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AMINO ACID SEQUENCE OF PHEROMONE-BIOSYNTHESIS-ACTIVATING NEUROPEPTIDE (PBAN) OF THE SILKWORM, BOMBYX MORI

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SUMMARY: We have isolated two distinct pheromone-biosynthesis-activating neuropeptides (PBAN), named PBAN-I and -II, as fully oxidized forms of Met residues from adult heads of the silkworm, Bombyx mori. PBAN-I was identical with the PBAN which we had isolated before. The complete amino acid sequence of PBAN-I, a total of 33 amino acid residues, was determined as H-Leu-Ser-Glu-Asp-Met-Pro-Ala-Thr-Pro-Ala-Asp-Gln-Glu-Met-Tyr-Gln-Pro-Asp-Pro-Glu-Glu-Met-Glu-Ser-Arg-Thr-Arg-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2. Synthetic PBAN-I after oxidation with H_2O_2 was chromatographically identical with the isolated PBAN-I. Examination of PBAN activity of synthetic analogues indicated that the carboxyl-terminal portion of PBAN-I was important for biological activity.
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It was first demonstrated in 1984 that a brain neuropeptide, later termed pheromone-biosynthesis-activating neuropeptide (PBAN), stimulates sex pheromone production in the female corn earworm, Heliothis zea (1). Since then, the presence of PBAN has been reported in other several lepidopteran insects (2-7) including the silkworm, Bombyx mori (3). PBAN was first purified from the extracts of brain-suboesophageal ganglion-corpora cardiaca complexes of H. zea to a single peak on RP-HPLC, and its amino acid composition and some chemical properties were reported (8,9): its molecular weight was about 4,200 and it was inactivated by digestion with trypsin or carboxypeptidase Y but not with aminopeptidase M. Recently, we have isolated PBAN from adult heads of B. mori, and determined amino-terminal 10 amino acid residues (10). We now report

ABBREVIATIONS: PBAN, pheromone-biosynthesis-activating neuropeptide; RP-HPLC, reverse-phase high performance liquid chromatography; TFA, trifluoroacetic acid; Met(O), methionine sulfoxide; FAB, fast atom bombardment; HFBA, heptafluorobutylic acid; PTH, phenylthiohydantoin.

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the isolation of two PBANs, PBAN-I and -II, and sequence determination of PBAN-I together with some data on structure-activity relationship.

MATERIALS AND METHODS

Isolation of PBAN: PBAN was purified from 6 x 10^5 male adult heads of B. mori (fresh weight, 4.48 kg) essentially according to the 10-step procedure reported previously (10) with a slight, but important modification: purification by RP-HPLC on a Senshu Pak VP318 column at step 9 was repeated twice. The active fractions obtained after the first run of RP-HPLC were combined, oxidized with H_2O_2, as described below, and subjected to the second run of RP-HPLC under the same condition as in the first run. The active fractions were pooled and subjected to the final step of purification as before (10).

Endoproteinase Glu-C digestion of PBAN-I: PBAN-I (15 μg, 3.8 nmol) was digested with 4 μg of endoproteinase Glu-C (EC 3.4.21.19) (ICN ImmunoBiologicals) in 100 μl of 0.1 M Tris-HCl buffer (pH 8.0) at 37°C for 15 hr. The digestion was stopped by adding 5 μl of 5% TFA. The resulting fragment peptides were separated by RP-HPLC (Fig. 3).

Sequence analysis: Amino acid sequences of intact PBAN-I, its fragment peptides generated by enzyme digestion and synthetic peptides were analyzed on an Applied Biosystems (ABI) model 470A gas-phase sequencer with an on-lined ABI model 120A phenylthiohydantoin (PTH) amino acid analyzer, or on a Shimadzu PSQ-1 gas-phase sequencer with an on-lined Shimadzu LC-6A HPLC system for identification of PTH amino acids. Reagents were purchased from ABI or Wako.

Oxidation with hydrogen peroxide: H_2O_2 was added to the eluate from RP-HPLC at purification step 9 or to the synthetic PBAN-I in 0.1% TFA containing about 20% CH_3CN to give a final concentration of 0.4 M, and the reaction mixture was left for 2 hr at room temperature.

