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Production and Some Properties of Chitinolytic Enzymes by Antagonistic Bacteria

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길항세균들이 생산하는 Chitin 분해효소의 특성

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ABSTRACT : Production and some properties of chitinolytic enzymes were investigated by 80% ammonium sulfate precipitates (crude enzymes) from culture supernatant of antagonistic bacteria, *Chromobacterium violaceum* strain C-61 and strain C-72, *Aeromonas hydrophila*, *Aeromonas caviae*, and *Serratia marcescens*. The maximum production of chitinase was obtained from the 3-day culture at 28°C in *C. violaceum* strains, the 6-day culture in *S. marcescens*, and the 2-day culture in *A. hydrophila* and *A. caviae*. In the optimum culture periods, chitinase activity of *C. violaceum* strain C-61 was 1.5, 5.5, 12.0 and 11.3 times higher than those of strain C-72, *S. marcescens*, *A. hydrophila* and *A. caviae*, respectively. However, N,N'-diacetylchitobiase activity was 3.2 times higher in *S. marcescens* than in *C. violaceum* strain C-61, and that of *Aeromonas* spp. was very low. On gels containing glycol chitin, chitinase of *C. violaceum* strains showed four isoforms of 54-, 52-, 50- and 37-kDa, whereas there were four isoforms of 58-, 52-, 48- and 38-kDa in *S. marcescens*, three isoforms of 70-, 58- and 54-kDa in *A. hydrophila* and six isoforms of 90-, 79-, 71-, 63-, 58- and 38-kDa in *A. caviae*. The chitinase of *C. violaceum* strain C-61 was most active at pH 7.0 and at 50°C and was stable in ranges of pH 5.0~10.0 for 2 hours and of 0~50°C for 30 min.

***Key words* : *Chromobacterium violaceum* strain C-61, chitinase activity, N,N'-diacetylchitobiase activity.**

Chitin is a major cell wall component of important pests including insects, fungi and nematodes (14). Many chitinolytic bacteria were reported to suppress several plant diseases caused by pathogenic fungi (4, 13, 17, 18, 20, 23). Recently we isolated chitinolytic bacteria, *Chromobacterium violaceum* strains, *Serratia marcescens*, *Aeromonas hydrophila* and *A. caviae* from soils (19). Among the bacteria, *S. marcescens* has already been studied in the field of biological control (4, 17, 18). In our experiments, however, *C. violaceum* strains showed very high antagonistic activity against several soilborne plant pathogens, compared with *S. marcescens*, *A. hydrophila* and *A. caviae* (20).

Production and properties of chitinolytic enzymes have been studied in several chitinolytic bacteria (1, 2, 5, 15, 16, 24, 27), especially in *Serratia* spp. (6, 7, 11, 21, 22) and *Aeromonas* spp. (9, 11, 27). Characteristics such as optimum culture period for chitinase production, and numbers and molecular weights of chitinase isozymes were reported to vary according to bacterial species or strains (8, 11, 21, 27). The kinds of enzymes associated with chitin degradation; chitinase (EC 3.5.1.14) and N-acetyl-β-glucosaminidase (EC 3.2.1.30) or N,N'-diacetylchitobiase which hydrolyzes the chitin into N-acetylglucosamine, and chitin deacetylase (EC 3.5.1.41), chitosanase and chitobiase which hydrolyze the chitin into glucosamine, were also known to vary depending on bacterial genera and species (1, 26). However, chitinolytic enzymes of *C. violaceum* have not been studied yet.

The major objective of this study was to make clear some properties of chitinolytic enzymes produced from

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C. violaceum, and to compared with those of *S. marcescens*, *A. hydrophila* and *A. caviae*.

