Tissue localization and partial characterization of pheromone biosynthesis activating neuropeptide in *Achaea janata*

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Female sex pheromone production in certain moth species have been shown to be regulated by a cephalic endocrine peptidic factor: pheromone biosynthesis activating neuropeptide (PBAN), having 33 amino acid residues. Antisera against synthetic *Heliothis zea*-PBAN were developed. Using these polyclonals, immunoreactivity was mapped in the nervous system of *Achaea janata*. Three distinct groups of immunopositive secretory neurons were identified in the suboesophageal ganglion; and immunoreactivity was observed in the corpora cardiaca, thoracic and in the abdominal ganglia. From about 6000 brain sub-oesophageal ganglion complexes, the neuropeptide was isolated; and purified sequentially by Sep-pak and reversed phase high performance liquid chromatographic methods. Identity of purified PBAN fraction was confirmed with polyclonal antibody by immunoblotting. Molecular mass of the isolated peptide was determined by matrix-assisted laser desorption/ionization mass spectrometry, and was found to be 3900 Da, same as that of known *H. zea*-PBAN. Radiochemical bioassay confirmed the pheromonotropic effect of the isolated neuropeptide in this insect.

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1. Introduction

In lepidopteran insects, females produce and release sex pheromone to attract males for mating. The existence of a neuropeptide in the brain-suboesophageal ganglion (Br-SOG) complex which stimulates pheromone production in *Heliothis zea* was already reported (Raina and Klun 1984). The peptidic nature of this neuropeptide led to coining of the term 'pheromone biosynthesis activating neuropeptide' (PBAN). Later PBANs were isolated from several other lepidopteran species as well (Kitamura *et al* 1989, 1990; Masler *et al* 1994). Molecular cloning of PBAN-encoding cDNA has been carried out in different species of insects (Choi *et al* 1998; Duportets *et al* 1998; Jacquin Joly *et al* 1998; Lee *et al* 2001; Iglesias *et al* 2002). A high degree of sequence homology exists in these peptides from different species of insects.

PBANs are 33 or 34 amino acid peptides with an amidated C-terminus (Raina *et al* 1989). The C-terminal pentapeptide fragment called FXPRL (X = Gly,Ser,Thr,Val) amide of PBAN is homologous in all which represents the active core capable of eliciting physiological activity in the insect *Heliothis zea* (Raina and Kempe 1990). PBANs are synthesized as part of a large precursor from which biologically active peptides are derived by post-translational processing (Sato *et al* 1993; Ma *et al* 1994; Jacquin Joly *et al* 1998). Besides regulation of pheromone production, peptide sequences encoded in pre-proPBAN

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Abbreviations used: AG, Abdominal ganglia; Br-SOG, brain-suboesophageal ganglia; CA, corpora allata; CC, corpora cardiana; DAB, 3,3′diamino benzidene; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; MW, molecular weight; PBAN, pheromone biosynthesis activating neuropeptide; PLI, PBAN-like immunoreactivity; SOG, suboesophageal ganglia; TG, thoracic ganglia.

appear to serve multiple physiological functions in insects: these include cuticular melanization in larvae (Matsumoto *et al* 1990), embryonic diapause (Imai *et al* 1991) and myotropic functions (Holman *et al* 1986; Schoofs *et al* 1991). Pheromonotropic activity has been reported in the head of cockroaches *Blatta orientalis* and cricket *Gryllus bimaculatus* (Sreng and Roelofs 1988; Sreng *et al* 1990). PBAN-like peptides were localized in the neuroendocrine system of a heteropteran insect *Dysdercus cingulatus*, and was isolated and purified (Ajitha and Muraleedharan 2001a,b). For the first time PBAN family of peptides were found to occur in a crustacean *Penaeus vannamei* (Torfs *et al* 2001).

Sex pheromones are critical factors for reproductive success in almost all species of insects. Since the emission of sex pheromone is necessary to attract a mate, failure to produce and emit pheromone can be utilized as a potential strategy for manipulating adult moth behaviour. Informations obtained from different studies suggest that PBAN may be useful in controlling pest insects since it regulates a key physiological role in pheromone production.

Molecular characterization of neuropeptides and their structure-activity studies present unique opportunities for developing specific, eco-friendly control measures, including formulation of antagonists and superagonists. Over-expression of neuropeptide genes at an inappropriate time could also lead to catastrophic consequences (Scharrer 1989). Genomic DNA and PBAN-encoding cDNA of *H. zea* and *Bombyx mori* have been sequenced (Sato *et al* 1993; Ma *et al* 1994). Based on evidence from Northern blot analyses, the Hez-PBAN gene is transcribed in the SOG of adult *H. zea* (Ma *et al* 1994). Recently an insect neuropeptide-based antagonist insecticide was developed, using PBAN family as a model (Altstein 2004).

Our lab undertook a detailed study of PBAN in *Achaea janata* the major pest of castor plants, which is one of the most economically important annual herb in India. Being polyphagous and voracious feeder, caterpillars of *Acheae* destroy tea, rose, sugarcane, mustard, ground nut, tamarind, etc. The adult moths attack soft fruits like citrus, tomato, grapes, banana, etc.

Furthermore, using immunohistochemical techniques, the major site of PBAN synthesis in *A. janata*, the castor semilooper, was examined. Isolation, purification and partial characterization of this neuropeptide were also undertaken to pave way for sequencing of the peptide and carrying out of further cloning experiments.

2. Materials and methods

2.1 *Insects*

Achaea janata Linn (Noctuidae) larvae were maintained in individual plastic cups supplied with fresh castor leaves until pupation under a 16 L : 8 D photoperiod and at 21 ± 1°C and 80 ± 5 relative humidity. Pupae were observed each day for newly emerged adults. Adults were held in wire-meshed cages and had access to 20% sucrose solution.

