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Purification of Chitinase from an Antagonistic Bacterium *Bacillus* sp. 7079 and Pro-Inflammatory Cytokine Gene Expression by PCTC

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Abstract Chitinase was purified from an antagonistic bacterium *Bacillus* sp. 7079 by ammonium sulfate precipitation, QAE-Sephadex anion exchange chromatography, Sephadex G-100 gel filtration, and SP-Sephadex cation exchange chromatography. The molecular weight of purified chitinase (PC-1) was approximately 66.5 kDa on SDS-PAGE. PC-1 exhibited optimum pH and temperature of pH 7.5 and 45°C, respectively. More than 80% of PC-1 was stable at pH 5.0 to 9.0, and more than 90% at 40°C. Fe^{2+} and Ca^{2+} inhibited the chitinase activity about 20%, and EDTA and ρ-CMB by about 30%, whereas Ag^{+} inhibited the activity up to 65%. The K_m value of PC-1 was 1.215 mg/ml with colloidal chitin as a substrate. We also investigated the effect of PC-1 treated chitin (PCTC) on the pro-inflammatory cytokine gene expression in macrophage RAW 264.7 cells. The expression of IL-1α and IL-1β mRNA gene was investigated using reverse transcriptase polymerase chain reaction (RT-PCR). IL-1α and IL-1β mRNA were induced by the treatment of PCTC and chitin only in RAW 264.7 cells. These expressions were induced as early as 2 h and sustained up to 24 h in RAW 264.7 cells. IL-1α and IL-1β mRNA were more strongly expressed by the treatment of PCTC than chitin treatment alone in RAW 264.7 cells.

Key words: Antagonistic bacterium, *Bacillus*, antifungal, chitinase, cytokine

Chitin, an insoluble linear β-1,4-*N*-acetyl-D-glucosamine polymer, is the second most abundant polysaccharide in nature and represents an important potential source of renewable biomass. It is a major component of most fungal cell walls, insect exoskeletons, and the shells of crustaceans [35]. Recently, chitin and its derivatives have been studied for food, pharmaceutics, agriculture, textile, polymers, and wastewater treatment due to their specific physicochemical and biological properties [11, 12, 14, 26, 28, 30]. However, even though chitin and its derivatives have very strong biological activity, their high molecular weights and high viscosity may restrict the uses *in vivo*. Thus, in order to prepare oligomers, increasing attention has recently been given to converting chitin and its derivatives. Chitin and its derivative oligomers can be obtained by either chemical or enzymatic hydrolysis. They have low molecular weights, lower viscosity and short chains, and are soluble in neutral aqueous solutions. Consequently, they seem to be readily absorbed *in vivo* [32]. Chitin and its derivative oligomers are known to have various biological activities including antitumor activities [32], antimicrobial activities [15], and immuno-enhancing effects [33].

Chitinase (E.C.3.2.1.14), which hydrolyzes the β-1,4-linkages of *N*-acetyl-D-glucosamine polymers of chitin, is carried out by three separate enzymes: endochitinase, which produces multimers of *N*-acetylglucosamine; exochitinase, which releases soluble low molecular weight dimers; and chitobiase, which hydrolyses chitobiose to *N*-acetylglucosamine [6]. Chitinase is commonly found in a wide variety of organisms including bacteria, fungi, higher plants, insects, crustaceans, and some vertebrates [23], and especially can be produced by many microorganisms, such as *Aeromonas* [20], *Aspergillus* [13], *Bacillus* [2, 9], *Enterobacter* [29], *Penicillum* [31], *Pseudomonas* [19], *Serratia* [5, 36], *Streptomyces* [21], *Trichoderma* [4], and *Vibrio* [34]. Many chitinases derived from different strains were purified, and several genes encoding chitinase have been cloned and characterized in detail, together with their gene structure [1, 3, 10, 17, 35].

Since some fungal cell walls are rich in chitin, chitinases have recently received increased attention due to their wide range of biotechnological applications, especially in the biocontrol of fungal phytopathogens. Thus, chitinase-producing strains have been extensively studied as biocontrol agents [6, 7, 18, 23, 25].

