

Molecular cloning, characterization, and homologous expression of an endochitinase gene from *Bacillus thuringiensis* serovar *morrisoni*

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Abstract: The endochitinase gene (*chi3023*) of *Bacillus thuringiensis* (Bt) serovar *morrisoni* strain 3023 was amplified via polymerase chain reaction (PCR) and cloned in *Escherichia coli*. The ORF of *chi3023* (GenBank Accession Number: DQ993175) consists of 2031 nucleotides encoding a 676-residue protein with a calculated molecular mass of 74.5 kDa and a *pI* value of 6.0. The amino acid sequence of Chi3023 was compared with previously sequenced Bt chitinases and the phylogenetic relationships among them were determined. The deduced N-terminal 34 amino acids of the premature Chi3023 exhibited a typical signal peptide. The *E. coli*-Bt shuttle vector pHT315 was used for homologous expression of *chi3023*. Introduction of recombinant pHT315BTC, carrying *chi3023* into Bt serovar *morrisoni* 3023, resulted in a 23-fold increase in endochitinase activity (0.185 U/mg versus 4.256 U/mg).

Key words: *Bacillus thuringiensis*, characterization analysis, endochitinase, gene cloning

Bacillus thuringiensis serovar *morrisoni* endokitinaz geninin moleküler kopyalanması, karakterize edilmesi ve homolog ifadesi

Özet: *Bacillus thuringiensis* (Bt) serovar *morrisoni* 3023 suşuna ait endokitinaz geni (*chi3023*) polimeraz zincir reaksiyonu (PCR) ile çoğaltılıp *E. coli*'ye kopyalandı. *chi3023* geni (Gen Bankası Erişim Numarası: DQ993175), 676 aminoasit kodlayan 2031 nükleotitten oluşmaktadır. Moleküler ağırlığı 74.5 kDa, *pI* değeri de 6.0 olarak hesaplandı. Chi3023'ün aminoasit dizisi daha önce dizilenmiş Bt kitinazları ile karşılaştırıldı ve aralarındaki filogenetik ilişki belirlendi. Prematüre Chi3023'ün N-ucundaki 34 aminoasit, tipik sinyal peptidi özelliği göstermektedir. *chi3023* geninin homolog ifadesi için *E. coli*-Bt vektörü pHT315 kullanıldı. *chi3023* genini taşıyan rekombinant pHT315BTC'nin Bt serovar *morrisoni* 3023'e aktarılması sonucunda endokitinaz aktivitesi 23 kat (0,185 U/mg'dan 4,256 U/mg'a) arttı.

Anahtar sözcükler: *Bacillus thuringiensis*, karakterizasyon analizi, endokitinaz, gen kopyalanması

Introduction

Chitin, a polysaccharide composed of β -1,4-linked N-acetyl-D-glucosamine (GlcNAc), is highly distributed in nature as a major structural component of the exoskeleton of insects and crustaceans (1). It is

also found in the cell walls of a variety of fungi (1) and the peritrophic membranes of insects (2). Hydrolysis of chitin to disaccharides and larger oligomeric saccharides usually takes place extracellularly, by the action of chitinases (EC 3.2.1.14). For

biotechnological interests, in addition to the potential applications of chitinase itself, the chito oligosaccharides [(GlcNAc)_n] have been found to function as antibacterial agents, elicitors, lysozyme inducers, and immunoenhancers (3). These enzymes have been shown to play an important role in the biological control of plant pathogens (4).

The strains of the spore-forming bacterium *Bacillus thuringiensis* (*Bt*) are among the most important entomopathogens (5). They produce crystal (Cry) toxins with highly selective modes of action (6). Chitinase has been found to increase the efficacy and potency of *Bt* Cry toxins in insect control (7). At least 27 chitinase gene sequences from *Bt* have been deposited in the GenBank database (www.ncbi.nlm.nih.gov); however, only 9 of the encoded proteins have been characterized. The endochitinase gene from *Bt* serovar *morrisoni* has not been characterized yet. In our previous study (8), the chitinase A (*chiA*) gene of *Serratia marcescens* Bn10 was expressed in *Bt* serovar *morrisoni* 3023. The effects of pH, temperature, and metal ions on both chitinases produced by recombinant and parental *Bt* strains were investigated. The present study reports molecular cloning and sequence analysis of an endochitinase gene (*chi3023*) from *Bt* serovar *morrisoni* strain 3023 as well as its homologous expression.

