaining activities were measured with the method of Somogyi and Nelson (1952).

Effect of metal ions and some reagent

The effects of metal ions and some reagents were examined under the standard assay conditions except that each reagent was added to the enzyme solution at a final concentration of 2 mM of each method ion solution.

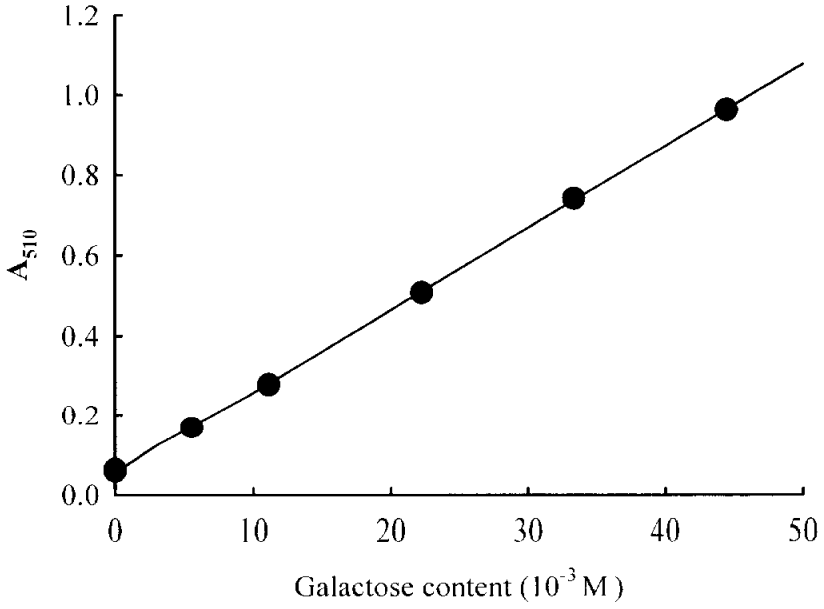

Fig. 2. Standard curve for the determination of galactose contents

RESULTS and DISCUSSION

1. Identification of agarase producing bacterium

The strain colonies softened the agar and produced halos of clearing after 48 hr of incubation at 25℃. Each isolates was separated on the modified marine medium plate and confirmed agarase activity with hydrolysis of agar after incubation at 25℃ for 3 days. Among them one isolated showing highest agarase activity was selected for this study and designated AP5333. The result of the phylogenetic analysis showed that strain AP5333 belongs to the genus *Pseudomonas*. When compared with sequenced available in the database, AP5333 showed 98% homology with *Pseudomonas* sp. PE 1 (Fig. 3).

2. Optimum culture conditions

Effect of various carbon sources on the production of agarase were resulted in Table. 1. As shown in Table. 1, production of agarase was affected by agar supplementation in the medium. The result of this study suggested that the agarase production was not related to the cell growth. Other carbon sources except agar were not produced or little level of agarase activity. Different concentration of agar in culture medium was performed for determining the production of agarase activity. As shown in Fig. 4, agarase activity was highest at the concentration of 0.2% in culture medium. Until 0.2% of agar, the enzyme activity was increased with the concentration of agar. However, the enzyme activity was decreased over 0.3% of agar in the medium. Determination of following optimum medium composition

of the isolated was determined with the presence of 0.2% agar. *Alteromonas* sp. strain C-1 (Leon et al., 1992), for which agar liquefaction appears to be dependent on the production of high concentration of agarase. The effects of different kinds of nitrogen sources on the cell growth and agarase activity were illustrated Table. 2. As shown in Table. 2, beef extract showed the highest agarase activity among 8 kinds of nitrogen source. Cell growth of yeast extract, casein, peptone, and tryptone in culture media showed higher than beef extract. However, agarase activity of them showed much lower then beef extract. To determine optimum concentration of beef extract on the production of agarase, 0 to 1.0% of beef extract was added in the culture medium. As shown in Fig. 5, 0.5% of beef extract showed the highest enzyme activity and the enzyme activity was decreased with the higher concentration of beef extract in the medium. Similar result was reported that the optimum nitrogen source of agarase production by *Bacillus cereus* ASK202 N3 was highest in the presence of 0.3% of yeast extract (Hwang et al., 1999)

The isolated AP5333 was inoculated in the modified marine medium containing 0.2% of agar and 0.5% of beef extract as a carbon and nitrogen source, respectively and each inoculum was incubated at 20, 25, 30, 37℃ for 72 hr for 3 days. Cell growth was not affected by the incubation temperature, however, enzyme activity was highest at the incubation at 25℃ (Fig. 6). Kim et al. (1998) reported that optimum conditions for the enzyme production with *Bacillus cererus* ASK202 was observed to be temperature 40℃, which is different

with the result in this study.

Effect of initial medium pH on the cell growth and agarase production was determined and cell growth was higher in the neutral and weak alkaline pH range (Fig. 7). Cell growth and agarase activity were highest at pH 7.5. Agarase activity increased with the increase of pH until 7.5 and decreased in the alkaline pH range as shown in Fig. 7. Kim et al. (1998) reported that optimum conditions for enzyme production of *Bacillus cererus* ASK202 was observed to be temperature 40℃, initial pH 7.5 and agar concentration 0.4% (w/v).

