Tech. Bull. Fac. Agr. Kagawa Univ., Vol.59, 71～77, 2007

Antimicrobial Activity of Recombinant Family 19 Chitinase Highly Purified from *Escherichia coli* Carrying *Streptomyces* Chitinase Gene

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Abstract

A recombinant family 19 chitinase was highly purified from the cell extract of *Escherichia coli* JM109 transformed by plasmid carrying the gene encoding *Streptomyces* chitinase by column chromatogramphies on DEAE- and CM-Sepharose. The bacterial growth after 24 hr cultivation in well of 96-well microplate by measurement of absorbance at 595 nm showed that the purified chitinase half inhibited growth of *Bacillus subtilis*, but not that of *Escherichia coli*. When the yeast growth after 48 hr cultivation was monitored by measurement of dry weight, the purified chitinase inhibited 95% of *Saccharomyces cerevisiae* (budding fashion) growth and 25% of *Schizosaccharomyces pombe* (binary fashion) growth. The family 19 chitinase of the *Streptomyces* had similarity to plant family 19 chitinases (class IV type) in their catalytic domains. Therefore, this and previous our studies that the chitinase exhibits growth inhibitory activity of some bacteria, yeast, and fungi suggested that the plant family 19 chitinases (class IV) have a significant role in defense against certain microbial infections.

Key words: recombinant family 19 chitinase; *Streptomycs* sp.; antimicrobial activity

Introduction

Chitinase (EC 3.2. 1.14) hydrolyzes β-1,4-bonds in chitin to produce *N*-acetylglucosamine (GlcNAc) and *N,N'*-diacetylchitobiose as sole reaction products. Among 91 glycosyl hydrolase families based on amino acid sequence similarities, chtinases are classified into families 18 and 19.[1] Family 19 chitinases are found mostly in higher plants, and rarely in microorganisms. All bacterial chitinases belong to family 18 except for several family 19 chitinases from species such as *Streptomyces* and *Nocardiopsis*. Since the amino acid sequences of bacterial family 19 chitinases have similarity to those of plant chitinases (class IV type) in their catalytic domains, bacterial family 19 chitinases alone exhibit antifungal activity against *Tricoderma reesei*.[2-4] Recently, we cloned the gene encoding family 19 chitinase (298 amino acids) consisting of a signal peptide (29 amino acids) and a mature protein (269 amino acids) from *Streptomyces* sp. (strain J-13-3), and constructed plasmid pUC19 carrying the family 19 chitinase gene for expression in *Escherichia coli* cells.[5] The purified chitinase from the *E. coli* cells had the identical N-terminal amino acid sequence of the mature protein by removal of the signal sequence by *E. coli* signal peptidase and inhibited the growth of *Trichoderma reesei* and *Aspergillus niger*.[6] However, effect of the recombinant chitinase on bacterial and yeast growth remains to be examined. This paper describes the highly purification and antimicrobial activity against some bacteria and yeast of the recombinant chitinase expressed in *E. coli* cells.

Materials and Methods

Substrate and chemicals

The sources of materials used in this study were as follows: *N*-acetylgluco samine (GlcNAc) and *N,N',N''*-triacetylchitotriose from Seikagaku Kogyo Co. (Tokyo), β-D-thiogalactopyranoside (IPTG) from Wako Pure Chemical Industries, Ltd. (Osaka), DEAE-Sepharose Fast Flow and CM-Sepharose Fast Flow from Amersham Biosciences (Uppsal, Sweden), and BugBuster protein extraction reagent (Novagen, Darmstadt, Germany). The strains JM109 of *E. coli* and plasmid pUC19 from Takara Shuzo (Kyoto) were used as host and vector for expression of the *Streptomyces* chitinase gene, respectively. Transformant of *E. coli* was grown at 37℃ in Luria-Bertani (LB) medium containing 100 μg/ml of ampicillin. Other reagents were obtained at analytical grade from commercial sources.

