Molecular Entomology Symposium, July 49-55, 1990

PBAN: FROM INSECTS TO SYNTHETIC PEPTIDE

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INTRODUCTION

Why do insects with a short reproductive life such as moths need neuroendocrine control of sex pheromone production, a key factor in their mating behavior? This was the question posed by Barth (1965) to support his argument that there is no neurohormonal control of pheromone production in moths. However, pheromone production in females of the majority of moth species follows a diel periodicity. For example, *Heliothis zea* females produce and release pheromone only during scotophase (Raina *et al.*, 1986). It was this observation that led to the investigation and discovery of neurohormonal control of pheromone production in *H. zea* (Raina and Klun, 1984).

Riddiford and Williams (1971) had reported that calling in *Hyalophora cecropia* and *Antheraea polyphemus* was controlled by a hormone released from the corpora cardiaca (CC) into the hemolymph. Cardé and Webster (1980) reported that decapitation of female *Platynota stultana* resulted in a decline in the pheromone titer. Raina and Klun (1984) showed that ligation of female *H. zea* between head and thorax completely shut down the pheromone production and that production could be induced in such ligated insects by the injection of brain suboesophageal ganglion (brain-SOG) homogenates. This experiment also formed the basis for a very sensitive and rather simple bioassay. The pheromone was extracted from the ovipositor and quantified by capillary gas chromatography. Because the brain and SOG in adult moths are closely joined, the initial report described the hormone as a brain factor. Subsequently, it was shown that the hormone is a peptide produced in the SOG and released into the hemolymph *via* the CC at the onset of scotophase (Raina and Menn, 1987; Raina *et al.*, 1987). It was also reported that the pheromone biosynthesis activating neuropeptide (PBAN) was present in very small amounts in larvae and pupae and that the titer of the hormone increased significantly in pharate adults, reached its maximum in 2–3 day old females and then gradually decreased. Raina *et al.* (1987) also reported that crude brain-SOG homogenates injected into ligated females rapidly induced pheromone production. Noticeable amounts of pheromone were detected within 15 minutes after the injection, the pheromone attained normal level in one hour, and peaked in four hours. Whereas, 0.5 brain-SOG equivalents was enough to induce a normal titer of the pheromone, it showed significant activity even at 0.125 equivalents. The activity was also shown to be associated with brain-SOG from the male moths.

ISOLATION AND IDENTIFICATION

Brain-SOG complexes were dissected from both male and female *H. zea* adults. Batches of about 2,500 brain-SOG complexes were accumulated and stored at −80°C in Bennett's buffer [5% formic acid, 15% trifluoroacetic acid (TFA), 1% NaCl, and 1 N HCl]. The tissues were homogenized with a polytron homogenizer (Brinkman Instruments) equipped with a PT-7 microprobe. The homogenate was centrifuged at 4,000 rpm at 5°C for 30 minutes. The supernatant was absorbed on a C-18 Sep-Pak (Waters) that had been washed before use with acetonitrile and 0.1% TFA. Sep-Pak was washed with 3 ml 0.1% TFA to remove the buffer. Peptides were eluted with 2 ml 80% acetonitrile+0.1% TFA, and concentrated in a Speed Vac (Savant). The material was diluted to 500 μl with 0.1% TFA, and extracted 2–3 times with ethyl acetate to remove the lipids. Excess ethyl acetate was removed by Speed Vac and the solution filtered through a Millex-HV filter.