The isolated AP5333 had optimum salt concentration for growth and enzyme activity in the range of 1-2% NaCl (Fig. 8). Cell growth of the strain was not influenced with the concentration of NaCl in the tesred range in this study. However, enzyme activity was highest in the presence of 2% NaCl in the culture medium and increased concentration of NaCl in the medium decreased agarase activity.

Overnight culture of glycerol stock (20 ㎖) was inoculated in a 250 ㎖ flask containing 50 ㎖ modified marine broth adjusted initial pH to 7.5 containing 0.2% agar, 0.5% beef extract, and 2% NaCl and incubated until 4 days at 25℃ on a ratary shaker with 200 rpm. At indicated time interval, cell growth and enzyme activity determined. Cell growth was maximum after 30 hr incubation and enzyme activity was highest at 72 hr incubation as shown in Fig. 9. Prolonged fermentation until 5 days did not changed agarase activity. In the optimal culture conditions, the agarase production was increased to 3.0 folds as compared to that by the basal medium.

TAGAGTTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGG
CCTTACACATGCAAGTCGAGCGCGAACGTTCCTTCGGGAACT
ATTAGAGCGGCGGACGGGTGAGTAACGCGTGGGAATCTGCC
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CATACGCCCTACGGGGGAAAGCAGGGGATCTTCGGACCTTGC
GCTATTGGATGAGCCCGCGTCGGATTAGTTTGTTGGTGGGG
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GATGATCAGCCAGACTGGAACTGAGACACGGTCCAGACTCC
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TAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGC
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TGCAGTTAGAACTGGCAAGCTAGAGTACTAGTAGAGGGGTG
TGGAATTTCTAGGTGTAGCGGTGAAATGCGTAGATATCTGA
AGGAACATCAGGGCGAAGGCGAC

Fig. 3. BLAST research result of 16s rDNA sequence

Table. 1. Effect of various carbon sources on the agarase production by *Pseudomonas* sp. AP5333

C-sources	Cell growth ($O.D_{660nm}$)	Final pH	Relatively activity (%)
Control	1.905	7.92	0
Agar	**1.550**	**7.61**	**100**
Agarose	1.728	7.45	20
Glucose	1.406	7.43	0
Fructose	0.538	7.62	0
Galactose	0.898	7.13	31
Maltose	1.747	7.53	5
Lactose	1.661	7.53	0
Corn starch	1.688	7.53	1
Potato starch	1.995	7.50	3
Wheat starch	1.826	7.60	2
soluble starch	1.860	7.56	0
Saccharose	1.835	7.48	5
D-Sorbitol	0.334	7.82	0
Mannitol	1.952	7.88	9

Table. 2. Effect of various organic nitrogen sources on the agarase production by *Pseudomonas* sp. AP5333

N-sources	Cell growth ($O.D_{660nm}$)	Final pH	Relatively activity (%)
Contol	0.703	7.73	0
Yeast extract	2.525	7.80	76
Casein	2.240	7.65	52
Bacto peptone	1.562	7.84	23
Tryptone	1.669	7.62	24
Beef extract	**1.441**	**7.70**	**100**
Gelatin	0.672	7.87	9
Urea	0.570	8.39	0

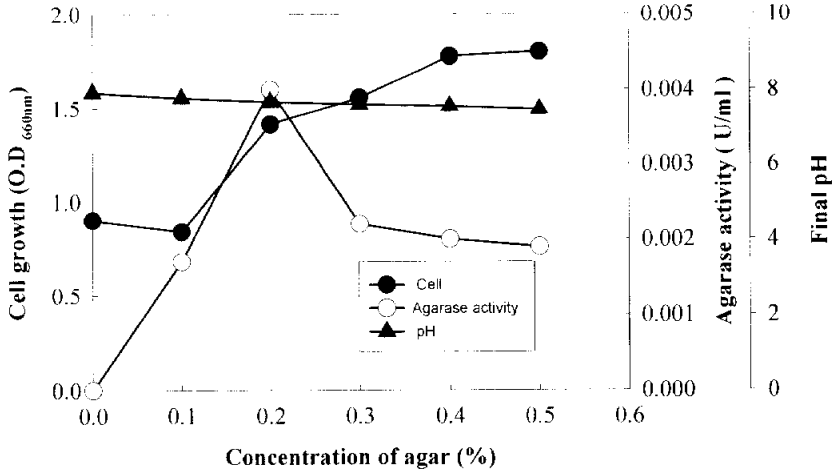


Fig. 4. Effect of agar concentration on the cell growth and the agarase production by *Pseudomonas* sp. AP5333

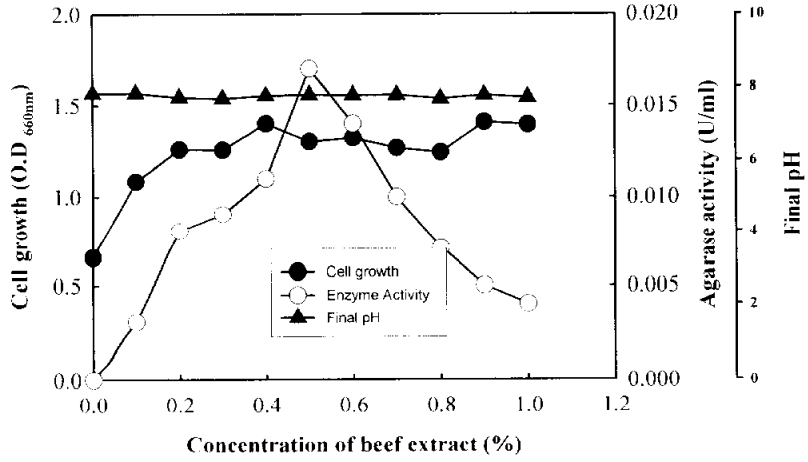


Fig. 5. Effect of beef extract concentration on the cell growth and the agarase production by *Pseudomonas* sp. AP5333

