Cloning and Expression of a *Bacillus circulans* KA-304 Gene Encoding Chitinase I, Which Participates in Protoplast Formation of *Schizophyllum commune*

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KA-prep, a culture filtrate of *Bacillus circulans* KA-304 grown on a cell-wall preparation of *Schizophyllum commune*, has an activity to form protoplasts from *S. commune* mycelia, and a combination of α-1,3-glucanase and chitinase I, isolated from KA-prep, brings about the protoplast-forming activity.

The gene of chitinase I was cloned from *B. circulans* KA-304 into pGEM-T Easy vector. The gene consists of 1,239 nucleotides, which encodes 413 amino acids including a putative signal peptide (24 amino acid residues). The molecular weight of 40,510, calculated depending on the open reading frame without the putative signal peptide, coincided with the apparent molecular weight of 41,000 of purified chitinase I estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The C-terminal domain of the deduced amino acid sequence showed high similarity to that of family 19 chitinases of actinomycetes and other organisms, indicating that chitinase I is the first example of family 19 chitinase in *Bacillus* species.

Recombinant chitinase I without the putative signal peptide was expressed in *Escherichia coli* Rosetta-gami B (DE 3). The properties of the purified recombinant enzyme were almost the same as those of chitinase I purified from KA-prep, and showed the protoplast-forming activity when it was combined with α-1,3-glucanase from KA-prep. Recombinant chitinase I as well as the native enzyme inhibited hyphal extension of *Trichoderma reesei*.

Key words: *Bacillus circulans* KA-304; *Schizophyllum commune*; protoplast; family 19 chitinase; α-1,3-glucanase

The 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 (CWP) of *S. commune* as an inducer. The cell-wall of basidiomycetes has been reported to consist of three layers: the outer layer with water-soluble β-glucan, the second with an alkaline-soluble α-1,3-glucan (S-glucan), and the inner with an alkaline-insoluble highly branched glucan with β-1,3 and β-1,6 linkages (R-glucan) in which chitin microfibrils are embedded. The finding that the filtrate of *B. circulans* KA-304 grown on CWP (KA-prep) showed the protoplast-forming activity indicated that KA-prep contained all the components necessary for the protoplast formation. Actually, KA-prep contained several kinds of α-glucan-hydrolyzing enzyme (α-glucanase), β-glucan-hydrolyzing enzyme (β-glucanase), and colloidal chitin-hydrolyzing enzyme (chitinase), which might correspondingly degrade the polysaccharide components of the cell-wall. But, the protoplast-forming activity decreased or disappeared when KA-prep was fractionated by ammonium sulfate, for example to the fractions 0–30% saturation (showing a slight protoplast forming activity), 30–60% (no activity), and 60–90% (no activity). In a previous study, we found that α-1,3-glucanase purified from the ammonium sulfate fraction of 0–30% saturation of KA-prep gave the protoplast-forming activity to the ammonium sulfate fraction of 30–50% saturation, which contained quite a part of chitinase(s) and β-glucanase(s) in KA-prep. The N-terminal amino acid sequence of the enzyme showed 88% identity with that of mutanase (α-1,3-glucanase) from *Bacillus* sp. RM1.

Subsequently we purified chitinase I from the ammonium sulfate fraction of 30–50% saturation, which showed the protoplast-forming activity with α-1,3-glucanase. This finding indicated that chitinase I and α-1,3-glucanase are the minimum requirements for the protoplast formation, and chitinase I was characterized to compare it with enzymes from other sources. But, the N-terminal amino acid sequence of chitinase I, in contrast with the case of α-1,3-glucanase, had no identity with those of known microbial chitinases, suggesting a need for further investigation of the enzyme.

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Materials and Methods

Microorganisms and cultivation. B. circulans KA-304 was grown at 30°C for 6 d on Medium B\(^1\) consisting of 0.5% polypeptone, 0.5% yeast extract, 0.1% K\(_2\)HPO\(_4\), 0.03% MgSO\(_4\)·7H\(_2\)O, 0.5% NaCl, and 0.5% CWP of S. commune, pH 7.0.

