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Expression of a Variant Chitinase Transcript of *Helicoverpa armigera* in *E. coli*

Esther Shoba R[1], Athmaram T N[2], Manjulakumari D[3]

[1, 3]Department of Microbiology and Biotechnology, Bangalore University, Bengauluru-560056, INDIA

[2]Advanced Research Products, LLC, New Jersey, USA

Abstract: ***Gene encoding for chitinase was isolated and sequenced from the cotton bollworm Helicoverpa armigera, using the polymerase chain reaction technique directed by primers designed from the chitinase cDNA of the same insect reported by Ahmad et al. (2003). The nucleotide sequence analysis suggested that the gene is of 1737 bp showing homolgy of 96% with the reference chitinase sequence (AY325496) and 80% homology with that of Manduca sexta, the first cloned insect chitinase gene (U02270). The cDNA encoded a polypeptide of 578 amino acids, short of 10 amino acid residues compared to reference sequence. The major differences in the protein sequence to the one reported earlier was in proline threonine linker region where four threonine residues were replaced by alanine and one threonine by serine and further, condensation of protein towards the C-terminal region. Surprisingly the protein sequence of chitin binding domain shows 98% homolgy to Lymantria dispar chitinase (KP337328.1) and 96% homology to Phyllonorycter ringoniella chitinase (JN607321.1) and 100% homology to the reference protein(AAQ91786.1). Expression of the gene in bacterial system resulted in the formation of inclusion bodies retaining enzymatic activity. Bioassay for insecticidal activity against Helicoverpa armigera showed 60% mortality for injection assay, reduction in larval weight gain in case of oral application and 40% mortality for topical application.***

Keywords: Chitinase, Helicoverpa armigera, Lepidoptera, Chitin binding domain, DNA sequence

1. Introduction

Chitin a linear polymer of N-acetyl-β-D-glucosamine is the second largest insoluble macromolecule polymer available in nature. It is synthesized by chitin synthase and secreted into extracellular space to form a scaffold framework in the form of microfibrils. They serve as a structural support for cell walls of fungi, cuticle and intestinal peritrophic matrices of insects and shells of arthropods.

In insects, chitinases are involved in molting and digestion. Insects achieve their growth by periodically shedding their old cuticles and resynthesize new ones. Cuticle consitutes chitin that accounts for 40% of exuvial dry mass of the exoskeleton(Kramer *et al.*,1995). During molting, the chitin in the old cuticle is broken down by the chitnases that is secreted in the molting fluid. This molting fluid gets accumulated inbetween the old cuticle and epidermis during the apolysis stage of ecdysis. The product of hydrolysis are recycled for the synthesis of the new cuticle and this is performed by chitin synthases. Thus insects consistently synthesize and degrade chitin in a highly controlled manner wherein chitin synthases and chitnolytic enzymes work hand in hand in remodelling chitinous structures that allow ecdysis and therby the growth of the insect (Kramer and Koga 1986, Cohen 1987, Kramer *et al.*, 1997).

Insects are the major pest of agricultural crops posing a big threat for the food security of human population across the globe. Use of chemical insecticides, to protect crops, though brought some relief to the problem, left cosiderable toxic residues in the environment posing a problem for human health. As chemical insecticides cannot discriminate between beneficial and harmful insects, several of non-target organisms were also killed in addition to, insects developing resistance to insecticides. As a result the need remains for the scientists to continually seek for new, effective and environmental friendly ways of controlling insects (Powell *et al.*,1990). Targeting the physiological process that interfere with growth, development, molting and metamorphosis is a modern approach to control the insects. Efforts in this direction are quite promising, and many biomolecules may serve as an alternative to the use of chemicals (Cook,1993). Chitinase is one such promising biomolecule which is being explored by several researchers. Considerable developments in the study of chitin and its metabolism which includes details of their physical, chemical, kinetic and biocidal properties and their role in the involvement of defense against pestiferous organisms such as insects has stimulated the researchers to use chitinase to degrade the vital structures that are made up of chitin and there by control the pests.

During 1986-1991, many workers reported the use of insect pathogeinc fungi that produce chitinolytic enzyme and proteolytic enzyme which penetrate the cuticle and facilitate the infection to the insect. The fungi used for insect toxicity tests were mainly *Beauveria spp., Nomuraea rileyi, Aschersonia aleyrodis, Verticillium lecanii, Metarhizium anisopliae* (St Leger *et al.*,1986, El Sayad *et al.*,1989). Similarly nematode chitinase (Fuhrman *et al.,* 1992) that break down the protective sheath of the mosquito host, insect venoms that contain chitinase (Krishnan *et al.*,1994), and bacterial chitinases have also been used. While bacterial chitinases, mixed with *Bacillus thuringenesis* enhanced the activity of microbial insecticides (Smirnoff 1974, Lysenko 1976, Sneh *et al.,* 1983). Regev *et al,* (1996) reported enhanced toxicity towards *Spodoptera littoralis* after administration of low levels of truncated recombinant *Bt* toxin and a bacterial endochitinase at a concentration of 10µg/ml. While chitinases from *Streptomyces griseus* mixed with the blood meal of the mosquito *Anopheles freeborni* (Shahabuddin *et al.,* 1993) prevented the formation of peritrophic membrane.

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Insect chitinases expressed in plants and Baculovirus have also been tested for insect control. In both the systems *Manduca sexta* chitinase has been expressed. A growth retardation was observed in tobacco bud worm *Heliothis virescens* larvae fed on *Manduca sexta* chitinase expressed tobacco plant leaves (Ding *et al.,* 1997). In *Spodoptera frugiperda* non occluded-AcMNPV (*Autographa californica* multiple nuclear polyhedrosis virus) carrying *Manduca sexta* chitinase were showed to accelerate the rate of killing (Gopalakrishnan *et al.*,1995) as compared to wild type virus. Fitches and group (2004) used yeast expressed *Laconobia olareacea* insect chitinase against the same insect to demonstrate 100% larval mortality. Use of insects own product might prove to be more efficient, least resistance and environment friendly. Chitinases can be used either to arrest the growth or to kill the larvae by introducing them at inappropriate time and in inappropriate levels to target the plant pest, ie., insect.

