Molecular Cloning and Expression of the Gene Encoding Family 19 Chitinase from *Streptomyces* sp. J-13-3

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Received August 11, 2003; Accepted October 7, 2003

The gene encoding chitinase from *Streptomyces* sp. (strain J-13-3) was cloned and its nucleotide structure was analyzed. The chitinase consisted of 298 amino acids containing a signal peptides (29 amino acids) and a mature protein (269 amino acids), and had calculated molecular mass of 31,081 Da. The calculated molecular mass (28,229 Da) of the mature protein was almost same as that of the native chitinase determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometer. Comparison of the encoded amino acid sequences with those of other chitinases showed that J-13-3 chitinase was a member of the glycosyl-hydrolase family 19 chitinases and the mature protein had a chitin binding domain (65 amino acids) containing AKWWTQ motif and a catalytic domain (204 amino acids). The J-13-3 strain had a single chitinase gene. The chitinase (298 amino acids) with C-terminal His tag was overexpressed in *Escherichia coli* BL21(DE3) cells. The recombinant chitinase purified from the cell extract had identical N-terminal amino acid sequence of the mature protein in spite of confirmation of the nucleotide sequence, suggesting that the signal peptide sequence is successfully cut off at the predicted site by signal peptidase from *E. coli* and will be a useful genetic tool in protein engineering for production of soluble recombinant protein. The optimum temperature and pH ranges of the purified chitinase were at 35–40°C and 5.5–6.0, respectively. The purified chitinase hydrolyzed colloidal chitin and trimer to hexamer of *N*-acetylglucosamine and also inhibited the hyphal extension of *Tricoderma reesei*.

Key words: family 19 chitinase; *Streptomyces* sp.; chitinase gene; expression

Chitin, an insoluble linear β-1,4-linked polymer of *N*-acetylglucosamine (GlcNAC), is one of the most abundant polysaccharides in nature because of major component of outer shells of crustaceans, skeletons of insect, and fungal cell wall. Chitinase (EC 3.2.1.14) hydrolyzes β-1,4-bonds in chitin to produce GlcNAC and (GlcNAC)2 as sole reaction products. Microbial and plant chitinases act as chitin degradation in soil and defense against fungal infection, respectively. Chitinases are classified into families 18 and 19 of glycosyl hydrolases based on the amino acid sequence similarities of their catalytic domains.1) 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,2) but not in family 19 chitinases. The family 19 chitinases are mostly found in higher plants, but rarely in microorganisms. *Streptomyces* are known as major producers of chitinase and its chitinase genes have been cloned and sequenced. *Streptomyces griseus* HUT 6037 produces a family 19 chitinase first found in an organism other than higher plants3,4) and the chitinase alone exhibits antifungal activity against *Tricoderma reesei*.5) Thereafter, seven genes encoding the family 19 chitinases have been cloned and characterized from *S. coelicolor*,6,7) *S. thermoviolaceus*,8) *S. olivaceoviridis*,9,10) allosamidin-producing *Streptomyces* (strain AJ9463)11,12) and *Noocardiosis prasina*.13,14) Furthermore, two chitinases belonging to the family 19 are also found in *Aeromonas* sp. No.10S-2415) and *Burkholderia gladioli*16) other than actinomycetes.

Our previous study demonstrated that *Streptomyces* sp. J-13-3 secretes two similar chitinases (Chi-A and Chi-B) into a medium containing powdered chitin and that the two purified chitinases with different isoelectric

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\[ \text{Received August 11, 2003; Accepted October 7, 2003} \]

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The nucleotide sequence reported in this study appears in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession number AB116547.

Abbreviations: DIG, digoxigenin; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria-Bertani; RBS, ribosome-binding site; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

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points are very similar to each other in biochemical and enzymatic properties such as molecular weight (31,000), optimum pH 6.0 and temperature (45°C), amino acid composition, N-terminal amino acid sequence, and immunological cross-reactivity. A concomitant decrease of Chi-A and increase of Chi-B is also observed in their productions of medium during cultivation. Therefore, the Chi-A and Chi-B may be derived from a single gene. In this report, molecular cloning and the encoded amino acid sequence of the J-13-3 chitinase gene showed that the enzyme belongs to the family 19 and the J-13-3 strain has a single chitinase gene. Furthermore, we describe purification, some properties, and a signal peptide processing of the recombinant chitinase expressed in Escherichia coli cells.

Materials and Methods

Materials, bacterial strains, and plasmids.

