Purification and Characterization of Chitinase A of *Streptomyces cyaneus* SP-27: An Enzyme Participates in Protoplast Formation from *Schizophyllum commune* Mycelia

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A culture filtrate of *Bacillus circulans* KA-304 grown on a cell-wall preparation of *Schizophyllum commune* mycelia has an activity to form protoplasts from *S. commune* mycelia. α-1,3-Glucanase and chitinase I, which were isolated from the filtrate, did not form the protoplast by itself while a mixture of them showed protoplast-forming activity.

*Streptomyces cyaneus* SP-27 was isolated based on the productivity of chitinase. The culture filtrate of *S. cyaneus* SP-27 did not form *S. commune* protoplasts, but addition of it to α-1,3-glucanase of *B. circulans* KA-304 brought about protoplast-forming activity. Chitinase A isolated from the *S. cyaneus* SP-27 culture filtrate was more effective than chitinase I of *B. circulans* KA-304 for the protoplast formation in combination with α-1,3-glucanase. The N-terminal amino acid sequence of chitinase A (MW 29,000) has a sequential similarity to those of several Streptomycete family 19 chitinases. Chitinase A adsorbed to chitinous substrate and inhibited the growth of *Trichoderma reesei* mycelia. Anomer analysis of the reaction products also suggested that the enzyme is a family 19 chitinase.

**Key words:** *Schizophyllum commune*; protoplast; family 19 chitinase; α-1,3-glucanase

Basidiomycetes are valuable not only as food or feed, but also as microbial resources for the production of bioactive substances, the transformation of lignocellulosic biomass, the improvement of polluted environments, and so on. 1–3 However, they have some disadvantages, such as slow growth and susceptibility to fungal invasion. Hence breeding with modern biotechnology has become significant, and this requires efficient formation of reproducible protoplasts. Several attempts have been made to form protoplasts from various basidiomycetes by using commercially available cell-wall lytic enzyme preparations, 4–7 most of them from *Trichoderma* species. However, these preparations show low protoplast-forming activity when they are used individually, and hence they are always used in combination. This indicates a need for further development of cell-wall lytic enzymes from diverse sources as well as more information on the basidiomycete cell-wall.

We have found that a culture filtrate of *Bacillus circulans* KA-304 showed an activity to release protoplasts from intact mycelia of *Schizophyllum commune* when the bacterium was grown on a cell-wall preparation of *S. commune* as an inducer. 8 Previous studies revealed that α-1,3-glucanase and chitinase I, 9,10 which were isolated from the culture filtrate (KA-prep), did not form *S. commune* protoplasts by itself, and that a mixture of them showed the protoplast-forming activity: the protoplasts increased during the early incubation period but scarcely increased during the later period. This finding indicated that chitinase I and α-1,3-glucanase are minimum requirements for protoplast formation.

The gene of α-1,3-glucanase has been cloned from *B. circulans* KA-304 and expressed in *Escherichia coli. 11* The deduced amino acid sequence of the enzyme showed approximately 80% homology with that of α-1,3-glucanase of *Bacillus sp.* RM1. 12 The chitinase I gene was also cloned and expressed in *E. coli. 13* The amino acid sequence indicated that the N-terminal moiety of the enzyme had only slight sequence homology with the linker domain of chitinase A1 of *B. circulans* WL-12, which contains two fibronectin type III domains. 14 The sequence also showed homology of the C-terminal moiety of chitinase I with the incomplete configuration.

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catalytic domain of glycosyl hydrolase family 19 chitinases. Family 19 chitinases were found initially only in plants (class I, II, and IV chitinases of plants), and were thought to constitute part of the defense system of plants against fungal pathogens. In 1996, chitinase C of Streptomyces griseus HUT 6037 was described as the first family 19 chitinase from a prokaryote.\textsuperscript{15} Subsequently, family 19 chitinases have been found in several Streptomyces species,\textsuperscript{15–17} Aeromonas sp.,\textsuperscript{18} Burkholderia gladioli\textsuperscript{19} etc., and chitinase I of B. circulans KA-304 was the first example of a family 19 chitinase in Bacillus.