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Macrophages participate in innate cellular immunity, and synthesize a variety of immunomodulatory factors including cytokine, leukocyte adhesion, and nitric oxide. Macrophages have secretory capacity for various kinds of mediators, such as IL-1, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNFα, which lead to secondary immune responses, such as proliferation of T and B cells, activation of macrophages for phagocytosis, and killing of microorganisms. Among theses mediators, pro-inflammatory cytokines such as IL-1 and IL-6 can be generated in response to immunological reaction, inflammation, and microbial invasion. Among them, the IL-1 family comprises several structurally-related ligands, and the most extensively characterized include IL-1α and IL-1β. IL-1 is a major pro-inflammatory cytokine with multiple activities in the regulation of immune, inflammatory, endocrine, and neuronal systems.

In a previous study, we selected and identified a novel antagonistic bacterium, *Bacillus* sp. 7079, which can produce chitinase with high antifungal activity as a powerful biological control agent [8]. In this study, we purified the antifungal chitinase from *Bacillus* sp. 7079 and investigated the characteristics of PC-1. Additionally, to search the useful application of PC-1, we investigated the effect of the pro-inflammatory cytokine gene expression after treating PC-1 with chitin in RAW 264.7 macrophage cells.

Materials and Methods

Microorganisms and Culture Conditions

An antagonistic bacterium, *Bacillus* sp. 7079, was isolated from soil in Gyeongju, Korea, and used in this study [8]. The bacterium was cultivated on nutrient agar medium at 30°C for 2 days, and then stored at 4°C. For the production of chitinase, *Bacillus* sp. 7079 was cultivated for 3 days at 30°C with rotary shaker in the chitin-yeast extract medium (CYM) containing 0.7% K_2HPO_4, 0.2% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.05% sodium citrate, 0.01% $MgSO_4 \cdot 7H_2O$, 0.1% yeast extract, and 0.1% colloidal chitin. The pH of the medium was adjusted to 6.8 prior to autoclaving. Colloidal chitin was prepared by a previously published method [8].

Enzyme Assay and Protein Estimation

Chitinase activity was measured by the DNS method [27] using colloidal chitin as a substrate. The assay mixture containing 0.5 ml colloidal chitin, 1 ml 1/15 M phosphate buffer (pH 7.5), and 0.1 ml enzyme solution was incubated in a shaker at 45°C for 2 h, and the reaction was then stopped by adding 0.5 ml 3,5-dinitrosalicylic acid (DNS) solution. After the reaction mixture was boiled for 10 min, it was allowed to stand at room temperature. The reaction mixture then was centrifuged at 3,000 rpm for 15 min, and absorbance of the supernatant was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme which produced 1 μmole glucose equivalent for 1 h from colloidal chitin under the above condition. Protein concentration was determined by measuring absorbance at 280 nm during purification. All other protein was measured by the Lowry method [24] using bovine serum albumin as a standard.

Purification Procedures of Chitinase

Chitinase-producing *Bacillus* sp. 7079 was cultivated in CYM for 3 days at 30°C and 170 rpm on a shaking incubator. After cultivation, the cells were removed by centrifugation at 6,000 rpm for 20 min. The supernatant was precipitated with 75% ammonium sulfate, and the precipitate was obtained by centrifugation at 6,000 rpm for 20 min. The pellet was dissolved in a minimal amount of 1/15 M phosphate buffer (pH 7.5) and dialyzed overnight against 2× volumes of the same buffer. The final enzyme solution was concentrated by lyophilization. The concentrated sample was placed on a QAE-Sephadex column (5.0× 75 cm) equilibrated with 1/15 M phosphate buffer (pH 7.5), and eluted with stepwise of 0–0.6 M NaCl in the same buffer. The active fractions were obtained and concentrated by lyophilization. The concentrated chitinase fraction was placed on a Sephadex G-100 column (2.5×100 cm) equilibrated with the same buffer, eluted in the same buffer. The active fractions were collected and lyophilized. For further purification, the lyophilized chitinase was put on a SP-Sephadex column (2.5×75 cm) equilibrated with 1/15 M phosphate buffer (pH 6.0), eluted in the same buffer. The active fractions were collected and lyophilized to concentrate.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [16], using 5% stacking and 8% resolving gels. After electrophoresis, the gels were stained with 0.25% Coomassie brilliant blue R-250, and then destained with 7% acetic acid and 5% methanol mixture. The marker proteins used as a standard molecular weight were phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), glutamate dehydrogenase (55 kDa), ovalbumin (42.7 kDa), and aldolase (40 kDa).

