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IDENTIFICATION OF CARDIOMODULATORY NEUROPEPTIDE IN COTTON BUG, *Dysdercus cingulatus*

Siny G Benjamin^{1*}, Muraleedharan Damodaran²

¹Department of Zoology, All Saints' College, Thiruvananthapuram, Kerala

²Amrita Institute of Medical Sciences, Kochi, Kerala, drdmuralee@gmail.com

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ABSTRACT

Neuropeptides regulate diverse physiological activities in invertebrate and vertebrates. Central nervous system is the major site for the production of neuropeptides. The function of neuropeptide varies in different insects even though they have structural similarities. Current study was carried out to characterize corazonin neuropeptide and its cardio acceleration bioassay in cotton pest bug. From 5000 Brain-CC-CASOG complexes, the neuropeptide was isolated; and purified by HPLC fractionation. Results of study revealed corazonin immunoreactivity in three to five neurosecretory cells in each hemisphere of the deutocerebrum of *Dysdercus cingulatus*. Identity of purified corazonin was confirmed with polyclonal antibody by immunoblotting. Molecular mass of the isolated peptide was determined as 1368.69 Da by MALDI-TOF mass spectrometry. Amino acid sequence of the purified peptide deduced the presence of [Arg7] - corazonin in the insect. Bioassay with the bug aorta and in aorta of *Periplaneta americana* confirmed its function as a cardio accelerator. Endocrine disruption of this cotton pest can be opted as a tool in integrated pest management. Synthetic analogues and compounds which mimics the neuropeptides can be effectively utilized as an alternative against synthetic pesticides without affecting non-target organisms or environment. Thus modified peptidomimetic compounds can be effectively utilized in pest control.

* Corresponding author

E-mail: sinyben@gmail.com (Siny G Benjamin)

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1 Introduction

Corazonin neuropeptide, an eleven amino acid peptide, has been isolated from various insects with specific physiological functions. Various isoforms of this highly conserved neuropeptide has been reported. The distribution across different invertebrate orders depicts its evolutionary significance (Zandawala et al., 2018). The wide spread corazonin isoforms are one with amino acid arginine at the seventh position, [Arg⁷-corazonin] and amino acid histidine at the seventh position, [His⁷-corazonin]. The physiological function of neuropeptide varies from one organism to another. In *Periplaneta americana* and in the tobacco horn worm, *Manduca sexta* [Arg⁷-corazonin] has cardiomodulatory in function (Veenstra, 1989; Slama & Rosinki, 2005). Presence of His⁷-corazonin has been identified in grass hoppers, *Schistocerca americana*, *S. gregaria*, *Locusta migratoria* and stick insect *Carausius morosus* (Veenstra, 1991; Predel et al., 1999; Tawfik et al., 1999). In social insects like ants corazonin controls the caste regulation. Corazonin down regulate egg laying by inhibiting the synthesis of vitellogenin in ants (Janko et al., 2017).

The cardio acceleratory function by the conserved peptide is not shown in all the insects such as moths, cockroaches and crickets in which it is identified (Veenstra, 1991; Hua et al., 2000; Predel et al., 2007). The gene and cDNA encoding for corazonin were identified in *Drosophila melanogaster* and wax moth *Galleria mellonella*, respectively. Preprocorazonin of *D. melanogaster* and *G. mellonella* contains corazonin along with longer, nonamidated peptide, which is of unknown function (Veenstra, 1994; Hansen et al., 2001). Further, [His⁷]-corazonin isolated from corpora cardiaca that induces pigmentation has a dual role of phase polymorphism in an albino mutant and normal nymphs of *Locusta migratoria* (Tanaka & Pener 1994). [Arg⁷]-corazonin is as potent as [His⁷]-corazonin in terms of dark color inducing activity in locusts (Hua et al., 2000). The imbalance in corazonin exhibited a delay in maturation and reduced growth rate and reproduction in marine worm, *Platynereis dumerilii* (Andreatta et al., 2020)