Enzyme and protein assay

N,N',N''-Triacetylchitotriose was used as a substrate for chitinase activity. In a standard assay, 25 μl of appropriately diluted enzyme solution was incubated with 50 μl of 0.4%

substrate solution and 25 μl of 0.2 M phosphate buffer (pH 6.0) at 40℃ for 10 min. After the reaction was stopped by boiling for 30 second, liberated GlcNAc was measured by the method of Reissig *et al.*[7] One unit (U) of enzyme was defined as the amount that liberated 1 mmol of GlcNAc equivalent per min under standard assay conditions. Protein was measured by the method of Lowry *et al*. with bovine serum albumin as the standard.[8]

Antimicrobial activity

Bacterial growth inhibition of the purified recombinant chitinase against *E. coli* JM109 and *Bacillus subtilis* ATCC1659 was determined by automated quantitative assay.[9] The bacteria (1,000 of colony forming unit) with and without the chitinase in 200 μl of LB medium were grown in the wells of a flat-bottom 96-well microplate (Sumitomo Bakelite, Tokyo) at 25℃ for 24 hr on a rotary shaker operating at 180 rpm. Bacterial growth was monitored by measurement of absorbance at 595 nm on a microplate reader (Model 450, Bio-Rad). Yeast growth inhibition of the purified chitinase against *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* was determined by measurement of dry weight. The yeast (5,000 of colony forming unit) with and without the chitinase in 400 μl of YPD medium (2% glucose, 1% yeast extract, and 2% peptone, pH 5.8) was grown in the wells of a flat-bottom 48-well microplate (Sumitomo) at 25℃ on a rotary shaker operating at 180 rpm. After 48 hr after inoculation, yeast cells were harvested in microtube from the microplate cultures, washed three times, and then dried under vacuum at 60℃.

Results

Highly purification of the recombinant chitinase expressed in *E. coli* cells

The expression plasmid (pUSC-7) was constructed by inserting the PCR-amplified DNA for family 19 chitinase (298 amino acids) of the strain J-13-3 into the *Hin*dIII-*Eco*RI site of pUC19, as described previously.[5] Nucleotide sequencing of the insert in pUSC-7 indicated that the constructed chitinase had the same deduced amino acid sequence as the J-13-3 chitinase gene and an additional 8 amino acids (MTMITPSF) in the N-terminus. The chitinase is modular enzyme consisting of the signal sequence (29 amino acids), the chitin-binding domain (65 amino acids), and the catalytic domain (204 amino acids) (Fig. 1).

The *E. coli* JM109 cells harboring pUSC-7 were inoculated into 10 ml of Luria-Bertani (LB) medium containing ampicillin (100 μg/ml). The seed culture was incubated at 37 ℃ overnight with shaking and then transferred to a 500-ml shaking flask containing 90 ml of LB medium with ampicillin (100 μg/ml). Cultivation was continued at 30℃ on a rotary shaker operating at 180 rpm. After 3 hr of incubation, isopropyl IPTG was added to the culture at a final concentration of 0.1 mM. The cells were harvested at 24 hr after the addition of IPTG from the culture (3,600 ml). A recombinant chitinase was extracted twice with BugBuster from the JM109 cells.

After the extraction, 360 ml of the crude enzyme solution was divided three portions. One portion was dialyzed against water, buffered with 10 mM Tris-HCl buffer (pH 8.0), and then put on a column (2 × 45 cm) of DEAE-Sepharose Fast Flow equilibrated with the same buffer. The column was eluted with the same buffer (300 ml) and with a stepwise gradient of NaCl (200 ml at each step). Chitinase activity was detected in the fraction eluted with 0.3 M NaCl and the active fractions were pooled (Fig. 2). The DEAE-Sepharose chromatographies of the other portions were also done under the same conditions. The enzyme solutions from three experiments were dialyzed against water, buffered with 10 mM malate-NaOH buffer (pH 6.0), and then put on a column (2×45 cm) of CM-Sepharose Fast Flow equilibrated with the same buffer. The column was eluted with the same buffer (120 ml) and with a stepwise gradient of NaCl (120 ml at each step). Chitinase activity was detected in the fraction eluted with 0.1 M NaCl and the active fractions were pooled (Fig. 3). The enzyme was purified about 350-fold from the crude extract. The enzyme protein (310 μg) and highly specific activity (35 U/mg) were obtained. In native polyacrylamide

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MFRRIMGLLAALGAVVAGLVVLPATTASAADCAAAWNASSVYTGGGSASY  50
|←——— Signal peptide ———→|
NGHNWTAKWWTQNETPGSSDVWADQGACGNGGGTDPGDPGSSFGFVVSEA 100
——— Chitin binding domain ———→|
QFNQMFPGRSSFYTYSGLVAALDAYPGFANTGSDTVKKQEAAAFLANVSH 150

ETGGLVYIVEQNTANYPHYCDSSQPYGCPAGQAAYYGRGPIQLSWNFNYK 200
——— Catalytic domain ———
AAGDALGIDLLGNPYLVEQDAAVAWKTGLWYWNTQNGPGSMTAHNAMVNG 250

AGFGETIRSINGSIECNGGNPAQVQSRIDKYQAFVQILGTTPGSNLSC   298
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Fig. 1. Amino acid sequence and domain structure of *Streptomyces* family 19 chitinase.

