

Faculty of Education  
Department of Biological and Geological Sciences

## **Isolation and Molecular Characterization of Some Common *Bt* Cry Protein Receptor Genes from Certain Living Organisms.**

A THESIS  
SUBMITTED FOR PhD FOR TEACHER PREPARATION IN SCIENCE  
(Zoology)

BY

**HEBA MOHAMED YASSIN ABDEL- GHAFAR**

General Diploma for Teacher Preparation in Science (Zoology) - 2002

Special Diploma for Teacher Preparation in Science (Zoology) – 2003

Master Degree for Teacher Preparation in Science (Zoology) – 2007

**SUPERVISED BY**

**Prof. Dr. MOHAMED ABD ELHAMID SHAHIN**

Professor of Experimental Embryology  
Department of Biological and Geological Sciences  
Faculty of Education – Ain Shams University.

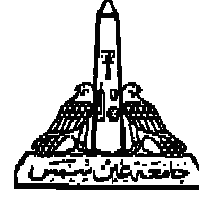
**Prof. Dr. MOHAMED SAYED SALAMA**

Professor of Molecular Biology and Vice President of Ain Shams University  
for Postgraduate studies and Research.

**Prof. Dr. SALAH ALI MOSTAFA**

Chief Researcher of Molecular Microbiology and  
Head of Molecular Microbiology Department –  
Agriculture Genetic Engineering Research Institute (AGERI)

**2011**



كلية التربية  
قسم العلوم البيولوجية والجيولوجية

# عزل وتوصيف جزيئي لبعض جينات مستقبلات بروتينات الـ *Bt* من كائنات حيه معينة.

رسالة مقدمة من

هبة محمد يس عبد الغفار

للحصول على درجة دكتوراه الفلسفة لإعداد المعلم في العلوم  
(تخصص علم الحيوان)

تحت إشرافه

أستاذ علم الأجنه التجريبي - قسم العلوم البيولوجية  
والجيولوجية - كلية التربية - جامعة عين شمس

أ.د. / محمد عبد الحميد شاهين

أستاذ البيولوجيا الجزيئية ونائب رئيس جامعة عين  
شمس لشئون الدراسات العليا والبحوث.

أ.د. / محمد سيد سلامة

رئيس بحوث ورئيس قسم الميكروبيولوجيا  
الجزيئية - معهد بحوث الهندسه الوراثيه  
الزراعيه - مركز البحوث الزراعيه

أ.د. / صلاح على مصطفى

2011

# ABSTRACT

*Bacillus thuringiensis* (*Bt*) crystal proteins are effective in controlling agriculturally harmful insects. However, the mechanism of *Bt* pesticidal action is fairly understood. It is assumed that the pesticidal protein has affinity for specific receptors in the midgut of the susceptible larvae and binds irreversibly to create holes in the gut leading to eventual death of the target larvae.

The main aim of the research is to identify and characterize *Bt*  $\delta$ -endotoxin receptor in an important pest, Egyptian Cotton leafworm (*Spodoptera littoralis*).

Biological activities of crystal protoxins of *BtkHD1* strain and Cry1C were determined by *in vitro* mortality assay on neonate larvae of *S. littoralis*. Also, Brush border membrane vesicles (BBMVs) were prepared from the 5<sup>th</sup> instar larvae of the same insect. Subsequently, immunoblotting experiments were performed using Cry1A *Bt*  $\delta$ -endotoxins to detect the presence of specific toxin binding proteins on BBMVs. The toxin binds to proteins of 108.58 kDa on brush border membrane vesicles. It is therefore a strong candidate for *in vitro* *S. littoralis* Cry1 toxin receptor.

Specific primers for *S. littoralis* APN (*SlAPN*) were designed according to the nucleotide sequence of *Spodoptera litura* APN; GenBank accession number (AF320764). The full length of APN gene from that insect was obtained and it was cloned in pGEMT-easy vector and pET-30a and pPICZ A expression vectors.

The cloned gene was characterized through sequencing and studying expression in *E. coli* and *Pichia*

---

---

**ABSTRACT**

*pastoris*. The expressed protein was analyzed by Western blotting with anti- Cry toxin antibody. The Western blotting showed that the expression of target protein was detected by toxin. This analysis proved that APN protein exhibited binding properties towards Cry1A toxin. The sequence of *SlAPN* gene was deposited in GenBank under accession number JF509138.