The cuticle melanization property of corazonin is restricted to locust species and this effect does not appear to extend beyond this group. Implantation of CNS ganglia or injection of their extracts from insects of thirteen orders induced melanization of cuticle in albino locust, but this physiological effect was not noticed in the donor insects (Tanaka, 2000; Roller et al., 2003). These data indicate that most insects produce corazonin, but its role in cuticle melanization is specific for locusts (Tanaka, 2001). Injection of [Arg⁷], [His⁷] and [Thr⁴, His⁷]-corazonins altered the developmental period of *Bombyx mori* in the fifth stage larval instar and delayed adult development (Tanaka et al., 2002; Roller et al., 2006). The three isoforms induced melanization of cuticle in *Locusta migratoria* (Roller et al., 2006). Even though all the three isoforms of corazonin evoked coloration, [Thr⁴, His⁷]-corazonin

characterized from the honey bee *Apis mellifera*, is relatively less active in the dark color inducing assay on albino locusts (Verleyen et al., 2006). Corazonin isoforms has been identified late during evolution in different insect orders. The occurrence of corazonin like substance in the primitive insect, the silver fish *Ctenolepisma lineatae* (Roller et al., 2003) and crustacean species, Jonah crab *Cancer borealis* (Li et al. 2003) and *Platynereis dumerilii* (Andreatta et al., 2020) implies on ancient origin of this neuropeptide. Identification of corazonin in many insect groups, together with its localization by immunohistochemical staining and bioassays suggest that corazonin is a highly conserved and widespread insect neuropeptide, but its physiological function is still unclear. Ecdysis in moths which is regulated by Pre Ecdysis Triggering Hormone (PETH) and Ecdysis Triggering Hormone (ETH) produced from the ink cells are significantly regulated in the initial stages by the neuropeptide corazonin (Kim et al., 2004). Present study focused to gain insight on the biochemical and functional characterization of corazonin in a heteropteran cotton bug *D. cingulatus* which is pest on cotton plant. Current study used the statistical evaluation of data on cardioacceleration to confirm the cardio regulatory role of the peptide.

2 Materials and Methods

2.1 Insects

The red cotton bug *D.cingulatus* Fabr. (Heteroptera: Pyrrhocoridae) were reared at a temperature of 29±30°C, R.H.90 % and 12:12 light/dark regime in plastic containers. The insects were fed *ad libitum* on soaked cotton seeds. Soaked cotton pads were also provided to maintain ideal R.H. Newly moulted adult insects were collected daily from the stock culture and maintained in separate marked containers. American cockroaches, *P. americana*, were reared at 27±2°C. They were fed on beaten rice and had access to water.

2.2 Immunohistochemistry

Cephalic endocrine complex including the Brain-CC-CA-SOG complex and the ventral nerve cord ganglia together were processed for whole mount immunostaining following the method of Schooneveld & Veenstra (1988). The CNS complex was dissected out under chilled phosphate buffered saline (PBS; pH 7.4), fixed in 4% formaldehyde at 4°C overnight and washed in PBS containing 1% Triton X-100 (PBST). After subsequent washes with PBST and preincubation with 10% normal goat serum in PBST for 1h, the tissues were then incubated in primary antibody at 4°C for 2-3 days. Polyclonal antibody of Arg-corazonin was employed in the study at a dilution of 1:1000. After several washes with PBST, the tissues were incubated with goat anti-rabbit IgG labeled with horseradish peroxidase (HRP) at 4°C overnight. The HRP was stained with 0.05% 3,3'-diaminobenzidine tetra

hydrochloride in 0.1M Tris-HCl buffer (pH 7.6) and 0.01% H₂O₂ under visual control. Stained tissues were washed sequentially in 50%, 70% and 90% glycerol in PBS and mounted in glycerol.