MATERIALS AND METHODS

Organisms and cultivation. Chitinolytic bacteria, *C. violaceum*, *S. marcescens*, *A. hydrophila* and *A. caviae* were isolated from soil, and the plant pathogen, *Rhizoctonia solani*, was isolated from diseased eggplant (20). The chitinolytic bacteria were identified as such based on their morphological and physiological characteristics (19). The chitinolytic bacteria were maintained on nutrient agar slants at 4°C and were subcultured every month. *R. solani* was maintained on potato dextrose agar (PDA) slants at 4°C and was subcultured every 2 months.

Bacterial growth and preparation of crude enzyme. The culture cells (100 μl) of the chitinolytic bacteria grown in nutrient broth medium for 1 day were inoculated in flasks (500 ml) containing 100 ml of the chitin medium (20, 23), and then were incubated with shaking (180 rpm) at 28°C. The cell growth was determined by dilution plate on nutrient agar medium containing ampicillin (50 ppm) at intervals of 12 hours.

The culture solution (30 ml) was collected every day, and cells and residual chitin were removed by centrifugation at 10,000 g for 20 min. Solid ammonium sulfate was slowly added to the culture supernatant at 4°C to give 80% saturation. After incubating overnight with continuous stirring, the precipitate was collected by centrifugation at 10,000 g for 30 min, and dissolved in 300 μl of 20 mM sodium acetate buffer, pH 5.0. The solutions were used as crude enzymes, and their protein concentrations were determined by the protein assay kit (Sigma, P 5656) using purified bovine serum albumin as a reference protein.

Enzyme assays. Activity of chitinase, N,N'-diacetylchitobiase and chitosanase produced from the chitinolytic bacteria, and stability and activity of *C. violaceum* strain C-61 chitinase at pH 2.0~11.0, and at 10~100°C, were investigated using the crude enzymes. Chitinase activity was determined by measuring the amount of N-acetylglucosamine (NAG) released from colloidal chitin. A reaction mixture (1.5 ml) containing 0.5% colloidal chitin and an enzyme solution in 20 mM sodium acetate buffer, pH 5.0, was incubated for 30 min at 37°C. After boiling for 5 min, pellets were removed by centrifugation at 10,000 g for 5 min. A mixture of 1.3 ml color reagent solution (0.05% potassium ferricyanide in 0.5 M sodium carbonate) and 1.0 ml supernatant solution was boiled for 15 min and was measured at 420 nm. For N,N'-diacetylchitobiase and chitosanase assay, colloidal chitin was replaced by 2 mM N,N'-diacetylchitobiose (Sigma, D 1523) and 0.5% (v/w) glycolchitosan (Sigma, G 7753), respectively, as the reaction substrates. The reaction was stopped by boiling for 4 min after adding color reagent solution. The OD values were calculated as the amounts of reducing sugar using a standard curve for N-acetyl-D-glucosamine (NAG) (Sigma, A 8625). A unit of enzyme activity is defined as the amount of enzyme required to produce 1 mole of NAG under the described conditions.

SDS-PAGE. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (10) in 10% acrylamide gels containing 0.01% glycol chitin. Samples were boiled for 1 min with 15% (v/w) sucrose, 2.5% (v/w) SDS in 125 mM Tris-HCl (pH 6.8) and 0.01% (v/w) bromophenol blue without β-mercaptoethanol. The proteins were stained with silver nitrate (6). The protein molecular weights were determined by the LMW calibration kit (Pharmacia Inc.).

Detection of chitinase activity after SDS-PAGE. After electrophoresis, gels were incubated at 37°C for 12 hours with slow shaking in 100 mM sodium acetate buffer, pH 5.0, containing 1% (v/w) Triton X-100. Gels were then stained with 0.01% (v/w) Calcoflour White M2R (Sigma, F 6259) in 500 mM Tris-HCl, pH 8.9 for 5 min in the dark and destained for more than 1 hour at room temperature in distilled water. Lytic zones were observed under the UV-transilluminator.

RESULTS

Cell growth and chitinase production. In chitin medium, *C. violaceum* strain C-61, strain C-72, *A. hydrophila* and *A. caviae* reached to its maximum growth after 1-day culture, and then decreased; slowly in *C. violaceum* strains, rapidly in *Aeromonas* spp., and most rapidly in *A. caviae* (Fig. 1). On the other hand, *S. marcescens* reached to the maximum growth after 2-day culture and maintained similar density until 7-day culture.