The sources of materials used in this study were as follows: N-acetylchitotriosylasacharides (dimer to hexamer) from Seikagaku kogyo Co. (Tokyo), Bio-Gel P-100 and Quantam Prep Plasmid Miniprep kit from Bio-Rad (Hercules, CA, U.S.A.), GFX Gel Band Purification kit, HiBond-N+, and HiTrap Chelating HP column from Amersham Biosciences (Uppsal, Sweden), Immobilon-P from Millipore Corp. (Bedford, MA, U.S.A.), restriction endonucleases, LaTaq DNA polymerase with GC buffer I, and DNA ligation kit from Takara Shuzo (Kyoto), DIG-High Prime DNA labeling kit and reagents for luminescent detection from Roche Diagnostics (Mannheim, Germany), and ampicillin sodium salt, IPTG, X-gal, and all other chemicals from Wako Pure Chemical Co. (Osaka). Three strains [JM109, DH5α, and BL21(DE3)] of E. coli and two plasmids [pUC19 and pET23a (+)] were used as hosts and vectors for cloning and expression of the chitinase gene, respectively. Transformants of E. coli were grown at 37°C in LB medium containing 100 μg/ml of ampicillin. Streptomyces sp. J-13-3 was used as the gene source.

Internal amino acid sequences and molecular mass determinations of purified chitinases. Two chitinases were purified from culture filtrate of the strain J-13-3 by successive chromatographies of DEAE-Sephadex A-50, DEAE-cellulose, andMono Q, as described previously. The purified two chitinases (30 μg of Chi-A, 25 μg of Chi-B) were denatured by boiling for 2 min and mixed with V8 protease (1 μg of protein, Miles Inc., Kankakee, IL) in 62.5 mM Tris-HCl buffer (pH 6.8). The resulting peptide fragments were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and then were electrotherrhetically transferred to a polyvinylidene difluoride membrane (Immobilon-P). After staining with the sheet with 0.1% Ponceau 3R in 2% glacial acetic acid, the peptide bands were cut out from the sheet, and then the N-terminal amino acid sequences of the peptides were analyzed by Edman degradation with Milligen/Biosearch model 6625 ProSequencer. In analysis of the N-terminal amino acid sequence of a recombinant chitinase, Procise Protein Sequencer (model 491, Applied Biosystems, Foster City, CA) was used. Molecular masses of the native and recombinant chitinases (0.1 μg of protein) were determined with matrix-assisted laser desorption ionization time-of-flight (MALDI TOF)-mass spectrometer (Bruker Daltonics, Germany).

DNA amplification of partial chitinase gene. Chromosomal DNA of the strain J-13-3 was extracted from mycelia as described previously and partial chitinase gene was amplified from the J-13-3 DNA by polymerase chain reaction (PCR). A sense primer (5'-GAATTC-GCSWSTACACGGSCACAAC-3') and an antisense primer (5'-GAATTC-GTAGTTSGCACGTTGC-TC-3') containing an underlined EcoRI site were designed on the basis of the N-terminal (ASYNGHN) and the internal amino acid sequences (EQNVANY) and the GC-rich genome of Streptomyces. PCR was done in reaction mixture (50 μl in GC buffer I) containing 600 ng of genome template DNA, 30 pmol of each primer, 200 μM of each dNTP, and 2.5 units of DNA polymerase (Takara LaTaq). After a cold start (10 min at 4°C) and initial denaturation (1 min at 94°C), amplification protocol (30 s at 94°C, 30 s at 55°C, and 2 min at 72°C) was repeated for 30 cycles, and followed by the final 72°C extension step for 5 min. The amplified DNA fragment (375 bp) was digested with EcoRI, extracted from 0.8% agarose gel with GFX Gel Band Purification kit, and used as a probe to isolate the J-13-3 chitinase gene. The fragment was also cloned into the EcoRI site of pUC19, which was then used to transform E. coli JM109. The construction plasmid was designated pUSC-12.

Southern hybridization and construction of the gene library. The PCR-amplified DNA fragment was labeled with DIG-conjugated dUTP using the DNA labeling kit. The DIG labeled probe hybridized to genomic DNA fragments treated with various restriction enzymes on the nylon membrane (HyBond-N+) at 68°C for 18 h, and then detected with alkaline phosphatase-conjugated anti-DIG antibody and luminescent substrate, as described previously. Genomic DNA (10 μg) of the strain J-13-3 was completely digested with PstI and the DNA fragments were ligated into PstI site of pUC19 to create a genomic library. The competent E. coli JM109 cells (Takara Shuzo) were transformed with the ligation mixture and placed on LB agar plates containing ampicillin (100 μg/ml), IPTG (0.1 mM), and X-gal (40 μg/ml). After screening of the transformants by colony and Southern hybridizations with the DIG labeled probe, a recombinant plasmid with an about 9.4-kb inserted fragment carrying the chitinase gene was obtained and designated pUSC-613.
**Nucleotide sequence and homology search.** The nucleotide sequences of recombinant plasmids isolated with Quantum Prep Plasmid Miniprep kit were analyzed for both strands by the primer walking method with a BigDye terminator cycle sequencing ready reaction kit using the ABI PRISM 310 genetic analyzer (Applied Biosystems). The nucleotide sequence data were analyzed using DNASIS-Mac (Hitachi Software Eng. Co., Tokyo). A computer assisted homology search of J-13-3 chitinase was done using programs of FASTA and BLAST. The nucleotide and amino acid sequences were aligned using CLUSTAL W.21)

