Volume 219, number 2, 415–418 FEB 04891 July 1987

Microanalysis of the amino acid sequence of the eclosion hormone from the tobacco hornworm *Manduca sexta*

Thomas Marti*, Koji Takio+, Kenneth A. Walsh, Gail Terzi° and James W. Truman°

Departments of Biochemistry and °*Zoology, University of Washington, Seattle, WA 98195, USA*

Received 15 May 1987

The amino acid sequence of the eclosion hormone from the tobacco hornworm *Manduca sexta* has been determined, using less than 500 pmol of protein and microanalytical techniques. The protein contains 62 amino acid residues and has a molecular mass of 6813 Da. The amino-terminal sequence is similar to that of a 13-residue segment at the amino terminus of the eclosion hormone of the silkworm *Bombyx mori*, but the hormone is not otherwise homologous with other hormones or proteins.

Amino acid sequence; Microsequence

1. INTRODUCTION

The structures of insect neuropeptides have been difficult to determine because so little material is usually available. The first two such peptides to be sequenced were very small, viz. the pentapeptide proctolin [1] and the decapeptide adipokinetic hormone [2]. More recently the complete sequence of a 48-residue peptide, the prothoracicotropic hormone of *Bombyx mori*, has been described. This hormone, which initiates the molting process in insects, is composed of two nonidentical peptide chains which display sequence homology to vertebrate insulin [3]. A second brain peptide associated with molting is the eclosion hormone [4]. It controls the events associated with ecdysis, the shedding of the old cuticle at the end of each molt. Its cellular targets and actions are diverse, ranging from triggering specific stereotyped behavior to inducing the programmed degeneration of various muscle groups.

This report describes the determination of the amino acid sequence of the eclosion hormone of the tobacco hornworm *Manduca sexta*, using protein microsequencing techniques.

Correspondence address: K.A. Walsh, Department of Biochemistry, SJ-70, University of Washington, Seattle, WA 98195, USA

* Present address: Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

+ Howard Hughes Medical Institute; present address: International Frontier Research Systems, RIKEN, 2-1 Hirosawa, Wako-shi, 351-01, Japan

Abbreviations: CM-, carboxymethyl; HPLC, high-performance liquid chromatography

2. MATERIALS AND METHODS

Brain neurohemal organs were dissected from the heads of over 17000 pharate adult tobacco hornworms (*M. sexta*). The glands were extracted with acidified methanol and the active protein partially purified by reversed-phase HPLC on a Baker RP-WP C_{18} column followed by fractionation on a Vydac C_4 column (Terzi, G. and Truman, J.W., unpublished).

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

The partially purified protein was reduced with dithiothreitol and *S*-carboxymethylated with iodoacetic acid [5]. Separation from reagents by reversed-phase HPLC on an Aquapore BU-300 column (30 × 2.1 mm, Brownlee) yielded pure eclosion hormone as judged by the single amino-terminal sequence. Fragments were generated by cleaving 80–120 pmol of *S*-CM-protein at methionyl bonds with cyanogen bromide [5] or at aspartyl bonds in dilute acid [6]. Lysyl residues were cleaved with *Achromobacter* protease I [7] (a gift of Dr T. Masaki, Ibaraki, Japan) in 50 mM Tris buffer, pH 9.0, at 37°C for 6 h. Digestion at glutamyl residues used *Staphylococcus aureus* V8 protease (Miles) in 0.1 M NH_4HCO_3 at 37°C for 24 h. All peptide separations were performed by reversed-phase HPLC with a Hewlett-Packard model 1090 instrument fitted with an Aquapore RP-300 column (30 × 2.1 mm, Brownlee) and using an acetonitrile gradient in dilute aqueous trifluoroacetic acid [8]. Amino acid sequences were analyzed with an Applied Biosystems 470 A sequencer [9], equipped with a model 120A PTH on-line analyzer. The amino acid composition was determined with a Waters Picotag system [10].

3. RESULTS

The entire sequence was determined with less than 3.5 μg (500 pmol) of protein that had been isolated from approx. 85 kg of hornworms. Nine cycles of Edman degradation of 3 pmol established the purity of the protein and its similarity to the amino-terminal sequence of the eclosion hormone of *B. mori* [4]. However, in later digests it became evident that approx. 20% of the protein lacked the amino-terminal Asn-Pro.

