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A Novel Enzyme, λ-Carrageenase, Isolated from a Deep-Sea Bacterium

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A λ-carrageenan–degrading *Pseudoalteromonas* bacterium, strain CL19, was isolated from a deep-sea sediment sample. A λ-carrageenase from the isolate was purified to homogeneity from cultures containing λ-carrageenan as a carbon source. This is the first report of the isolation of λ-carrageenase together with the gene sequence for the enzyme. The molecular mass of the purified enzyme was approximately 100 kDa on both SDS-PAGE and gel-filtration chromatography, suggesting that the enzyme is a monomer. The optimal pH and temperature for activity were about 7 and 35°C, respectively. The enzyme had specific activity of 253 U/mg protein. The enzyme required monovalent salts for the activity. Carbohydrates, such as sorbitol, sucrose, trehalose, improved the enzyme stability. The pattern of λ-carrageenan hydrolysis showed that the enzyme is an endo-type λ-carrageenase, and the final main product was a tetrasaccharide of the λ-carrageenan ideal structure with galactose 2,6-disulfate at the reducing end, indicating the enzyme cleaves the β-1,4 linkages of its backbone structure. Furthermore, the gene (*cglA*) encoding the enzyme was sequenced. It encoded a mature protein of 103 kDa (917 amino acids). Remarkably, the deduced amino acid sequence showed no similarity to any reported proteins.

Key words: cloning, endo-β-1,4-carrageenose 2,6,2′-trisulfate-hydrolase, λ-carrageenan, λ-carrageenase, sequence.

Generally, marine microorganisms are considered to be a major component of global nutrient cycle (*1*). Seventy percent of the earth's surface is covered by the sea, about 90% of which has a depth of more than 1,000 m. In this context, we intended to find a biodegradation activity toward λ-carrageenan from marine microorganisms of deep-sea origin. Deep sea is common with regard to its volume, yet an extreme environment. Extreme conditions such as high pressure, low or high temperature, or high concentrations of inorganic compounds are considered lethal to many organisms. However, microorganisms have infiltrated virtually every space of the earth's biosphere, because they can adapt to a wide range of environmental conditions. Many extremophilic bacteria, such as alkaliphiles, thermophiles, psychrophiles, halophiles and piezophiles, have been isolated from the deep-sea as well as mesophiles. Some of them were categorized as new species (*2*). Deep-sea organisms are either metabolically active or dormant to obtain capability of survival or thriving throughout history of life. Previous studies have revealed the diversity and novelty in their microbiota (*3*). We have been conducting extensive screening for unique new enzymes from deep-sea microorganisms as abundant sources of unexplored properties (*4, 5*).

Carrageenans are a generic name for a family of water-soluble, sulfated galactans extracted from the cell walls of marine red algae. They consist of D-galactose residues bound by alternate α-1,3 and β-1,4 linkages. Their common types of them are called ι-, κ-, and λ-carrageenan based on the structure of major disaccharide units, corresponding to carrageenose 2,4′-disulfate, carrageenose 4′-sulfate, and carrageenose 2,6,2′-trisulfate (*6*), respectively. Natural carrageenans are often hybrids of more than one of these repeating units with some modifications. Whereas ι-, κ-, and other types of carrageenans are produced at cystocarpic stages in members of Gigartinceae, λ-carrageenan is produced at tetrasporic stages in them. Several carrageenan-degrading bacteria were detected from marine environments in earlier studies (*7–9*). Carrageenan-degrading enzymes from marine bacterium such as *Pseudoalteromonas carrageenovora, Zobellia galactanovorans* and *Alteromonas fortis* have been described with respect to their enzymatic properties and gene sequences (*8, 10, 11*). They display strict substrate specificity and hydrolyze either ι- or κ-carrageenan. In contrast, to the best of our knowledge, there has been no report on the isolation and characterization of λ-carrageenan–degrading enzyme together with the gene sequence.

Recently, we detected high λ-carrageenan hydrolytic activity produced by a deep-sea isolate. In this report, we describe the purification and characterization of λ-carrageenase. The gene encoding λ-carrageenase was sequenced for the first time.