Materials and methods

Bacterial strains, plasmids, and media

A list of the strains, plasmids, and their sources is given in the Table. Luria broth (LB; Qbiogene) was used for cultivation of the strains. The cultures were incubated by shaking (200 rpm) at 30 °C for *B. thuringiensis* and *B. subtilis* and 37 °C for *Escherichia coli*. For endochitinase production, 1.6% (w/v) nutrient broth (Merck) containing 0.2% (w/v) colloidal chitin (9) was used. Cultures grown at 30 °C for 16 h were centrifuged at 12,000 rpm for 10 min, and the supernatants were used as the source of crude enzyme.

Gene cloning

For the amplification of *chi3023*, the endochitinase gene of *Bt* serovar *morrisoni*, forward (5'

AAGCTTTTTCCTCCCATACCAACCTT 3') and reverse primers (5' GCATGCAAGCCTTT CCTAACAGGTGA 3'), were designed according to the nucleotide sequence of the chitinase gene of *Bt* serovar *israelensis* (GenBank accession number AF526379). The primer design was made to include the promoter region for further expression of the gene. For cloning into pHT315, the restriction enzyme sites *Hind*III and *Sph*I were included in the 5' region of the forward and reverse primers, respectively (underlined bases). Chromosomal DNA used as a template was isolated from *Bt* serovar *morrisoni* according to the methods of Cutting and van der Horn (10). PCR reaction mixtures contained 34 µL of dH₂O, 5 µL of 10× buffer for Taq polymerase (Fermentas), 5 µL of 25 mM MgCl₂, 50 pmol of each primer, 1 µL of 10 mM dNTP mix (Fermentas), 0.5 µg of chromosomal DNA and 3 units (1 µL) of Taq polymerase (Fermentas). PCR was carried out in 40 cycles: 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. The initial denaturation was carried out at 94 °C for 3 min. The final extension was performed for 10 min at 72 °C. Reaction mixtures were run in a 0.8% agarose gel. PCR products were extracted from the gel and ligated to pGEM-T Easy Vector (Promega). Ligation product was then transferred into the competent *E. coli* DH5α cells, which were prepared according to the protocol described by Sambrook et al. (11) with slight modifications. White colonies were selected on X-gal IPTG-containing selective LB agar plates. *E. coli* plasmid DNA was isolated using the Plasmid Miniprep Purification Kit (GeneMark) as specified by the manufacturers. The *chi3023* gene, together with its promoter region, was cloned into *Hind*III-*Sph*I sites of *E. coli*-*Bt* shuttle vector pHT315 and the recombinant vector was named as pHT315BTC. Transformation of competent *B. subtilis* 168 cells was performed according to the methods of Klein et al. (12). Transformants were selected in the presence of 25 µg/mL of erythromycin. The pHT315BTC molecules isolated from *B. subtilis* 168 were introduced into *Bt* serovar *morrisoni* 3023 via electroporation, as described by Lereclus et al. (13).

DNA sequencing

DNA sequencing was carried out at Iontek Company (İstanbul, Turkey) by using the chain

Table. List of the bacterial strains and plasmids used in this study and their sources.

Bacterial strains or plasmids	Description	Sources and references
Strains		
<i>B. thuringiensis</i> serovar <i>morrisoni</i>	Chitinase producer	Prof. F. Gadani; Kaelin et al. (18)
<i>B. subtilis</i> 168	Standard strain; <i>trpC2</i> , <i>thyA1</i> , <i>thyB1</i>	Dr. D. Zeigler, BGSC
<i>E. coli</i> DH5 α	F' ϕ dlacZ Δ (lacZY A-argF)U169 <i>supE44</i> ⁻ <i>thi-1 gyrA recA1 relA1 endA1 hsdR17</i>	ATCC
Plasmids		
pGEM-T	<i>amp</i> , <i>lacZ</i>	Promega Inc. (Madison, WI)
pHT315	<i>erm</i> , <i>lacZ</i>	Prof. D. Lereclus; Arantes and Lereclus (19)
pHT315BTC	pHT315 carrying <i>Bt</i> serovar <i>morrisoni</i> chitinase	This study

termination method with dye-labeled dideoxy terminators of the DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare). Deduced nucleotide and amino acid sequence data were compared with the National Center for Biotechnology Information (NCBI) database using the NCBI website's BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST>).