2.2 *Immunohistochemistry*

Polyclonal antibodies against synthetic Hez-PBAN (Peninzula Lab Inc., Belmont, CA, USA) were raised in New Zealand strain of white rabbit. The antiserum was subjected to affinity purification to improve its specificity according to the method of Kingan *et al* (1992).

Immunocytochemical techniques were employed on the central nervous system of day-4 females following the method of Schooneveld and Veenstra (1988). Brain-SOG, corpora cardiaca-corpora allata (CC-CA), thoracic ganglia (TG) and abdominal ganglia (AG) were dissected out in Ringer solution and fixed in Bouin's fluid at room temperature. Tissue sections were incubated overnight at 4°C in anti-PBAN diluted 1 : 1500 in PBS containing 0·3% Triton X-100. The secondary antibody used was peroxidase-conjugated goat anti-rabbit IgG (Bangalore Genei). Immunovisualization was achieved by using 3,3′ diamino benzidene tetra hydrochloride (DAB-HCl) in the presence of H_2O_2.

2.3 *Prepurification and HPLC fractionation*

Brain-SOG complexes (about 6000) that found to contain maximum amount of PBAN-like immunoreactivity (PLI) were dissected out in homogenizing medium containing methanol, water and acetic acid (90 : 9 : 1) and stored at – 20°C. Later tissues were homogenized, sonicated and after centrifugation (16,000 g, 20 min, 4°C), the concentrated supernatant was applied to a C-18 Sep-pak preparative catridge (MERCK, Germany) and eluted with 60% acetonitrile containing 0·1% TFA in water. The catridge was finally washed with 100% methanol to recover all proteins and sample was vacuum concentrated to 500 µl.

The HPLC system used was a reversed phase C-18 (Shimadzu, Japan) dual pump delivery system. Mobile phase comprised of 0·1% TFA in water (solution A), and 60% acetonitrile containing 0·1% TFA (solution B). A linear gradient 0–100% of solution B was applied in 40 min at a flow rate of 1 ml/min.

The standard peptide, Hez-PBAN was run at the above parameters and the retention time was noted. The prepurified sample (20 µl) was injected into the column and the fraction eluting at the same retention time was collected automatically in a fraction collector. The same fractions from multiple runs were pooled together, concentrated and injected as second step. The process was

repeated until a single peak of the purified peptide was obtained.

2.4 *Tricine-SDS-PAGE and immunoblotting*

The low molecular weight neuropeptide was resolved in modified Tricine-SDS-PAGE following the procedure of Schagger and Von Jagow (1987). It consisted of a 16·5% T, 6% C small pore separation gel, overlaid by a 10% T, 3% C spacer gel and a 4% T, 3% C stacking gel. Both synthetic Hez-PBAN and purified tissue sample were dissolved in sample buffer and heated to 100°C for 3 min. At the end of the electrophoretic run, electroblotting and immunostaining were carried out according to the method of Jacquin Joly and Descoins (1996). Transfer was performed for 90 min at a constant power supply of 20 V. The membrane was taken out from the *trans*-blot apparatus and incubated in primary antibody at a dilution of 1 : 1500 overnight at 4°C. After washing with PBS, the membrane was treated with secondary antibody for 1 h and visualized with DAB staining solution.

2.5 *Bioassay*

In vitro radiochemical assay was conducted according to the method of Soroker and Rafaeli (1989). The pheromone gland from adult virgin female moths were dissected out and washed under saline. The gland was incubated in a 5 µl drop of saline containing 0·5 µCi ^{14}C-sodium acetate (sp. act. 41·8 m Ci/m mol, Bhabha Atomic Research Centre, Mumbai) with and without isolated neuropeptide fraction from *A. janata*. After appropriate incubation period at room temperature, the glands were analysed for label incorporation into the pheromone.

Radiolabel incorporation was determined by thin-layer chromatography (TLC) using hexane: ethyl ether (85 : 15) as the developing solvent. The position of the acetate esters in the TLC plate was determined by co-migrating a standard (tri decanyl acetate) along with the sample and exposing the plate to iodine vapours. The spots were scraped out, placed in a scintillation vial with cocktail and counted using Liquid Scintillation Counter (LKB, Wallac).

2.6 *Matrix-assisted laser desorption/ionization mass spectrometry*

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was employed to determine the molecular weight (MW) of the isolated peptide. Ten µg of 2-cyano 4-hydroxy cinnamic acid (HCCA) matrix was dissolved in 1 ml of acetonitrile containing 0·1% TFA in H_2O. 0·3 µl of sample in 0·3 µl 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 Kompact SEQ, voyager elite time-of-flight mass spectrometer (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 20 kV. Insulin from bovine pancreas (5734 Da, Sigma, USA) was used to calibrate the mass spectrometer. Hez-PBAN was also processed to ensure the reliability of the method.

3. Results

3.1 *Immunohistochemistry*

Figure 1A is a diagramatic representation of the organization of the *A. janata* moth central nervous system and the locations of neuronal cell bodies and neurohemal structures exhibiting PBAN-like immunoreactivity. The richest source of PLI in *Achaea* nervous system was observed in the SOG. Insect SOG comprises the fused ganglia of the primitive mandibular, maxillary and labial segments or neuromeres. In *Achaea* moth SOG itself is fused to the brain (figure 1B).

Serial sections of Br-SOG complex revealed three clusters of PBAN-ir somata in the SOG (figure 2A). The anterodorsal mandibular cell group contained large immunoreactive somata (figure 2B). The maxillary cluster contained a group of six-eight cells showing PBAN-immunoreactivity (figure 2C). The labial group occupied the most posteroventral position in the SOG and contained two-four ir-neurons (figure 2D). The bands of immunoreactivity found in the SOG are attributable to axons arising from the mandibular and maxillary clusters (figure 2A, arrowhead). In the corpora cardiaca (CC) immunoreactive axons and immunoreactive cells could be located (figure 2E). Fused TGs and other three AGs showed PLI containing neurons. Immunopositive neurons were also localized in the TAG of adults (figure 2F).

