ijlpr 2020; doi 10.22376/ijpbs/lpr.2020.10.4.L50-59

International Journal of Life science and Pharma Research

Research Article

Biotechnology for Prospective Medical Science

A Comparative Study on Toxicity of Recombinant Chitinase on A Polyphagous Pest, *Helicoverpa Armigera*

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Abstract: Holometabolous insects complete their life cycle in four stages: egg, larva, pupa and adult. The larval stage is characterized by instar and on an average most larvae has 4- 6 instar stages that occurs between molts. One of the essential enzyme for the process of moulting is chitinase. Expression of chitinase gene takes place when the insect is ready to moult, wherein the old exoskeleton being made of chitin is replaced with the new exoskeleton. Reports indicate that the introduction of the chitinase enzyme at inappropriate time and at inappropriate concentration will prevent the formation of chitin, resulting in no moult, thus growth retardation of the insect and finally death. Insects own chitinase has shown to be a potential insecticide. In the present study, gene encoding for *Helicoverpa armigera* chitinase was isolated and the sequence was found to be of 1734bp as compared to the reference sequence. The gene was expressed in a prokaryotic and eukaryotic host system. The protein was purified using Ni- NTA column as the protein was tagged with histidine. Chitinase activity was determined and the obtained enzyme was used for toxicity studies against the same insect. Injection assay, topical application and oral ingestion studies showed that *Pichia pastoris* expressed recombinant chitinase was more effective as compared to the *Escherichia coli* expressed recombinant chitinase. In case of injection assay, *P. pastoris* expressed recombinant chitinase recorded 77% mortality for the highest concentration used. Topical application showed 58% and 40% of malformed pupae for *Pichia pastoris* end *E. coli* expressed recombinant chitinase. An significant decrease in weight gain was observed on 2nd day for *P. pastoris* expressed recombinant chitinase and from 7th day for *E.coli* expressed recombinant chitinase in the oral ingestion studies. Effectiveness of *Pichia pastoris* expressed recombinant chitinase might be because of post translational modifications in the eukaryotic system.

Keywords: *Helicoverpa armigera*, Chitin, Chitinase, *Pichia pastoris* and *E. coli*

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Recieved On 10 February 2020
Revised On 11 March 2020
Accepted On 01 June 2020
Published On 02 October 2020

Funding This research did not receive any specific grant from any funding agencies in the public, commercial or not for profit sectors.

Citation Dr. Esther Shoba R , A Comparative Study on Toxicity of Recombinant Chitinase on A Polyphagous Pest, Helicoverpa Armigera.(2020).Int. J. Life Sci. Pharma Res.10(4), L50-59 http://dx.doi.org/10.22376/ijpbs/lpr.2020.10.4.L50-59

1. INTRODUCTION

A highly polyphagous pest *Helicoverpa armigera* attacks more than 182 plants of agricultural importance all over the world.[1] It is a cosmopolitan, multivoltine insect and commonly known as *Heliothis*. Residing in 300 plant species across 47 families, in the Indian subcontinent, 56 are heavily damaged and 126 are rarely affected.[2] In India, crop losses due to *H. armigera* are commonly more than half the yield. Bollworm induced damage can range from 50 to 90 percent of the yield, depending on the crop.[3] Frequent outbreak of *H. armigera* has led to severe social disturbances with several farmers losing their life because of crop failure especially in regard to the cotton.[4] The principle structural component of insects exoskeleton is **c**hitin, an abundant, insoluble polysaccharide composed of linear chains of β-1,4 -N-acetylglucosamine (GlcNAc) residues that are highly cross-linked by hydrogen bonds.[4] It is also found in the foregut , hindgut, midgut lining of the peritrophic membrane. Insects grow through successive stages of egg, larva, pupa and adult. The first immature stage of the insect hatches from the egg and passes through several instars to reach pupal stage, a dormant condition. Differences between instars can often be seen in altered body proportions, colours, patterns, or changes in the number of body segments.[5] In insects chitinase an moulting enzymes plays a important role in morphogenesis as the insects exoskeleton is made of chitin (85%). Shedding of old exoskeleton and synthesis of a new one is by a process known as chitin metabolism. Disruption of exoskeleton, which shields the organism from external environmental vagaries, fails to protect the insect anymore. Targeting chitinases or the use of chitinase enzyme to hydrolyse the chitin is explored to insect control. Chitinases of insect origin (native or recombinant) have been employed either at laboratory level or field level to control the pest insects.[4] To date, only a few studies have been reported on directly used insect-derived chitinases as biopesticides for the control of pests. A recombinant AcMNPV baculovirus expressing group I chitinase from *Manduca sexta* (tobacco bornworm) under the control of the polyhedrin promoter liquefied infected *S. frugiperda* larvae significantly earlier that is three quarters of the time required than when the insects were infected with a wild-type virus, indicating increased insecticidal activity.[6] Fitches and group in 2004 produced a recombinant chitinase from the tomato moth, *Lacanobia oleracea* in the yeast, *Pichia pastoris*, and injected it into *L. oleracea* larvae. They observed 100 % mortality at a comparable low dose and a reduction in cuticle thickness when injected prior to moulting. When fed orally, larval growth and food consumption were reduced.[7] Similarly, Assenga and team in 2006 used recombinant AcMNPV expressing a group III chitinase from the hard tick *Haemaphysalis longicornis*. A mixture of recombinant virus and the purified recombinant protein on topical application was found to be more efficient in killing the ticks than the recombinant virus and pure chitinase alone.[8] Toxicity potential of purified chitinase (75kDa) from *Bombyx mori* was evaluated against *Monochamus alternatus* [Japanese pine sawyer (JPS)]. A concentration of 11µg/50µL of purified chitinase when ingested orally to JPS resulted in high mortality as well as significant decrease in feed consumption and slight reduction of body weight was observed.[9] In the present work *Helicoverpa armigera* chitinase is expressed in two different host systems- *E. coli* and *Pichia pastoris* and assess the insecticidal activity of the expressed chitinase on *Helicoverpa armigera*.