Helicoverpa armigera, a polyphagous pest which feeds on more than 182 plants (Sharma *et al.,* 2005) damage the crops extensively and is a serious economic threatening pest. The loss caused by this pest has resulted in the social disturbances by the suicide of farmers (Patil *et al.,* 1996, Kranthi 2001a). As the first step in order to curb this pest we have cloned and expressed the *Helicoverpa armigera* chitinase and an attempt is made to evaluate the insecticidal efficacy of purified chitinase.

2. Materials and Methods

2.1. Materials and General Cloning Methods

Oligonucleotide primers were designed using Bioedit tool and Integrated DNA Technologies (Oligo DNA analyser) and synthesized by Eurofins Genomics India Private Limited. Cloning and subcloning was carried out using pET expression and host systems purchased from Novagen. Restriction endonucleases and T_4 DNA ligase were from Fermentas. All DNA sequencing was carried out using dideoxygenated chain termination protocols on Applied Biosystems automated DNA sequencers by Eurofins Genomics India Private Limited. Sequences were checked and assembled using Blastn software on NCBI site (www.ncbi.nlm.nih).

2.2. Insect Culture

Helicoverpa armigera (NBAII – MP-NOC-001) was reared continuously on artificial diet (Shobha *et al.,* 2009a) at 28ºC and 25ºC under scotophase and photophase respectively. Larvae were dissected under physiological saline to collect integument, midgut and fat bodies. All tissues were snap frozen in liquid nitrogen and stored at -80ºC.

2.3. Isolation and Sequencing of chitinase cDNA

Total RNA was isolated from the integument of day-3 sixth instar larvae following Guanidine method (Kingston *et al.,* 2001) with few modifications. Tissue of 100 mg added to1ml of denaturation solution (4M GITC- guanidine isothiocyanate, 25mM sodium citrate, 0.5% N-lauryl sarcosine and 0.1M β mercaptoethanol) was frozen in liquid nitrogen and homogenized. To the homogenate, 0.1ml of 2M sodium acetate (pH 4.0), 1ml of water saturated phenol, 0.2ml of chloroform : isoamyl alcohol (49:1) was added and mixed gently 25 times by inversion. The vials were then incubated on ice for 10 minutes and centrifuged at 12,000 rpm at 4ºC for 20 minutes. Resulting supernatant was mixed with 1µl glycogen (20mg/ml) and precipitated with equal volume of isopropanol. Glycogen was used as a carrier that helps in nucleic acid precipitation. RNA was pelleted on centrifugation and washed with 70% ethanol. The pellet was air dried and dissolved in 20µl of DEPC treated water and quantified. Three micrograms of total RNA was used to synthesize first strand cDNA with M-MuLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase) and oligo dT primer according to the manufactures instructions (RevertAid™ M-MuLV Reverse Transcriptase Kit, Fermentas Life Sciences). Gene specific primers (F_1: 5´acc atg gga atg aga gtg ata cta gcg acg ttg 3´ and R_1:5´actc gag agg cgt cct gtt cat gag ccg gca 3´) were synthesized for the cDNA fragment encoding the putative chitinase ORF (AY325496, Ahmad *et al.,* 2003) along with restriction sites (*Nco*I and *Xho*I) inclusive of start codon but devoid of stop site to enable cloning, expression and purification. Standard PCR was followed with the gene specific primers in Master cycler® Thermocycler (Eppendorf, Germany) using the following program: initial denaturation of 94°C for 2 minutes followed by denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. PCR was performed for 40 cycles. Final extension was carried out at 72°C for 15 min. The obtained PCR product was cloned into a T7 expression vector pET28a, transformed into an expression host BL21 DE3 and the positive clones were selected. Two clones were sequenced using the universal primers (T7 promoter and T7 terminator primer) along with internal primers. Sequence of the clone (pET28a+HAchi) was analysed using Blastn software on NCBI site (www.ncbi.nlm.nih).

From the sequencing results and protein translation tool, it was inferred that the gene was terminated beforehand as compared to the reference protein sequence (AAQ91786.1), thereby plasmid PCR was performed with *E.coli* forward primer (F1) and with a newly designed *E.coli* reverse primer (R_2: 5´ gac tcg ag acg gtc ggc gtt gt 3´) for terminated region. The resulting PCR product was once again cloned into pET28a and transformed into BL21 DE3 and screening was done.

2.4. Heterologous expression of *H.armigera* chitinase

On sequence confirmation, the recombinant clone was induced for protein expression. Induction was performed with IPTG-isopropyl thio galactosidase at a final concentration of 1mM and the culture was allowed to grow for 4 hours at 37ºC on a shaker at 200rpm. The cells were collected by centrifugation, resuspended and sonicated to disrupt the cell membrane. The soluble and insoluble fractions were separated by centrifugation at 12,000 rpm and the induced proteins were resolved on 10% SDS-PAGE.

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2.5. Sub-cloning into pET32a

As the protein of required size was not obtained, the insert was released from pET28a+HA chi and then subcloned into pET32a vector using the same restriction site. Ligated product was transformed into BL21 DE3 and the positive clones were screened and selected. Positive clones (pET32a+HA chi) were induced for protein expression. To check for protein expression, different expression hosts namely C43 DE3 and Rosetta^{TM} DE3 were used and expression analysis was done as mentioned before.

2.6. Purification and quantification of the protein

The recombinant protein produced with 6X Histidine-tag towards the carboxy terminal was purified using IMAC-immobilized metal affinity chromatography technique. The protein was purified using Ni-NTA resin (Nickel-nitrilotriacetic acid, Novagen) as per the manufacturer's instructions. The purified chitinase was quantified by Bradford method and assayed for enzyme activity.

