Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2019; 7(6): 304-310

Received: 09-09-2019
Accepted: 13-10-2019

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Exploitation of insect neuropeptides in pest management as a novel strategy: A review

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Abstract
Neuropeptides are produced in the cell body of neuron and released into the haemolymph which regulate major bio-chemical, physiological and behavioural activities in insects. Proctolin was the first neuropeptide isolated from *Periplanta americana* L. during 1975. Modifying the normal function of G protein coupled receptor (GPCR) by blocking or over stimulating its action may either results in the pest death or its normal functions gets disrupted. The novel insect control agents are also developed based on backbone cyclic (BBC) peptidomimetic antagonists of insect-neuropeptides. In this review, backbone cyclic PK/ PBAN neuropeptide antagonist (BBC-25) is discussed. The databases for neuropeptides are DINeR, NeuropPedia, NeuroPep, NeuroPID, NeuroPred and NeuroPP. NeuroPIpred is a tool to predict, design and scan insect neuropeptides. The research in the field of neuroendocrinology is still limited, hence the fundamental studies on the neuropeptides as a novel insecticidal agent is vital for the development to proceed in future.

Keywords: Neuropeptides, proctolin, PBAN, *Periplanta americana*, *Helicoverpa peltigera*

Introduction
The enormous use of insecticides had led to the development of resistance in insects. Therefore, there is a need to move on to an eco-friendly management measures. In insects, the basic biochemical and physiological processes like growth, development, reproduction, energy metabolism, fat mobilization, homeostasis and the behavioural activities like mating, migration, oviposition are controlled by insect neuropeptides, hence it can be commercially exploited as a novel insecticidal agent [15].

Neuropeptides are the protein molecules released from the cell body of the axon in the central nervous system. Neuropeptides originating from the brain are produced by neuro-secretory cells. They are used to communicate the impulse from the stimulant to the receptor. Neuropeptides act as neuro-transmitters as well as neuromodulators. They are small peptides consists of 5 to 80 amino-acids, each amino acid is linked together by peptide bond. The prepropeptides are the large inactive precursor of neuropeptides composed of 90 aminoacids. The bioactive peptide can be produced by the removal of the signal sequence from the neuropeptide precursor [6]. The other insect hormones are polypeptides whereas the moulting hormones are polyhydroxylated steroids and the juvenile hormones are sesquiterpenes.

History
Proctolin was the first neuropeptide isolated from *Periplanata americana* in the year 1975 by Starratt and Brown. It is the myotropic pentapeptide *i.e.*, having myostimulatory activity which helps in the muscle contractions of hindgut, reproductive, skeletal and heart muscle [16]. Adipokinetic hormone (AKH) peptide is the second neuropeptide which was isolated from *Locusta migratoria* in the year 1976 by Stone and his co-workers [17]. In the year 1989, Pheromone bio-synthesis activating neuropeptide (PBAN) was identified which is known to regulate the sex pheromone synthesis in female moths [13]. At present, 4782 insect neuropeptides have been identified and characterized after the development of various separation and identification methods like High Performance Liquid Chromatography (HPLC), Amino acid sequencing, Protein mass spectrometry, High-field Nuclear Magnetic Resonance Spectroscopy, Recombinant DNA technology and PCR. Thus it paves the way for the development of novel insecticides.

Table 1: Difference between neuropeptides and neurotransmitters [11]

S. No.	Particulars	Neuropeptides	Neurotransmitters
1.	Definition	They are composed of short chains of amino acids and act as neurotransmitters.	They are endogenous chemicals that enable neurotransmission.
2.	Release	Releases into the haemolymph	Releases into the neuro-neuro junction or neuro-muscular junction.
3.	Size	Larger molecule	Smaller molecule
4.	Molecular weight	High	Low
5.	Activity	Slow acting	Fast acting
6.	Duration	Prolonged action	Short term response
7.	Concentration	Synthesized in low concentrations	High concentration
8.	Receptor proteins	Acts on a number of receptor proteins	Act only on a specific receptor
9.	Genes	Alter the expression of specific genes	Do not alter gene expression
10.	Synthesis	Synthesized in rough endoplasmic reticulum and Golgi apparatus	Synthesized in the cytosol of pre-synaptic neuron terminals
11.	Location	Found all over the neurons	Found only in the axon terminals of pre-synaptic neurons
12.	Storage	Stored in large dense-core vesicles (LDCV'S)	Small secretory vesicles (SSV'S) Both type of vesicles may be found in the same neuron
13.	Duration of release	Axonal streaming occurs in few cm/ day	Released within few milliseconds upon an arrival of an action potential
14.	Released with	Released to the synaptic cleft along with another neurotransmitter	Released individually depending on the action potential
15.	Fate	Vesicles are autolysed without reusing; once released, they do not undergo reuptake.	Destroyed by enzymes in the synaptic cleft or are reuptaken by pre-synaptic terminal or neuroglia by active transport.
16.	Production	Cell body of neuron	Axon terminal of presynaptic neuron
17.	Example	Proctolin	Acetyl choline