The material was subjected to reverse phase-high performance liquid chromatography (RP-HPLC) on a Supelcosil LC-18 DB column with a Pelliguard guard column (Supelco) fitted on a Waters Model 840 instrument. A convave gradient (Waters curve #7) starting with 10% acetonitrile and 90% aqueous 0.1% TFA and ending with 60% acetonitrile and 40% aqueous 0.1% TFA was used. The flow rate was 1 ml/min for 60 minutes. Bioassay of the fractions in ligated *H. zea* females indicated the activity to be associated with 44–52 minute fractions. However, the bulk of the activity was associated with 44 and 45 minute fractions (Fig. 1a). Active fractions were pooled, filtered through Milex HV filter, and analyzed on four Waters Protein-Pak 125 HP size exclusion columns, eluted isocratically with 40% acetonitrile+0.1% TFA at 1.0 ml/minute. Effluent was monitored at 214 nm. The columns were calibrated with 12 standards ranging in molecular weight from 556–67,000. Bioassays indicated activity in 33–34 minute fractions. The molecular weight of PBAN was estimated at 4,200 (Jaffe *et al.*, 1986). Active fractions from HP-SEC were pooled and rerun on Vydac 218 TP 54 C-18 column, eluted with a linear gradient of 10–50% acetonitrile containing 0.1% TFA against 0.1% aqueous TFA over 1 hour at 0.4 ml/minute on a model 1090 M HPLC with photodiode array detector and chemstation (Hewlett-Packard). A single biologically active peak was obtained at 29.2 minutes (Fig. 1b).

Amino acid analysis of about 25 pmol of the peptide was carried out with an Applied Biosystems Model 420 A derivatizer-analyzer. A first attempt at determining the amino acid sequence was carried out on an Applied Biosystems Model 470 A gas-phase sequencer with about 50 pmol of pure peptide. Sequence data was obtained for the first 14 amino acid residues. A second attempt to obtain the amino acid sequence was carried out with about 200 pmol of peptide on an Applied

