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Eclosion hormone of the silkworm *Bombyx mori*

Expression in *Escherichia coli* and location of disulfide bonds

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A gene encoding eclosion hormone (EH) from the silkworm, *Bombyx mori* was chemically synthesized, inserted into a secretion vector and expressed in *Escherichia coli*, leading to the production of biologically active EH. Sequence analysis of cystine-containing peptides in a thermolysin digest of this EH established the locations of 3 disulfide bonds in the molecule. Evidence was also obtained that the 6 residues at the NH_2-terminal are dispensable but 4 residues at the COOH-terminal play an important role in EH activity.

Eclosion hormone; Insect peptide hormone; Synthetic gene; Disulfide bond; *Bombyx mori*

1. INTRODUCTION

Eclosion hormone (EH) is a neurosecretory, peptide hormone that triggers eclosion behavior in lepidopteran insects [1]. Previously we purified EH from 770000 pharate adult heads of the silkworm *Bombyx mori* and determined its 61-residue primary structure [2]. Concurrently, two groups independently reported the 62-amino-acid sequence of EH from the tobacco hornworm *Manduca sexta* [3,4]. This latter sequence is about 80% identical with that of *B. mori* EH. Analysis of a genomic clone for *Bombyx* EH has shown that its incomplete reading frame encodes Leu-62 in addition to the segment spanning Ala-5 through Lys-61 [5], but our recent reexamination of the COOH-terminal structure of *Bombyx* EH purified from 180000 *Bombyx* heads unambiguously established the whole sequence of *Bombyx* EH consisting of 61 amino acids and the processing of the COOH-terminal Leu is suggested (unpublished data).

Since *Bombyx* EH contains six cysteine residues, it is likely that the polypeptide has three intramolecular disulfide bonds, as suggested from its biological activity [6]. However, nothing is as yet known of the putative disulfide bonds because of the extremely small amount of EH isolated from *B. mori*.

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Abbreviations: EH, eclosion hormone; RP-HPLC, reverse-phase high-performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay

In this study, we succeeded in expressing *Bombyx* EH in *Escherichia coli* and purified the expressed hormone. By using the preparation thus purified, we could show that the EH actually possesses three disulfide bonds between Cys-14 and Cys-38, Cys-18 and Cys-34, and Cys-21 and Cys-49. Evidence was also obtained that 4 residues at the COOH-terminal play an important role in the hormone activity.

2. MATERIALS AND METHODS

2.1. *Synthesis of the EH gene and construction of secretion vector pEH2063*

All DNA manipulations were carried out as described in a laboratory manual [7]. Seven deoxynucleotides (see Fig. 1, 1–3 for coding strand and 4–7 for complementary strand) that together encode *Bombyx* EH were synthesized by the solid-phase phosphoamidite method on an Applied Biosystems model 380B DNA synthesizer. In designing these nucleotides the codon usage in *E. coli* [8] was taken into account. After purification, nucleotides were mixed, annealed and ligated into pUC18 with T4 DNA ligase. The double-stranded DNA with expected sequence was excised and inserted between *Hin*dIII and *Bam*HI sites of pYK331 to construct a hybrid gene of alkaline phosphatase signal sequence and EH structural gene, which was termed pEH2063. The vector pYK331 used for expression and secretion was derived from pTA529 [9] and has an alkaline phosphatase promoter, its signal sequence and a multiple cloning site near the signal sequence cleavage site.

2.2. *Expression in* E. coli *and purification of* B. mori *EH*

E. coli YK537 [10] transformed with pEH2063 was grown in 200 ml of 121 medium [11] containing 1 mM KH_2PO_4 at 30°C for 16 h. Cells were collected by centrifugation, resuspended in 200 ml of 121 medium containing 0.05 mM KH_2PO_4 and further cultivated at 30°C for 2 h. Cells were collected by centrifugation, washed with 20% sucrose solution, and rapidly mixed with 50 ml of cold water to prepare the periplasmic fraction [12]. After each centrifugation, the

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supernatant was assayed for EH activity either by bioassay using *Bombyx* pharate adults [13] or by competitive ELISA using a monoclonal antibody to the COOH-terminal portion of the hormone [14]. Native EH has a specific activity of 5–10 units/ng. EH activity was mainly recovered in 20% sucrose washing and cold osmotic shock fractions. These fractions were combined, boiled for 10 min, and centrifuged. The supernatant was subjected to DEAE-Sepharose CL-6B chromatography in the presence of 0.05% Triton X-100 and two more steps of column chromatography on SP-Sephadex C-25 and Sephadex G-50 were done, essentially following the procedure used for the purification of native EH [6]. Then EH was further purified by semi-preparative RP-HPLC using VP304 (10 × 250 mm, Senshu Kagaku) with a linear acetonitrile gradient (20–40%) in 0.1% trifluoroacetic acid, yielding two (major and minor) active fractions.