Bio-synthesis of neuropeptides

Neuropeptides are produced from larger precursor proteins, known as prepropeptides, which are encoded in the genome. The prepropeptide comprises of signal peptide (which directs the protein to the secretary pathway), progenitors of mature peptides (bio-active peptides), spacer peptides (peptide fragments with no known biological function and non-conserved sequences) and cleavage sites (monobasic and dibasic). Larger pre-propeptides which are cleaved and modified post-translationally into smaller bioactive peptide [19].

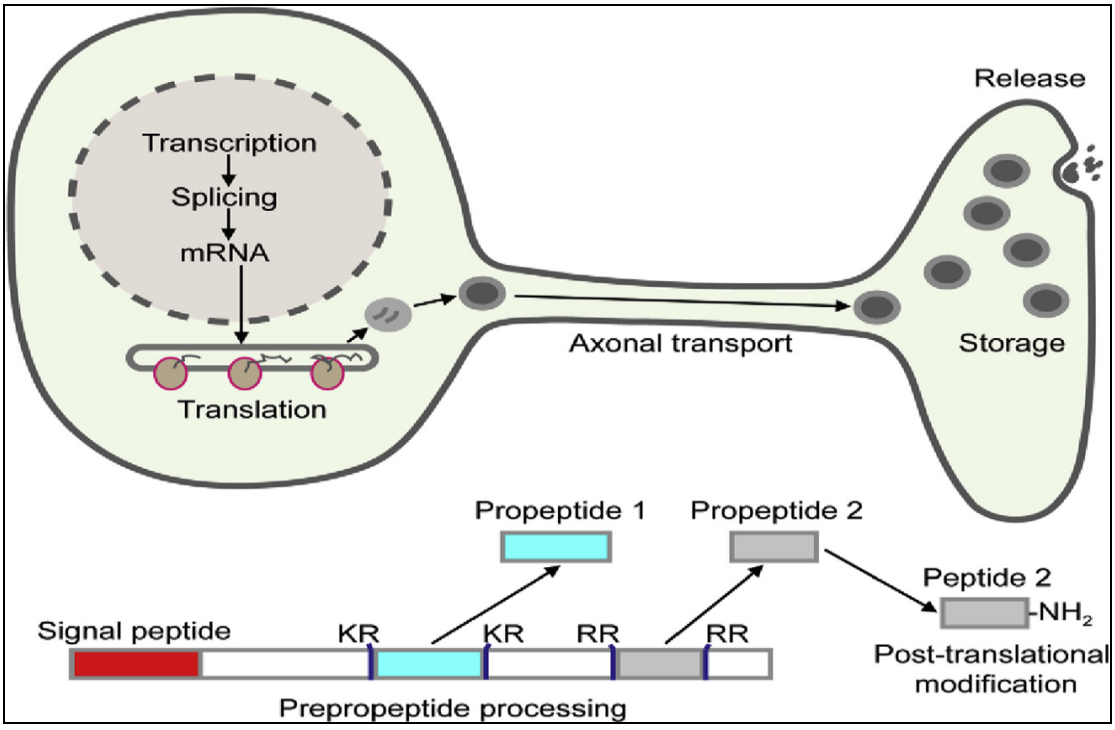


Fig 1: Schematic representation of bio-synthesis of neuropeptide [19]

Steps involved in the bio-synthesis and secretion of neuropeptides [1]

1. **Nucleus**

- The production of neuropeptide starts from the nucleus and ends in the dense core vesicle.
- Transcription of prepropeptide (PPP) gene.
- Post-transcriptional modification of prepropeptide mRNA.
- Transport of prepropeptide mRNA to Endoplasmic reticulum.