3.2 *RP-HPLC purification*

PBAN–like peptide was purified from 6000 Br-SOG complex of adult female insect by a three-step RP-HPLC fractionation. Under specific parameters, synthetic Hez-PBAN (used as standard) shows a retention time of about 21 min (figure 3A). Twenty µl Sep-pak purified Br-SOG extract of *A. janata* was injected into the column under same parameters (figure 3B). A fraction eluted showing a peak corresponding to that of the standard PBAN retention time was collected (figure 3D). This HPLC profile shows along with our peptide of interest,

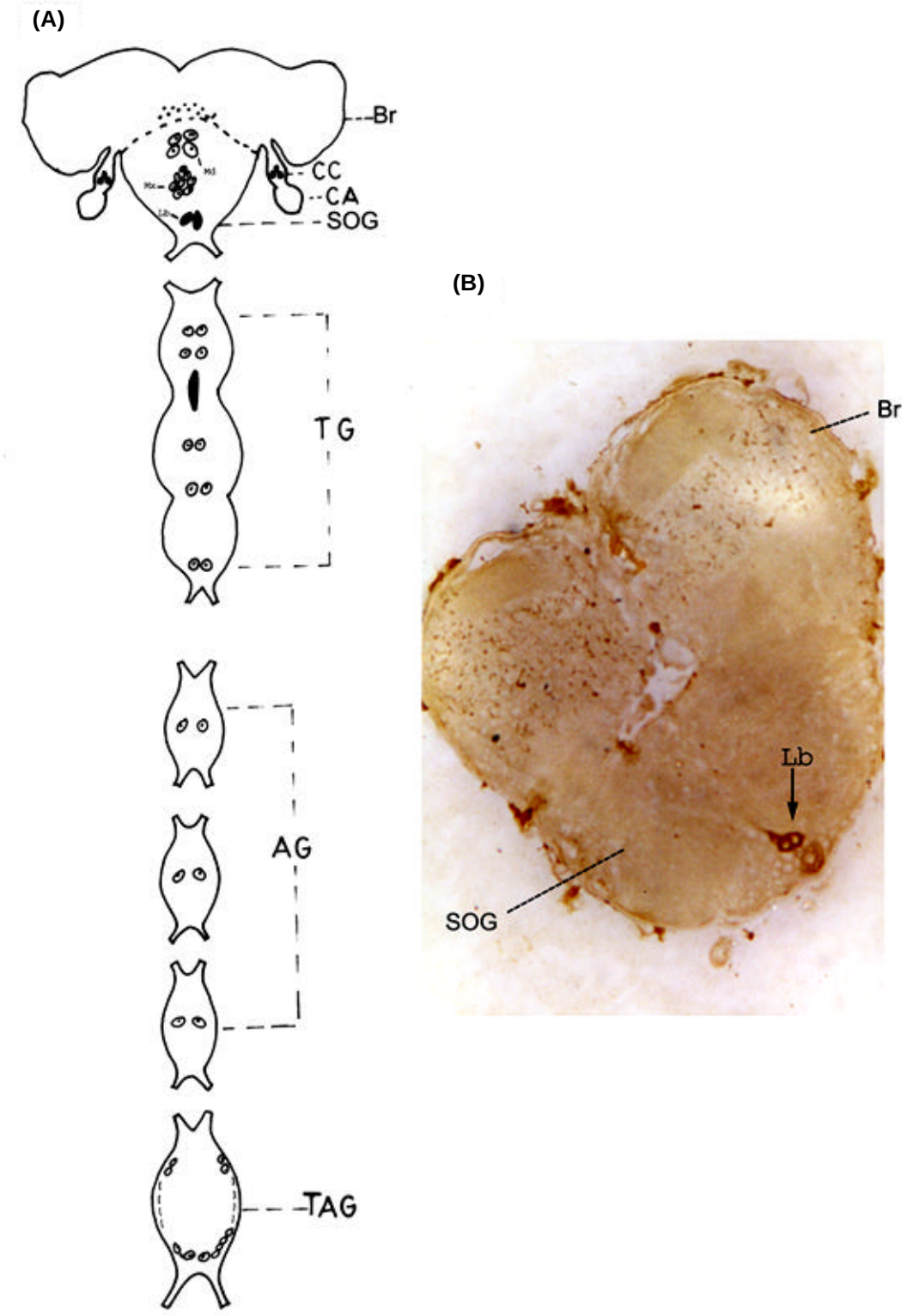

Figure 1. (**A**) Diagrammatic representation of *A. janata* moth central nervous system (CNS) and the distribution pattern of immunoreactive somata. Br, Brain; CC, corpus cardiacum; CA, corpus allatum; SOG, suboesophageal ganglion; TG, fused thoracic ganglia; AG, abdominal ganglia; TAG, terminal abdominal ganglion. (**B**) Sagittal section of Br-SOG complex showing PBAN immunoreactive (ir) labial cels in the SOG (arrow), no ir-cells in the brain.