MATERIALS AND METHODS

Isolation of a λ-Carrageenan–Degrading Bacterium and 16S rDNA Analysis of the Isolate—A sediment sample from Suruga Bay, Japan, at a depth of 2409 m was collected using the remotely operated submersible *Kaiko*. The sample was suitably diluted and spread on the solid medium

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containing artificial sea water (Nihon Pharmaceutical, Tokyo, Japan), 0.2% polypepton, 0.1% yeast extract, 0.5% λ-carrageenan from *Gigartina aciculaire* and *Gigartina pistillata* (Type IV; Sigma-Aldrich, St. Louis, MO, USA), and 1% agar, and followed by incubation at 20°C for several days. After flooding the solid medium with 10% cetylpyridinium chloride, the colonies with clear zones against a white background were considered to be candidate λ-carrageenan–degrading microorganisms. The candidates were cultivated at 20°C for 3 days in Difco marine broth 2216 (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing 0.2% λ-carrageenan. λ-Carrageenan hydrolytic activity in the culture broth was assessed by measuring the reducing sugars produced after incubation with 0.5% λ-carrageenan at 20°C for 3 h. Reducing sugar was quantified following dinitrosalicylic acid method (*12*). One of the bacteria exhibiting λ-carrageenan hydrolytic activity was selected and designated as strain CL19. 16S rDNA analysis was performed following the method described previously (*13*).

Enzyme Assay—A suitably diluted solution of enzyme preparation was incubated at 35°C in 50 mM 3-morpholinopropanesulfonic acid–NaOH buffer (MOPS, pH 7.0), containing 0.5% λ-carrageenan and 0.4 M NaCl. One unit (U) of enzymatic activity was defined as the amount of protein that produced 1 μmol of reducing sugar as D-galactose per minute under the assay conditions. Reducing sugar was quantified as described above. The activity was determined according to the initial rate of the reaction.

Purification of λ-Carrageenase from Strain CL19—Strain CL19 was propagated in 500 ml of marine broth 2216 containing 0.4% λ-carrageenan with shaking at 120 rpm in a rotary shaker for 24 h at 23°C. The following procedures were carried out at temperatures below 4°C. The centrifugal supernatant of the culture was brought to 0.5 M sorbitol and 1.5 M $(NH_4)_2SO_4$. The resulting solution was applied to a Butyl-Toyopearl 650M column (2.5 × 15 cm; Tosoh, Tokyo, Japan) previously equilibrated in 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 M sorbitol and 1.5 M $(NH_4)_2SO_4$. The column was washed with buffer to remove the unbound material and eluted with a linearly decreasing gradient of $(NH_4)_2SO_4$ (1.5–0.9 M). Active fractions were pooled and desalted using an Amicon concentrator with an ultrafiltration membrane (10,000-M_r cutoff; Millipore, Bedford, MA, USA). The resulting solution was applied to a DEAE-Toyopearl 650M column (2.5 × 15 cm; Tosoh), and eluted with a linear gradient of NaCl (0.1–0.35 M) in 20 mM Tris/HCl, pH 7.5 including 0.5 M sorbitol. The active fractions were pooled and desalted as described above. The desalted solution was further purified on a Heparin-sepharose column (HiTrap Heparin HP 5 ml; Amersham Bioscience, Piscataway, NJ, USA) pre-equilibrated with 20 mM Tris/HCl, pH 7.5, containing 0.5 M sorbitol and 0.2 M NaCl and eluted with a linear gradient of NaCl (0.2–0.5 M) in 20 mM Tris/HCl, pH 7.5 including 0.5 M sorbitol. The active fractions were pooled, concentrated to 1 ml, and the concentrate was used as the final preparation of purified enzyme (CglA) throughout the experiments.

SDS-PAGE and Activity Staining—SDS-PAGE of the purified enzyme was performed essentially as described by Laemmli (*14*). After electrophoresis, SDS in the gels was removed by soaking the gels in 50 mM MOPS (pH 7.0) containing 0.4 M NaCl three times for a total of 30 min. The gels were then overlaid onto the λ-carrageenan sheets containing 0.5% λ-carrageenan, 1.0% agarose and 50 mM MOPS (pH 7.0) and incubated for 3 h at 25°C. Following incubation, the λ-carrageenan sheets were stained by flooding with 10% cetylpyridinium chloride. λ-Carrageenase activity was visualized as clear zones on a white background. Proteins were visualized by staining with Coomassie brilliant blue. The molecular mass of the enzyme was estimated on SDS-PAGE and by gel filtration on a YMC-pack Diol-120 column (7.8 × 500 mm, YMC, Kyoto, Japan) with Precision-plus dual standards (Bio-Rad, Hercules, CA, USA) and a High-molecular-mass protein kit (Amersham Bioscience) as molecular mass standards, respectively.

Biochemical Properties of CglA—The pH optimum for the activity of the enzyme was examined in various buffers including 0.4 M NaCl. The buffers used were 50 mM 2-morpholinoethanesulfonic acid–NaOH (MES), pH 5.5–7.0; 3-morpholinopropanesulfonic acid–NaOH (MOPS), pH 6.5–8.0; 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid–NaOH (HEPES), pH 6.5–8.0; *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid–NaOH (TAPS), pH 7.5–9.0; and *N*-cyclohexyl-2-aminoethanesulfonic acid–NaOH (CHES), pH 8.5–10.0. The effect of temperature for the enzyme activity and stability was examined in 50 mM MOPS, pH 7.0, containing 0.4 M NaCl.