The residue number of the last amino acid in each line is shown on the right.

gel electrophoresis of the purified recombinant enzyme, a single protein band was detected (data not shown).

Antimicrobial activity of the recombinant chitinase

We examined inhibitory effect of the purified recombinant chitinase on growth of bacteria (Gram positive *B. subtilis* and Grame negative *E. coli*) and yeast (*S. cerevisiae* and *S. pombe*). After 24 hr cultivation, about 25% and 50% of the *B. subtilis* growth were inhibited by 10 mU (0.28 μg of protein) and 30 mU (0.84 μg of protein) of the purified chitinase, respectively (Fig 4A). However, no growth inhibition by the chitinase was observed in *E. coli* (Fig 4B). The purified chitinase (30 mU) inhibited 95% of *S. cerevisiae* growth and 25% of *S. pombe* growth (Fig 5).

Discussion

Our previous study showed that the recombinant chitinase is purified from the cell extract of *E. coli* JM109 transformed by plasmid pUC19 carrying the gene encoding family 19 chitinase of Streptomyces sp. J-13-3 by column chromatography on DEAE-Sepharose, CM-Sepharose, and Bio-Gel P-100 and that the additional 8 amino acids (MTMITPSF) and the signal peptide in the N-terminus of the purified recombinant chitinase with molecular weight of 32,000 are removed in cells of *E. coli* JM109.[6] Therefore, the purified recombinant chitinase in present study is same as native chitinase produced in *Streptomyces* sp. J-13-3.[10] As for the column chromatography on CM-Sepharose equilibrated with buffer at pH 4.5, the chitinase seems to be unstable in the previous study.[6] In this study, we purified the recombinant chitinase with minor modification of CM-Sepharose equilibrated with at pH 6.0 of buffer. The recombinant chitinase was detected in adsorbed fraction and specific activity of the present purified chitinase was about 10-fold higher than that of the previous purified chitinase. In addition, further purification step of the Bio-Gel P-100 column could be omitted. Thus the recombinant family 19 chitinase was highly purified from *E. coli* cells.

Chitinases are classified into families 18 and 19 of glycosyl hydrolases based on the amino acid sequence similarities of their catalytic domains.[1] The family 18 chitinases are widely distributed in a variety of organisms such as bacteria, fungi, viruses, animals, and higher plants (classes III and V). In the catalytic domains, the consensus sequence containing two Asp and one Glu (DXDXE motif) as essential residues for chitinase activity is present in family 18 chitinases.[11] On the other hand, the family 19 chitinases were found only in higher plants (classes I, II and IV) [12] until several chitinases of *Streptomyces* have been identified as the family 19 chitinases. [2,3,5,13] The amino acid sequences of *Streptomyces* family 19 chitinases had similarity to those of plant chitinases (class IV) in their catalytic domains, suggesting that family 19 chitinases of *Streptomyces* species were acquired from plants by horizontal gene transfer.[2] Therefore, the amino acid sequences of our Streptomyces and representative plant (classes I, II and IV) family 19 chitinases were aligned in their catalytic domains (Fig. 6). Four conserved regions (C 1-4) and two Glu residues (catalytic amino acids) were found in all sequences, as described by recent report. [14,15] Plant chitinase of class I had high sequence similarity to that of

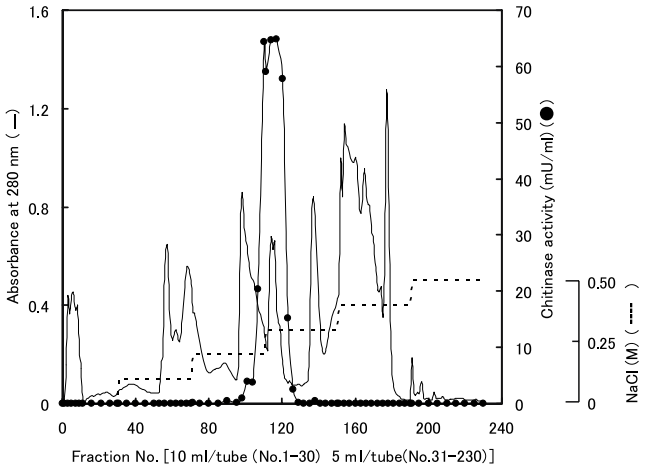

Fig. 2. Elution profile of recombinant family 19 chitinse from DEAE-Sepharose Fast Flow column.

