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Isolation and Characterization of *Marinobacter* sp. KS-1, which Produces a Chondroitin Sulfate-like Mucopolysaccharide

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In an effort to isolate a bacterium producing chondroitin sulfate (CS), a marine bacterium, KS-1, which produces mucopolysaccharides, was isolated from seawater and identified as *Marinobacter* sp. based on analyses of its morphological and biochemical traits and 16S rDNA sequence. Agarose-gel electrophoresis showed that the KS-1 strain produces a CS-like mucopolysaccharide. Structural analysis using Fourier transform infrared spectroscopy revealed that the structure of the CS-like mucopolysaccharide produced by *Marinobacter* sp. KS-1 is similar to that of dermatan sulfate (CS B). However, the molecular mass of the CS-like mucopolysaccharide is higher than that of standard chondroitin sulfates. Considering the above results, we conclude that the *Marinobacter* sp. KS-1 produces a CS-like mucopolysaccharide that differs somewhat from CS B in molecular mass.

Key words: Chondroitin sulfate, Marine bacterium, *Marinobacter* sp., Mucopolysaccharide

Introduction

Chondroitin sulfates (CSs) are a type of mucopolysaccharide that consist of D-glucuronic acid, *N*-acetyl-D-galactosamine, and a sulfate group. Depending on the location of the sulfate group in the basic skeleton of the disaccharide, the molecules are classified as chondroitin 4-sulfate (CS A), dermatan sulfate (CS B), and chondroitin 6-sulfate (CS C) (Kang et al., 1994). CSs are known to be effective in the control of cell electrolytes (Tanaka, 1978), the protection of articular tissue and the formation of bone by calcification, the suppression and prevention of atherosclerosis with blood clarification (Prince and Navia, 1983), corneal protection (Mac-Rae et al., 1983), the suppression of bacterial infections (Smith and Schurman, 1983), anticoagulation of blood (Bjornsson et al., 1982; Mourao et al., 1996), and anti-inflammatory and pain relief effects (Fontenele et al., 1996). In addition, CSs are also effective against cancer (Moon et al., 1998), asthma, miasma, thirst, trench mouth, and laryngitis (Hjerpe et al., 1979; Krueger et al., 1992; Ha and Kim, 1999). Currently, as the physiological activities of CSs are in the spotlight, many studies are being conducted on CSs for use in medical products, cosmetics, and functional food materials. The domestic consumption of CS products relies heavily on imports, and the amount of imports is increasing every year.

Many suppliers obtain CSs commercially from mammalian species, which are more cost-effective (Im et al., 2009). However, the frequent occurrence of animal epidemics, such as mad-cow disease, foot and mouth disease, and hog cholera, has been a serious issue that mitigates the use of CSs from mammalian sources (Sim et al., 2005; Im et al., 2009; Kim et al., 2010). Thus, CSs are currently purified from marine sources (Im et al., 2009). Among them, shark cartilage is the one most commonly used as a commercial source (Im et al., 2009; Kim et al., 2010). However, several problems are likely to occur with this source, such as overfishing, rising prices of this raw material, and environmental pollution caused by

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the dumping of unused shark parts into the ocean after fishing. Therefore, other organisms need to be investigated as alternative sources of CS production. The objective of this study was to evaluate the possibility of CS production using a microorganism.

Materials and Methods

Screen for a mucopolysaccharide-producing microorganism

To isolate a bacterium producing CSs, seawater was collected from the Haeundae Coast, Korea, and the samples were smeared on PPES-II agar medium (Taga, 1968; 0.2% polypeptone, 0.1% proteose peptone, 0.1% yeast extract, 0.1% soytone, 0.001% ferric citrate, and 2.5% NaCl). After incubation at 25°C for 5 days, each bacterial colony on the plate was cultured for 48 h at 25°C in PPES-II broth medium. A screen for CS-producing bacteria was carried out as described by Tandavanitj et al. (1989). The bacterial cells were harvested by centrifugation (7,000×*g*, 30 min) and suspended in 1% phenol solution. Two volumes of ethanol were then added after removing the supernatant by centrifugation (30,000×*g*, 2 h). The polysaccharide strands formed by the previous treatment were twined around a glass rod and dissolved in water. The solution was subjected to the carbazole-sulfuric acid test, and bacteria that caused a dark red color in the test were selected as candidate strains producing a mucopolysaccharide.

