Reports

Identification of a Neuropeptide Hormone That Regulates Sex Pheromone Production in Female Moths

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A pheromone biosynthesis activating neuropeptide (PBAN) hormone that controls sex pheromone production in female moths was identified from the brain-subesophageal ganglion complexes of the adult corn earworm, *Heliothis zea*. PBAN has 33 amino acid residues and a molecular weight of 3900. Its amino acid sequence has no significant homology with any of the fully characterized peptide hormones. The synthetic peptide, at a dose of between 2 and 4 picomoles, induced production of a normal quantity of sex pheromone in ligated *H. zea* females. The peptide also induced pheromone production in six other species of moths, thus indicating that this or similar peptides may be responsible for the regulation of pheromone production in moths.

FEMALE MOTHS OF MANY SPECIES produce and release sex pheromones to attract conspecific males for mating. In the corn earworm, *Heliothis zea*, and several other species of moths, pheromone production follows a diel periodicity (*1*). Sex pheromone production in *H. zea* was shown to be controlled by a brain factor (*2*). Subsequently the factor was reported to be a peptide hormone produced in the subesophageal ganglion of both males and females and released through the corpora cardiaca into the hemolymph at the onset of scotophase to induce pheromone biosynthesis (*3*).

We report the isolation, characterization, and synthesis of the pheromone biosynthesis activating neuropeptide from *H. zea* [Hez-PBAN (*4*)] and demonstrate its hormonal activity in six species of moths in four families by means of a simple and highly sensitive bioassay developed for this hormone (*2*). The chemistry of the sex pheromone of these species is different from that of *H. zea*. This peptide or ones structurally similar to it may also regulate pheromone production in these and other species of moths. The partial sequence of PBAN from the silkworm, *Bombyx mori*, which is very similar to that of Hez-PBAN (*5*) supports this speculation.

PBAN was isolated from two sets of about 2500 brain-subesophageal ganglion complexes dissected from male and female *H. zea* adults. We used a three-step isolation procedure (*6*) that was a modification of a previously reported method used for the purification of this peptide (*7*). Fractions from high-performance liquid chromatography (HPLC) with a Supelcosil LC-18DB column (step 1) showed biological activity between 44 and 52 min with three distinct fractions eluting at 44 through 45, 49, and 51 min (*8*). Most activity was associated with the 44- through 45-min fractions, and these were purified further. Step 2 used four Waters Protein-Pak 125 high-performance size exclusion columns. Biological activity was found in fractions eluting at 33 through 34 min. These fractions were pooled and rerun on a Vydac C-18 column (step 3) to yield a single biologically active peak eluting at 29.2 min. Amino acid analysis of about 25 pmol of the peptide with an Applied Biosystems Model 420A derivatizer-analyzer revealed the following composition: Asn or Asp, 5.2 (5); Gln or Glu, 4.5 (5); Ser, 2.2 (2); Gly, 2.6 (0); Arg, 2.7 (3); Thr, 1.6 (2); Ala, 2.2 (2); Pro, 3.5 (4); Tyr, 1.7 (2); Met, 1.0 (2); Ile, 0.8 (1); Leu, 1.6 (2); Phe, 0.8 (1); and Lys, 0.9 (1) (values in parentheses indicate the number of residues subsequently found in complete sequence). Determination of the amino acid sequence was first carried out with about 50 pmol of purified peptide with an Applied Biosystems Model 470A gas-phase sequencer. Sequence information was obtained only for the first 14 amino acid residues, and it was also revealed that the amino terminus of the peptide was not blocked. A second analysis was performed with about 200 pmol of the peptide with an Applied Biosystems Model 477A pulsed liquid-phase sequencer on line with a Model 120A phenylthiohydantoin (PTH) analyzer. Data obtained from 33 cycles indicated the following sequence: Leu-Ser-Asp-Asp-Met-Pro-Ala-Thr-Pro-Ala-Asp-Gln-Glu-Met-Tyr-Arg-Gln-Asp-Pro-Glu-Gln-Ile-?-Ser-Arg-Thr-Lys-Tyr-Phe-Ser-Pro-?-Leu (Fig. 1) (*9*). However, the residues at positions 23 and 32 were not determined. A third analysis with the same instrument confirmed the earlier sequence and established the presence of Asp and Arg at positions 23 and 32, respectively. The amino acid composition was in good agreement with sequence data, except for extraneous Gly and low Met. The low yield of Met commonly results from hydrolysis (*10*), whereas the Gly is apparently a common contaminant of isolation protocols. Carboxyl-terminal sequencing revealed that Leu was the carboxyl terminus, as was evident by its complete release at the 2-min time point (Fig. 2). The next residue released was Arg followed by Pro, Ser, Phe, Tyr, and Lys. The results confirmed the sequence data for the last seven residues. However, the data did not establish whether Leu was present as a free acid or an amide.