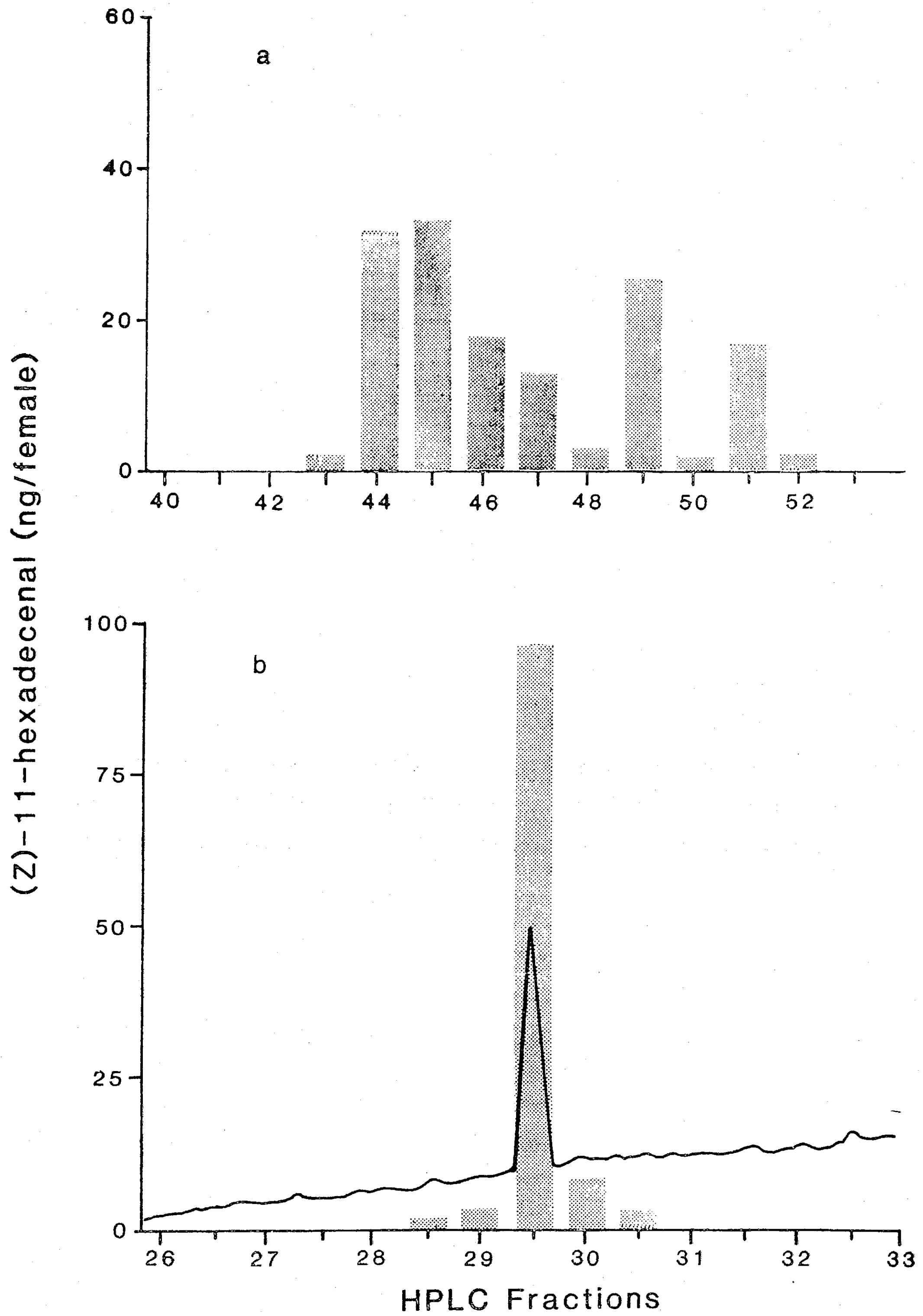

Fig. 1. a. Pheromonotropic activity associated with various HPLC fractions after the first chromatographic step of purification; b. Final purification of PBAN and biological activity associated with a single peak eluting at 29.2 min.

Biosystems Model 477 A pulse liquid phase sequencer and an on-line Model 120 A PTH analyzer. Sequence data was obtained for 33 residues with cycles 23 and 32 as blanks.

Molecular weight of the natural peptide was determined on a Californium-252 time-of-flight plasma desorption mass spectrometer. Two peaks were observed suggesting molecular weights of 3933 and 3930 respectively. By substituting cysteine at positions 23 and 32, we came up with a calculated molecular weight of 3933. However, we could not confirm if the carboxyl-terminal was amidated or free acid. Consequently, two peptides each with 33 residues but differing in the C-terminus (amide or acid) were synthesized by solid phase synthesis with benzhydrylamine resin, on a Biosearch 9600 peptide synthesizer. All amino acids were protected with N-tert-butyloxycarbonyl group. The dried peptide-resin was treated with the low-high HF method of cleavage (Tam *et al.*, 1983). Neither of these synthetic peptides had any biological activity. A third attempt at sequencing revealed Asp and Arg at positions 23 and 32 respectively. C-terminus sequencing confirmed the identity of the last seven residues. However, we still were not sure whether the C-terminus was amidated or free acid. Once again two versions of the peptide were synthesized. Both of these had biological activity but their HPLC-retention times and UV spectra did not match with the natural peptide. The amide had a retention time of 31.5 minutes and the acid 33.5 minutes. During