2. MATERIALS AND METHODS

2.1 Isolation and Expression of Chitinase Encoding Gene Construction of the expression plasmid

Total RNA was extracted from *H. armigera* during the prepupal stage (day three sixth instar larvae) using guanidine isothiocyanate method as described by Kingston and group[9] with necessary modifications and was subjected to reverse transcription using Revert Aid™ M-MuLV RT [Moloney Murine Leukemia Virus Reverse Transcriptase (Fermentas Life Science)]. Based on the reference mRNA sequence coding for chitinase in *Helicoverpa armigera* (GenBank accession number: AY325496), primers were synthesized for the chitinase gene expression in two different host with the compatible vectors: bacterial – pET32a /Rosetta DE3 (using *Nco* I and *Xba* I restriction sites) and yeast pPICZαB/ X-33 (using *Eco*R I and *Xba* I restriction sites). Gene specific primers for bacterial expression (F1: 5´ACC ATG GGA ATG AGA GTG ATA CTA GCG ACG TTG 3´ and R1:5´ACTC GAG AGG CGT CCT GTT CAT GAG CCG GCA 3´) and for yeast expression (F1: 5' CAG TCA GAA TTC ATG AGA GTG ATA CTA GCG ACG T --3' and R1: 5'- TGA CAC TCT AGA ACG GTC GGC GTT GT -3') was used.[10] Care was taken to include 6X His terminal tag for protein purification.

2.2 Heterologous expression of H. armigera chitinase

On sequence confirmation, the recombinant clones were induced for protein expression. For bacterial expression, induction was carried out using IPTG-isopropyl thio galactosidase at a final concentration of 1mM and the culture was allowed to grow for 4 h at 37 °C in a shaker at 200 rpm. The cells were harvested by centrifugation, resuspended and sonicated to disrupt the cell wall and 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. For yeast expression, positive colony (on confirmation with genomic DNA PCR with gene specific primers) was inoculated into 25ml of YPD-Yeast Extract Peptone Dextrose taken in a sterile 250 mL flask and incubated at 30 °C in a shaking incubator (250-300 rpm) until culture reached an OD_{600}-6. As the cells reached log-phase growth, the culture was centrifuged at 4500 rpm for 5 minutes at room temperature and the pellet was washed twice with BMMY- Buffered methanol Complex Medium to remove traces of YPD medium. To induce expression, the cell pellet was resuspended to an OD_{600} -1 in BMMY medium and the culture was transferred to 1 litre baffled flask and incubated at 30 °C. At an interval of every 24 h for 5 days, 0.5% methanol was added to maintain induction. Samples were withdrawn regularly every 24 h for analysis of extracellular expression (the protein was secreted extracellular because of presence of α- secretion signal sequence from *Saccharomyces cerevisiae* in the pPICZαB vector) by SDS- PAGE.

2.3 *Purification of the recombinant protein*

Affinity chromatography, in specific IMAC was performed for the purification of the recombinant protein produced with 6X Histidine-tag towards the carboxy terminal. Ni-NTA column was used and the protocol was followed as per the

manufacturer's instructions (Novagen). The purified chitinase was quantified by Bradford method and assayed for enzyme activity. [11]

2.4 *Western blotting*

Bacterial expressed purified protein was used to raise polyclonal antibodies in rabbit (Bhat-Biotech India Private Limited). Western blotting was performed using these antibodies (1: 8000) against the purified protein (both bacterial and yeast expressed recombinant protein) that act as an antigen. For the reaction, diaminobenzidine (DAB) as substrate, Tris buffered saline (TBS containing 0.05% (v/v) Tween-20) as the blocking reagent and secondary antibody pro- Rec A. was used.

2.5 Determination of chitinase activity

The chitinase enzyme activity was determined by Miller's (1959) method.[12] Colloidal chitin as substrate was prepared from commercial chitin according to Roberts and Selitrennikoff (1988).[13] 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 concentrations of N-acetyl glucosamine levels using dinitrosalicylic acid-DNSA reagent.

2.6 Determination of optimum pH and temperature for enzyme activity

Optimum pH for the chitinase activity was determined by using 100mM sodium acetate buffer (pH 3.0-5.0), 100mM sodium phosphate buffer (pH 6.0-8.0) and 100mM 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 1 h. Similarly, optimum temperature was determined at different temperatures viz., 0, 10, 20, 30, 40, 50, and 60 °C with sodium phosphate buffer pH 7.0 as constant. The reaction was incubated at the above temperatures for 1 hour and enzyme activity was assayed as described earlier.[12]

2.7 Bioassay of recombinant chitinase for toxicity

Recombinant chitinase was assayed for its toxicity on *Helicoverpa armigera* larvae by oral (leaf dip method), topical applications and injection methods. For injection method and oral application, *Helicoverpa armigera* larvae of third instar stage weighing approximately in the range of 80-110 mg were used and larvae of weight 310-370 mg were used for topical application. For each test 20 samples were used and was done in triplicates.

