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Purification and Characterization of Extracellular Chitinase Produced by Marine Bacterium, *Bacillus* sp. LJ-25

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Abstract Extracellular chitinase was purified from the culture liquid of the marine bacterium, *Bacillus* sp. LJ-25, and its enzymatic properties were examined. The purified chitinase exhibited a single band on SDS-PAGE and the molecular weight was estimated to be approximately 50 kDa. The optimum pH and temperature for the enzymatic activity were 7.0 and 35°C, respectively. The activity of the chitinase was strongly inhibited by Zn^{2+} and slightly inhibited by Ba^{2+}, Co^{2+}, Mn^{2+}, and Cu^{2+}. The purified chitinase did not hydrolyze *p*-nitrophenol-N-acetyl-β-D-glucosaminide $(GlcNAc)_2$ and *Micrococcus lysodeikticus* cells, which are known to be the substrates for exo-type chitinase. Among the hydrolyzates of colloidal chitin, $(GlcNAc)_2$ was in the highest concentration with small amounts of GlcNAc and $(GlcNAc)_3$.

Key words: Marine bacterium, *Bacillus* sp. LJ-25, extracellular chitinase, purification and enzymatic properties

Chitin, a homopolymer of N-acetyl-D-glucosamine (GlcNAc) residues linked by β-1,4 bonds, is abundant in nature, especially in marine invertebrates, insects, fungi, and yeasts. Chitin and its derivatives are of interest because they have varied biological functions, e.g., as immunoadjuvants, flocculants of wastewater sludge, and agrochemicals [19]. The number and nature of the chemical derivatives of chitin can be complemented and expanded by the use of chitin-degrading and modifying enzymes.

Chitinase (EC 3.2.1.14) plays an important role in the decomposition of chitin and potentially in the utilization of chitin as a renewable resource [13, 25]. Chitinase-producing bacteria are expected to inhabit the oceans, because a high amount of chitin are produced by sea animals such as crabs, shrimp, and squid.

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Although there have been many reports on the isolation, purification, and properties of chitinase from microorganisms, animals, and plants, studies related to marine bacteria have been limited so far [3, 8, 15, 24, 28, 29]. Recently, we isolated a chitinolytic bacterium, *Bacillus* sp. LJ-25, from the viscera of Korean bony fish, and examined its optimal culture conditions to produce an extracellular enzyme [9].

Consequently, the present study describes the purification and characterization of the extracellular chitinase from *Bacillus* sp. LJ-25.

Materials and Methods

Chemicals

Colloidal chitin was prepared from commercial crab chitin by the method of Jeuniaux [4]. *N*-Acetylchitooligosaccharides $(GlcNAc_{2\text{-}6})$ were purchased from Wako Pure Chemical Industries, Ltd., and chitosan (minimum 85% deacetylated), *Micrococcus lysodeikticus* cells, and *p*-nitrophenol-N-acetyl-β-D-glucosaminide were from Sigma Chemical Company. All other chemicals were of analytical grade.

Organism and Culture Conditions

Bacillus sp. LJ-25, which we selected as the most potent chitinolytic enzyme producer, was used for the production of chitinase. It was cultivated in a liquid medium containing 0.3% (w/v) colloidal chitin, 1% nutrient broth, and 1% NaCl (pH 7.0) at 30°C for 168 h, as the optimal conditions described in a previous paper [9]. After cultivation, the culture broth was centrifuged for 10 min at 12,000 ×g and the supernatant was used for purification of the enzyme.

Purification Procedures

All operations in the purification of the enzyme were carried out at 4°C. Solid ammonium sulfate was added to the culture liquid of *Bacillus* sp. LJ-25 to a final concentration

of 85%. After standing overnight, the resultant precipitate was collected by centrifugation and dialyzed against a 30 mM Tris-HCl buffer (pH 7.0) for 20 h. The dialysate was centrifuged for 10 min at 12,000 ×g to remove any insoluble material, and the supernatant was concentrated by ultrafiltration using an ultrafilter (Advantec Inc., U.S.A.). The chitinase was further purified from this concentrated supernatant by column chromatography on DEAE-cellulose, Sephadex G-100, and CM-Sephadex C25.