**Gene expression and enzyme purification.** The complete open reading frame of the chitinase with a signal peptide was amplified from the pUSC-613 DNA using a sense Hind III linker primer (5'-CCCAAGCTTCATGTTCCGGTCGCATCAT-3') and an antisense EcoRI linker primer (5'-GGAATTC-CTTATTACCTACTCA-GCAAGCTCAAGTTGGG-3') by PCR as described above. The specific product (0.9 kb) was purified and cloned into the Hind III and EcoRI sites of pUC19, which was then used to transform E. coli JM109. The construction plasmid was designated pUSC-7. For overexpression of the chitinase gene in E. coli BL21(DE3) cells, PCR was done with a sense Nhel-linker primer (5'-TTGCTAGCAGTTCCGGTCGCATCAT-3') and an antisense XhoI-linker primer (5'-CCCTGAG-GCAAGCTCAAGTTGGG-3') using pUC-7 DNA as a template. The amplified DNA fragment was cloned into the Nhel and XhoI sites of pET23a(+) carrying a C-terminal six His tag, which was then used to transform E. coli DH5α by electroporation. The resulting plasmid was designated pESC-17. The E. coli BL21(DE3) cells harboring the pESC-17 was isolated and inoculated into 10 ml of LB medium containing ampicillin (100 μg/ml). The seed culture was incubated at 37°C 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 25°C on a rotary shaker operating at 180 rpm. After 5 h of incubation, IPTG was added to the culture at a final concentration of 0.25 mM. The cells were harvested at 48 h of cultivation, suspended in 10 ml of 20 mM Tris-HCl buffer containing 150 mM NaCl (pH 7.5), and then lysed by sonication on ice. The recombinant chitinase was purified from supernatant of the lysate by HiTrap Chelating HP and Bio-Gel P-100 columns.

**Chitinase assay and protein measurement.** N,N',N'-Triacetylchitotriose [(GlcNAc)₃] was used as a substrate for chitinase activity. The (GlcNAc)₃ was more sensitive substrate for chitinase activity than (GlcNAc)₂ and (GlcNAc)₄ because we used endoprotein (GlcNAc) assay, but did not reducing sugar assay. 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°C for 30 min. After the reaction was stopped by boiling for 30 s, liberated GlcNAc was measured by the method of Reissig et al.22) One unit (U) of enzyme was defined as the amount that liberated 1 μmol of GlcNAc equivalent per min under the standard assay conditions. Protein was measured by the method of Lowry et al.23) with bovine serum albumin as the standard.

**Analysis of hydrolysis products and antifungal activity.** High-performance liquid chromatography (HPLC) analysis was used for the hydrolysis products from colloidal chitin by the purified recombinant chitinase. Sugars were separated on Radial-PAK μBondapak NH₂ column (8 × 100 mm, Waters, Milford, MA) with water-acetonitrile (3:7) as an elution solvent mixture at a flow rate of 1 ml/min and detected by monitoring the absorbance at 210 nm. The hydrolysis products of N-acetylchitooligosaccharides by the recombinant chitinase were analyzed by thin-layer chromatography (TLC). The concentrated hydrolysate was spotted on a silica gel plate (Whatman, Linear-K6, 20 × 20 cm). After development three times using a solvent system of chloroform-methanol-water (90:65:15, v/v), sugars on the plate were detected by heating at 120°C for 15 min after spraying with 30% sulfuric acid.24) Antifungal activity was estimated using the hyphal extension-inhibition assay.5) The mycelium of Trichoderma reesei NBRC (formerly IFO) 31326 was directly inoculated on to the center of a potato dextrose agar (Eiken Chemical Co., Ltd., Tokyo) plate. After incubation at 25°C for 24 h, paper disks were placed around the edge of the T. reesei culture and 50 μl of the recombinant chitinase solution was put onto the disks. The plate was further incubated at 25°C for 48 h and the inhibition of hyphal extension around the disks was observed.