These findings suggest that the chitinase species necessary for protoplast formation is family 19 chitinase, not family 18 chitinase. This might be beneficial in prescribing a lytic enzyme system for efficient formation of protoplasts, indicating the need for further structural and functional information on effective chitinases.

This paper deals with a chitinase (chitinase A) produced by chitin-assimilating Streptomyces cyaneus SP-27. The organism was selected by chitinase productivity in its culture filtrate, and also by ability of the filtrate to form S. commune protoplasts in a mixture containing α-1,3-glucanase. Isolated chitinase A formed more S. commune protoplasts than chitinase I of B. circulans KA-304 in the presence of α-1,3-glucanase.

Materials and Methods

Selection of chitinase-producing actinomycetes. Two procedures were used in selecting microorganisms. (i) The first was done by using an agar plate containing colloidal chitin. A soil sample was suspended in sterilized water, and heated at 60 °C for 10 min. The upper phase of the suspension (0.1 ml) was spread on an agar plate containing 0.5% colloidal chitin, 0.05% K\textsubscript{2}HPO\textsubscript{4}, 0.05% KH\textsubscript{2}PO\textsubscript{4}, 0.02% MgSO\textsubscript{4}•7H\textsubscript{2}O, 0.05% yeast extract, and 1.5% agar. After incubation at 30 °C for 7 to 14 d, all tough and leathery colonies were isolated, which formed a clear zone on the plate. (ii) The second selection was by chitinase productivity in liquid medium. Each isolate was inoculated into 5 ml of a medium in a test tube. The medium consisted of 0.5% glucose, 0.05% K\textsubscript{2}HPO\textsubscript{4}, 0.05% KH\textsubscript{2}PO\textsubscript{4}, 0.02% MgSO\textsubscript{4}•7H\textsubscript{2}O, and 0.05% yeast extract. The culture, which was incubated for 2 d at 30 °C with shaking, was transferred to a 2-liter Sakaguchi flask containing 1-liter of the medium with 0.5% powdered chitin, 0.05% K\textsubscript{2}HPO\textsubscript{4}, 0.05% KH\textsubscript{2}PO\textsubscript{4}, 0.02% MgSO\textsubscript{4}•7H\textsubscript{2}O, and 0.05% yeast extract, pH 7. During incubation at 30 °C on a reciprocal shaker (100 rpm), the culture filtrate obtained by 20 min of centrifugation at 10,000 × g was used to measure chitinase activity.

16S ribosomal DNA analysis. 16S ribosomal DNA was amplified by polymerase chain reaction (PCR) using primers 20f (5’-AGTTTGATCCTGGCTC-3’) and 1510r (5’-GGCTACCTTGTTACGA-3’). PCR was done in a reaction mixture (50 µl in EX-Taq buffer) containing 10 ng of chromosomal DNA, 20 pmol of each primer, 200 µM each of deoxynucleoside triphosphates (dNTP), and 2.5 units of EX Taq polymerase (Takara, Shiga, Japan). Thermocycling was 1 cycle of 94 °C for 1 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The sequence of the PCR products was analyzed with an ABI-Prism Big Dye terminator cycle sequencing ready reaction kit and an ABI-prism 377 sequencer (Applied Biosystems, Tokyo, Japan). A homology search of the DNA sequences was done with the BLAST program\textsuperscript{20} on the NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST/).

Purification and Characterization of Chitinase A of S. cyaneus SP-27. All operations of the enzyme purification was carried out at 4 °C with 10 mM potassium phosphate buffer (pH 7.0, buffer A) or 10 mM Tris–HCl buffer (pH 8.0, buffer B) unless otherwise stated.

Step 1. S. cyaneus SP-27 was cultivated for 12 d with 1-liter of the medium described above, and the culture was centrifuged at 12,000 × g for 20 min. About 5-liter of the culture filtrate was used as a starting material.

Step 2. To the culture filtrate, was added solid ammonium sulfate to reach 90% saturation. After the mixture was stored overnight, the precipitate, collected by centrifugation (12,000 × g for 20 min), was dissolved in and dialyzed against buffer A.