Sequencing of the N-Terminal and Internal Regions of CglA—The enzyme sample was blotted on a polyvinylidene difluoride membrane (Prosorb; Perkin-Elmer, Foster City, CA, USA), which had been wetted with methanol. The N-terminal sequence of the protein was determined directly with a protein sequencer (model 476A; Perkin-Elmer). The enzyme was digested with lysyl endopeptidase (Wako Pure Chemical, Osaka, Japan) and applied to SDS-PAGE. After blotting on a polyvinylidene difluoride membrane, the 79-kDa and 39-kDa fragments were sequenced to determine the internal amino acid sequence.

Sequencing of the λ-Carrageenase Gene—The complete λ-carrageenase gene and its flanking region were sequenced using the cassette ligation–mediated PCR method with a TaKaRa *LA* PCR *in vitro* Cloning kit, according to the manufacturer's instructions (TaKaRa Bio, Ohtsu, Japan). First, we designed primers A and B based on the N-terminal and internal amino acid sequences NRTITKVRTG and SMEMSV, respectively, of purified λ-carrageenase isolated from the culture of strain CL19. The nucleotide sequence of primer A was 5′-AAYMGNACNATHACNAARGTNMGNACNGG-3′ and that of primer B was 5′-NACNSWCATYTCCATNS-3′. PCR was carried out using genomic DNA (0.5 μg) of strain CL19 as a template, and primers A and B. A 1.6-kb DNA fragment was amplified and subcloned into the *Sma*I site of plasmid DNA pUC18 (TaKaRa Bio) and then sequenced. Based on the nucleotide sequence of the amplified fragment, primers for the amplification of its flanking regions were designed. The genomic DNA was digested with *Eco*RI, *Hind*III, or *Xba*I and ligated to the cassette DNA fragments in the kit. PCR was performed to amplify the fragment using the ligation mixture of *Eco*RI digestion as the template and primers C (5′-GGATATGAGAGTAGCGATGG-3′) and C1 (provided in the kit). The

PCR-amplified DNA was used as a template for the second round of PCR with primers D (5′-GATAGAAGGACATG-GAGACG-3′) and C2 (provided in the kit). To determine the complete sequence of the *cglA* gene, primers were synthesized based on the results of the successive sequencing of the PCR-amplified DNA fragments. Nucleotide sequencing was performed on an automated DNA sequencer (Model 377; Applied Biosystems) using a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Both strands of DNA were sequenced, and computer analysis was performed with the GENETYX program (SDC Software Development, Tokyo, Japan). A database homology search was performed with the BLAST program provided by the US National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Expression of the CglA Gene in Escherichia coli—The structural gene for CglA was amplified by PCR using primers E (5′-TCCCTACTGCTCGAGCTTCTCAATCGGC-3′) and F (5′-CGGAATTCTTACAATGTTGAACTTTGCATG-3′), and inserted into the *Xho*I and *Eco*RI sites of expresseion vector, pRSETC (Invitrogen, San Diego, CA, USA). This recombinant plasmid, designated pECL113, was used for the expression of the *cglA* gene in *E. coli* BL21(DE3)pLysS (Invitrogen). The transformation and gene expression were carried out according to the manufacture's instruction.

Analysis of the Main Product of λ-Carrageenan Hydrolysis—Enzymatic hydrolysis of λ-carrageenan with purified enzyme was carried out under the standard conditions at 25°C for 48 h. The sample was fractionated according to the method of Ohta *et al*. (*15*), using 50 mM $NaNO_3$ as a mobile phase. The fraction of the main product was pooled and freeze-dried. The samples were dissolved in distilled water and desalted on an Asahipak GS220 G7 column (6.7 × 500 mm; Asahi Kasei, Tokyo, Japan) using distilled water as a mobile phase. The oligosaccharide fraction was freeze-dried and then dissolved in methanol. The molecular mass of the sample was analyzed using electro spray ionization mass spectrometry (ESI-MS) (LCT Premier XE; Waters). The structure was analyzed in D_2O with NMR including

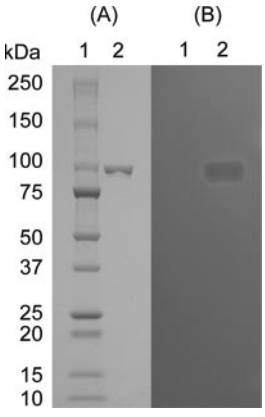


Fig. 1. **SDS-PAGE of λ-carrageenase purified from the culture of strain CL19.** Molecular mass markers (Lane 1) and purified λ-carrageenase (Lane 2) were visualized with Coomassie Brilliant Blue staining (A) and activity staining (B). The activity was visualized as a clear zone by flooding the λ-carrageenan sheet with 10% cetylpyridinium chloride.