S. commune IFO 4928 was grown at 30°C with shaking on Medium C\(^1\) containing 2% glucose, 1% polypeptone, 0.3% yeast extract, 0.3% K\(_2\)HPO\(_4\), and 5 \(\mu\)g/l thiamine, pH 7.0. The mycelial pellet after 4 d of cultivation with 5 ml of Medium C in a test tube was fragmented with a blender (Waring No. 7009) at full speed for 1 min. The suspension was inoculated into 250 ml of Medium C in a 500-ml Sakaguchi flask and incubated on a reciprocal shaker for 24 h (100 strokes/ min). The fine mycelial pellets were collected by filtration with cotton cloth and washed several times with water, twice with 50 mM potassium phosphate buffer (pH 6.5), and finally with the same buffer containing 0.5 mM mannitol as an osmotic stabilizer.

Escherichia coli JM 109 was used as a host for constructing various recombinant plasmids, and was grown at 37°C in LB medium containing 100 \(\mu\)g/ml ampicillin. E. coli Rosetta-gami B (DE3) carrying the recombinant plasmid was grown at 30°C in LB medium containing 100 \(\mu\)g/ml ampicillin, 10 \(\mu\)g/ml chloramphenicol, 25 \(\mu\)g/ml kanamycin, and 15 \(\mu\)g/ml tetracycline.

Purification of chitinase I of B. circulans KA-304. Chitinase I was purified from KA-prep by ammonium sulfate fractionation, DEAE-cellulose A-500 column chromatography, and CM-cellulose column chromatography, as described previously.\(^9\)

Internal amino acid sequence. Chitinase I (2.5 \(\mu\)g/ml, 40 \(\mu\)l) was denatured by immersing in boiling water for 3 min, and mixed with lysyl endopeptidase (1 \(\mu\)g/ml, 5 \(\mu\)l) and 5 \(\mu\)l of 500 mM Tris–HCl buffer (pH 9.0) containing 0.1% sodium dodecyl sulfate. The peptide fragments were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the band of the protein was transferred onto polyvinylidene difluoride membrane by electroblotting. Several peptide bands were detected on the sheet by staining with 0.1% ponceau S. A peptide band (MW 5,000) was cut out from the sheet, and then its N-terminal amino acid sequence was determined according to the method of Edman\(^10\) using peptide sequencer PPSQ-10 (Shimadzu).

Amplification of partial chitinase I gene. Chromosomal DNA was purified from B. circulans KA-304 by the phenol/chloroform extraction method,\(^11\) and part of the chitinase I gene was amplified from the DNA by polymerase chain reaction (PCR). All the primers used in the cloning of the chitinase I gene are listed in Table 1. A sense primer, chi1n1, was designed on the basis of the N-terminal amino acid sequence of chitinase I described previously,\(^9\) and an antisense primer, chi1c1, on the basis of the internal amino acid sequence. PCR was done in a reaction mixture (50 \(\mu\)l in GC buffer I) containing 10 ng of chromosomal DNA, 40 pmol of each primer, 200 \(\mu\)M each of deoxynucleoside triphosphates (dNTP), and 2.5 units of LA Taq polymerase (Takara). Thermocycling was one cycle of 94°C for 1 min followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min. A DNA fragment of 0.8-kb obtained by the PCR was cloned into pGEM-T Easy (Promega) and sequenced with the ABI-Prism Big Dye terminator cycle sequencing ready reaction kit and the ABI-prism 377 sequencer (Perkin Elmer, Applied Biosystems). This fragment was described as “partial chitinase I gene”.

### Table 1. Oligonucleotide Primers Used in This Study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>position</th>
<th>Sequence*(5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>chi1n1</td>
<td>Sense</td>
<td>109–126</td>
<td>GCNACICCCGACIYTGAAY</td>
</tr>
<tr>
<td>chi1c1</td>
<td>antisense</td>
<td>922–944</td>
<td>GGICCNCICCRARTAYTGYTT</td>
</tr>
<tr>
<td>chi1n51</td>
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<td>782–801</td>
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<td>chi1n52</td>
<td>antisense</td>
<td>740–759</td>
<td>TGCAAGGAATGAGATAAAG</td>
</tr>
<tr>
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<td>sense</td>
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<td>AAATATCCGGTGCAACAGGCTAT</td>
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<tr>
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<td>sense</td>
<td>533–572</td>
<td>TACACCGTGATCCGCACTAAA</td>
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<td>553</td>
<td>GAACTCCGGACGCAAGAT</td>
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<tr>
<td>chi1c2</td>
<td>antisense</td>
<td>—b</td>
<td>GAACTCCCCAATAAACCTGCT</td>
</tr>
<tr>
<td>chi1nNde</td>
<td>sense</td>
<td>82–110</td>
<td>TACTCGTTCACCGCTATAGCTTTG</td>
</tr>
<tr>
<td>chi1cXho</td>
<td>antisense</td>
<td>1270–1290</td>
<td>CGAATGTAAGCTGAGCTTTA</td>
</tr>
<tr>
<td>cassette C1</td>
<td></td>
<td></td>
<td>GTCATATTTGTCGTTAAGACCCGTATAACGACTCA</td>
</tr>
<tr>
<td>cassette C2</td>
<td></td>
<td></td>
<td>CGTTAAGACCCGTATAACGACTCA</td>
</tr>
</tbody>
</table>