Identification of a mucopolysaccharide-producing microorganism

The strains isolated in this study were identified by morphological observations, conventional biochemical tests, and 16S rDNA sequence analysis. Morphological observations and conventional biochemical tests were performed using methods described by Smibert and Krieg (1994). Analysis of the 16S rDNA sequence was carried out based on Moyer et al. (1994). Polymerase chain reaction (PCR) was performed using intact cells treated for 5 min at 95°C as a template. The thermal profile used was 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 1 min at 72°C. A final extension step consisting of 5 min at 72°C was included. The 16S rDNA amplified by PCR was purified, inserted into the pGEM-T Easy vector (Promega, Madison, WI, USA), and sequenced. The resulting sequence data were analyzed using BLASTN (http://www.ncbi.nlm.nih.gov/BLAST). The sequences were aligned using the program CLUSTAL W, and a phylogenetic tree was generated using the MEGA 2.0 program.

Agarose-gel electrophoresis

According to the method of Volpi (1999), mucopolysaccharides produced by the isolated strain were further separated by agarose-gel electrophoresis. Agarose gels were made as 1% agarose in 0.04 M barium acetate buffer (pH 5.8). Using 0.05 M 1,2-diaminopropane (pH 9.0 in the form of acetic acid) as the tank buffer, the gel was electrophoresed for 80 min at 50 mA. After electrophoresis, the gel was dyed for at least 6 h in 0.1% cetyltrimethylammonium bromide. The solution was observed in a dark background and dyed again in toluidine blue (2% in 50:49:1 ethanol:water:acetic acid) for 15-30 min. The gel was then decolored using a decoloring agent (50:49:1 ethanol:water:acetic acid) to verify the existence of mucopolysaccharides.

Fourier transform infrared spectroscopy (FT-IR) analysis

To verify the structure of the CS extract, a chemical structural analysis was conducted using an FT-IR-5300 spectrophotometer (JASCO; Oklahoma, USA) at the Cooperative Laboratory Center, Pukyong National University.

CS content

The CS content was tested according to the method of Tandavanitj et al. (1989). The harvested cell pellets (150 mg of wet weight) were mixed with water to 50 ml. The solution (10 ml) was then passed through filter paper (Advantec 5A; Toyo, Tokyo, Japan) and used as a test solution. Five milliliters of borax sulfate solution was placed in Nessler tubes and cooled with ice water. One milliliter each of the sample and standard solutions was carefully added and cooled with mixing. After 10 min of heating in a water bath, the solutions were immediately cooled using ice water. To each solution, 0.2 ml of carbazole was added and mixed. After 15 min of heating in a water bath, the solutions were cooled to room temperature using ice water. A water solution was prepared as a control using the same procedure. The absorption of each sample at 530 nm was measured, and the mucopolysaccharide content was calculated according to the method of Tandavanitj et al. (1989).

Results and Discussion

Isolation and identification of a mucopolysaccharide-producing microorganism

Mucopolysaccharides, including CSs and heparan sulfate, were obtained commercially from marine sources such as whale shark, skate, squid, salmon, king crab, and sea cucumber (Im et al., 2009). Among these, shark cartilage is the most commonly used commercial source (Im et al., 2009), but overfishing and rising prices of this raw material will likely occur in the future. Therefore, other organisms need to be developed as alternatives to replace this marine resource.

We attempted to isolate a microorganism that produces CS. In the current study, we isolated a total of 150 colonies with distinguishable shapes from seawater samples. Among them, one species, KS-1, was selected as a candidate strain producing mucopolysaccharide as revealed by the test of Tandavanitj et al. (1989). To identify this strain, morphological, biochemical, and genetic analyses were performed. Gram staining revealed that this strain is Gram negative (data not shown). The results of biological and physiological tests are listed in Table 1. For the genetic characterization of KS-1, PCR was carried out to amplify the 16S rDNA as described above. The 16S rDNA sequences (1,476 bases) of KS-1 were aligned in comparison with available sequences from GenBank (Fig. 1). The sequences of KS-1 shared the greatest similarity with those of *Marinobacter* sp. R-28768 (99% similarity; data not shown). A phylogenetic tree based on bacterial 16S rDNA sequences showed close relationships of KS-1 with the genera *Marinobacter* (Fig. 2). Based on these results, the isolated KS-1 was identified as *Marinobacter* sp.