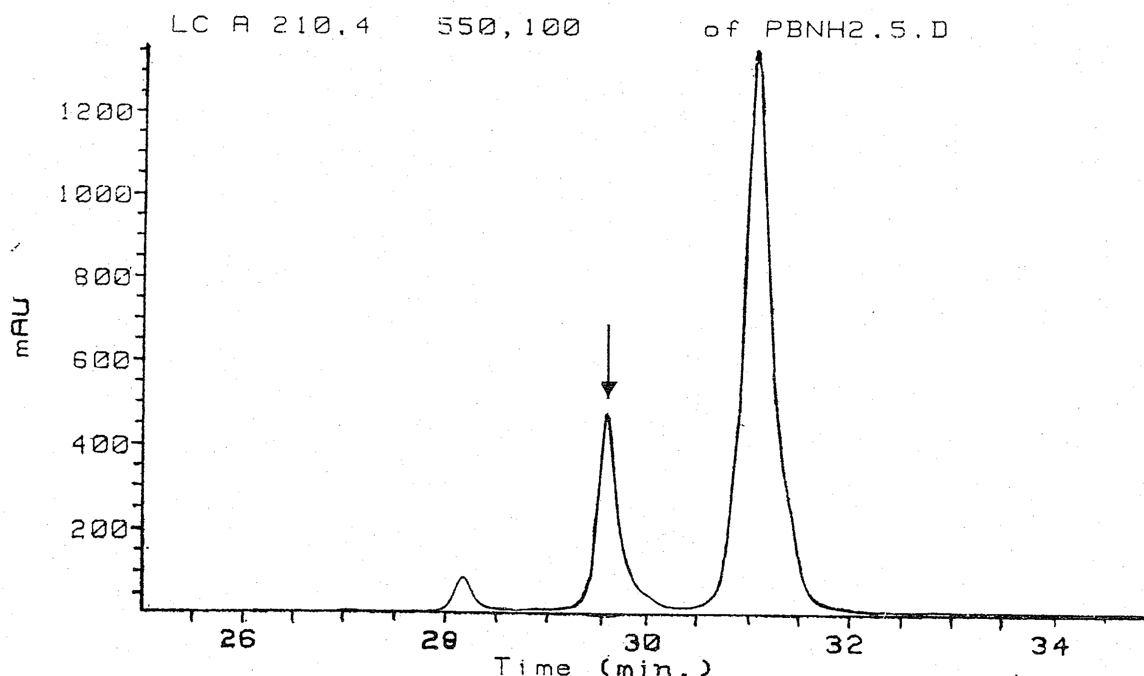

Fig. 2. HPLC purification of synthetic PBAN with C-terminus amide showing the production of two additional compounds, one of which (arrow) matched the purified natural PBAN.

the purification of the peptide-amide, it split into 3 peaks (Fig. 2), one of which had the same retention time as the natural peptide and also matched the UV spectrum.

It was noted from the literature that oxidation of Met to methionine sulfoxide occurs during the isolation of peptides and proteins from their natural sources (Vale *et al.*, 1981). The peptide amide was oxidized with a mixture of dimethylsulfoxide, 9N HCl and acetic acid (4:15:30) for 30 minutes at room temperature (Wagner and Fraser, 1987). The resulting product demonstrated a retention time and UV spectrum identical to that of the natural peptide.

BIOLOGICAL ACTIVITY

The Hez-PBAN, named according to a recent system of insect peptide nomenclature (Raina and Gäde, 1988), is a C-terminal amidated, 33-amino acid residue peptide (Fig. 3) having a molecular weight of 3900. Hez-PBAN induced pheromone production in *H. zea* and six other species of moths (Raina *et al.*, 1989). Approximately 2.5 pmol of the peptide caused a pheromone production equivalent to one brain-SOG. The free acid version was between 100 and 1000 fold less active (Fig. 4).

Nagasawa *et al.* (1988) have reported partial sequence of PBAN isolated from the silk worm *Bombyx mori.* Of the 10 amino acids in their sequence, nine are exactly the same as in Hez-PBAN. It is possible that slight variation in the structure of PBAN from various families of moths may exist. This is also supported by the fact that we got low biological activity from Hez-PBAN in the gypsy moth, *Lymantria dispar*, (Raina *et al.*, 1989). We also do not have any confirmation of its role in the males. With the increasing evidence of presence of male pheromones, it may be that PBAN also controls pheromone production and/or other mating related behaviors in males. There is also evidence that at least in one moth species, the cabbage looper, *Trichoplusia ni*, PBAN does not appear to regulate pheromone production (Tang *et al.*, 1989). However, when

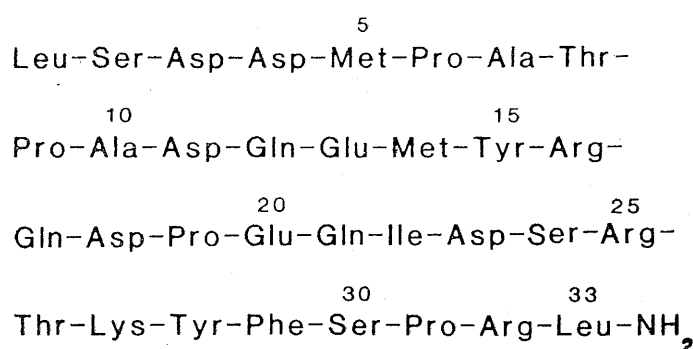

Fig. 3. Amino acid sequence of Hez-PBAN.