Immunolocalization in whole mount tissues with FITC (fluorescein isothiocyanate) was done for con-focal imaging of corazonin-positive secretory neurons following the procedure of Verleyen et al. (2004) and the control preparations lacked the primary antibody. Stained preparations were viewed and imaged with an inverted confocal laser scanning microscope (Leica He-NE) with both 10X and 20X objective and excitation wavelength 500-580nm extended by Z-stacks of serial optical sections.

2.3 Pre-purification and HPLC fractionation

Brain-SOG-CC-CA complexes (about 5000 nos) from two day-old adult females were dissected out in the homogenizing medium containing methanol, water and acetic acid (90:9:1) and was stored at -20°C. Accumulated tissues in bulk were homogenized, sonicated and centrifuged (10,000g, 20min, 4°C). Pellets were re-extracted again after sonication and centrifugation. Pooled supernatants of brain-SOG complexes were filtered through an Amicon spindown filter (5 kDa cut off) (Amicon, Millipore). The vacuum-concentrated supernatant extract was used for subsequent HPLC fractionation.

HPLC was carried out on a Shimadzu purifier (Japan) using a Sephasil C-18 reverse phase analytical column (4.6 × 250 mm, 12 µm particle 300 Å pore size). Mobile phase comprised of 0.1% TFA in water (solution A), and 80% acetonitrile containing 0.08%TFA (solution B). A linear gradient 0-100% of solution B was applied for 60 min at a flow rate of 1ml/min. HPLC fractionation was done according to Baumann & Gersch (1982).

The standard peptide [Arg⁷]-corazonin (Sigma) was run at the above parameters and the retention time was noted. The pre-purified sample (20µl) was injected into the column and the fraction eluting at the same retention time was collected automatically in the fraction collector. Same fractions from multiple runs were pooled together, concentrated and injected as the second step. The process was repeated until a single peak of the purified peptide was obtained following the same parameters as above.

2.4 Tricine-SDS-PAGE and immunoblotting

The low molecular weight neuropeptide was resolved in modified Tricine-SDS-PAGE following the procedure of Schägger & von Jagow (1987). Electrophoretic run was performed both with synthetic and purified peptide tissue samples. At the end of the electrophoretic run, electro blotting and immunoassay was done according to the method of Jacquin Joly & Descoins (1996). Transfer was performed for 90 min at a constant power supply of

20V on a transblot apparatus (Broviga, India). The membrane was taken out from the trans-blot apparatus and incubated with primary antibody [Arg⁷]-corazonin (GeneI) at a dilution of 1:1500 overnight at 4°C. After washing with PBS, the membrane was treated with secondary antibody (Goat anti-rabbit IgG, GeneI) for 1hr and visualized with chloronaphthol staining solution.

2.5 Matrix-assisted laser desorption/ionization mass spectrometry

The molecular weight of the isolated peptide was determined using MALDI-TOF-MS as suggested by Hillenkamp & Karas (2007). Ten mg of 2-cyano 4-hydroxy cinnamic acid (HCCA) matrix was dissolved in 1ml of acetonitrile containing 0.1% TFA in water. 1µl of sample (containing 1/80th portion of isolated peptide) in 1µl of matrix was coated on MALDI target plate and allowed to air dry at room temperature prior to analysis. MALDI-MS spectra were recorded on MALDI-TOF MS Kompact (Kratos analytical Manchester UK). A nitrogen laser 659 nm was used as the desorption/ionization source and positive ions were detected in linear modes. The instrument was operated with an acceleration voltage at 20kV. Angiotensin (1046Da, Sigma, USA) was used to calibrate the mass spectrometer.