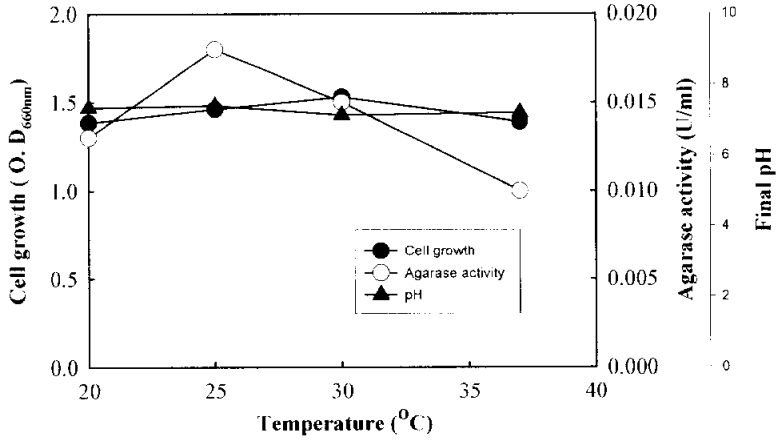


Fig. 6. Effect of temperature on the cell growth and the agarase production by *Pseudomonas* sp. AP5333

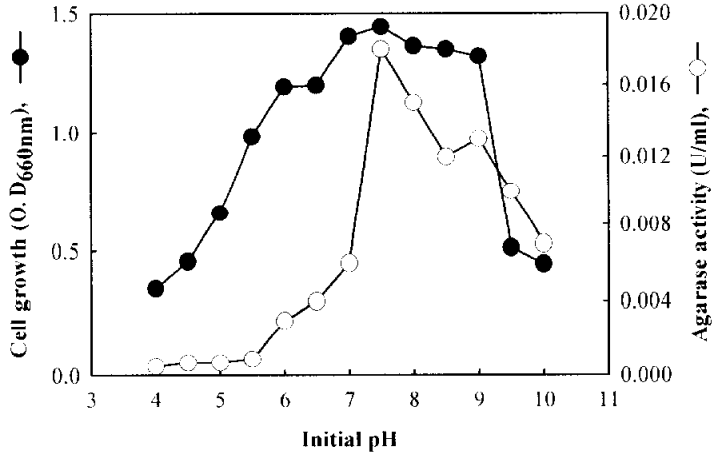


Fig. 7. Effect of initial pH on the cell growth and the agarase production by *Pseudomonas* sp. AP5333

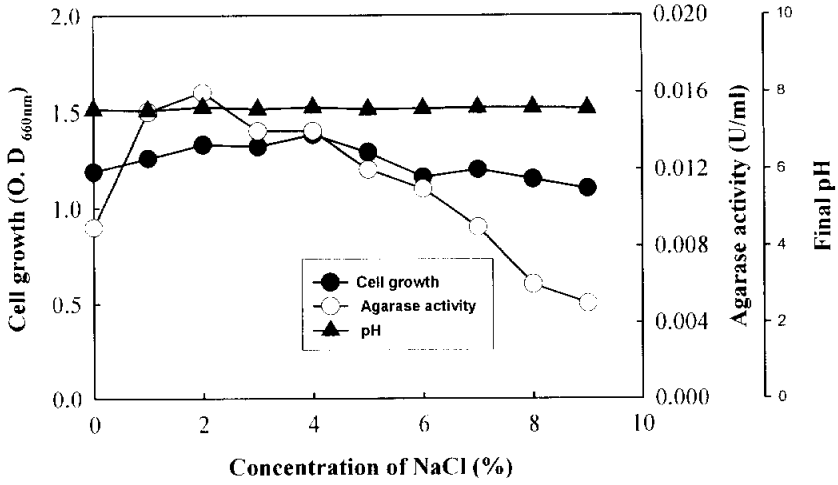


Fig. 8. Effect of NaCl concentration on the cell growth and the agarase production by *Pseudomonas* sp. AP5333