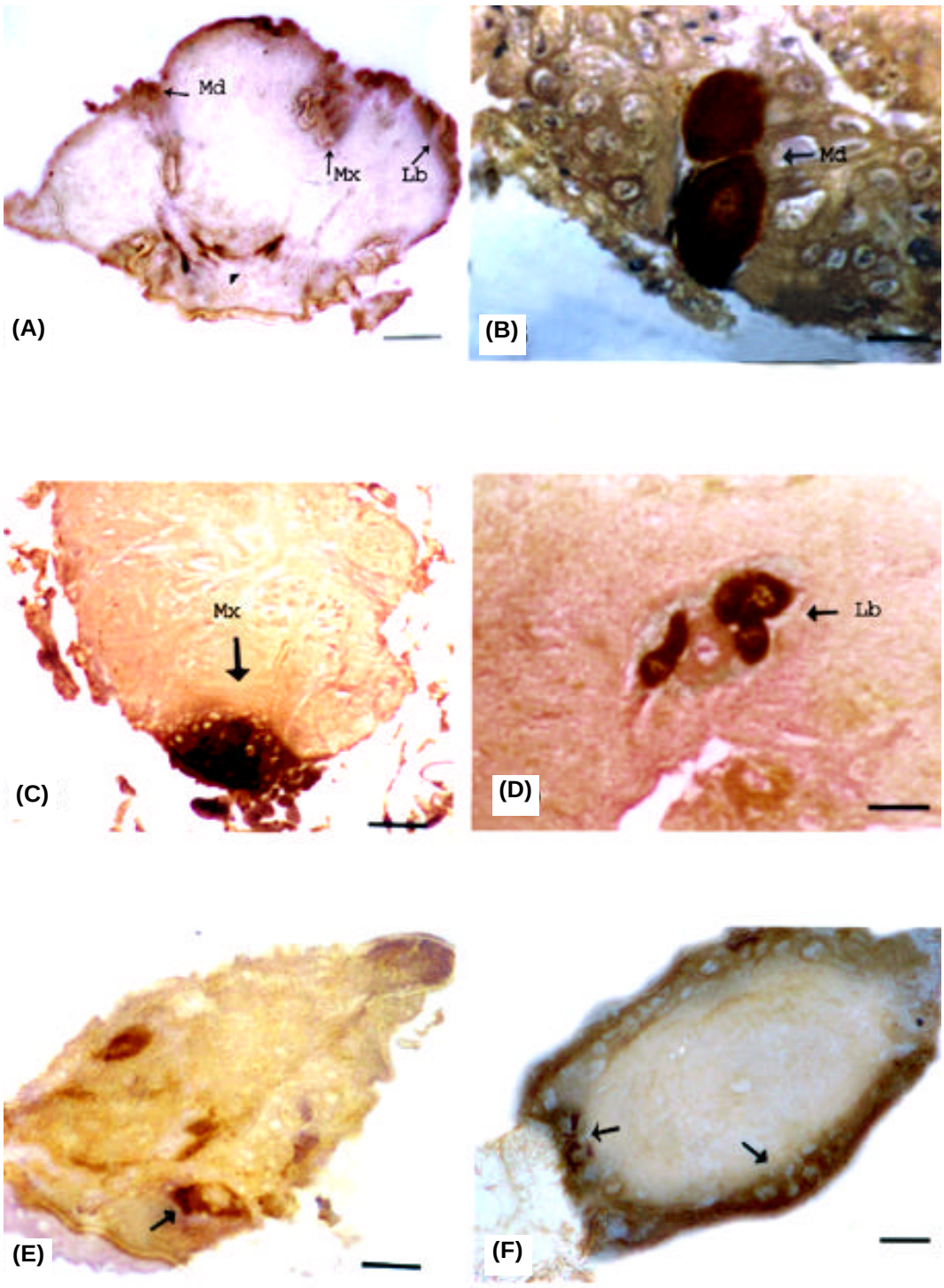

Figure 2. (**A**) Sagittal section of SOG showing three clusters of PBAN positive cells – mandibular; maxillary; and labial (arrows) and reveals tracts (arrowheads) comprising axons from neurons. (**B**) Enlarged view of two mandibular PBAN-positive cells in the SOG. (**C**) Maxillary groups of small immunopositive cells in the TS of SOG. (**D**) TS of SOG showing labial ir-somata. (**E**) Section of CC showing PBAN-immunoreactive cells (arrow) and axonal endings. (**F**) Immunoreactive neurons and arborizarions in the TS of terminal abdominal ganglion. (Scale bars: **A**, **E**, **F**, 20 µm; **B**, **C**, **D**, 10 µm.)

other peaks corresponding to several other peptides that might be present in the same tissue. The particular fractions showing almost same retention time were collected from multiple runs, pooled and concentrated. This was subjected to second and third HPLC purification steps. Figure 3C shows second step purification in which fewer peaks can be observed along with our peptide of interest which appeared as a major peak after 21 min. This particular fraction was collected from five successive runs, lyophilized and injected into the column. The final step HPLC profile shows the single major peak corresponding to the isolated peptide from Br-SOG extract of *A. janata* (figure 3D).

3.3 *Immunoblotting*

Using Western blot, antibodies raised against synthetic Hez-PBAN proved to be specific for the corresponding peptide (figure 4, lane 1). The antibodies were then tested

