The present invention relates to a novel insecticidal protein named XnGroEL isolated and purified from Xenorhabdus nematophilus having GENE BANK ACCESSION No. AY18449. The process for the purification of novel insecticidal protein XnGroEL is also disclosed. The invention also relates to the process for preparation and purification of the recombinant novel insecticidal XnGroEL protein. The process involves purification of a novel insecticidal protein Xenorhabdus nematophilus, construction of genomic DNA library, screening of library using probes with N-terminal amino acid sequence, cloning in an expression vector followed by its transformation in E. coli cells and purification of the recombinant protein. Both the novel native and recombinant insecticidal XnGroEL protein have growth inhibitory and insecticidal property for Helicoverpa armigera larvae. The present invention also discloses that the novel native and recombinant insecticidal XnGroEL protein has larval gut brush border membrane vesicles binding activity and α-chitin binding activity.
INSECTICIDAL XnGroEL PROTEIN OF Xenorhabdus nematophila

FIELD OF THE INVENTION
The present invention relates to the identification and purification of a novel nucleic acid fragment encoding orally insecticidal XnGroEL protein from Xenorhabdus nematophila, its cloning and expression and process for the production of recombinant XnGroEL protein having growth inhibitory and insecticidal property for Heliothrips armigera larvae. In particular, the present invention relates to identification, isolation and purification of XnGroEL protein having oral insecticidal property. The present invention also relates to a method for the manufacture of the said protein by recombinant techniques.

BACKGROUND OF THE INVENTION
Pests infestation can cause farmers a huge financial loss annually either in crop loss or in purchasing expensive pesticides to keep check on pests. The loss is heavy in terms of reduced agriculture production which includes, reduced crop yield and reduced crop quality. Insects are also a threat to vegetable growers, to producers of ornamental flowers and home gardeners. Over the years, use of high level of nitrogen fertilizers and crop rotation has partially addressed the problem of agricultural pests. But inadequate resources, excessive demand of certain crop, and lack of information has restricted the use of crop rotation and made it prone to pest infection.

Synthetic chemical insecticides have been heavily relied upon by farmers all across the globe to tackle the pest problem. However, heavy use of chemicals has adversely affected the beneficial insects on one hand and gave rise insect pest resistance against several pesticides on the other hand. Disproportionate use of chemical pesticides for a given crop and improper calibration of pesticides has resulted in poor pest control. The heavy use of chemical insecticides has raised environmental concerns about contaminating the water supply, fertilized soil, and residues remained on treated fruits and vegetables. Working with the insecticide can be hazardous to the persons applying them and also consumption of contaminated fruit or vegetables by humans.

Stringent new restrictions on use of pesticides and elimination of few effective pesticides could limit effective options for controlling damaging pests.

Use of biopesticides alone or in combination with synthetic chemical pesticides, could reduce the level of toxic chemicals in the environment. More environmentally sound approaches are being developed using Bacillus thuringiensis (Bt) and other Bacillus species. One approach involves the Bacillus thuringiensis toxin protein. This protein all by itself is harmless, but is converted to a potent toxin in the gut of certain kinds of moths (depending on the bacterial strain the toxin was isolated from) and of mosquito larvae. Initially Bt toxin proteins were formulated as spray for insect control. Later, the gene coding for this protein has been cloned from various Bacillus thuringiensis strains, and has been incorporated into several plants. The moth against which the toxin is active dies after eating the transformed plant. Transgenic plants producing these toxins are quite
efficacious and usage is quite high for some crops (US patent no. 6313378, RE039580). The major advantage of the *Bacillus thuringiensis* (Bt) toxin is that it is harmful to only a few species of insects, while it is essentially harmless to other animals and humans. These biological pesticides also degrade rapidly in the environment. Thus, the use of such biological pesticides appears to be a significantly more environmentally safe solution to pest control than the classical (synthetic chemical) pesticides.