Enzyme Activity Assay

The chitinase activity was assayed by the following method using colloidal chitin as the substrate. A reaction mixture containing 3.0 ml of 0.3% colloidal chitin solution and 2.0 ml of an enzyme solution diluted in a 30 mM Tris-HCl buffer (pH 7.0) was incubated at 35°C for 1 h, and the reaction was then stopped by boiling for 5 min. The released reducing sugars were measured using the method of Ressing *et al.* [21].

Protein Determination

The protein content was estimated by the method of Lowry *et al.* [10] using bovine serum albumin as the standard protein.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to check the protein purity and determine the molecular mass of the purified enzyme under denaturing conditions using 11% acrylamide gel, as described by Laemmli [7]. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250, and then destained in the same solution without the dye. The marker proteins used as the standard molecular weights were bovine albumin (66,000), egg albumin (45,000), pepsin (34,700), trypsinogen (24,000), and β-lactoglobulin (18,400).

Analysis of Chitinase Reaction Products

The hydrolysis products were separated by reverse-phase HPLC with a Tosoh TSK gel NH_2-60 column (Shimadzu LC 6A). The column was eluted with a mixture of acetonitrile and water (55:45 by v/v) as the mobile phase, and monitored by the absorbance at 210 nm.

Results and Discussion

Purification of Chitinase

The dialyzed and concentrated enzyme solution, prepared by the method described in Materials and Methods, was put on a DEAE-Cellulose column (3.0×50 cm), previously equilibrated with 30 mM Tris-HCl buffer (pH 7.0). The enzyme was then eluted with a linear gradient of NaCl (0-1.0 M) at a flow rate of 30 ml/h. As shown in Fig. 1A, the active fractions were obtained as a broad peak. The active fractions were concentrated by ultrafiltration and put on a Sephadex G-100 (2.6×50 cm) column, previously equilibrated with 30 mM Tris-HCl buffer, pH 7.0, and then eluted with the same buffer containing 0.15 M NaCl. Two peaks with chitinase activity were found on the Sephadex G-100

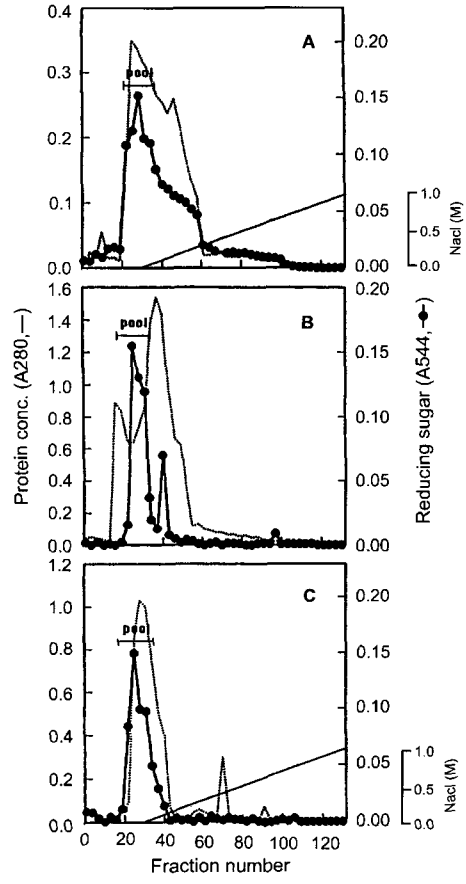

Fig. 1. Chromatographs of chitinase produced by *Bacillus* sp. LJ-25.
Details are described in the text. A, DEAE-Cellulose chromatography; B, Sephadex G-100 chromatography; C, CM-Sephadex C25 chromatography.

