Prediction of the biologically active sites in eclosion hormone from the silkworm, *Bombyx mori*

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The structure–activity relationship of eclosion hormone from the silkworm, *Bombyx mori*, was analyzed. First, the probable active residues in silkworm eclosion hormone and also tobacco hornworm eclosion hormone were predicted by the average distance map method. To examine the contributions of those residues to the activity of silkworm eclosion hormone, Gly-substituted mutants for those predicted residues were produced by site-directed mutagenesis and their activities were evaluated by a bioassay. Finally, Glu12, Met24 and Phe25 were estimated to be the crucial residues for the eclosion hormone activity. The possibility of the development of a blocker of an eclosion hormone receptor on the basis of the present work is also discussed.

Keywords: average distance map/biologically active sites/eclosion hormone/insecticide/structure–activity relationship

Introduction

Eclosion hormone (EH) is an insect neurosecretory peptide which plays a crucial role in insect ecdysis (Truman, 1987). EH isolated from the silkworm, *Bombyx mori*, consists of 62 amino acid residues of which primary structure already has been elucidated and turned out to have 80% homology with that of the tobacco hornworm, *Manduca sexta* EH (Kataoka et al., 1987; Kono et al., 1987; Terzi et al. 1988). Biochemical and physiological studies showed that the EH-induced signal transduction is mediated by the rapid turnover of phospholipids and the activation of Ca2+-dependent nitric oxide synthase (Shibanaka et al., 1991, 1993, 1994; Morton and Giunta, 1992). On the other hand, it has been found that EHs distribute in a wide variety in invertebrate but not vertebrate species (Truman, 1987; Horodyski et al., 1993). This restricted distribution of the peptide in insects leads us to an idea of novel insect control by blocking the EH action. Thus, we suggest the possibility of the development of a new type of insecticide by rational design of a blocker of an EH receptor.

For design of a blocker, we need information on the structure–activity relationship as well as the tertiary structure of EH. However, few studies have been performed on these problems. Only the intracellular three disulfide linkages of silkworm EH have been elucidated (Kono et al., 1990). Therefore, it is necessary to predict the location of residues required for the EH action.

In this study, as the first step towards the elucidation of the structure–activity relationships of silkworm and tobacco hornworm EHs, we tried to predict structurally compact regions in these peptides from only their sequences. It was observed that compact regions in a biologically active peptide can be regarded as probable sites which contain residues required for its activity (Kikuchi, 1992). The residues in the predicted compact regions in silkworm EH were examined to identify the most crucial residues for the biological activity by means of the Gly substitution method developed by Konishi et al. (1987) for structure–activity studies of peptides. In this work, we used site-directed mutagenesis to produce Gly-substituted EHs and their activities were evaluated by the bioassay for the peptide using silkworm pharate adults.

Materials and methods

Prediction of active sites in EHs

Predictions of compact regions were carried out with the average distance map method developed by Kikuchi et al. (1988a–c). This method is based on average distances between Cα atoms of residues in proteins with known structures calculated from the Brookhaven protein data bank (Bernstein et al., 1977). According to the separation of regions with 

\[ |i-j| \]

in an amino acid sequence of a protein (i and j denote two residues in the protein), interactions between i and j in the protein were divided into several ranges. Within each range, average spatial distances between every pair of amino acid residues were computed. A predicted average distance map (ADM) was constructed using these average distances. We analyzed the ADM for the occurrence of regions with high densities of contacts (compact regions). Scanning plots of densities on the map were used to determine rapid changes of contact density between various parts of the map. (Kikuchi et al., 1988a,c). The distributions of compact regions were predicted by these locations. Thus, this method was used to predict the location of domains or smaller compact regions in a protein.

We made ADMS for both EHs from the silkworm (*Bombyx*) and the hornworm (*Manduca*). We employed values of two adjustable parameters of \( D = 0.25 \) and \( C = 8.39 \) for the construction of ADMS as described by Kikuchi et al. (1988a,b). These values should provide an ADM with a density of contacts close to a real distance map constructed with a 10 Å cut-off distance (Kikuchi et al., 1988b). The location of core regions (sub-domains) and short-range compact regions (i.e. compact regions formed by interactions in the short-range) in each EH were predicted by the scanning plot analysis of each map.