Measurement of enzyme activity

The endochitinase activity was measured as described by Brants and Earle (14). 4-methylumbelliferyl- β -D-N, N', N''-triacylchitotrioside [4-MU- β -(GlcNAc)₃] (Sigma) solution was prepared as a substrate at a concentration of 25 μ g/mL in 0.1 M potassium phosphate buffer (pH 6.6). Then 15 μ L of enzyme solution was added to 200 μ L of the substrate and incubated at 30 °C for 20 min; later 30 μ L of aliquots were added to 1.9 mL of 0.2 M Na₂CO₃ stopping buffer. Fluorescence after the enzyme activity was measured by using a Varian Cary Eclipse Fluorescence Spectrophotometer (Varian Chrompack) at 360 nm excitation and 450 nm emission wavelengths. Enzyme assays were performed in triplicates. One unit of enzyme activity (U) was defined as the amount of enzyme able to release one micromole of reducing sugar per minute.

Results and discussion

Sequence of the endochitinase gene from *Bt* serovar *morrisoni*

An approximately 2.1 kb endochitinase gene was amplified via PCR from the chromosomal DNA of *Bt* serovar *morrisoni* (Figure 1). The PCR product was cloned in a pGEM-T Easy Vector and sequenced (GenBank Accession Number: DQ993175). Computer-aided analysis (EditSeq, DNASTAR) indicated that the ORF of *chi3023* consists of 2031 nucleotides encoding a 676-residue protein with a calculated molecular mass of 74.5 kDa and a pI value of 6.0. The mean G+C content of the gene is 37.6%. A potential ribosome-binding (Shine-Dalgarno) sequence (5'GAAAGG3') precedes 4 bp upstream from the initiation codon. The putative promoter consensus sequences are identical to those reported for *chiA71* of *Bt* subsp. *pakistanii*, *chiA74* of *Bt* subsp. *kenyae*, and *chi255* of *Bt* subsp. *kurstaki* (15). The -10 sequence (5'TTAATA3') is 165 bp upstream from the initiation codon and is 14 bp distant from the -35 sequence (5'TTGAGA3').

The alignment of the deduced amino acid sequence of Chi3023 from *Bt* serovar *morrisoni* with other *Bt* chitinase sequences (MegAlign, DNASTAR)

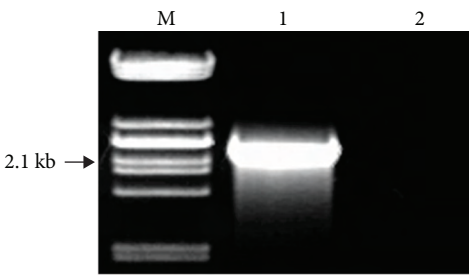


Figure 1. Amplification of *chi3023* from genomic DNA of *Bt* 3023 via PCR. M: DNA ladder (Lambda DNA/*Pst*I, Fermentas), Lane 1: PCR product for amplification of *chi3023*, Lane 2: Negative control.

showed that the sequence was 98.2%-92.6% identical to those from *Bt* serovar *sotto* (AAM94024, 98.2%), *Bt* serovar *alesti* (AAR19092, 97.8%), *Bt* serovar *toumanoffi* (AAR19091, 97.8%), *Bt* serovar *kurstaki* (AAO34713, 97.2%), *Bt* serovar *israelensis* (AAM88400, 97%), *Bt* serovar *colmeri* strain 15A3 (ABF57674, 96.8%), *Bt* serovar *canadensis* strain HD224 (AAR21234, 96.7%), *Bt* serovar *entomocidus* (AAR19226, 96.6%), *Bt* serovar *kurstaki* (CAG25670, 95.7%), and *Bt* serovar *konkukian* strain 97-27

(AAT60699, 92.6%). The phylogenetic relationship of these chitinases belonging to different *Bt* serovars is shown in Figure 2, for the first time in this study.

The deduced N-terminal 34 amino acids of the premature Chi3023 exhibited typical signal peptide characteristics of bacterial extracellular enzymes. SignalP 3.0 Server (<http://www.cbs.dtu.dk/services/SignalP>) prediction results showed that the most likely cleavage site of Chi3023 is between Ala³⁴ and Asp³⁵ (Figure 3).

Accordingly, the cleavage of the signal peptide generates a 70.6 kDa protein with a *pI* value of 5.73. The amino terminus of the mature protein is Asp-Ser-Pro-Lys-Gln, which is identical to that of *Bt* serovar *alesti*, *Bt* serovar *kenyae*, *Bt* serovar *pakistani* (16), and *Bt* subsp. *kurstaki* (15). The Chi3023 N-terminal region (residue 147 to 222) showed similarity to catalytic domains of chitinases belonging to the glycosyl hydrolase family 18. Although the catalytic region is conserved in *Bt* chitinases, 4 residues (Arg¹⁵⁰, Gln¹⁵¹, Thr¹⁵⁶, and Leu²⁰⁶) are unique to Chi3023 (Figure 4). It contains the characteristic DXDXE