2.7.1 *Injection Assay*

Chitinase of concentrations ranging from 0.5 µg/10µl to 3.0 µg/10µl were injected into the hemolymph of the larvae. The larvae were observed for the effects upon survival over the next 48 h. As control, 10 µl of 0.1M Sodium phosphate buffer of pH 7.0) was used.

2.7.2 Oral ingestion Assay

Alcohol sterilized cotton leaves of 5cm in diameter were dipped in enzyme preparation of increased concentrations (10 µg, 30 µg, 50 µg, 70µg and 90 µg/10 µl) for 1 min and air dried. The oral ingestion assay was carried out by starving the larvae for 24 hours and then the experiment was carried out. For each concentration, chitinase treated cotton leaf discs were given as feed for 3 days, after which normal feeding was resumed. As control leaf discs dipped in 0.1M, pH 7.0 sodium phosphate buffer was used. The larvae were monitored for weight gain, pupation and death over a period of 14 days.

2.7.3 *Topical application*

For topical application, fifth instar larvae were used and enzyme preparations of 10 µg, 30 µg, 50 µg, 70 µg and 90 µg/10µl was applied topically on the dorsal side of the thorax. The treatment was continued for 3 days and thereafter larvae were left undisturbed. Same volume of 0.1M 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. STATISTICAL ANALYSIS

The data for oral ingestion were represented as mean ± standard deviation (SD) and were analysed using Single way ANOVA (Bonferroni - Dunn) and for injection and topical application studies the data were represented using MS Excel bar graph with error bars.

4. RESULTS

Total RNA isolated from the larval integument tissue showed concentration of 4.588 µg/µL with 2.077 purity. On 1% agarose gel electrophoresis, bands were observed indicating the intactness and integrity of RNA. Total RNA isolated when reverse transcribed with the oligo $(dT)_{18}$ primer and M-MuLV RT resulted in first strand cDNA and on normal PCR along with gene specific primers resulted in the amplification of approximately 1.8kb fragment (Figure 1). On sequencing the gene showed homology with the reference sequence and the deposited in the Genebank with the accession number KT894380.1.

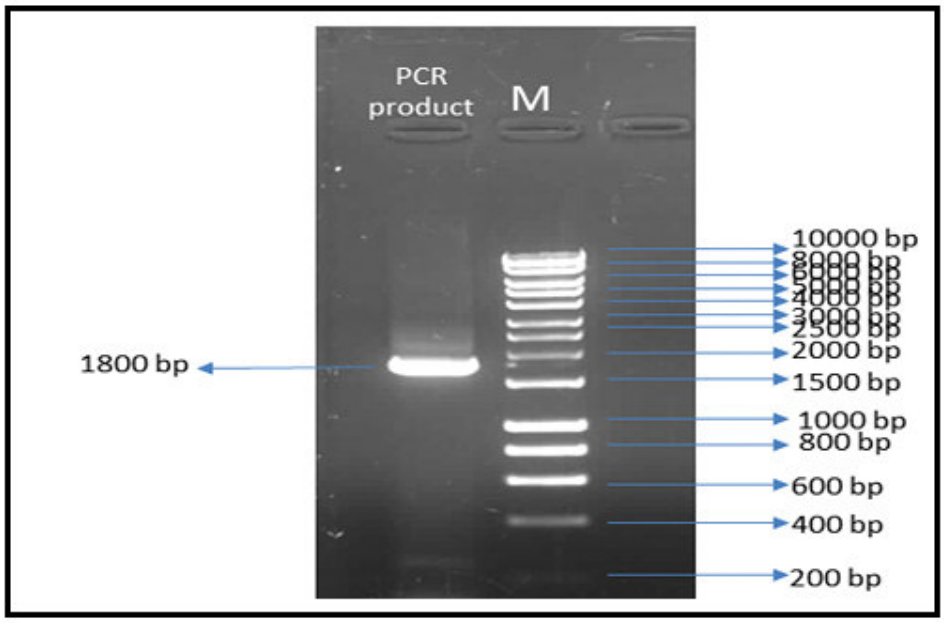

Fig 1. Amplification of *H. armigera* chitinase gene of approximately 1800bp

4.1 Heterologous expression of H. armigera chitinase

Positive clones of (pET32a + HA chi) in Rosetta (DE3) on induction showed an extra band of ~78kDa in the denaturation sample as compared with the uninduced culture. Expression studies of the yeast positive clones were confirmed by SDS-PAGE analysis with an extra band at ~66kDa as compared to the uninduced culture. Protein band of ~66 kDa was observed after 48h induction in the supernatant sample indicating that the protein was extracellular because of the presence of α-signal secretion factor present in the vector. Studies conducted to optimize the expression level revealed the protein expression until 120 hrs of induction period with 0.5% methanol concentration.

4.2 Purification of the target protein

Purification of the expressed protein with Ni-NTA column resulted in a single band of ~78kDa and ~66kDa protein in the eluted fraction when eluted with buffer having 500mM imidazole concentration for *E. coli* and *Pichia pastoris* expression system respectively.

4.3 Western Blotting analysis of E. coli and Pichia pastoris expressed protein

Western blotting analysis of *E. coli* expressed *H. armigera* chitinase and *Pichia pastoris* expressed *H. armigera* chitinase revealed the presence of ~66 kDa and ~78 kDa band respectively with anti chitinase antibodies raised in rabbit (Figure 2).