Few insects like *BoH weevils*, *Black cutworm*, *Helicoverpa zea* as well as some adult insect species are virtually insensitive to these pesticidal proteins. Resistance development among insects against Bacillus pesticide proteins is another potential obstacle in efficient pest control. The wide-spread use of Bt transgenic plant could be a matter of concern as insects could develop resistance more quickly than traditional sprayable applications. *Helicoverpa armigera* and *Diamondback moth* (*Plutella xylostella*) has also demonstrated resistance in field setting recently (Robin et al., 2005, App. Environ. Microbiol. 71: 2558-2563; Jurat_Fuentes ., 2002, Appl. Environ. Microbiol. 68: 5711-5717; Ferre et al., 2002, Annu. Rev. Entomol. 47: 501-553). Several strategies have been employed for resistance management in Bt transgenic plants for efficient use of *B. thuringiensis* toxins. These strategies include high expression of toxin proteins, use of different toxin proteins alternatively and co-expression with different toxins (McGaughey et al., 1998, Nature Biotechnology, 16: 144-146). The need of hour is to find new genes in addition to Bt genes which can be used in transgenic plants in order to control various insects.

*Xenorhabdus* and *Photorhabdus* species are gram negative gamma proteobacteria that form entomopathogenic symbioses with soil nematodes of families Steinernematidae and Heterorhabditidae respectively (Owuama, World. J. Microbiol. Biotechnol. 17: 505-515). These bacteria undergo a complex life cycle that involves symbiotic and pathogenic stages. During the symbiotic stage, the bacteria are carried in the gut of the nematode, but after infection of an insect host, the nematodes inject the bacteria into the insect hemocoel. Over several days, the combined actions of the nematode and bacteria kill the insect. Within the hemocoel of the insect carcass, the bacteria grow to stationary phase while the nematodes develop and sexually reproduce. The final stage of development is the re-association of the bacteria and nematodes to form non-feeding infective juveniles, which emerge from the insect carcass to find new hosts.

The naturally occurring bacterial symbionts found in the gut of the nematodes are called phase I cells. Variant forms, called phase II, are rarely observed in vivo but are often observed under laboratory conditions (Forst et al., 1997, Annu. Rev. Microbiol. 51: 47-72) and also in the free living clinical isolates of *Photorhabdus*. The phase II bacteria are still toxic to insects such as *Galleria mellonella*, despite having many altered properties, including motility and lipase, phospholipase, and protease activities (Akhurst, R. J., and Dunphy, G. B., 1993, In Parasites and Pathogens of Insects (Beckage et al., eds, pp. 1-23, Academic Press, Inc., San Diego, CA; Forst and Nealson, 1996, Microbiol. Rev. 60: 21-43; Smigielski and Akhurst, 1994, J. Invertebr. Pathol. 64: 214-220).
Throughout their life cycle, the bacteria and the nematodes produce a variety of metabolites to enable them to colonize and reproduce in the insect host. These metabolites often have overlapping functions, a strategy that is likely to contribute to the success of the nematode-bacteria association against a variety of insect hosts. The metabolites produced include molecules to help evade the insect immune system, enzymes such as proteases, Upases, and phospholipases to maintain a food supply during reproduction, and antifungal and antibacterial agents to prevent degradation or colonization of the insect carcass while the bacteria and nematodes reproduce. The bacteria and nematodes also produce toxins that are responsible for killing the insect host. (Steven et al., Ann. Rev. Microbiol. 51: 47-72). Xenorhabdus can be cultured from their nematode host as described by Frost and Nealson (Frost et al., 1996, Microbiol. Rev. 1: 21-43). Xenorhabdus and Photorhabdus bacteria secrete a wide range of virulent proteins in culture medium (FFrench-Constant et al., 2000, Appl. Environ. Microbiol. 66: 3310-3329). To efficiently exploit the insecticidal properties of the nematode and bacterial association, the proteins which can have oral activity can be formulated as a sprayable insecticide. The gene encoding the oral toxic protein can be isolated and can be used in the production of transgenic plants.