Step 3. The dialyzed solution was put on a DEAE-cellulofine A-500 column (3 × 15 cm) equilibrated with buffer A. Chitinase activity was detected in the unadsorbed fractions (chitinase A, see Fig. 2), and another was eluted with the buffer containing 0.2 M NaCl (chitinase B). Each preparation was concentrated with ammonium sulfate (90% saturation) and dialyzed against buffer B. The unadsorbed chitinase preparation was active in protoplast formation in the presence of α-1,3-glucanase of B. circulans KA-304.

Step 4. The unadsorbed chitinase preparation (chitinase A) was put on a DEAE-Toyopearl 650M column (3 × 15 cm) equilibrated with buffer B. After being washed, the column was developed with buffer B containing 0.2 M NaCl. Chitinase activity was eluted with the buffer containing 0.2 M NaCl. The fractions was concentrated with ammonium sulfate (90% saturation), and dialyzed against buffer B.

Step 5. To the dialyzed precipitate, ammonium sulfate was added to 5% saturation, and the solution was applied to a column of Butyl-Toyopearl 650M (2 × 3 cm) equilibrated with buffer B containing 5% ammonium sulfate. After washing with buffer B containing 5% ammonium sulfate, the column was developed with the buffer containing 2.5% ammonium sulfate, and then with buffer B without ammonium sulfate. Chitinase activity was found in buffer B without ammonium sulfate. The active fractions were concentrated with ammonium sulfate, and the solution was dialyzed against buffer B.
Assay of chitinase activity. Chitinase was assayed colorimetrically using ethylene glycol chitin as a substrate. A reaction mixture (0.4 ml) containing 0.1% ethylene glycol chitin, 50 mM potassium phosphate buffer (pH 6.5), and appropriate amounts of enzyme was incubated at 30 °C, and the reaction was stopped by immersing the mixture in boiling water for 10 min. The reducing sugar formed in the mixture was measured as N-acetylgalactosamine by the method of Shales21) or the modified method of Reissig.22) One unit of the enzyme was defined as the amount of enzyme that released 1 nmol of reducing sugar per min.

Assay of α-1,3-glucanase activity. α-1,3-Glucanase activity was determined as described previously,11) using mutan as a substrate. One unit of the enzyme was defined as the amount of enzyme releasing 1 nmol of reducing sugar (as glucose) per min.

Assay of protoplast-forming activity. Protoplast-forming activity was determined according to methods described previously,8) using Schizophyllum commune IFO 4928 as a test strain.

Assay of chitin-binding activity. The binding assay mixture contained 0.1 mg/ml of chitinase, 1% substrate, 100 mM NaCl and 50 mM potassium phosphate buffer (pH 6.5). After 1 h incubation on ice with occasional stirring, the mixture was centrifuged, and the protein concentration in the supernatant was determined. The amount of bound protein was estimated by subtracting the amount of free protein in the supernatant from the initial amount of protein, and chitin-binding efficiency was shown as percentages of the initial amount of protein.

Assay of antifungal activity. The antifungal activity of chitinase was estimated by hyphal extension inhibition assay, as described previously33) using Trichoderma reesei NBRC 31326 as a test strain.

HPLC analysis of enzymatic products. The anomic form of the hydrolytic products was determined by HPLC.23) The reaction mixture contained 5 μg/ml chitinase A, 5 mM N-Acetylglucosamine pentasaccharide (GlcNAc)₅, and 50 mM potassium phosphate buffer (pH 6.5), and the enzymatic reaction was conducted at a lower temperature (25 °C) to suppress mutarotation. In order to terminate the enzymatic reaction, the mixture was mixed with the same volume of chilled acetone (−20 °C). The resulting solution was applied onto a column of TSK-Gel amido-80 (4.6 × 250 mm, Tosoh, Tokyo). The elution solvent was 70% acetone, and the flow rate was 0.7 ml/min. The substrate and the products were monitored by the absorbance at 210 nm. N-Acetylglucosamine disaccharide (GlcNAc)₂, N-Acetylglucosamine trisaccharide (GlcNAc)₃, N-Acetylglucosamine tetrasaccharide (GlcNAc)₄, and (GlcNAc)₅ were used as standards.