Enzyme Characteristics

Characteristics of chitinase were studied by modified Lee and Kim [17] methods. The optimum pH for the enzyme reaction was investigated after incubation in various pHs at 45°C for 2 h. The buffers used were: 1 M sodium acetate-HCl buffer (pH 2.0–5.0), 1/15 M phosphate buffer (pH 5.0–8.0), 1/20 M $Na_2B_4O_7$-HCl buffer (pH 8.0–9.0), and 1/20 M $Na_2B_4O_7$-NaOH buffer (pH 9.0–12.0). pH stability of the enzyme was measured from the residual activity

after preincubation in buffers with various pHs for 12 h at 4°C. The optimum temperature was investigated at various temperatures between 40°C and 70°C for 2 h at pH 7.5 (1/15 M phosphate buffer). Thermal stability was measured from the residual activity after preincubation at various temperatures (40°C, 50°C, 60°C, and 70°C) for 10–60 min. The effects of several metal ions and chemicals on the activity was measured. The enzyme solution at a final concentration of 1 mM was preincubated at 4°C for 12 h in the presence of metal ions and at 40°C for 1 h in the presence of chemicals, and then the residual activity was measured using the standard assay conditions.

Cell Culture and Preparation of PCTC

Murine macrophage cell line, RAW 264.7 cell, was obtained from American Tissue Culture Collection (ATCC; Rockville, MD, U.S.A.). RAW 264.7 cells were suspended at concentration of 1×10^6/ml in Dulbecco's modified essential medium (DMEM; GibcoBRL, U.S.A.) containing 10% fetal bovine serum (FBS; GibcoBRL, U.S.A.), 50 mg/ml of streptomycin, and 50 U/ml of penicillin G (GibcoBRL, U.S.A.). RAW 264.7 cells were plated in 60-mm dishes and samples were added. After then, the cells were incubated for 24 h at 37°C in an atmosphere of 5% CO_2.

Colloidal chitin was used at concentration of 100 μg/ml and stored at 4°C. In the case of PCTC, 100 μg/ml of colloidal chitin was incubated with 1% PC-1 solution at 45°C for 2 h, and they were added to RAW 264.7 cells (1×10^6/ml). Subsequently, the cells were incubated for 24 h at 37°C in an atmosphere of 5% CO_2. After incubation, RNA was isolated from RAW 264.7 cells using Trizol.

Total RNA Isolation and RT-PCR

Murine macrophage RAW 264.7 cells in Trizol (GibcoBRL, U.S.A.) reagent were well homogenized and vortexed after 1/10 volume of chloroform was added. After incubating the mixture on ice for 15 min, the samples were centrifuged at 12,000 rpm and 4°C for 15 min. The aqueous phase was transferred to a new 1.5 ml micro-centrifuge tube. Total RNA from the aqueous phase was precipitated by mixing with the same volume of isopropyl alcohol and centrifuged at 12,000 rpm for 15 min at 4°C following 30 min of incubation on ice. Precipitated total RNA pellets were washed once with DEPC-treated 70% ethyl alcohol and were redissolved in DEPC-treated water.

Table 1. Primer sequence used for detection of cytokine gene expression.