There has been substantial progress in the cloning of gene encoding insecticidal toxins for both Photorhabdus luminescence and Xenorhabdus nematophila. Analysis of the genome of P. luminescens identified more predicted toxin genes than any other bacteria sequenced to date, including potential gene products with homology to hemolysin A, chitinase, Rtx (repeats-in-toxin)-like toxin, and ^-endotoxin (US patent no. 6590142, Duchaud et al., 2003, Nat. Biotechnol. 21: 1307-1313). The only toxins studied in detail are the Tc toxins from P. luminescens strain W14 (Guo et al., 1999, J Biol. Chem. 274: 9836-9842; Bowen et al., Appl. Environ. Microbiol. 64: 3029-3035), although a small amount of work has also been done on a 39-kDa toxin from X. nematophila (US patent no. 6841165; Rye et al., 2000, Biotechnol. Bioprocess Eng. 5: 141-145), the large Xin toxin from X. nematophila strain BJ (Pan et al., (2002) Perg Nat. Sci. 12: 310-312), and the PhIA hemolysin from P. luminescens strain TTOI (Brillard et al., J. Bacteriol. 184: 3871-3878).

Xenorhabdus nematophila secretes number of proteins in culture supernatant in enclosed outer membrane vesicles (OMV). OMV proteins have shown oral toxicity to Helicoverpa armigera. OMV proteins have also shown cytotoxicity on Sf21 insect cell lines (Khandewal et al., 2004, J. Bacteriol. 186: 6465-6476). One of the predominantly secreted proteins was sequenced and identified as XnGroEL, a chaperone homologue. The sequence similarity of protein with E. coli GroEL is 88% at amino acid level and was 84% at nucleotide sequence.


The present invention discloses a novel insecticidal protein isolated from *Xenorhabdus nematophila*. The present invention further discloses the process for purification of the novel insecticidal protein from *Xenorhabdus nematophila* and the cloning and expression of the gene encoding the novel insecticidal protein of *Xenorhabdus nematophila* and production of the novel recombinant insecticidal protein using recombinant DNA technology. The present invention also discloses that the purified recombinant protein binds to larval gut brush border membrane vesicles in insect mid-gut epithelium and has α-chitin binding activity.

**OBJECTS OF THE INVENTION**

It is an important object of the present invention to isolate and purify novel oral insecticidal protein from *Xenorhabdus nematophila*.

Another object of the present invention is to screen the genomic DNA library of the novel insecticidal protein from *Xenorhabdus nematophila*.

Another object of the present invention is to clone and express the DNA encoding the novel oral insecticidal protein from *Xenorhabdus nematophila*.

Yet another object of the present invention is to produce novel recombinant insecticidal protein from *Xenorhabdus nematophila*.

Still another object of the present invention is to provide a novel recombinant protein which has brush border membrane vesicles binding activity and α-chitin binding activity.

Yet another object of the present invention is to provide a novel recombinant protein which has brush border membrane vesicles binding activity and α-chitin binding activity.

**SUMMARY OF THE INVENTION**

The present invention discloses relates to a novel insecticidal protein named XnGroEL isolated and purified from *Xenorhabdus nematophila* having GENE BANK ACCESSION No. EF 451158.

In another embodiment of the present invention, the process for the purification of novel insecticidal protein named XnGroEL is disclosed.

In still another embodiment of the present invention, purification of the protein is done both from culture supernatant and cell lysate.

In yet embodiment of the present invention, a process for preparation and purification of recombinant novel insecticidal protein named XnGroEL isolated and
purified from *Xenorhabdus nematophila* is disclosed. The process involves construction of genomic DNA library, cloning in an expression vector, transformation of the cloned cells and purification of the recombinant protein.

In another embodiment of the present invention, a method for inhibiting the growth and controlling mortality of insects by administering novel insecticidal protein named XnGroEL against larvae of *Helicoverpa armigera* is disclosed.

In still another embodiment of the present invention, the recombinant insecticidal protein binds to larval gut brush border membrane vesicles proteins.

In yet another embodiment of the present invention, the recombinant novel insecticidal protein has $\alpha$-chitin binding activity.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Fig 1. Coomassie SDS-PAGE showing Q-Sepharose purified native XNGroEL fractions from *X. nematophila* cell lysate (Intracellular).

Fig 2. Chromatogram of FPLC purified intracellular XnGroEL.

Fig 3. Coomassie stained purified intracellular XnGroEL protein fractions.

Fig 4. Coomassie SDS-PAGE showing Q-Sepharose purified XnGroEL fractions from *X. nematophila* culture supernatant (Extracellular).

Fig 5. Chromatogram of FPLC purified extracellular XnGroEL.

