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* endochitinaz geninin moleküler kopyalanması, karakterize edilmesi ve homolog ifadesi

Özet: *Bacillus thuringiensis* (*Bt*) serovar *morrisoni* 3023 suşuna ait endochitinaz 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 dizilnen *Bt* kitinazları ile karşılaştırıldı ve aralarındaki filogenetik ilişkiye belirledi. 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 sonuçunda endochitinaz aktivitesi 23 kat (0.185 U/mg’dan 4.256 U/mg’a) arttı.

Anahtar sözcükler: *Bacillus thuringiensis*, karakterizasyon analizi, endochitinaz, 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 immuno-enhancers (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’ AAGCTTTTTCTCCCCATACCCAACCTT 3’) and reverse primers (5’ GCATGCAAGCCTTCTCTAACAGGTGA 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 HindIII and SphI 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 HindIII-SphI 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
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''-triacetylchitotrioside [4-MU-β-(GlcNAc)₃] (Sigma) solution was prepared as a substrate at a concentration of 25 μg/mL in 0.1 M potassium phosphate buffer (pH 6.6). Then 15 μL of enzyme solution was added to 200 μL of the substrate and incubated at 30 °C for 20 min; later 30 μL 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. pakistani, chiA74 of Bt subsp. kenyaе, 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)
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 Ala \(^{34}\) and Asp \(^{35}\) (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 kenyaee, 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 (Arg\(^{150}\), Gln\(^{151}\), Thr\(^{156}\), and Leu\(^{206}\)) are unique to Chi3023 (Figure 4). It contains the characteristic DXDXE
sequence motif (Asp<sup>207</sup>-Leu<sup>208</sup>-Asp<sup>209</sup>-Trp<sup>210</sup>-Glu<sup>211</sup>), 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 Lys<sup>350</sup> to Tyr<sup>435</sup>, and FLD2, from Ile<sup>479</sup> to Thr<sup>574</sup>, by analogy with those reported for Chi<sub>A74</sub> of <i>Bt</i> subsp. <i>kenyae</i> and Chi<sub>255</sub> of <i>Bt</i> subsp. <i>kurstaki</i> at the same positions (15). At the C-terminal region, the residues Trp<sup>591</sup>, Tyr<sup>595</sup>, and Trp<sup>626</sup> correspond to a chitin-binding domain (CBD) from Thr<sup>588</sup> to Ser<sup>628</sup> (15,17).

**Homologous expression of chi<sub>3023</sub>**

The recombinant plasmid pHT315BTC, containing <i>chi</i>3023, was constructed and introduced first to <i>E. coli</i> DH5α and then to <i>B. subtilis</i> 168 cells. The pHT315BTC molecules isolated from recombinant <i>B. subtilis</i> cells were introduced into <i>Bt</i>...
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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**References**