Chitinase production in *C. violaceum* strains was maximum after 3-day culture, and then was decreased rapidly (in strain C-61) or slowly (in strain C-72) (Fig. 1B). Chitinase production from *A. hydrophila* and *A. caviae* reached maximum after 2-day culture, and then

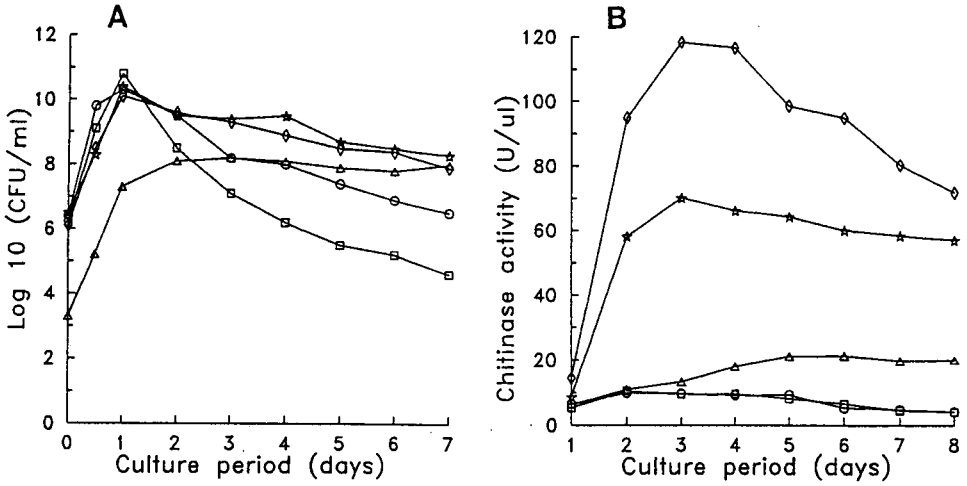

Fig. 1. Time course of (A) cell growth and (B) chitinase production by chitinolytic bacteria; *C. violaceum* strain C-61 (◇), strain C-72 (☆), *S. marcescens* (△), *A. hydrophila* (○), and *A. caviae* (□). The cell suspension (100 μl) grown in nutrient broth for 1 day was inoculated in the chitin medium (100 ml), and then cultured at 28°C. A unit of chitinase activity is defined as the amount of enzyme required to produce 1 μmole of NAG under the described conditions. Each value represents the mean of three separate determinations.

Table 1. Protein concentration and activity of chitinolytic enzymes secreted by chitinolytic bacetria in chitin medium[a]

Chitinolytic bacteria	Protein concentration (mg/ml)[b]	Enzyme activity (U/μl)[c]		
		Chitinase	Chitobiase[d]	Chitosanase
C. violaceum C-61	4.1	118.4	7.3	0.7
C. violaceum C-71	2.8	70.2	3.6	0.3
S. marcescens	1.6	21.4	23.1	0.4
A. hydrophila	0.7	9.9	1.0	0
A. caviae	0.7	10.5	0.8	0

[a] *C. violaceum* strains, *S. marcescens* and *Aeromonas* spp. were cultured at 28°C for 3, 6 and 2 days, respectively.

[b] Determined by protein assay kit (Sigma, P 5656) using purified bovine serum albumin as reference protein.

[c] A unit represents the amount of enzyme required to produce 1 μmole of NAG under the described conditions. Each value represents the mean of three separate determinations.

slowly decreased. On the other hand, chitinase production from *S. marcescens* reached maximum after 6-day culture and maintained the similar level until a 8-day culture (Fig. 1B).