Fig 6. Coomassie stained purified extracellular XnGroEL protein fractions.

Fig 7. PCR amplified 1.7kb XnGroEL gene.

Fig 8. Coomassie stained SDS-PAGE of induced and un-induced pellet.

Fig 9. Coomassie stained gel of purified fraction of recombinant XnGroEL protein by Ni-NTA chromatography.

Fig 10. XnGroEL protein Fed larvae. A (1) Control (2) 5µg (3) 10µg (4) 20µg.

B. Table-1 shows observed larval weight and mortality till pupation.

Fig 11. BBMV proteins interaction with XnGroEL protein.

Fig 12. Chitin binding assay.

**DETAILED DESCRIPTION OF THE INVENTION**

The present invention discloses a novel insecticidal protein named XnGroEL isolated and purified from *Xenorhabdus nematophila* having GENE BANK ACCESSION No. EF 451158. *X. nematophila* grown in 2L of LB medium yielded ~2mg/L of pure homogenous XnGroEL protein.

The process for the isolation of novel insecticidal protein named XnGroEL comprises of growing *X. nematophila* in a suitable media; harvesting the cells by centrifugation to obtain culture supernatant and cell lysate. Both the culture supernatant and cell lysate are processed separately to obtain purified protein.

The medium used for growing *Xenorhabdus nematophila* is Lysogeny Broth (LB) medium at particular temperature with moderate shaking. In yet another embodiment of the present invention, the centrifugation is done at 5,000 x g for 15 min. at 4°C.
The purification of the protein from the culture supernatant comprises of precipitating the supernatant with continuous stirring; centrifuging the precipitate; dialyzing the centrifugate against buffer A overnight at 4°C; loading the dialyzed protein on a 25 ml Q-Sepharose buffer A pre-calibrated column, followed by extensive washing with buffer A, buffer B and buffer C; eluting the fractions; checking the fractions on SDS-PAGE, pooling the desired protein, and dialyzing against Buffer A; concentrating the dialyzed by lyophilization; and further purifying the protein by Superose 12 gel filtration column in an FPLC system.

The purification of the protein from the cell lysate comprising washing and re-suspending the cells in 20 ml of buffer A; disrupting the cells by sonication; centrifugation of the cells; loading the centrifuged cells on a 25 ml Q-Sepharose buffer A pre-calibrated column; further purifying the protein. Further purification is done as in case of culture supernatant.

A process for preparation and purification of recombinant novel insecticidal protein named XnGroEL isolated and purified from Xenorhabdus nematophila is disclosed. The process comprising isolating genomic DNA from Xenorhabdus nematophila and digesting the DNA with restriction enzyme; cloning the DNA fragment of ~3.8kb with probe N-terminal amino acid sequence in a cloning vector; transforming the clone in a transformation vector; screening the positive clones by sequencing; amplifying the 1.7kb DNA fragment encoding novel insecticidal protein named XnGroEL by PCR using 3.8 kb DNA fragment as template; gel purifying the PCR amplified 1.7 kb fragment; cloning the gel purified PCR amplified 1.7 kb fragment in PCR cloning vector pGEMT-easy producing plasmid pMJ1; digesting the pMJ1 plasmid with restriction enzymes; cloning the 1.7 kb insert in an expression vector producing pMJ2 plasmid; transforming the pMJ2 plasmid in a transformation vector producing pMJ2 strain; growing the pMJ2 strain containing the said novel insecticidal protein in suitable medium; harvesting, washing and re-suspending the cells in buffer A; lysing the cells by sonication and purifying the protein from cell free supernatant by metal affinity chromatography.

A method for inhibiting the growth and controlling mortality of insects which comprises administering to insects novel insecticidal protein named XnGroEL from Xenorhabdus nematophila having GENE BANK ACCESSION No. EF 451 158 is disclosed. The novel insecticidal protein is effective against larvae of Heliothis armigera, when given orally in artificial diet, 1µg to 20µg of novel insecticidal protein in the artificial diet resulted in 15% to 50% reduction in weight of the larvae of Heliothis armigera and 1µg to 50µg of novel insecticidal protein in the artificial diet resulted in 35% to 95% mortality of the larvae of Heliothis armigera.