The predicted amino acid sequence of *SlAPN* cloned gene encoded a putative 952 amino acid residues. The sequence possesses three conserved regions which are zinc-binding/gluzincin motif HEX<sub>2</sub>HX<sub>18</sub>E, the third zinc-binding ligand and gluzincin aminopeptidase motif GAMEN. Furthermore, five potential N-linked glycosylation sites (NXS/T) beside four highly conserved Cys residues are also observed. Finally, a highly conserved 64 amino acid residues from Leu 129 to Pro 193 was also detected.

Based on computational alignment, a theoretical 3-D model structure of *SlAPN* was obtained. Whereas, about 814 aa residues of whole suggested 3-D model were corresponded to residues 58-871 of the primary structure. The present *SlAPN* model contained four structural domains, which spread from N-terminal domain I to C-terminal domain IV over the regions Asn<sup>58</sup>-Ile<sup>266</sup>; Ser<sup>267</sup>-Gly<sup>506</sup>; Asn<sup>507</sup>-Leu<sup>581</sup> and Ser<sup>582</sup>-Ala<sup>871</sup>, respectively. The recorded overall dimensions of this model were 91A° x 55 A° x 65A° forming together a hook like structure.

---

Key words:

*Bacillus thuringiensis*; δ-Endotoxin; Aminopeptidase N receptor; *Spodoptera littoralis*; 3-D homology modeling; *Pichia pastoris*.

# CONTENT

<b>ABSTRACT</b> .....	
<b>ACKNOWLEDGMENT</b> .....	
<b>ABBREVIATIONS</b> .....	I
<b>LIST OF FIGURES</b> .....	IV
<b>LIST OF TABLES</b> .....	IX
<b>INTRODUCTION &amp; AIM OF THE WORK</b> .....	1
<b>REVIEW OF LITERATURE</b> .....	3
Control of <i>Spodoptera littoralis</i> .....	3
General aspects of <i>Bacillus thuringiensis</i> ( <i>Bt</i> ) .....	4
Diversity and structure of Cry $\delta$ -endotoxin proteins.....	5
Mechanism of action of Cry toxin.....	7
Models for insertion of insecticidal Cry toxins into insect receptors.....	9
Receptor molecules of Cry toxins in lepidopteran BBMVs.....	10
The structure and main functions of GPI-anchored aminopeptidase-N receptors.....	11
APN as a Cry toxin receptor.....	14
Sites on Cry toxins that binds to APN receptors.....	15
Morphological and functional description of larval midgut cells.....	17
The role of midgut pH of Lepidoptera in activation of Cry protoxin.....	19

The effect of gut enzymes in proteolytic activation of Cry protoxin.....	20
<i>Bt</i> as a microbial control agent.....	21
Homology modeling.....	22
<i>Escherichia coli</i> verses <i>Pichia pastoris</i> expression systems.....	24
<b>MATERIALS AND METHODS.....</b>	<b>26</b>
<b>1- Identification of a <i>Spodoptera littoralis</i> BBMV's receptor protein that binds to Cry toxin.....</b>	<b>26</b>
1-1- Insect rearing and sample collection.....	26
1-2- Bacterial culture of <i>Bacillus thuringiensis</i> .....	26
1-3- Preparation and solubilization of parasporal crystal toxins.....	27
1-4- Insect toxicity assays.....	28
<b>2- Binding characteristics of receptor protein on BBMV's of <i>Spodoptera littoralis</i>.....</b>	<b>28</b>
2-1- Midgut isolation and tissue preservation.....	28
2-2- Preparation of midgut brush border membrane vesicles (BBMV's) .....	29
2-3- SDS-PAGE electrophoresis.....	30
2-4- Immunodetection of specific Cry toxin receptors.....	33
<b>3-Cloning, sequencing and characteristics of <i>Spodoptera littoralis</i> APN (<i>SlAPN</i>) Cry toxin receptor gene.....</b>	<b>34</b>
3-1- RNA preparation.....	35
3-1-a- Isolation of the Total RNA from the midgut of <i>Spodoptera littoralis</i> and <i>Bombyx mori</i> .....	35
3-1-b- RNA concentration and purity determination....	35
3-2- Preparation of cDNA.....	36
3-3- primers and PCR reaction designs.....	37