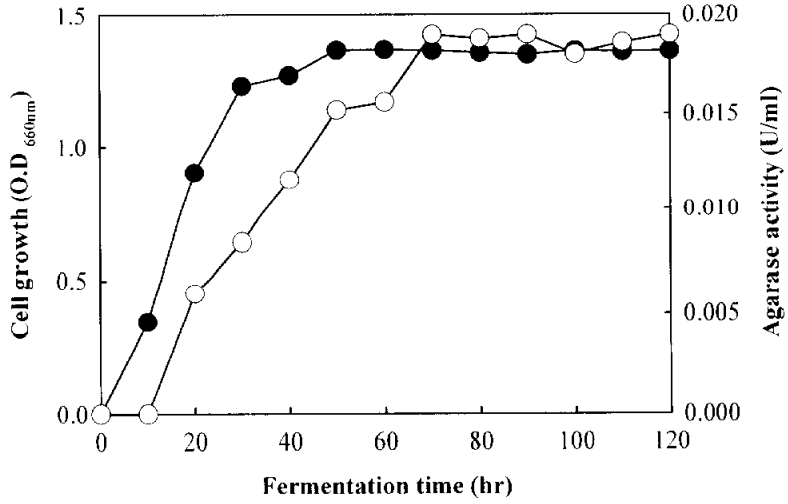


Fig. 9. Effect of fermentation time on the growth of *Pseudomonas* sp. AP5333

3. Purification of agarase from *Pseudomonas* sp. AP5333

After a 3 day culture of the isolated strain, culture medium was separated with the centrifuge at 12,000xg for 20 min, The supernatant was passed through S-Sepharose column (2.5×12 cm) equilibrated with 20 mM Tris-HCl (pH 7.5) to remove contaminated proteins in the culture. The unbound protein was load onto a Q-Sepharose column (2.5×12 cm) equilibrated with 20 mM Tris HCl buffer (pH 7.5) and enzyme was eluted with the linear gradient of 0.0 to 1.0 M NaCl concentration. Fig. 10 shows a Q Sepharose chromatogram of the flow-through from S Sepharose column with the culture supernatant from *Pseudomonas* sp AP5333. Flow rate was 120 ㎖/ hr and the fraction volume was 5 ㎖/ tube. The agarase was eluted around salt concentration of 0.5-0.6 M in 20 mM Tris - HCl buffer (pH 7.5). The absorbance at 280 nm was measured after 100 ㎕ of each fraction was diluted by 10 times with the same buffer. The fractions (fraction number 33-45) having agarase activity were pooled and diluted with 2 volumes of 20 mM Tris-HCl buffer (pH 7.5) for further purification by Mono-Q ion exchange chromatography.

Enzyme solution was loaded onto a Resource-S column in order to remove contaminated proteins, which is an anionic property. The flow throught showed the total activity of the agarase, which means the enzyme is not bound to cationic exchange column at pH 7.5. To obtain enough protein concentration of samples for subsequent step, the first Mono Q chromatogrphy was conducted with a salt gradient.

Flow rate was 0.5 ㎖/min and the fraction volume was 1 ㎖/tube. In every case, agarase activity was eluted at 0.5 0.53 M NaCl concentration. As shown in Fig. 11 a. protein peak indicated by an arrow showed high enzyme activities, however, the fraction contained several contaminated proteins determined by SDS-PAGE. For further purify the 1'st Mono-Q fractions, enzyme fraction was diluted to two times with 20 mM Tris-HCl buffer and did a second Mono-Q chromatography. In the second Mono Q step, proteins were eluted with a same salt gradient and still enzyme fraction had minor contaminated proteins (Fig. 11-b). To remove unseparated proteins, the enzyme fractions were taken to a third Mono-Q chromatography with previous conditions. Flow rate was 0.5 ㎖/min and the fraction volumn was 500 ㎕/tube. Although Mono-Q chromatogram showed still a single protein peak (Fig. 11 c), whereas minor proteins were still detected on SDS/PAGE. The specific activity and purification of the agarase were 5.8 units/㎍ of protein and 8.7 fold, respectively. Compared with culture supernatant.

Two hundred microliters of enzyme fractions were injected to FPLC with GPC column and minor protein was separated by GPC chromatography. Two hundred microliters from the GPC fraction was concentrated by TCA precipitation and run on SDS-PAGE. A single protein band having 27 kDa was shown on the SDS-PAGE gel (Fig. 11). The purified enzyme was identified as a single subunit peptide. This value is close to those reported *P. atlantica* ATCC 19292 (32 kDa), *Pseudomonas* sp. strain PT 5 (31 kDa), and *Cytophaga*

flevensis (26.5 kDa), but different with the molecular weight of agarase from *Vibrio* sp. strain JT0107 whose molecular weight is 107 kDa (Sugano et. al., 1993). The MW of agarase from *Alteromonas* sp. E 1 was also reported to be about 82 kDa (Kirimura et al., 1999). Results from the purification of agarase from *Pseudomonas* sp. AP5333 are summarized in Table. 3. The agarase was purified 13.5 fold with 1% yield against culture supernatant. Purity of agarase from microorganism showed a various purity such as 40.9-fold from *Alteromonas* sp. E-1 (Kirimura et al., 1999), 45 fold from *Vibrio* sp. strain JT0107 (Sugano et al., 1993), 23.5-fold from *Vibrio* sp. AP-2 (Aoki et al., 1990), 29-fold from *Bacillus* sp. MK03 (Suzuki et al., 2003), and 23.6-fold of agarase from *Alteromonas agarolyticus* (Richard et al., 1993). Specific activity of the purified agarase showed 9 U per ㎎ protein at 40℃ and pH 7.5.