Mass spectra of the peptide were obtained on a californium-252 time-of-flight plasma

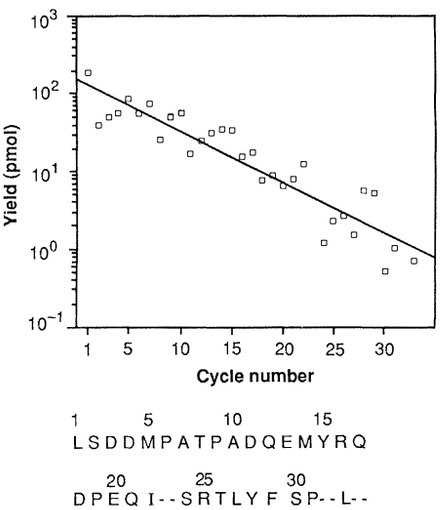

Fig. 1. Smithies plot of the sequence analysis of Hez-PBAN. Background corrected data. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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desorption mass spectrometer (*11*). Two peaks were observed, each containing about 100 ions in its central channel. The first peak was at a mass-to-charge ratio (*m/z*) of 3934 $[(M + H)^+]$, and the second, broader peak at *m/z* 1966 $[(M + 2H)^{2+}]$, suggesting molecular weights of 3933 and 3930, respectively.

Because the majority of the peptides identified from insects are amidated at the carboxyl terminal, a 33–amino acid peptide with a carboxyl-terminal amide was synthesized by solid-phase synthesis (*12*). Final purification of the synthetic peptide was carried out by HPLC [same as step 3 in isolation (*6*)]. The peptide eluted at 32.2 min. Its ultraviolet light (UV) spectrum was similar to but not identical to that of the native peptide, and the retention times were also significantly different. It was noted from the literature that oxidation of Met to methionine sulfoxide occurs during the isolation of peptides and proteins from natural sources (*13*). Consequently, when the synthetic peptide was oxidized (*14*), the resulting product demonstrated a retention time and UV spectrum identical to that of the native peptide. The molecular weight for the native peptide calculated from the most abundant ion in its isotope cluster is 3899.8. Addition of two oxygens, one for each Met, gave 3931.8, in good agreement with the experimental value of 3930 to 3933. The synthetic unoxidized peptide showed a very broad $[(M + H)^+]$ ion at *m/z* 3902 to 3906. On the basis of all the data presented, the following primary structure is proposed for Hez-PBAN: Leu-Ser-Asp-Asp-Met-Pro-Ala-Thr-Pro-Ala-Asp-Gln-Glu-Met-Tyr-Arg-Gln-Asp-Pro-Glu-Gln-Ile-Asp-Ser-Arg-Thr-Lys-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2. It was observed that purified samples of synthetic Hez-PBAN were easily converted to a mixture of oxidation products resulting from the oxidation of Met^5 or Met^{14}, or both, to methionine sulfoxide. We speculate that the continuum of biologically active HPLC fractions, originally observed (*8*) between 44 and 52 min after step 1, represented Hez-PBAN and its various oxidation products, all of which had biological activity. To our knowledge, Hez-PBAN does not have significant sequence homology with any of the fully characterized invertebrate or vertebrate peptide hormones. However, there is a striking similarity between this peptide and the partial amino-terminal sequence of the melanization and reddish coloration hormone (MRCH) isolated from *B. mori* (*15*). Recently, it was suggested that PBAN from *B. mori* is very close to, or identical with, MRCH (*5*).