Analytical method. Protein was measured by Lowry’s method24) with egg albumin as the standard. In column chromatography, protein was followed by the absorbance at 280 nm. SDS–PAGE was performed by the method of Laemmli.25) Determination of the N-Terminal amino acid sequence of the isolated enzyme was done as described in a previous paper.9)

Reagents. α-1,3-Glucanase and chitinase I were prepared according to methods described previously from cell-free extract of E. coli harboring plasmid pET22-agl or pET22-chi1. Colloidal chitin was prepared by the method of Berger and Reynolds.20) Other reagents were chemically pure grades of commercial products.

Results and Discussion

Selection of chitin-degrading actinomycetes

From among chitin-assimilating actinomycetes isolated by the procedure described in “Materials and Methods,” four organisms (strains SP-7, SP-9, SP-16, and SP-27) were selected. They formed large clear zones on an agar plate containing colloidal chitin.

They were cultivated in a 2-liter Sakaguchi flask on a 1-liter of a medium containing 0.5% powdered chitin, and the chitinase activity of the culture filtrates was measured. Strain SP-7, strain SP-9, and strain SP-27 produced chitinase during the later incubation period (6 to 12 d), and maximum activity was observed in the 12-d culture filtrate (SP-7, 280 units/ml; SP-9, 180 units/ml; SP-27, 340 units/ml). In the case of strain SP-16, the maximum enzyme activity was obtained in 7 d culture filtrate (140 units/ml), and activity lowered in the later cultivation period.

To the filtrate of each organism with maximum activity, was added solid ammonium sulfate to reach 90% saturation. The precipitate was collected by centrifugation (12,000 × g for 20 min), and then dissolved in and dialyzed against 10 mM potassium phosphate buffer (pH 7.0). All the dialysate (ammonium sulfate concentrate) formed protoplasts from S. commune mycelia in the mixture containing α-1,3-glucanase, and their ability, which was evaluated by the numbers of released protoplast per crude protein, was almost the same (data not shown).

Strain SP-27, which showed the highest chitinase productivity, was chosen for further study. The physiological and morphological characteristics of strain SP-27 indicated that the strain belongs to genus Streptomyces (data not shown). The 16S ribosomal DNA sequence of Strain SP-27 (DDBJ accession no. AB361037) showed 99% similarity to that of Streptomyces cyaneus. On the basis of these findings, strain SP-27 is expressed as Streptomyces cyaneus SP-27 in this study.
Protoplast formation using ammonium sulfate concentrate of S. cyaneus SP-27 culture filtrate

The filtrate of 12 d culture of S. cyaneus SP-27 was concentrated with ammonium sulfate (90% saturation) as described in the preceding paragraph, and the concentrated filtrate was confirmed and characterized by its ability to form protoplasts in the mixture containing \( \alpha \)-1,3-glucanase.

The ammonium sulfate concentrate of the culture filtrate did not contain \( \alpha \)-1,3-glucanase, and did not form protoplasts from Schizophyllum commune mycelia (Table 1). However, when \( \alpha \)-1,3-glucanase (0.2 mg/ml) of B. circulans KA-304 was added to the concentrate (0.025 mg protein/ml), protoplast-forming activity appeared as in the case of the mixture of \( \alpha \)-1,3-glucanase and chitinase I of B. circulans KA-304. Increase of ammonium sulfate concentrate (0.05 to 0.1 mg/ml) was effective for increasing protoplasts. These results suggest that family 19 chitinase(s), like chitinase I, occur in the culture filtrate of S. cyaneus SP-27.

Separation of chitinase effective for protoplast formation

To identify chitinase(s) participating in protoplast formation, the ammonium sulfate concentrate of the culture filtrate was chromatographed on a DEAE-cellulofine A-500 column at pH 7.0. As shown in Fig. 1, chitinase activity was detected in the unadsorbed fractions (tentatively named chitinase A, 443,000 units), and another was eluted with the buffer containing 0.2 M NaCl (chitinase B, 260,000 units). The chitinase A fraction maintained the ability to impart protoplast-forming activity to \( \alpha \)-1,3-glucanase of B. circulans KA-304, but the chitinase B fraction did not (data not shown).

