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Purification and Characterization of Chitinase from a Marine Bacterium, *Vibrio* sp. 98CJ11027

Shin Hye Park, Jung-Hyun Lee, and Hong Kum Lee*

Microbiology Laboratory, Korea Ocean Research & Development Institute, Ansan P. O. Box 29, Seoul 425-600, Korea

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Chitin-degrading marine bacterial strain 98CJ11027 was isolated from bryozoa from the coastal area of Cheju Island, Korea, and identified as a member of the genus *Vibrio*. The molecular mass of the main extracellular chitinase (chitinase I), purified from strain 98CJ11027, was estimated to be 98 kDa. The optimal condition for chitinase I activity is pH 6.0 and 45°C. The activity was inhibited by Fe^{+2} and Cu^{+2}. Chitinase I displayed the hydrolysis type of chitobiosidase and catalyzed reversed hydrolysis leading to the synthesis of tetraacetylchitotetraose.

Key words: chitinase, purification, marine bacterium, *Vibrio* sp.

Chitin is a polymer of β-1,4-linked N-acetylglucosamine (GlcNAc) and a very abundant natural polymer. It is the main structural compound of cell walls of fungi, insect exoskeletons and the shells of crustaceans (29). Chitin hydrolysate can be used as the carbon and nitrogen source in the production of single-cell proteins (28). Chitinase is involved in the process of producing mono- and oligosaccharides from chitin. Furthermore, chitinase is a potential antifungal agent through its chitin degradation activity (15, 20, 25, 29).

Chitinase is widely distributed in bacteria, actinomycetes, and plants (26, 33, 35, 36). Chitinase-producing marine bacteria play an important role in the degradation of chitin in the oceans (11, 37). Chitinases from marine bacteria have been isolated and their properties reported (10, 13, 14, 23, 24, 31). However, chitinases produced by marine bacteria isolated from coastal areas of Korea have not been studied extensively.

In this paper, we describe the isolation of a chitinase-producing bacterium from bryozoa of Cheju Island, Korea, and the characteristics of chitinase produced by the isolate, *Vibrio* sp. 98CJ11027.

Materials and Methods

Sample collection and screening of chitin-degrading microorganisms

Seawater, sediment and marine animals were collected from the coastal area of Cheju Island, Korea. Samples were diluted 10 to 1,000 fold in sterile seawater and spread on chitin-containing minimal agar plates (colloidal chitin 12 g, $(NH_4)_2SO_4$ 2 g, KH_2PO_4 0.7 g, $Na_2HPO_47H_2O$ 0.2 g, $FeSO_47H_2O$ 1 mg, $MnSO_45H_2O$ 1 mg, agar 15 g, distilled water 500 ml, aged sea water 500 ml, pH 7.0). After incubation for 2 weeks at room temperature, clear-hole forming bacteria were selected as the chitinase producer. Among the bacteria showing the chitinase activity, strain 98CJ11027 isolated from a bryozoa, was selected for the production and characterization of chitinase.

Identification of the isolate

The isolate was identified through its morphological and physiological properties according to Bergey's Manual of Systematic Bacteriology (2). The nearly complete nucleotide sequence of 16S rDNA was determined as described previously (17). The 16S rDNA sequence was compared to the sequences in the Ribosomal Database Project (19) by using the SEQUENCE_MATCH (v. 2.7) option and GenBank nucleotide database by using Basic Local Alignment Search Tool (BLAST) (1). Unaligned sequence data from the GenBank database and this study were aligned manually with the pre-aligned sequences downloaded from the RDP. Phylogenetic relationships were estimated using the Phylogenetic Inference Package (PHYLIP version 3.57c) (7). Jukes-Cantor evolutionary distances were calculated using the DNADIST program and dendrograms depicting phylogenetic relationships were derived using the FITCH program.