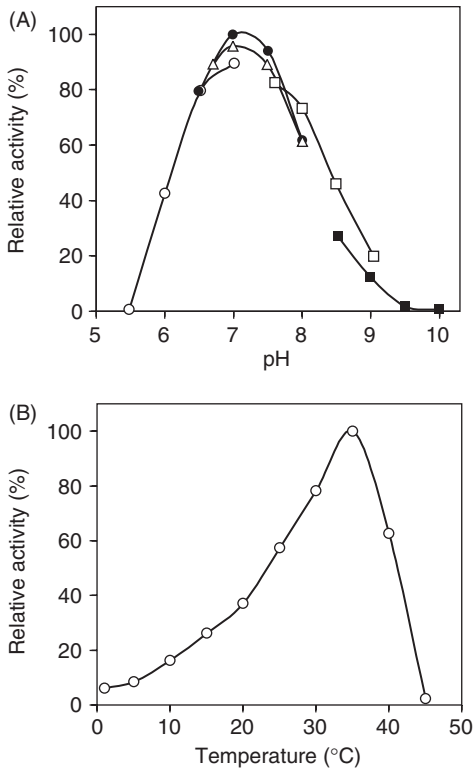


Fig. 2. **Effects of pH and temperature on activity of λ-carrageenase.** (A) The pH-activity curves of the purified λ-carrageenase are shown. The buffers used were 50 mM MES (pH 5.5–7.0; open circles), MOPS (pH 6.5–8.0; closed circles), HEPES (pH 6.5–8.0; open triangles), TAPS (pH 7.5–9.0; open squares), CHES (pH 8.5–10.0; closed squares). The activity was measured in the buffers including 0.4 M NaCl at 35°C. The values are shown as percentages of the maximal activity of λ-carrageenase observed at pH 7, which is taken as 100%. (B) The temperature-activity curve of the purified λ-carrageenase is shown. The initial activity at indicated temperature was measured at pH 7.0 in 50 mM MOPS including 0.4 M NaCl. The values are shown as percentages of the maximal activity of λ-carrageenase observed at 35°C, which is taken as 100%.

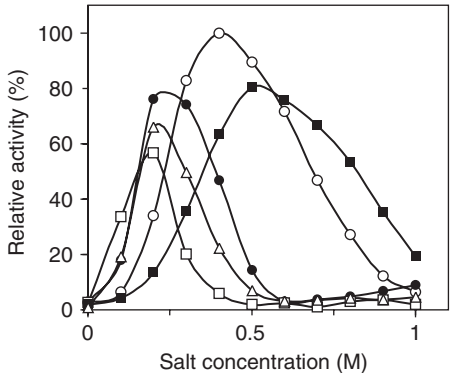
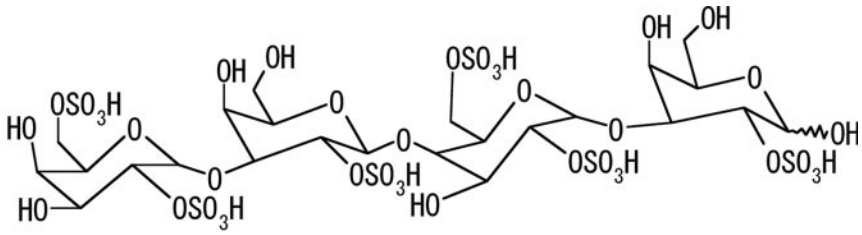


Fig. 3. **Effects of monovalent salts on activity of λ-carrageenase.** The reactions were carried out with monovalent salts at various concentrations for 15 min at pH 7.5 in 50 mM Tris/HCl. The salts tested were NaCl (open circles), KCl (closed circles), CsCl (open squares), LiCl (closed squares), and RbCl (open triangles). The values are shown as percentages of the maximal activity of CglA observed in the presence of 0.4 M NaCl, which is taken as 100%.



α-D-Gal*p*2,6*S*-(1→3)-β-D-Gal*p*2*S*-(1→4)-α-D-Gal*p*2,6*S*-(1→3)-α,β-D-Gal*p*2*S*

Fig. 4. **Structure of the main product of λ-carrageenan hydrolysis by CglA determined by NMR.**