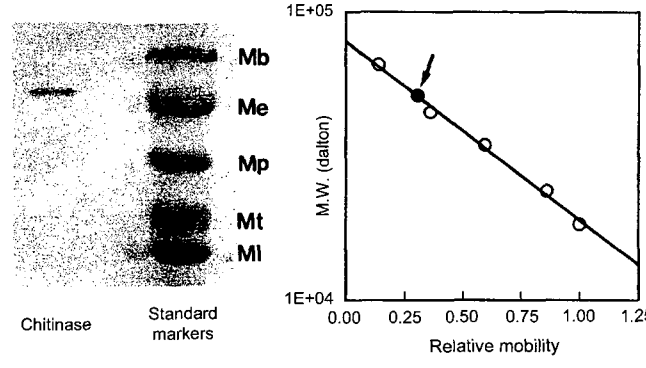

Fig. 2. Determination of the molecular weight of purified chitinase by SDS-polyacrylamide gel electrophoresis.
The conditions of SDS-PAGE are described in Material and Methods. Standard markers (kDa): Mb, bovine (66); Me, egg white ovalbumin (45); Mp, pepsin (34.7); Mt, trypsinogen (24); Ml, β-lactoglobulin (18.4).

Table 1. Summary of the purification procedure of chitinase from *Bacillus* sp. LJ-25.

Purification step	Total protein (mg)	Total activity (unit)*	Specific activity (unit/mg)	Yield (%)	Purification (fold)
Culture filtrate	2,281	13,686	6.0	100	1
Ammonium sulfate ppt.	246.9	2,664	10.8	19.5	1.8
DEAE-Cellulose chromatography	52.7	1,490	28.2	10.9	4.7
Sephadex G-100 chromatography	17.6	576.5	32.8	4.2	5.5
CM-Sephadex C25 chromatography	2.7	336	124.4	2.5	20.7

*One unit of chitinase activity was defined as the amount of the enzyme which liberated 1 μmole of N-acetylglucosamine per 1 h at 35°C.

chromatograph (Fig. 1B). The main active fractions, indicated by bars in Fig. 1B, were concentrated by ultrafiltration, and then further purified by chromatography on a CM-Sephadex C25 (1.5×50 cm), equilibrated with the same buffer as mentioned above (Fig. 1C). The enzyme was completely separated from the other proteins, and migrated as a single band on SDS-PAGE (Fig. 2). Accordingly, it was identified as the purified enzyme. The purification procedures and results are summarized in Table 1. The chitinase was purified about 21-fold with 2.5% recovery from the culture medium, and the specific activity of the chitinase was about 124 U/mg protein.

Molecular Weight of Chitinase

As shown in Fig. 2 the molecular weight of the purified chitinase was determined to be approximately 50 kD by SDS-PAGE. Most of the chitinases from *Bacillus* sp. reported so far have shown to have molecular weights within the range of 20,000 to 90,000. Among these *Bacillus* sp., the molecular weight of the presently described enzyme is similar to those of the chitinases from *Bacillus licheniformis* TP-1 [21], *Bacillus licheniformis* B-6839 [24], and *Bacillus circulan* WL-12 [28].

Effects of pH on Activity and Stability of Chitinase

The enzyme was most active at around pH 7.0 and relatively stable at pH 7.0, but unstable under pH 5.0 and over pH 8.0 (Fig. 3). It was previously reported that the optimum pH for the chitinases of the *Alteromonas* sp. strain O-7 [24], *Enterobacter* sp. G-1 [18], and *Vibrio* sp. [16] was neutral. In contrast, differences have been observed in the optimum pH between bacterial and non-bacterial chitinases isolated from marine fish: It has been recognized that the optimum pH of an enzyme of bacterial origin is higher than that of non-bacterial origin [6].

Effects of Temperature on Activity and Stability of Chitinase

When the pH of the reaction mixture was maintained at pH 7.0, the optimum temperature of the enzyme was around 35°C, as shown in Fig. 4. This value, in terms of thermostability, was significantly lower than those of several chitinases from the *Bacillus* species [1, 19, 22, 23]. In contrast, the chitinase was stable at temperatures below 40°C, however, about 50% of the original activity was lost at 50°C (Fig. 4), probably due to the thermal denaturation of the enzyme.