**Results**

**Internal amino acid sequences and molecular mass of the native chitinases**

Our previous study demonstrated that the chitinases (Chi-A and Chi-B) purified from strain J-13-3 had same molecular mass of 31 kDa estimated by SDS-PAGE and an identical N-terminal amino acid sequence, which was ADXAAAWNASSVYTGGGSASYNGHN (X, not identified).25) To obtain further information for cloning the gene encoding the chitinase, we analyzed their internal amino acid sequences and determined their exact molecular masses. After digestion with the V-8 protease, separation on a 12.5% polyacrylamide gel, and electroblotting on a membrane of the purified chitinases, the two peptide bands (17 kDa and 25 kDa) were detected in both enzymes and the N-terminal amino acids of the fragments on the membrane were sequenced. Identical amino acid sequences were found to be THHLVYIEQNVANYPGYXD for 17 kDa and AQFNMFGXXSFYTYSGLV for 25 kDa in Chi-A and THHLVYIVE for 17 kDa and AQFNMQMF for 25 kDa.
in Chi-B. The molecular masses were determined to be 28,210 Da for Chi-A and 28,225 Da for Chi-B by MALDI TOF-mass spectrometry, indicating overestimation as 31 kDa in a previous paper.\textsuperscript{17}

**Cloning and sequencing of the chitinase gene**

For probe to isolate chitinase gene from a genomic library of the strain J-13-3, PCR primers were synthesized on the basis of the N-terminal and internal amino acid sequences (ASYNGHN in N-terminus and EQN-VANY in the 17 kDa peptide of Chi-A). DNA fragment was specifically amplified and its sequence (375 bp) was determined. The deduced 119 amino acid sequence of the insert in the construction plasmid pUSC-12 showed high similarity to family 19 chitinases such as \textit{S. griseus} ChiC.\textsuperscript{4} suggesting that our chitinase is a member of family 19 chitinases. Southern hybridization using the PCR-amplified DNA probe labeled with DIG against J-13-3 DNA digested with various restriction endonucleases demonstrated that one hybridizing band was detected in digestions with various enzymes other than with \textit{Smal} (Fig. 1, lane 1 to 7 and lane 9). Total DNA was digested with \textit{Psrl} and its DNA fragments were ligated into \textit{Psrl} site of pUC19 to create a genomic library. A recombinant plasmid pUSC-613 carrying an about 9.4-kb insert was selected from the resulting library. A recombinant plasmid (pUSC-613) carrying the insert was digested with \textit{PstI} and its DNA fragments were hybridized to the insert fragment of the pUSC-613 by Southern hybridization, indicating the existence of the chitinase gene in the insert.

After 6 or 9 times sequencings for unknown regions of 5'- and 3'-ends of the chitinase gene by the primer walking using the pUSC-613 as a template, the complete nucleotide and deduced amino acid sequences of the chitinase gene could be decided in 1,289-bp sequenced region (Fig. 2). The N-terminal and two internal amino acid sequences obtained by analysis of the chitinase purified form strain J-13-3 were found in an open reading frame (underlined in Fig. 2). The frame sequence was composed of 897 nucleotides with the ATG initiation codon at position 355 and the TGA termination codon at position 1,249. The initiation codon was preceded by a potential RBS (AAGGAG) which complement the sequence at the 3' end of 16S rRNA of \textit{Streptomyces lividans}.\textsuperscript{25} Possible promoter sequences (TTGACA for the –35 region and TTGAGT for the –10 region) were identified upstream from the initiation codon.\textsuperscript{26} The chitinase was 298 amino acids with a calculated molecular mass of 31,081 Da. The coding sequence had a high overall G+C content (69 mol%) and strong tendency (95 mol%) to have G or C in the third position of the triplet, which were characteristic of many \textit{Streptomyces} genes.\textsuperscript{27}

The chitinase was composed of a signal peptides of 29 amino acids and a mature protein of 269 amino acids with a calculated molecular mass of 28,229 Da which was almost same as those of the purified Chi-A and Chi-B determined by MALDI TOF-mass spectrometer. In addition, four amino acids (GGTH, shaded in Fig. 2) deduced from the nucleotide sequence were different from those sequenced by Edman degradation of the 17 kDa peptide of the Chi-A. The gene cloned and sequenced in this study was designated \textit{chiA}.