	Oligonucleotide sequence
G3PDH	5'-CCA CCC AGA AGA CTG TGG ATG GC-3'
	5'-CAT GTA GGC CAT GAG GTC CAC CAC-3'
IL-1α	5'-CAC TAT CTC AGC ACC ACT TG-3'
	5'-CTG GAA GTC TGT CAT AGA GG-3'
IL-1β	5'-CCG TGG ACC TTC CAG GAT GA-3'
	5'-GAT CCA CAC TCT CCA GCT GC-3'

RT reaction of 6 μg of total RNA was performed in a 20 μl RT reaction mixture containing 0.5 μl of MMLV reverse transcriptase (200 U/μl, Promega), 4.0 μl of 5× MMLV RT buffer (Promega), 2.0 μl of dNTP mixture (10 mM, BM), 0.5 μl of RNase inhibitor (40 U/μl, Promega), and 2.0 μl of oligo dT (50 μM) in DEPC-treated water. The reaction was performed under the conditions of 42°C for 1 h and 95°C for 5 min. PCR was carried out with the use of 1.0 μl of RT products as templates: 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 57°C for 45 sec, and elongation at 72°C for 45 sec. The last cycle was followed by a 10 min extension step at 72°C. The amplified products were analyzed by ethidium bromide-stained agarose gel electrophoresis. The PCR primer sequences are shown in Table 1.

Results and Discussion

Purification and Molecular Weight of PC-1

The chitinase from *Bacillus* sp. 7079 was purified by ammonium sulfate precipitation, QAE-Sephadex anion exchange chromatography, Sephadex G-100 gel filtration, and SP-Sephadex cation exchange chromatography, and the results of the purification are summarized in Table 2. The chitinase was purified 12.6-fold from the culture broth and overall recovery yield was 4.2%. The molecular weight of PC-1 was estimated to be approximately 66.5 kDa by SDS-PAGE (Fig. 1). The molecular weight of PC-1 is similar to the chitinases from *Aspergillus* (50 kDa) [13], *Penicillum* (54.9 kDa) [31], *Enterobacter* (60 kDa) [29], *Serratia* (62 kDa) [17], and *Streptomyces* (70 kDa) [6]. However, it is larger than the chitinolytic enzyme isolated

Table 2. Summary of purification process of chitinase from *Bacillus* sp. 7079.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification fold	Yield (%)
Culture broth	24,343.9	10,573.9	0.4	-	100
Salting out	1,997.6	2,951.5	1.5	3.4	27.9
QAE-Sephadex	438.5	1,756.5	4.0	9.3	16.6
Sephadex G-100	146.1	1,332.3	9.1	21.2	12.6
SP-Sephadex	81.5	440.5	5.4	12.6	4.2

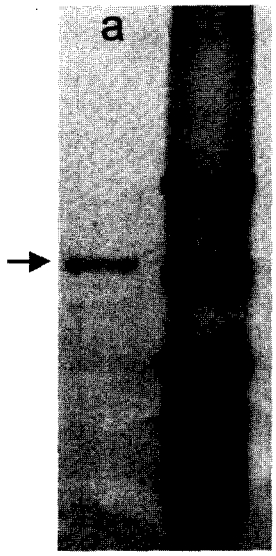

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified *Bacillus* sp. 7079 chitinase.
The purified chitinase was electrophoresed on a 8% polyacrylamide slab gel with 0.1% SDS. Line a: PC-1; Line b: Molecular mass markers [Phosphorylase B (97.4 kDa), Bovine serum albumin (66.2 kDa), Glutamate dehydrogenase (55 kDa), Ovalbumin (42.7 kDa), Aldolase (40 kDa)].

from other strains of *Bacillus* [35], *Ewingella* [10], and *Trichoderma* [4], which have molecular weights between 27.5 and 36 kDa, and is smaller than the chitinase isolated from *Aeromonas* [20], which has a calculated molecular size of 200 kDa.