The recombinant insecticidal protein binds to larval gut brush border membrane vesicles proteins and has α-chitin binding activity.

The present invention is illustrated and supported by the following examples. These are merely representative examples and optimization details and are not intended to restrict the scope of the present invention in any way.
ISXAMPLE - 1: Purification of native XnGroEL protein (Extracellular) purification from culture supernatant

X. nematophila was grown in 2L of LB medium for 180 rpm at 28 (+/-1)°C with moderate shaking at 180 rpm. Cells were harvested by centrifugation (5,000 x g for 15 min at 4°C) and both the pellet and supernatant was processed separately for purification of the GroEL protein. The culture supernatant was precipitated by 70% ammonium sulfate (472gm per liter) with continuous stirring for 16 hrs at 4°C. The precipitate was collected by centrifugation (12,000 x g for 30 min at 4°C) and dialyzed against Buffer A (50mM Sod. Phosphate buffer pH-7.0 with ImM (PMSF) overnight at 4oc. The dialyzed proteins were loaded on a 25 ml Q-Sepharose Buffer A pre-equilibrated column. The column was washed extensively with buffer A and followed by buffer B (Buffer A + 150 mM NaCl) and Buffer C (Buffer A + 30OnMNaCl) to remove the unbound proteins. GroEL protein was eluted with 100ml of a NaCl gradient (0.3N-1.0 N) (Fig 1).

The fractions were checked on SDS-PAGE; those fractions containing desired protein were pooled and dialyzed against Buffer A for overnight at 4°C. The dialyzed protein was concentrated by lyophilization and further purified by Superose 12 gel filtration column in an FPLC system. The column was equilibrated with Buffer B and 0.5ml of concentrated proteins from previous steps were loaded on the column and eluted with 1 bed volume of buffer B. 1ml fractions were collected and checked by 12% SDS PAGE (Fig 3) XnGroEL protein was eluted in the void volume fractions in oligomeric form (Fig 2). The void volume fractions 5-7 contained pure homogeneous XnGroEL protein with a yield of~2mg/L.

EXAMPLE - 2: Purification of native XnGroEL protein (Intracellular) protein purification from Cell lysate

The X. nematophila cell pellet after removal of the supernatant in the previous step was used for isolation of intracellular GroEL protein. Cells were washed and resuspended in 20 ml of Buffer A, the cells were disrupted by sonication (Vibro Sonics) for 15 min with an intermittent pulse of 5.0 sec. on and 5.0 sec off at 25% amplitude. The sonicated suspension was centrifuged (12,000 X g for 30 min at 4°C) and the supernatant was loaded on a 25ml Q-Sepharose column Buffer A equilibrated. Further purification was carried out as described earlier for extracellular protein. The purified protein yield was~3mg/L (Fig 4, 5 and 6)

EXAMPLE - 3: Construction of Genomic DNA library and cloning of GroEL gene

High molecular weight Genomic DNA was isolated from X. nematophila strain 19061 as described previously (Hammer 1961). Genomic DNA fragments were generated with different restriction enzyme and probed with N-terminally amino acid sequence deduced 45bp nucleotide fragment. DNA fragment of ~3.8kb reacting with probe in the EcoRI digested DNA was cloned in pUC18 cloning vector and transformed in to E. coli DH5αcells. Colonies were screened and positive clones were confirmed by sequencing.

1.7kb DNA encoding the GroEL gene was amplified by PCR using primer designed from the 5’ and 3’ end of the gene and the 3.8 kb cloned fragment as template. 1.7kb DNA fragment was gel purified and cloned in pGEMTeasy (Promega) vector resulting in pMJ1 plasmid. Positive clones were confirmed by sequencing.

EXAMPLE - 4: Expression and Purification of recombinant GroEL protein
pMJ1 plasmid was digested with BamHI and HindIII and the 1.7kb insert was cloned in pET28a expression vector resulting in pMJ2 plasmid and transformed in E.coli BL21DE3 and pMJ2 strain was produced. PMJ2 cells were grown in 5ml LB medium containing 50µg/ml Kanamycin at 37°C for 16hr with shaking at 200rpm and subcultured in 50ml fresh medium till an OD600~0.5, then induced with 1mM IPTG (Isopropyl-thiogalactopyranoside) and incubated for 3-4 hours. The cells were harvested, washed and resuspended in 5ml of bufferA (50mM Sod. Phosphate buffer, pH-7.0). The cells were lysed by sonication and the protein from cell free supernatant was purified by Ni-NTA agarose affinity matrix in cold using standard protocol checked by SDS-PAGE. All the procedures were carried out at 4°C.