2.7. Western Blotting

Protein purified was used to raise polyclonal antibodies in rabbit (Bhat-Biotech India Private Limited). Western Blotting was performed using this antibodies (1: 8000) against the purified protein that act as an antigen. Secondary antibody used was pro- Rec A. Reactivity of the recombinant protein was done with anti-chitinase antibodies using blocking reagent and Tris buffered saline (TBS containing 0.05% (v/v) Tween-20) and the substrate diamino benzidine (DAB).

2.8. Determination of Enzyme Activity

The chitinase enzyme activity was determined by Miller's (1959) method using colloidal chitin as substrate prepared from commercial chitin according to Roberts and Selitrennikoff (1988). The reaction mixture contained 0.5 ml of 0.2% colloidal chitin in 100mM sodium phosphate buffer (pH 7.0) and 0.5 ml enzyme solution. The reaction mixture was incubated for 1 hour at 37°C. The reducing sugars liberated were determined using DNSA-dinitrosalicylic acid reagent. Standard curve was drawn by measuring the absorbance of solutions containing varied N-acetylglucosamine levels using dinitrosalicylic acid-DNSA reagent.

2.9. Determination of optimum pH and temperature for enzyme activity

Optimum pH for the chitinase activity was determined by using 100 mM sodium acetate buffer (pH 3.0-5.0), 100mM sodium phosphate buffer (pH 6.0-8.0) and 100 mM Tris-HCl buffer (pH 8.0 - 9.0). Chitinase activity was studied using 1.5 ml of reaction mixture containing 0.5 ml of buffer, 0.5 ml of enzyme and 0.5 ml of 0.2% colloidal chitin as a substrate at 37°C for 1hour. The concentration of all the buffers used is 0.1M.

The optimum temperature was determined at different temperatures viz., 0, 10, 20, 30, 40, 50, and 60°C. Chitinase activity was studied using 1.5 ml of reaction mixture containing 0.5 ml of sodium phosphate buffer pH 7.0, 0.5 ml of enzyme and 0.5 ml of 0.2% colloidal chitin as a substrate. The reaction was incubated at the above temperatures for 1 hour and enzyme activity was assayed as described earlier.

2.10. Bioassay of chitinase for toxicity

Recombinant chitinase was assayed for its toxicity on *Helicoverpa armigera* larvae by oral (leaf dip method), topical applications and injection methods. Third instar larvae (80-110mg) for injection method and oral application and fifth instar larvae (310-370mg) for topical application were respectively used for toxicity tests.

2.10.1. Injection method

Different concentrations varying from 0.5µg/10µl to 3.0µg/10µl were injected in to the hemolymph of the larvae. For each concentration 20 larvae in triplicates were used and effects upon survival were monitored over the next 48 h. Sodium phosphate buffer (100mM, pH 7.0) was used as control.

2.10.2. Oral application

Discs of (5cm in diameter) cotton leaves sterilised in 70% alcohol were dipped in enzyme preparation of different concentrations (10µg, 30µg, 50µg, 70µg and 90µg/10µl) for 1 min. The discs were air dried and fed to the larvae that was starved for 24 hours before feeding. Each experimental group consisted of 20 larvae in triplicates and feeding with treated leaves continued for 3 days after which normal feeding were resumed. Leaf discs similarly treated in sodium phosphate buffer (100mM, pH 7.0) was used as control. The weight of larvae, number of larvae pupated and died were recorded every day for 14 days.

2.10.3. Topical application

Enzyme preparation of different concentrations (10µg, 30µg, 50µg, 70µg and 90µg/10µl) was applied topically on the dorsal side of the thorax of the fifth instar larvae using a brush. Each experimental group consisted of 20 larvae in triplicates. Topical application was continued for 3 days after which the larvae were left undisturbed. 100mM sodium phosphate buffer (pH 7.0) served as control. The weight of larvae, number of larvae pupated, adult emergence and death were recorded every day for 14 days.

3. Results

3.1 Isolation of a cDNA encoding a chitinase gene from *Helicoverpa armigera* larvae

Gene specific primers corresponding to the *Helicoverpa armigera* chitinase (Ahmad *et al.*, 2003) were used to amplify a region of ~1800bp as PCR product (Fig.1). The amplified product showed homology of 96% with the reference sequence - AY325496 in Blastn analysis. It also showed similarity of 96% to two more lepidopteran insect chitinases. The nucleotide sequence when translated into protein sequence using ExPASy Translate tool, a stop codon was observed towards the carboxy terminal at 1737bp (578 amino acids) rather ending at 1767bp (588 amino acids) as reported by Ahmad *et al*. (2003). Sequences were verified

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from different clones and confirmed. The *H.armigera* chitinase cDNA sequence has been submitted in GenBank under Accession No. KT894380. High level of homology was observed for the conserved motif among the insect chitinases, especially the lepidopteran, with reference to central core of the catalytic region and glycosylation sites and chitin binding region.

3.2. Expression and purification of recombinant chitinase

Construct encoding the mature *H. armigera* chitinase was cloned into the expression vector pET28a and then subcloned into pET32a. The predicted protein product was arranged in frame with the 6X His Tag of expression vector. Transformed clones in BL21 DE3, C43 DE3 and Rosetta^{TM} DE3 were induced for protein expression. Of three hosts used, BL21 DE3 showed truncated protein, C43 DE3 showed no expression whereas Rosetta^{TM} DE3 expressed full length protein along with Trx.Tag. Inducible expression of the recombinant protein gave a yield of 3-5 mg/l. Recombinant protein was purified by immobilised affinity chromatography technique on Ni-NTA resin. The proteins in insoluble form were eluted using 500mM imidazole in the elution buffer. Purified recombinant chitinase gave a single major band after analysis by SDS-PAGE, at the predicted molecular weight of more than 76 kDa that includes ~64 kDa of chitinase and ~12 kDa of thioredoxin tag (Fig.2). The *E.coli* expressed chitinase was catalytically active and hydrolysed the colloidal chitin substrate.

3.3. Western Blotting

Western blotting results showed that the purified protein showed immune reactivity with anti chitinase antibodies (Fig. 3).