\(a\) L, A/G; W, A/T; S, C/G; Y, C/T; D, A/G/T; N, A/C/G/T.

\(b\) 523–503 bp upstream of position 1 in Fig. 1.

\(c\) 543–503 bp downstream of position 1350 in Fig. 1.

Nucleotides underlined indicate restriction sites by endonuclease.
Nucleotide sequence of chitinase gene. The LA PCR in vitro cloning kit (Takara) was used to clone the upstream and the downstream regions of the partial chitinase I gene. *B. circulans* KA-304 DNA was digested with *Eco*RI, and the fragments were ligated with the double-stranded ligation cassette (*Eco*RI cassette) containing the respective sticky ends for ligation and the nucleotide sequence complementary to two primers (cassette C1 and cassette C2). Two antisense primers (chi1nS1 and chi1nS2) were designed to be complementary to the opposite side of the partial chitinase I gene. The upstream side of the partial chitinase I gene was amplified by two rounds of PCR reactions. The first PCR was done in a reaction mixture (50 μl in GC buffer I) containing 200 μM of each dNTP, 15 pmol of each primer (cassette C1 and chi1nS1), 2.5 units of LA *Taq* polymerase, and the ligation products, which were used as templates. Thermocycling was one 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 3 min. The second PCR was done in a reaction mixture (50 μl in GC buffer I) containing 200 μM each of dNTP, 1 μl of the first PCR product, 15 pmol of each primer (cassette C2 and chi1cS2), and 2.5 U of LA *Taq* polymerase. Thermocycling was one 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 2 min. A 1.4-kb PCR product located on the upstream region of the partial chitinase gene was amplified. A 1.0-kb DNA fragment located on the downstream side of the partial chitinase I gene was also amplified by two rounds of PCR reactions with the use of oligonucleotides, chi1cS1 and chi1cS2, as sense primers. These PCR products were cloned and their nucleotide sequences were determined. The determined upstream and downstream sequences of the partial chitinase I gene allowed to design PCR primers, chi1n2 and chi1c2, for amplification of the sequence containing the complete chitinase I gene. PCR was done in a reaction mixture (50 μl in GC buffer I) containing 10 ng of genome template DNA, 40 pmol of each primer, 200 μM of each dNTP, and 2.5 units of LA *Taq* polymerase. Thermocycling was one 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 2 min. A PCR product (2.0-kb) was cloned and its nucleotide sequence was determined.

Nucleotide sequence accession number. The nucleotide sequence of chitinase I was assigned to the DDBJ accession number AB196732.

Expression of the chitinase I gene. The complete open reading frame (ORF) of chitinase I without a signal peptide was amplified from *B. circulans* KA-304 DNA using a sense *Nde*I-linker primer (chi1nNde) and an antisense *Xho*I-linker primer (chi1cXho) by PCR. PCR was done in a reaction mixture (50 μl in GC buffer I) containing 10 ng of genome template DNA, 20 pmol of each primer, 200 μM each of dNTP, and 2.5 units of LA *Taq* polymerase. Thermocycling was one 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. A DNA fragment of 1.2-kb was produced. The fragment was cloned into the *Nde*I and *Xho*I sites of pET-22b, which was indicated as pET22-chi1, and was transformed into *E. coli* Rosetta-gami B.