Circular dichroism (CD) measurements

The CD spectra were measured for the fragment of silkworm EH consisting of residues 1–34 with a JASCO J600 spectropolarimeter at 25°C. The fragment was resolved in three solvents, i.e. 10 mM sodium phosphate buffer, MeOH–H2O (1:1 v/v) and TFE–H2O (1:1 v/v) at pH 6.8. The final concentration of the peptide was 78.1 mM. The molar ellipticity of the peptide in the solutions was measured in the range 200–300 nm.

Peptide synthesis

The fragment of silkworm [Cys14,21(Acm)]–EH 1–34 was synthesized by a solid-phase procedure on an Applied Biosystems
Fig. 1. ADMs for eclosion hormones (EHs). (a) ADM for EH from the silkworm (*Bombyx mori*); (b) ADM for EH from the tobacco hornworm (*Manduca sexta*). A triangle along the diagonal on a map implies a short-range compact region. A part formed by several overlapping triangles is also regarded as a short-range compact region. The hooked lines denote the final assignments of the predicted short-range compact regions. The region lying between the zigzag and the diagonal lines on a map means the short-range area (i.e. the separation of \(i\)th and \(j\)th residues along a chain, \(|i - j|\), is between 1 and 8). The lines drawn on a map parallel to an ordinate or an abscissa means the location of the peaks defined by the scanning plots analysis of the ADMs, which denotes a boundary of a core region. For details, see Kikuchi et al. (1988a–c).

Table I. Predicted short-range compact regions in silkworm EH (*Bombyx mori*) and tobacco hornworm EH (*Manduca sexta*)

<table>
<thead>
<tr>
<th>Silkworm EH</th>
<th>Tobacco hornworm EH</th>
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Model 431A automated peptide synthesizer applying the Boc-strategy. The peptide was constructed on 0.25 mmol Boc-Cys (4MeBzl) linked to a PAM resin. Each Boc group was removed by treatment with 50% TFA in methylene chloride before the condensation reaction step. The peptide chain was elongated with *in situ*-prepared 1-hydroxybenzotriazole ester using a four-fold excess of Boc-amino acid derivatives. The fully protected peptide resin was treated with anhydrous liquid hydrogen fluoride in the presence of anisole to remove all the protecting groups except Acm groups of Cys14,21 and the resin. The disulfide bridge was formed by the air oxidation method. The desired peptide was obtained after several purification steps by using gel filtration and preparative RP-HPLC.

Site-directed mutagenesis

The complete coding sequence of silkworm EH (Hayashi et al., 1990) was ligated into BamHI–EcoRI sites in M13mp18 which can generate single-stranded DNA, a useful tool for site-directed mutagenesis. Ten mutagenic oligodeoxynucleotides devised for the substitution of the following amino acid
Prediction of biologically active sites

Fig. 2. Helical wheel representations of the portions predicted as α-helices. (a) 9–26 in EH from silkworm; (b) 9–26 in the EH from tobacco hornworm. An arrow indicates a polar residue sitting on the surface of a peptide. Residues indicated by underlined letters were examined by Gly substitution. Three cysteine residues in each helix link to the other parts of the peptides through the disulfide bonds.

Fig. 3. Schematic drawing of the disulfide topology of the silkworm EH.

residues to Gly were synthesized using a DNA synthesizer (Applied Biosystems Model 394); 12E, 16E, 17E, 20Q, 22K, 23K, 24M, 25F, 36E and 37S; the numbers represent the sequential numbers of each amino acid in EH. Mutagenesis was performed using an in vitro mutagenesis kit (Amersham). Each mutant gene was subcloned into pPL-Mu, an expression vector bearing lambda PL promoter (Buell et al., 1985). The constructed plasmids were designated pPL-MEHx (x = 12, 16, 17, 20, 22, 23, 24, 25, 36 and 37) and used for the transformation of Escherichia coli.

Expression of pPL-MEHx in E.coli
LC137, a strain of E.coli, was cotransfected with pPL-MEHx and pIC857 (Buell et al., 1985), and grown in LB-broth containing 100 mg/ml ampicillin and 50 mg/ml kanamycin at 30°C for 16 h. Since the plasmid pIC857 involves the temperature-sensitive repressor gene for PL promoter, expression of EH genes from the PL promoter was induced by increasing the cultivation temperature from 30 to 42°C. Further cultivation was carried out for 4.5 h to maintain the de novo biosynthesis of recombinant peptide.