Peptide synthesis: Peptides, PBAN-I, PBAN-I(1-32), PBAN-I(24-33)NH_2 and PBAN-I(24-33)OH, were synthesized by the solid-phase method on an ABI model 430A peptide synthesizer, using a t-butoxycarbonyl (Boc) protocol. All reagents and protected amino acids were purchased from ABI. p-Methylbenzhydrylamine resin was used for synthesizing PBAN-I and PBAN-I(24-33)NH_2, and 4-(oxymethyl)-phenylacetamidomethyl (PAM) resin for PBAN-I(1-32) and PBAN-I(24-33)OH. Side chain protecting groups were: Arg, mesitylenesulfonyl; Asp and Glu, benzyl ester; Met, sulfoxide; Ser and Thr, benzyl; Tyr, 2-bromobenzyloxycarbonyl. After completion of stepwise elongation of peptide chain, the dried peptide-resins were cleaved by the low-high (for Met(O)-containing peptides) or high trifluoromethanesulfonic acid (TFMSA) procedure (11). The resulting crude peptides were purified by RP-HPLC using a 4.6 x 250 mm Vydac C_{18} column with a lenear gradient elution of 10-30% CH_3CN in 0.1% TFA in 40 min. In all cases, each desired peptide was obtained as a major component from the crude mixture. The identity of the synthetic peptides was checked by sequence analysis, peptide mapping and FAB mass spectrum.

Bioassay: The bioassay was performed using Bombyx decapitated female moths as reported previously (3).

Fast atom bombardment (FAB) mass spectometry: FAB mass spectra were recorded on JMS-DX303 (JEOL) using glycerol as matrix.

RESULTS AND DISCUSSION

PBAN was extracted again from a much larger number of adult heads of B. mori than before and purified according to the previous method (10) with slight modifications for large scale purification. In this experiment, however, at step 9, second RP-HPLC, PBAN activity was eluted over many consecutive fractions (18-24% CH_3CN). Since our preliminary experiments showed

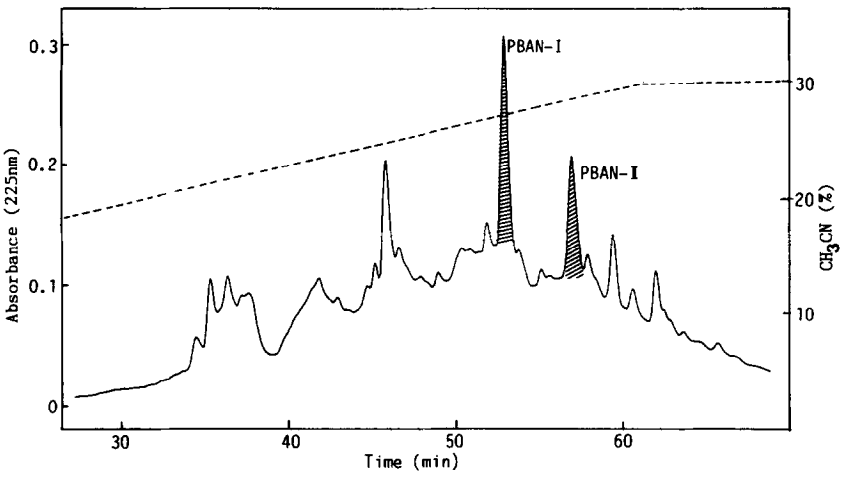

Fig. 1 Final purification of PBAN.
Sample: active fractions after purification step 9 (about 6 x 10^5 heads).
Column: Vydac C_{18} (4.6 x 250 mm). Flow rate: 1.0 ml/min.
Solvent A: 0.1% HFBA, Solvent B: 40% CH_3CN, 0.1% HFBA, Lenear gradients of 20–40% B (5 min), 40% B (5–10 min) and 40–75% B (10–52 min). Shaded peaks, named PBAN-I and -II, showed PBAN activity.

that PBAN existed as a mixture of various oxidized molecular forms at Met residues, the active fractions were pooled, oxidized with H_2O_2, and rechromatographed under the same condition. As expected, PBAN activity appeared in a smaller number of fractions (18–20 % CH_3CN) without any marked changes in total activity. The active fractions thus obtained were combined and then subjected to the final step of purification by RP-HPLC as before (10). PBAN activity was recovered in two peaks (Fig. 1). The peptides in these two peaks were found to be sufficiently pure and named PBAN-I (20 μg) and -II (7 μg). The major component, PBAN-I, was identical with the previously isolated PBAN (10) by coelution on RP-HPLC. Sequence analysis was done only with PBAN-I.