As shown in Table 2, chitinase A was purified 16-fold with an overall yield of 9.3%, and SDS–PAGE analysis indicated that the final preparation was homogeneous and its molecular mass was 29,000 (Fig. 2). The molecular weight of chitinase A was estimated to be 27,000 by HPLC-gel filtration on TSK-GEL G2000SW (7.5 × 600 mm) standardized with ribonuclease A (MW

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**Table 1.** Protoplast Formation in the Mixture Containing \( \alpha \)-1,3-Glucanase and Ammonium Sulfate Concentrate of Culture Filtrate of S. cyaneus SP-27

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Number of released protoplasts ((\times 10^3/ml)) at an incubation time of</th>
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<tr>
<td></td>
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<tr>
<td>Ammonium sulfate concentrate (0.1 mg/ml)</td>
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<tr>
<td>( \alpha )-1,3-Glucanase (0.2 mg/ml)(^a)</td>
<td>0</td>
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<tr>
<td>Ammonium sulfate concentrate (0.025 mg/ml) + ( \alpha )-1,3-glucanase</td>
<td>0</td>
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<tr>
<td>Ammonium sulfate concentrate (0.05 mg/ml) + ( \alpha )-1,3-glucanase</td>
<td>0</td>
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<tr>
<td>Ammonium sulfate concentrate (0.1 mg/ml) + ( \alpha )-1,3-glucanase</td>
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Protoplast formation was done in a mixture containing 50 mM potassium phosphate buffer (pH 6.5) containing 0.5 M mannitol and several enzyme preparations as described in the Table. The numbers of protoplasts released in 1 ml of the mixture are indicated. The released protoplasts were counted microscopically with a hemocytometer.

\(^a\)An amount of 0.2 mg/ml corresponds to 100 units/ml.

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**Fig. 1.** Separation of Chitinases by DEAE-Cellulofine A-500 Column Chromatography.

The ammonium sulfate concentrate of the culture filtrate of S. cyaneus SP-27 was put on a DEAE-cellulofine A-500 column (3 × 15 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.0). After being washed, the column was developed with the buffer containing 0.2 M NaCl. Symbols: —, chitinase activity; —, protein; ——, NaCl concentration in the elution buffer.
13,700), carbonic anhydrase (MW 29,000), ovalbumin (MW 43,000), and albumin (MW 67,000). These results suggest that chitinase A consists of a single polypeptide.

Properties of chitinase A

Chitinase A was stable in a pH range of 5.5–8.5, and retained its full activity after 10 min incubation at 60°C and pH 6.5. The optimum pH for the reaction of chitinase A was 7.0. The enzyme hydrolyzed ethylene glycol chitin (100% relative activity), colloidal chitin (30%), and powdered chitin (4.5%). Analysis of reaction products revealed that chitinase A cleaved (GlcNAc)₄ into (GlcNAc)₂ and (GlcNAc)₃ (see Fig. 5). The enzyme was inert toward (GlcNAc)₂, (GlcNAc)₃, and p-nitrophenyl-β-D-N-acetylglucosaminide, suggesting that chitinase A is an endo-type enzyme.

The N-terminal amino acid sequence of chitinase A (A A X S S Y P A W A A G; X, not identified) showed high similarity to those of *Streptomyces olivaceoviridisis* chitinase 30₂₇) and *Streptomyces coelicolor* A₃(2) chitinase F₁₆) (Fig. 3). These enzymes are classified as family 19 chitinases, and composed of an N-terminal chitin binding domain and a C-terminal catalytic domain.