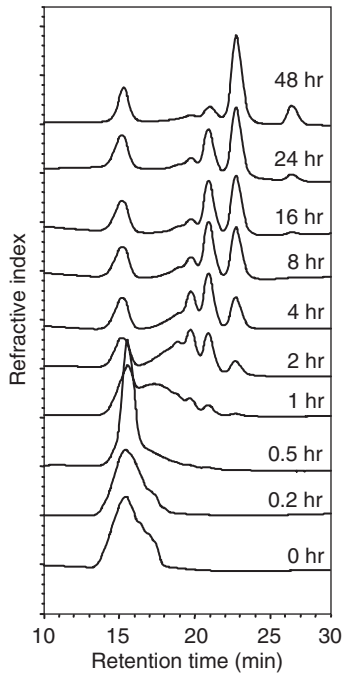


Fig. 5. **HPLC analysis of the λ-carrageenan hydrolysis pattern of CglA.** The enzyme (0.2 U/ml) was incubated with 1% λ-carrageenan at 25°C and at pH 7.0 in 50 mM MOPS including 0.4 M NaCl for up to 48 h. Samples were taken at the indicated intervals and boiled for 5 min to terminate the reaction, and analyzed on a YMC-Pack Diol-120 column (YMC).

DEPT HSQC, COSY, TOCSY, and HMBC analyses at 20–33°C on a Varian Unity Inova 600 system (Palo Alto, CA, USA), operating at 599.9 MHz for ^{1}H-NMR and 150.8 MHz for ^{13}C-NMR, essentially as described by Masuhiro *et al.* (*16*).

Analysis of λ-Carrageenan Hydrolysis Pattern and Substrate Specificity—Enzymatic hydrolyses of λ-carrageenan were carried out under the standard condition using 1.0% of each substrate at 25°C for up to 48 h. Aliquots of the reaction mixtures were analyzed on a YMC-pack Diol-120 column (YMC) with the HPLC system described above using 50 mM $NaNO_3$ as a mobile phase. To examine the substrate specificity of the enzyme, ι-carrageenan (from *Eucheuma spinosa*, Type V) and κ-carrageenan (from *Eucheuma cottonii*, Type III) were purchased from Sigma-Aldrich. Agarose was purchased from Bio-Rad. Porphyran was prepared from *Porphyra yezoensis*, according to the method of Takahashi *et al.* (*17*). Each polysaccharide was used as a substrate under the standard conditions.

Nucleotide Sequence Accession Numbers—The 16S rDNA and *cglA* gene nucleotide sequence data of strain CL19 have been submitted to the DDBJ, EMBL, and GenBank databases under accession numbers AB261170 and AB261169, respectively.

RESULTS AND DISCUSSION

16S rDNA Sequence Analysis of the Isolate—To determine the phylogenetic position of the λ-carrageenan–degrading bacterium strain CL19, its 16S rDNA sequence was determined and analyzed using comparative sequence analysis against known 16S rDNA sequences. The 16S rDNA sequence of the isolate showed a relationship (99%) to *Pseudoalteromonas haloplanktis* str. TAC125 (CR954246), *Pseudoalteromonas distincta* KMM638^{T} (AF082564), *Pseudoalteromonas elyakovii* KMM 162^{T} (AF082562), and *Pseudoalteromonas agarovorans* KMM 255^{T} (AJ417594), suggesting that the isolate is a novel member of the genus *Pseudoalteromonas*. Bacteria within the genus *Pseudoalteromonas* are known to display notable phenotypic diversity reflecting their ecological habitats (*18*). Strain CL19 was also expected to adapt to its habitat. Further genetic and physiological analyses of strain CL19 are in progress.

Purification of λ-Carrageenase—Addition of λ-carrageenan to the medium increased the enzyme activity in culture broth of strain CL19 by about 200-fold compared to that in the absence of additional carbohydrates to marine broth 2216 medium, although the growth of the strain CL19 was almost unchanged. Maximal activity was observed in the presence of 0.4% λ-carrageenan, which corresponded to 4,600 U/liter. The enzyme was inactivated by a column chromatography without addition of high concentration (>0.5 M) of carbohydrate such as sorbitol, sucrose, and trehalose to the buffers. Then, we used sorbitol as a stabilizer for the enzyme during all the purification steps. The enzyme was purified homogeneously (160-fold) by three steps of chromatographies. The purified enzyme had a specific activity of 253 U/mg protein at 35°C. The molecular mass of the enzyme was determined to be 100 kDa using gel-filtration chromatography. The SDS-PAGE of the purified enzyme gave a single band with an apparent mass of 100 kDa (Fig. 1A), consistent with the result of gel-filtration chromatography. Consequently, the enzyme is a monomeric protein. The hydrolytic activity of the protein was visualized by activity staining with cethylpyridinium chloride (Fig. 1B). Cetylpyridinium chloride forms insoluble salt with acidic polysaccharide like alginic acid and carrageenans. A clear zone against white background was formed at