Amino-terminal sequence analysis of PBAN-I identified 33 amino acid residues except for 3 residues at positions 23, 27 and 32 (Fig. 2). Digestion

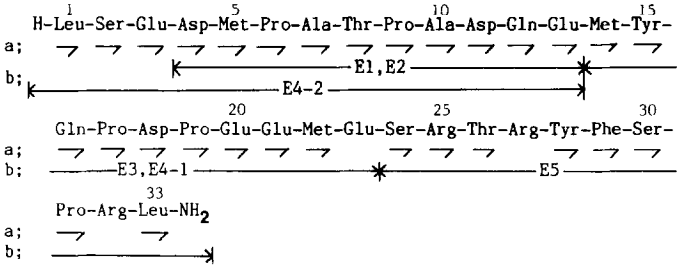

Fig. 2 Amino acid sequence of PBAN-I.
a: Amino-terminal amino acid sequence analysis by a gas-phase sequencer. → means identified residues. b: Assignment of fragment peptides generated by digestion with endoproteinase Glu-C.

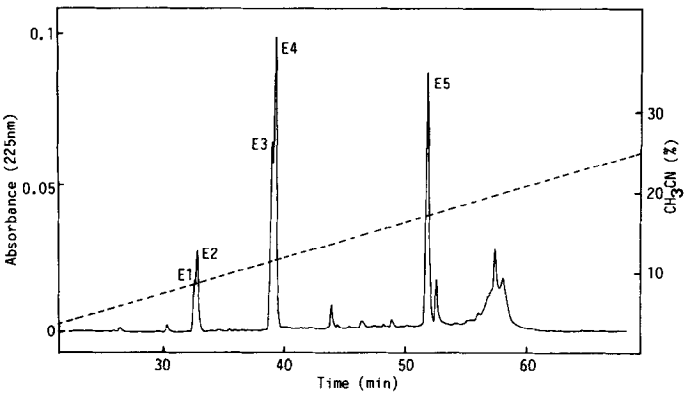

Fig. 3 RP-HPLC of fragment peptides after digestion of PBAN-I with endoproteinase Glu-C.
Column: SenshuPak VP318 (4.6 x 250 mm). Flow rate: 1.0 ml/min.
Solvent A: 0.09% TFA, Solvent B: 50% CH_3CN, 0.08% TFA. Linear gradient of 0-60% B. Results of sequencing of each peak are shown in Fig. 2.

of PBAN-I with endoproteinase Glu-C followed by RP-HPLC purification resulted in separation of 5 peaks including two shoulder peaks, named E1-E5 (Fig. 3). The results of sequencing of each fragment are summarized in Fig. 2. Thus, Glu at position 23 and two Arg residues at positions 27 and 32 were unambiguously identified. Both E1 and E2 were decapeptide with an identical sequence [4-13]. In sequence analysis, Met(O) residue could not be distinguished by Met residue, because Met(O) was converted to, and identified as PTH-Met by reduction with dithiothreitol. Both FAB mass spectra of E1 and E2 showed an $(M+H)^+$ ion at m/z 1,090, which was larger than the calculated mass number from the sequence by 16 mass units, indicating that the Met was oxidized to Met(O). Therefore, they were considered to be diastereomers which differ only in the chirality of sulfoxide of Met(O) due to the stereo-nonselective oxidation with H_2O_2. Sequence analysis of E4 gave two different sequences, [1-13] and [14-23], which were named E4-1 and E4-2, respectively. This was in accord with the observation of two major ion peaks at m/z 1,418 and 1,300 $(M+H)^+$ in FAB mass spectrum of E4. These FAB mass spectral data also indicated that two Met residues in E4-1 and a Met residue in E4-2 were all oxidized to Met(O). E3 showed a single sequence identical with E4-1. Though FAB mass spectrum of E3 failed to be measured successfully, E3 was presumed to have Met(O) and to be a diastereoisomer of E4-1. Therefore, a total of four possible diastereomers in relation to two Met(O) residues for this sequence [14-23] might occur in the peaks of E3 and E4. E4-2 was generated by an incomplete cleavage of Glu^3-Asp^4 and was also considered to be a mixture of two diastereomers.

E5, different from the other fragments, had Leu at the carboxyl-terminus, indicating that E5 was the carboxyl-terminal fragment of the intact peptide. FAB mass spectrum of E5 showed an $(M+H)^+$ ion peak at m/z 1,281, revealing that the carboxyl-terminal Leu was amidated. In order to confirm this amidated