3-3-a- Primers used for screening APN and Cadherin receptors.....	37
3-3-b- Preparation of PCR Reactions.....	38
3-3-c- PCR Program and temperature profile.....	39
3-3-d- Agarose Gel Electrophoresis.....	39
3-3-e- Purification of desired APN fragments from the agarose using QIAGEN gel extraction kit.....	40
3-4- Cloning of desired APN fragments into appropriate cloning vector.....	41
3-4-a- Preparation of cloning vectors.....	41
3-4-b- Ligation of Vector and Insert.....	41
3-4-c- Preparation of competent cells (Calcium Chloride Method).....	43
3-4-d- Transformation of Competent Cells with plasmid DNA.....	44
3-4-e- Master plate preparation and rapid screening of recombinant clones.....	45
3-4-f- Isolation of Plasmids from <i>E. coli</i> by Boiling Lysis.....	45
3-4-g- Spectrophotometric Determination of Plasmid Concentration.....	47
3-4-h- Screening of Recombinant clones using restriction enzyme digestion analysis.....	47
3-5- Sequencing and analysis.....	48
<b>4- Bioinformatics analysis.....</b>	<b>49</b>
4-1- Multiple nucleotide sequence alignment analysis....	49
4-2- Bioinformatics analysis of the deduced amino acid sequence.....	49
4-3- The prediction of 3-D structure of <i>SlAPN</i> using homology modeling.....	50
<b>5- Expression of <i>Spodoptera littoralis</i> APN (<i>SlAPN</i>) Cry toxin receptor gene.....</b>	<b>51</b>

5-1-	Expression of <i>SlAPN</i> receptor protein in enterobacterium <i>Escherichia coli</i> .....	51
5-1-a-	Subcloning of 2185 bp APN fragment excised from pGEM-T easy vector into pET30a vector.....	51
5-1-b-	Screening Recombinant DNA.....	51
5-1-c-	IPTG induction for recombinant protein expression.....	53
5-1-d-	Screening of recombinant protein in bacterial cells using SDS-PAGE.....	53
5-2-	Expression of <i>SlAPN</i> receptor protein in methylotrophic yeast <i>Pichia pastoris</i> .....	54
5-2-a-	Construction of vector pPICZA/ F1 <i>SlAPN</i> .....	54
5-2-b-	Preparation of pPICZA/ F1 <i>SlAPN</i> plasmid.....	55
5-2-c-	Preparation of <i>P. pastoris</i> competent cells for electroporation.....	55
5-2-d-	Integration of pPICZA/ F1 <i>SlAPN</i> vector into the <i>P. pastoris</i> genome.....	55
5-2-e-	Isolation of genomic DNA from <i>P. pastoris</i> .....	56
5-2-f-	PCR amplification analysis of F1 <i>SlAPN</i> fragment from genomic DNA of <i>P. pastoris</i> transformant...57	
5-2-g-	Expression of recombinant <i>Pichia</i> Strains.....	58
5-2-h-	Expression analysis of recombinant <i>Pichia</i> Strains by using SDS-PAGE electrophoresis and Western blotting.....	59
	<b>RESULTS AND OBSERVATIONS.....</b>	<b>60</b>
<b>1-</b>	<b>Identification of a <i>Spodoptera littoralis</i> BBMV's receptor protein that binds to Cry toxin.....</b>	<b>60</b>
1-1-	Toxicity of Cry protoxin of <i>BtkHD1</i> strain and Cry1C to <i>S. littoralis</i> .....	60
<b>2-</b>	<b>Binding characteristics of receptor protein on BBMV's of <i>Spodoptera littoralis</i>.....</b>	<b>62</b>



2-1- Identification of BBMV's protein that binds to Cry1 toxin.....	62
<b>3- Cloning, sequencing and characteristics of <i>Spodoptera littoralis</i> APN (SIAPN) Cry toxin receptor gene.....</b>	<b>63</b>
3-1- RNA isolation, Primer selection and reverse transcriptase polymerase chain reaction (RT-PCR)..	63
3-2- Cloning of <i>SIAPN</i> fragments and screening for recombinant clones.....	67
3-3- Reconstructing of Full-length cDNA of <i>SIAPN</i> .....	70
3-4- Cloning of the reconstructed full-length cDNA ( <i>Sifl</i> ) of <i>SIAPN</i> .....	72
3-5- Sequencing of <i>SIAPN</i> .....	75
<b>4- Bioinformatics analysis.....</b>	<b>75</b>
4-1- <i>SIAPN</i> nucleotide sequence and their BLAST search results.....	75
4-2- Phylogenetic analysis of the <i>SIAPN</i> sequence.....	78
4-3- Characterization of deduced protein sequence of <i>SIAPN</i> .....	84
4-4- Prediction of the three-dimensional structure of <i>SIAPN</i> protein using homology modeling.....	87
<b>5- Expression of <i>Spodoptera littoralis</i> APN (SIAPN) Cry toxin receptor gene.....</b>	<b>92</b>
5-1- Expression of truncated <i>SIAPN</i> in <i>E. coli</i> .....	92
5-2- Expression of <i>SIAPN</i> receptor protein in methylotrophic yeast <i>Pichia pastoris</i> .....	99
<b>DISCUSSION.....</b>	<b>106</b>
1-Identification of a <i>Spodoptera littoralis</i> BBMV's receptor protein that binds to Cry toxin.....	106
2-Binding characteristics of receptor protein on BBMV's of <i>Spodoptera littoralis</i> .....	110