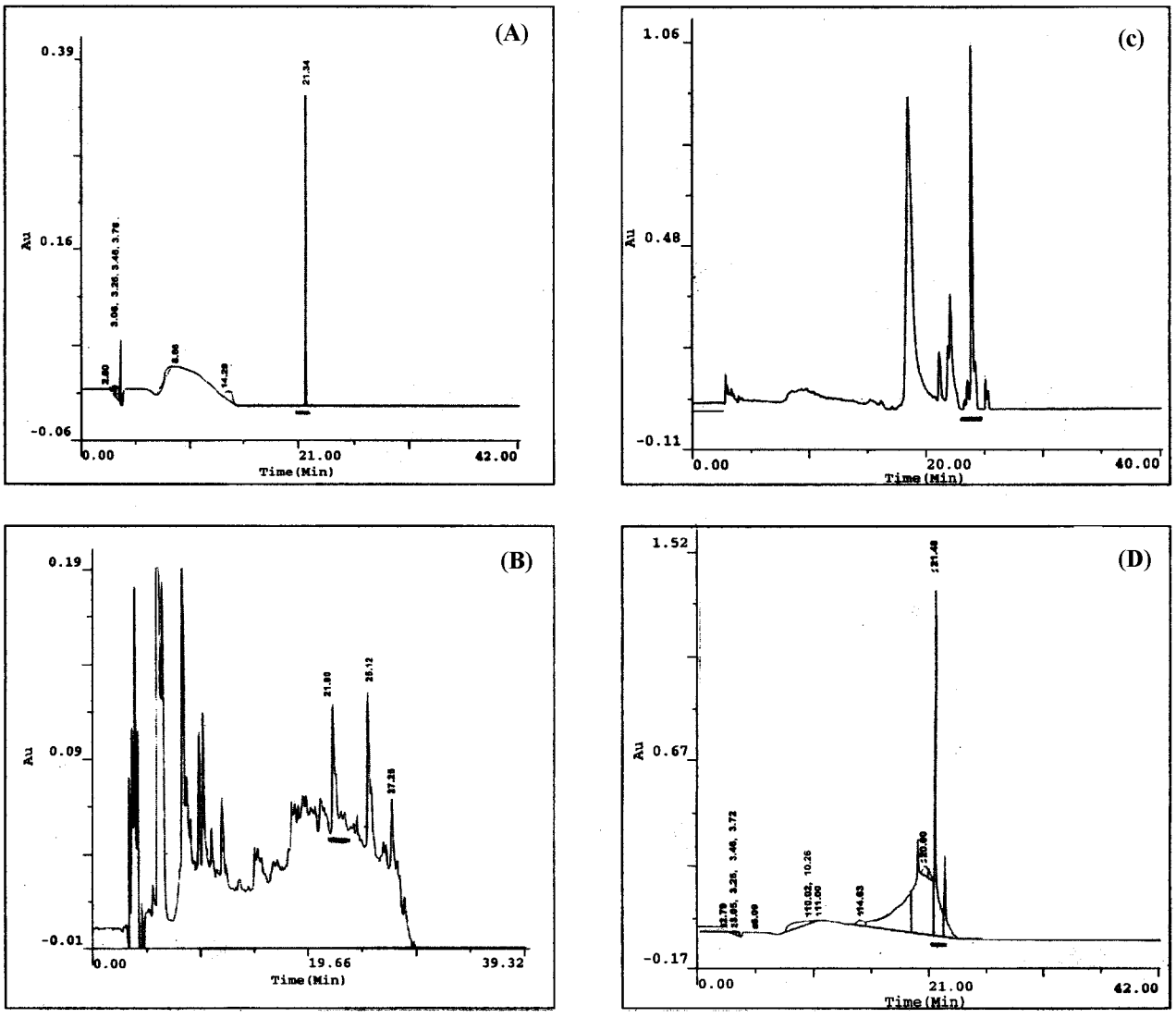

Figure 3. (**A**) RP-HPLC profile of synthetic Hez-PBAN standard (solid bar) indicates the peak corresponding to PBAN. (**B**) Fractionation of Sep-pak purified Br-SOG extract from *A. janata* on RP-HPLC-first step isolation profile of PBAN (solid bar) indicates fraction showing PBAN-like activity. (**C**) Second step fractionation of active fractions eluted at about 21 min in first step (**B**). Particular fractions were pooled from 10 runs. HPLC profile shows fewer peaks and solid bar represents the PBAN-like fraction. (**D**) Final step reverse-phase fractionation of active fractions from second step HPLC. Active fractions were pooled from five runs, concentrated and injected. Pheromonotropic activity was determined in the fraction eluting at 21·48 min which represents purified PBAN from *A. janata* (solid bar).

against purified PBAN-like fraction from *A. janata*. These antibodies specifically recognized a peptide with almost same electrophoretic mobility as the PBAN standard (figure 4, lane 2).

3.4 *Bioassay*

Addition of the purified peptide fraction into the incubation medium containing pheromone glands resulted in higher label incorporation than the controls, indicating higher pheromone production. Radioactivity (cpm/gland) was measured and it was observed to be higher in experimental samples containing 1 pmol of PBAN fraction than the control (figure 5).

3.5 *Molecular weight analysis*

MALDI-MS analysis of the HPLC-purified peptide fraction from *A. janata* showed a single peak of molecular weight 3900 Da (figure 6).

4. Discussion

In the adults of *A. janata*, the SOG itself is fused to the brain. Serial sections of Br-SOG complex revealed no PLI in the brain. This finding is similar to that with other lepidopteran insects where ir-cells are not detected in the brain (Blackburn *et al* 1992; Ichikawa *et al* 1995; Davis *et al* 1996). Three clusters of ir-somata are detected in the SOG which parallels the findings with *H. zea* (Blackburn *et al* 1992; Kingan *et al* 1992) with *B. mori* (Ichikawa *et al* 1995), *Manduca sexta* (Davis *et al* 1996), *Lymantria dispar* (Golubeva *et al* 1997) as well as with results in *Agrotis ipsilon* (Duportets *et al* 1998). In all these reports, the three clusters of cells are mandibular, maxillary and labial cell groups.

In the present study axonal endings could be detected in the CC along with ir-cells which may suggest dual role of the CC – the glandular and neuronal functions. Presence of putative PBAN in the CC and its fluctuating titer between photophase and scotophase is quantified by *in vivo* bioassay (Raina *et al* 1991), led to the assumption that PBAN is continuously produced in the SOG and transported to the CC and released into the haemolymph at the onset of scotophase.

In the present study, in addition to Br-SOG, certain cells of TG and AG reacted positively to anti-PBAN. Similar results were obtained in gypsy moth *L. dispar* where the prothoracic ganglion and meso- and metathoracic neuromeres of the pterothoracic ganglion each contained a pair of ir-somata (Golubeva *et al* 1997). The posterior part of the TAG in *A. janata* contained ir-cells and a field of ir-arborizations not found in other ganglia. Similar distribution of PLI could be located in *H. zea* also (Kingan *et al* 1992).