Culture conditions for chitinase production

The isolate 98CJ11027 was pre-cultured in the same medium as described above without agar for 3 days at 30

* To whom correspondence should be addressed.
(Tel) 82-31-400-6241; (Fax) 82-31-406-2495
(E-mail) hklee@kordi.re.kr

°C with stirring at 140 rpm. The medium (500 ml) was inoculated with 1% of preculture in a 2-liter Erlenmeyer flask and incubated for 6 days at 30°C on a reciprocal shaker.

Preparation of colloidal chitin

Colloidal chitin was prepared by the method of Rodriguez-Kabana *et al.* (30), by partial hydrolysis of chitin (Sigma, St. Louis, USA) with 10 N HCl for 2 h at room temperature. The colloidal chitin was washed several times with large volumes of distilled water to adjust the pH to 7.0.

Assay of chitinase activity

Chitinase activity was measured by determining the release of *p*-nitrophenol from *p*-nitrophenyl β-D-N-acetylglucosaminide (PNG) on the basis of the method of Roberts and Selitrennikoff (29) with modification. 100 µl of enzyme solution was added to 100 µl of 10 mM PNG (Sigma) and 300 µl of 0.1 M citrate-phosphate buffer (pH 6.0). After incubation at 37°C, the reaction was stopped with the addition of 500 µl of 1 M Na_2CO_3. The amount of p-nitrophenol was measured at 405 nm. One unit of chitinase was defined as the amount of enzyme needed to release one mmol of p-nitrophenol per hour.

Effect of pH and temperature optima on the activity

The effect of pH on the purified chitinase was determined at different pH values (4.5 to 10.0) under standard assay conditions. The buffer systems used were as follows: 0.1 M citrate-phosphate buffer, pH 4.5-6.5; 0.1 M phosphate buffer, pH 6.5-8.5; 0.1 M glycine-NaOH buffer, pH 8.5-10.0. The optimal temperature for the chitinase activity was determined in the range of 20 to 50°C under standard assay conditions. The purified enzyme was added to 0.1 M citrate-phosphate buffer (pH 6.0), which was preincubated for 5 min at the temperature tested. The reaction was started with the addition of PNG.

Effect of metal ions on chitinase activity

The influence of metal ions on the activity of chitinase was investigated in 0.1 M imidazole-HCl (pH 7.0). The purified chitinase was incubated with 1 or 5 mM of metal salt for 30 min at room temperature, and the remaining activity was measured under the standard assay conditions.

Protein estimation

Protein concentrations were measured according to Bradford's method (4) using bovine serum albumin as the standard.

Purification of chitinase

After cultivation, the cells were removed by centrifugation at 5,000 × *g* and 4°C for 20 min. Proteins in the cell-free culture broth (900 ml) were precipitated with ammonium sulfate (30%, w/v). The precipitate was obtained by centrifugation (16,000 × *g*, 30 min, 4°C) and suspended in 3 ml of 0.1 M citrate-phosphate buffer (pH 6.0). The suspension was eluted through a Sephadex G-25 (Pharmarcia, Uppsala, Sweden) column (1 by 6 cm) to remove low molecular compounds and then lyophilized. The enzyme sample was applied onto a Q-Sepharose (Pharmarcia) column (1.6 by 13 cm) equilibrated with 30 mM Tris-HCl (pH 9.0). Proteins were eluted with a linear gradient of 0 to 1 M KCl in 30 mM Tris-HCl (pH 9.0) at a flow rate of 4 ml/min. The active fractions were pooled and lyophilized. The concentrated sample was dissolved in 200 µl of 25 mM Tris-HCl (pH 7.0) and loaded onto a Sephadex G-200 (Pharmarcia) column (1.5 by 60 cm) equilibrated with 25 mM Tris-HCl (pH 7.0). Proteins were eluted with 25 mM Tris-HCl (pH 7.0) at a flow rate of 0.2 ml/min.

