Original Research Article

Isolation, computational characterization and structure prediction of chitinase gene-*Bbchit*of *Beauveriabassiana*

ABSTRACT

Beauveriabassiana is an entomopathogenic fungus that is used as a biopesticide for the control of many insect pests in agriculturally important crops. *B. bassiana* produces many extracellular hydrolytic enzymes that degrade the insect cuticle. Among them, the fungal exochitinase mainly assists in the infection by degrading the chitin present in the insect cuticle. To characterize the exochitinase from *B. bassiana*, the chitinase gene (*Bbchit*) of 1050bp was amplified from the genomic DNA through PCR using gene-specific primers. Thischitinasegene was cloned into the pJET1.2 cloning vector, confirmed through sequencing. The sequence (GI; OP114061) has been deposited in the NCBI database and used for bioinformatics analysis. The various Physico-chemical properties of chitinase protein such as pI, EC, AI, GRAVY and instability index were predicted using primary structure analysis. Using MEGA11 software, the translated chitinase protein- *Bbchit* showed that it closely resembles with the QJE37897.1 (chitinase Chit37). Primary and secondary structure analysis helps to characterize the functionality of chitinase protein (*Bbchit*).The 3D structure of *B. bassiana*was predicted using Robetta, an abinitio prediction method and confirmed through in-silicousing the SAVESv6.0 (PROCHECK) server. The isolated exochitinasegene from*B. bassiana*has been characterized for use as abio-control agentfor the control of insect pests in crop plants.

Keywords:Bbchit, Beauveriabassiana (Bb), Chitinase,Physico-chemical, protein structure prediction.

1. INTRODUCTION

Beauveriabassiana (Balsamo) Vuillemin belongs to the class Sordariomycetes and is one of the well-known genera of entomopathogenic fungi, used as a biopesticide to control a wide range of plant-insectpests[1]. Agostino Bassi di Lodi (1835) discovered that *B. bassiana* was responsible for the white muscardine disease of silkworms (*Bombyxmori*). As cosmopolitan fungi found on infected insects in both temperate and tropical regions, *B. basisiana* habitats range from desert soils to forest and cultivated soils[2]. *B. bassiana*secretes many extracellular enzymes includingchitinase, proteases, lipases, amylase, laccase and others[3]. Chitin, a homopolymer chain of N-Acetylglucasamine (GlcNAc) linked by β-1,4glucosidic linkage, is cleaved by chitinase, an enzyme that has been extensively studied as a pest control agent[4]. In addition,*B. bassiana*also produces a plethora of biologically

active secondary metabolites such as polyketides, peptides and oxalic acid, all of which are useful in parasitizing and killing hosts [5].

Earlier studies reported the isolation of an endochitinase gene (*Bbchit1*) from *B.bassiana*[6].Increased aphid pathogenicity was observed when the *Bbchit1* gene was overexpressed in transgenic *B. bassiana*[7].Thetransformation of the *B. bassiana*chitinase gene into Chinese white poplar (*Populustomentosa*) resulted in resistance to infection by the fungal pathogen, *Cytosporachrysoperma*[8].The genome of *B. bassiana*strain ARSF2860 was sequenced for abetter understanding of pathogenesis and interactions between insects and plants [9]and high throughput RNA-seq transcriptomic analysis revealed that *B. bassiana* can survive in a variety of environmental niches by stimulating well-defined gene sets. One integrated crop pest management tool employs *B.bassiana* as a mycoinsecticide, which aids research into improving fermentation and formulation technologies [10].

In our present investigation, we have taken up to isolate and characterize an insect cuticle degrading chitinasegene, *Bbchit* from the entomopathogenic fungus *B.bassiana*. Using the various bioinformatics tools, the Physico-chemical properties, as well as the primary, secondary and tertiary structures of *B. bassiana*chitinase protein was determined. Phylogenetic analysis was done to study the evolutionary comparisons with other accessions of *B. bassiana*chitinase. This computational study will aid in understanding the biological function of the chitinase protein and the mechanism of chitin degradation.