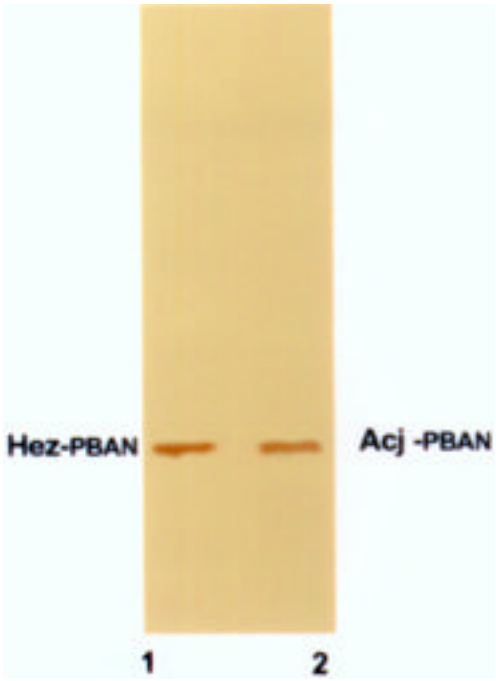

Figure 4. Immunoblot of both Hez-PBAN (lane 1) and purified PBAN from *A. janata* (lane 2) using antibody against synthetic Hez-PBAN.

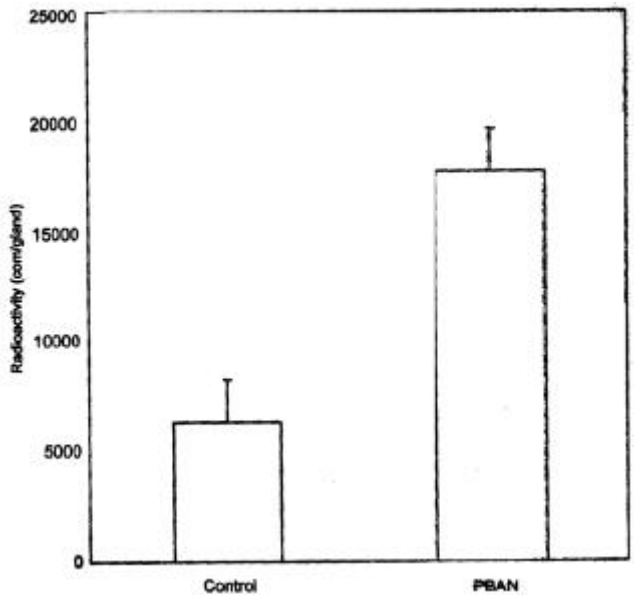

Figure 5. Pheromonotropic effect of isolated PBAN from *A. janata*.

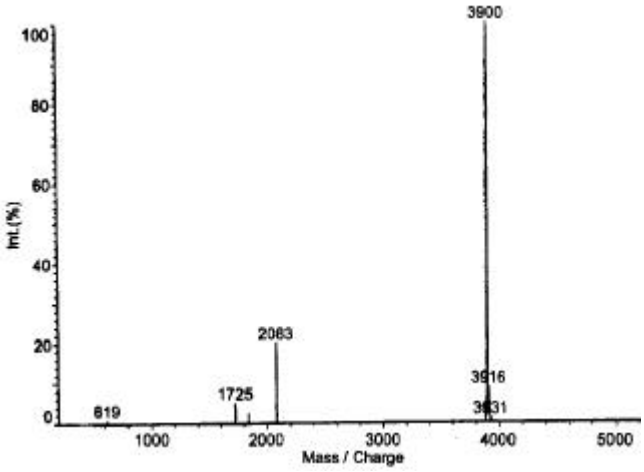

Figure 6. MALDI-MS showing the molecular mass of isolated PBAN.

Pheromonotropic activity could be recovered from *A. janata* Br-SOG complex through RP-HPLC fractionation. Hez-PBAN, a 33 amino acid peptide with a molecular mass of almost 3900 Da was purified by several HPLC steps (Raina *et al* 1989). Later Bom-PBAN I and II which were purified from 6×10^5 heads of adult silk worm, *B. mori* using multi-step purification procedure (Kitamura *et al* 1989 1990). A much shorter peptide with pheromonotropic activity has been isolated from 32000 heads of the larvae of army worm, *Pseudaletia separata* by HPLC purification (Matsumoto *et al* 1992). Lyd-PBAN was isolated from about 2000 Br-SOG complexes in a 5 step HPLC purification protocol (Masler *et al* 1994).

Using Western blot, polyclonal antibodies raised against synthetic Hez-PBAN (MW 3900 Da) specifically recognized a peptide with the same electrophoretic mobility as that of the standard. The estimated MW of this HPLC-purified immunoreactive peptide in *A. janata* employing MALDI-MS was found to be 3900 Da. This result is in accordance with the observation that showed pheromonotropic activity in Br-SOG extracts from *M. brassicae* and *H. zea* using western blot (Jacquin-Joly *et al* 1994; Jacquin-Joly and Descoins 1996). *M. brassicae* and *H. zea* Br-SOG extracts showed two different immunoreactive peptides with MW 3·8 and 5·4 kDa (Jacquin-Joly *et al* 1994). In all the spodopteran species studied, only one peptide with a MW close to that of PBAN was visible after immunodetection (Jacquin-Joly *et al* 1994).

During isolation and characterization of a neuropeptide, bioassay has to be employed to confirm its activity in the insect. In this study radiolabel (^{14}C acetate) incorporation into the pheromone was found to be higher in experimental samples containing isolated peptide fraction than that in the control not having it.