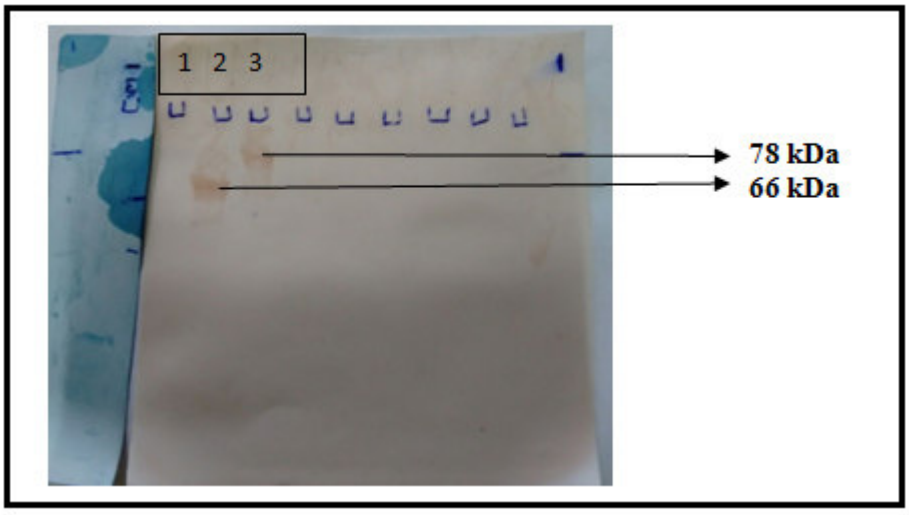

Fig 2. Western blotting analysis for confirmation of expressed chitinase. Band of ~66kDa and ~78kDa was observed for chitinase expressed in *Pichia pastoris* and *E. coli* expression system respectively.

4.4 Analytical Methods

4.4.1 Determination of protein concentration

The concentration of *E. coli* expressed chitinase protein was 3-5mg/litre and *Pichia pastoris* expressed protein was found to be 1.2 mg/litre as determined by Bradford assay.

4.4.2 Determination of enzyme activity and optimum pH and temperature

The enzyme activity of purified recombinant chitinase measured colorimetrically using colloidal chitin as substrate was found to be 2.28 µmol/min/mL for *E. coli* expressed chitinase and 18.56µmol/min/mL for *Pichia pastoris* expressed chitinase protein. The optimum pH and temperature at which the enzyme activity was maximum, when investigated for both *E. coli* expressed chitinase protein and *Pichia pastoris* expressed chitinase protein, was found to be almost similar to both expressed proteins. The maximum activity was at pH 7.0 and decreased sharply after that for *E. coli* expressed *H. armigera* chitinase. In case of *Pichia pastoris* expressed *H. armigera* chitinase, enzyme activity of about 90% was observed at pH 8.0, however maximum activity was at pH 7.0. Enzyme activity increased with increase in temperature and peaked at 40°C and declined with further increase in temperature.

4.5 Bioassay for chitinase toxicity

4.5.1 Injection method

Chitinase injections to *H. armigera* larvae showed mortality which increased with the increase in concentration in case of both *E. coli* expressed chitinase protein and *P. pastoris* expressed chitinase protein. However mortality of larvae was comparatively more in *P. pastoris* expressed chitinase protein as compared to *E. coli* expressed chitinase protein. In case of *E. coli* expressed chitinase protein, the toxicity was observed at concentration as low as 0.5µg recording about 25% mortality after 48 hours and recorded 60% mortality over the same period for the concentrations 2.5 and 3.0 µg/10µL (31, 37.5µg/g body weight respectively). *Pichia pastoris* expressed *H. armigera* chitinase protein also showed toxicity at 0.5µg recording about 28% mortality after 48 hours and 77% mortality over the same period for the concentration 3.0 µg/10µL. There was no mortality recorded in case of control over a period of 48 hours (Figure 3).

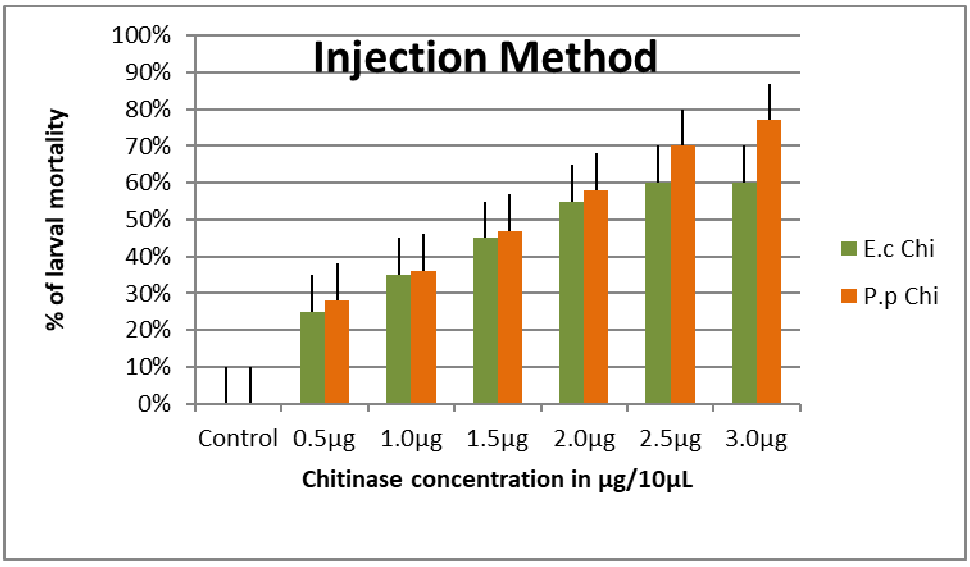

Fig 3. Effect of injection of *E. coli* expressed (E.c.Chi) and *P. pastoris* expressed *H. armigera* chitinase (P.p Chi). Highest percentage of mortality (60 and 78%) was observed in both the recombinant expressed chitinase for the highest concentration used.