3.4. Determination of Enzyme Activity

The enzyme activity of purified recombinant chitinase measured colorimetrically using colloidal chitin as substrate was found to be 2.28µmol/min/ml. The maximum activity was at pH 7.0 and decreased sharply after that (Fig. 4). Enzyme activity increased with temperature from 0 to 40ºC and decreased at 50ºC (Fig. 5).

3.5. Bioassay for Chitinase Toxicity

3.5.1. Injection Method

Chitinase toxicity was observed at concentration as low as 0.5µg recording about 25% mortality after 48 hours. The larval mortality increased with the increase in concentration of chitinase and recorded 60% mortality over the same period for the concentrations 2.5 and 3.0µg/10µl (31, 37.5µg/g body weight respectively) (Fig. 6).

3.5.2. Oral Application

No mortality was recorded in oral application mode, however, a retarded growth rate was observed at all concentrations as compared to the control. The gain in larval body weight though decreased with increase in concentration of chitinase, a significant ($p<0.05$) decrease was observed at 90µg/10µl concentration (Fig.7). All the larvae entered in to pupation stage and attained adulthood.

3.5.3. Topical Application

Different concentrations of chitinase were applied on the dorsal side of the fifth instar larvae. In the test insects no significant decrease in larval weight gain, compared to control, was observed. However, percentage of pupation decreased in proportion to the concentration of chitinase. The percentage of successful pupation measured on 14th day recorded indicated 100% (30% pupae + 70% adult) in the lowest concentration (10µg) that was similar to control (20% pupae + 80% adult), and the lowest i.e., 60% (20% pupa + 40% adult) in highest concentration (90µg) of chitinase administered, whereas remaining 40% of the larvae of 90µg chitinase treated showed incomplete pupation (malformed) (Fig.8).

4. Discussion

Chitinases are hydrolytic enzymes that catalyse the degradation of chitin, a major component of insect exoskeleton. These enzymes which facilitates the moulting process in an insect can inhibit moulting when introduced at inappropriate time and at inappropriate levels; detrimental to insect but advantageous to crops when target insect is a pest on that crop. Making these chitinases available to insects in the fields can prevent the crop damage. Two approaches have been considered by researchers to deliver the chitinases to insects in the fields. First approach under consideration is to express these chitinases in systems such as yeast and bacteria for abundant harvest of the enzyme which can be delivered through an appropriate base (solvent) system. Second approach is to express in baculovirus that can be directly used as a carrier system.

Chitinase genes have been isolated, cloned and characterised from various insects, belonging to the orders, especially, lepidoptera: *Manduca sexta* (Kramer *et al*., 1993), *Bombyx mori* (Kim *et al*., 1998; Mikitani *et al*., 2000; Abdel-Banat and Koga, 2001; Daimon *et al*., 2003), *Hyphantria cunea* (Kim *et al*., 1998), *Spodoptera litura* (Shinoda *et al*., 2001), *Choristoneura fumiferana* (Zheng *et al*., 2002), *Helicoverpa armigera* (Ahmad *et al*., 2003) *Lacanobia oleracea* (Fitches *et al*., 2004), *Ostrinia furnacalis* (Wu *et al*., 2013); diptera: *Aedes aegypti* (de la Vega *et al*., 1998), *Anopheles gambiae* (Shen and Jacobs-Lorena, 1997), *Glossina moristans*, *Drosophila melanogaster* (de la Vega *et al*., 1998), *Chelonus* sp. (Krishnan *et al*., 1994), *Lutzomyia longipalpis* (Ramalho-Ortigao and Traub-Cseko, 2003) and *Chironomus tentans* (Feix *et al*., 2000); and coleoptera: *Phaedon cochleriae* (Shinoda *et al*., 2001) and *Tenebrio molitor* (Royer *et al*., 2002). In all these insects except *Manduca sexta* and *Lacanobia olareceae* chitinase has been cloned in bacterial system, while plant and baculovirus system is used for *Manduca sexta* and yeast in case of *Lacanobia oleracea* chitinase expression.

For expression of chitinase of *Haemaphysalis longicornis*, a tick, baculovirus expression system has been used. Use of baculovirus expressed chitinase as a bioacaricide against the tick was found to be effective in nymphal stages. Though effective, the baculovirus were found to be sensitive to

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temperature above 30ºC and infrared red radiations and thus may not be viable in the field applications (Assenga *et al.,* 2006). Gopalakrishnan *et al.,* (1995) constructed a recombinant AcMNPV baculovirus expressing a group I chitinase from *M. sexta* under the control of polyhedrin promoter and injected into the fourth instar larvae of *Spodoptera frugiperda.* Killing of the larvae was accelerated significantly earlier than with the wild type virus. Though efficient, expression of chitinase in baculovirus is costly and their sensitivity to UV light is a limitation. Considering this, expression in yeast or bacterial system is more feasible option for chitinase expression.

Helicoverpa armigera being a major pest of several crops has drawn attention of several researchers who have been investigating various ways of controlling this pest. Use of chitinase was also one of the options to control this pest. Binod *et al.* (2007) used fungal chitinase sourced from *Trichoderma harzianum* to demonstrate the toxicity against *Helicoverpa armigera.* Use of fungal culture filtrate of *Trichoderma harzianum,* shown to be human pathogen, off the late and sometimes fatal in cases of immunocompromised individuals, is a concern of safety (Guarro *et al.,* 1999). Use of insect's own chitinase may have edge over use of fungal chitinase as for as safety is concerned. Though, Ahmad *et al.* (2003) expressed the chitinase of *Helicoverpa armigera* in bacterial system their option was to characterize the protein but not to investigate the insecticidal activity of the recombinant chitinase. Hence, the present work was aimed at producing insect recombinant chitinase using a bacterial system and to study its toxicity against *Helicoverpa armigera.*

Gene encoding for chitinase was isolated from the integument of day-3 sixth instar larvae of *H. armigera.* The gene of ~1.8kb was cloned into pET vector (pET28a) and transformed into BL21 DE3 expression host. The positive clone was sequenced and taken for Scan prosite analysis which revealed a two-domain structure, consisting of a glycohydrolase family 18 chitinase domain, cysteine-rich region (peritrophin A chitin-binding domain) and an active site typical of insect chitinases (Kramer and Muthukrishnan, 1997). The gene sequence reported has three glycosylation sites located at 86-89, 304-307 and 408-410 similar to the reference chitinase gene, (Ahmad *et al.,* 2003).