2. MATERIAL AND METHODS

2.1 Isolation of genomic DNA from *Beauveriabassiana*

The fungal genomic DNA from *B. bassiana*was isolated by the CTAB method [11]. One gram of the *B. bassiana* mycelial mat was homogenized to a powder with a pre-chilled pestle and mortar using liquid nitrogen (-196°C). About 700µl of pre-warmed CTAB buffer containing β-mercaptoethanol was added to the powdered sample. Then the mixture was transferred to a 2ml microfuge tube and incubated at 65°C for 40 min in a water bath (mixed by inversion at 10min intervals). It was then centrifuged at 4°C for 10 min at 13000 rpm. The supernatant was transferred to a new microfuge tube and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) mixturewas added. After thorough mixing, the mixture was centrifuged at 4°C for 10 min at 13000 rpm and theaqueous layerwas transferred to a new microfuge tube. To the above mixture,an equal volume of ice-cold isopropanol was added and incubated at -20°C overnight. The DNA was pelleted by centrifuging at 12000 rpm for 10 min at 4°C. After

discarding the supernatant, the pellet was washed twice with ice-cold 70% ethanol followed by centrifuging at 12000 rpm for 5 min at 4°C. Nuclease-free water (50µl)was used to re-suspend air-dried DNA pellets.

2.2 Amplification of *B. bassiana*chitinase gene

The chitinase-specific primers Bbchit_F (5'-ATGGCTCCTTTTCTTCAAACCAGCC-3') and Bbchit_R (5'-TTACGCAGTCCCCAAAGTCCCCT-3') were designed using the *B. bassiana*nucleotide sequence (NCBI Accession No. XM 008601414.1). The PCR reactions contained 50 ng of *B.bassiana* genomic DNA, 1 µmol forward and reverse primers, 1X PCR buffer, 1 unit of Taq DNA polymerase (Takara), 0.25 mMdNTPs and nuclease-free water to make up the volume of 40 µl. The amplification was performed by following PCR profile; initial denaturation (5 min at 95 °C), followed by 30 cycles (denaturation (45 sec at 95 °C), annealing (45 sec at 55 °C), extension (45 sec at 72 °C)) and final extension (10 min at 72°C). The PCR productswere then resolved in 1.0% agarose gel and the amplified product of ~1.0 kbp was eluted, purified and used for cloning.

2.3 Cloning of *Bbchit* gene of *B. bassiana* into pJET1.2 vector

The purified PCR product was cloned into pJET1.2/bluntcloning vector. The ligation reaction contains 50ng of purified PCR product, 2X reaction buffer, 1 µl of pJET1.2 cloning vector (50 ng), 1 unit of T_4 DNA ligase and nuclease-free water to make up the volume of 20 µl. The cocktail is vortexed and centrifuged for 3-5 seconds and incubated at 22 °C for 20 min. The entire ligated product is used for transformation into *E.coli*- DH5α competent cells.

2.4 Sequence retrieval and phylogenetic analysis

For computational analysis, the amino acid sequences of eight chitinases for different accessions of *B. bassiana* with 100% query coverage were obtained in FASTA format from theNCBIdatabase[12]. MEGA11 software was used to generate the phylogenetic tree for the retrieved sequences and their evolutionary studies were determined using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method.

2.5 Physico-chemical characterization of chitinase

The Protoparam[13]tool was used to calculate the Physico-chemical parameters of deduced chitinase protein sequence. Isoelectric point (pI), the total number of amino acids (+ve and -ve charged residues), instability index (II), molecular weight, extinction coefficient (EC), aliphatic index (AI) and grand average hydropathy (GRAVY) were all calculated [14].

2.6 Functional and secondary structure analysis

The Prosite, Pfam, and SOSUI servers were used to perform functional annotation of the chitinase protein. The length of amino acid residues of proteins with specific profiles and patterns was recorded in Expasy-prosite[15,16], a database of protein families and domains. The conserved domain of *B. bassiana*chitinase was checked using Pfam[17].
Self-Optimized Prediction Method with Alignment(SOPMA) server[18, 19] was used to predict the secondary structure of chitinase with default parameters such as Similarity threshold 8 and Window width 17. It computes the content of α-helix, β-sheets, turns, random coils, and extended strands based on the amino acid sequence.

2.7 Molecular modelling of chitinase

The protein three-dimensional structure of *B. bassiana* full-length chitinase was not available in the PDB. As a result, the tertiary structure of the protein- chitinase was modeled using the Robetta web server [20]. The quality of the predicted 3D model was validated by the SAVES v6.0 server to study the overall stereo-chemical property of protein ie.to evaluate the energetically allowed regions.

3. RESULTS AND DISCUSSION

3.1 PCR amplification of the *Bbchit* gene of *Beauveriabassiana*

The *Bbchit*gene was amplified by using designed chitinase gene-specific primers. The amplified fragment of the *Bbchit*gene when analyzed by agarose gel electrophoresis (Fig.1), the expected size *Bbchit*gene was observed. The amplified DNA was eluted and cloned into the pJET1.2 vector (Thermo Scientific).