4.5.2 Oral application

No mortality was recorded in oral application for both *E. coli* and *Pichia pastoris* expressed *H. armigera* chitinase, however, a retarded growth rate was observed at all concentrations as compared to the control. Decrease in the larval weight gain was recorded for a duration of 14 days. The gain in larval body weight though decreased with increase in concentration of chitinase, a significant ($p<0.05$) decrease was observed at 90 µg/10µL concentration after the 7th day in case of *E. coli* expressed *H. armigera* chitinase (Figure 4). A significant decrease at $p<0.05$ was observed from 2nd day onwards in case of *P. pastoris* expressed *H. armigera* chitinase for the concentration of 90 µg/10µL **(**Figure 5**)**. Larvae treated with *E. coli* expressed *H. armigera* chitinase entered into the pupation stage and attained adulthood, however in case of larvae treated with *Pichia pastoris* expressed *H. armigera* chitinase most of them were deformed in the adult.

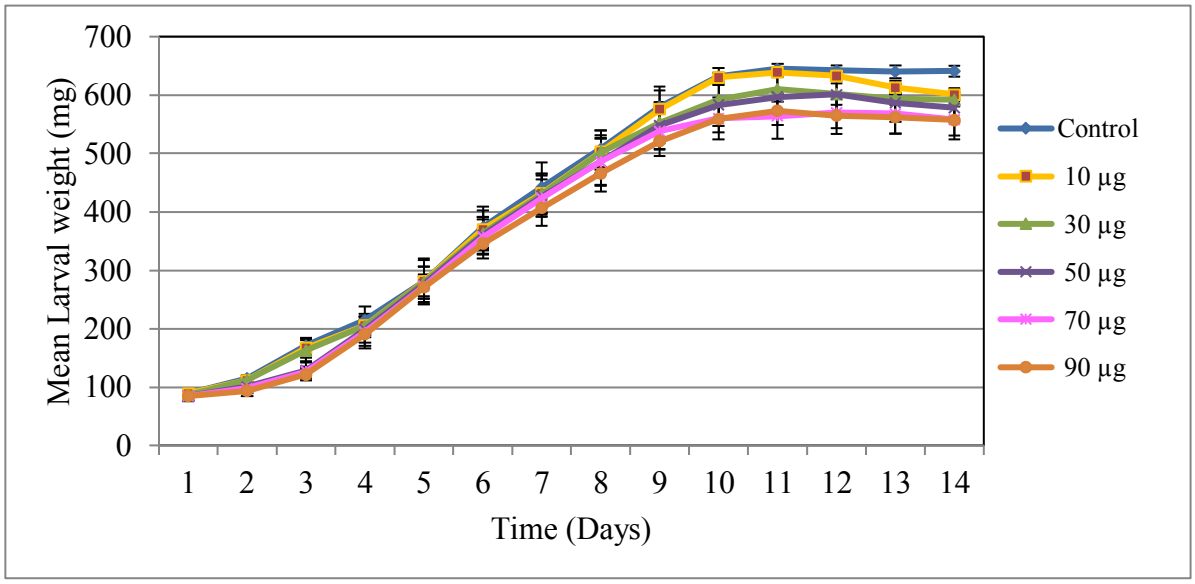

Figure 4a

Days	Concentration of bacterial recombinant chitinase in µg/10µl					
	Control	10	30	50	70	90
1	89.0±5.67^{a}	89.0±5.67^{a}	87.0±6.74^{a}	85.0±8.49^{a}	85.0±7.07^{a}	85.0±7.07^{a}
2	115.0±5.27^{b}	112.0±6.32^{b}	112.0±9.18^{b}	101.0±5.67^{a}	98.4±8.88^{a}	94.0±8.43^{a}
3	171.0±13.70^{b}	167.0±16.36^{b}	163.0±18.28^{b}	129.0±13.70^{a}	127.0±8.23^{a}	122.0±10.32^{a}
4	215.0±23.21^{b}	206.0±20.11^{ab}	205.0±15.81^{ab}	199.0±22.33^{ab}	195.0±24.15^{ab}	191.0±24.24^{a}
5	281.0±39.28^{a}	281.0±25.14^{a}	280.0±37.71^{a}	276.0±30.62^{a}	273.0±20.57^{a}	271.0±19.69^{a}
6	374.0±35.02^{a}	370.0±32.99^{a}	363.0±28.69^{a}	360.0±30.91^{a}	357.0±29.83^{a}	346.0±25.03^{a}
7	442.0±42.63^{a}	432.0±33.92^{a}	431.0±34.46^{a}	427.0±34.65^{a}	424.0±32.04^{a}	407.0±30.56^{a}
8	508.0±31.55^{b}	503.0±36.53^{b}	502.0±29.73^{b}	486.0±41.15^{ab}	486.0±39.21^{ab}	466.0±31.34^{a}
9	580.0±34.31^{b}	576.0±32.04^{b}	553.0±35.29^{ab}	548.0±39.94^{ab}	538.0±31.19^{a}	521.0±25.14^{a}
10	632.0±14.75^{c}	630.0±8.16^{c}	592.0±31.55^{b}	583.0±36.53^{ab}	560.0±36.20^{a}	559.0±23.30^{a}
11	645.0±8.49^{d}	639.0±5.67^{cd}	610.0±21.08^{bc}	597.0±34.97^{ab}	564.0±38.64^{a}	573.0±24.06^{a}
12	639.0±8.75^{c}	633.0±6.74^{c}	602.0±18.13^{b}	601.0±32.12^{b}	570.0±26.66^{a}	565.0±31.71^{a}
13	633.0±10.59^{e}	613.0±11.59^{de}	594.0±10.47^{cd}	587.0±32.33^{bc}	568.0±33.92^{ab}	562.0±28.20^{a}
14	618.0±9.18^{d}	601.0±11.00^{cd}	592.0±11.35^{bc}	578.0±24.40^{b}	558.0±34.25^{a}	557.0±26.26^{a}