Effect of pH on Activity and Stability of PC-1

To investigate the optimum pH of PC-1, chitinase activity was measured at various pHs ranging from 2.0 to 12.0. The optimum pH of PC-1 was 7.5, and it was stable at pH 6.5–8.0, as shown in Fig. 2. The optimum pH of PC-1 is similar to *Enterobacter* (pH 7.0) [29] and *Streptomyces* (pH 8.0) [6], but different from *Penicillum* (pH 5.0) [31] and *Trichoderma* (pH 3.5) [4] with acidic optimum pHs. To investigate the effect of pH on the stability of PC-1, the enzyme was preincubated at various pHs for 12 h at 4°C, and the remaining activity was then determined. The chitinase was stable between pH 5.0 and 9.0, and more than 80% of the original activity remained (Fig. 3). Some workers have reported a broad optimum pH, such as 5.0–9.0, 4.0–9.0, and 2.0–8.0 of the chitinases from *Pseudomonas* [31], *Streptomyces* [6], and *Trichoderma* [4], respectively. Taken together, these results indicate that PC-1 is stable in a relatively broad pH range. Therefore, PC-1 activity could be very stable and strong in the soil of nature, which has a weak acidic pH range.

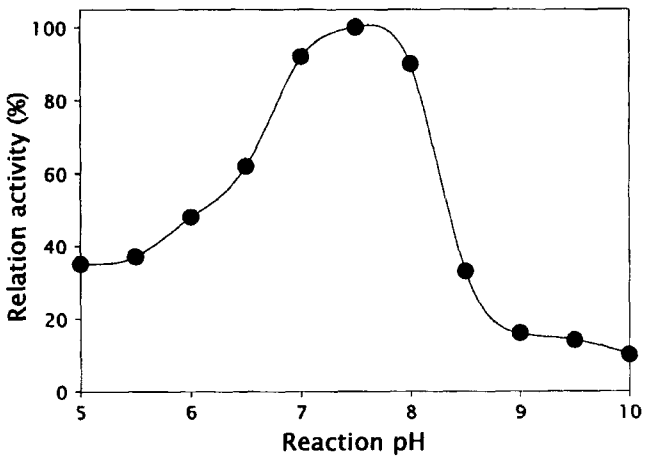

Fig. 2. Effect of pH on the activity of PC-1.
The enzyme activity was assayed in a 1/15 M phosphate buffer (pH 5.0 – 8.0), 1/20 M $Na_2B_4O_7$-HCl buffer (pH 8.0–9.0), and 1/20 M $Na_2B_4O_7$-NaOH buffer (pH 9.0–10.0) at various pHs under the standard conditions.

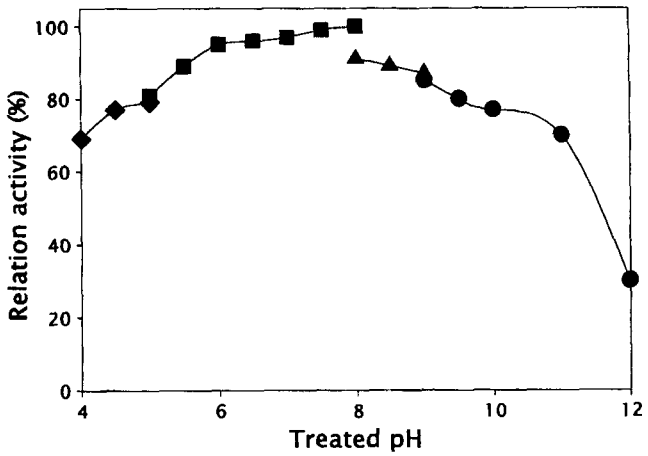

Fig. 3. pH stability of PC-1.
The enzyme solution was preincubated for 12 h in a 1 M sodium acetate-HCl buffer (◆; pH 4.0–5.0), 1/15 M phosphate buffer (■; pH 5.0–8.0), 1/20 M $Na_2B_4O_7$-HCl buffer (▲; pH 8.0–9.0), and 1/20 M $Na_2B_4O_7$-NaOH buffer (●; pH 9.0–12.0) of various pHs, and the remaining activity was assayed under the standard conditions.