The *E. coli* Rosetta-gami B harboring pET22-chi1 was inoculated into 10 ml of LB medium containing ampicillin (100 μg/ml). After incubation at 37°C overnight with shaking, the culture was transferred to 2-liter Sakaguchi flasks containing 1-liter of LB medium with 100 μg/ml ampicillin, 10 μg/ml chloramphenicol, 25 μg/ml kanamycin, and 15 μg/ml tetracycline, and then incubated at 30°C on a reciprocal shaker (100 strokes/min). After 15 h of incubation, isopropyl-β-d-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.4 mM, and the culture was incubated for another 5 h.

Purification of recombinant chitinase I. All operations were done at 0–4°C, and 10 mM Tris–HCl buffer (pH 8.0, buffer A) or potassium phosphate buffer (pH 6.5, buffer B) were used, unless otherwise stated. The cells harvested from 3-liter culture medium by centrifugation (10,000 × g for 30 min) were suspended in 50 ml of buffer A and then disrupted by sonication on ice. The cell debris was removed by centrifugation and the supernatant was dialysed against buffer A. The dialysate (cell-free extract) was put on a DEAE-cellulose column (3 × 5 cm) equilibrated with buffer A, and the column was washed with the buffer. The enzyme was found in the unadsorbed fractions, and the active fractions were concentrated with ammonium sulfate (80% saturation). The precipitate was dissolved in and dialyzed against buffer B, and then put on a CM-cellulose column (2 × 20 cm) equilibrated with the same buffer. After it was washed, the column was developed with buffer B containing 0.2 M NaCl. Recombinant chitinase was eluted with buffer B containing 0.2 M NaCl.

Assay of chitinase activity. Chitinase activity was determined as described previously using colloidal chitin as a substrate. One unit of chitinase was defined as the enzyme amount that released 1 nmol of reducing sugar per min.

Assay of protoplast-forming activity. Activity was determined according to the methods described by Mizuno et al. The washed *S. commune* mycelia (0.2 g of fresh weight) were suspended in a mixture (1 ml) containing 50 mM potassium phosphate buffer (pH 6.5), 0.2 mg/ml sodium azide, 0.5 mM mannitol, and the enzyme preparation. After incubation at 30°C with moderate shaking (100 rpm), the released protoplasts were counted microscopically with a hemocytometer.
Assay of antifungal activity. The antifungal activity of recombinant chitinase I was assayed by hyphal extension inhibition assay. Mycelium of *Trichoderma reesei* NBRC 31326 was inoculated on the center of a potato dextrose agar plate. After incubation at 20 °C for 36 h, paper disks were placed around the edge of the *T. reesei* colony, and appropriate amounts of recombinant chitinase I were put onto the disks. The plate was incubated at 20 °C for 48 h, and the inhibition of hyphal extension around the disks was observed.

Analytical method. Protein was measured by Lowry’s method with egg albumin as a standard. In column chromatography, it was followed by absorbance at 280 nm. SDS–PAGE was performed by the method of Laemmli. Molecular Weight Standard Mixture, Recombinant (Sigma) was used as a molecular weight marker.

Reagents. CWP of *S. commune* and α-1,3-glucanase were prepared according to the methods described in the previous paper. Colloidal chitin was prepared by the method of Berger and Renolds. Other reagents were the chemically pure grade of commercial products.

Results

Cloning of the chitinase I gene

Our previous study found that the N-terminal amino acid sequence of chitinase I is A L A T P T L N V S A S S G M, which has no similarity to those of known chitinases. To obtain information for cloning the chitinase I gene, the internal amino acid sequence was analyzed. Lysyl endopeptidase digestion of the enzyme yielded one major peptide fragment (MW 5,000), and its N-terminal amino acid sequence was found to be Q Y Y G R G P I Q. Based on the N-terminal and the internal amino acid sequences, two PCR primers (chi1n1 and chi1c1) were designed. After PCR amplification, a fragment of 837 bp was obtained and sequenced, which indicated that the fragment contained an ORF corresponding to 279 amino acid residues (partial chitinase I gene).

Subsequently, to clone the complete chitinase I gene, amplification of the upstream and downstream sequences of the partial chitinase I gene was done by the procedure described in “Materials and Methods”. After determination of the upstream and downstream regions of the partial chitinase I gene, PCR was done again with *B. circulans* KA-304 chromosomal DNA as the template using the primers, chi1n2 and chi1c2, which were outside the ORF. A single DNA fragment (approximately 2.0-kb) was cloned into pGEM-T Easy vector to generate pCHI1, and its nucleotide sequence was determined.