2. **Endoplasmic reticulum**

- Translation of prepropeptide mRNA.
- Propeptide (PP) formation.
- Post-translational modification of propeptide.
- Transport of propeptide to Golgi body.

3. **Golgi bodies and vesicles**
- Post-translational modification of propeptide
- Endoproteolysis
- Exoproteolysis
- Amidation
- Acetylation

4. Transport of active neuropeptide carrying mature vesicles towards the axon terminals.
5. Neuropeptide storage and release

Mechanism of neuropeptide synthesis

The single peptide can be synthesized from one protein E.g., Prothoracicotropic hormone (PTTH), Adipokinetic hormone (AKH). Otherwise several different peptides are produced from a single protein E.g., In *Diploptera punctate*, 13 different allatostatin (AST) peptides were identified. In *Bombyx mori*, a single precursor protein is the source of diapause hormone (DH) and pheromone bio-synthesis activating neuropeptide (PBAN), but their production and function is being separated in time and space [7].

Receptors of neuropeptides

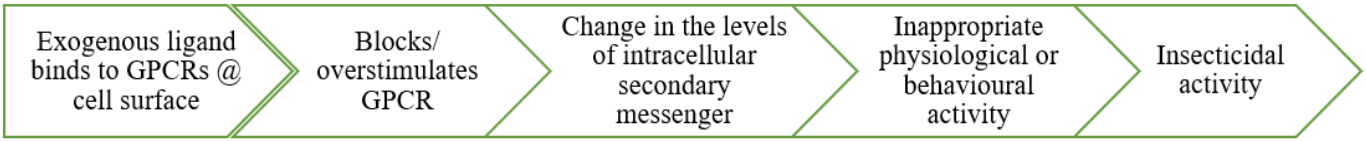
The signal transducing proteins of neuropeptides are categorized into heptahelical (7 transmembrane protein) or GPCR and single transmembrane containing receptors.

The most identified and described receptors are *Drosophila melanogaster* tachykinin-like peptide receptor (TKr), *Manduca sexta* diuretic peptide receptor (DPr) and *Drosophila* allatostatin receptor (ASTr) [10, 14, 5].

The other receptors controlling insect development are A type allatostatin receptor which was first discovered in the cockroach, *P. americana*, B type allatostatin receptor was first noticed in cricket, C type allatostatin receptor was identified in the moth, *Manduca sexta*, Leucine-rich repeat containing GPCR, Diuretic hormone receptors as water and salt regulators, Adipokinetic hormone (AKH) receptors are involved in the control of energy metabolism, Tachykinin receptors and NPF/NPY receptors control the behaviour of insects, receptors controlling ecdysis behaviour and the receptors controlling insect reproduction (PK/PBAN receptors) [18].

GPCR as potential pesticidal target

The insect's major processes like biology, behaviour, physiology, growth and development are being modulated by G protein coupled receptors (GPCRs). The death of the pest can be obtained by modifying the normal function of GPCR receptor. Hence GPCRs would be considered as a potential targets for the development of next generation pesticides. The studies on the Red flour beetle, *Tribolium castaneum* suggest that GPCRs involved in growth and development (eclosion hormone, ecdysis triggering hormone and crustacean cardio acceleratory peptide receptors) as well as the dopamine-2 like, latrophilin-like, starry night, frizzled-like, methuselah-like and the smoothened receptors may be suitable pesticide targets [4].



Nomenclature

The abbreviation of genus/ species name (source), followed by primary action extended with Roman number.

E.g., 1. Mas-AST - *Manduca sexta* allatostatin

2. Bom-PBAN I and II - *Bombyx mori* pheromone biosynthesis activating neuropeptide [12].

Classification of neuropeptides

At present, only four neuropeptides (Proctolin, kinin, PBAN & allatostatin) are studied thoroughly among 54 insect neuropeptide families which covers 23 insect orders and tested for their bioassay activity against various insects.