2.6 Bioassay

Accelerating heart beat activity was tested on newly-isolated abdominal hearts from adult males of *P. americana* as well as in *D. cingulatus* maintained in MEM following the procedure of Veenstra (1989). Hearts were kept in 1ml of MEM. After 30 minutes the rate of heart beat was determined by counting the number of heart beats per minute. These measurements were repeated every 30 seconds. Only regularly beating hearts were used for the assays, which consisted of exposing the isolated heart to increasing doses of corazonin. Each subsequent dose was only applied after the heart beat had stabilized.

2.7 Sequence Analysis

Amino acid sequencing of the isolated peptide was done using PTH-21A Shimadzu Protein sequencer. The machine was calibrated using standard PTH (Sigma) and 1pmol of isolated peptide was used for amino acid sequencing.

3 Results

3.1 Immunohistochemistry and Immunoblotting

Corazonin-positive immunostaining was noticed in three to five pairs of secretory neurons at the deutocerebral lobe in the lateral region of brain. The pterothoracic ganglion showed positive immunoreactivity in one pair of neurosecretory cells. In *D. cingulatus* ventral nerve cord comprises of one distinct prothoracic

Figure 1 Confocal images of corazonin immunoreactivity in the CNS of *Dysdercus cingulatus*, both FITC conjugated secondary antibody and DAB stained (a) Corazonin immunopositive secretory neurons in the deutocerebral region of brain of *Dysdercus cingulatus*. Arrows indicate immunoreactivity (b) The axonal innervations of corazonin neuropeptide in the dorsal aorta (c) Immunopositive median neurosecretory cells in the brain- DAB stained (d) One pair of corazonin positive neurosecretory cells in the compound pterothoracic ganglion. e. Corazonin immunoreactivity in the corpora cardiaca of *Dysdercus cingulatus* a,b- FITC conjugated and (c,d,e) DAB stained. The bars indicate 25µm f. Distribution pattern of corazonin immunoreactive cells in the CNS of *D. cingulatus*. Innervations of the axonal endings in the dorsal aorta (A) are shown by dotted lines. Br- brain, CC- corpora cardiaca, CA- corpora allata, A- aorta

Figure 2 Immunoblot of both Synthetic corazonin [Syn-Crz, 1] and purified Bug corazonin [Bug-Crz, 2] from *Dysdercus cingulatus*

ganglion, and the terminal pterothoracic ganglion formed by the fusion of mesothoracic, metathoracic and abdominal ganglia together. The axonal innervations were also prominent in the dorsal vessel. Strong immune reactivity was also observed in many arborizations from the brain to the dorsal vessel (Figure 1).

In Western blot analysis, positive antigenic activity was noticed against [Arg⁷] corazonin antibodies indicating the bug Corazonin peptide band with similar electrophoretic mobility as the synthetic peptide (Figure 2).

3.2 Peptide Purification

Corazonin was purified from acid methanolic extract of 5000Br-CC-CA-SOG complexes of adult female insects by RP-HPLC fractionation. Under specific parameters synthetic-corazonin (Sigma) showed a retention time of 23.2min. The pre purified tissue extracts was used for RP-HPLC fractionation with different runs using Sephasil C-18 column [Figure 3a]. The eluants with the same retention time as that of the synthetic peptide was collected from multiple runs, pooled together and lyophilized. The HPLC profile of the lyophilized peptide showed a single major peak (Figure 3b). The amount of isolated corazonin was estimated from 5000 Brain-CC-CA-SOG complexes and peak height as ~ 12ng of corazonin.

3.3 Molecular mass and Amino acid sequence analysis of Bug Corazonin

To determine the molecular weight of the fractionated bug corazonin, the purified peptide was analyzed by MALDI-TOF-MS which showed a single peak of molecular weight 1368.68 Da (Figure 4). Automated Edmann degradation of the peptide in a protein sequencer determined the amino acid sequence of Bug Corazonin as pGlu-Thr-Phe-Gln-Tyr-Ser-Arg-Gly-Trp-Thr-Asn-amide.