DNA sequence and deduced amino acid sequence

Figure 1 shows the complete nucleotide sequence of the chitinase I gene. It consists of 1,239 nucleotides encoding a protein of 413 amino acids with a predicted molecular weight of 43,090. The ORF has an ATG start codon at position 31 preceded by a possible ribosome-binding site (CATGAA) at position 18. The N-terminal amino acid sequence of purified chitinase I was found in amino acid residues 25–39 of the deduced ORF, suggesting that 24 amino acid residues in the upstream correspond to a signal peptide. The ORF without the putative signal peptide encodes 389 amino acids, and the predicted molecular weight of the peptide (40,500) was consistent with the value of 41,000 determined by SDS–PAGE. In addition, the

Fig. 1. Nucleotide Sequence of Chitinase I Gene and Deduced Amino Acid Sequence.
proteolytic fragment of chitinase I (MW 5,000) with lysyl endopeptidase digestion also existed in the ORF.

A homology search of the deduced amino acid sequence of the chitinase I gene with the BLAST program on the NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST/) indicated that the C-terminal region of the deduced sequence showed a similarity to the catalytic domain of family 19 chitinases of actinomycetes and other organisms. Especially, as shown in Fig. 2, the sequence of catalytic domain of chitinase I was significantly similar to those of chitinase C from Streptomyces griseus HUT6037,17) chitinase F from Streptomyces coelicolor A3(2),18) chitinase 35 from Streptomyces thermoviolaceus OPC-520,19) chitinase B from Nocardiopsis prasina OPC-131,20) and chitinase B from Burkholderia gladioli CHB101.21) Chitinase I preserved four amino acid residues (two Glu, one Asn, and one Ser for catalysis),22) which have been recognized to participate in the reaction of family 19 chitinase. The enzyme also preserved four Cys residues forming two disulfide bonds.22) These results indicate that chitinase I is a family 19 chitinase.

Nevertheless, the N-terminal amino acid sequence deduced from the chitinase I gene had no identity with those of known family 18 and 19 chitinases, and only a slight identity was found with the C-terminal region of chitinase A1 of B. circulans WL-12,23) which consists of two fibronectin type III domains (Fig. 3).

Expression of the chitinase I gene and purification of the recombinant protein

The chitinase I gene without the putative signal sequence was ligated into the expression vector, pET-22b. The generated pET22-chi1 was designed for its expressed protein to contain additional N-terminal methionine residue, and was transformed into E. coli Rosetta-gami B (DE3). Significant chitinase activity was detected in the cell-free extract of the organisms treated with an inducer (IPTG). As Fig. 4 shows, a remarkable band of the recombinant protein with a molecular weight of 41,000 occurred in both the insoluble and the soluble fractions of the cells.

The cells were disrupted by sonication, and the recombinant enzyme was purified from cell-free extract by column chromatography on DEAE-cellulose A-500 and CM-cellulose, as described in “Materials and Methods”. The recombinant enzyme was purified 16-fold with an overall yield of 46% (Table 2). The final preparation was homogeneous on SDS–PAGE, and the molecular mass (41,000) agreed with that of chitinase I purified from KA-prep (Fig. 4, lanes 6 and 7).

Properties of recombinant chitinase I

The recombinant enzyme was stable in a pH range of 5–9, and retained its full activity after 10 min of incubation at 50°C and pH 6.5. The reaction with colloidal chitin as a substrate proceeded at pH 5.0–7.0

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Fig. 2. Alignment of the Catalytic Domain of Chitinase I with Those of Other Family 19 Chitinases.

Amino acids conserved in at least 4 of the 6 sequences are shaded. Two Glu for the catalytic site of family 19 chitinases are indicated by solid circles. One Ser proposed to hold a water molecule is indicated by a solid triangle. Two Cys forming disulfide bonds are indicated by line. The abbreviations are: Bc, chitinase I of B. circulans KA-304; Sg, chitinase C of Streptomyces griseus HUT6037; Sc, chitinase F of Streptomyces coelicolor A3(2); St, chitinase 35 of Streptomyces thermoviolaceus OPC-520; Np, chitinase B of Nocardiopsis prasina OPC-131; bg, chitinase B of Burkholderia gladioli CHB101. The residue numbers of the first and the last amino acids in each line are shown on left and right sides.
with an optimum of 6.0. The enzyme hydrolyzed colloidal chitin, ethylene glycol chitin, and powder chitin, but not \( p \)-NP-GlcNAc. Ethylene glycol chitin was the best substrate. TLC of the reaction mixture with colloidal chitin as a substrate indicated that the predominant products were \( N,N',N'' \)-triacetylchitotriose and \( N,N'-\)diacetylchitobiose.