Effect of Temperature on Activity and Stability of PC-1

To obtain optimum temperature for the reaction, the enzyme activity was measured at between 40°C and 70°C for 2 h. The optimum temperature of PC-1 was observed to 45°C (Fig. 4). Most of the chitinases from other strains, including *Bacillus* [35], *Ewingella* [10], *Pesudomonas* [19], *Streptomyces* [6], and *Trichoderma* [4], showed the optimum temperature in the range of 40°C to 50°C. To investigate the heat stability of PC-1, the enzyme was preincubated at various temperatures (40°C, 50°C, 60°C, and 70°C) for 10–60 min and then the remaining activity was determined. About 80% of the initial activity remained after incubation for 60 min at 40°C. But, PC-1 rapidly lost the activity at above 50°C, and it was virtually inactivated by preincubation at 70°C (Fig. 5). Other groups have reported that the chitinase from *Ewingella* [10] was stable at 40°C and inactivated at

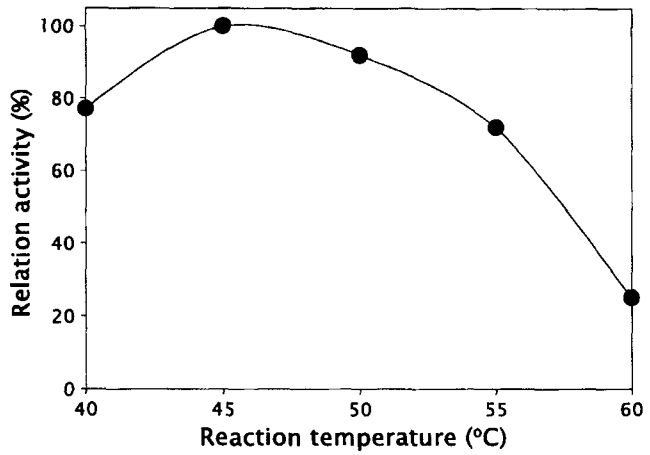

Fig. 4. Effect of temperature on the activity of PC-1.
The enzyme activity was assayed in 1/15 M phosphate buffer (pH 7.5) at various temperatures under the standard conditions.

70°C, and the exochitinase from *Trichoderma* [4] was stable at temperature below 50°C and inactivated at 70°C. The above results indicated that PC-1 activity was stable at temperatures above 40°C, suggesting that PC-1 could carry out the function as an antifungal biocontrol agent in hot summer condition.

Effects of Metal Ions and Chemical Reagents of PC-1

To investigate the effect of several metal ions and chemicals on the activity of PC-1, the enzyme solution at a final concentration of 1 mM was preincubated at 4°C for 12 h in the presence of metal ions and at 40°C for 1 h in the presence of chemicals, and the residual activity was measured. Fe^{2+} and Ca^{2+} ions inhibited the enzyme activity by about 20% and Ag^{+} ion inhibited up to 65% (Table 3). EDTA and ρ-CMB inhibited the activity by about 30%, but it was not inhibited by iodoacetate, thiourea, and hydroxyurea (Table 4).

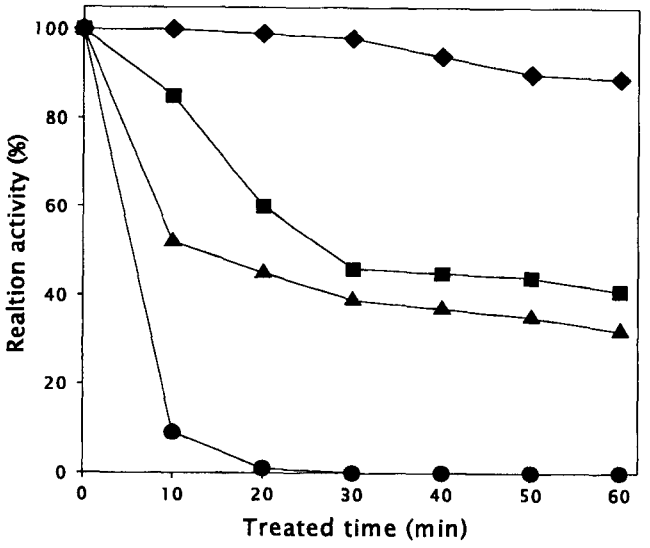

Fig. 5. Thermal stability of PC-1.
The enzyme solution was preincubated in 1/15 M phosphate buffer (pH 7.5) for 10–60 min at various temperatures of 40°C (◆), 50°C (■), 60°C (▲), and 70°C (●), and the remaining activity was assayed under the standard conditions.