Table 3 shows that Chitinase A bound to colloidal chitin and powdered chitin, slightly bound to avicel, but not to chitosan. Figure 4 indicates that a slight amount of chitinase A (2.5 µg/disc) inhibited the hyphal extension of *T. reesei* as the known family 19 chitinases do.₁₇,₂₈,₂₉)

It has been reported that family 19 chitinases form the α-anomer as an initial product from *N*-acetylchitooligosaccharide.₃₀) To identify the anomeric form of hydrolysis products from a reaction catalyzed by chitinase A, the reaction with (GlcNAc)₅ as a substrate was analyzed. As shown in Fig. 5A, the ratios of α-anomer to β-anomer of naturally occurring (GlcNAc)₂, (GlcNAc)₃, (GlcNAc)₄, and (GlcNAc)₅ were respectively 1:0.69, 1:0.65, 1:0.64, and 1:0.60. Figure 5B shows that chitinase A cleaved (GlcNAc)₅ into (GlcNAc)₂ and (GlcNAc)₃ with ratios of α-anomer to β-anomer of 1:0.27 and 1:0.37 respectively. The ratios of anomeric indicate that the enzyme hydrolyzes the second and third glycosidic linkages from the nonreducing end of (GlcNAc)₅, with almost the same reactivity. The accumulation of α-anomer suggests that the enzyme catalyzes the hydrolysis through an inverting mechanism.₃¹)

These results suggest that chitinase A is a family 19 chitinase with a chitin-binding domain.

Protoplast formation using chitinase A

Figure 6 compares protoplast formation by chitinase A with that by chitinase I of *B. circulans* KA-304 in a mixture containing 1,3-glucanase. The addition of chitinase A to 1,3-glucanase caused the appearance of protoplast-forming activity, and the number of protoplasts increased with increase in the chitinase A concentration from 100 to 200 units/ml. The addition of 200 units/ml chitinase A to the mixture with α-1,3-glucanase resulted in the formation of approximately 1.0 x 10⁷ protoplasts (per ml) in 18 h incubation.
It is notable that protoplast formation continued through the entire incubation period.

In the case of chitinase I of \textit{B. circulans} KA-304, the number of protoplasts formed was less than with chitinase A (addition of 100 units/ml chitinase I, approximately $2.5 \times 10^6$ protoplasts/ml), and an increase in chitinase I to 200 units/ml was not effective for increasing the number of protoplasts (200 units/ml chitinase I, approximately $3.8 \times 10^6$ protoplasts/ml). Protoplast formation in the mixture of chitinase I and \(\beta\)-1,3-glucanase proceeded only in the early incubation period with the declined rate in the later period, which has been observed in previous studies.\(^{10,13}\)

The present study suggests that chitinase A is a family 19 chitinase with a chitin binding domain whereas chitinase I was described to have no amino acid sequence similarity to known chitin binding domains.\(^{13}\)

Cloning and analysis of the chitinase A gene might reveal the cause of the different functions in protoplast formation of chitinases I and chitinases A. Comparative studies of their reactivity toward various chitinous complexes are also important. These are now underway, and the results should appear soon.

Acknowledgment

We would like to thank Mr. Takahiko Nakao for valuable support.
Fig. 6. Effect of Chitinase A or Chitinase I on Protoplast Formation in a Mixture Containing α-1,3-Glucanase.

The activity of chitinase I has been measured in a mixture containing colloidal chitin as a substrate. In contrast, chitinase A in the present study was determined with ethylene glycol chitin as a substrate. To compare these enzymes, chitinase A was redefined with colloidal chitin as a substrate. A reaction mixture containing colloidal chitin (final turbidity at 610 nm, 0.4), 50 mM potassium phosphate buffer (pH 6.5), and appropriate amounts of enzyme was incubated at 30 °C, and the reducing sugar formed in the mixture was measured as N-acetylglucosamine. One unit of the enzyme was defined as the amount that released 1 nmol of reducing sugar per min. For protoplast formation, chitinase A or chitinase I was added to a reaction mixture of protoplast formation containing 0.2 mg/ml α-1,3-glucanase. The number of protoplasts released in 1 ml of the mixture was determined. Symbols: —, chitinase A (100 units/ml, 0.013 mg/ml protein); —, chitinase A (200 units/ml, 0.026 mg/ml protein); —, chitinase I (100 units/ml, 0.06 mg/ml protein); —, chitinase I (200 units/ml, 0.12 mg/ml protein).

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