3.4 Cardio-acceleration bioassay

Isolated bug corazonin showed strong cardio-acceleratory effect as evidenced by number of heart beats per minute. At very low concentrations such as 0.1nM the rate of heart beat increased significantly in both *P. americana* and *D. cingulatus* *in vitro*. The cardiac rate up regulation was observed. This confirms the function of corazonin as a strong cardio accelerator (Figure 5). For each bioassay five different heart preparations each from *P. americana* and *D. cingulatus* were used. Descriptive statistics and test of significance (ANOVA) of mean heart at different concentrations showed significant difference between respective pairs of means at 0.001% level (Duncan's multiple range test).

Fig: 3b

Fig: 3a

Figure 2 Immunoblot of both Synthetic corazonin [Syn-Crz, 1] and purified Bug corazonin[Bug-Crz, 2] from *Dysdercus cingulatus* Figure 3 (a) Preliminary RP-HPLC profile of Br-CC-CA-SOG complex of *D.cingulatus*. The

Figure 4 MALDI-TOF-MS showing the mass of isolated Bug corazonin as 1368.69Da.

Figure 5 Error bar diagram showing the effect of corazonin on the heart beat of *Dysdercus cingulatus* and *Periplaneta americana* (Significance assessed by Duncan's multiple range test)

4 Discussion and Conclusions

Corazonin a highly conserved neuropeptide is widespread among insects except in all coleopterans studied (Tanaka, 2000; Tanaka, 2005; Predel, 2007). Even though it is an essential physiological regulator for various biological events such as development, metabolism, and sexual behavior, it is not having a universal specific role. [Arg⁷]-corazonin, the firstly isolated corazonin is widespread in different insect orders. It is also reported from the decapod crustacean crabs, *Cancer productus* and *C. borealis* (Li et al., 2003; Fu et al., 2005). In the marine worm *Platynereis dumerilii* corazonin directly controls carbohydrate metabolism and homeostasis (Andreatta et al., 2020).

Corazonin isolated in different species ranged from 1350 Da-1397 Da. Table 1 shows a list of insect corzonins. The molecular mass

of isolated corazonin from *D. cingulatus* is 1368.68Da. Peptide immunoreactivity noticed in the CNS of *D. cingulatus* revealed the presence of corazonin neuropeptide in the brain neurosecretory cells and also in the ventral ganglia. Immuno-positive axonal innervations were also noticed at the dorsal vessel and aorta indicating the supply of this neuropeptide to the cardiac region of the heteropteran bug. Corazonin is also reported as a sperm ejaculation peptide that shows its presence in abdominal ganglia of *Drosophila melanogaster* (Timothy et al., 2012) and located in three neuron pairs in CNS of the adult oriental fruit fly, *Bactrocera dorsalis* (Qiu et al., 2018).

Corazonin produced from the neurons in central nervous system of *D. melanogaster* regulates the pupal body growth by regulating the ecdysteroid biosynthesis (Imura et al., 2020). Expression of corazonin mRNA in four pairs of lateral neurosecretory cells was confirmed in *Galleria mellonella* by *in situ* hybridization (Hansen et al., 2001). The distribution of corazonin receptor cells in the crab, *Carcinus maenas* stressed the role of corazonin as a pigment concentrating hormone (Alexander et al., 2018). The presence of [Arg⁷]-corazonin in three heteropteran insect species was reported by Predel et al (2007) using MALDI-TOF MS. But its cardio acceleratory function in the bug has not been reported so far. The study confirmed its cardiac rate up regulation in the bug and is confirmed with *P. americana*. So this is the first report of the peptide as a cardio accelerator in a bug. The reverse pattern of heart beat by corazonin is shown in diapausing *Manduca sexta* (Slama, 2004). In *Schistocerca gregaria* and *Locusta migratoria*, both [His⁷]-corazonin as well as [Arg⁷]-corazonin stimulate induction of cuticular darkening and phase related morphological changes (Hoste et al., 2002a; Hoste et al., 2002b; Tanaka, 2003; Yamamoto et al., 2004; Maeno et al., 2004; Tanaka, 2006). Co-