Table 1. Biological and physiological characteristics of the isolate KS-1

Test	Result	Test	Result
Gram staining	-	Glucose	+
Motility	+	Lactose	-
Indole	-	Sucrose	+
MR	-	Mannitol	-
VP	-	Ducitol	-
Oxidase	+	Solicin	-
Catalase	+	Adonitol	-
Citrate	-	Inocitol	-
Gelatine	-	Sorbitol	-
Dnase	-	Arabinose	-
KIA	K/A	Raffinose	-
Arginine	+	Growth at	
Lysine	-	37°C	+
Orinithine	-	41°C	-
Maltose	+	Growth at	
Xylose	-	0% NaCl	-
Trehalose	-	2% NaCl	+
Cellobiose	-	5% NaCl	+

Identification of mucopolysaccharides using agarose-gel electrophoresis

Agarose-gel electrophoresis is used for the identification and separation of various mucopolysaccharides, such as slow- and fast-moving heparin, heparan sulfate, and CSs (Volpi, 1993; 1999). Analysis of KS-1 by this technique indicated the presence of a mucopolysaccharide similar to CSs (Fig. 3). However, no other mucopolysaccharides, including heparin, heparan sulfate, hyaluronic acid, or CS B, were found (Fig. 3). In addition, the gel profiles indicated that the molecular mass of the CS-like mucopolysaccharide produced by KS-1 strain is larger than those of the standard CSs (CS A, CS B, and CS C).

FT-IR analysis of the CS-like mucopolysaccharide produced by *Marinobacter* sp. KS-1

To determine the chemical structure of the mucopolysaccharide produced by KS-1, the molecule was analyzed by FT-IR spectrophotometry in comparison with the standard CSs. The results of the FT-IR analysis are shown in Fig. 4. Comparing the peak absorption wavelengths of the mucopolysaccharides from KS-1 and standard CSs, the FT-IR analysis revealed that the structure of the mucopolysaccharide produced by *Marinobacter* sp. KS-1 is similar to that of CS B, which is known to be a bioactive compound. Therefore, we conclude that the *Marinobacter* sp. KS-1 strain reported here produces a CS like-mucopolysaccharide that differs slightly from CS B in molecular mass.

Increasing mucopolysaccharide content with longer treatment times with 1% phenol

To obtain mucopolysaccharides, organic solvents are usually used to precipitate and remove protein (Tandavanitj et al., 1989). The yield of mucopolysaccharide increases as the time of treatment with phenol is extended. Here, the effect of the phenol treatment time on the yield of mucopolysaccharide from KS-1 pellets was investigated. To measure the mucopolysaccharide content, mucopolysaccharide was extracted from *Marinobacter* sp. KS-1 as described in the Materials and Methods. The content was measured in accordance with the Health Functional Food Code (KFDA; Korea Food and Drug Administration, 2009). As expected, the yield of CS-like mucopolysaccharide increased as the treatment