---

---

**CONTENT**

3-Cloning, sequencing and characteristics of <i>Spodoptera littoralis</i> APN (SlAPN) Cry toxin receptor gene.....	112
4-Bioinformatics analysis using homology modeling to predict the three-dimensional structure of <i>S. littoralis</i> APN receptor.....	116
5-Expression of <i>Spodoptera littoralis</i> APN (SlAPN) Cry toxin receptor gene.....	120
<b>SUMMARY</b> .....	124
<b>REFERENCES</b> .....	127
<b>ARABIC SUMMARY</b> .....	155

# LIST OF ABBREVIATIONS

<b>A260</b>	Absorbance at 260 nanometer
<b>A280</b>	Absorbance at 280 nanometer
<b>AIP</b>	Alkaline Phosphatase
<b>Amp</b>	Ampicillin
<b>APN</b>	Aminopeptidase-N
<b>APS</b>	Ammonium peroxosulphate
<b>ATP</b>	Adenosine triphosphate
<b>BBMV</b>	Brush Border Membrane Vesicle
<b>BCIP</b>	5-bromo 4-chloro 3-indolyl phosphate
<b>BLAST</b>	Basic local alignment search tool
<b>BLASTn</b>	Nucleotide blast (Search a nucleotide database using a nucleotide query)
<b>BLASTx</b>	Search protein database using translated nucleotide query
<b><i>Bm</i></b>	<i>Bombyx mori</i>
<b>bp</b>	Base pair
<b><i>Bm</i>APN</b>	<i>Bombyx mori</i> Aminopeptidase-N
<b>BSA</b>	Bovine Serum Albumin
<b><i>Bt</i></b>	<i>Bacillus thuringiensis</i>
<b><i>Btk</i></b>	<i>Bt</i> subsp. <i>krustaki</i>
<b>cDNA</b>	Complementary DNA
<b>Cry toxin</b>	Crystal toxin
<b>C- terminal</b>	Carboxy terminal

---



---

**LIST OF ABBREVIATIONS**

<b>DTT</b>	Dithiothreitol
<b>DEPC</b>	Diethylpyrocarbonate
<b>dH<sub>2</sub>O</b>	Distilled Water
<b>DNA</b>	Deoxyribonucleic Acid
<b><i>E. Coli</i></b>	<i>Escherichia coli</i>
<b>F1 <i>SlAPN</i></b>	A segment of <i>SlAPN</i> (from bp 1 to 2185) was excised from recombinant vector pGEM T-Easy/ <i>Slfl</i> with <i>EcoR</i> I
<b>GPI</b>	glycosylphosphatidyl-inositol
<b>IPTG</b>	Isopropyl-β-D-thiogalactopyranoside
<b>Kb</b>	Kilobase(s)
<b>KDa</b>	Kilodalton(s)
<b>LB broth</b>	Luria-Bertani broth
<b>LC50</b>	50% lethal concentration
<b>μg</b>	Microgram(s)
<b>min</b>	Minute(s)
<b>MW</b>	Molecular Weight
<b>NBT</b>	Nitro blue tetrazolium chloride
<b>OD</b>	Optical density
<b>ORF</b>	Open Reading Frame
<b>PCR</b>	Polymerase Chain Reaction
<b>PEG</b>	Polyethylene glycol
<b>PFT</b>	Pore-Forming Toxins
<b><i>P. pastoris</i></b>	<i>Pichia pastoris</i>
<b>PM</b>	Peritrophic Membrane
<b>PMSF</b>	Phenylmethylsulfonyl fluoride
<b>Ppm</b>	Part per million