The results of Edman degradation of eight peptides derived from *S*-carboxymethylated eclosion hormone are summarized in fig.1. Together these analyses provide an overlapping set of information that describes the unique sequence of the 62-residue protein. Cleavage at methionyl residues provided three fragments (fig.2), yielding most of the sequence to residue 57. Cleavage at lysyl residues generated peptides K1 and K3, which linked M1 to M2 and M2 to M3, respectively. Peptide K6 overlapped the carboxyl terminus of M3 and extended the sequence to Lys-61. The carboxyl-terminal leucine was assigned by sequence analysis

Fig.1. Summary of the proof of sequence. The sequences of specific peptides (names are underlined) are given in one-letter code (table 1) below the summary sequence (bold type). Prefixes M, K, E and D denote peptides generated by cleavage at the carboxyl side of Met, Lys, Glu and Asp, respectively. Lower-case letters indicate tentatively identified residues; dashes denote unidentified amino acids. Methionine residues in M1 and M2 were recognized as their homoserine derivatives.

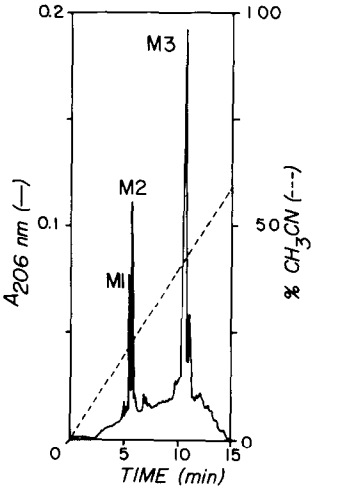

Fig.2. Separation of peptides after digestion of 100 pmol *S*-CM-protein with cyanogen bromide. The flow rate was 0.3 ml/min through an Aquapore RP-300 column.

of peptide D3, generated by dilute acid treatment. Other peptides from this digest were not recovered in sufficient yield, due to partial oxidation of Met and CM-Cys. Digestion at glutamyl residues provided peptide E3, verifying the alignment of M2 and M3.

The carboxyl terminus was not confirmed by direct analysis since incomplete cleavage at glutamyl residues 36, 48 and 50 generated several fragments from this region in low yield. Indirect

support for the proposed carboxyl terminus was achieved by accounting for all of the peptides in the lysyl digest. In addition to the fractions containing K1, K3 and K6, a break-through fraction appeared by sequence analysis to contain only lysine (residue 23) and two dipeptides, Phe-Lys (residues 41–42) and Gly-Lys (residues 43–44). No short peptide with an amino-terminal Leu was found in any fraction. Based on these results we conclude tentatively that Leu-62 represents the carboxyl terminus of the protein. The amino acid composition of the unreduced eclosion hormone was determined only once and with 40 pmol of protein. It is in reasonable agreement with the data from the sequence analysis (table 1), although traces of contaminants may be present.

4. DISCUSSION

The amino acid sequence of eclosion hormone from the hornworm *M. sexta* has been established by the strategy outlined in fig.1, although only minute amounts of protein were available. Several improvements in sensitivity were made in order to perform digests at the 100 pmol level. The most important of these were the use of microbore column cartridges (2 mm i.d.) in the HPLC and slow flow rates (0.3 ml/min). For cleavage, *Achromobacter* protease and cyanogen bromide turned out to be most effective, yielding peptides in 50–60% yield, as calculated from the recovery of phenylthiohydantoins in the first cycles of sequence analysis.

Table 1

Amino acid composition of eclosion hormone[a]

Asp/Asn (D/N)	5.6 (5)
Glu/Gln (E/Q)	7.3 (7)
Ser (S)	2.3 (2)
Gly (G)	4.2 (4)
Arg (R)	0.5 (0)
Thr (T)	1.2 (1)
Ala (A)	6.7 (7)
Pro (P)	4.3 (5)
Tyr (Y)	0.9 (1)
Val (V)	0.4 (0)
Met (M)	2.1 (2)
Cys (C)	N.D. (6)
Ile (I)	5.7 (6)
Leu (L)	5.0 (5)
Phe (F)	3.9 (4)
Lys (K)	6.1 (6)
Trp (W)	N.D. (1)

[a] Residues per molecule by amino acid analysis of 40 pmol unreduced protein or (in parentheses) from the sequence

The sequence at each end of the 62-residue chain remains somewhat tentative. At the amino terminus, peptides M1 and K1 included a component (~20%) that lacked the terminal dipeptide Asn-Pro. It is not clear whether the shorter molecule represents a natural isotype or simply an artifact of partial proteolysis during purification. At the carboxyl terminus, Leu-62 is placed only within peptide D3, which was a product of cleavage at Asp. No peptide with amino-terminal Leu-Asp was found in the lysyl digest. Its apparent absence is consistent with the proposed structure if free Leu is not recovered from the lysyl digest or if the carboxyl terminus of peptide K6 is Asn-Lys-Leu-COOH, which may not be cleaved by the *Achromobacter* protease at the penultimate Lys.