The sequence homology was searched using the sequences deposited in NCBI and analysed using Blastn and Blastp software. Sequence similarity found this protein to have greatest similarity to Lepidopteran chitinases; strongest similarity (96%) was observed to chitinases from *Helicoverpa armigera, Phyllonorycter ringoniella, Lymantria dispar* as the best hits based on the multiple sequence alignment and conserved protein domains. Whereas a similarity of about 90%, 89%, 88%, 88% and 87% was observed respectively for chitinases of other lepidopteran insects such as *Lacanobia olaraceae, Spodoptera litura, Spodoptera exugia, Spodoptera frugiperda* and *Spodoptera littoralis* respectively. Comparison of the chitinase sequence against the GH18 domains showed similarity to all the four signature sequences which are highly conserved in insect chitinases: motifs I- IV that reside in the β-strand 3, 4, 6, and 8 respectively, and have the consensus sequences, motif I-KXX(V/L/I)A(V/L)GGW, where X is a non-conserved amino acid, motif II-FDG(L/F)DLDWE(Y/F)P, motif III - M(S/T)YDL(R/H)G, and motif IV-GAM(T/V)WA(I/L)D (Arakane and Muthukrishnan, 2010). Importantly, similarity was also observed in the 3 acidic amino acids present in motif II D143, D145, and E147 which is critical for the catalytic activity of the enzyme (Choi *et al.,* 1997, de la Vega *et al.,* 1998). Similar sequence of alternating acidic aminoacids in the position D145, D147 and E149 has been reported in the structural model of group I chitinase of *Anopheles gambiae* (Lu *et al.,* 2002). As presence of glutamate E147 is responsible for catalytic activity, presence of W146 is necessary for optimal enzyme activity within motif II (Huang *et al.,* 2000, Lu *et al.,* 2002, Royer *et al.,* 2002) and both are conserved in this protein indicating the protein being catalytically active.

Though the enzyme was showed to be catalytically active by structural analysis and later confirmed by enzyme assay, the protein size was 10 amino acids short of the reference chitinase.

The PEST-proline threonine linker region starting from 393-516 contains similar number of proline, residues, but 23 residues of threonine instead of 28 compared to the reference chitinase. Wherein, 4 residues of threonine are replaced by alanine and one by serine resulting in 5 amino acid changes in this region. Together a change of 10 amino acids was observed in the chitinase protein of the present study. Further, the linker region did not have stretches of five residues of PTTAK in-tandem four times though the length of linker region was not compromised compared to the reference chitinase. The arrangement of the amino acid sequence in the PEST region as well as length of the region is not very critical as the main function of linker region is to connect catalytic domain to the chitin binding domain. The stretch with tandem repeats of PTTAK is not found in other lepidopteran chitinases, however, Ahmad *et al.* (2003) has claimed that this region is unique to *Helicoverpa armigera*. In case of *Bombyx mori,* however, a homology is observed in this region in all the transcripts, though, the amino acids in tandem are different (Kim *et al.,* 1998, Abdel-Banet and Koga, 2001, Suetsugu *et al.,* 2013).

The protein size of chitinase reported in the present work is 578 amino acids which is 10 short of the reference protein (AAQ91786.1) towards the C-terminus. The chitin binding domain (CBD) of insect chitinases belongs to carbohydrate-binding module family 14 (CBM14), which consists of approximately 70 residues (Coutinho and Henrissat, 1999) towards the C-terminus that is well characterised. Detailed study of the domain of tachycitin an antimicrobial polypeptide in an invertebrate *Tachypleus tridentatus* (horse shoe crab) reveals the presence of 10 cysteine residues (5 disulfide bridges) that stabilizes the protein. However in insect chitinases only 6 conserved cysteines are found in the CBM14 domain forming three disulphide bridges. The present protein, though shortened by 10 amino acids still showed the presence of six cysteine residues towards the C-terminal. Some of the reports Jasrapuria *et al.* (2010), Suetake *et al.* (2000), Aseniso *et al.* (1998) suggest that the

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characteristic spacing between these cysteines is $CX_{13-20}CX_5CX_{9-19}CX_{10-14}CX_{4-14}C$. In the present protein the characteristic spacing between cysteine amino acids is highly conserved except between the first and second cysteine amino acid where 12 residues were observed, however, it is similar to the reference protein reported by Ahmad *et al.,* (2003) but he reported 15 residues between the fifth and sixth cysteine residue.

Even though the chitinase protein reported was of 578 amino acids it still possessed functional chitin binding domain towards the C-terminal which is evident by the fact that the CBM 14 region lies between 523-572 amino acid positions which is same for both the reference sequence chitinase gene reported. With respect to, alignment of chitin binding region similarity of 100%, 98% and 96% respectively, has been observed in other lepidopteran species: *Lymantria dispar* and *Phyllonorycter ringoniella.*

Abdel Banat *et al.* (1999), put forth an interesting point that even if the chitin binding domain is lost from the C-terminal side due to proteolytic activity it may result in chitinase variants that are still active. The proposed function of the chitin binding domain is to anchor chitin but though this domain was deleted in the experiments performed by Zhu *et al.* (2001) on *M. sexta* chitinase, the enzyme still showed activity on polymeric substrate like glycol chitin. Ding *et al.* (1998) and Wang *et al.* (1996) in *Manduca sexta* and Venegas *et al.,* (1996) in *Brugia malayi*, a nematode have demonstrated that the truncated chitinase especially in C-terminal are enzymatically active.