---

---

**LIST OF ABBREVIATIONS**

<b>PVDF</b>	Polyvinylidene difluoride
<b>RE</b>	Restriction Endonucleases
<b>RNA</b>	Ribonucleic Acid
<b>rpm</b>	Revolution per minutes
<b>RT-PCR</b>	Reverse transcription- PCR
<b>SDS-PAGE</b>	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
<b><i>Sl</i></b>	<i>Spodoptera littoralis</i>
<b><i>SlAPN</i></b>	<i>Spodoptera littoralis</i> APN
<b><i>Slfl</i></b>	Full-length cDNA of <i>SlAPN</i>
<b><i>Sl7</i></b>	5'-truncated 1601 bp fragment of <i>SlAPN</i>
<b><i>Sl8</i></b>	3'- truncated 1359 bp fragment of <i>SlAPN</i>
<b>TEMED</b>	Tetramethylethylenediamine
<b>U</b>	Unit
<b>UV</b>	Ultraviolet
<b>W/V</b>	Weight Per Volume
<b>X-gal</b>	5-bromo 4-chloro 3-indolyl $\beta$ - D- galactopyrannoside

## LIST OF FIGURES

<b>Fig. No.</b>	<b>Figure Title</b>	<b>Page. No.</b>
<b>1</b>	pGEM®-T Easy Vector circle map and sequence reference points.	<b>42</b>
<b>2</b>	pUC 18 Vector circle map and multiple cloning sites.	<b>43</b>
<b>3</b>	pET30a Expression Vector circle map and sequence reference points.	<b>52</b>
<b>4</b>	The map summarizes the features of the pPICZA vectors.	<b>54</b>
<b>5</b>	Toxicity regression lines of nonactivated protoxin of <i>Btk</i> HD1 and Cry1C proteins to the 1 <sup>st</sup> larval instar of <i>Spodoptera littoralis</i> .	<b>61</b>
<b>6</b>	SDS-PAGE gel with coomassie blue staining of <i>B. thuringiensis</i> subsp. <i>kurstaki</i> HD-1.	<b>62</b>
<b>7</b>	SDS-PAGE and immunoblot analysis of <i>Bm</i> (lanes 1, 3, 5) and <i>Sl</i> midgut proteins (lanes 2, 4, 6).	<b>63</b>
<b>8</b>	Electrophoretic patterns of total RNA from 5 <sup>th</sup> larval stage midgut tissue of <i>S. littoralis</i> (lane1) and <i>B. mori</i> (lane2).	<b>64</b>
<b>9</b>	RT-PCR analysis of <i>Sl</i> APN.	<b>65</b>
<b>10</b>	RT-PCR analysis of <i>Bm</i> APN.	<b>66</b>

<b>Fig. No.</b>	<b>Figure Title</b>	<b>Page. No.</b>
<b>11</b>	RT-PCR screening of Cadherin gene of <i>B. mori</i> .	<b>66</b>
<b>12</b>	PCR amplification of the <i>Sl7</i> (1600 bp) APN fragment.	<b>68</b>
<b>13</b>	PCR amplification of the <i>Sl8</i> (1359 bp) APN fragment.	<b>68</b>
<b>14</b>	Screening of recombined plasmid pGEM T-Easy vector/ <i>Sl7</i> using <i>EcoR1</i> digestion.	<b>69</b>
<b>15</b>	Screening of recombined plasmid pUC18 vector/ <i>Sl8</i> using <i>EcoR1</i> digestion.	<b>70</b>
<b>16</b>	Schematic representation of full length cDNA of <i>SlAPN</i> ( <i>Slfl</i> ) reconstructing strategy.	<b>71</b>
<b>17</b>	Full-length cDNA of <i>SlAPN</i> ( <i>Slfl</i> ).	<b>72</b>
<b>18</b>	The strategy for construction of pGEM T- Easy vector with full-length cDNA of APN of <i>S. littoralis</i> ( <i>Slfl</i> ).	<b>73</b>
<b>19</b>	PCR amplification of the 2882 bp APN fragment.	<b>74</b>
<b>20</b>	Screening of recombined plasmid pGEM T-Easy vector/ <i>Slfl</i> using <i>EcoR1</i> digestion.	<b>74</b>
<b>21</b>	Schematic representation of primers used in sequencing of full-length <i>SlAPN</i> .	<b>75</b>