Table 2. Purification of Recombinant Chitinase I Expressed in \( E. coli \)

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total Protein (mg)</th>
<th>Total Activity (units)</th>
<th>Specific activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>352</td>
<td>36,600</td>
<td>104</td>
</tr>
<tr>
<td>DEAE-cellulofine A-500</td>
<td>150</td>
<td>30,000</td>
<td>200</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>10</td>
<td>16,700</td>
<td>1670</td>
</tr>
</tbody>
</table>

\( N,N',N'' \)-triacetylchitotriose. These properties were almost the same as those of purified chitinase I.\(^9\) In addition, recombinant chitinase I showed the protoplast-forming activity with \( \alpha-1,3 \)-glucanase (Table 3) as purified chitinase I.\(^9\)

It has been reported that family 19 chitinases generally show antifungal activity. As shown in Fig. 5, the recombinant as well as the native chitinase I preparation (5–20 \( \mu \)g/disc) inhibited the hyphal extension of \( T. reesei \).

Discussion

Chitinase I of \( B. circulans \) KA-304 is an indispensible enzyme for protoplast formation from \( S. commune \) mycelia. Our findings suggest that detailed analysis of chitinase I brings valuable information for efficient formation of basidiomycetes protoplast.

The N-terminal amino acid sequence of chitinase I did not show any similarity to those of known chitinases. But, the chitinase I gene in the present study indicated that the deduced N-terminal moiety (Fig. 3) has a slight sequence homology with the C-terminal region of
chitinase A1 of *B. circulans* WL-12, which contains two fibronectin type III domains. The fibronectin type III domain, one of the most common modular proteins, was initially characterized in fibronectin, and then was found in many kinds of intracellular, extracellular, and membrane-receptor proteins as well as in bacterial chitinases, cellulases, and amylases. Investigation of chitinase I modified in the fibronectin type III-like region should allow discussion of the function of the region.

On the other hand, the C-terminal domain of chitinase I showed sequence homology with family 19 chitinases such as the enzymes of actinomycetes, plants (class IV type), and other organisms. Chitinases are classified into two families (18 and 19) of glycosyl hydrolase on the basis of the amino acid sequence similarity in their catalytic domains. Family 18 chitinases have been found widely in bacteria, fungi, viruses, animals, and some plants (class III and V chitinases of plants).

On the other hand, family 19 chitinases were isolated initially only from plants (class I, II, and IV chitinases). However, a chitinase belonging to family 19 was demonstrated in Streptomyces griseus in 1996,\(^\text{17}\) and subsequently found in many bacteria, viruses, and nematodes, etc. Kawase et al. analyzed these family 19 chitinases from the viewpoint of enzyme phylogeny and separated them into five clusters of family 19 chitinases.\(^\text{22}\)

In our knowledge, chitinase I of *B. circulans* KA-304 is the first example of a family 19 chitinase in the *Bacillus* species. As indicated in Fig. 2, chitinase I shows high sequence similarity to chitinases of several actinomycetes and chitinase B of Burkholderia gladioli CHB101, which are in the same cluster of family 19 chitinases with plant IV chitinase.\(^\text{22}\) Family 19 chitinases usually show antifungal activity.\(^\text{24}\) This activity has been understood to be due to the fungal cell-wall being weakened with respect to change in osmotic pressure, which is caused by hydrolysis of chitin in the cell-wall by the enzymes.\(^\text{25}\) The finding that chitinase I of *B. circulans* KA-304 inhibited fungal growth might correlate with its contribution in forming protoplast, which was accomplished by combination with α-1,3-glucanase. Its action should be investigated considering the heterogeneity of the cell-wall of multi-cellular basidiomycetes. This is now underway, and the results should appear soon.

### Acknowledgment

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### References

1) Mizuno, K., Kimura, O., and Tachiki, T., Protoplast formation from *Schizopyllum commune* by a culture