Work by Suetsugu *et al.* (2013) on *B.mori* revealed the presence of different transcripts for chitinase as chitinase transcript 1, 2 and 4 encoding for proteins of 566, 565 and 543 amino acids respectively. Variation in sequence and similarity in function is evident enough to say the present protein which is shorter is not actually a shorter version of the reference chitinase but is a variant. Whether the variant is tissue specific or stage specific is not clear, however, the reference chitinase reported by Ahmad *et al.* (2003) was isolated from pupal integument and the present chitinase, was from late sixth instar larvae of *H. armigera.*

The chitinase was expressed in different strains of *E.coli* expression host. Of the 3 hosts used, Rosetta™ DE3 expressed the recombinant chitinase with a migrated molecular weight higher than its predicted molecular weight. The observed shift has been attributed to the presence of Trx.tag of 109 amino acids in the pET32a vector. Chitinase expressed in *E.coli* was in the form of inclusion bodies (IBs) but still retained catalytic activity in the presence of colloidal chitin as the substrate. This indicates that the protein aggregation, as bacterial inclusion bodies, does not necessarily imply loss of biological activity. Natalia S´anchez de Groot, Salvador Ventura (2006) demonstrated that their protein expressed as inclusion bodies was active. The results from this report indicate that the accumulation of active protein in IBs is not anecdotic but that it could be a general feature in recombinant protein production. It is not necessarily an unspecific and passive process but rather a kinetically controlled event which depends specifically on the polypeptide nature and probably mainly on the sequence of certain aggregation-prone regions.

E.coli expressed recombinant chitinase showed activity with colloidal chitin as the substrate at an optimum pH of 7 and at optimum temperature of 40º C. Normally chitinases are active at pH range of 7-10 an alkaline environment (Zheng *et al.,* 2002).

Insects, nematodes, fungi, viruses and other organisms use chitinolytic enzymes for moulting or penetration of structural barriers. These enzymes are potentially employable in pest management. The pathogen or pest can be exposed to chitinases at their developmental stages to increase their vulnerability to host defense. Few reports are available on use of insect derieved chitinases as biopesticides for the control of pests. Baculovirus expressed chitinases of *Manduca sexta* against fourth instar larvae of *Spodoptera frugiperda* (Gopalkrishnan *et al.,* 1995) and *Haemaphysalis longicornis* against ticks showed insecticidal activity through topical applications (Assenga *et al.,* 2006). Further, You and Fujisaki (2009) injected baculovirus expressed *Haemaphysalis longicornis* chitinase to mice and found that the mice showed a specific protective anti-tick immune response affecting tick molting. Purified chitinase (75kDa) from *Bombyx mori* on oral ingestion (11µg/50µl) caused high mortality as well as significant decrease in feed consumption and slight reduction of body weight in Japanese pine sawyer (JPS), a beetle *Monochamus alternatus* (Kabir *et al.,* 2006). These studies have shown that chitinase is capable of negatively affecting growth and moults of the larvae and thereby, can act as insecticides.

E. coli expressed *H. armigera* chitinase tested on *H. armigera* larvae did show insecticidal activity in spite of being expressed as an inclusion protein. Injection bioassays with different concentration of chitinase resulted in 60% of larval mortality as compared to control whereas in case of topical and oral application no mortality was observed; however, in topical application though, all the larvae entered in to pupation 40% of them were malformed and in oral application, a retarded growth rate was observed as compared to the control.

There are no reports available on use of insect chitinase against *Helicoverpa armigera* for insecticidal activity but against some other insects has been reported by Fitches *et al.* (2004), Ding *et al.* (1998), and Gopalkrishnan *et al.* (1995). The earlier two have used eukaryotic recombinant chitinase against *Laconobia oleracea* and *Manduca sexta* respectively; while the latter one used baculovirus as a carrier system for insect chitinase of *Manduca sexta* against the same insect. Through injection bioassays Fitches *et al.* (2004), recorded 100% mortality in tomato moth *Laconobia oleracea* suggesting that the eukaryotic expressed chitinases are fully functional compared to the prokaryotic recombinant chitinases. However, studies by Natalia S´anchez de Grootand Salvador Ventura (2006) have shown that the prokaryotic expressed chitinases, though as inclusion bodies, too have retained activity which is evident from the present study which recorded 60% mortality.

Paper ID: NOV162023 DOI: 10.21275/v5i3.NOV162023

Both Fitches *et al.* (2004) and Binod *et al.* (2007) have reported no mortality by oral delivery of chitinase, but have observed a decrease in weight gain in the test insect larvae similar to the present study. Insect gut enzymes are known to degrade the toxic substances entering their body through diet, a defensive mechanism evolved over a period of time. This possibly explains the fact that no mortality and only decrease in the larval weight gain was observed in the above studies as some amounts of chitinase might be degraded by gut proteases. On the other hand the destruction of peritrophic membrane of the gut by the chitinase might have decreased the feeding efficiency of the larvae resulting in slow growth.

Binod *et al.* (2007) recorded 75% mortality on topical application of chitinase on *H. armigera* larvae whereas in the present study about 40% of them were malformed. Malformed pupae with topical application are indicating chitinase toxicity. This indicates the effective dose for insecticidal activity should be much higher, as what is evident from earlier work where they have used 2000U/ml.

Chitinase are found to be more effective, even in small quantities, when delivered to haemolymph than when it is topically applied. Possible explanation is that, chitin synthase which is active underneath the exoskeleton is probably counteracted by chitinase, inhibiting the formation of new cuticle resulting in the death of larva while with topical application chitinase may be successful in damaging cuticle by degrading the chitin but chitin synthase underneath keeps repairing by synthesis of new cuticle.