The molecular mass calculated for the 62-residue sequence is 6813 Da, which is significantly lower than the value of 8400–8500 Da previously estimated by gel filtration on Sephadex G-50 for the *Manduca* hormone [4] or for the corresponding hormone from *B. mori* [11]. Although the protein contains at least 18 hydrophobic amino acids, no Val residues are present; additionally, it lacks His and Arg. The present data confirm that the eclosion hormone from *Manduca* is an acidic peptide with a calculated isoelectric point of pH 4.7.

The protein is inactivated upon reduction [12], and it is likely that the six Cys residues form disulfide bonds in the active conformation. Nagasawa et al. [11] have previously determined the sequence of the amino-terminal 13 residues of the eclosion hormone of the silkworm *B. mori*, using 1.3 nmol protein. Their data show nine identities with the *Manduca* hormone (69%) but differences at residues 1 (Ser), 6 (Ser), 7 (Ser) and 10 (Ala). The insulin-related prothoracicotropic hormone from the same organism [11] differs from the *Manduca* eclosion hormone in size and amino acid sequence, as well as function. A search of the December 1986 Protein Databank (National Biomedical Research Foundation) did not reveal any other vertebrate or invertebrate peptide or

protein homologous with the eclosion hormone.

The present elucidation of the amino acid sequence of eclosion hormone should provide a basis for understanding more precisely its mode of action and its role in regulating aspects of insect behavior and development.

ACKNOWLEDGEMENTS

The authors are grateful for the assistance of Donavon Wilhelm, Roger D. Wade, Susanne Rösselet and Maria Harrylock with the dissections, amino acid analysis, peptide separations and sequence analyses, respectively. This work was supported by National Institutes of Health grants GM-15731 (to K.A.W.) and NS-13079 (to J.W.T.) as well as a National Science Foundation grant DCB-8316055 (to J.W.T.).

REFERENCES

[1] Starratt, A.N. and Brown, B.E. (1975) Life Sci. 17, 1253–1256.

[2] Stone, J.V., Mordue, W., Batley, K.E. and Morris, H.R. (1976) Nature 263, 207–211.

[3] Nagasawa, H., Kataoka, H., Isogai, A., Tamura, S., Suzuki, A., Mizoguchi, A., Fujiwara, Y., Suzuki, A., Takahashi, S.Y. and Ishizaki, H. (1986) Proc. Natl. Acad. Sci. USA 83, 5840–5843.

[4] Reynolds, S.E. and Truman, J.W. (1983) in: Endocrinology of Insects (Downer, R.G.H. and Laufer, H. eds) pp.217–233, Alan R. Liss, New York.

[5] Titani, K., Sasagawa, T., Ericsson, L.H., Kumar, S., Smith, S.B., Krebs, E.G. and Walsh, K.A. (1984) Biochemistry 23, 4193–4199.

[6] Inglis, A.S. (1983) Methods Enzymol. 91, 324–332.

[7] Masaki, T., Tanabe, M., Nakamura, K. and Soejima, M. (1981) Biochim. Biophys. Acta 660, 44–50.

[8] Mahoney, W.C. and Hermodson, M.A. (1980) J. Biol. Chem. 255, 11199–11203.

[9] Hunkapiller, M.W., Hewick, R.M., Dreyer, W.J. and Hood, L.E. (1983) Methods Enzymol. 91, 399–413.

[10] Bidlingmeyer, B.A., Cohen, S.A. and Tarvin, T.L. (1984) J. Chromatogr. 336, 93–104.

[11] Nagasawa, H., Kamito, T., Takahashi, S., Isogai, A., Fugo, H. and Suzuki, A. (1985) Insect Biochem. 15, 573–578.

[12] Nagasawa, H., Fugo, H., Takahashi, S., Kamito, T., Isogai, A. and Suzuki, A. (1983) Agric. Biol. Chem. 47, 1901–1906.