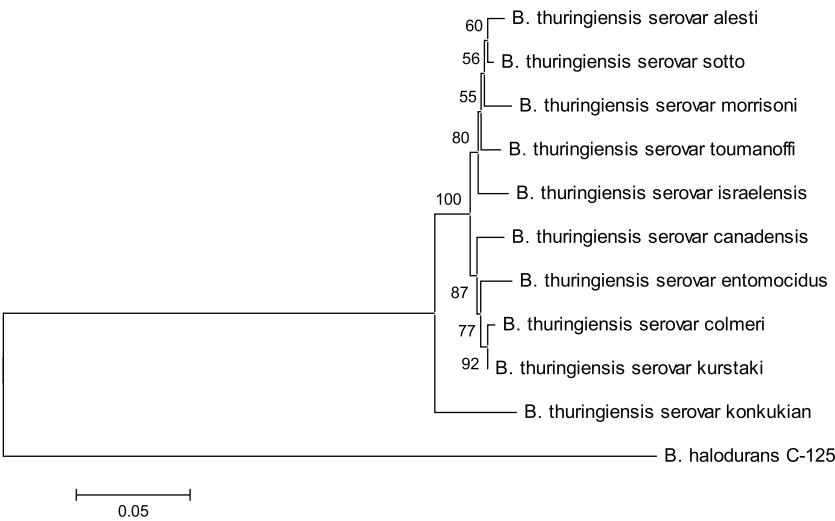


Figure 2. Evolutionary relationships of *Bt* chitinases. The evolutionary history was inferred using the neighbor-joining method (20). The optimal tree with the sum of branch length = 0.61751910 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (21). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 589 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (22).

MAMRSOKFTLLLSLLFLPLFLTNFITPNLALADSPKQSQKIVGYFPSWGVYGRNYQVADI
 DASKLTHLNYAFADICWNGKHGPNSTHPDNPKNQTNWCKESGVPLQNKEVPNGTLVLGEPWA
 DVTKSNPVSQTTWEDCDKYARCGNFROLKRLTAKYPHLKTIIISVGGWTWSNRFSDMAADEKT
RKVFAESTVSFLRAYGFDGLDLDWEYPGVETIPGGSYRPEYKQNFNTLLQDVRNALNKAGAE
 DGKQYLLTIASGASQRYADHTELKKISQILDWINIMTYDFHGGWEATSNHNAALYKDPNDPA
 ANTNEYVDGAIDVYTNEGVPVDKLVGVFPYGRGWKSCGKENNGQYQPGKPGSDGKLASKGT
WDDYSTGDTGVYDYGDLAANYWNKNGFWRWYNDTAKVPLYNATGTFTLSYDDNESMKYKTD
YIKTKGLSGAMFWELSGDCRTSPKYSCSGPKLLDTLVKELLGGPISQKDEPPTNVKNIVVT
NKNSSSVQLSWTASTDNVGVTEYEITAGEEKSSTTNSITIKNLKPNTTEYTFSVIAKDASGN
KSQPTALTVKTDDEANTTPPDGNGTATFSVTSNWGSGYNFSIIKNNGTTPIKNWKLEFDYSG
NLTQVWDSKINSKTNNHVITNAGWNGEIPPGGSITIGGAGTGNPAELLNAVISEN

Figure 3. Deduced amino acid sequence of Chi3023. The secretion signal peptide is underlined. The conserved catalytic domain is shaded and underlined, FLD1 is boxed and dotted, FLD2 is boxed, and the chitin-binding domain is shaded.

Bt morrisoni	GNF <u>R</u> OLKRLTAKYPHLKTIIISVGGWTWSNRFSDMAADEKTRK
Bt alesti	GNFGELKRLKAKYPHLKTIIISVGGWTWSNRFSDMAADEKTRK
Bt canadensis	GNFGELKRLKAKYPHLKTIIISVGGWTWSNRFSDMAADEKTRK
Bt colmeri	GNFGELKRLKAKYPHLKTIIISVGGWTWSNRFSDMAADEKTRK
Bt entomocidus	GNFGELKRLKAKYPHLKTIIISVGGWTWSNRFSDMAADEKTRK
Bt israelensis	GNFGELKRLKAKYPHLKTIIISVGGWTWSNRFSDMAADEKTRK
Bt konkukian	GNFGELKRLKAKYPHLKTIIISVGGWTWSNRFSDMAADEKTRK
Bt kurstaki	GNFGELKRLKAKYPHLKTIIISVGGWTWSNRFSDMAADEKTRK
Bt sotto	GNFGELKRLKAKYPHLKTIIISVGGWTWSNRFSDMAADEKTRK
Bt toumanoffi	GNFGELKRLKAKYPHLKTIIISVGGWTWSNRFSDMAADEKTRK