**EXAMPLE - 5:** Evaluation of oral insecticidal activity

For larvicidal assays, different concentrations of native as well as recombinant GroEL protein preparations were diluted in 10 mM sodium phosphate buffer (pH 8.0) mixed in artificial diet as described before. Each group contained 30 neonates of *Heicoperpa armigera*. Larvae were placed on the surface of the diet and the plates were incubated at 25°C (16hrs-day-length period) with 80% relative humidity. Mortality and larval weight were recorded periodically over the entire larval period. The dose of protein shown in the results was the amount of protein mixed into the diet and was not always the actual amount consumed by the larvae. Heat-inactivated GroEL protein, BSA, GroEL homologue from E. coli K-12, and buffer were used as controls. The 50% lethal dose (LD50) was determined by probit analysis and statistical analysis of the data was done by using ANOVA.

**EXAMPLE - 6:** Binding of GroEL protein with BBMV proteins

1µg of BBMV proteins were incubated with 1µg of XnGroEL or mutant proteins and incubated at room temperature for 30 min followed by centrifugation at 12,000 x g for 5 min in cold to remove the unbound protein. The pellet was washed with 30µl of PBS twice and resuspended in 20µl of PBS. The samples were boiled in laemmli's sample dye for 5 min and resolved by SDS-PAGE. The proteins were transferred on nitrocellulose membrane and blotted with anti-GroEL antibodies diluted 1:40,000.