Table 1 Insect corazonins and its characteristics

Corazonin types	Peptide sequence	Molecular weight (m/z)	Descriptions
[Arg ⁷]-corazonin (cockroach)	pQTFQYSRGWTN-NH ₂	1368.68 Da	Veenstra, 1989
[His ⁷]-corazonin (Locust corazonin)	pQTFQYSHGWTN-NH ₂	1350.00 Da	Tawfik et al., 1999
[Arg ⁷]-corazonin (Cricket corazonin)	pQTFQYSRGWTN-NH ₂	1368.68 Da	Hua et al., 2000
[Thr ⁴ ,His ⁷]-corazonin (Apime corazonin)	pQTFITYSHGWTN-NH ₂	1322.57 Da	Verleyen et al., 2006
[Tyr ³ ,Gln ⁷ ,Gln ¹⁰]-corazonin (Bombyx corazonin)	pQTYQYSQGWQN-NH ₂	1384.59 Da	Predel et al., 2007
[His ⁴ ,Gln ⁷]-corazonin (Austro corazonin)	pQTFHYSGWTN-NH ₂	1350.59 Da	Predel et al., 2007
[Gln ¹⁰]-corazonin (Tipula corazonin)	pQTFQYSRGWQN-NH ₂	1396.64 Da	Predel et al., 2007
[Arg ⁷]-corazonin Bug corazonin	pQTFQYSRGWTN-NH ₂	1368.68 Da	Present study <i>Dysdercus cingulatus</i>

elution of the bug corazonin with synthetic corazonin on HPLC and sequence analysis suggests that the bug corazonin is identical with [Arg⁷]-corazonin, pGlu-Thr-Phe-Gln-Tyr-Ser-Arg-Gly-Trp-Thr-Asn-amide. Very low concentrations of bug corazonin increased the rate of heart beat of the cotton stainer, *Dysdercus cingulatus* which confirmed its function as well.

Hence all the results from immunohistochemistry, HPLC fractionation, Western blot analysis, MALDI-TOF-MS, sequence analysis and bioassay account for confirmation and the presence of [Arg⁷]-corazonin in *D. cingulatus*. The identified corazonin in the bug *D. cingulatus* is found specifically localized in the neurosecretory cell groups at the deutocerebral region of brain and also only in one pair of secretory neurons in the pterothoracic ganglion. This bug corazonin [Arg⁷]-corazonin is likely to be present in several other insect species as well. Cardio acceleratory role of this neuropeptide [Arg⁷]-corazonin also is hereby further confirmed in the heteropteran bug.

The neuropeptide corazonin in the heteropteran bug is confirmed as the one with the amino acid arginine in the seventh position. The physiological function of the peptide is cardioacceleration and it is confirmed by bioassay in the insect itself. Mapping of corazonin is done in central nervous system, ganglia in the ventral nerve cord and dorsal aorta of the insect and molecular mass determined.

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Abbreviations

CA- corpora allata; CC- corpora cardiaca; CNS- central nervous system; ETH- Ecdysis Triggering Hormone; FITC- fluorescein isothiocyanate; HCCA- 2-cyano 4-hydroxy cinnamic acid; HPLC- high performance liquid chromatography; HRP- horseradish peroxidase; MALDI-TOF-MS- Matrix-assisted laser desorption/ionization mass spectrometry; PBS- phosphate buffered saline; PBST- phosphate buffered saline triton X-100; PETH- Pre Ecdysis Triggering Hormone; RP-HPLC- reverse phase high performance liquid chromatography; SDS-PAGE- sodium dodecyl sulphate-poly acrylamide gel electrophoresis; SOG- suboesophageal ganglion; TFA- trifluoroacetic acid.

Conflict of interest

The authors declare no conflict of interest.

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