When the synthetic Hez-PBAN was injected into *H. zea* females that were ligated between the head and the thorax, it caused normal production of the sex pheromone, with all the components being present in the correct proportion. A dose-response study indicated that a dose of 1 pmol or less did not cause significant production of the sex pheromone (Fig. 3). There was also a significant decrease in pheromone production above the 4-pmol dose, but the response showed a second peak at 32 pmol and then leveled off. A crude extract of brain-subesophageal ganglion caused production of pheromone [99.0 ± 17.1 ng (SEM), $n = 9$] equivalent to between 2 and 4 pmol of the synthetic peptide. Hez-PBAN, when injected into ligated females of six other species of moths, caused production of significant amounts of their respective pheromones (Table 1). In two of the six species, the quantity of pheromone produced was six to seven times that found in normal females. The expression of pheromonotropic activity of Hez-PBAN in species with different pheromone chemistry suggests that PBAN may be activating an early step in the biosynthetic pathway of these pheromones. The avail-

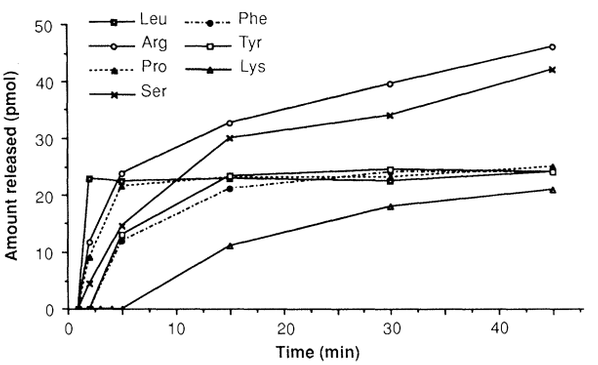

Fig. 2. Carboxyl-terminal sequencing of Hez-PBAN. Approximately 200 pmol of the peptide was digested with carboxypeptidase P (Boehringer Mannheim). Digest proceeded in 10 m*M* sodium acetate, *p*H 5.25, at room temperature for the designated time intervals. Enzyme-to-substrate molar ratio was 1:20. The values as plotted were normalized to the recovery of norleucine, used as an internal standard.

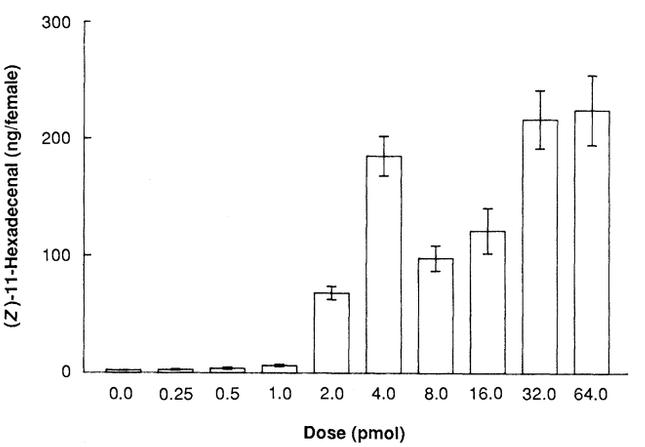

Fig. 3. Dose response of Hez-PBAN. Synthetic peptide was dissolved in sucrose phosphate buffer (*3*), and injected into ligated 3-day-old females during scotophase. Pheromone was extracted 3 hours after injection and quantified by capillary gas chromatography (*1*). Each data point represents the mean of five observations ± SEM.

Table 1. Cross-reactivity of Hez-PBAN in six other species of moths. Bioassay procedure is described under Fig. 3. Each female was injected with 5 pmol of the synthetic peptide in 10 μl of buffer. The major component in the pheromone of *Heliothis virescens* (tobacco budworm), *Spodoptera frugiperda* (fall armyworm), *Diaphania nitidalis* (pickleworm), *Ostrinia nubilalis* (European corn borer), *Manduca sexta* (tobacco hornworm), and *Lymantria dispar* (gypsy moth) are (*Z*)-11-hexadecenal, (*Z*)-9-tetradecen-1-ol acetate, (*E*)-11-hexadecenal, (*E*)-11-tetradecen-1-ol acetate, (*E, Z*)-10,12-hexadecadienal, and *cis*-2-decyl-3-(5-methylhexyl)oxirane, respectively. Sex pheromone titer is expressed in nanograms of the major component per female ± SEM, $n = 5$.