2.3. *Thermolysin digestion of EH*

About 70 μg of the purified EH (major fraction) was digested with 2 μg of thermolysin (Boehringer-Mannheim) in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 6.5) at 55°C for 20 h. The peptide fragments produced were then separated by RP-HPLC using a VP318 column (4.6 × 250 mm, Senshu Kagaku) with a linear gradient of acetonitrile (0–40%) in 0.1% trifluoroacetic acid for 80 min. The elution of peptides was monitored by absorbance at 225 nm. The amino acid sequence was analyzed on an Applied Biosystems model 470A sequencer or on a Shimadzu PSQ-1 sequencer.

2.4. *Carboxypeptidase A digestion of EH*

About 30 μg of the purified EH (major fraction) was digested with 1 μg of carboxypeptidase A (Sigma) in 0.1 M Tris-HCl (pH 8.2) at 30°C for 1 min or 2 h. To characterize the COOH-terminal sequence of the digested peptide, each digest was subjected to reductive carboxymethylation and further digested with V8 protease. The peptide fragments were separated by RP-HPLC and the amino acid sequence of the COOH-terminal fragment was analyzed as above.

3. RESULTS AND DISCUSSION

In attempting the expression of a synthetic *B. mori* EH gene in *E. coli*, we used the expression vector pYK331, because this vector has been successfully used in expressing a synthetic human epidermal growth factor (hEGF) gene [10]. Furthermore, hEGF has a similar molecular size to EH and contains three intramolecular disulfide bonds [15]. The *B. mori* EH gene synthesized in this study (Fig. 1) encodes 62 amino acids including the COOH-terminal Leu, the presence of which has been deduced from the nucleotide sequence of the cloned gene [5]. This strategy was adopted because the COOH-terminal Leu might have a role in folding of the polypeptide and/or formation of disulfide bonds after translation, though it does not exist in the mature peptide (unpublished data).

After inducing EH synthesis in *E. coli* harboring pEH2063 by lowering the phosphate concentration, EH activity was mainly recovered in 20% sucrose washing and cold osmotic shock fractions. EH was purified from the combined two fractions as described in section 2, yielding two biologically active fractions. NH_2-terminal sequence analysis showed that the active material in the major fraction has the same sequence as native EH, indicating that the expected cleavage between the alkaline phosphatase signal sequence and EH

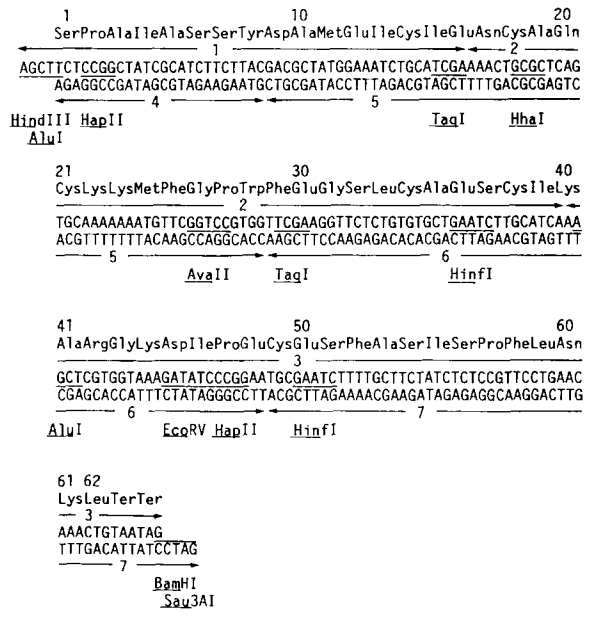

Fig. 1. Design of the synthetic *Bombyx* EH gene. The seven oligodeoxynucleotides synthesized are indicated by arrows above and below the nucleotide sequences. Residue numbers above the amino acid sequence are those for *Bombyx* EH. Major restriction sites are underlined.

had occurred correctly. The polypeptide in the minor fraction had a sequence starting from Ser-7 of EH, presumably because of a wrong cleavage between signal sequence and EH. This shorter EH was as active as