Sodium dodecyl sulfate-polyacrylamine gel electrophoresis

The molecular mass of the purified chitinase was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel, according to Laemmli's method (16). The sample, obtained from Sephadex G-200 column chromatography and then concentrated by freeze-drying, was applied onto a gel and run at 20 mA. The gel was stained with 0.2% silver nitrate (27).

Thin layer chromatography

Hydrolysis products of colloidal chitin and N-acetyl-chitooligosaccharides were analyzed by thin layer chromatography (TLC) according to the method of Lewis and Smith (18). The purified chitinase I of the isolate (0.5 U) was incubated with 1 mg of colloidal chitin or N-acetyl-chitiooligosaccharides (Sigma) in 1 ml of 0.1 M citrate-phosphate buffer (pH 6.0) for 0.5 to 6 h at 37°C. The hydrolysates were spotted onto a Silica gel 60 F_{254} sheet (Merk) and developed in a solution of n-butanol:methanol:25% ammonia solution:water (5:4:2:1). The spots were detected with aniline phthalate reagent.

Nucleotide sequence accession number

The sequence of 16S rDNA of strain 98CJ11027 has been submitted to GenBank database under accession number AF246980.

Results and Discussion

Selection of chitinase-producing marine bacteria

Thirty-eight chitinase-producing bacteria were isolated from marine sources on the selective medium containing colloidal chitin, and strain 98CJ11027 was selected for further study of chitinase due to its formatim of the largest clear zone on the chitin agar plate.

Taxonomic characteristics of chitinase-producing bacterium

The morphological, physiological and biochemical characteristics of the isolate were investigated (Table 1). The 98CJ11027 strain colony was rhizoid, smooth, translucent, and cream-colored. The nearly complete 16S rDNA sequence (1505 bases, positions 28 to 1521, *E. coli* numbering) of strain 98CJ11027 was determined. The SEQUENCE_MATCH option from the RDP and BLAST analysis of the GenBank was used to identify the most similar sequences available in the database. A phylogenetic tree based on 16S rDNA sequence data was constructed (Fig. 1). The strain closely resembled its nearest neighbors, *Vibrio alginolyticus* CIP 75.03 (X74690) (99.55 % similarity), *V. pelagius* ATCC 25916T (X74722) (99.24 % similarity), *V. natriegens* CIP 103193T (X74714) (99.24 % similarity), and *V. carchiariae* ATCC 35084T (X74693) (98.86% similarity). The morphological, physiological, and biochemical characteristics and 16S rDNA sequence indicated stain 98CJ11027 is a member of the genus *Vibrio*.

Table 1. Taxonomic studies on strain 98CJ11027

Characteristics	Strain 11027	*V. alinolyticus*
Shape	Rod	rod
Gram-staining	-	-
Motility	-	+
O/F test	O, F	O, F
Nitrate reduction to nitrite	+	+
Catalase	+	+
Oxidase	+	+
Growth in NaCl	1-7%	3-8%
Acetoin production	-	+
β-galactosidase	+	-
Arginine dihydrolase	-	-
Lysine decarboxylase	+	+
Ornithine decarboxylase	+	+
Citrate utilization	+	-
Gelatinase	+	+
Chitinase	+	+
Utilization : Glucose	+	+
Manitol	+	+
Inositol	-	-
Sorbitol	-	-
Rhamnose	-	-
Sucrose	+	+
Arabinose	-	-

Purification of chitinase I

Extracellular chitinase from strain 98CJ11027 was efficiently concentrated by a salting-out method with ammonium sulfate. Two fraction peaks containing chitinase activity (chitinase I and II, respectively) were obtained through Q-Sepharose column chromatography (not shown). Chitinase I, the main enzyme, was purified by gel filtration on a Sephadex G-200 column. Extracellular chitinase I was purified up to 33 fold from culture supernatant in 27% recovery (Table 2). The low purification factor of the enzyme may be caused by the non-efficient assay method. The enzyme exhibited chitobiosidase activity (discussed later), and therefore the specific activity of the purified enzyme,