The neuropeptides are categorized based on their functions *viz.*, Growth and development e.g., Mas-AST, Mas-AT, Reproduction e.g., Hez-PBAN, Metabolism and homeostasis e.g., Mas-AKH and Muscle movement e.g., Proctolin. When the neuro-peptides elicit more than one above biological action, it is called as Pleiotropic neuropeptides [7].

Table 2: Functions of neuropeptides [19, 6]

Neuropeptide	Isolated from	Function
Adipokinetic hormone	*Locusta migratoria*	Development and ecdysis.
Anti-diuretic Factor	*Tenebrio molitor*	Inhibit fluid secretion in Malphigian tubules.
Allatostatin A,B,C	Cockroach, Cricket, Moth	Inhibits JH synthesis.
Allatotropin	*Manduca sexta*	Stimulates JH biosynthesis.
Bursicon	*Drosophila melanogaster*	Cuticle tanning.
CCH amide	*Bombyx mori*	Increases the motivation to feed.
Pigment-dispersing factor	*Romalea microptera*	Pigment movements in response to light.
Diuretic Hormone	*Diploptera punctata, Manduca sexta*	Fluid secretion in Malphigian tubules.
Eclosion hormone	*Manduca sexta, Bombyx mori*	Ecdysis behaviour.
Ecdysis-triggering hormone	*Manduca sexta*	Triggers ecdysis.
FMRF amide	*Drosophila melanogaster*	Ecdysis, myostimulatory in action.
Insulin-like Peptide	*Bombyx mori*	Growth, metabolism and reproduction.
Ion transport peptide	*Apis mellifera*	Modulate ion transport.
Kinin	*Leucophaea maderae*	Myotropic, diuretic activities.
Myosuppressin	*Leucophaea maderae*	Inhibit heart and visceral muscle.

Neuropeptide F	*Drosophila*	Helps in foraging and feeding.
Neuropeptide-like precursor	*Drosophila*	Role in development.
PBAN	*Leucophaea maderae*	Regulating pheromone biosynthesis.
Diapause hormone	*Leucophaea maderae*	Regulation of insect diapause.
Pre-ecdysis triggering hormone	*Manduca sexta*	Tracheal air filling and triggering ecdysis.
Pyrokinin	*Leucophaea maderae*	Myostimulatory activity and regulates hormone biosynthesis.
Proctolin	*Periplaneta americana*	Stimulate muscle contractions.
Prothoracicotropic hormone	*Bombyx mori*	Regulates moulting and metamorphosis.

Neuropeptides as insecticidal agent

The novel insect control agents are developed based on backbone cyclic (BBC) peptidomimetic antagonists of insect-neuropeptides. The cyclisation of the active hexapeptide, PBAN called Backbone cyclic neuropeptide-based antagonist (BBC-NBA). At present, four different neuropeptides are studied thoroughly and their biologically active sequences are identified. Using this sequence peptidomimetic analogues (agonists or antagonists) are synthesized in automated peptide synthesizer and tested for their efficacy as insecticide. The following steps are involved in the development of neuropeptide insecticide [2].

1. Gene cloning: The DNA contains thousands of different genes in which one particular gene encodes for one particular protein. The genes responsible for particular neuropeptide will be isolated and identified through Gene libraries.

2. Protein sequencing: After identifying the gene, the protein of targeted hormone will be synthesized using protein sequencer.

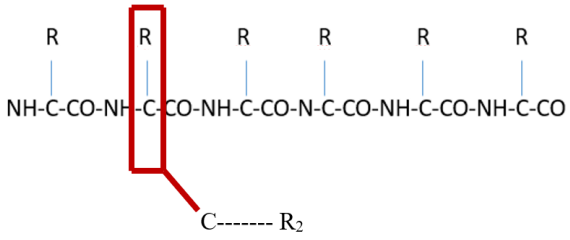
3. Structural elucidation: By using Nuclear Magnetic Resonance (NMR) spectroscopy, the structure of particular hormone is elucidated.

4. Artificial peptide synthesis: Artificial synthesis of linear peptide can be done by Automated peptide synthesizer.

5. *In vitro* study of protein docking using bio-informatic tools: The *in vitro* study of protein docking is used to identify the binding of ligand with the receptor. The protein docking softwares are 1-Click Docking, Automated active site detection, docking, and scoring (AADS), Blaster, BSP-SLIM, Docking Server, EADock, Fitted, FlexPepDock, iScreen and MEDoc. About hundreds of proteins can be analysed within 1 hour.