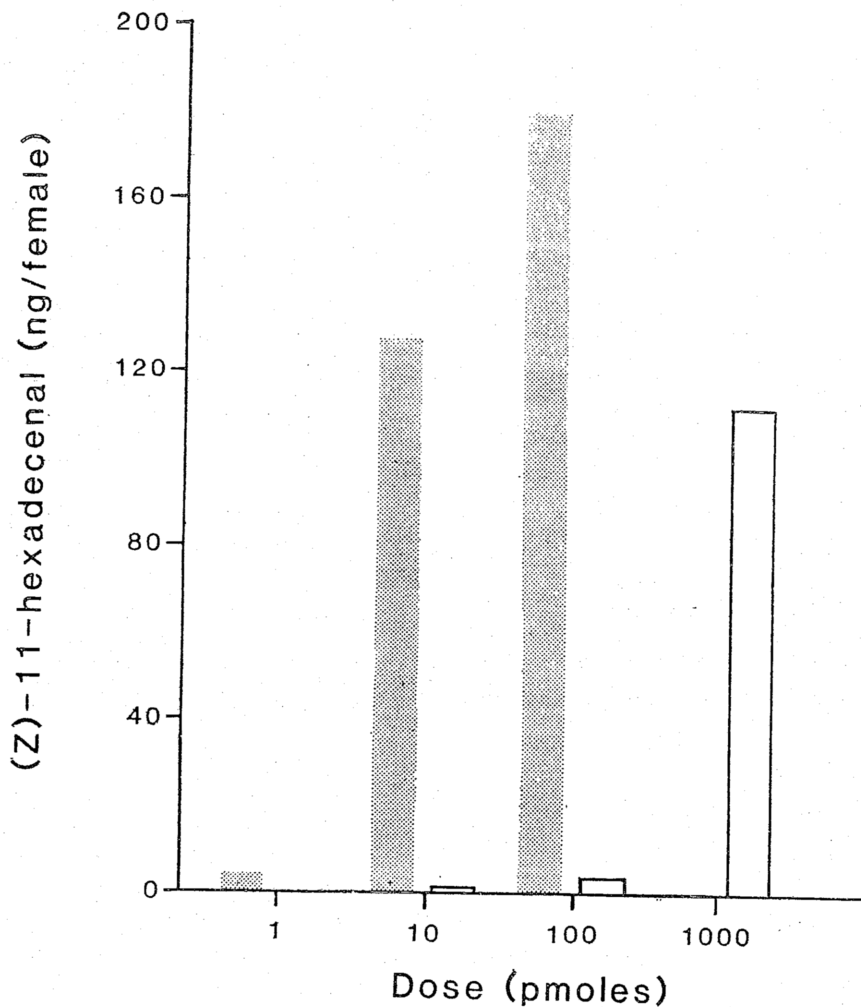

Fig. 4. Biological activity of Hez-PBAN (hatched bars) and its C-terminus acid analog (open bars) in *Heliothis zea* females.

brain-SOG homogenates of *T. ni* were injected into ligated *H. zea* females, significant amount of pheromone was produced (Raina, unpublished). A preliminary study by Tang *et al.* (1989) on the redbanded leafroller, *Argyrotaenia velutinana*, has indicated that PBAN acts by increasing the substrate supply for fatty acid synthesis. There is still a lot to be learned about the sites of PBAN production, release, and action, and the mechanism through which it stimulates pheromone production. We have generated monoclonal antibodies to Hez-PBAN and plan to use these to answer some of the above questions.

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