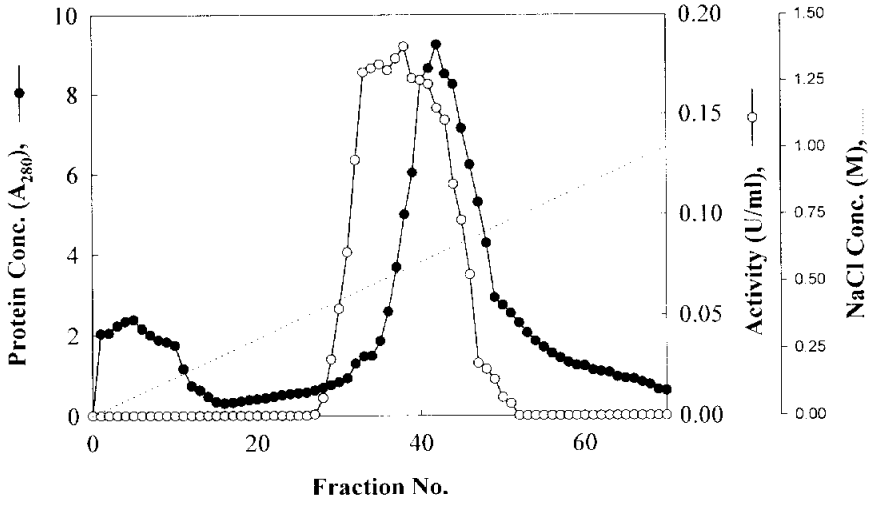
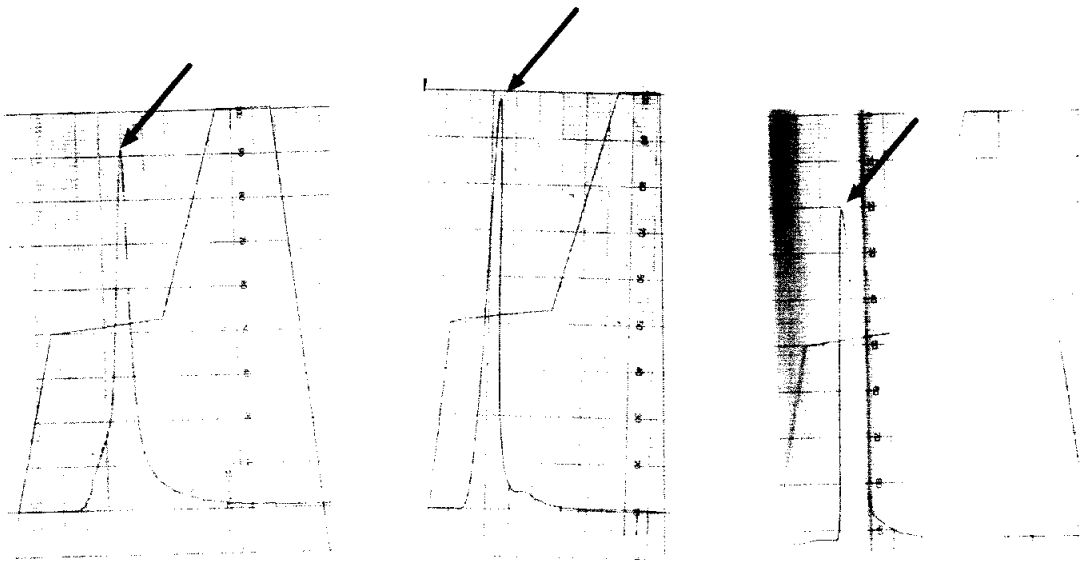


Fig. 10. Chromatogram of a Q-Sepharose ionic exchange chromatography of the flow-through from S-Sepharose column. Flow rate was 120ml/hr and fraction volumn was 5ml/tube. The agarase was eluted with a gradient of 0.1 - 1.0 M NaCl in 20mM Tris-HCl buffer (pH 7.5) The protein concentration was measured at 280 nm after 100ul of each fraction was diluted 10 times with the same buffer



a ;Mono Q (1'st)　　b; Mono Q (2'nd)　　c; Mono-Q(3'rd)

Fig.11. Chromatogram of mono-Q column chromatography of agarase from the Q-sepharose chromatography

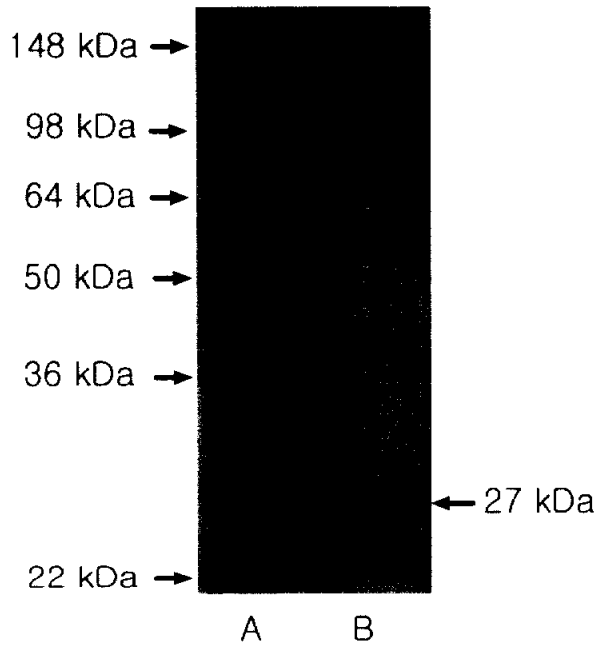


Fig. 12 SDS-PAGE of purified agarase from GPC

Lane A, molecular marker

Lane B, purified agarase (TCA precipitation of 200 $\mu\ell$)

Table. 3. Purification of agarase from *Pseudomonas* sp. AP5333

Fraction	Volume (ml)	Protein conc. (ug/ml)	Activity (U/ml)	Specic activity (U/ug)	Purity (fold)	Yeield (%)
Culture fluid	1010	24	0.016	0.667	1	100
Q-Sep	60	38	0.128	3.36	5.04	47.5
Mono-Q(1st)	30	43	0.169	3.93	5.89	31.4
Mono-Q(2nd)	22	26	0.123	4.73	7.09	16.7
Mono-Q(3rd)	17	10	0.070	5.8	8.7	7.3
GPC	10	2	0.018	9	13.5	1