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   1 AGAATTCTTACACATGCAAGTCGAGCGGTAACAGGGGGAGCTTGCTCCCC
  51 GCTGACGAGCGGCGGACGGGTGAGTAATGCATAGGAAACTGCCCAGTAGT
 101 GGGGGATAGCCCGGGGAAACCCGGATTAATACCGCATACGCCCTTTGGGG
 151 GAAAGCAGGGGATCTTCGGACCTTGCGCTATTGGATGTGCCTATGTCGGA
 201 TTAGCTAGTTGGTGGGGTAAGAGCCTACCAAGGCGACGATCCGTAGCTGG
 251 TCTGAGAGGATGATCAGCCACATCGGGACTGAGACACGGCCCGAACTCCT
 301 ACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTGATCCA
 351 GCCATGCCGCGTGTGTGAAGAAGGCTTTCGGGTTGTAAAGCACTTTCAGT
 401 GAGGAGGAAAACCTTACGATTAATACTCGTGAGGCTTGACGTTACTCACA
 451 GAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCCGCGGTAATACGGAGGG
 501 TGCAAGCGTTAATACGGAATTACTGGGACGTAAAGCGCGCGTAGGTGGTT
 551 TGATAAGCGAGATGTGAAAGCCCCGGGCTCAACCTGGGAACGGCATTTCG
 601 AACTGTCAGGCTAGAGTATGGTAGAGGAGTGTGGAATTTCCTGTGTAGCG
 651 GTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGGCACTCT
 701 GGACCAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTA
 751 GATACCCTGGTAGTCCACGCTGTAAACGATGTCAACTAGCCGTTGGGACT
 801 CTTGAAGTCTTAGTGGCGCAGCTAACGCACTAAGTTGACCGCCTGGGGAG
 851 TACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGC
 901 GGTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTGGCC
 951 TTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCT
1001 GACACAGGTGCTGCATGGCCGTCGTCAGCTCGTGTCGTGGGATGTTGGGT
1051 TAAGTCCCGTAACGAGCGCAACCCCTATCCCTAGTTGCTAGCAGTTCGGC
1101 TGAGAACTCTAGGGAGACTGCCGG-TGACAAACCGGAGGAAGGTGGGGAT
1151 GACGTCAGGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAA
1201 TGGTGCGTACAGAGGGCTGCAAACCCGCGAGGGGGAGCTAATCTCACAAA
1251 ACGCATCGTAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCGGA
1301 ATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCC
1351 TTGTACACACCGCCCGTCACACCATGGGAGTGGATTGCACCAGAAGTGGT
1401 TAGTCTAACCTTCGGGAGGACGATCACCACGGTG-GGTTCAGACTGGGGT
1451 AAGTCGTAACAAGGAACCGGATCCAC
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Reference (accession no.)	Identity (%)
Marinobacter sp. R-28768 (AM944524.1)	99
Marinobacter sp. R-28770 (AM944523.1)	99
Marinobacter sp. ASs2019 (DQ665806.1|)	99
Marinobacter sp. SCSWC22 (FJ461447.1)	99

Fig. 1. 16S rDNA sequences (1476 bp) of strain KS-1 and homology search based on 16S rDNA sequences.

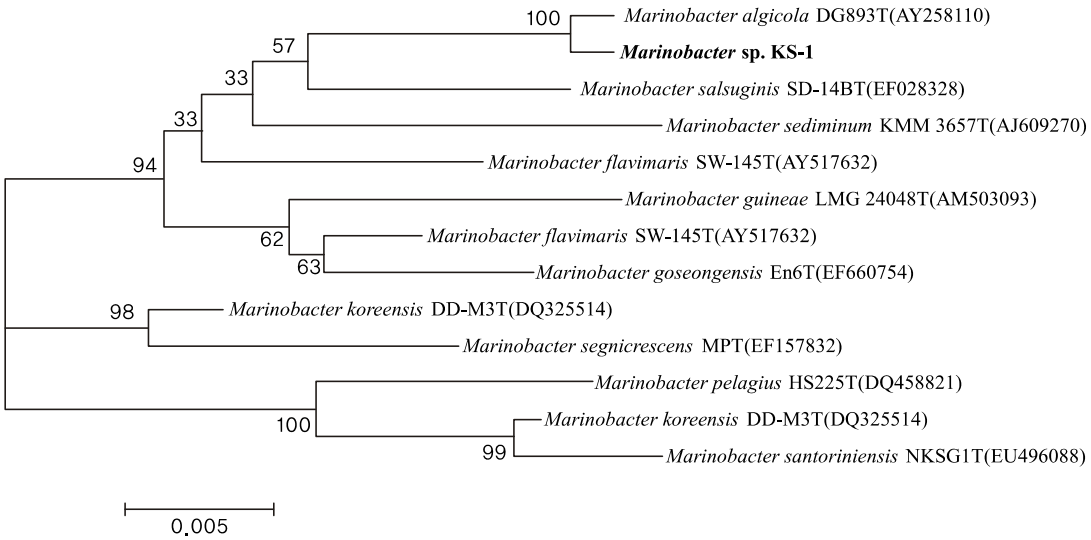

Fig. 2. Phylogenetic tree based on 16S rDNA sequences of *Marinobacter* sp. KS-1 and closely related members of the genus *Marinobacter*. Numbers at nodes are levels of bootstrap support based on neighbour-joining analyses of 1000 replications.