Test species	Family	Quantity of the major component in the pheromone of		
		Normal female	Ligated female	Hez-PBAB–injected female
H. virescens	Noctuidae	177.9 ± 21.3	5.7 ± 1.6	189.2 ± 12.2
S. frugiperda	Noctuidae	6.9 ± 1.1	1.5 ± 0.2	42.9 ± 12.2
D. nitidalis	Pyralidae	17.5 ± 5.0	2.3 ± 1.0	49.2 ± 16.3
O. nubilalis	Pyralidae	4.0 ± 0.3	0.0	6.7 ± 1.1
M. sexta	Sphingidae	9.0 ± 1.7	3.2 ± 0.4	65.4 ± 11.9
L. dispar	Lymantriidae	9.3 ± 2.3	0.1 ± 0.06	2.5 ± 0.4

ability of the synthetic peptide will greatly enhance our ability to investigate the biochemical processes involved in hormonal control of sex pheromone production in moths. The knowledge thus gained could lead to the development of new methods for the control of insect pests through the interruption of pheromone biosynthesis and reproduction.

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6. Brain-subesophageal ganglion complexes dissected from about 2500 insects and stored at −80°C in 5% formic acid v/v, 15% trifluoroacetic acid (TFA) v/v, 1% NaCl w/v, 1*N* HCl [H. P. J. Bennett, C. A. Brown, S. Solomon, *Anal. Biochem.* **128**, 121 (1983)]. The tissues were homogenized as described previously (*7*). The defatted extract was subjected to an HPLC purification procedure based on a sequence of three chromatographic steps: (i) Supelcosil LC-18 DB with Pelliguard guard column (Supelco) eluted with a concave gradient (Waters Curve no. 7) of 10 to 60% acetonitrile containing 0.1% v/v TFA in 0.1% aqueous TFA over 1 hour at ambient temperature and 1 ml/min on a Model 840 HPLC with autosampler (Waters). The eluant was monitored at 214 nm. (ii) Four Waters Protein-Pak 125 HP-SEC columns plumbed in series were eluted isocratically with a 40% aqueous acetonitrile (0.1% TFA) at ambient temperature and 1 ml/min on the same instrument as in the first step. (iii) Vydac 218 TP 54 C-18 column (Separations Group) was eluted with a linear gradient of 10 to 50% acetonitrile containing 0.1% TFA in 0.1% aqueous TFA, over 1 hour at 28°C at 0.4 ml/min on a Model 1090M HPLC with photodiode array detector and Chemstation (Hewlett-Packard).
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10. The hydrochloric acid used for hydrolysis contained 0.1% sodium sulfite, a reducing agent that may have partially converted the methionine sulfoxide to methionine. The sulfoxide was not determined under the chromatographic conditions used.
11. The sample was applied in the form of a 1% TFA solution containing about 30 pmol of peptide to a nitrocellulose-covered [G. P. Jonsson *et al.*, *Anal. Chem.* **58**, 1084 (1986)] aluminized Mylar foil. The sample was run at 15-kV accelerating volts with a 30-cm flight path, and the signal was allowed to accumulate for 19 hours.
12. Solid-phase synthesis of the peptide amide was done with benzhydrylamine resin, on a Biosearch 9600 peptide synthesizer. All amino acids were protected with *N-tert*-butyloxycarbonyl group. Side chain–protecting groups were: Arg, 4-toluenesulfonyl; Asp, cyclohexyl ester; Glu, benzyl ester; Lys, 2-chlorobenzyloxycarbonyl; Met, sulfoxide; Ser and Thr, benzyl; and Tyr, 2-bromobenzyloxycarbonyl. The dried peptide-resin was treated with the low-high HF method of cleavage [J. P. Tam, W. F. Heath, R. B. Merrifield, *J. Am. Chem. Soc.* **105**, 6442 (1983)]. The crude peptide was extracted from the resin with aqueous acetic acid (20%) and then initially purified by HPLC (Ultrasphere column, 4.6 by 250 mm) with a gradient of 0 to 60% acetonitrile (0.1% TFA) at a flow rate of 1.5 ml/min.
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14. The synthetic peptide amide was oxidized with dimethylsulfoxide/9*N* hydrochloric acid/acetic acid (4:15:30, v/v/v) for 30 min at ambient temperature [R. M. Wagner and B. A. Fraser, *Biomed. Mass Spectrom.* **14**, 69 (1987)]. The product containing the sulfoxide was isolated by HPLC.
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16. We thank J. C. Davis, E. C. Uebel, and C. Michels for technical assistance, C. Woods for doing the final amino acid sequence, and E. Sokoloski for mass spectrometry.