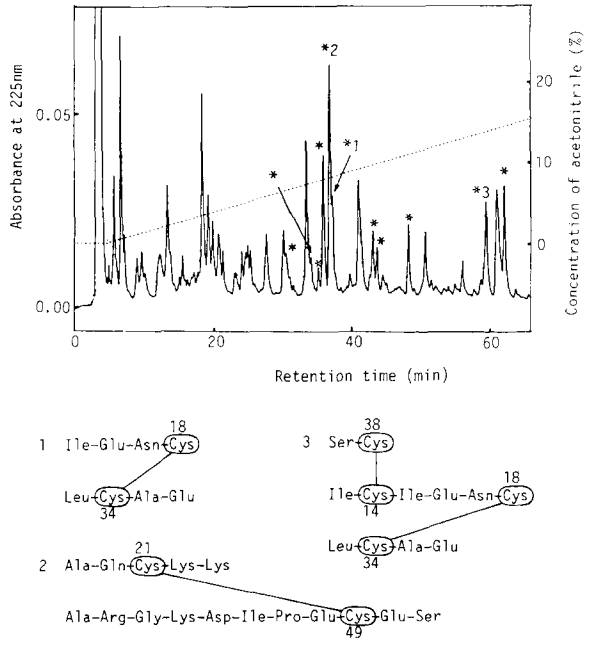

Fig. 2. RP-HPLC analysis of a thermolysin digest of EH synthesized in *E. coli*. Peaks, elution positions of which were altered after reductive carboxymethylation, are marked with asterisks. The covalent structures of peaks 1, 2 and 3 are shown at the bottom. All the structures of the other asterisked peaks were consistent with those of the numbered peaks.

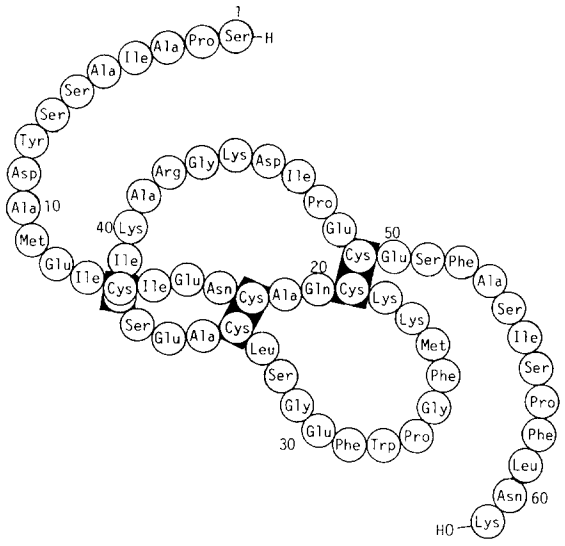

Fig. 3. Complete covalent structure of *Bombyx* EH.

native EH, suggesting that the six residues at the NH_2-terminal are dispensable for EH activity. About 200 μg of purified EH was obtained from 8 liters of culture. A weak activity was also detected in the culture supernatant, from which about 10 μg of EH was isolated by almost the same purification procedure. To our knowledge, this is the first success in producing an insect neurosecretory hormone by recombinant DNA technique.

The purified EH synthesized in *E. coli* was digested with thermolysin and the peptide fragments produced were separated by RP-HPLC, as shown in Fig. 2. The peaks, elution positions of which were altered after reductive carboxymethylation, were thought to contain disulfide bridges and are marked with asterisks. Sequence analysis of these peptides indicated that they did actually contain disulfide bridges. The structures of three of them are shown in Fig. 2. It was thus evident that there are three disulfide bonds between Cys-14 and Cys-38, Cys-18 and Cys-34, and Cys-21 and Cys-49. In view of the finding that the specific activity of this EH preparation was approximately the same as that of native EH, it is highly likely that native EH has disulfide bridges at the same positions. The complete covalent structure of *Bombyx* EH is shown in Fig. 3. Incubation of the EH synthesized in *E. coli* with 2-mercaptoethanol decreased its activity to about 1/100, which was restored completely either by aerobic dialysis against 0.1 M Tris-HCl buffer (pH 8.1) or by the glutathione regeneration method [16].

To study the contribution of the COOH-terminal portion to the biological activity, the EH preparation was digested with carboxypeptidase A for 1 min and 2 h. The short incubation removed only the COOH-terminal Leu, whereas the longer incubation produced a product that had lost the five residues at the COOH-terminal (Phe-Leu-Asn-Lys-Leu). Although the former product showed a specific activity (5–10 units/ng) that was nearly equal to that of the intact molecule, the activity of the product after longer digestion was weaker than the original level by two orders of magnitude (0.05 units/ng). These findings indicate that the 4-residue segment -Phe(58)-Leu-Asn-Lys(61)- in the COOH-terminal portion plays an important role in maintaining the EH activity, though it is still unclear whether all of the four residues are essential or not.

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