6. Modification of peptide structure

i) Linear replacement/ side chain modification



ii) Cyclization (changing N group and changing C group)

In cyclization process, the conformational change can be done to restructure the peptide. In the case of PBAN, heptapeptide H-Arg-Tyr-Phe-**Ser**-Pro-Arg-Leu-NH_2 was used as an active sequence for the discovery of an antagonist. The library of seven peptides was developed by replacing each sequential residue in the heptapeptide by D-Phe. The agonistic/antagonistic activity of each peptide was tested through bioassay using *H. peltigera*. Among the seven peptides, the highest antagonistic activity was achieved in H-Arg-Tyr-Phe-D-Phe-Pro-Arg-Leu-NH_2 peptide.

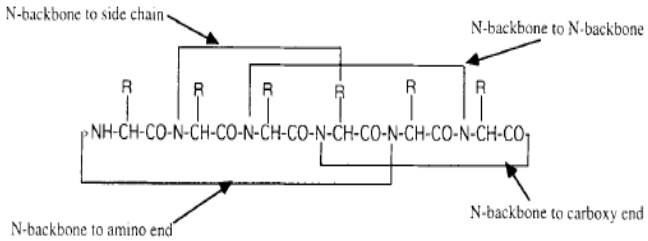


Fig 2: Modes of N-backbone cyclisation [8]

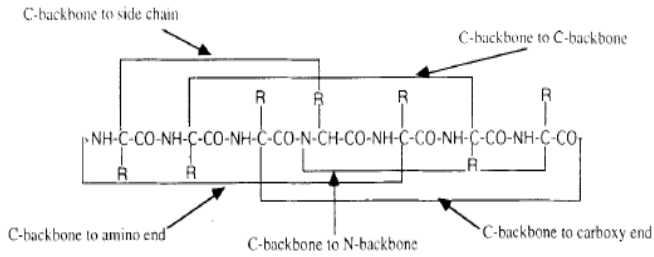


Fig 3: Modes of C-backbone cyclisation [8]

7. Preparation of formulation

The synthesized neuropeptides are formulated as wettable powder. A requirement while designing a molecule is that an exogenously applied peptide should be able to reach the haemolymph compartment unaltered, either via cuticle or upon ingestion by crossing intact epithelium of the gut [3].