**EXAMPLE - 7:** Chitin binding assays

Binding of the wild type GroEL protein with α-chitin (Sigma Aldrich) was evaluated. In initial binding, reaction mixture (1ml) containing α-chitin (0.5mg/ml) substrate was incubated with a 5, 25, 50, 100, 150 and 200 µg/ml protein in 50mM Tris. Cl buffer, pH 7.0. The reaction mixture was rotated at 120 rpm at room temperature for 16hr. The samples were centrifuged for 4 min at 13,000 rpm to pellet the chitin, and the A280 values of supernatant were measured. Apparent extinction co-efficient calculated from the respective A280 standard curves were subsequently used to convert A280 values to protein concentration. All assays were performed in triplicates and with a blank buffer. The equilibrium dissociations constants, Kd (µM), and substrate binding capacities, B_max (µmol/gm) were determined by fitting the binding isotherm to one site binding equation where p stands for protein: \[ [\text{Pbound}] = B_{\text{max}} [\text{Pfree}]/K_d + [\text{Pfree}] \], by non-linear regression using the Graphpad Prism software (Demo version, GraphPad Software Inc., San Diego, CA) (Fig 12). The Kd value of XnGroEL protein is 0.6273 µM and has a binding capacity 4.721( µmol/gm).
We claim:
1. A novel insecticidal protein named XnGroEL isolated and purified from Xenorhabdus nematophila having GENE BANK ACCESSION No. EF 451 158.
2. The novel oral insecticidal protein as claimed in claim 1, wherein the said protein is isolated from Xenorhabdus nematophila ATCC 1906 1.
3. The polynucleotide sequence as claimed in claim 2, wherein the size of polynucleotide sequence is 1.7 kB.
4. A process for the isolation and purification of novel insecticidal protein named XnGroEL from Xenorhabdus nematophila comprising:
   a. growing Xenorhabdus nematophila in suitable medium;
   b. harvesting the cells obtained from step (a) by centrifugation to obtain culture supernatant and cell lysate;
   c. separately purifying the protein from culture supernatant and cell lysate obtained from step (b).
5. The process as claimed in claim 5, wherein said growing of step (a) is done in Lysogeny Broth (LB) medium at 28°C ±1 with moderate shaking at 180 rpm.
6. The process as claimed in claim 5, wherein said centrifugation of step (b) is done at 5,000 x g for 15 min. at 4°C.
7. The process as claimed in claim 5, wherein the said purifying of the protein of step (c) from the culture supernatant comprising:
   a. precipitating the supernatant with continuous stirring;
   b. centrifuging the precipitate obtained from step (a);
   c. dialyzing the centrifugate obtained from step (b) against buffer A overnight at 4°C;
   d. loading the dialyzed protein obtained from step (c) on a 25 ml Q-Sepharose buffer A pre-calibrated column, followed by extensive washing with buffer A, buffer B and buffer C;
   e. eluting the fractions from the column of step (d);
   f. checking the fractions obtained from step (e) on SDS-PAGE, pooling the desired protein, and dialyzing against Buffer A.
   g. concentrating the dialyzed protein obtained from step (f);
   h. further purifying the protein obtained from step (g).
8. The process as claimed in claim 8, wherein said precipitating of step (a) is done by 70% ammonium sulphate with continuous stirring for 16 hrs at 4°C.
9. The process as claimed in claim 8, wherein said centrifuging of step (b) is done at 12,000 x g for 30 min. at 4°C.
10. The process as claimed in claim 8, wherein buffer A of step (c) is 50 mM sodium phosphate buffer with 1 mM phenylmethysulphonyl fluoride at pH 7.0.
11. The process as claimed in claim 8, wherein buffer B of step (d) is buffer A with 150 mM sodium chloride and buffer C is buffer A with 300 mM sodium chloride.
12. The process as claimed in claim 8, wherein said eluting of step (e) is done with sodium chloride gradient (0.3N-1.0N).
13. The process as claimed in claim 8, wherein said concentrating of step (g) is done by lyophilization.
14. The process as claimed in claim 8, wherein said further purifying of step (h) is done by Superose 12 gel filtration column in an FPLC system.
15. The process as claimed in claim 5, wherein said purifying of the protein of step (c) from the cell lysate comprising:
   a. washing and re-suspending the cells in 20 ml of buffer A;
   b. disrupting the cells obtained by step (a) by sonication;
   c. centrifugation of the cells obtained by step (b);
   d. loading the centrifuged cells obtained by step (c) on a 25 ml Q-Sepharose buffer A pre-calibrated column;
   e. further purifying the protein as described in claims 8 (d) to (h).
16. The process as claimed in claim 16, wherein the sonication of step (b) is done for 15 min. with a intermittent pulse of 5.0 sec. on and 5.0 sec. off at 25% amplitude.
17. The process as claimed in claim 16, wherein the centrifugation of step (c) is done at 12,000 x g for 30 min. at 40°C.
18. A process for preparation and purification of recombinant novel insecticidal protein named XnGroEL isolated and purified from Xenorhabdus nematophila comprising:
   a. isolating genomic DNA from Xenorhabdus nematophila and digesting the DNA with restriction enzyme.
   b. cloning the DNA fragment of ~3.8kb obtained by step (a) with probe N-terminal amino acid sequence in a cloning vector;
   c. transforming the clone obtained by step (b) in a transformation vector;
   d. screening the positive clones obtained by step (c) by sequencing;
   e. amplifying the 1.7kb DNA fragment obtained by step (d) encoding novel insecticidal protein named XnGroEL by PCR using 3.8 kb DNA fragment as template;
   f. gel purifying the PCR amplified 1.7 kb fragment obtained by step (e);
   g. cloning the gel purified PCR amplified 1.7 kb fragment obtained by step (f) in PCR cloning vector pGEMT-easy producing plasmid pMJ1;
   h. digesting the pMJ1 plasmid obtained by step (g) with restriction enzymes;
   i. cloning the 1.7 kb insert obtained by step (h) in an expression vector producing pMJ2 plasmid;
   j. transforming the pMJ2 plasmid obtained by step (i) in a transformation vector producing pMJ2 strain;
k. growing the pMJ2 strain containing the said novel insecticidal protein obtained by step (j) in suitable medium;

m. lysing the cells obtained by step (i) by sonication and purifying the protein from cell free supernatant by meted affinity chromatography.