Table 3. Effect of various metal ions on the activity of PC-1.

Metal ion	Residual activity (%)
KCl	62.1
$BaCl_2 \cdot 2H_2O$	77.8
$FeSO_4 \cdot 7H_2O$	81.2
$CaCl_2$	80.8
$CuSO_4 \cdot 5H_2O$	68.2
$MgSO_4 \cdot 7H_2O$	68.5
$ZnSO_4 \cdot 7H_2O$	72.4
$MnSO_4$	69.1
$AgNO_3$	35.6
None	100.0

The enzyme solution at a final concentration of 1 mM was preincubated in 1/15 M phosphate buffer (pH 7.5) containing the various metal ions at 4 °C for 12 h, and enzyme activity was measured under the standard assay conditions.

K_m Value

The effect of increasing substrate concentration on the activity of PC-1 was investigated. The Michaelis-Menten constant, (K_m) value, was determined by the Lineweaver-Burke method using colloidal chitin as a substrate. As shown in Fig. 6, the K_m of PC-1 was calculated to be 1.215 mg/ml. This result indicates that *Bacillus* sp. 7079 chitinase was similar to *Aeromonas* with a K_m value of 1.276 mg/ml [20].

Table 4. Effect of various chemical inhibitors on the activity of PC-1.

Chemicals	Residual activity (%)
EDTA	72.1
SDS	98.5
CDTA	83.5
L-Cysteine	98.0
Iodoacetate	107.2
Sodium azide	97.1
ρ-CMB	68.4
Thiourea	128.3
AHA	80.6
Hydroxyurea	138.7
None	100.0

The enzyme solution at a final concentration of 1 mM was preincubated in 1/15 M phosphate buffer (pH 7.5) containing the various chemicals at 40°C for 1 h, and enzyme activity was measured under the standard assay conditions. Abbreviation: EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; CDTA, *trans*-1,2-diaminocycloheane-N,N,N',N'-tetraacetic acid; ρ-CMB, ρ-chloromercuribenzoic acid; AHA, acetohydroxamic acid.

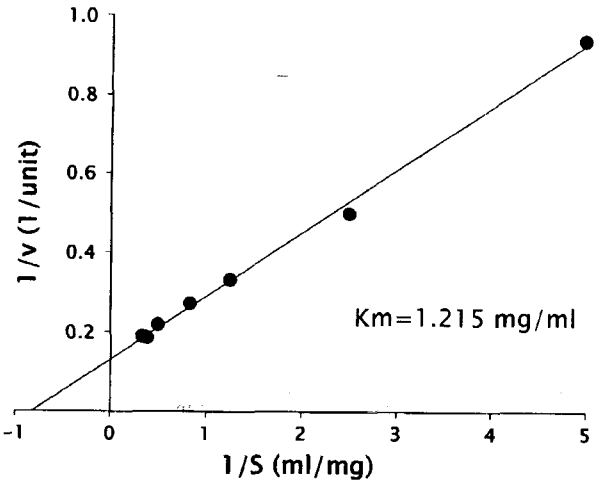

Fig. 6. Lineweaver-Burk plots of colloidal chitin hydrolysis by PC-1.

Effect and Time Course Analysis of Chitin and PCTC on IL-1α and IL-β mRNA Gene Expression in RAW 264.7 Cells

To further utilize PC-1, we examined the difference of pro-inflammatory cytokine gene expression between chitin-only and PCTC-treated RAW 264.7 cells. Thus, murine macrophage RAW 264.7 cells were treated with chitin alone and PCTC at 37°C for 24 h in an atmosphere of 5% CO_2 in air. After incubation, total RNA was isolated from the cells, and the expression of IL-1α and IL-1β mRNA were examined using RT-PCR. As shown in Fig. 7, both chitin alone and PCTC induced IL-1α and IL-1β mRNA gene expression in RAW 264.7 cells, but the expression in RAW 264.7 cells was more strongly induced by PCTC than with chitin alone.