Purification of mutant EHs
Bacterial pellets were washed with 20 mM Tris–HCl (pH 7.5) solution containing 20% sucrose and 1 mM EDTA and resuspended in PBS buffer containing 5 mM EDTA, 1 mg/ml leupeptin and 0.5 mM PMSF. Cells were lysed by sonication with Sonifier II (Branson) and centrifuged. The pellet was washed with buffer W (100 mM Tris–HCl, pH 8.5, 5 mM EDTA, 3 M urea) and then solubilized in buffer S (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 7 M urea) for 1 h (Halenbeck et al., 1989). After subsequent centrifugation, the supernatant was refolded by oxidation in 0.1 M ammonium acetate buffer containing 1 mM oxidized glutathione and 0.1 mM reduced glutathione for 24 h at 4°C (Umemura et al., 1993). This preparation was used as a crude extract for the evaluation of mutant EH activity (primary assay).

For more precise quantitative analyses of the biological activities of the mutants which showed significant changes in the primary assay mentioned above, we purified the mutants from crude extracts as follows: each crude extract was treated on a Bond Elut C18 disposable solid-phase extraction column (Varian) and the eluate obtained with 40% acetonitrile in 0.1% TFA was further purified by RP-HPLC (C18 column; TOSOH). The amount of mutant EH fractionated in 33% acetonitrile in 0.1% TFA was estimated by measuring the UV absorbance at 215 nm. Synthetic EH (wild-type) was used as a standard for calibration (Umemura et al., 1993). The amino-terminal sequences were analyzed using an automated gas-phase sequencer (PSQ-2; Shimazu).

Bioassay of the wild-type and mutant EHs
The fifth instar larvae of silkworm, Bombyx mori, were purchased from Katakura Industrial (Saitama). They were
reared at 25°C with a 16 h light/8 h dark photoperiod. Pharate adults were selected by their hatched antennas as a development marker and utilized for the bioassay (Hayashi et al., 1990).

The amount of recombinant EH in each crude extract was quantified by the densitometry (Pharmacia Image Master 1) of the SDS–polyacrylamide stained with Coomassie Brilliant Blue after gel electrophoresis.

Results and discussion
Prediction of active sites in EHs

The ADMs for silkworm EH and tobacco hornworm EH are shown in Figure 1a and b, respectively. A region enclosed by a triangle denotes a short-range compact region (see Kikuchi et al., 1988c, for definition) in a peptide structure. It was confirmed that the short-range compact regions correspond to α-helices and β-turns, i.e. small compact regions, in globular proteins.

The ADM for silkworm EH indicates the existence of the short-range compact regions at positions 9–24, 28–30 and 33–36 in the sequence. Similarly, the positions of the short-range compact regions are predicted as 11–30, 33–36 and 44–46 from the ADM for tobacco hornworm EH. The lines drawn on the ADMs parallel to the ordinates and abscissas denote the location of boundaries of core regions (subdomains) determined from the scanning plot analyses of the maps (Kikuchi et al., 1988a). From the boundaries in Figure 1a, we can infer the gross features of the folding of silkworm EH as follows. The short-range compact regions, 9–24, 28–30 and 33–36, fold into larger compact regions, 13–39, by long-range interactions. This region is regarded as a core region of the peptide structure. This region would grow further taking the portion 40–49 in the folding process. Finally, the whole peptide structure formation would be completed with the N- and C-terminal parts. A similar inference can be made for tobacco hornworm EH.

In Table I, we summarize the locations of the predicted short-range compact regions in silkworm and tobacco hornworm EHs. We assume that the relatively large short-range compact regions 9–24 of silkworm EH and 11–30 of tobacco hornworm EH form α-helices, and the other smaller short-range compact regions consisting of three or four residues are in β-turns.

Figure 2a shows the helical wheel representation of the region 9–26 in silkworm EH which covers the predicted α-helix. This hormone contains three disulfide bonds as shown in Figure 3 and three half cysteines of the disulfide bonds are arranged on the same side of the α-helix. On the other hand, some polar residues such as Glu, Asp and Lys are on the opposite side of the helix. This result strongly indicates that the side of the helix which contains three cysteines is toward the inside of the peptide, and the opposite side is exposed to solvent, i.e. this side can be regarded as a part of the surface of the peptide. In the same way, Figure 2b represents the character of the helix in tobacco hornworm EH, which is similar to that of the silkworm EH.