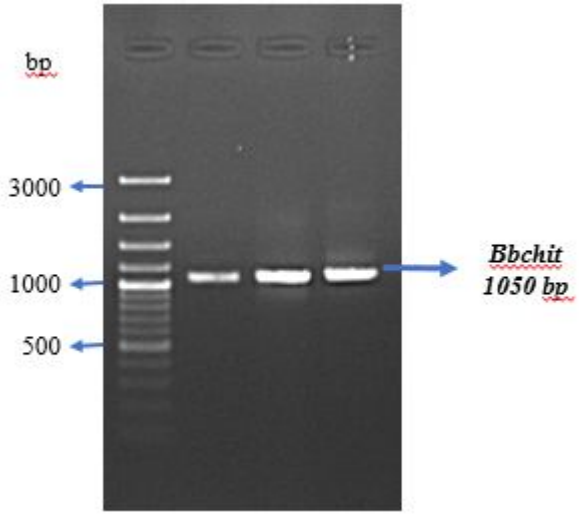

Fig. 1. PCR amplified *Bbchit*gene from *B. bassiana*

3.2 Sequence analysis of the *Bbchit* gene

To verify the nucleotide sequence of a cloned PCR product in the pJET1.2 vector. The plasmid DNA was isolated and then sequenced with both vector-specific and gene-specific primers.The sequence of the cloned *Bbchit*gene is 1050 bases (Annexure. 1).

The cloned exochitinase gene sequence was deposited in the NCBI database (accession number: OP11406). This gene sequence was used for further bioinformatics analysis.The translated protein sequence revealed an open reading frame encoding the exochitinase gene of 1047 bases. Performing an NCBI protein blast search with translated protein revealed that one of the *B. bassiana* gene deposited from Colombia had 100% sequence homology. However, at the nucleotide level, the isolated chitinase*Bbchit*gene showed 98.38 % sequence similarity with accession no. MN871410.1.The dissimilarity in nucleotide sequence helped to proceed to check the evolutionary relationship at the protein level.

3.3 Sequence retrieval and phylogenetic analysis

Using the Expasy tool – translate [21], the nucleotide sequence of *B. bassiana*chitinase (GI; OP114061) was translated to amino acid sequence. The translated sequence was tested for similarity using BLASTP. The full-length translated protein sequences of *B. Bassiana*were used for the phylogenetic analysis with other *B. bassiana*chitinase genes deposited in NCBI

database and they are QJE37897.1 (chitinase Chit37), ADP44708.1 (chitinase), ACF32998.1 (chitinase), XP 008599636.1 (chitinase-like protein), AEG21075.1 (chitinase), ACZ28129.1 (endochitinase), UQK86746.1 (chitinase) and AAN41259.1 (chitinase). The translated chitinase protein- *Bbchit* closely resembles the QJE37897.1 (chitinase Chit37) and was distantly related (out cluster) to the ACZ28129.1 (endochitinase).Based on the amino acid sequences of the chitinase protein from different *B. bassiana* strains, the clusters with the highest similaritywere observed in comparison analysis (Fig.2).Except the ACZ28129.1 (endochitinase) strain, translated chitinase and other chitinase strains belong to the exochitinase subgroup.

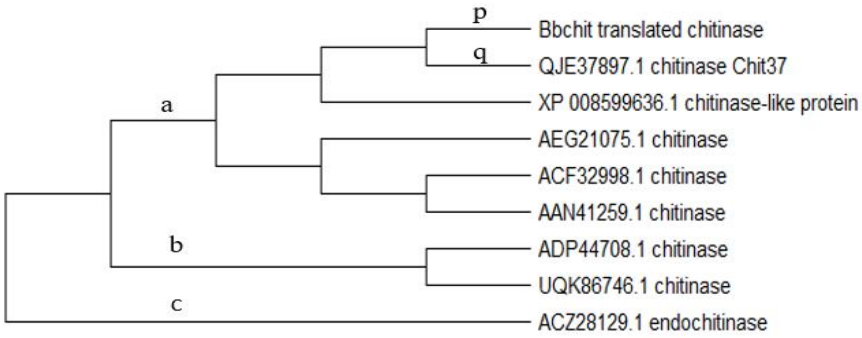

Fig. 2. Phylogenetic analysis of chitinase from different accessions of *B. bassiana:* In this fig, a and b are 2 clusters, c is the out-group, p and q are closely related.