Protein concentration and chitinolytic enzymes activity. In the optimum culture period for chitinase production, total protein of *C. violaceum* strain C-61 was 4.1 mg/ml, which was 1.4, 2.5, 5.5 and 5.8 times higher than those of strain C-72, *S. marcescens*, *A. hydrophila* and *A. caviae*, respectively (Table 1). On the other hand, chitinase activities of *C. violaceum* strain C-61 was 118.4 U/μl, which was 1.7, 5.5, 12.0 and 11.3 times higher than those of strain C-72, *S. marcescens*, *A. hydrophila* and *A. caviae*, respectively. However, N, N'-diacetylchitobiase activity was 3.2~6.4 times higher in *S. marcescens* with 23.1 U/μl than in *C. violaceum* strains, and those of *A. hydrophila* and *A. caviae* were very low. Chitosanase activity was very low in all of the isolates (Table 1).

Numbers and molecular weight of chitinase isoforms. Protein bands stained by silver nitrate after SDS-PAGE varied according to the tested isolates (Fig. 2A). Among the protein bands, chitinase activities (chitinase isoforms) of *C. violaceum* strain C-61 and strain C-72 were detected in four bands of about 54-, 52-, 50- and 37-kDa. On the other hand, chitinase activities of *S. marcescens* were detected in four bands of about 58-, 52-, 48-, 38-kDa, which were the same as commercial chitinase (Sigma, C 7809). *A. hydrophila* and *A. caviae* had chitinase activity in three bands of 70-, 58-, 54-kDa and six bands of 90-, 79-, 71-, 63-, 58-, 38-kDa, respectively (Fig. 2B, Table 2).

Stability and activity of *C. violaceum* chitinase on pH and temperature. *C. violaceum* strains, which have strong antagonistic activity to soil borne plant

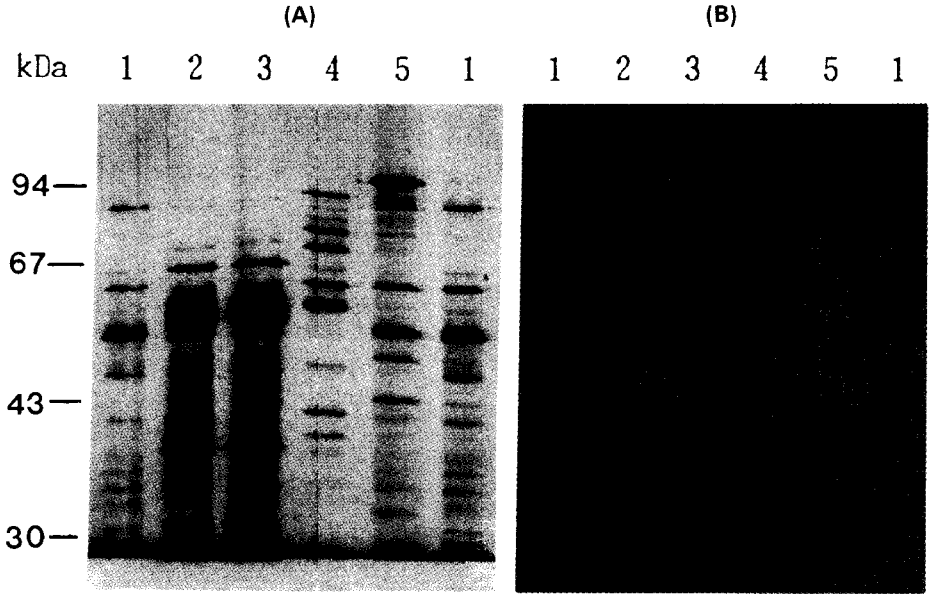

Fig. 2. SDS-PAGE of (A) protein and (B) chitinase secreted by chitinolytic bacteria; Commercial chitinase from *S. marcescens* (Sigma, C 1650) (lane 1), *C. violaceum* strain C-61 (lane 2), strain C-72 (lane 3), *A. caviae* (lane 4) and *S. marcescens* (lane 5). The crude enzymes prepared by 80% ammonium sulfate precipitate from the culture supernatants were loaded on gels containing 0.01% glycol chitin. (A) Protein bands were detected after silver nitrate staining and (B) bands with chitinase activities were detected under UV illumination after staining with Calcofluor White M2R.