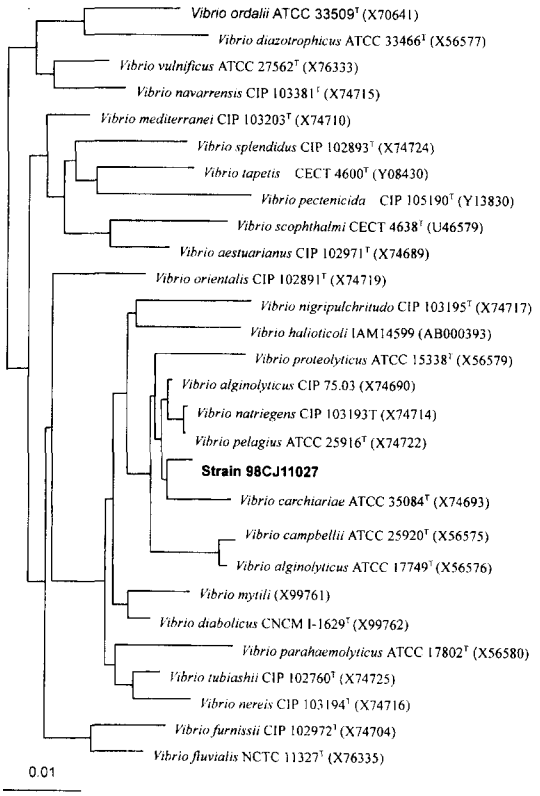

Fig. 1. Phylogenetic tree based on almost colmplete 16S rDNA sequences data (36 to 1427 in *Escherichia coli* numbering), showing the location of strain 98CJ11027. The scale bar represents 0.01 substitution per base position.

Table 2. Purification of chitinase I from strain 98CJ11027

Purification step	Activity		Protein (mg)	Purification Fold	Yield (%)
	Total (U)	Specific (U/mg)			
Crude	259.2	1.3	199.4	1	100
$(NH_4)_2SO_4$ precipitation	304.8	5.9	51.7	4.5	117
Q-Sepharose	184.0	20.0	9.2	15.4	71
Sephadex G-200	68.8	43.0	1.6	33.1	27

was not increased enough compared with that of the crude culture broth by the assay method using PNG as substrate.

Molecular weight of chitinase I

The molecular weight of the enzyme was calculated to be 91kDa by gel filtration on Sephadex G-200. SDS-PAGE analysis of the purified enzyme revealed one protein band with an estimated molecular weight of 98 kDa (Fig. 2). The molecular weights of chitinases from marine bacteria are mostly around 60 kDa (10, 24, 31, 32, 34). However, the molecular weight of the chitinase I from strain 98CJ11027 was similar to that of endochitinase of *Chromobacterium violaceum* (5).

pH optimum of chitinase I

The effect of pH on the chitinolytic activity was studied with citrate-phosphate buffer (pH 4.5-6.5), phosphate buffer (pH 6.5-8.5), and glycine-NaOH buffer (pH 8.5-10.0) under the standard conditions. The pH-activity profile was bell shaped with maximum value at pH 6.0 (Fig. 3). The pH optimum of the chitinase produced by strain 98CJ11027 is similar to that of the chitinase from enteric bacterium *Ewingella americana* (12) and from kidney beans (3). Other marine bacterial chitinases showed broader pH optima (10, 24) or were more active in neutral or slightly alkaline conditions (32, 34). Acidic amino acids may be present and act significantly in the active site of the chitinase I of strain 98CJ11027 catalyzing the acid hydrolysis of glycosidic bonds, as suggested by Inglis and Peberdy (12).

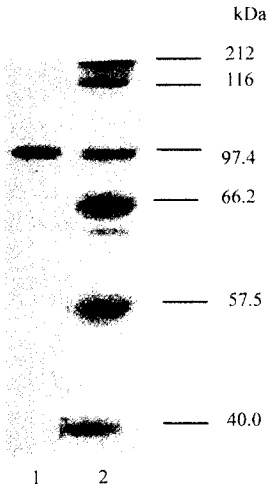

Fig. 2. SDS-PAGE of the purified chitinase I from strain 98CJ11027.

