Short Communication

Molecular Heterogeneity of Eclosion Hormone in Adult Heads of the Silkworm, *Bombyx mori*

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Eclosion hormone (EH), a polypeptide with an apparent molecular mass of 8,400, elicits a series of stereotyped eclosion behavior in insects.^{1,2)} Purification of EH has been tried from two kinds of lepidopteran insects, *Manduca sexta*³⁾ and *Bombyx mori*.^{4,5)} *Bombyx* EH was first purified partially from adult heads by a 14-step purification procedure of solvent extraction, heat-treatment, fractional precipitations, and conventional liquid chromatographies.⁴⁾ Subsequently, *Bombyx* EH purified to homogeneity from pharate adult heads, which contained 5~10 times more EH than adult heads, through 2 steps of purification by high performance liquid chromatography (HPLC) after purification by the 14-step procedure modified slightly; its amino-terminal 13 amino acid residues were H-Ser-Pro-Ala-Ile-Ala-Ser-Ser-Tyr-Asp-Ala-Met-Glu-Ile-.⁵⁾ Recently we have tried again to purify *Bombyx* EH from adult heads, which can be collected in large amounts much more easily than pharate adult heads.

EH was extracted from 470,000 male adult heads of a Chinese race of *Bombyx* called "Showa" (fresh weight, about 3.5 kg) and purified partially by the 14-step procedure.⁴⁾ Hormonal activity was monitored by an ability

to elicit precocious adult eclosion behavior and one EH unit was defined as the amount which caused a positive response in 50% of the assayed insects.^{4,6)} The EH preparation thus obtained weighed about 14 mg and was active at a dose of 0.32 μ g. Comparison of these values with the earlier ones (21 mg from 264,000 adult heads, 1.8 μ g/EH unit)⁴⁾ indicated that this purification was more efficient. This preparation was applied to reversed phase HPLC on a TSKgel ODS-120T column (Toyo Soda, 4.6 $\times$ 250 mm) in three batches (step 15). The materials were eluted with a 40-min gradient of 10~50% acetonitrile in 0.1% aqueous trifluoroacetic acid at a flow rate of 1 ml/min. Figure 1a shows a typical chromatogram. EH was recovered from two discrete fractions, A and B, with an approximate activity ratio of 2:1. Only fraction A was purified further. The fraction A combined from three batches was then applied to reversed phase HPLC on a column of Hi-Pore RP-304 (Bio-Rad, 4.6 $\times$ 250 mm), and eluted with a 30-min gradient of 27.5~35% acetonitrile in 0.05% aqueous heptafluorobutyric acid at a flow rate of 1 ml/min (step 16). EH activity was eluted at a concentration of acetonitrile of about 30%. Since most of the impurities were eluted later, this HPLC was extremely efficient, and attained about 30-fold purification. The above fraction was applied to ion-exchange HPLC on a TSKgel DEAE-5PW column (Toyo Soda, 7.5 $\times$ 75 mm) (step 17). A 30-min gradient elution of 0~0.1 M NaCl in 0.02 M Tris-HCl buffer (pH 7.8) containing 10% acetonitrile at a flow rate of 0.8 ml/min was performed. On the chromatogram monitored by UV absorption at 225 nm, two large, sharp peaks, A1 and A2, appeared; they were eluted at 0.45 and 0.49 M NaCl, respectively. EH activity was detected only in these two peaks. A1 and A2 were finally chromatographed separately by reversed phase HPLC using a column of Hi-Pore RP-304 (4.6 $\times$ 250 mm) (step 18). Elution was done with a 30-min gradient of 25~40% acetonitrile in 0.1% aqueous trifluoroacetic acid. Each chromatography gave pure EH, tentatively named EH-I (0.49 μ g) from A1

FIG. 1. Purification of EH by HPLC.

Cross-hatched areas indicate EH activity. Dotted lines illustrate the concentration of acetonitrile. (a): Reversed phase HPLC of the EH preparation after step 14 on TSKgel ODS-120T (step 15). (b) and (c): Reversed phase HPLC on Hi-Pore RP-304 (step 18).

(Fig. 1b) and EH-II (0.80 μ g) from A2 (Fig. 1c), each of which was active at a dose of 0.10 ng. These EH's, EH-I and -II, seem to be more active than the EH previously isolated from pharate adult heads, since it showed activity at a dose of 0.83 ng.⁵⁾ However, because the pharate adult EH which had been stored at -20°C for more than 1 year was still active at the same dose (0.10 ng) when injected into the same lot of insects assayed for EH-I and -II, the difference in the specific activity of these EH's seems to be accounted for simply by the difference in sensitivity of the test

EH-I H-Ala-Ile-Ala-Ser-Ser-Tyr-Asp-Ala-Met-Glu-Ile-
EH-II H-Ser-Pro-Ala-Ile-Ala-Ser-Ser-Tyr-Asp-Ala-Met-Glu-Ile-

FIG. 2. Amino-terminal Amino Acid Sequences of EH-I and EH-II.

insects.

EH-I and -II were sequenced using an Applied Biosystems gas-phase sequencer (model 470A) and an on-line phenylthiohydantoin amino acid analyzer (model 120A). All samples of EH-I and -II were subjected to amino-terminal sequence analyses because of their small amounts. As shown in Fig. 2, the amino-terminal sequence of 13 residues of EH-II was identical with that of EH previously isolated from pharate adult heads, while EH-I was shorter than EH-II by an amino-terminal dipeptide, Ser-Pro. The repetitive yields in sequencing were 76% for EH-I and 87% for EH-II. Such heterogeneity of two peptides different in amino-terminal chain length has been found in one of the other insect neurohormones, melanization and reddish coloration hormone, also purified from *Bombyx* adult heads.⁷⁾ Considering the EH in fraction B which has not been purified yet, at least three molecular forms of EH are present in *Bombyx* adult heads.

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