**Comparisons of promoter region and deduced gene product with other family 19 chitinases of actinomycetes**

The deduced amino acid sequence of our chitinase had high similarity to ChiIS from \textit{Streptomyces} sp. strain AJ9463 (91% identity),\textsuperscript{12} ChiC from \textit{S. griseus} (79% identity),\textsuperscript{4} ChiB from \textit{N. prasina} (72% identity),\textsuperscript{10} ChiF from \textit{S. coelicolor} (70% identity),\textsuperscript{14} and Chi30 from \textit{S. olivaceoviridis} (67% identity).\textsuperscript{10} Our chitinase was also modular enzyme consisting of the signal sequence (29 amino acids), the chitin-binding domain (65 amino acids), and the catalytic domain (204 amino acids).\textsuperscript{6} Above six family 19 chitinases were aligned in their promoter and chitin-binding regions (Fig. 3). The possible promoters (boxed in Fig. 3A) of the chitinase genes were highly conserved [TTGACN for –35 hexamer and T(A/T)(G/A) NNT for –10 hexamer].\textsuperscript{26} The distance between –10 hexamer and the initiation codon in the upper four genes (\textit{chiA}, \textit{chiI S}, \textit{chiC}, and \textit{chiB}) was about twice shorter than that in the lower two genes (\textit{chiF} and \textit{chi30}). The upper and lower chitinase genes had one and two sequences of a 12-bp direct repeat (horizontal arrows in Fig. 3A) involved in both chitin induction and glucose repression,\textsuperscript{28} respectively. In chitin-binding domain (Fig. 3B), the amino acid sequence of our chitinase had high similarity to ChiIS (89% identity), ChiC (72% identity), and ChiB (67% identity) but had low similarity (28% identity) to ChiF and Chi30 in spite of well conserved 10 amino acid residues (shaded in Fig. 3B). The AKWWTQ motif

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**Fig. 1.** Southern Blot Analysis of Genomic DNA from J-13-3 Strain. The genomic DNA (10 μg) digested with \textit{Bam HI} (lane 1), \textit{EcoRI} (lane 2), \textit{Hin dIII} (lane 3), \textit{KpnI} (lane 4), \textit{Psrl} (lane 5), \textit{SacI} (lane 6), \textit{SalI} (lane 7), \textit{SmaI} (lane 8), or \textit{SphI} (lane 9) was electrophoresed on a 0.8% agarose gel. After the gel was denatured and neutralized, the digestion products were electrophoretically transferred to a nylon sheet. The DNA fragments on the sheet were hybridized with the DIG-labeled size markers from Roche Diagnostics.
boxed in Fig. 3B) was conserved in the upper chitinases, but not in the lower chitinases. Furthermore, ChiF had high similarity to Chi30 (79% identity) in amino acid sequence of their chitin-binding domain and only one different nucleotide was observed in the promoter regions of the \( \text{chiF} \) and the \( \text{chi30} \). Thus the six family 19 chitinases can be divided into two groups with respect to the sequences in their promoter region and chitin-binding domain. On the other hand, the catalytic domain (204 amino acids) of our chitinase showed high sequence similarity without gap penalty to those (204 amino acids) of eight family 19 chitinases including ChiIS (93% identity), ChiF (88% identity), Chi30 (88% identity), ChiC (82% identity), ChiB (77% identity), ChiG (82% identity) without a chitin-binding domain from \( S. \ coelicolor \),6) Chi25 (80% identity) without a chitin-binding domain from \( S. \ thermoviolaceus \),8) and Chi35 (78% identity) with a novel type of polysaccharide-binding domain from \( S. \ thermoviolaceus \)8) (Fig. 4).

Fig. 2.  Nucleotide and Deduced Amino Acid Sequences of J-13-3 Chitinase Gene.

The deduced amino acid sequence of chitinase is shown below the nucleotide sequence. The putative ribosome-binding sequences and \(-10/-35\) hexamers of the possible promoter are underlined and boxed, respectively. The stop codon is indicated by an asterisk. The double underlines indicate \( Smal \) sites. The signal peptide cleavage site is shown by a vertical arrow. Amino acid sequence regions matched to the N-terminal amino acid residues of the purified chitinases (N-terminus) and two peptide fragments (V8-25kDa and -17kDa) analyzed with a protein sequencer are indicated by underlines. Four deduced amino acids that differed from the determination of the fragment of V8-17kDa are shaded.
Purification of the recombinant chitinase

The *E. coli* JM109 harboring pUSC-7 produced a low chitinase activity (30 mU/mg of protein) and no induced band was detected on SDS-PAGE in soluble fraction of the cells after 3 h of cultivation at 30°C in the presence of 1 mM IPTG. Therefore, the chitinase with a signal

Fig. 3. Alignment of the Promoter Region (A) and the Deduced Chitin-binding Amino Acid Sequence (B) of J-13-3 Chitinase Gene with Those of Five Family 19 Chitinase Genes.