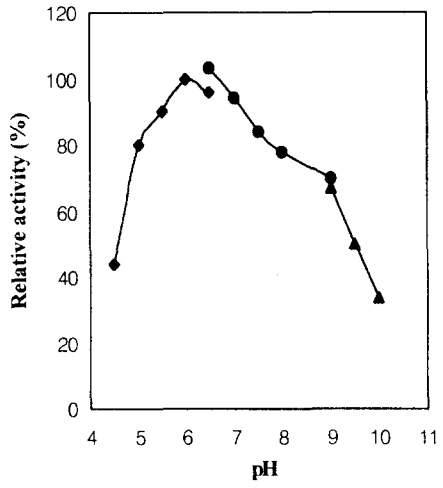

Fig. 3. Effect of pH on activity of chitinase I. ◆, citrate-phosphate buffer; ●, phosphate buffer; ▲, glycine-NaOH buffer.

Effect of temperature on chitinase I

The optimum temperature for chitinase I was 45°C under the standard assay conditions, above which the activity sharply declined (Fig. 4). Chitinase I of strain 98CJ11027 showed a similar temperature optimum to those of other marine bacteria (*Aeromonas hydrophila* H-2330 (10), *Alteromonas* sp. O-7 (32), and *Pseudomonas aeruginosa* K-187 (34).

Effect of metal ions on chitinase I

Certain metal ions affected activity of chitinase I of strain 98CJ11027 (Table 3). The enzyme was precipitated by the addition of Mn^{++} and Zn^{++} at the concentration of 1 mM and could not be used in further assays. Fe^{++} and Cu^{++} inhibited the chitinase I activity. Other chitinases from *A. hydrophila* H-2330 (10), *Alteromonas* sp. O-7 (32), *E. americana* (12), *P. aeruginosa* K-187 (34), and *Fusarium chlamydosporum* (20) were also inhibited by Fe^{++}, Fe^{+++} and/or Cu^{++}. Chitinase from *Alteromonas* sp. strain O-7 (9) was activated by Na^{+} and Ca^{++}. In chitinase I from *Vibrio* sp. 98CJ11027, however, activation of enzyme activity was not observed under the assay conditions.

The inhibition of chitinase I by Fe^{++} and Cu^{++} could be

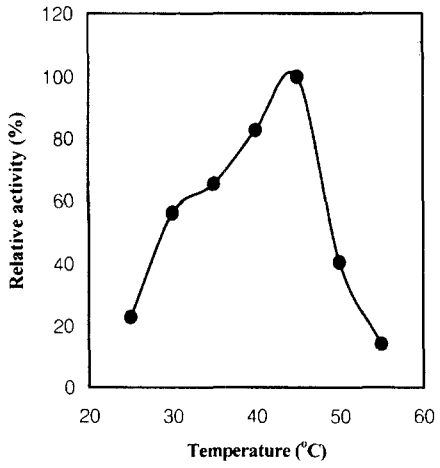

Fig. 4. Effect of temperature on chitinase I activity.

Table 3. Effect of metal ions on chitinase I activity.

Compound	Concentration (mM)	Relative activity (%)[a]
KCl	5	97 ± 7
KNO_3	5	98 ± 8
NaCl	5	97 ± 3
$NaNO_3$	5	95 ± 6
NH_4Cl	5	98 ± 7
$(NH_4)_2SO_4$	5	99 ± 11
$CaCl_2$	5	99 ± 8
$MnCl_2$	1	N.D.[b]
$MgCl_2$	5	101 ± 1
$ZnSO_4$	1	N.D.[b]
$NiCl_2$	5	102 ± 6
$CoNO_3$	5	101 ± 7
$CuSO_4$	1	93 ± 6
	5	57 ± 3
$FeSO_4$	1	67 ± 8
	5	N.D.[b]
EDTA	5	106 ± 9
none	0	100

[a]Results are the mean of triplicate tests.
Activities calculated as a percentage of the control treatment which was taken as 100%.
[b]Not determined due to interference with the assay.