A previous study (Kikuchi, 1992) revealed that biologically active sites in peptides are contained in the short-range compact regions predicted by ADMs with high probability. Hence, we assume that those regions in Table I include the active residues required for the biological activity.

CD spectra for the fragment of silkworm EH

The CD spectra of the fragment 1–34 of silkworm EH dissolved in the three solvents are presented in Figure 4. This fragment contains the predicted α-helical region. In particular, EH 1–34 in MeOH and in TFE (Figure 4, traces 2 and 3, respectively) show typical spectra of an α-helix, namely the negative Cotton bands at 208 and 222 nm. Even the spectrum of EH 1–34 in water (Figure 4) exhibits weak signals at these wavelengths. These results indicate that this part of EH tends to form an α-helix especially in hydrophobic solvents, which supports our predictions. It is considered that the α-helix in this part is stabilized in the hydrophobic environment of the peptide structure. The existence of the α-helix in EH 1–34 has been confirmed by NMR measurement (A.Nosaka and K.Kanaori, personal communication).

Expression of the mutagenic EH genes

In this work, we particularly took into account the residues with functional groups because such residues seem to make
specific interactions (e.g. electrostatic interactions, hydrogen bonds) with a receptor. Those residues are targets for the Gly substitution. We chose the polar residues in the common part of the predicted α-helices in silkworm and tobacco hornworm EHs, i.e., 11-24. These are Glu12, Glu16, Asp17, Gln20, Lys22 and Lys23. In addition, Met24 and Phe25 were also selected for the examination. A hydrophobic residue facing the inside of the peptide such as Phe25 shown Figure 2a might be required to maintain its tertiary structure. Met might also undergo a specific interaction with other residues. Therefore, Met24 which is near to the putative peptide surface was also included. Those residues are indicated in Figure 2a. Furthermore, the polar Glu36 and Ser37 located near the predicted β-turn (33-36) were also substituted by Gly. This predicted region also appears in both the silkworm and tobacco hornworm EHs. We selected Glu36 and Ser37 for this study. These two residues are conserved in both sequences of Bombyx and Manduca EHs and might also be important for the interaction with a receptor. These ten mutants are designated as E12G, E16G, N17G, Q20G, K22G, K23G, M24G, F25G, E36G and S37G. [We noted that cross-reactivity of EHs occurs in several insect species, including lepidoptera, diptera and semiptera (Truman, 1987)].

Western blot analyses showed that each EH gene was expressed in the E.coli transformed with pPL-MEHx, correctly (data not shown). By varying the temperature at which the transformed E.coli was grown, the expression of wild-type and mutant genes could be induced very efficiently so that the expressed protein is an insolubilized and inactive form (reduced form). On the other hand, Umemura et al. (1993) have demonstrated that synthetic EH mutants are denoted by filled squares and those of Ala-substituted mutants by open squares. Each point on the abscissa denotes each EH gene. The amount of recombinant EH was quantified by densitometry (see Materials and methods).

High levels of a recombinant protein lead to the formation of inclusion body in E.coli, and the expressed protein is an insolubilized and inactive form (reduced form). On the other hand, Umemura et al. (1993) have demonstrated that synthetic EH was refolded with the correct disulfide pairs by the glutathione-stimulated oxidation. We therefore used glutathione to oxidize the crude extracts for their refolding. The HPLC profile of the refolded recombinant EH 24 h after starting the random oxidation indicates that a single peak at A215 appeared at a similar retention time to the synthetic EH. The ED50 value of the oxidized and purified recombinant EH was 0.4 ng/body, a lower potency than for the native EH, with an ED50 value of 0.1-0.2 ng/body. The reduced activity might be due to an addition of methionine in the amino-terminus, since it was reported that the modification of the amino-terminus of EH showed the biological potency slightly (Hayashi et al., 1990). (Fig. 5).

Table II. ED50 values of the Gly-substituted mutant silkworm EHs relative to the ED50 value of the wild-type

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Each crude extract from E.coli transformed with pPL-MEHx was subjected to the EH bioassay. The amount of recombinant EH was quantified by densitometry (see Materials and methods). Table III. ED50 values of the Ala-substituted mutant silkworm EHs relative to the ED50 value of the wild-type

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<th>WT</th>
<th>D9A</th>
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Each crude extract from E.coli transformed with pPL-MEHx was subjected to the EH bioassay. The amount of recombinant EH was quantified by densitometry (see Materials and methods).