Through the development of *in vitro* bioassay, isolated pheromone glands of *H. armigera* and *H. zea* were found to be the target tissues for PBAN (Soroker and Rafaeli 1989; Rafaeli *et al* 1990). Injection of pheromone precursor ^{14}C-hexa decanoic acid with and without PBAN into decapitated *B. mori* resulted in pheromone production only after administration of PBAN (Arima *et al* 1991). In *M. brassicae* PBAN stimulated the incorporation of radiolabel acetate into the main pheromone component (Jacquin-Joly *et al* 1994). High pheromone titers were also observed in *Plodia interpunctella* after injection of Hez-PBAN (Rafaeli and Gileadi 1995). Our *in vitro* incubation result also fully agrees with the fact that PBAN is active in isolated pheromone glands in *A. janata*. The *in vitro* results obtained after culturing *S. litura* pheromone gland also favour our results that the target organ of PBAN seems to be the pheromone gland (Fonagy *et al* 1992). PBAN receptors in pheromone gland cells of *Heliothis peltigera* females were demonstrated with photoaffinity biotinilated ligands (Altstein *et al* 2003).

This study suggests that the naturally occurring factor isolated from Br-SOG extract of *A. janata* belongs to the PBAN family of peptides and it controls pheromone production in this species.

Acknowledgements

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References

Ajitha V S and Muraleedharan D 2001a Identification of PBAN-like immuno-reactivity in the neuroendocrine system and midgut of *Dysdercus cingulatus* (Heteroptera, Pyrrhocoridae); *Eur. J. Entomol.* **98** 159–165

Ajitha V S and Muraleedharan D 2001b Isolation and Characterisation of PBAN in *Dysdercus cingulatus* (Heteroptera: Pyrrhocoridae); *Entomon* (*Spl. Issue*) **26** 173–181

Altstein M 2004 Novel insect control agents based on neuropeptide antagonists: The pyrokinin/PBAN family as a model; *J. Mol. Neurosci*. **22** 147–158

Altstein M, Ben–Aziz O, Bhargawa K, Li Q and Martins-green M 2003 Histochemical localization of PBAN receptor in the pheromone glands of *Heliothis peltigera*; *Peptides* **24** 1335–1347

Arima R, Takahara K, Kadoshima T, Numazaki F and Ando T 1991 Hormonal regulaton of pheromone biosynthesis in the silkworm moth *Bombyx mori*; *Appl. Entomol. Zool.* **26** 137–147

Blackburn M, Kingan T G, Raina A K and Ma M C 1992 Co-localization and differential expression of PBAN – and FMRF amide – like immuno reactivity in the suboesophageal ganglion of *Heliothis zea* during development; *Arch. Insect Biochem. Physiol*. **21** 225–238

Choi M Y, Tanaka S, Kataoka H, Boo K S and Tatsuki S 1998 Isolation and identification of the CDNA encoding the pheromone biosynthesis activating neuropeptide and additional neuropeptides in the oriental tobacco budworm *Helicoverpa assulta*; *Insect Biochem. Mol. Biol.* **28** 759–766

Davis N T, Homberg U, Teal P E A, Altstein M, Agricola H J and Hildebrand J G 1996 Neuroanatomy and immunocytochemistry of the median neuroendocrine cells of suboesophageal ganglion of the tobacco hawkmoth, *Manduca Sexta*. Immunoreactivities to PBAN and Other peptides; *Microsc. Res. Tech.* **35** 201–229

Duportets L, Gadenna C, Dufour M C and Couillaud F 1998 Pheromone biosynthesis activating neuropeptide (PBAN) of black cutworm moth, *Agrotis ipsilon*: Immunochemistry, molecular characterization and bioassay of its peptide sequence; *Insect Biochem. Mol. Biol.* **28** 591–599

Fonagy A, Matsumoto S, Schoofs L, De Loof A and Mitsui T 1992 *In vivo* and *in vitro* pheromonotropic activity of two locusta tachykinin peptides in *Bombyx mori*; *Biosci. Biotechnol. Biochem.* **56** 1692–1693

Golubeva E, Kingan T G, Blackburn M B, Masler E P and Raina A K 1997 The distribution of PBAN-like immunoreactivity in the central nervous system of gypsymoth *Lymantria dispar*; *Arch. Insect Biochem. Physiol.* **34** 391–408

Holman G M, Cook B J and Nachman R J 1986 Isolation, primary structure and synthesis of two neuropeptides from *Leucophaea maderae*: Members of a new family of cephalomyotropins; *Comp. Biochem. Physiol.* **84** 205–211

Ichikawa T, Hasegawa K, Shimizu L, Katsuno K, Kataoka H and Suzuki A 1995 Structure of neurosecretory cells with immuno reactive diapause hormone and PBAN in the silkworm *Bombyx mori*; *Zool. Sci.* **12** 703–712

Iglesias F, Marco P, Francois M-C, Camps F, Fabrias G and Jacquin-Joly E 2002 A new member of the PBAN family in *Spodoptera littoralis*: molecular cloning and immunovisualisation in scotophase haemolymph; *Insect Biochem. Mol. Biol.* **32** 901–908

Imai K, Konno T, Nagasawa Y, Komiya T, Isobe M Koga K, Hasegawa K and Yamashita O 1991 Isolation and structure of diapause hormone of the silkworm, *Bombyx mori*; *Proc. Jpn. Acad. Sericul.* **B67** 98–101

Jacquin-Joly E, Burnet M, Francois M C, Ammar D, Nagman L M P and Descoins C 1998 cDNA cloning and sequence determination of pheromone biosynthesis new member of the PBAN family; *Insect Biochem. Mol. Biol.* **B2** 251–258

Jacquin-Joly E and Descoins C 1996 Identification of PBAN-like peptides in the brain-suboesophageal ganglion complex of Lepidoptera using Western blotting; *Insect Biochem. Mol. Biol.* **26** 209–216

Jacquin-Joly E, Jurenka R A, Ljungnberg H, Nagnan P, Lofstedt C, Descoins C and Roelofs W L 1994 Control of sex pheromone biosynthesis in the moth, *Mamestra brassicae* by the pheromone biosynthesis activating neuropeptide; *Insect Biochem. Mol. Biol.* **24** 203–211