***8. In vivo* study using live insects**

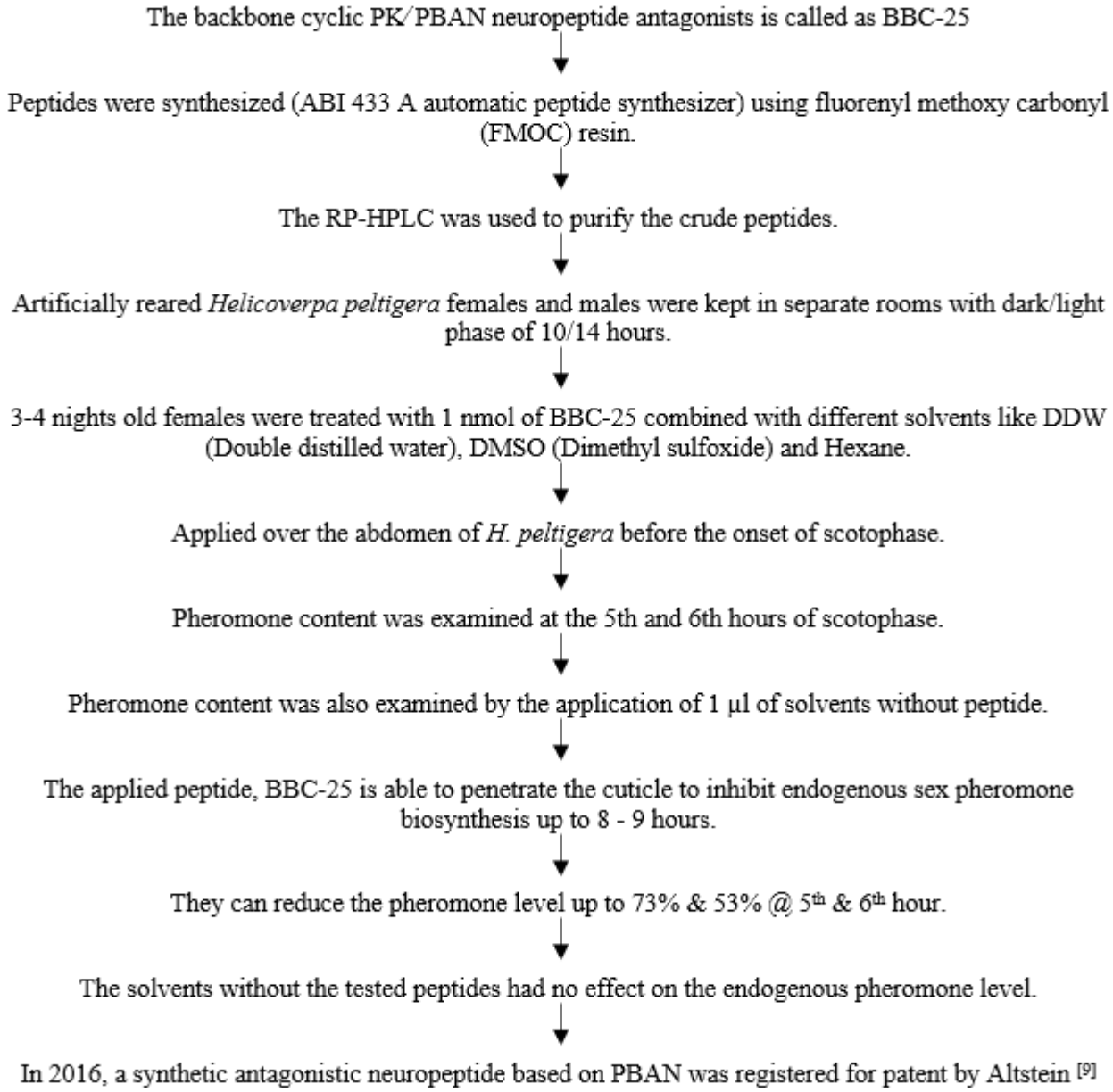
Direct analysis of neuropeptides with insect by topical application.

Case study: PBAN as insecticide

The highly potent, linear, selective, metabolically stable backbone cyclic (BBC) pure antagonists was developed based on the substitution of L-amino acids with D-Phe followed by backbone cyclization. The highest antagonistic activity was exhibited in the peptide sequence H-Arg-Tyr-Phe-D-Phe-Pro-Arg-Leu-NH_2. The injection of 100 pmol concentration of this peptide, inhibited sex pheromone biosynthesis by 63% after 2 hours.

The two BBC pyrokinin/ pheromone biosynthesis-activating neuropeptide (PK/PBAN) antagonistic peptides was tested for their cuticle penetration to reach the target organ and to exert bioactivity by applying them topically to *Helicoverpa peltigera* females and the inhibition of sex pheromone production was monitored during scotophase. The peptides are applied at different time intervals before the onset of scotophase and pheromone content was examined at the 5th or 6th hour of scotophase. Both peptides penetrated the cuticle efficiently and inhibited sex pheromone biosynthesis for 8 to 9 hours after application. The degree of inhibition was more when applied in double-distilled water (DDW) than those applied in dimethyl sulfoxide (53-73%, 15-38% inhibition for BBC-25 and 46-67%, 36-40% inhibition for BBC-28).

Peptides applied in dimethyl sulfoxide and hexane exhibited slightly more persistent inhibitory activity than those applied in DDW. The solvents themselves did not affect pheromone production. Multiple applications (at 2, 0, +2 and +4 h) resulted in almost complete (87%) inhibition of sex pheromone biosynthesis compared with single application (52%).



Databases for neuropeptides

For effective usage of data by scientific community, various web resources have been designed such as DINeR, Neurop Pedia, NeuroPep, NeuroPID, NeuroPred and NeuroPP.

DINeR

DINeR is abbreviated as 'A Database for Insect Neuropeptide Research'. It is a web-based database-application for insect neuropeptides wherein it gives details of sequence, functions, and receptor binding sites of the neuropeptides. The neuropeptide information of many insects can be retrieved. It includes 50 well described neuropeptide families from over 400 different insect species. Approximately 4700 FASTA formatted, neuropeptide isoform amino acid sequences and over 200 records of physiological functionality have been recorded based on published literature.