Effect of Ionic Strength on Activity

The effect of ionic strength on the chitinase activity was investigated by varying the concentrations of NaCl in the reaction mixture at pH 7.0 and 35°C. As a result (Fig. 5), the activity of the chitinase was drastically increased up to 1.0%

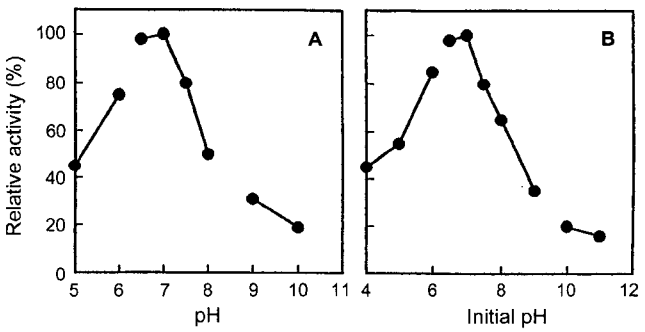

Fig. 3. Effects of pH on the activity and stability of chitinase.
A: Chitinase activity was measured at various pHs at 35°C for 1 h. B: The enzyme solution was incubated at various pHs at 35°C for 2 h and the remaining activity was measured by the method described in Materials and Methods. Buffers used were sodium acetate-acetate (pH 5.0–6.0), Tris-HCl (pH 6.5–9.0), and carbonate-sodium hydroxide carbonate (pH 9.0–11.0), at 0.1 M each.

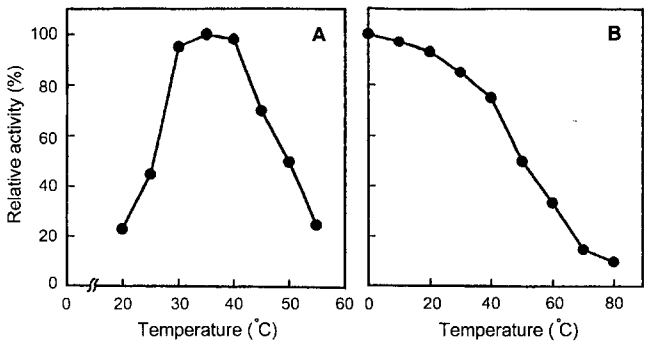

Fig. 4. Effects of temperature on the activity and stability of chitinase.
A: Chitinase activity was measured at various temperatures at pH 7.0. B: The enzyme solution was incubated at various temperatures at pH 7.0 for 30 min and the remaining activity was measured under standard assay conditions.

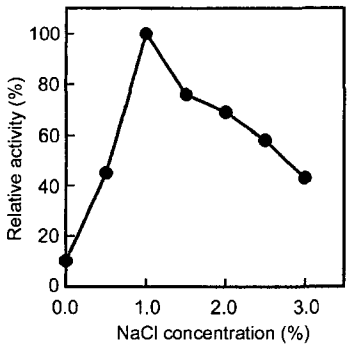

Fig. 5. Effect of ionic strength on chitinase activity.
The chitinase activity was measured at various NaCl concentrations at 35°C and pH 7.0 for 1 h.

NaCl, however, above this concentration, the activity gradually decreased. This result would suggest that the chitinase from *Bacillus* sp. LJ-25 requires NaCl for its maximum activity.

Effect of Metal Ions on the Chitinase Activity

The effect of metal ions on the chitinase activity is summarized in Table 2. Among them, Zn^{2+} inhibited 80% of the original chitinase activity and Ba^{2+}, Co^{2+}, Cu^{2+}, and Mn^{2+} caused 39-80% inhibiton. Ulhoa and Peberdy [26] also reported that the chitinase from *Trichoderma harzianum* was strongly inhibited by Zn^{2+}.

Substrate Specificity

The purified chitinase was examined for its ability to hydrolyze some chitin-related substrates (Table 3). Colloidal chitin, which is a substrate of endo-type chitinases [17], was effectively hydrolyzed by the chitinase, however, the activity was significantly lower towards chitin and chitosan. In contrast, the chitinase did not hydrolyze *N,N'*-diacetylchitobiose, *p*-nitrophenol-N-acetyl-β-D-glucosaminide, and *Micrococcus lysodeikticus* cells which are known to be the substrates of exo-type chitinases [5]. From the above

Table 2. Effect of metal ions on the activity of chitinase.