Irrespective of the intensity of activity, in all 3 modes of application, chitinase was found to inflict damages to *Helicoverpa armigera* larvae. Damages in terms of decrease in weight gain, larval mortality and decrease in pupation were evident in the present study and were dose dependent. The difference noted between the control and chitinase treated insects attracts further investigation to use them as a pesticide. The less efficiency of protein expressed as inclusion bodies can overcome by using a eukaryotic expression system. Additionally, many factors are to be considered for investigation such as, effectiveness in the presence of temperature, radiation, dose, and different larval stages and more importantly its ability to act in field conditions, mode of application. Mediating chitinase as part of formulation along with other biocontrol products rather chitinase alone can also be evaluated.

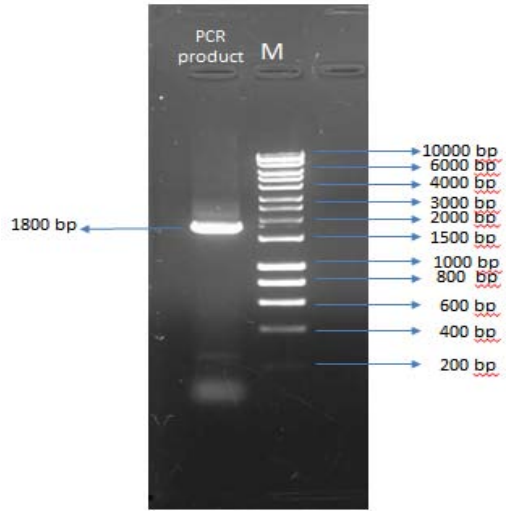

Figure 1: PCR amplification of *H. armigera* chitinase. Lane M- DNA molecular weight marker (Hyperladder ^{TM} 1)

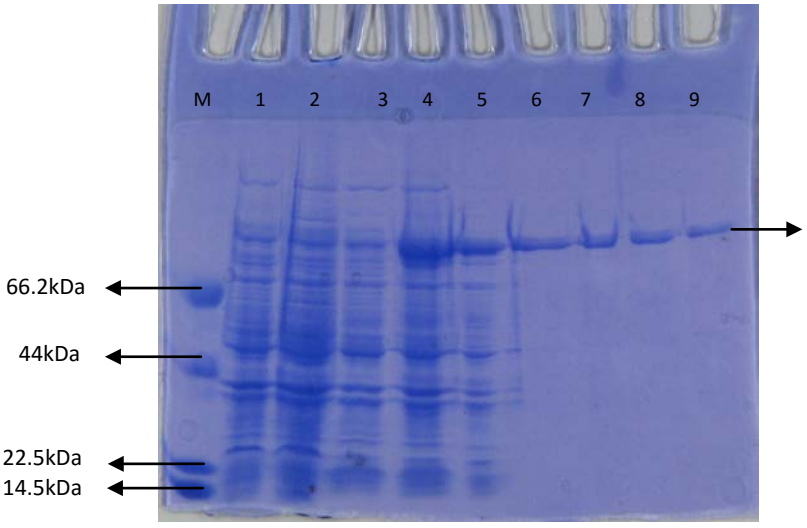

Figure 2: SDS-PAGE analysis of expressed proteins. Lane M, Medium Range Marker; lane 1, Induced tranformed vector control (pET32a); lane 2, Induced Rosetta host without vector; lane 3, Uninduced pET32a + HA chi; lane 4, Induced pET32a+ HAchi, lane 5, insoluble fraction; lane 6,7,8,9 purified protein. Expressed chitinase is denoted by arrow on right hand.

Paper ID: NOV162023 DOI: 10.21275/v5i3.NOV162023

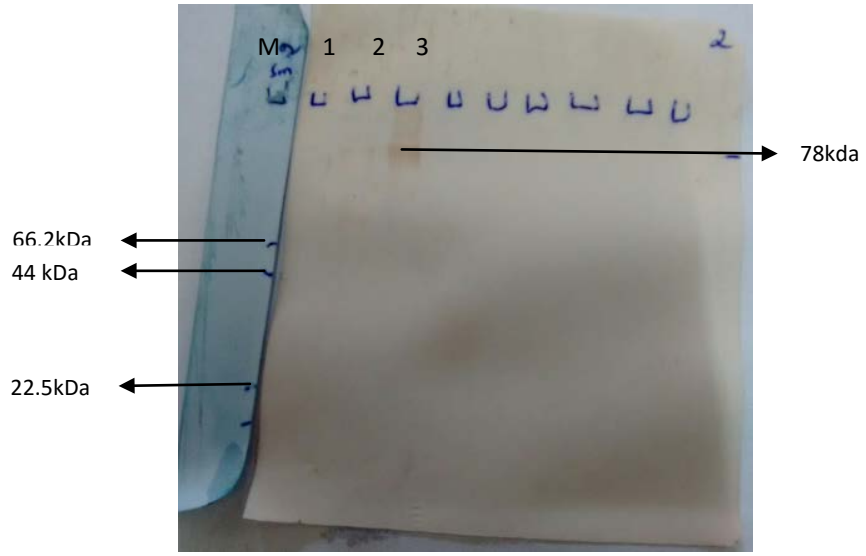

Figure 3: Western Blotting of purified protein. Lane1: Empty, Lane 2: Uninduced culture, Lane 3: Purified chitinase

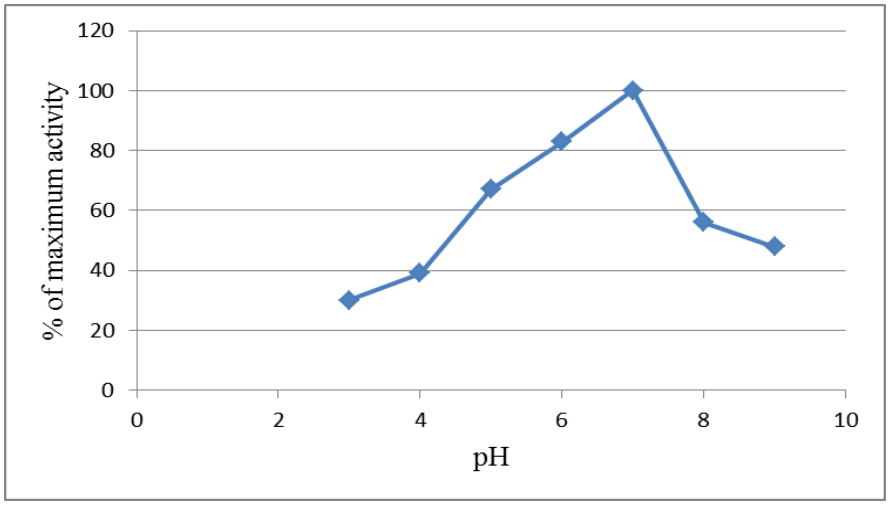

Figure 4: Effect of pH of recombinant chitinase (pET32a+HA chi) using colloidal chitin as a substrate