Also the images of neuropeptide receptor locations are available. Moreover, in this database they have adopted a standardized nomenclature to address inconsistent classification of neuropeptides. It is available at the website:

http://www.neurostresspep.eu/diner/ [19].

NeuroPedia provides information about neuropeptide sequence and its mass spectra libraries not only for insects but also for humans and other mammals. The information can be easily downloadable from this database.

NeuroPep maintains information of about 5949 peptides obtained from 493 organism belonging to 65 neuropeptide families. It also maintains information about human neuropeptides. In addition, there are few prediction methods, which predict the cleavage site in the prepropeptide, which may lead to potential neuropeptides.

NeuroPID is one such machine learning based method, which predicts the neuropeptide precursors from the metazoan proteome.

NeuroPred predicts the cleavage site in the neuropeptide precursors. Recently, another tool NeuroPP has been published which utilizes compositional features (single, dipeptide and tripeptide) to predict neuropeptide precursors.

NeuroPIpred is a tool to predict, design and scan insect neuropeptides [1].

Functions of NeuroPIpred

- To discriminate the insect neuropeptides and non-neuropeptides with high accuracy and allow users to generate mutant analogs of neuropeptides, which can be potential neuropeptide-based insecticides.
- Predict the insect neuropeptides.
- Provides structure and physicochemical properties of neuropeptides.
- Known neuropeptides constitute the 'positive' set whereas the 'negative' sequences have functions unrelated to neuropeptides.
- Positive data is extracted from DINeR database whereas the negative data is extracted from SwissProt and SATPDB.

Two models with two datasets

1. NeuroPIpred_DS1

- The positive dataset of this model is called as NeuroPep_NR and the negative dataset is called as Random_Pep.
- 86.50% accuracy was achieved in this model.
- NeuroPIpred_DS1 consists of natural neuropeptides. Hence, it is also called as natural model.

2. NeuroPIpred_DS2

- The positive dataset of this model is called as NeuroPep_CTM and the negative dataset is called as BioPep_CTM.
- 97.47% accuracy was achieved in this model.
- This model consists of C terminal modified neuropeptides - having amide in C terminal. Hence, it is also called as modified model.

Residue composition analysis

There are 20 natural amino acids present in a peptide, it is important to analyse the frequency of an amino acids present in insect neuropeptides. The percent average composition of each residue present in the datasets (positive and negative peptides) should be calculated.

Implementation of web server

The server consists majorly of five modules;

1. **Predict**: This module is used to predict the entered peptide sequence.
2. **Design**: Using this module, user can design the customized neuropeptides.
3. **Protein Scan:** The protein scan module is used find out probable regions in the protein which can be neuropeptides.
4. **BLAST**: BLAST is abbreviated as Basic Local Alignment Search Technique. It allows user to check the similar peptides in the existing database of experimentally verified neuropeptides with its input peptides.
5. **Download:** The datasets used in the study can be easily downloaded from this module.

Web server for NeuroPIpred is available at https://webs.iiitd.edu.in/raghava/neuropipred [1].

Advantages

1. Neuropeptides are insect specific which kills insect by controlling various related physiological activities and behavioural activities.
2. Development of resistance is slow.
3. Long term storage compared to bio control agents.
4. It ensures environment safety.
5. It can be used as a component in IPM.

Constraints

1. Difficulty in the improvement of antagonistic/agonistic activity.
2. Identification of receptor site serves as a major problem.
3. Cost of production is high and time consuming.
4. Poor solubility in organic and aqueous solutions.
5. Rapid degradation in the insect digestive tract.

Conclusion

- Targeting the precursor of neuropeptide instead of direct neuropeptide.
- Inactivate the secondary messenger produced by neuropeptide at receptor site.
- Research on insect neuroendocrinology is still limited.
- Increased knowledge in the fields of neurochemistry, biotechnology, physiology of insects is necessary.
- At present, the large scale synthesis of such "peptides" is still very expensive therefore price reduction is of prime importance, although the minute amount required for necessary effect is, however, a big advantage.

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