Table 2. Numbers and molecular weights of chitinase isoforms secreted by chitinolytic bacteria

Crude enzyme from bacteria	Chitinase isoforms	
	Number	Molecular weight (kDa)
Chitinase[a]	4	58 – 52 – 48 38
S. marcescens	4	58 – 52 – 48 38
C. violaceum C-61	4	– 54 52 50 – 37
C. violaceum C-72	4	– 54 52 50 – 37
A. hydrophila	3	70 – 58 54 – – – –
A. caviae	6	90 79 71 63 58 – – – – 38

[a] Commercial chitinase from *Serratia marcescens* (Sigma, C 1650).

pathogens (20) but were not studied in the enzyme production, were investigated on the stability and activity of the enzymes in various pH and temperature conditions. The chitinases were most active at pH 7 and at 50°C, and stable in ranges of pH 5 to pH 10 at 30°C for 2 hours and in ranges of 0~50°C for 30 min (Fig. 3A, 3B).

DISCUSSION

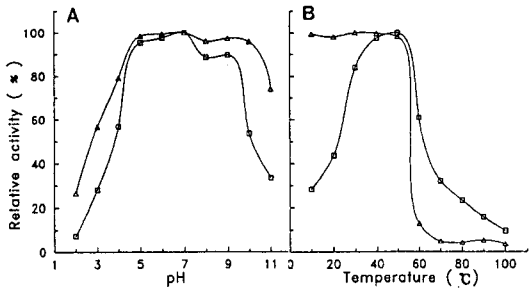

Fig. 3. Effect of (A) pH and (B) temperature on activity (□) and stability (△) of *C. violaceum* strain C-61 chitinase. The standard assay of chitinase activity was carried out at 37°C for 30 min at pH 5.0. The stability on pH and temperature was determined by the standard assay method after preincubation of the enzymes at various pH's for 2 hours and at various temperatures for 30 min, respectively. Relative activity was converted from the chitinase activity.

The optimum culture period for chitinase production was reported to vary according to species or genera of chitinolytic bacteria (1, 8, 11, 12, 16, 27), which was consistent with our results; 3 days in *C. violaceum* strains, 2 days in *A. hydrophila* and *A. caviae*, and 6

days in *S. marcescens*. The optimum culture periods of *A. hydrophila* and *S. marcescens* were similar to those in the reports (8, 12, 22, 27) showing 3 days in *A. hydrophila* and 6~7 days in *S. marcescens*. It is not clear at present why the optimum culture period differs with bacterial genera or species; however, it may be associated with the bacterial growth. For example, *S. marcescens* with a longer culture period for the enzyme production grew more slowly.

Many chitinolytic bacteria produce both chitinase and N,N'-diacetylchitobiase (1, 15, 22, 27). In our experiments, all of the tested isolates also produced both chitinase and N,N'-diacetylchitobiase, but did not produce chitosanase. This suggests that the isolates convert chitin into N-acetylglucosamine, because a chitin polymer is hydrolyzed by chitinase into small oligosaccharides, especially diacetylchitobiose (1), which is then hydrolyzed by N,N'-diacetylchitobiase into N-acetylglucosamine (26). However, to determine whether chitosanase or another chitinolytic enzymes were produced or not, further studies must be conducted in basal media containing each substrate, because chitinolytic enzymes are generally induced by the substrates (9, 11, 12, 22, 26, 27).

Ability of chitinolytic bacteria for disease suppression was not studied in relation to their chitinase production in our experiments. However, *C. violaceum* strain C-61, which had higher ability of inhibiting soilborne plant pathogens *in vitro* and suppressing damping-off diseases (20), produced 5.5, 12.0 and 11.3 times higher chitinase than the less antagonistic bacteria, *S. marcescens*, *A. hydrophila* and *A. caviae*, respectively. Therefore, it is considered that there may be some correlations between chitinase production and antagonistic ability in *C. violaceum* strain C-61, although other factors such as competition for nutrients and production of antagonistic materials should not be excluded in relation to the antagonism of the bacteria. More studies on the relationships between chitinase production and antagonistic ability are needed to make clear the importance of chitinase in the suppression of plant diseases.