Table 2. Effect of phenol treatment time on the yield of chondroitin (CS) like mucopolysaccharide

Treatment time of 1% phenol	Wet weight of cell pellets (g)	Total polysaccharide mass (mg)	CS-like mucopolysaccharide content (%)
15 h	3	137.5	20.7
18 h	3	157.2	22.1
21 h	3	185	26.0

The CS content was measured according to the method of Tandavanitj et al. (1989).

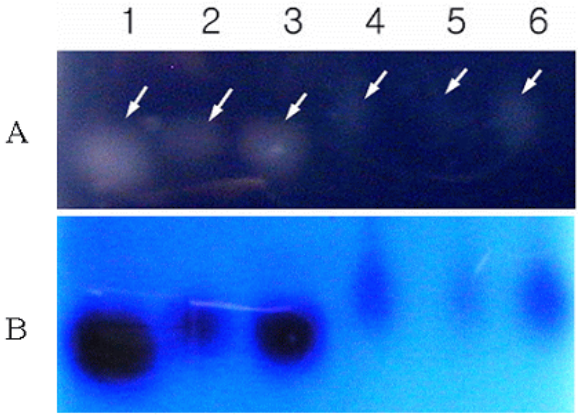

Fig. 3. Agarose-gel electrophoresis. A, Precipitation of bands in cetyltrimethyammonium bromide for 6 h; B, Bands stained with toluidine blue. Lane 1, CS A; lane 2, CS B; lane 3, CS C; lane 4-6, extraction of mucopolysaccharides produced by *Marinobacter* KS-1.

time with 1% phenol was extended (Table 2). After 15, 18, and 21 h of treatment using 3 g of harvested cell pellet, the yields of CS-like mucopolysaccharide were 20.7%, 22.1%, and 26.0%, respectively (Table 2). That is, the content of the CS-like mucopolysaccharide increased with the increasing treatment time of 1% phenol.

Im et al. (2009) reported that the yields of CS from various marine organisms ranged from 1.3% to 12.5%. CS from shark cartilage and skate cartilage exhibited relatively high yield (9.7% and 12.5%, respectively). In comparison, the CS-like mucopolysaccharide from KS-1 showed higher efficiency in terms of yield. However, the content of the CS-like mucopolysaccharide from the microorganism in the overall specimen was lower than that from animal or marine sources.

In the current study, we isolated a marine bacterium that produces a CS-like mucopolysaccharide, and defined the characteristics of the mucopolysaccharide produced by the isolated strain. We hope that the current study will contribute to future studies on CS production to replace that from shark cartilage, a main commercial CS source.

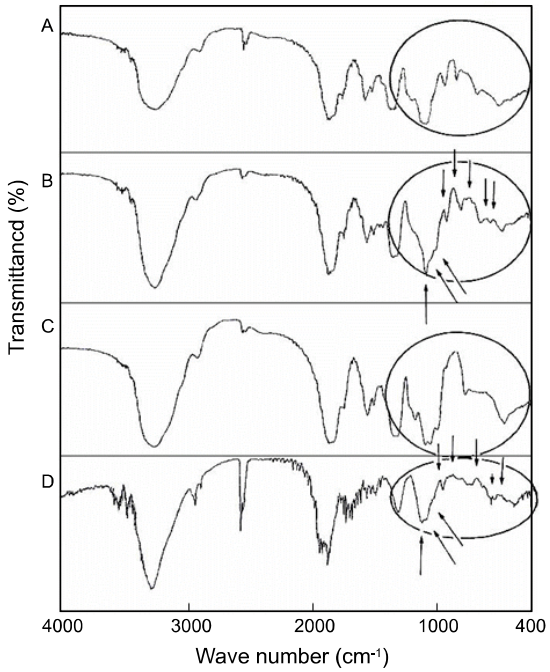

Fig. 4. Fourier transform infrared spectroscopy (FT-IR) spectrums. (A) CS A; (B) CS B; (C) CS C; (D) mucopolysaccharides produced by *Marinobacter* KS-1.

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