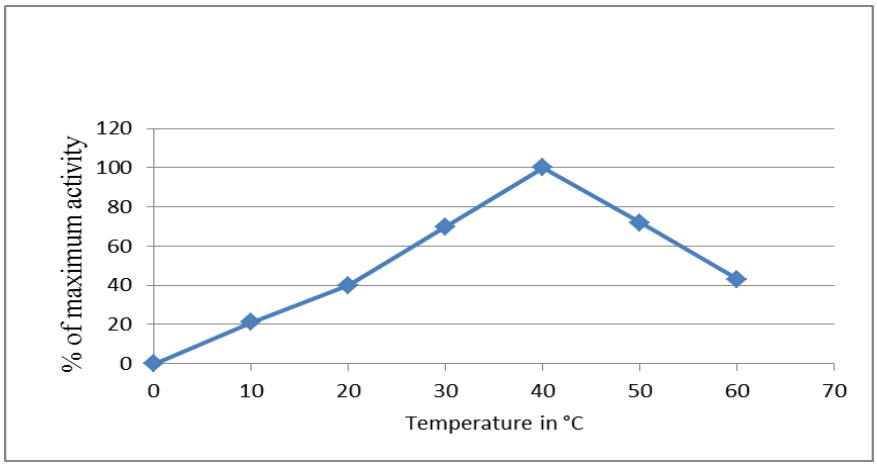

Figure 5: Effect of temperature of recombinant chitinase (pET32a+HA chi) using colloidal chitin as a substrate

Paper ID: NOV162023 DOI: 10.21275/v5i3.NOV162023

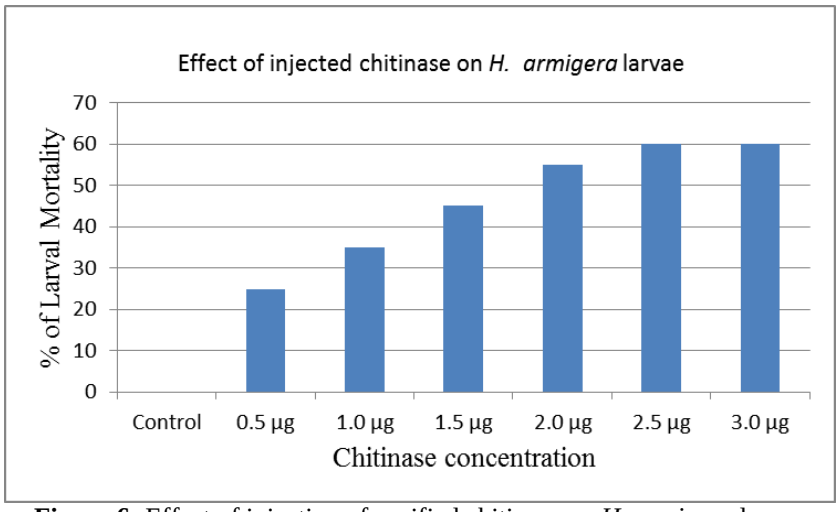

Figure 6: Effect of injection of purified chitinase on *H. armigera* larvae

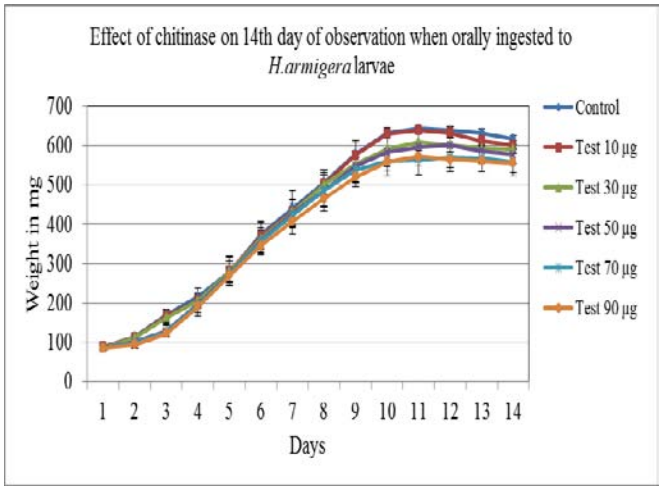

Figure 7: Effect of oral ingestion of purified chitinase on *H. armigera* larvae

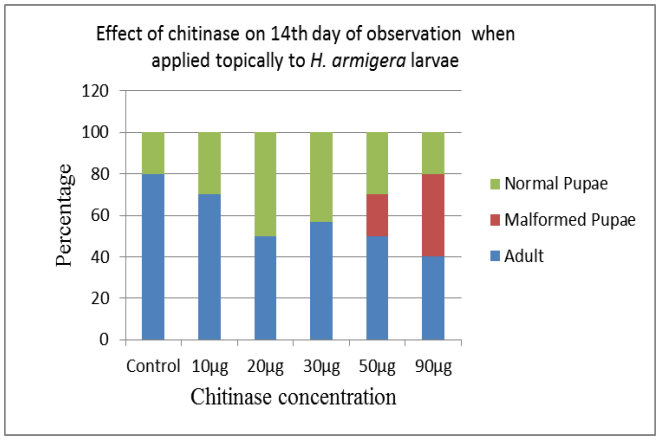

Figure 8: Effect of topical application of purified chitinase on *H.armigera* larvae

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