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Figure 4b

Fig 4. Effect of oral ingestion of *E. coli* expressed *H. armigera* chitinase. 4a: Decrease in the larval weight gain was recorded for a duration of 14 days. 4b: A significant ($p<0.05$) decrease was observed at 90 µg/10µL concentration after the 7th day. Mean values followed by different letters a,b,c and d in the same row are significantly different at $p<0.05$.

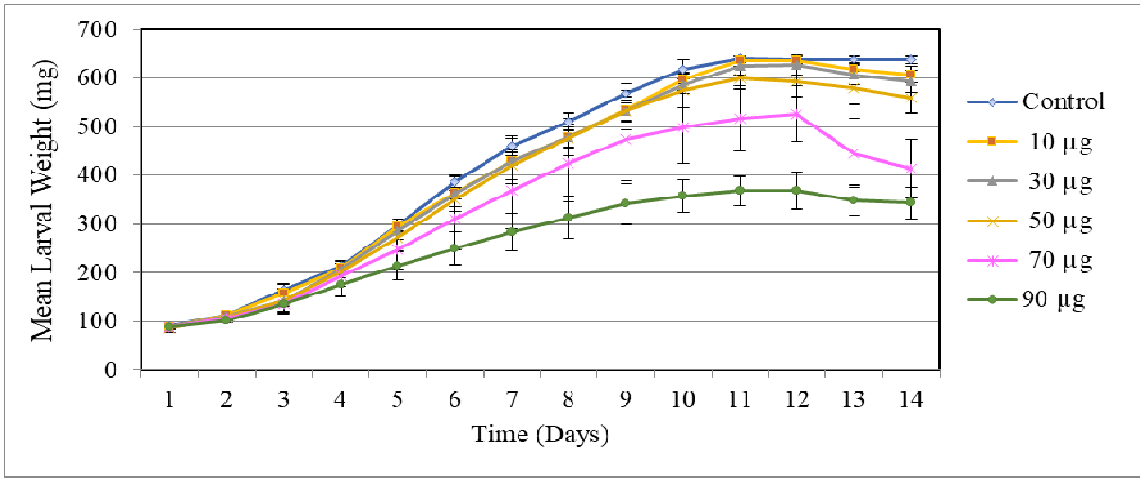

Figure 5a

Days	Concentration of *Pichia pastoris* recombinant chitinase in µg/10µl					
	Control	10	30	50	70	90
1	90.3±2.54^{a}	89.3±3.05^{a}	89.5±3.68^{a}	86.0±9.66^{a}	89.0±5.67^{a}	89.0±5.67^{a}
2	113.8±3.82^{d}	112.5±5.25^{cd}	111.7±6.49^{cd}	109.0±3.77^{bc}	105.3±3.91^{ab}	102.5±4.55^{a}
3	165.0±14.33^{c}	159.0±17.91^{bc}	142.0±24.40^{abc}	142.0±10.32^{abc}	136.0±20.65^{ab}	135.0±15.81^{a}
4	214.0±8.43^{c}	210.0±14.90^{bc}	208.0±11.35^{bc}	204.0±14.29^{bc}	195.0±23.21^{b}	177.0±26.26^{a}
5	297.0±14.18^{c}	295.0±11.78^{c}	287.0±20.02^{c}	273.0±28.30^{bc}	247.0±38.60^{ab}	215.0±29.15^{a}
6	387.0±11.59^{d}	364.0±12.64^{cd}	363.0±36.53^{cd}	352.0±16.19^{c}	311.0±63.32^{b}	250.0±33.33^{a}
7	462.0±20.43^{c}	430.0±47.84^{c}	429.0±23.78^{c}	420.0±27.48^{c}	369.0±79.08^{b}	284.0±38.64^{a}
8	511.0±17.91^{c}	479.0±37.54^{c}	478.0±20.43^{c}	476.0±17.76^{c}	426.0±79.74^{b}	314.0±43.51^{a}
9	568.0±20.43^{c}	535.0±41.16^{c}	534.0±21.70^{c}	532.0±20.43^{c}	475.0±86.31^{b}	341.0±42.28^{a}
10	619.0±20.24^{d}	599.0±11.00^{bc}	587.0±18.88^{bc}	575.0±35.97^{c}	500.0±75.86^{b}	358.0±33.26^{a}
11	641.0±3.16^{d}	637.0±4.83^{d}	624.0±17.76^{cd}	601.0±23.78^{c}	517.0±67.66^{b}	368.0±29.73^{a}
12	638.0±7.88^{d}	637.0±4.83^{d}	627.0±19.46^{d}	594.0±30.98^{c}	527.0±56.57^{b}	370.0±38.00^{a}
13	638.0±7.88^{d}	618.0±13.16^{d}	607.0±19.46^{cd}	580.0±33.33^{c}	446.0±70.42^{b}	349.0±32.12^{a}
14	638.0±7.88^{d}	608.0±16.86^{d}	593.0±22.13^{d}	560.0±31.62^{c}	413.0±60.56^{b}	344.0±32.72^{a}