The abbreviations were as follows: Sj, *Streptomycyes* sp. J-13-3; Sa, *Streptomycyes* sp. strain AJ9463; Sg, *S. griseus*; Np, *N. prasina*; Sc, *S. coelicolor*; So, *S. olivaceoviridis*; chi, chitinase gene; Chi, chitinase. (A) The −10/−35 hexamers of the each possible promoter are boxed. Horizontal arrows indicate the 12 bp-direct repeat sequences and nucleotide sequences identical to TGGTC(C/T)(A/G)GACC(T/A) proposed as consensus sequence of the direct repeats are shaded. The nucleotide numbers of upstream from the initiation codon are shown on both sides in each line. (B) Amino acid residues conserved in products of all six genes are shaded. The AKWWTQ motif (consensus sequence in bacterial chitin-binding domains) is boxed. The residue number of the first and last amino acid in each line is shown on the left and right, respectively.

Fig. 4. Alignment of the Amino Acid Sequence in the Catalytic Domain of J-13-3 Chitinase with Those of Eight Family 19 Chitinases.

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 at least 6 of the 9 sequences are shaded. The strains have been abbreviated in the same way as in Fig. 3 other than *S. thermoviolaceus* (St).

**Purification of the recombinant chitinase**

The *E. coli* JM109 harboring pUSC-7 produced a low chitinase activity (30 mU/mg of protein) and no induced band was detected on SDS-PAGE in soluble fraction of the cells after 3 h of cultivation at 30°C in the presence of 1 mM IPTG. Therefore, the chitinase with a signal...
peptide was cloned into the vector pET23a (+) to obtain high-expression strain. When *E. coli* BL21(DE3) harboring pESC-17 was cultured at 25°C for 48 h, respectively; lanes 3 and 4, molecular mass markers (Nakarai Tesque, Kyoto); lane 5, purified chitinase (9 μg of protein). An arrow indicates the recombinant chitinase.

The enzyme activity was eluted with 200 mM imidazole and the active three fractions were pooled. The recombinant chitinase was further purified from the insoluble and soluble fractions of the cells (Fig. 5, lane 5) and its molecular mass of 32 kDa was detected on SDS-PAGE in both soluble and insoluble fractions of the cells (Fig. 5, lane 1 and 2). The soluble extract was put on a column of HiTrap Chelating HP. The column was eluted with 20, 50, and 200 mM buffers adjusted to various pHs. The optimum pH range for the enzyme activity was 5.5–6.0. The enzyme was stable from pH 3.0 to 8.0 after incubation at 4°C for 24 h in various 50 mM buffers. The optimum temperature range for the enzyme was 35–40°C. When the enzyme was kept at various temperatures for 10 min, it was stable up to 40°C, and 70% and 20% of the original activity remained after incubation at 45°C and 55°C, respectively. After incubation with 0.9 ml of colloidal chitin suspension (1.0 of absorbance at 610 nm) and the chitinase (150 μU) at 40°C, the absorbance of the reaction mixture was reduced 10% for 3 h, 15% for 5 h, and 21% for 24 h. HPLC analysis demonstrated that the hydrolysis products from colloidal chitin were mainly GlcNAc and (GlcNAc)₂ in the ratio of about 1:9 after 24 h of reaction. The purified chitinase (10 μU) was reacted with various N-acetylchitooligosaccharides (0.4 mg) at 40°C for 10 min and then the products were analyzed by TLC (Fig. 6). The enzyme hydrolyzed (GlcNAc)₀ to GlcNAc and (GlcNAc)₂ (Fig. 6, lane 3), and (GlcNAc)₆ to (GlcNAc)₁₂ (Fig. 6, lane 4). The (GlcNAc)₂ and (GlcNAc)₆ were hydrolyzed to GlcNAc, (GlcNAc)₂, and (GlcNAc)₆, and to GlcNAc, (GlcNAc)₁₂, (GlcNAc)₁₈, and (GlcNAc)₃₀, respectively (Fig. 6, lane 5 and 6). However, the purified chitinase did not hydrolyzed (GlcNAc)₆ (Fig. 6, lane 2).