4. Characterization of agarase purified from *Pseudomonas* sp. AP5333

pH dependence of the purified agarase

The pH dependence of the purified agarase was shown in Fig. 13. The highest activity appeared at pH 7.5 and agarase activity at pH 4.0 showed about 50% of its maximum activity, which indicates that the agarase was unstable under acidic conditions. The purified enzyme had a broad optimum pH range from pH 6.0 to pH 9.0. Enzyme activity was abruptly decreased at alkaline pH range and activity was not shown at pH 10.0. Several bacterial enzymes were relatively stable at neutral pH or slightly acidic range compared with agarases from other microorganism (Kim et al., 1999). But, agarase from *Vibrio* sp. AP-2 have the optimal pH of 5.5 (Aoki et al., 1990).

Temperature dependence of the purified agarase

Agarase activity was measured at different temperature. The enzyme activity was gradually increased in the temperature range of 25-50℃ and the optimum temperature was found to be 50℃. The enzyme showed 25% of its maximum activity at 20iÉ, which is an average temperature of Korean coast. However, the enzyme lost most of activity at the temperature over 55℃ possibly due to thermal denaturation of the enzyme. The agarase from *Pseudomonas* sp. AP5333 showed its maximal activity at 50℃, and this temperature is much higher than the optimal temperature of *Alteromonas* sp. PT-5 agarase which is the most active at 30℃ (Morrice et al., 1983).

Optimum temperature of β-agarase from *Vibrio* sp. PO-303 was over 40℃ (Araki et al., 1998). Agarase activity seems to have the most suitable characteristics to express maximum activity in a marine environment.

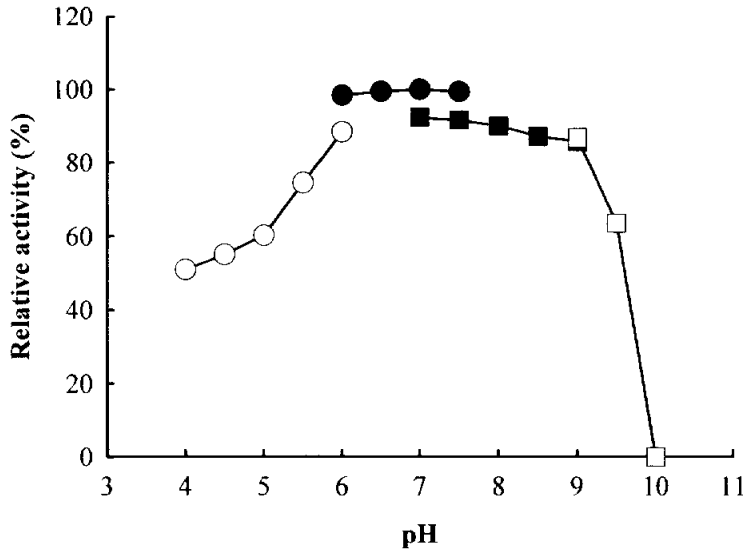


Fig. 13. pH dependence of the activity of agarase with various buffer solution. The buffers used: open circles, 10mM citric acid-sodium citrate buffer (pH 4.0 to 6.0); solid circles, 10mM sodium phosphate buffer (pH 6.0 to 7.5); sold squares, 10mM Tris-HCl buffer (pH 7.0 to 9.0); open squares, 10mM sodium bicarbonate buffer (pH9.0 to 10.0). The agarase reaction was carried outvarious pH 7.5 for 30min at 30^{0}C

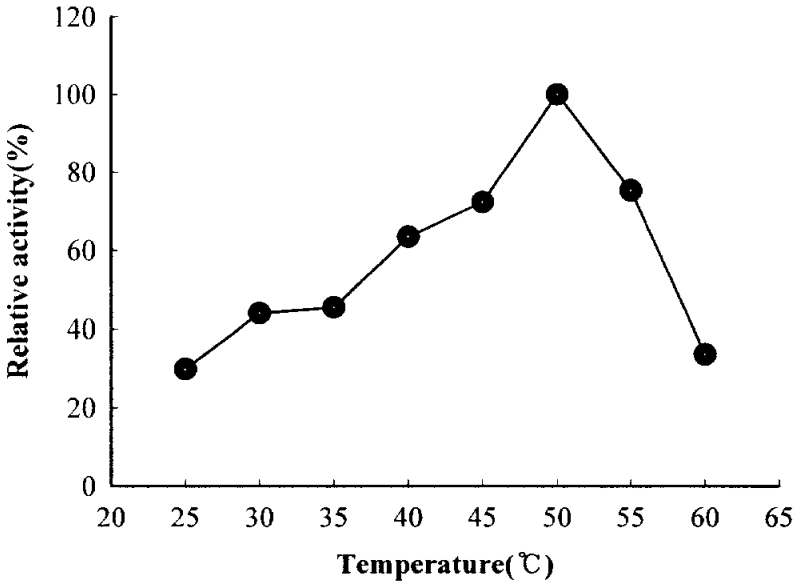


Fig. 14. Effect of temperature on the agarase activity.
Enzyme activity was measured at various temperatures in 10mM sodium phosphate buffer, pH 7.5 for 30min, respectively

pH-stability of the purified agarase

To confirm pH stability of the agarase, enzyme was incubated 4℃ for 12 hr in various pH value of buffers and the remaining activity was measured at pH 7.5 at 50℃. As shown in Fig. 15, the enzyme activity was stable over the pH range from 7.0 to 8.5. Activity of agarase retained only 51% of maximal activity after incubation at pH 6.0. Agarase from *Pseudomonas* sp. PT-5 and from *Vibrio* sp. PO-303 were stable after incubation in the pH ranges of 6 to 9 and 5 to 8, respectively.