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Figure 5b

Fig 5: Effect of oral ingestion of *P. pastoris* expressed *H. armigera* chitinase. 5a: Decrease in the larval weight gain was recorded for a duration of 14 days. 5b: A significant ($p<0.05$) decrease was observed at 90 µg/10µL concentration after 2nd day. Mean values followed by different letters a, b. c and d in the same row are significantly different at $p<0.05$.

4.5.3 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, the percentage of adult emergence decreased and the rate of malformed pupae increased in proportion to the increased concentration of chitinase. The percentage of adult emergence was high on 14th day in the lowest concentration (10µg) for both the recombinantly expressed chitinase and was low in highest concentration (90µg) of chitinase administered. *P. pastoris* expressed chitinase concentration of 50µg/10µL, 70µg/10µL and 90µg/10µL on *H. armigera* larvae showed 20%, 40% and 58% pupae malformation respectively however *E. coli* expressed chitinase showed malformed pupae of 20 and 40% for 50µg/10µL,70µg/10µL respectively (Figure 6). With respect to this, the percentage of adult emergence decreased with increase in chitinase concentration (Figure 7).

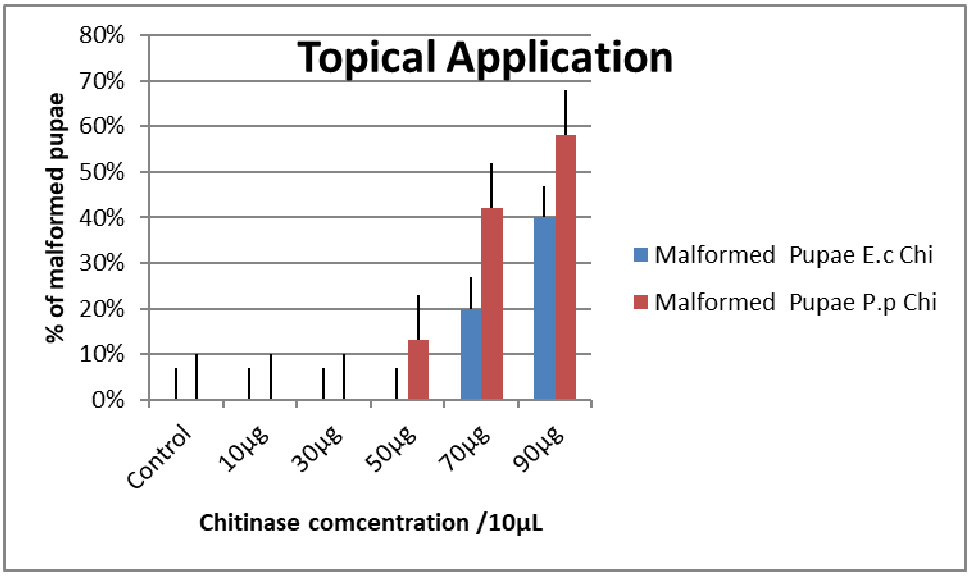

Fig 6. Malformed pupae on topical application of *E. coli* and *P. pastoris* expressed *H. armigera* chitinase (E.c Chi and P.p Chi). Increase in malformed pupae with increase in chitinase concentration.

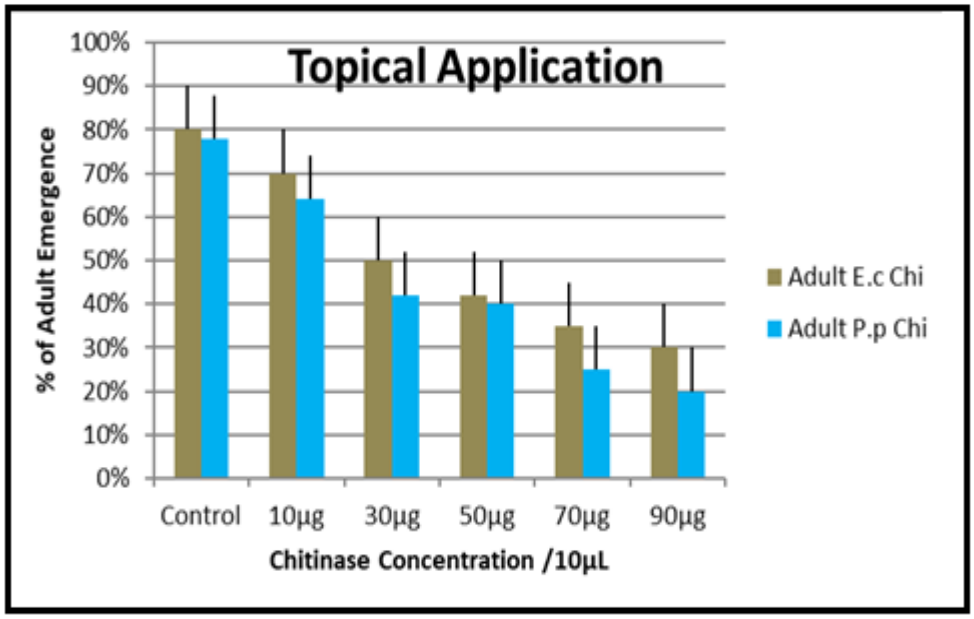

Fig 7. Adult emergence on topical application of *E. coli* and *P. pastoris* expressed *H. armigera* chitinase (E.c Chi and P.p Chi). Decrease in adult emergence with increase in chitinase concentration.