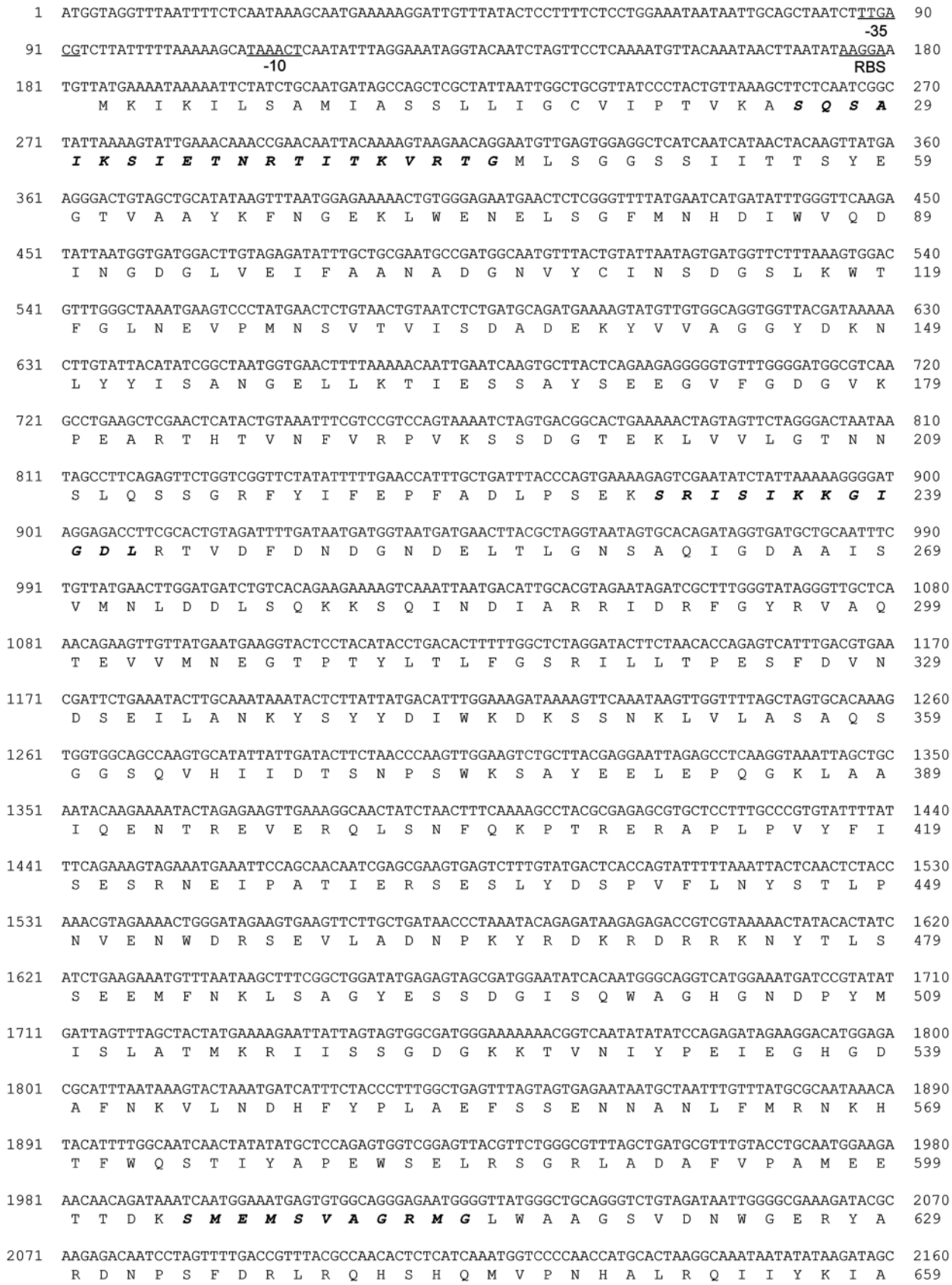


Fig. 6. ***Continued***

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2161  GTCTGGTGCCAGATATATAAATAACTTCGGCTTCAATCAGGAGTATATGAGCCTTGCTTGGGAGTTAATTGGTAAAGGCGCTCTTTATGT  2250
       S  G  A  R  Y  I  N  N  F  G  F  N  Q  E  Y  M  S  L  A  W  E  L  I  G  K  G  A  L  Y  V   689

2251  ACCAAAGCGTGAAGAATTATTAAGCTTGTCACCAGTTCACATAAGTATGAAAGAACCAGATCCAATCTACAGAGAAACATCAAATAATGT  2340
       P  K  R  E  E  L  L  S  L  S  P  V  H  I  S  M  K  E  P  D  P  I  Y  R  E  T  S  N  N  V   719

2341  GAAGTGGACTACGTTCTATGATGAAGAAAAAGACTCTATTCCATATGTGTTTAGTCGCTTAAATGGTACTTGGCCAGGTGCAAAAACTTT  2430
       K  W  T  T  F  Y  D  E  E  K  D  S  I  P  Y  V  F  S  R  L  N  G  T  W  P  G  A  K  T  L   749