19. The process as claimed in claim 19, wherein the restriction enzyme of step (a) is EcoRI.

20. The process as claimed in claim 19, wherein the cloning vector of step (b) is pUC18.

21. The process as claimed in claim 19, wherein the transformation vector of step (c) is E. coli DH5α cells.

22. The process as claimed in claim 19, wherein the restriction enzymes of step (h) BamHI and Hind III.

23. The process as claimed in claim 19, wherein the expression vector of step (i) is pET28a.

24. The process as claimed in claim 19, wherein the transformation vector of step (j) is E. coli BL21DE3.

25. The process as claimed in claim 19, wherein medium of step (k) is Lysogeny Broth medium containing kanamycin at 37°C for 16 hrs with shaking at 200 rpm, sub-culturing in fresh medium till O.D600~0.5, inducing with ImM isopropylthiogalactopyranoside, and incubating for 3-4 hrs.

26. The process as claimed in claim 19, wherein buffer A of step (i) is 50 mM sodium phosphate buffer with 1 mM phenylmethylsulphonyl fluoride at pH 7.0.

27. The process as claimed in claim 19, wherein the metal of step (m) is nickel.

28. A method for inhibiting the growth and controlling mortality of insects which comprises administering to insects novel insecticidal protein named XnGroEL from Xenorhabdus nematophilus having GENE BANK ACCESSION No. EF 451 158.

29. The method as claimed in claim 29, wherein the said novel insecticidal protein is native GroEL protein of Xenorhabdus nematophilus.

30. The method as claimed in claim 29, wherein the said novel insecticidal protein is recombinant GroEL protein of Xenorhabdus nematophilus.

31. The method as claimed in claim 29 to 31, wherein the said insect is Helicoverpa armigera.

32. The method as claimed in claim 29 to 32, wherein the said novel insecticidal protein is effective against larvae of Helicoverpa armigera.

33. The method as claimed in claim 29 to 33, wherein the said step of administration comprises oral administration.

34. The method as claimed in claim 29 to 34, wherein the said step of administration comprises oral administration in artificial diet.
35. The method as claimed in claim 29 to 35, comprising:
   a. diluting the said novel insecticidal protein in a buffer;
   b. mixing the protein obtained by step (a) in artificial diet;
   c. placing the larvae of Helico\textit{v}erpa \textit{armigera} on the surface of the diet of step (b) in plates;
   d. incubating the plates of step (d).

36. The method as claimed in claim 36, wherein the buffer of step (a) is 10mM sodium phosphate buffer at pH 8.0.

37. The method as claimed in claim 36, wherein the incubation of step (d) is done at 25°C at 16 hrs-day-length period with 80% relative humidity.
Fig 1.
Fig 2.
Void Volume fractions

98 kda  →  66 kda  →  45 kda  →  35 kda  →  21 kda  →  14 kda

Fig 3.
Fig. 4:
Fig 5.
Fig 6.
Fig 8.
Fig 9.
A.

1.  

2.  

3.  

4.  

B.

Fig 10.
Table 1

<table>
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<th>Conc. (µg)</th>
<th>6th Day observation</th>
<th>8th Day observation</th>
<th>Pupation</th>
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<td></td>
<td>Mortality</td>
<td>Avg. weight (mg)</td>
<td>Mortality</td>
</tr>
<tr>
<td>Buffer</td>
<td>2/24</td>
<td>106.8</td>
<td>2/24</td>
</tr>
<tr>
<td>Heated</td>
<td>2/24</td>
<td>104.2</td>
<td>2/24</td>
</tr>
<tr>
<td>U.T</td>
<td>1/24</td>
<td>112.2</td>
<td>1/24</td>
</tr>
<tr>
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<td>4/24</td>
<td>95.5</td>
<td>6/24</td>
</tr>
<tr>
<td>5 µg</td>
<td>6/24</td>
<td>90.2</td>
<td>8/24</td>
</tr>
<tr>
<td>10 µg</td>
<td>8/24</td>
<td>75.4</td>
<td>12/24</td>
</tr>
<tr>
<td>20 µg</td>
<td>10/24</td>
<td>69.5</td>
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<tr>
<td>50 µg</td>
<td>10/24</td>
<td>60.5</td>
<td>16/24</td>
</tr>
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</table>

B. Table 1 shows the observed larval weight and mortality till pupation.

Fig 10. (Contd.)
Fig 11.
Fig 12.