Temperature-stability of the purified agarase

To confirm thermostability of the purified agarase, enzyme was preincubated for 30 min at different temperature range from 0℃ to 60℃ and immediately cooled on ice. Residual activity was determined at 50℃ at pH 7.5. As displayed in Fig. 16, the activity was stable up to heat treatment at 20℃ since the relative activity was 92% after 30 min incubation at this temperature range. In contrast to the agarase from *Pseudomonas atlantica* ATCC 19292 and *Pseudomonas.* sp. PT-5, the enzyme was rapidly inactivated at temperature above 30℃ (Vera et al., 1998).

Substrate specificity on marine polysaccharides

Substrate specificity of the agarase from *Pseudomonas* sp. AP5333 was determined with several marine polysaccharides. As shown in Table 4, the agarase had a particularly high activity for agar and

agarose, however, enzyme activity was not shown against sodium alginate, or κ-, λ-, and ι-carrageenan that are composed of D-galactose and 3, 6-anhydro-D-galactose with β-1, 4 and α-1, 3 linkages. Therefore, these results suggest that the agarase selectively hydrolyzed the bond between D-galactose and 3, 6-anhydro-L-galactose on the agarose structure.

Substrate specificity on synthetic substrate

The purified agarase was reacted with 10 mM of synthetic substrate such as ρ-nitrophenyl α-D-galactopyronoside, ρ-nitrophenyl β-D galactopyronoside, ρ-nitrophenyl β-D-fucopyranoside, ρ-nitrophenyl α-L-fucopyranoside, ρ-nitrophenyl α-D-mannopyranoside, and β-D-mannopyranoside. As shown in Table 5, the agarase showed unique activity on ρ-nitrophenyl α-D-galactopyronoside, which suggest that the enzyme hydrolyzed the linkage of the α-glycosidic linkages in synthetic substrate. With this result, the agarase from *Pseudomonas* sp. AP5333 was confirmed to be α-agarase without any other glycolytic activities.

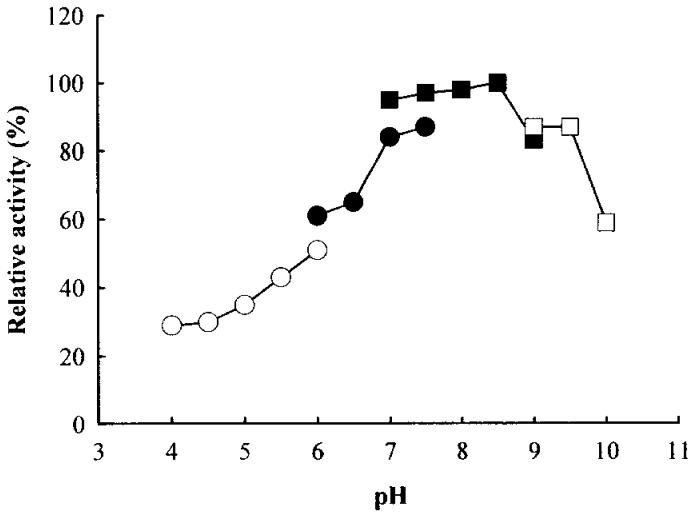


Fig. 15. pH stability of the activity of agarase with various buffer solution. The buffers used: open circles, 10mM citric acid-sodium citrate buffer (pH 4.0 to 6.0); solid circles, 10mM sodium phosphate buffer (pH 6.0 to 7.5); sold squares, 10mM Tris-HCl buffer (pH 7.0 to 9.0); open squares, 10mM sodium bicarbonate buffer (pH9.0 to 10.0). The agarase reaction was carried outvarious pH 7.5 for 30min at 30°C

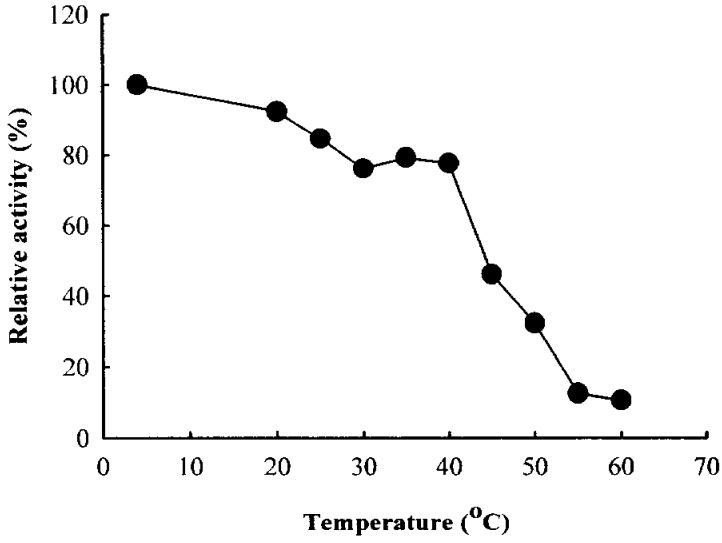


Fig. 16. Stability of the agarase activity at various temperature.
Enzyme activity was measured at various temperature
in 10mM sodium phosphate bufer, pH7.5 for 30min, respectively