2431  GCCGTGGGATTACTCTAATTATGCTGCAGATACTAAAGAAAGGCGATTAGATTTTATACCAAAATTTCCTAAAGGATTAGTTTTAATTAC  2520
       P  W  D  Y  S  N  Y  A  A  D  T  K  E  R  R  L  D  F  I  P  K  F  P  K  G  L  V  L  I  T   779

2521  GCCTGTTCAGCAAGGTAAATTTAAAGATGAAGGCACCGTTAGAGGCACATTGGCTGATAACATGCACCCTATCTATAAAGATATAATGAA  2610
       P  V  Q  Q  G  K  F  K  D  E  G  T  V  R  G  T  L  A  D  N  M  H  P  I  Y  K  D  I  M  K   809

2611  AGAGTATATCACTGACGGTAAAAACTACTATAACGCTAATGGTGAGCAAGTAATGGCTGCTGATAGTGTTAGATACAGACAAATTAAAAA  2700
       E  Y  I  T  D  G  K  N  Y  Y  N  A  N  G  E  Q  V  M  A  A  D  S  V  R  Y  R  Q  I  K  N   839

2701  TAAGATTGAAGAAAAATCCAATCTTTTGCCAATGACTGTATCAGGAGAAGCTGCTTGGGTTGTTGCTCAATCTGCTAGAAAGCATTTGCG  2790
       K  I  E  E  K  S  N  L  L  P  M  T  V  S  G  E  A  A  W  V  V  A  Q  S  A  R  K  H  L  R   869

2791  ACTGACGCTGGTTGATAGTGGTTATTTAAATCCGAGTAACAAAGTTGCTAAGGTTAAATTTAACTCTGTAACACCAGTAGCGATAGTAGA  2880
       L  T  L  V  D  S  G  Y  L  N  P  S  N  K  V  A  K  V  K  F  N  S  V  T  P  V  A  I  V  D   899

2881  CGTATTGTCAGGTGAGACATTTTCGCCAGATTCAAATGGGGTTGTAGAAATACCGGTCTTAGCTGGTGCTTTTAGGTTCATTGATGTGAA  2970
       V  L  S  G  E  T  F  S  P  D  S  N  G  V  V  E  I  P  V  L  A  G  A  F  R  F  I  D  V  K   929

2971  AATTACTGAAGACCTTAGAAACATGCAAAGTTCAACATTGTAATTACGGCGTTTAATTTATAGTGCGGGATTGAAGTTTATTAGTCCTGC  3060
       I  T  E  D  L  R  N  M  O  S  S  T  L  *                                                  942
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Fig. 6. **Nucleotide and amino acid sequences of CglA.** A putative ribosome-binding sequence is indicated by RBS. Sequences similar to the consensus promoter of *E. coli* are indicated by –35 and –10. The stop codon is marked with an asterisk. N-terminal and internal amino acid sequences of the mature CglA isolated from the culture of strain CL19 are shown in bold-oblique typeface.

corresponding position of the purified protein band. It showed the absence of carrageenan polymer in the zone due to enzymatic hydrolysis by the purified protein. The N-terminal sequence of the purified protein was determined to be SQSAIKSIETNRTITKVRTG. Two internal amino acid sequences were determined to be SRISIKK-GIGDL and SMEMSVAGRMG after lysyl endopeptidase treatment.

Biochemical Properties of the Enzyme—The pH optimum for the activity of the enzyme was examined (Fig. 2A). The enzyme showed maximal activity at pH 7.0. The optimal temperature for the activity of the enzyme was 3°C (Fig. 2B). In the low temperature range (<10°C), this enzyme showed significant activity. The thermostability of the enzyme was examined in the presence of 0.4 M NaCl. The enzyme was stable at up to 25°C during 120 min incubation. When 0.5 M sorbitol was added to the buffer, the enzyme was stable at up to 30°C during 120 min. The activity of the enzyme strongly depended on the concentration of monovalent salt (Fig. 3). Maximal activity was observed in the presence of 0.4 M NaCl, which is almost the same concentration with that in seawater. Other monovalent salts such as KCl, LiCl, CsCl, and RbCl are thought to be able to compensate for the role of NaCl. Divalent salts such as $CaCl_2$, $MgCl_2$, $FeCl_2$, $MnCl_2$, and $CoCl_2$ at 1 mM had no apparent effect on the enzyme activity.

Analysis of Hydrolysis Products and Substrate Specificity—After completion of enzymatic λ-carrageenan hydrolysis, the main product was purified and analyzed using ESI-MS and ^{1}H- and ^{13}C-NMR spectrometry. The results confirmed that the enzyme cleaved the β-1,4 linkages of the λ-carrageenan backbone, resulting in a tetrasaccharide of ideal structure with galactose 2,6-disulfate at the reducing end (Fig. 4).