Structure–activity relationship analysis

The biological activities determined by the bioassay for each crude extract are presented in Tables II and III. The ED50 value of the wild-type EH quantified by densitometry was 4.0 ng/animal. This value is 10-fold larger than that of purified EH, demonstrating that 10% of the EH in the crude extract are folded correctly by the random oxidation. From Table II, we note that the activities of E12G, M24G and F25G are less potent than that of the wild-type EH. All the rest show similar biological activities to the wild-type. D9G, E36G and S37G showed lower values. These can be explained by an efficient refolding process, probably due to the higher expression levels as mentioned above. We therefore do not think that these mutants are actually more active than the wild-type. Since Gly substitution for Glu12, Met24 and Phe25 might disturb the correct folding, owing to the helical breaking property of Gly, their crude extracts were subjected to further purification by RP-HPLC. The precise ED50 values of E12G, M24G and F25G mutants were calculated as 3.4, 28 and 12.4 g/body, respectively. M24G mutant was about 70 times less potent than the wild-type and showed the least activity of all mutants. Furthermore, we substituted these three residues to Ala independently, and analyzed the activities. As a result, the biological activity in the mutant of the twelfth residue was recovered by the substitution of Gly12 to Ala12. Since Glu and Ala possess strong helix formation properties [e.g. 1.53 and 1.45, respectively, according to the definition of Chou and Fasman (1974)], this demonstrates that the helical conformation around the twelfth residue disturbed in E12G is recovered in E12A accompanied by the recovery of the activity. That is, the helical structure in this part is required for the eclosion activity and
 activity. Two residues in the predicted. The helical structure is important for the biological

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References
the molecule to keep the active globular structure.
to be required to construct a hydrophobic interaction inside

pivotal roles; Met24 is important for maintaining the tertiary
structure–activity analyses based on the present predictions.

design of inhibitors. We are currently working on further
structure–activity relationship data are also necessary for the
model of the 9–24 region can be used for this kind of study,
more detailed structure–activity relationship data are also necessary for the
design of inhibitors. We are currently working on further
structure–activity analyses based on the present predictions.

Conclusion
From these data, we conclude that the neuropeptide EH contains an α-helix structure in the N-terminal region as predicted. The helical structure is important for the biological activity. Two residues in the α-helix, Met24 and Phe25, play pivotal roles; Met24 is important for maintaining the tertiary structure and interacting with a receptor and Phe25 is likely to be required to construct a hydrophobic interaction inside the molecule to keep the active globular structure.

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Glu12 is important for keeping the helical structure. Even though the activity in the mutant M24A recovered slightly on changing Gly24 to Ala24, it was still less potent by a factor of 10. The comparison of the ED50 values of mutants is shown in Figure 5. These results indicate that Met24 is important both to maintain the α-helical structure and to interact functionally with a receptor. F25A showed the least activity (about 100 times less potent) in the three Ala substitutions. In the tobacco hornworm and Drosophila EHs, the corresponding residues at Phe25 are Ile and Phe, respectively, suggesting that the 25th residue should be hydrophobic. This evidence, plus recent further work by T.Maekawa and N.Fujita (to be published), showing the requirement of hydrophobicity in a residue near the C-terminal region is as predicted, demonstrates that Phe25 is important to interact with the hydrophobic residue intramolecularly. Kono et al. (1990) have also reported that the C-terminal region of the silkworm EH plays an important role in the biological activity. These results indicate that the interaction between the C-terminal residues and Phe25 contributes to the formation of the conformation significant to the activity.

These results will be useful for designing an inhibitor of EH as a new type of insect control. Based on the information on the location of active residues, we can proceed to design non-peptidic inhibitors using molecular modeling tools, e.g. by searching for compounds that fit the geometry of the functional groups of the active site in EH through a three-dimensional structure database. Although the present helical model of the 9–24 region can be used for this kind of study, the precise tertiary structure of EH is desirable. More detailed structure–activity relationship data are also necessary for the design of inhibitors. We are currently working on further structure–activity analyses based on the present predictions.

Conclusion
From these data, we conclude that the neuropeptide EH contains an α-helix structure in the N-terminal region as predicted. The helical structure is important for the biological activity. Two residues in the α-helix, Met24 and Phe25, play pivotal roles; Met24 is important for maintaining the tertiary structure and interacting with a receptor and Phe25 is likely to be required to construct a hydrophobic interaction inside the molecule to keep the active globular structure.