Bt morrisoni	VFAESTVSFLRAYGFDGLDLDWEYPGVETIPGGS
Bt alesti	VFAESTVAFLRAYGFDGVDLDWEYPGVETIPGGS
Bt canadensis	VFAESTVAFLRAYGFDGVDLDWEYPGVETIPGGS
Bt colmeri	VFAESTVAFLRAYGFDGVDLDWEYPGVETIPGGS
Bt entomocidus	VFAESTVAFLRAYGFDGVDLDWEYPGVETIPGGS
Bt israelensis	VFAESTVAFLRAYGFDGVDLDWEYPGVETIPGGS
Bt konkukian	VFAESTVDFLREYGFDDGVDLDWEYPGVETIPGGS
Bt kurstaki	VFAESTVAFLRAYGFDGVDLDWEYPGVETIPGGS
Bt sotto	VFAESTVSFLRAYGFDGVDLDWEYPGVETIPGGS
Bt toumanoffi	VFAESTVSFLRAYGFDGADLDWEYPGVETIPGGS

Figure 4. Alignment of the catalytic domain of Chi3023 with the other *Bt* chitinases. The differences from the conserved sequence are shaded.

sequence motif (Asp²⁰⁷-Leu²⁰⁸-Asp²⁰⁹-Trp²¹⁰-Glu²¹¹), including a glutamate residue that protonates the oxygen in the scissile glycosidic bond. Two fibronectin type III-like domains (FLD) are present as FLD1, from Lys³⁵⁰ to Tyr⁴³⁵, and FLD2, from Ile⁴⁷⁹ to Thr⁵⁷⁴, by analogy with those reported for ChiA74 of *Bt* subsp. *kenyae* and Chi255 of *Bt* subsp. *kurstaki* at the same positions (15). At the C-terminal region, the residues Trp⁵⁹¹, Tyr⁵⁹⁵, and Trp⁶²⁶ correspond to a

chitin-binding domain (CBD) from Thr⁵⁸⁸ to Ser⁶²⁸ (15,17).

Homologous expression of *chi3023*

The recombinant plasmid pHT315BTC, containing *chi3023*, was constructed and introduced first to *E. coli* DH5a and then to *B. subtilis* 168 cells. The pHT315BTC molecules isolated from recombinant *B. subtilis* cells were introduced into *Bt*

serovar *morrisoni* 3023 via electroporation. Recombinants were selected on erythromycin-containing LB plates. Plasmids were isolated from putative recombinants and verification of recombination was performed by double digestion (Figure 5) and PCR. When Chi3023 activity was fluorometrically determined in overnight cultures of the parental *Bt* and its recombinants, it was found that homologous expression of the *chi3023* gene gave rise to an over 23-fold increase, as compared with the activity of parental *Bt* (0.185 U/mg versus 4.256 U/mg).

In summary, the endochitinase gene belonging to *Bt* serovar *morrisoni* 3023 was cloned and characterized in this study. Homologous expression of the endochitinase gene on a multicopy plasmid in *Bt* 3023 gave rise to a 23-fold increase in enzyme activity. Considering the synergistic effect of chitinases and Cry toxins, homologous expression of *chi3023* in Cry3A producer *Bt* serovar *morrisoni* 3023 at high levels seems promising as a better biological agent against plant pathogenic fungi and insects.

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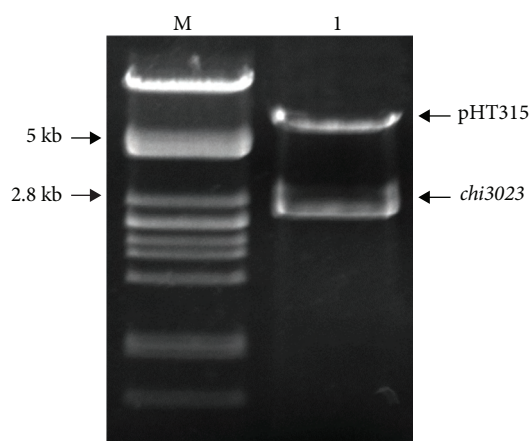


Figure 5. Verification of pHT315BTC by restriction enzyme digestion. M: DNA ladder (Lambda DNA/*Pst*I, Fermentas), Lane 1: Double digestion of pHT315BTC with *Hind*III and *Sph*I.

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