**Enzymatic properties and hydrolysis products of the recombinant chitinase**

The enzymatic properties were examined using about 2 μU of the purified recombinant chitinase under the standard conditions. The activity was measured in 50 mM buffers adjusted to various pHs. The optimum pH range for the enzyme activity was 5.5–6.0. The enzyme was stable from pH 3.0 to 8.0 after incubation at 4°C for 24 h in various 50 mM buffers. The optimum temperature range for the enzyme was 35–40°C. When the enzyme was kept at various temperatures for 10 min, it was stable up to 40°C, and 70% and 20% of the original activity remained after incubation at 45°C and 55°C, respectively. After incubation with 0.9 ml of colloidal chitin suspension (1.0 of absorbance at 610 nm) and the chitinase (150 μU) at 40°C, the absorbance of the reaction mixture was reduced 10% for 3 h, 15% for 5 h, and 21% for 24 h. HPLC analysis demonstrated that the hydrolysis products from colloidal chitin were mainly GlcNAc and (GlcNAc)₂ in the ratio of about 1:9 after 24 h of reaction. The purified chitinase (10 μU) was reacted with various N-acetylchitooligosaccharides (0.4 mg) at 40°C for 10 min and then the products were analyzed by TLC (Fig. 6). The enzyme hydrolyzed (GlcNAc)₀ to GlcNAc and (GlcNAc)₂ (Fig. 6, lane 3), and (GlcNAc)₆ to (GlcNAc)₁₂ (Fig. 6, lane 4). The (GlcNAc)₂ and (GlcNAc)₆ were hydrolyzed to GlcNAc, (GlcNAc)₂, and (GlcNAc)₆, and to GlcNAc, (GlcNAc)₁₂, (GlcNAc)₁₈, and (GlcNAc)₃₀, respectively (Fig. 6, lane 5 and 6). However, the purified chitinase did not hydrolyzed (GlcNAc)₆ (Fig. 6, lane 2).
Antifungal activity of the recombinant chitinase

The growth of T. reesei is inhibited by bacterial family 19 chitinase of ChiC,14 ChiB,14 and Chi35,8 but not by a family 18 chitinase A1 from Bacillus circulans.5 The antifungal activity appears to be a general property of the bacterial family 19 chitinases. Therefore, we also tested antifungal activity of our recombinant chitinase as an inhibitory effect on the hyphal extension of T. reesei (Fig. 7). The inhibitory effect was observed using 5 μg (Fig. 7, disk 3) but not 1 μg (Fig. 7, disk 2) of the recombinant chitinase.

Discussion

All bacterial chitinases are classified in family 18 of glycosyl hydrolases except several family 19 chitinases from the species of Streptomyces, Nocardiosis, Achromobacter, and Burkholderia. The amino acid sequences of the bacterial family 19 chitinases have similarity to those of plant chitinases (class IV type) in their catalytic domains, but not in chitin-binding domains, suggesting horizontal gene transfer from plants to the bacteria.5,8 In this study, we have cloned and analyzed the gene encoding family 19 chitinase from Streptomyces sp. (strain J-13-3) and the recombinant chitinase overexpressed in E. coli cells have been purified to homogeneity with a high yield and characterized to compare with the native chitinase.

The amino-terminal signal peptide found on most secretory proteins serves to initiate export across the cytoplasmic membrane in bacteria.29,30 As shown in Fig. 8, typical signal sequence was found in N-terminal regions (first 29 or 30 amino acids) of the six family 19 chitinases. The signal sequences consisted of a short positively charged segment (n-region) with the two Arg residues, a central hydrophobic segment (h-region), and a cleavage segment (c-region) started with the helix-breaker Pro residue. The sequence of Ala-X-Ala preceding the cleavage sites in the c-region was signal peptidase recognition sequence29 and conserved in these six chitinases. The chitinases other than ChiF with mRNA expression were produced in culture filtrate of actinomycetes.3,9,11,13,17 Therefore, the presence of typical signal sequence results in high chitinase productivity although family 19 chitinases have been rare in bacteria. The removal of signal peptide of the recombinant chitinase overexpressed in cells of E. coli BL21(DE3) harboring pESC-17 indicated that the signal peptidase of E. coli cleaved the position between the two Ala residues, amino acids 29 and 30 (vertical arrow in Fig. 2). The same result is also obtained in the recombinant chitinase of ChiC from S. griseus.5 Furthermore, similar signal sequence (30 amino acids), MRFRHK (n-region) AAAALATLALPLGLAS (h-region) PAQA (c-region), of chitinase C (family 18) from S. lividans31 is recognized by the signal peptidase of E. coli BL21(DE3, pLys S) and the fusion protein consisting of the signal peptide of chitinase C and mature protein of chitin deacetylase is produced in culture medium after removal of the signal peptide.32 These results suggested that the signal peptide sequences of the family 19 chitinases will be a useful genetic tool in protein engineering for production of soluble recombinant protein.