The λ-carrageenan hydrolysis pattern of the enzyme was monitored on HPLC on a gel-filtration column (Fig. 5). In the initial stage, CglA hydrolyzed λ-carrageenan to generate a series of large oligosaccharides. This hydrolysis pattern indicates that this enzyme acts on λ-carrageenan in an endo-manner. Undigested material was present throughout the reaction. To assess the substrate specificity of this enzyme, different types (ι-, κ-, and λ-) of carrageenan and red seaweed galactans were used as substrates. No activity was detected toward ι- and κ-carrageenans, agarose, and porphyran (highly sulfated agarose) under the standard assay conditions. Taken together, this enzyme is an endo-β-1,4-carrageenose 2,6,2′-trisulfate-hydrolase with strict substrate specificity.

Cloning and Sequence Analysis of the Gene for CglA—First, the N-terminal and two internal amino acid sequences of the purified enzyme were determined as described above. Two primers (primers A and B) designed from the amino acid sequences were used for PCR, and a 1.6-kDa DNA fragment coding partial *cglA* gene was amplified and sequenced. Based on the results of the sequence analysis, primers were designed and used to amplify the flanking regions that include the whole *cglA* gene, as described in "MATERIALS AND METHODS." Finally, the 3.1-kb DNA fragment containing the *cglA* gene was sequenced. The G+C content of the gene was 37.3%. The fragment contains a single open reading frame (ORF), which begins with an ATG codon at nucleotide 185 and ends with a TAA codon at nucleotide 3013, as shown in Fig. 6. The ORF encodes 942 amino acid residues. Upstream from this ORF, the putative ribosome-binding

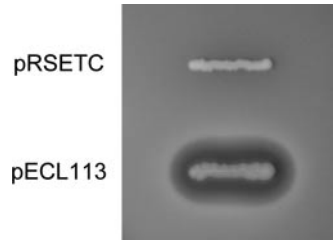

Fig. 7. **Heterologous expression of the *cglA* gene in *E. coli*.** *E. coli* harboring the plasmid pECL113 (containing structural gene for CglA) was streaked on a LB plate containing 0.5% λ-carrageenan and incubated at 25°C for 2 days. The activity of the λ-carrageenase expressed by the transformant was detected by flooding the solid medium with 10% cetylpyridinium chloride as a clear zone around the colony (lower). As a control, *E. coli* harboring the plasmid pRSETC was also cultured (upper).

sequence AAGGA was found, separated by 6 bp from the initiation codon ATG. It is remarkable that a database search using the BLAST program revealed that the nucleotide sequence of *cglA* has no significant similarity to nucleotide sequences deposited in the databases.

Amino Acid Sequence Analysis—As shown in Fig. 6, the N-terminal amino acid sequence SQSAIKSIETNRTITKVRTG and internal amino acid sequences SRISIKKGIGDL and SMEMSVAGRMG of the λ-carrageenase secreted by strain CL19 were all found in the deduced amino acid sequences at amino acids 26–45, 231–242, and 604–614, respectively. The molecular mass of the mature enzyme secreted by strain CL19 was calculated based on the deduced amino acid sequence (amino acids 26–942), to be 102,989 Da. A Database search using the BLAST program with the deduced amino acid sequence of CglA showed that this protein has no similarity to reported proteins, indicating this protein is a completely new enzyme.

Expression of the CglA Gene in E. coli—To clarify whether λ-carrageenan hydrolysis is catalyzed by a single protein, heterologous expression of the *cglA* gene was performed using *E. coli* as a host. We amplified a 2.8-kb fragment of the *cglA* gene corresponding to nucleotides 257–3013, which encode the mature λ-carrageenase purified from strain CL19, using appropriate primers and cloned the fragment into an expression vector, pRSETC in the frame to construct a plasmid, pECL113. The enzyme activity expressed by *E. coli* transformant harboring pECL113 was confirmed by cetylpyridinium chloride staining of a Luria-Bertani (LB) plate containing 0.5% λ-carrageenan after the transformant was cultured on the medium (Fig. 7). After 2-days incubation, a clear zone was formed around the colony by enzymatic hydrolysis of λ-carrageenan, showing heterologous and fuctional production of the λ-carrageenase was successful. The result demonstrated that λ-carrageenan hydrolysis is catalyzed by a single gene product.

In conclusion, we isolate a novel λ-carrageenan–degrading bacterium, and purified and characterized a novel enzyme, λ-carrageenase, that it produced. Moreover, the nucleotide sequence for the enzyme was determined. This protein is a new enzyme with no similarity to any reported proteins. These findings will provide a new category for enzyme.

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