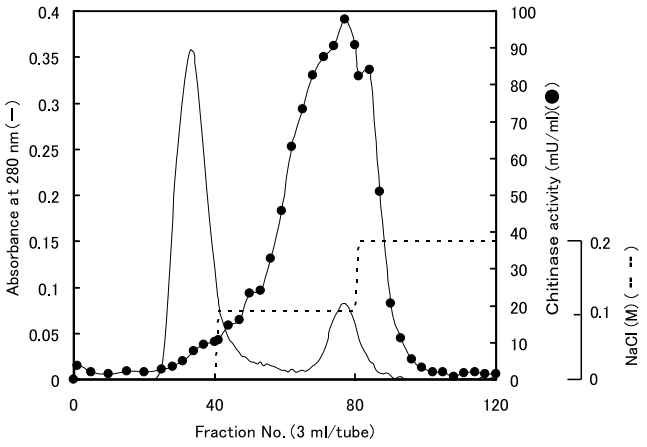

Fig. 3. Elution profile of recombinant family 19 chitinse from CM-Sepharose Fast Flow column.

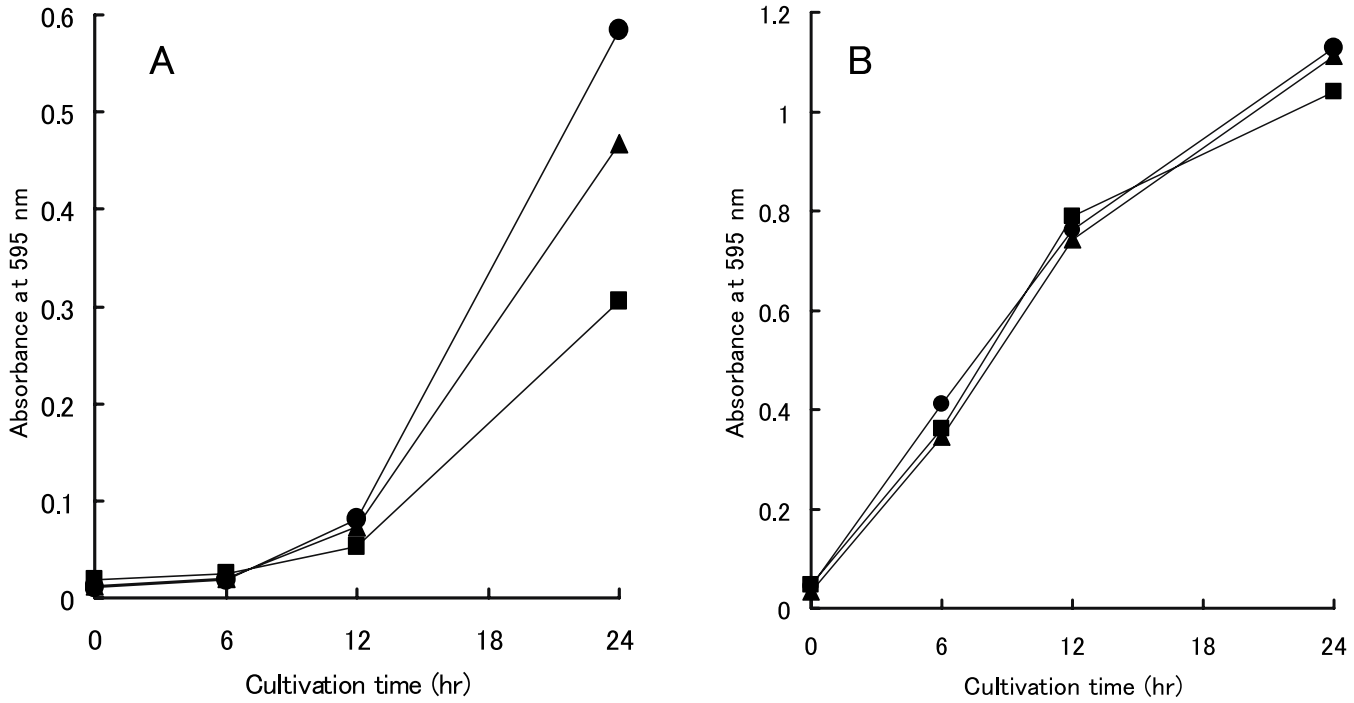

Fig. 4. Effect of purified recombinant family 19 chitinase on the growth of *B. subtilis* (A) and *E. coli* (B).
The bacteria were cultured at 25 ℃ with and without the purified recombinant chitinase. Symbols: ●, no chitinase control; ▲, 10 mU of chitinase (0.28 μg of protein); ■, 30 mU of chitinase (0.84 μg of protein).

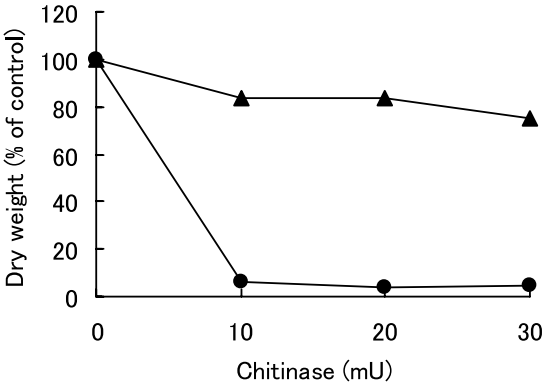

Fig. 5. Effect of purified recombinant family 19 chitinase on the growth of *S. cerevisiae* (●) and *S. pombe* (▲).
The yeast were cultured at 25℃ for 48 hr with and without the purified recombinant chitinase.

class II. Two deletions (D 1-2) were characteristic of class IV chitinase as compared to those of class I or II and our *Streptomyces* family 19 chitinase had also the deletions.

The highly purified chitinase had inhibitory effect on growth of *B. subtilis* but not that of *E. coli*. Bacteria have a complex surface structure with a rigid cell wall containing peptide glycan. Lysozyme hydrolyzes not only the glycan (linear β-1,4-linked polymer of GlcNAc and *N*-acetylmuramic acid) but also chitin. Glu and Asp residues (catalytic amino acids) are found in amino acid sequences of the lysozyme.[16] However, Gram-negative bacteria such as *E. coli* have outer membrane surrounding the cell wall. *S. cerevisiae* (budding fashion) was more sensitive to the purified chitinase than *S. pombe* (binary fashion) because of accumulation of chitin in the budding site. These results suggested that peptide glycan and chitin in bacterial and yeast cell wall are target of our recombinant family 19 chitinase. Bacterial family 18 chitinases do not exhibit antifungal activity.[17] However, family 19 chitinases of *Streptomyces* alone exhibit antifungal activity against *T. reesei*.[2-5] We also demonstrated that the recombinant family 19 chitinase from *Streptomyces* sp. J-13-3 inhibited the growth of *T. reesei* and *A. niger*.[6] Recent study showed that the recombinant family 19 chitinase of *Streptomyces coelicolor* A3(2) inhibited hyphal extension of *T. reesei, T. viride, Mucor javanicus, Fusarium solani*, and *A.niger*.[17] These and present studies that the *Streptomyces* family 19 chitinases exhibit growth inhibitory activity of some bacteria, yeast, and fungi suggested that the plant family 19 chitinases (class IV) have a significant role in defense against certain microbial infections.