5. DISCUSSION

Use of chitinase to control insect pests and as a potential biopesticide have been well appreciated by works of different groups.[6,7,8] Thus in this work an attempt is made to use *Helicoverpa armigera* chitinase to control the polyphagous pest. The present study was focused on the isolation of the gene that encodes chitinase from *Helicoverpa armigera* and its expression in a suitable system that enables the purification of the protein and its use for assessing the insecticidal activity against the plant pest insect *Helicoverpa armigera*. The gene encoding for chitinase was obtained from the integument of day three sixth instar larvae as increased expression levels of chitinase was reported.[10] The PCR product obtained using first strand cDNA as template and gene specific primers resulted in a single band of ~1.8kb. The expression of chitinase gene was carried out in both prokaryotic and eukaryotic system using *E. coli* and *Pichia pastoris* as host systems. The cloned *Helicoverpa* cDNA displayed a high degree of similarity of about 96% with the reference chitinase gene of the same insect *Helicoverpa armigera*. There are no reports available on use of insect chitinase against *Helicoverpa armigera* for insecticidal activity but against some other insects has been reported. Application of chitinase on *H. armigera* larvae, with different mode, did show insecticidal activity. Insecticidal efficacy of the bacterial expressed chitinase was as low as compared to the yeast expressed chitinase, the reason being that the bacterial chitinase was expressed in the form of inclusion bodies. Work by Natalia S´anchez de Groot and group, indicates that the protein expressed in the form of inclusion bodies is active.[14] Injection bioassays with different concentrations of chitinase resulted in 60% and 77% of larval mortality respectively at the maximum concentration used for *E. coli* expressed and *Pichia pastoris* expressed *H. armigera* chitinase, as compared to control. Till date only Fitches and group has demonstrated the use of yeast expressed *Lacanobia oleraceae* chitinase for insecticidal activity and reported 100% mortality in injection bioassays. [7] In case of oral application, reduction in larval weight gain was observed for higher concentration tested i.e., 90µg/10µL for both *E. coli* expressed *H. armigera* chitinase as well as *P. pastoris* expressed *H. armigera* chitinase, however no mortality was observed. This might be due to the fact that when chitinase are ingested insect gut enzymes are known to degrade the toxic substances entering their body through diet, a defensive mechanism evolved over a period of time. The loss in weight gain can be explained that the concentrations of chitinase used were sufficient enough only to disrupt the peritrophic membrane of the gut and thereby decreasing the feeding efficiency of the larvae. In topical application though, all the larvae entered in to pupation but 40% and 58% of them were malformed for the *E.coli and P. pastoris* expressed chitinase respectively, tested at higher concentration (90µg/10µL). Defects in pupation was observed in 5th-instar *O. furnacalis* larvae.[15] Binod *et al.* (2007) recorded 75% mortality on topical application of chitinase on *H. armigera* larvae by taking into account the malformed pupae.[16] Chitinase was 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. To overcome this probably higher doses of chitinase need to be used. Irrespective of the intensity of activity, in all 3 modes of application, chitinase was found to inflict damages to *Helicoverpa armigera* larvae. The damage caused by *Pichia pastoris* expressed *H. armigera* chitinase was more as compared to the *E. coli* expressed *H. armigera* chitinase. This is due to the formation of protein as inclusion bodies in *E. coli*. 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. This work is an attempt to lay the foundation that chitinase from the insect is indispensable to moulting as the results interfere that chitinase when administered at inappropriate time and levels prevents moulting.[17] With this initial reports mediating

chitinase as part of formulation along with other biocontrol products rather than chitinase alone can also be evaluated. However there, effectiveness in the presence of temperature, radiation, dose, and different larval stages and more importantly its ability to act in field conditions, and mode of application have to be explored.

6. CONCLUSION

Expression of chitinase was studied in two expression systems, bacterial and yeast systems. The investigation revealed the presence of a new variant of chitinase gene in *Helicoverpa armigera* expressing a protein of 578 amino acids. In bacteria though the protein obtained was in the form of aggregates it still showed biological activity but less when compared to the eukaryotic expressed protein. *Pichia pastoris* expression system was investigated for the production of protein. The major advantage of expressing heterologous protein as secreted proteins is that *P. pastoris* secretes very low levels of native proteins, which facilitates to design a simple and convenient purification process. Enzyme activity of *P. pastoris* purified protein was 8 fold higher compared to *E.coli* expressed purified protein suggesting the proper protein folding. However, the low yield obtained probably suggests the need for codon optimization. As a future perspective, codon optimization to improve the expression level of chitinase in *P. pastoris* can be considered. Also the optimisation of media that helps in maximum production of the protein can be tried. Chitinase obtained in the functional form can be used as a part of insecticide formulation but with a great deal of investigations in field trials. Studies on how these chitinases can be delivered in the field can be explored.

7. CONFLICT OF INTEREST

Conflict of interest declared none

8. REFERENCES

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