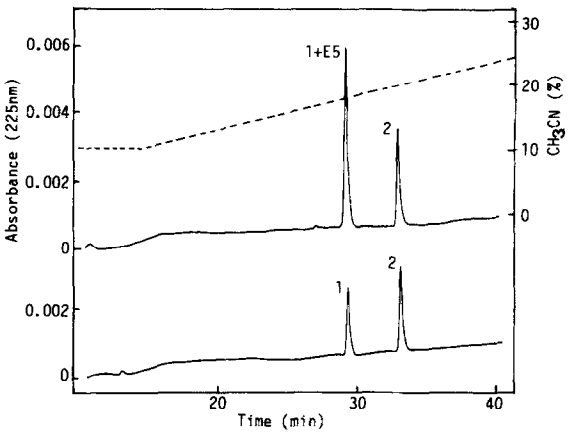

Fig. 4 Chromatographic comparison of the carboxyl-terminal fragment E5 with two synthetic peptides.
1: synthetic PBAN(24-33)NH_2, 2: synthetic PBAN(24-33)OH.
Top: coinjection of 1, 2 and E5, Bottom: coinjection of 1 and 2.
Column, flow rate and solvents were the same as those in Fig. 3. Linear gradient of 20-60% B in 40 min.

structure, PBAN(24-33)OH and PBAN(24-33)NH_2 were synthesized. These two synthetic peptides were eluted separately on RP-HPLC, and E5 was coeluted with the amidated peptide (Fig. 4). Based on these results, Bombyx PBAN was unambiguously identified as a carboxyl-terminally amidated peptide with 33 amino acid residues (Fig. 2).

PBAN-I was synthesized by the solid-phase method. The natural PBAN was coeluted not with this synthetic peptide but with its oxidized peptide. In addition, the identical peptide mapping patterns of natural and oxidized synthetic PBAN-I on RP-HPLC after endoproteinase Glu-C digestion assured the determined sequence, though small differences in peak height between the corresponding fragments containing Met(O) residue(s) was observed.

Table 1 shows PBAN activity of natural peptides and some synthetic peptides. PBAN-I showed clear activity at a dose of 0.25 pmol (1.0 ng) and

Table 1 Effect of various peptides on the extractable sex pheromone (bombykol) in decapitated female Bombyx mori

Peptide	Bombykol, mean(ng) ± S.D.(injected dose, pmol)	
PBAN-I (natural, oxidized)	61.6 ± 37.1 (0.25),	2.7 ± 1.9 (0.13)
PBAN-II (natural, oxidized)	37.7 ± 8.2 (0.50),	6.3 ± 2.1 (0.25)*
PBAN-I (synthetic, oxidized)	63.0 ± 17.0 (0.13),	15.3 ± 7.6 (0.06)
PBAN-I (synthetic, unoxidized)	43.0 ± 22.4 (1.02),	8.0 ± 1.4 (0.51)
PBAN-I(24-33)NH_2	66.7 ± 18.3 (12.5),	1.3 ± 1.9 (6.3)
PBAN-I(24-33)OH	0.0 ± 0.0 (39.0)	
PBAN-I(1-32)	0.0 ± 0.0 (13.2)	
oxidized PBAN-I(1-32)	0.0 ± 0.0 (13.0)	
control(0.1M Tris buffer)	0.0 ± 0.0	

n= 5. * The pmol values were calculated on the assumption that PBAN-II had the same molecular weight as PBAN-I (3,961).

still appreciable activity at its half dose. The isolated PBAN-I seems to be a little less active than oxidized synthetic PBAN-I. More interestingly, PBAN-I of oxidized form was about 10-times as active as that of reduced form. These findings suggest that either one of three Met(O) residues together with its chilarity of sulfoxide is responsible for increasing the activity. PBAN(1-32) was inactive even at a dose of 13 pmol (50 ng). PBAN(24-33)NH_2 had a low, but definite activity, while PBAN(24-33)OH was inactive. These data suggest that the carboxyl-terminal portion, especially carboxyl-terminal amide, is indispensable for the PBAN activity.

When we determined the amino-terminal 10 residues of PBAN, we found that the sequence was identical with that of melanization and reddish coloration hormone (MRCH)-I (10,12). Now we can compare the following 6 residues between these two peptides, because MRCH-I has been characterized only of its amino-terminal 16 residues, and they are still identical. Thus, it becomes more likely that PBAN-I is identical with MRCH-I. The amino-terminal sequence is homologous with insulin-like growth factor-II, as was pointed out already (11). On the other hand, the carboxyl-terminal portion first determined in this experiment was found to have considerable sequence homology including the carboxyl-terminal amide with leucopyrokinin isolated from locust heads (13).

Sequence analysis of PBAN-II is now under investigation.

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ADDENDUM

When we were preparing this manuscript, the complete amino acid sequence of the corn earworm, Heliothis zea, appeared (Raina, A.K. et al. (1989) Science, 244, 796-798). Heliothis PBAN has some structural similarity to Bombyx PBAN: (1) it has the same number of amino acid residues, (2) its carboxyl-terminus is amidated, (3) 27 out of 33 amino acid residues are identical, and (4) two Met residues were oxidized when it was isolated.

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