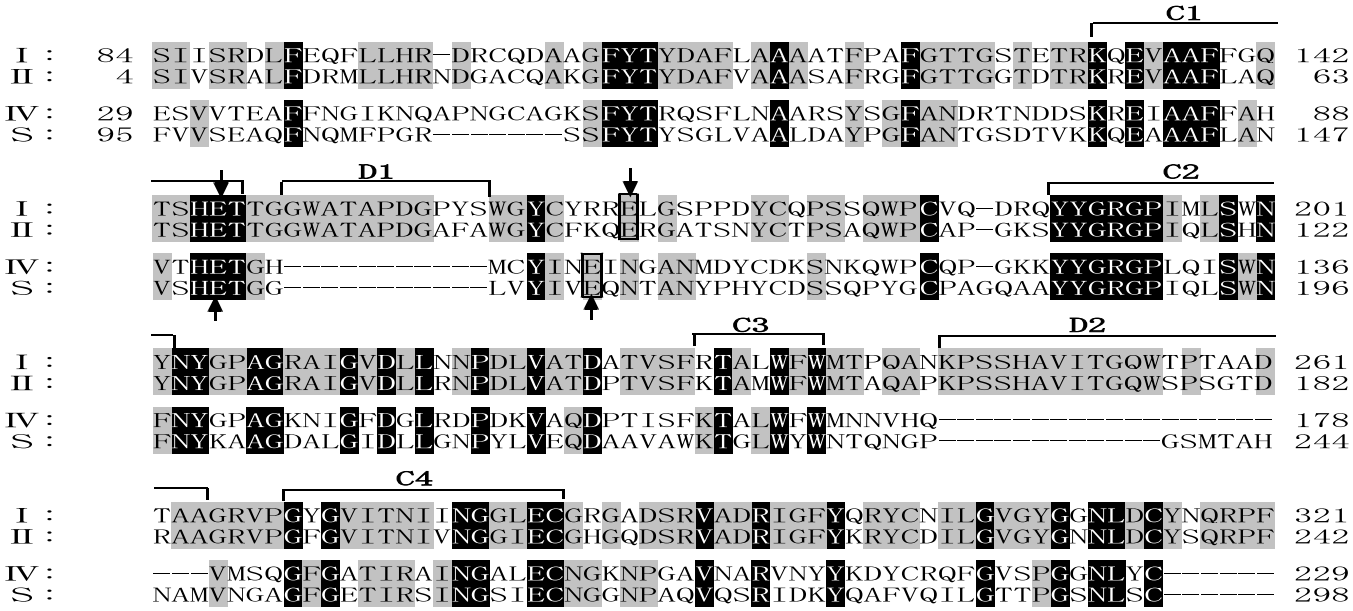

Fig. 6. Alignment of the amino acid sequence in the catalytic domain of *Streptomyces* family 19 chitinase with those of plant family 19 chitinases (classes I, II, and IV).

The abbreviations were as follows:I, barley class I chitinase (UniProt Accession No. Q42839);[14] II, barley class II chitinase (P11955);[14] IV, *Oryza. sativa* class IV chitinase (O04138);[14] S, *Streptomyces* chitinase (GenBank Accession No. AB116547).[5] The residue number of the first and last amino acid in each line is shown on the left and right, respectively. Amino acids conserved in 4 sequences are indicated by white type on a black background. Residues conserved in two classes (I and II or IV and S) are shaded. The two catalytic amino acid residues of family 19 chitinases are indicated by arrows. C1-4, conserved regions of family 19 chitinases; D1-2, deletions in classes Ⅳ chitinase with respect to the classes I and II chitinases.

Acknowledgment

We thank Prof. K. Takegawa of Faculty of Agriculture, Kagawa University for supplying *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*.

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(Received October 31, 2006)

放線菌のファミリー19キチナーゼ遺伝子を持つ大腸菌から高度に精製した組換えキチナーゼの抗菌活性

山下陽輔，岡崎勝一郎

要　　約

放線菌*Streptomyces*のファミリー 19キチナーゼ遺伝子を挿入したプラスミドで形質転換した大腸菌から組換えキチナーゼをDEAE-Sepharose及びCM-Sepharoseの各クロマトグラフィーで高度に精製した．マイクロプレートウェルで24時間培養した細菌の増殖を595 nmの吸光度で測定した結果，精製した組換えキチナーゼは*Bacillus subtilis*の増殖を50%阻害したが，*Escherichia coli*の増殖は阻害しなかった．48時間培養した酵母の増殖を乾燥菌体重量で測定した結果，組換えキチナーゼは出芽酵母*Saccharomyces cerevisiae* の増殖を95%，分裂酵母*Schizosaccharomyces pombe* の増殖を25%阻害した．放線菌ファミリー 19キチナーゼの触媒ドメインはファミリー 19に属する植物キチナーゼクラスⅣと相同性を持っていた．したがって，放線菌ファミリー 19キチナーゼがある種の細菌、酵母、カビに対して増殖阻害を示すことから，植物ファミリー 19キチナーゼ（クラスⅣ）はある種の微生物感染に対する防御に重要な役割を持つと考えられた．