Kingan T G, Blackburn M B and Raina A K 1992 The distribution of Pheromone biosynthesis activating neuropeptid (PBAN) immunoreactivity in the central nervous system of the corn earworm moth, *Heliothis zea*; *Cell Tissue Res.* **270** 229–240

Kitamura A, Nagasawa H, Kataoka H, Inoue T, Matsumoto S, Ando T and Suzuki A 1989 Aminoacid sequence of PBAN of the silkmoth, *Bombyx mori*; *Biochem. Biophys. Res. Commun.* **163** 520–526

Kitamura A, Nagasawa H, Kataoka H, Ando T and Suzuki A 1990 Aminoacid sequence of PBAN-II of the silkworm moth *Bombyx mori*; *Agric. Biol. Chem.* **54** 2495–2497

Lee J M, Choi M Y, Han K S and Boo K S 2001 Cloning of the cDNA Encoding pheromone biosynthesis activating neuropeptide in *Adoxophyes* Sp. (Lepidoptera: Tortricidae): a new member of the PBAN family; *Gen Bank* (Direct Submission)

Ma P W K, Knipple D C and Roelofs W L 1994 Structural organization of the *Heliothis zea* gene encoding the precursor protein for pheromone biosynthesis activating neuropeptides; *Proc. Natl. Acad. Sci. USA* **91** 6506–6510

Masler E P, Raina A K, Wagner R W and Kochansky J P 1994 Isolation and identification of a pheromonotropic neuropeptide from the brain–suboesophageal ganglion complex of *Lymantria dispar*: A new member of the PBAN family; *Insect Biochem. Mol. Biol.* **24** 829–836

Matsumoto S, Fonagy A, Kurihara M, Uchiumi K, Nagamine T, Chijimatsu M and Mitsui T 1992 Isolation and primary structure of a novel pheromonotropic neuropeptide structurally related to leucokinin from the armyworm larvae, *Pseudaletia separata*; *Biochem. Biophys. Res. Commun.* **182** 534–539

Matsumoto S, Kitamura A, Nagasawa H, Kataoka H, Orikasa C, Mitsui A and Suzuki A 1990 Functional diversity of a neurohormone produced by the sub-oesophageal ganglion – molecular identity of melanization and reddish coloration hormone and pheromone biosynthesis activating neuropeptide; *J. Insect Physiol.* **36** 427–432

Rafaeli A and Gileadi C 1995 Factors affecting pheromone production in the stored product moth, *Plodia interpunctella.* A preliminary study; *J. Stored Product Res.* **31** 243–247

Rafaeli A, Soroker V, Kamensky B and Raina A K 1990 Action of pheromone biosynthesis activating neuropeptide on *in vitro* pheromone glands of *Heliothis armigera* females; *J. Insect Physiol.* **36** 691–696

Raina A K, Jeffe H, Kempe T G, Keim P, Blacher R W, Fales H M, Riley C T, Klun J A, Ridgway R L and Hayes D K 1989 Identification of a neuropeptide hormone that regulates sex pheromone production in female moths; *Science* **244** 796–798

Raina A K and Kempe T G 1990 A pentapeptide of the C-terminal sequence of PBAN with pheromonotropic activity; *Insect Biochem.* **20** 849–851

Raina A K, Kempe T G and Jeffe H 1991 PBAN regulation of pheromone production in moths; in *Insect neuropeptides*: *Chemistry, biology and action* (eds) J J Menn, T J Kelly and E P Masler (Washington DC: American Chemical Society) pp 100–109

Raina A K and Klun J A 1984 Brain factor control of sex pheromone production in the female corn earworm moth; *Science* **225** 531–533

Sato Y, Oguchi M, Menjo N, Imai K, Saito Ikeda M, Isobe M and Yamashita O 1993 Precursor polyprotein for multiple neuropeptides secreted from the suboesophageal ganglion of the silkmoth, *Bombyx mori*: Characterization of the cDNA encoding the diapause hormone precursor and identification of additional peptides; *Proc. Natl. Acad. Sci. USA* **90** 3251–3255

Schagger H and Von Jagow G 1987 Tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis for the separation of proteins in the range of 1 to 100 kDa; *Anal. Biochem.* **166** 368–379

Scharrer B 1989 Neuropeptide research in historical perspective; in *Insect neuropeptides*: *Chemistry, biology and action* (eds) J J Menn, T J Kelly and E P Masler (2–5, ACS symposium series)

Schoofs L, Holman G M, Hayes T K, Nachman R J and De Loof A 1991 Isolation, primary structure and synthesis of locusta pyrokinin: a myotropic peptide of *Locusta migratoria*; *Gen. Comp. Endocrinol.* **81** 97–104

Schooneveld H and Veenstra J A 1988 Immunocytochemistry; in *Immunological techniques in insect biology* (eds) L I Gilbert and T A Miller (New York: Springer Verlag) pp 93–133

Soroker V and Rafaeli A 1989 *In vitro* hormonal stimulation of acetate incorporation by *Heliothis armigera* pheromone glands; *Insect Biochem.* **67** 1–5

Sreng L, Moreau R and Girardie A 1990 Locust neuropeptides stimulating sex pheromone production in female European corn borer moth, *Ostrinia nubilalis*; *J. Insect Physiol.* **36** 726–729

Sreng L and Roelofs W L 1988 Nouveaux neuropeptides de Blatte (Dictyoptera) et de Grillon (Orthoptera), Stimulant la production de pheromone chez la pyrale dur mais (Lepidoptera); *Abstracts in Annals Society of Entomology*, France 24, p. 453

Torfs P, Nieto J, Cerstiaens A, Boon D, Baggerman G, Poulos C, Waelkens E, Derua R, Calderon J, De Loof A and Schoofs L 2001 Pyrokinin neuropeptides in a Crustacean: Isolation and Identification in the white shrimp, *Penaeus vannamei*; *Eur. J. Biochem.* **268** 149–154

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