Metal ion* (2 mM)	Relative activity (%)
None	100
K^+	74
Li^+	76
NH_4^+	75
Ba^{2+}	40
Ca^{2+}	84
Co^{2+}	55
Cu^{2+}	60
Hg^{2+}	65
Mg^{2+}	80
Mn^{2+}	61
Zn^{2+}	20

*Chloride form.

Table 3. Substrate specificity of chitinase.

Substrate	Relative activity (%)
Colloidal chitin	100
Chitin	9
Chitosan	13
Micrococcus lysodeikticus cells	0
*p*NPGlcAc*	0
N,N'-diacetylchitobiose	0

**p*-Nitrophenol-N-acetyl-β-D-glucosaminide.

results, the purified chitinase in this study appeared to be an endo-type chitinase.

Hydrolysis Products of Colloidal Chitin

A solution of 0.3% colloidal chitin (pH 7.0) was incubated with the purified chitinase at 35°C, and the hydrolyzates of the colloidal chitin were analyzed by HPLC. Although the major product of the hydrolysis of colloidal chitin was $(GlcNAc)_2$, $(GlcNAc)_3$ was also produced by the purified chitinase (Fig. 6). The lack of higher-order chitooligosaccharides from the colloidal chitin may be due to the fibrous structure preformed with tight chitin hydrogen bonds, which may interfere with the binding of the enzyme and allow the enzyme to digest only exposed regions of the fibers [12]. Furthermore, some reports have indicated that endo-chitinases produce higher-order chitooligosaccharides from nascent chitin, which was synthesized by solubilized chitin synthetase rather than from preformed chitin [2, 11]. In contrast, the transglycosylation reaction of chitinase has been reported by several researchers [14, 22]. Therefore, we also examined whether this new enzyme had similar activities. When $(GlcNAc)_4$ was incubated with the enzyme at 35°C, no

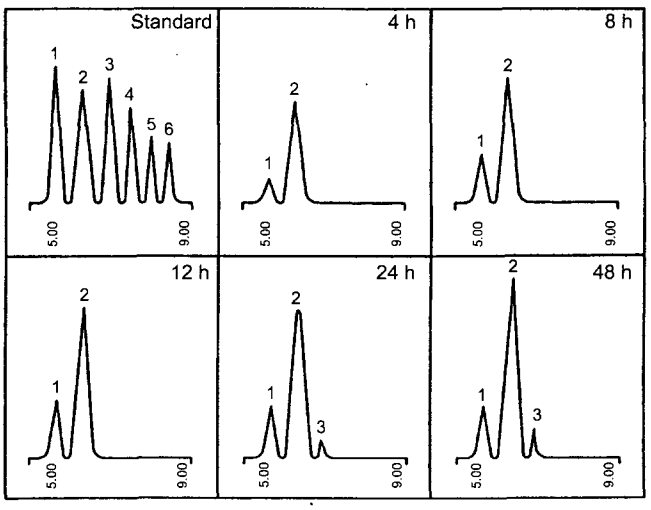

Fig. 6. HPLC analysis of reaction products from colloidal chitin by chitinase.
The chitinase was incubated with 0.3% colloidal chitin at pH 7.0 and 35°C for 4 h to 48 h. The analytic conditions of HPLC are described in Materials and Methods. Standard materials: 1, GlcNAc; 2, $(GlcNAc)_2$; 3, $(GlcNAc)_3$; 4, $(GlcNAc)_4$; 5, $(GlcNAc)_5$; 6, $(GlcNAc)_6$.

chitooligosaccharides of higher order than $(GlcNAc)_4$ appeared, thereby suggesting that the enzyme from *Bacillus* sp. LJ-25 does not have transglycosylation activity (data not shown).

The enzymatic properties mentioned above indicate that the chitinase from *Bacillus* sp. LJ-25 was uniquely different from the previously identified chitinases.

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