Previous studies shown that the chitin-binding domains of ChiC and ChiB have similarity to the binding domains of some bacterial family 18 chitinases, cellulases, and proteases.4,14 This study demonstrated that

| Sj | ChiA | 1 | MFERIMGLLAALGAVVAGLTVLPATTASA | 29 |
| Sa | ChiIS | 1 | MERRIMGLLAALGAVVAMVVLPATTAA | 29 |
| Sg | ChiC | 1 | VYRRVMSLLVALGAIYAIIVLPATTQA | 29 |
| Np | ChiB | 1 | VRIRLLQLAALGAIAVLYTVLPAAQA | 29 |
| Sc | ChiF | 1 | MSRERISAVYTLALAGAVPLMPANEASA | 30 |
| So | Chi30 | 1 | VPRRTSALLAALVISTAAPVLLPAAPAA | 30 |

Fig. 8. Comparison of Signal Peptide Sequences of Family 19 Chitinases.

The residue number of the first and last amino acid in each line is shown on the left and right, respectively. Amino acids conserved are shaded. The strains have been abbreviated as in Fig. 3. The boxes n, h, and c represent short positively charged segment (n-region), central hydrophobic segment (h-region), and cleavage segment (c-region), respectively.

Fig. 7. Inhibition of Hyphal Growth of T. reesei NBRC 31326 by the Purified Recombinant Chitinase.

Growth inhibition was assayed as described in the text. Amounts of the purified recombinant chitinase applied were as follows: disk 1, no chitinase control; disk 2, 1 μg; disk 3, 5 μg.
of chitin-binding domains of the six family 19 chitinases can be divided two distinct groups with respect to similarity of their amino acid sequences and presence of a conserved AKWWTQ motif, as reported for the chitin-binding domains of bacterial family 18 chitinases. \(^{33,31}\) As for essential residues for adsorption to chitin in the chitin-binding domains, it was recently reported that Trp-59 and Trp-60 in the AKWWTQ motif of ChiC (Fig. 3B) and Trp-687, which probably corresponded to Tyr-64 in ChiF and Chi30 (Fig. 3B), in a family 18 chitinase A1 from \textit{B. circulans} are important for the binding activity. \(^{34,35}\) The chitin-binding domain of ChiC contributes more importantly to antifungal activity against \textit{T. reesei} than to catalytic activity. \(^{36}\) Our purified recombinant chitinase also exhibited antifungal activity against \textit{T. reesei}. However, antifungal activities against fungi other than \textit{T. reesei} and the essential residue(s) for catalytic activity of the bacterial family 19 chitinases remain to be examined more detail.

The DIG-labeled probe corresponding to nucleotide positions from 496 to 852 (Fig. 2) hybridized one fragment in digestions of J-13-3 DNA with various restriction endonucleases other than with Smal (Fig. 1, lane 1 to 7 and lane 9). In the chitinase coding region, four Smal sites (double underlined in Fig. 2) were fond, but not the other restriction enzyme sites. The DIG-labeled probe contained the four Smal sites. Therefore, the strain J-13-3 had a single chitinase gene, indicating that the Chi-A and Chi-B must be products from same gene. However, the difference in the protein structures of the native Chi-A and Chi-B is still unclear, but may be due to the presence of a conserved AKWWTQ motif, as reported for the chitin-binding domains of \textit{Streptomyces griseus} \textit{HUT 6037}. \(^{11}\) The same results are also obtained in the recombinant chitinases of ChiIS, \(^{12}\) ChiC, \(^{3}\) ChiB, \(^{3}\) and Chi30. \(^{3}\) The results suggested that the slow migration on the gel is related to the protein structure of the family 19 chitinases. In addition, MALDI TOF-mass spectrometry proved to be useful for confirming exact molecular masses of our native and recombinant chitinases.

The temperature and pH optima of the purified recombinant chitinase were similar to those of the native chitinases. Properties of our native chitinases were also similar to those of ChiIS, \(^{11}\) ChiC, \(^{3}\) and ChiB \(^{13}\) in optimum pH 4–7, of ChiC and ChiB in stable temperature (up to 45–50°C), and of ChiB in inhibition of enzyme activity by N-bromosuccinimide, inhibitor of tryptophan residue. A large amount of the recombinant chitinase with antifungal activity could be purified with a high yield by simple isolation methods using affinity and gel filtration columns. More recent studies demonstrated that the ChiC from \textit{S. griseus} and the plant chitinase E (family 19 and class IV) from yam have increased resistance to the fungal disease in transgenic rice plants with the \textit{chi}C gene and in strawberry plants using spry method, respectively. \(^{37,38}\) Therefore, our chitinase gene and purified recombinant chitinase will be also useful for biocontrol of fungal infection of plants in addition to chitin degradation.

**Acknowledgment**

We would greatly thank Mr. Takashi Amano and Miss Yoshiko Yokoyama for their technical assistance.

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