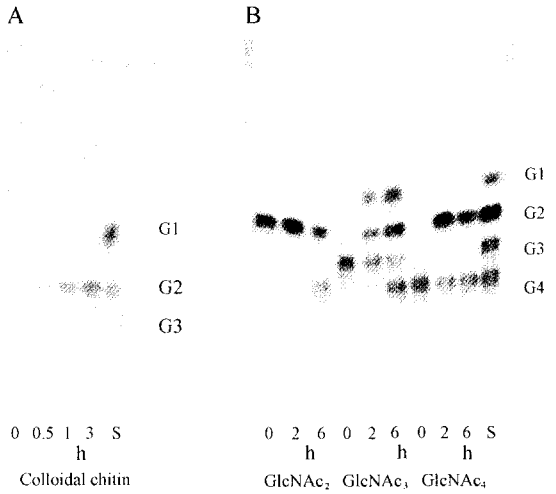

Fig. 5. TLC of the products from colloidal chitin (A) and N-acetylchitooligosaccharides (B) by chitinase I. S: standard markers; G1, GlcNAc; G2, $GlcNAc_2$; G3, $GlcNAc_3$; G4, $GlcNAc_4$.

related to the residues of aspartic and glutarmic acid in chitinases. It has been shown that these amino acids in the active sites of chitinases bind to certain divalent cations, thereby possibly inhibiting chitinases (22).

Action mode of chitinase I on chitin

Washing the SDS-gel with a buffer (40 mM Tris-HCl, pH 9.0, 2 mM EDTA, 0.02% sodium azide) containing 1% casein released no recovery of chitinolytic activity, while other chitinases are reactivated by washing the gels with casein-EDTA solution after SDS-PAGE (5, 8, 21). The hydrolysis of fluorescent substrates, 4-methylumbelliferyl chitooligosaccharides, by chitinase I could not be detected on the gel.

The hydrolysis of colloidal chitin with chitinase I was examined. The main product formed in the decomposition of colloidal chitin by the enzyme was diacetylchitobiose ($GlcNAc_2$) (Fig. 5A). Chitinase I cleaved triacetylchitotriose ($GlcNAc_3$) into $GlcNAc_2$ and GlcNAc, and tetraacetylchitotetraose ($GlcNAc_4$) was hydrolyzed into $GlcNAc_2$ (Fig. 5B). The chitinolytic enzymes are divided into three types. Endochitinases (EC 3. 2. 1. 14) catalyze the random hydrolysis of 1,4-β-linkages of GlcNAc at the internal sites of chitin. Exochitinase, also termed chitobiosidase, release $GlcNAc_2$ units in a stepwise mode. N-acetyl-β-1,4-D-glucosaminidases (EC 3. 2. 1. 30) act on $GlcNAc_2$ and higher analogs of chitin in exo-splitting fashion, resulting in GlcNAc monomers (6). On the basis of this suggestion, chitinase I produced by strain 98CJ11027 can be regarded as a chitobiosidiase (exochitinase).

After 6 h reaction of chitinase I in $GlcNAc_2$ and $GlcNAc_3$, $GlcNAc_4$ was detected on the TLC plate (Fig. 5B). This result suggested that the chitinase I of the isolate catalyzes also reversed hydrolysis. Chitobiosidases, which catalyzes the hydrolysis reaction in a reversed direction, have not been reported yet. From these results, the chitinase I from *Vibrio* sp. 98CJ11027 could be regarded as a novel enzyme and can be applied to use in the synthesis of derivatives of $GlcNAc_2$ and $GlcNAc_4$ as well.

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