Numbers and molecular weights of chitinase isoforms have been also reported to vary according to bacterial species or genera (12, 16, 22, 27), which are consistent with our results that chitinase isoforms varied with the tested bacterial isolates. Molecular weights of four chitinase isoforms in *S. marcescens* were almost agreed with the reports that show the production of five chitinase isoforms of 57-, 52-, 47-, 37- and 21-kDa (12, 22), except for 21-kDa. In our experiments, the isozyme of 21-kDa was not detected as in the commercial chitinase extracted from *S. marcescens*. On the other hand, chitinase isoforms from *Aeromonas* spp. were not similar to those in other studies that *A. hydrophilla* produced chitinases of 110-kDa (27) and 85-kDa (3), and *A. salmonicida* produced a 200-kDa isozyme (11). These suggest that chitinase isoforms from *Aeromonas* spp. are different with their species or isolates. The chitinase isoforms from *C. violaceum* could not be compared with other reports, since no study on the enzyme production has been reported yet.

The optimum pH for the activity and the stability of *C. violaceum* chitinase was pH 7 and pH 5~10, respectively, which was similar to those of other bacteria; *S. marcescens* (12, 22), *A. hydrophila* (27), *A. salmonicida* (11) and *Vibrio* sp. (15). Bacterial chitinases were known to be generally more active and stable around neutral, compared with *Streptmyces* spp. and fungi. The optimum temperature for the activity and stability of *C. violaceum* was also similar to that of other bacteria, which is reported to be around 50°C (11, 12, 16, 22, 27).

This study revealed that the production of chitinases differed with the bacterial species or genera, and that a strong antagonistic bacteria, *C. violaceum* strain C-61, produced the enzymes much higher than other well-known chitinolytic bacteria. The role of *C. violaceum* strain C-61 chitinase on *R. solani* inhibition is in progress.

요 약

길항세균 *C. violaceum* strain C-61과 strain C-72, *S. marcescens*, *A. hydrophila* 및 *A. caviae*의 배양여액을 ammonium sulfate으로 침전시켜 chitin 분해효소의 생산 및 특성을 조사하였다. *C. violaceum* strain들은 28°C에서 3일, *S. marcescens*는 6일, *A. hydrophila* 및 *A. caviae*는 2일 배양하였을 경우에 가장 많은 chitinase를 생산하였다. 최적 배양 조건에서, *C. violaceum* strain C-61의 chitinase 활성은 strain C-72 보다 약 1.5배, *S. marcescens* 보다 약 5.5배, *A. hydrophila* 및 *A. caviae* 보다 각각 12.0배 및 11.3배 더 높았다. 그러나, N,N'-diacetylchitobiase 활성은 *C. violaceum strain* C-61 보다 *S. marcescens*에서 3.2배 더 높았고, *A. hydrophila* 및 *A. caviae*에서는 매우 낮았다. glycol chitin이 함유된 gel에서, *C. violaceum* strain들의 chi-

tinase는 54-, 52-, 50-, 37-kDa의 4 isoform이 검출되었는데, *S. marcescens*는 58-, 52-, 48-, 38-kDa의 4 isoform, *A. hydrophila*는 70-, 58-, 54-kDa의 3 isoform, 그리고 *A. caviae*는 90-, 79-, 71-, 63-, 58-, 38-kDa의 6 isoform이 검출되었다. *C. violaceum* strain C-61의 chitinase는 pH 7.0과 50°C에서 가장 활성이 높았고, pH 5.0~10.0에 2시간, 0~50°C에 30분 동안 두었을 경우 활성의 변화를 나타내지 않았다.

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