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The Role of Excitatory Amino Acids and NMDA Receptors in Traumatic Brain Injury

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Brain injury induced by fluid percussion in rats caused a marked elevation in extracellular glutamate and aspartate adjacent to the trauma site. This increase in excitatory amino acids was related to the severity of the injury and was associated with a reduction in cellular bioenergetic state and intracellular free magnesium. Treatment with the noncompetitive *N*-methyl-D-aspartate (NMDA) antagonist dextrorphan or the competitive antagonist 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid limited the resultant neurological dysfunction; dextrorphan treatment also improved the bioenergetic state after trauma and increased the intracellular free magnesium. Thus, excitatory amino acids contribute to delayed tissue damage after brain trauma; NMDA antagonists may be of benefit in treating acute head injury.

EXCITATORY AMINO ACIDS (EAAs) have been implicated in delayed brain tissue injury after cerebral ischemia (*1*) and hypoglycemia (*2*) through actions mediated by NMDA receptors. Traumatic brain injury (TBI) also causes secondary neurochemical changes that are associated with delayed tissue damage, many of which are similar to those occurring after cerebral ischemia (*3*). However, it is not known whether EAAs play a role in TBI. We have used ^{31}P magnetic resonance spectroscopy (MRS), microdialysis, and behavioral measures to examine the hypothesis that EAAs are released after TBI and that they contribute to secondary tissue damage through effects at NMDA receptors.

Male Sprague-Dawley rats (400 ± 25 g) were anesthetized with sodium pentobarbital (60 mg per kilogram of body weight, intraperitoneally). Although pentobarbital may have weak nonselective antagonism for EAA receptors (*4*), barbiturates have commonly been utilized in electrophysiological studies that examine the activity of EAA antagonists (*5*). Animals were subjected to a lateral (parasagittal) fluid percussion–induced injury (2.0 or 2.85 atm) through a 2-mm craniotomy site centered over the left parietal cortex (*6*). This fluid percussion method causes transient brain deformation with resultant tissue damage (*6, 7*), leading to moderate (2.0 atm) or severe (2.85 atm) behavioral and histological abnormalities. Microdialysis techniques (*8*) were used to determine whether EAAs are released in response to TBI and whether changes are related to the severity of the injury. Microdialysis probes were implanted stereotaxically ipsilateral to the trauma site, so as to position the probe just below the site of fluid percussion injury; these regions, including the CA2 and CA3 areas of the hippocampus, show delayed cell death after trauma (*9*). Samples were collected at a flow rate of 2 μl/min before and after trauma. Amino acid levels were assayed with high-performance liquid chromatography (*10*). Animals that experienced severe injury ($n = 6$) exhibited a rapid and significant increase in extracellular aspartate and glutamate (Fig. 1), as compared to uninjured, control animals ($n = 4$). Changes were related to injury severity with moderately injured animals ($n = 4$) showing peak increases in glutamate and aspartate of 282% and 273%, respectively, as compared to severely injured animals, which showed increases of 940% and 1849%. Peak effects occurred within the first 10 min, with elevations sustained for more than 1 hour in the severely injured group.

Earlier studies of TBI have demonstrated that the ^{31}P MRS–determined ratio of phosphocreatine to inorganic phosphate (PCr/

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