3.4 Physico-chemical characterization of chitinase

Physico-chemical parameters analysis done with translated *B. bassiana*chitinase enzyme has a molecular weight of 36.78kDa. The total number of amino acids is 348 residues, of which 26 are negatively charged and 23 are positively charged. The chitinase enzyme has a calculated pI value of 5.94, which have acidic proteins and plays an important role in chitinase activity. The instability index value is 30.14, indicating that the protein is stable (index value is less than 40)[22]and has an *in vivo* half-life of more than 20 hours in yeast, >10 hours in *Escherichia coli* and 30 hours in mammalian reticulocytes.The aliphatic index is the relative volume occupied by the aliphatic side chains (alanine, valine, isoleucine and leucine) and it predicts protein stability over a wide temperature range. Chitinase protein has an aliphatic index of 84.17 and is predicted to be thermostable based on the report of Ikai, 1980[23]. The GRAVY value of -0.027 indicates that the chitinase protein is hydrophilic [14].

3.5 Functional and secondary structure analysis

The pattern and profile hit of the proteins were analyzed using ExpasyProsite. The chitinase protein has 18 active sites, including the N-myristoylation site (14 regions), Casein kinase II phosphorylation site (2 regions), Protein kinase C phosphorylation site (single) and N-glycosylation site (single). The domain identified by profile for the chitinase enzyme was the GH 18 catalytic domain (position; 41-348 residue), with glutamic acid as the active site at residue 163. The chitinase enzyme was classified as a glycosyl hydrolase using the Pfam tool (GH 18). The glycosyl hydrolase-18 family's chitinase is a hydrolytic enzyme that cleaves the α-1,4-bond, releasing oligomeric, dimeric (chitobiose), or monomeric (N-acetyl glucosamine, GlcNAc) products. The SOSUI online server indicates that the chitinase protein is water soluble.

The SOPMA tool is used to find the distribution of amino acids as helix, strand, turn, or coil. The results for the chitinase protein show that random coils dominate secondary structure elements, followed by anα-helix, extended strand and β-turns for all sequences (Table.1). High random coil studies lead to intrinsic confirmations that correlate with low energy confirmations and have implications for protein structure prediction and design.

Table 1. Secondary structure elements arecalculated by SOPMA.

Structures	α- helix	Extended strand	β- turn	Random coil
No. of residues	108	69	22	149
Distribution	31.03(%)	19.83(%)	6.32(%)	42.82(%)

3.6 3-D modeling and validation

The tertiary structure of proteins will aid in the study of the protein's function and active sites. Robetta online server was used to create a 3D model of the chitinase protein. Using the PyMOL molecular visualization tool, the predicted model for chitinase was visualized (Fig. 3). The SAVES server was used to evaluate the predicted model. The stereo-chemical quality of the model was classified using the PROCHECK tool. The Ramachandran plot (Fig. 4) depicts the statistical distribution of the possible combinations of backbone dihedral angles ϕ and ψ for all the amino acid [24]. The total number of residues distributed in the most

distributed region is approximately 91.5 %, indicating that the modeled structure is reliable and of high quality (Table. 2).

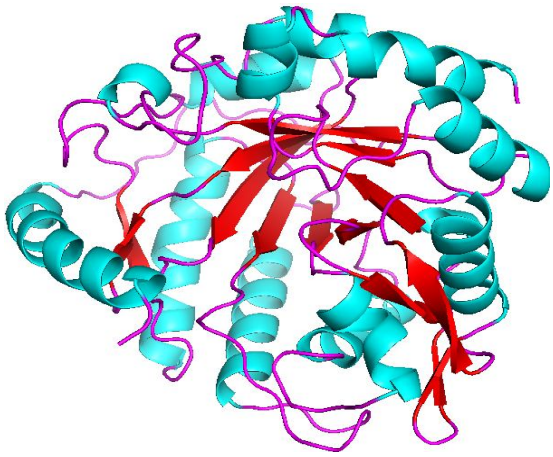

Fig. 3. 3D structure of chitinase protein visualized through PyMOL: The cyan color represents the α-helix, the red color represents the β-sheets and the magenta color represents random coils.