Table 4. Substrate specificity of the purified enzyme on natural polysaccharides

Substrate	Relatively activity (%)
Agar	100
Agarose	84.6
Alginate	N/D
Cellulose	N/D
κ -Carrageenan	N/D
λ -Carrageenan	N/D
ι -Carrageenan	N/D

Table. 5. Substrate specificity of the purified enzyme on synthetic substrate

Synthetic sbstrate	Relatively activity (%)
ρ nitrophenyl α-D-galactopyronoside	100
ρ nitrophenyl β-D-galactopyronoside	N/D
ρ nitrophenyl β-D-fucopyranoside	N/D
ρ nitrophenyl α-L-fucopyranoside	N/D
ρ nitrophenyl α-D-mannopyranoside	N/D
ρ nitrophenyl β-D-mannopyranoside	N/D

Effects of inhibitors on the agarase activity

Effects of inhibitors on the agarase activity were determined using PMSF, leupeptin, pepstatin, iodoacetate, and EDTA. The enzyme activity was not inactivated by 0.1 mM of inhibitors used in this study. However, 1 mM PMSF and iodoacetate reduced 21% and 31%, respectively, of its original activity. With this result, the enzyme has serine residue in active site.

Effect of various metal ions on enzyme activity

The effects of various metal ions on agarase activity were examined under the standard assay conditions including indicated concentration of metal ions. As shown in Table 7, the enzyme activity was strongly inhibited by bivalent cations such as Cu^{2+}, and Ni^{2+} and completely inhibited by Zn^{2+}. Enzyme activity was slightly inactivated by Mn^{2+}, Ca^{2+}, and Na^{+}, however, cations such as Mg^{2+}, K^{+}, Li^{2+} did not shown any activity change. Agarase from *Alteromonas* sp. E-1 was slightly activated by K^{+} and Na^{+} (Kirimura et al., 1999) and strongly inhibited by bivalent cations such as Mn^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+} and Zn^{2+}. An agarase from *Bacillus* sp. MK03 was increased to 163% by 2 mM Mg^{2+} and 128% by 2 mM Zn^{2+}, respectively (Suzuki et al., 2002).

Table 6. Effect of several inhibitors on agarase activity from *Pseudomonas* sp. AP5333

Inhibitors	Conc. (mM)	Relative activity(%)
Control		100
PMSF	0.1	100
	1	79
Leupeptin	0.1	100
	1	100
Pepstatin	0.1	100
	1	100
Iodoacetate	0.1	100
	1	65
EDTA	0.1	100
	1	100

Table 7. Effects of various metal ions on the enzyme activity

Reagent (2mM)	Relativity activity(%)
None	100
$MnSO_4$	84
$MgCl_2$	100
$CaCl_2$	95
KCl	100
$CuCl_2$	41
$FeCl_2$	75
$NiCl_2$	50
$LiCl_2$	100
NaCl	89
$ZnCl_2$	0

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Purification and characterization of α-agarase produced from a marine bacterium, *Pseudomonas* sp. AP5333

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본 연구에서는 해양자원으로부터 한천 분해능이 뛰어난 균주를 분리하여 *Pseudomonas* sp. AP5333으로 명명하였으며, 분리된 균주의 agarase 생산을 위한 배양조건을 분석한 결과 0.2% (w/v) agar, 0.5% (w/v) beef extract, 2.0% (w/v) NaCl, pH 7.5, 25℃에서 최적배양조건을 나타냈다. 최적배양조건에서의 효소생산은 기본배지에 비해 약 3배정도의 높은 효소 생산성을 나타내었다. Agarase생산 균주인 *Pseudomonas* sp. AP5333를 최적조건에서 3일간 배양하여 배양액으로부터 일련의 이온교환 크로마토그래피와 겔 여과를 통해서 효소를 분리하였다. 분리 결과 13.5배의 정제도를 나타내었으며 수율은 1%로 나타났다. 정제 효소는 SDS-PAGE에 의해서 분자량이 27 kDa으로 나타났으며, 효소활성에 대한 최적온도와 최적 pH는 각각 50℃와 7.5이었다. 분리된 효소는 agar, agarose와 특이적으로 반응하였으나 alginate, κ-, λ-, and ι-carrageenan을 분해하지 못하였다. 이 결과로부터 agarose구조를 이루는 D-galactose와 3, 6-anhydro-L-galactose결합을 절단한다는 것을 확인할 수가 있었으며, 합성기질의 특이성에 의해서 α-agarase로 분류되었다.

그리고 효소의 활성에 대한 여러 가지 효소 저해제의 영향을 살펴본 결과, 1 mM의 PMSF, iodoacetate에서 효소활성에 어느 정도 영향을 미쳤으나, leupeptin, peptatin, EDTA 등은 효소의 활성을 저해하지 않았다.

금속이온의 영향에서는 Mn^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+} and Zn^{2+} 에 의해서 효소 활성이 저해되었으나 , Mg^{2+}, Li^{2+}에 의해서는 저해되지 않았다.