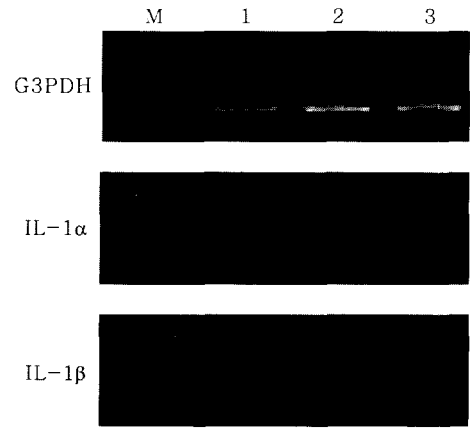

Fig. 7. Effect of IL-1α and IL-1β mRNA expression in RAW 264.7 cells by chitin and PCTC.
RAW 264.7 cells (1×10^6/ml) were incubated with 100 μg/ml of chitin or PCTC for 24 h. Total RNA was prepared from each sample using Trizol, and IL-1α and IL-1β mRNA were analyzed by RT-PCR. M: 100 bp size marker. 1, Control; 2, Chitin; 3, PCTC. G3PDH (glyceraldehyde-3-phosphate dehydrogenase) was used as control genes.

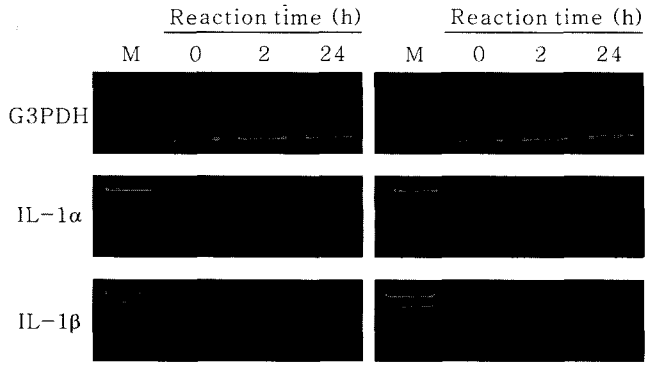

Fig. 8. Time course analysis of effect of either chitin or PCTC on cytokine expression in RAW 264.7 cells.
RAW 264.7 cells (1×10^6/ml) were cultured for 2 h and 24 h in the presence of 100 μg/ml each of chitin and PCTC. After stimulation, total RNA was isolated from the cultures cells using Trizol, and IL-1 α and IL-1β mRNA were analyzed by RT-PCR. M: 100 bp size marker. Left, Chitin; Right, PCTC. G3PDH (glyceraldehyde-3-phosphate dehydrogenase) was used as control genes.

To investigate the time course of the expressions of IL-1α and IL-1β mRNA, chitin only and PCTC were treated in RAW 264.7 cells, and then the cells were incubated at 37°C under an atmosphere of 5% CO_2 in air. Total RNA was isolated from the cells at 0, 2, and 24 h, respectively, and then the cytokine gene expressions were observed using RT-PCR. As shown in Fig. 8, the expression of IL-1α and IL-1β mRNA was detectable as early as 2 h in RAW 264.7 cells both by chitin and PCTC. IL-1α and IL-1β mRNA expressions by chitin were decreased at 24 h, whereas PCTC-induced IL-1α and IL-1β mRNA genes were strongly sustained up to 24 h.

Taken together, these results demonstrate that PCTC was more strongly involved than chitin only in the modulation of pro-inflammatory cytokine gene expressions in macrophage RAW 264.7 cells. PCTC may contribute to industrial utilization of chitin. Further characterization of the compounds and mechanism of PCTC as well as *in vivo* experiments will be further investigated.

Acknowledgment

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