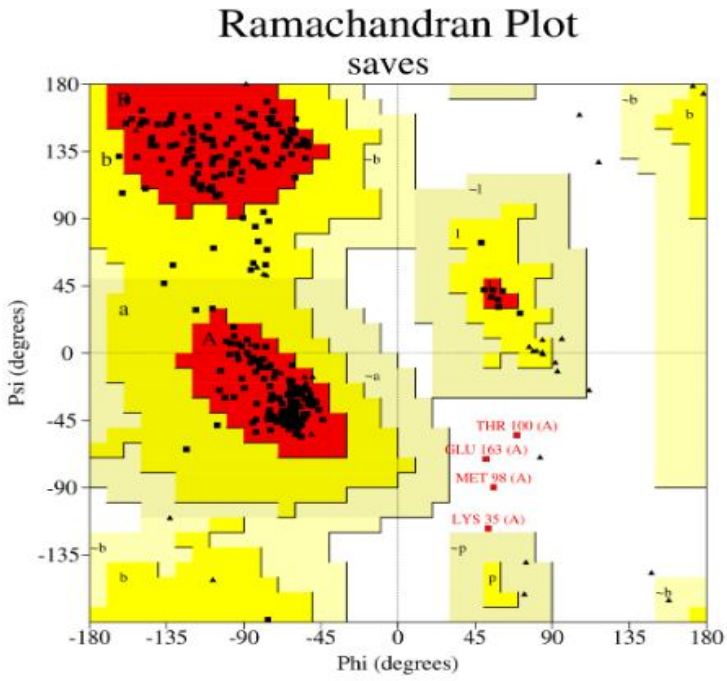

Fig. 4. Ramachandran plot showing the phi -psi torsion angles for all the residues of the chitinase protein: the distribution of amino acid residues to β-sheets (+psi, -phi), right-handed α-helix (-psi, -phi) and left-handed α-helix (+phi, +psi)

Table 2. Ramachandran plot calculation with Procheck program

Ramachandran plot statistics	Distribution
Residues in most favoured regions	91.5(%)
Residues in additionally allowed regions	7.1(%)
Residues in generously allowed regions	0.0(%)
Residues in disallowed regions	1.4(%)

4. CONCLUSION

Exochitinase gene of *B. bassiana* was amplified, cloned and sequenced. The translated chitinase enzyme shows that it was having 100% sequence homology with theQJE37897.1 (chitinase Chit37). The primary structure of chitinase protein reveals that it is acidic. According to the functional analysis, the protein is stable, hydrophilic, and soluble in water. The Ramachandran plot validated the 3D model of the chitinase protein created by Robetta, which has a 91.5 % distribution of amino acid residues in the most favoured region. This research on *B. bassiana*chitinase would be useful for *in vitro* molecular studies such as to know how the chitinase enzyme help to degrade the chitin polymer of many insects and fungal strains to act as a bio-control agent.

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Annexure.1. Sequence of the *Bbchit*gene (GI: OP11406)

TGGCTCCTTTTCTTCAAACCAGCCTCGCGCTCCTTCCATTGTTGGGCTTTCCAACCA
TGGTCAGCGCCTCGCCATTGGCGCCGCGAGCCGACACCTGCGCAACCAAGGGCC
GGCCGGCCGGCAAAGTGCTCCAGGGCTACTGGGAGAACTGGGACGGTGCCAAGA
ACGGCGTGCACCCTCCGTTTGGCTGGACGCCCATCCAAAACCCCGACATTCGCAA
GCACGGCTACAACGTCATCAATGCTGCCTTTCCCATCATCCAGCCCGACGGCACC
GCGCTCTGGGAGGACGGCATGGACACGGGCGTCAAGGTGGCGAGCCCGGCCGA
CATGTGCGAGGCCAAGGCAGCGGGCGCCACCATCTTGATGTCGATTGGCGGCGCT
ACTGCGGCCATTGACCTGAGCTCGTCGGCTGTGGCTGACAAGTTTGTCTCGACCAT
TGTGCCGATTCTGAAAAAGTACAACTTTGACGGCATTGATATCGACATTGAATCCGG
CCTCACAGGCAGCGGAAACATAAACACCCTGTCCACCTCGCAGACCAACCTGATTA
GAATCATTGACGGCGTTCTCGCGCAGATGCCCGCCAACTTTGGCTTGACCATGGC
GCCAGAGACTGCCTACGTTACCGGTGGGACGATTACGTACGGATCAATCTGGGGC
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CATGCAGTACTACAATGGCGAAATGTACGGCTGCTCCGGCGACTCGCACAAGGCC
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TCAGGGCGTGACAATCACGATTCCCTATGACAAGCAAGTGCCTGGCCTTCCTGCCC
AGCCTGGGGCTGGCGGCGGCCACATGTCCCCGTCCAACGTGGCGCAAGTTCTCTC
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